Cytokine Knockouts

Edited by Scott K. Durum and Kathrin Muegge
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...should be the most useful for everybody who wants to learn about those thrilling, novel leads into the biology of the immune system and the pathogenesis of human diseases.
—from the Foreword by Klaus Rajewsky, Institute of Genetics, Cologne, Germany

Surveying one of today’s most powerful new methodologies, Durum and Muegge assemble in *Cytokine Knockouts* a unique compendium of the latest information on cytokine knockout mice. Each chapter—written by the leading scientists who created or studied each knockout—illuminates the physiological consequences of deficiency in that cytokine and relates the phenotype to other knockout mice and to human diseases. The effects discussed range from diseases of the bowel, lung, heart, blood, kidney, and liver to immune deficiency, developmental defects, and autoimmunity. Most of the key knockouts of cytokines or receptors, including IL-1 through 8, IFNα, IFNβ, TNF, LT, CD40, LIF, CSFs, c-mpl, gp130, and ICE, are thoroughly reviewed.

*Cytokine Knockouts*’ many distinguished contributors crystalize their insights into the role of cytokines in health and disease, explain important new concepts in human and animal diseases, and indicate which cytokines should (and should not) become pharmacological targets. Their book offers an authoritative synthesis and review of this important new field and will immediately become a standard resource for today’s biomedical researchers.

- Identifies potential targets for new drugs
- Reviews the key studies that established the importance of cytokines
- Demonstrates the unique role of each cytokine in health and disease
- Highlights major unresolved questions, missing cytokines and receptors, and biochemical pathways
- Provides insights into the pathogenesis and treatment of arthritis and sepsis

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Cytokine Knockouts

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Foreword by
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Foreword

My personal history in the field of cytokines had an initial period of several years during which my student and then colleague, Werner Müller, tried in vain to attract me to them. My interest always vanished when I was confronted with complex data pointing to functional redundancy of cytokines in cell culture systems. When gene targeting in the mouse germline became possible, this frustration came to an end. We and others immediately embarked on analyzing the in vivo function of cytokines and the problem of functional redundancy with this powerful new approach. The early cytokine gene knockouts performed by colleagues in Würzburg (IL-2) and by ourselves (IL-4 and IL-10) seemed to give clear answers and at the same time led to surprises: Each of these cytokines apparently had its own special and irreplaceable function, and this function could be quite distinct from what had been anticipated from functional experiments in vitro. Although the latter finding is of course a wonderful incentive for further research, the former is pleasing in a general sense since it highlights the value of each of those one hundred thousand genes or so in our genome, cherished by evolution to become respectable members of the community. Even in the present era of “genomics” there will be no way around the careful functional analysis of each gene by itself.

At this stage, through the efforts of many groups worldwide, a large number of cytokine and cytokine receptor genes have been inactivated in the mouse germline, and the corresponding mutant mice have provided a wealth of novel information, assigning to almost all of these genes a unique function in vivo. Much of this information is assembled in Cytokine Knockouts, which thus marks a turning point in the history of cytokine research. Only now, because of the gene targeting approach, are we beginning to grasp the fundamental and,
to my understanding, amazingly general importance of cytokines in
the control of the immune system’s development from early on in
ontogeny and its function in immunological defense.

Immunological research always relates to medicine. It appears
natural in retrospect that cytokine deficiencies in mice often gave
rise to states of disease. Because of our earlier ignorance about the
true in vivo function of cytokines, these diseases were often unex-
pected and turned out to represent counterparts of human diseases
whose origins are not yet understood. The inflammatory bowel dis-
eyes in the early IL-2 and IL-10 knockouts (resembling ulcerative
colitis and Morbus Crohn, respectively) are examples of this kind.
The impact of such mouse mutants on medical research is obvious.

All these new insights and developments in the field of cytokine
research are amply documented in this timely book, although the
reader should not expect a complete account of what has been achieved
in this huge area. The book should be most useful for everybody who
wants to learn about those thrilling, novel leads into the biology of
the immune system and the pathogenesis of human diseases.

Klaus Rajewsky
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Preface

The technique of gene targeting or “knockout” has swept through biomedical research of the 1990s as if it were the Occam’s razor of biology. The technique provides an acid test of the function of a gene (for recent reviews of the knockout technique, see refs. 1 and 2). It involves creating deletions in one designated gene in an embryonic stem cell line, and then producing mice with just one damaged gene from that cell line. The mice are then tested for physiological abnormalities. The logic is “You don’t know what you got til it’s gone” (Joni Mitchell). Hundreds of genes have now been knocked out and the results have changed many paradigms.

This book is a collection of reviews on the major cytokine knockouts studied to date. A cytokine is a peptide that one cell uses to signal another cell. The cytokine binds to high affinity receptors on the target cell, eliciting intracellular responses. The target cell can be in intimate contact, as in the case of a T-lymphocyte signaling a B-lymphocyte (via CD40 ligand-CD40). The target cell can also be at a considerable distance, for example, IL-1 produced in a local inflammatory site travels via the blood and triggers a reaction in the hypothalamus leading to fever. There are hundreds of such cytokines mediating inflammation, growth, differentiation, apoptosis, adhesion, and chemotaxis.

Surprises

Cytokine research has been revolutionized by knockouts. There had accumulated an enormous body of literature on the effects of cytokines, much of which fell under sharp scrutiny in the wake of research using knockouts. This began with the first cytokine knockout, IL-2, published in 1991 (3). IL-2 had been discovered as a T-cell
growth factor and studied as such for over a decade: The knockout mice (Chapter 1) caused much chagrin everywhere by producing T-cells quite readily, instead of succumbing to a mysterious inflammatory process. IL-2 is indeed critical, but not for what was thought, since it appears to play a role, not in growth, but in programming CD4 cells for death.

GM-CSF was regarded as an important inducer of production of granulocytes and monocytes. The knockout results (Chapter 21) showed that this is not its vital activity, which appears instead to be activating macrophages to dispose of lung surfactant.

Lymphotoxin was studied as a cytotoxic factor, whose most likely roles would seem to be killing cells that were harboring intracellular pathogens or disposing of otherwise undesirable cells. It came as a complete surprise that lymphotoxin knockouts (Chapters 7 and 8) exhibited a lack of lymph nodes and Peyer’s patches, implicating an organogenic role.

Several knockouts were lethal, demonstrating a far broader function than anticipated from their discoveries in inflammatory processes. GP130 was initially discovered as an element in the signal transducing chain for receptors of a number of inflammatory cytokines. The knockout (Chapter 16) is lethal to embryos, producing failure in heart development and severe anemia, with the mice also showing deficiencies in primordial germ cells. LIF receptor, also discovered based on its inflammatory role, is embryonically lethal (Chapter 18). TGFβ3 knockout is perinatally lethal and causes cleft palate (Chapter 19). In all these cases of knockouts leading to early death, it has been difficult to test inflammatory processes to determine cytokine roles.

**Inflammatory Effects**

TGFβ1 knockout (Chapters 19 and 20) revealed its vital function as a kind of “speedtrap on the inflammation superhighway.” T-cells, unchecked by TGFβ1, trigger deadly cellular infiltration of vital organs. It remains perplexing just what critical process is controlled by TGFβ1—Are these self-recognizing T-cells whose self-tolerance is maintained by TGFβ1? Alternatively, do these T-cells recognize foreign antigens, then proliferate unchecked, or infiltrate
the wrong organs unchecked, because of increased integrin-mediated adhesion? Other surprising findings about TGFβ₁ were its role in maintaining genome stability and the phenomenon of maternal transfer of this cytokine.

IL-2 knockout (Chapter 1), contrary to expectation, resulted in overproliferation of T-cells and lethal bowel inflammation. It is suggested that IL-2 prepares activated T-cells for apoptotic death.

IL-6 knockout (Chapters 14 and 15) substantiated its role as a key mediator of inflammation. Several features of the global inflammatory response depend on IL-6 triggered by gram-positive bacteria or turpentine injection; these include the acute phase response of the liver and the fever response of the brain. Local inflammation itself, mimicked by turpentine injection, is also IL-6 dependent.

**Shock**

It was expected that septic shock, induced by high doses of endotoxin, would be dependent on cytokines, based on the efficacy of blocking the cytokines with antibodies, soluble receptors, and receptor antagonists. This had prompted development of anti-IL-1 and TNF reagents to protect septic patients. However, the knockouts of IL-1b (Chapter 9), IL-1RI (Chapter 10), and TNFRp55 (Chapters 5 and 6) did not protect mice from high doses of endotoxin. The simplest explanation is that endotoxin needs no intermediary cytokine to induce endothelial cells to produce nitric oxide, but instead directly relaxes smooth muscle and thus reduces blood pressure below a critical level. Similarly, the acute phase response, which requires IL-6 when the mouse is challenged with gram-positive bacteria, is triggered by endotoxin directly acting on hepatocytes with no cytokine intermediary (Chapter 14). On the other hand, cytokines could still be essential mediators of shock if there were several different lethal combinations produced, such that removing IL-1 alone or TNF alone would not be sufficient. Support for this comes from the knockout of IFNγ receptor, which reduced endotoxin shock (Chapter 13), perhaps because IFNγ acts synergistically in several different lethal pathways.

ICE knockout (Chapter 11) did have a protective effect on endotoxin shock. But the ICE knockout could not be simply blocking
IL-1β processing, the property for which ICE was discovered, since IL-1β knockout does not protect from shock. ICE does affect the production of several different cytokines, perhaps giving hope to the possibility of therapeutically blocking combinations of cytokines in shock. Or ICE may affect some (as yet unknown) noncytokine function in shock; for example, it has been shown to mediate apoptotic signals, but we do not yet know how that property would be involved in shock.

A different experimental system has been used to model shock in some studies: galactosamine priming, followed by the introduction of endotoxin. This is lethal to mice because of hepatotoxicity, rather than the vascular collapse that results from high doses of endotoxin given alone. TNFRp55 knockout (Chapters 5 and 6) did protect mice from this type of shock.

**Immunity**

Lymphocytes are far more oblivious to cytokine knockout than anticipated from their vigorous responses to them in vitro and in vivo. A large body of observations before the knockouts had shown potent effects of cytokines on lymphocytes, but most are apparently not critical.

The interactions of T-cells with antigen-presenting cells, T-cells, and B-cells, and of T-cell precursors with thymic stroma must now be viewed as primarily cognate. The major stimuli mediating these cellular interactions do not diffuse away from the producing cells, as was once widely believed; for example, soluble cytokines were thought to be critical products of antigen-presenting cells. CD40 ligand (Chapter 4) is a cognate signaling molecule, anchored to the T-cell membrane, and required for signaling B-cells and macrophages during cell contact. Whether CD40 ligand should be termed a "cytokine" or not does not seem important, but it is interesting that its gene family contains both anchored and soluble species. IL-6 knockout did reduce IgG and mucosal IgA production (Chapters 14 and 15), and it is proposed to act as a growth factor for plasmablasts; however, it is not clear whether the relevant IL-6 derives from T-cells since activated macrophages are also a good source. Other cytokines shown to augment Ig production are LTα (IgA) (Chapter 8), IFNγ (IgG2) (Chap-
ter 13), TNFR (IgG) (Chapters 5 and 6), and IL-4 (IgE) (Chapter 1). T-cell-dependent immunity against leishmania was impaired in CD40L (Chapter 4) and IFNγ (Chapter 13) knockouts, reflecting a deficiency in TH1 type immunity.

IL-7 and its α and γ receptor components (Chapters 2 and 3) are very important for lymphoid development and would appear to be an exception to the lymphocyte’s preference for immobilized signals, since IL-7 is secreted. However, IL-7 has a glycosaminoglycan binding site and most of it is probably anchored to extracellular matrix rather than being freely diffusible (Kitazawa et al., unpublished observations).

Nonlymphoid arms of the immune system, on the other hand, clearly require soluble cytokines. Defense against many types of virus requires the interferons (Chapter 13). Defense against listeria requires the activation of macrophages to kill the bacteria, and is defective in IFNγ (Chapter 13), TNF (Chapters 5 and 6), and IL-6 (Chapters 14 and 15) knockout mice. NF-IL6 (Chapter 17) knockout mice are also sensitive to listeria; their macrophages fail to lyse intracellular bacteria for unknown reasons, perhaps because a cytokine-induced gene is required. Antiviral immunity requires the interferons (Chapter 13) and IL-6 (Chapters 14 and 15). Neutrophil entry into an inflammatory site requires IL-8R (Chapter 12).

**Hematopoiesis**

gp130 is required to expand pluripotential stem cells in the embryo (Chapter 16); just part of this requirement is as a receptor for IL-6, which at least in the adult mouse, optimizes hematopoiesis (Chapters 14 and 15). G-CSF is required for normal levels of neutrophils (Chapter 23). A low level of neutrophils is produced in the G-CSF knockouts, suggesting that this cytokine could be required for expansion of the granulocytic precursors, but not for their differentiation. GM-CSF was surprisingly not required for hematopoiesis (Chapter 21). c-mpl is required for platelet production (Chapter 24). Eosinophil production after nematode infection required βc (Chapter 22).

Are the numbers of blood cells actually regulated, or is production stochastic? There are examples of regulation. For example, irra-
diated mice, depleted of progenitors, increase production of all lineages. Lineage specific regulation occurs for example via hypoxia, which induces erythropoietin and hence erythropoiesis. Neutrophil production appears to increase in mice transferred from germ-free to conventional environments (Chapter 23), suggesting that immune responses or activated phagocytes produce a cytokine with this activity. Lymphoid production, if it could be selectively stimulated, would be an obvious target for AIDS therapy. Lymphopoiesis depends on IL-7 (Chapters 2 and 3). Is IL-7 production regulated, or is the number of lymphoid cells controlled at much later stages?

G-CSF heterozygous mice (with one knockout and one normal allele), show an intermediate number of hematopoietic stem cells, (Chapter 23). In other words, the magnitude of G-CSF signaling controls the vigor of the proliferative response. However, a heterozygous effect was not observed for gp130 (Chapter 6) or c-mpl (Chapter 24) knockouts, even though they are required hematopoietic factors. What does this imply about hematopoietic regulation? There seem to be two possibilities. One, that the factor is made in excess, so half the production is still more than enough. Two, that the amount of factor is limiting, but its production is regulated by demand, as in the example of erythropoietin production and hypoxia. The second possibility appears to be supported by the effects of giving exogenous factor, which increases production of the cell type, but there could be other explanations for this effect, such as recruitment of progenitors that would normally not encounter the endogenous product.

**Effects of Germ-Free vs Conventional Housing**

Several of the knockouts, IL-2 (Chapter 1) and TGFβ1 (Chapters 19 and 20) show much more severe phenotypes in conventional environments than in clean ones. In these mice, normal flora or pathogens are not just replicating uncontrollably, as in immunodeficiency, but are probably inducing immune and inflammatory responses that are not controlled properly. Such uncontrolled immune responses are reminiscent of the murine strains with natural mutation in fasL and fas (reviewed in ref. 4), and knockout of CTLA-4 (5)—mutations that affect the life-span of lymphoid cells.
IL-8R knockout (Chapter 12) showed increased granulopoiesis in a conventional environment, but not in a germ-free one. This suggests that a site of inflammation calls for neutrophils, and when neutrophils do not arrive, calls for more production.

CD40L knockout (Chapter 4) showed neutropenia in a conventional environment, but not in a germ-free one. This suggests that granulopoiesis can be increased by immune responses controlled by the CD40–CD40L interaction.

Background Gene Effects

The severity of phenotype is influenced by background genes in several cases. Balb/c mice die early from IL-2 knockout, C57BL/6 mice die late (Chapter 1). TGFβ1 knockout induces colonic inflammation in 129xC57BL/6 mice versus stomach inflammation in 129xCF-1 mice (Chapters 19 and 20). This indicates that other genes can ameliorate or exacerbate these immune and inflammatory effects and has implications for the many human diseases that cannot be reduced to one simple gene.

LIFR knockout mice die at birth on a C57BL/6 background, but on a 129 background, they die at various times in utero (Chapter 18). gp130 knockout mice show variations in hematopoiesis among individuals, possibly because of the mixed genetic background (Chapter 16). It is indeed remarkable that biological processes as fundamental as hematopoietic development could differ between strains of mice.

Lack of Redundancy

Because cytokines have so many similar activities, it has become widely assumed that they are redundant, the idea being that T-cell proliferation, for example, is so important that several cytokines are endowed with mitogenic activity. Hence knockout of just one cytokine, perhaps IL-2 or IL-4, would not eliminate T-cell proliferation. But the double knockout of IL-2 and IL-4 also did not eliminate T-cell proliferation. Instead it now appears that T-cells do not need cytokines to proliferate. However, this is not an example of redundancy.
If cytokines were redundant, then combining knockouts of redundant cytokines would produce more than their additive effects. There is a biological precedent for redundancy among the src-family kinases: knockout of src, fyn, or yes alone produce subtly varied phenotypes, but combined knockouts of src + fyn or src + yes are perinatally lethal and fyn + yes shows glomerulosclerosis (6). So far, cytokine knockouts have not shown such synergistic effects. TNF and lymphotoxin (Chapters 5–8) might have been expected to be redundant, since they can use the same receptor; but their combined knockout produces a phenotype that is the sum of the two individual knockouts. Likewise for the potential redundancy of IL-1α and β, knockout of IL-1β (Chapters 9 and 10), resulted in a more subtle phenotype than many expected, so it could be presumed that IL-1α would cover for the β deficiency; but knockout of IL-1RI, which serves both IL-1α and β, produced nearly the same phenotype as the IL-1β knockout. Moreover, IL-1β knockout did not elicit a compensatory rise in IL-1α production.

Of course this does not mean that redundancies will not be observed with combined knockouts in the future. But the lesson to date is that, despite their extensive overlap in activities, each cytokine has its own unique importance.

**Human Disease Models**

A number of cytokine knockouts mimic human diseases. Human hyper-IgM syndrome was known to be based CD40 deficiency, which was verified by knockout of CD40L (Chapter 4). Congenital neutropenia can result from mutations in GCSF receptor, as reflected in the GCSF knockout (Chapter 23). Human X-linked severe combined immunodeficiency results from mutations in the common γ chain of cytokine receptors and a similar phenotype is observed in γ chain knockout mice (Chapter 1).

Other knockouts produce a phenotype like that for the human disease, although they are probably not the target gene in humans. NF-IL6 knockouts exhibit Castleman’s disease, perhaps because of constitutively high levels of IL-6 (Chapter 17). GM-CSF knockouts exhibit alveolar proteinosis (Chapter 21). IL-2 knockouts exhibit colitis (Chapter 1).
Knockout of IL-6 implicates this cytokine in several pathogenic states (Chapters 14 and 15). The mice do not develop oil-induced plasmacytomas and are resistant to osteoporosis following ovariectomy. These findings hold great promise for the use of cytokine knockouts in general to identify their roles in pathological processes, and hence to indicate targets for therapeutic intervention.

Open Ends

Overall, the knockouts have had great impact on cytokine research because they not only answer important questions, but they raise many more. How does IL-2 program T-cells for death and does this relate to its growth effect? How can colitis appear in IL-2 deficiency, but not receptor deficiency? What are T-cells reacting to in the absences of TGFβ1—self or environment? Why is GM-CSF such a good stimulus of hematopoiesis—Is it really not used? How does LT control lymph node, Peyer’s patch, and spleen follicle formation; what cells make the LT that triggers these formations and what cells respond? How does IL-6 within the brain mediate the fever response? The IL-1 system is so elaborate, containing two cytokines, α and β, IL-1β-converting enzyme, two inhibitors, IL-1RA and IL-1RII, and is a target of poxvirus, yet the knockouts have subtle phenotypes in local inflammatory responses—is this the survival value of this complex system, one that has been preserved for one hundred million years of evolution? What does ICE control that mediates shock? How do CD40L and IL-8R regulate granulopoiesis? How do αβ intraepithelial lymphocytes develop normally in IL-7 knockout mice whereas all the other lymphocyte populations are blocked? What are the ligands for gp130 and LIFR that are essential for embryonic development, regulate glycogen storage, and the proliferation of hematopoietic stem cells and primordial germ cells? What genes are controlled by NF-IL6 that are required for killing listeria in phagosomes or killing tumor cells? How does IRF-1 control the numbers of CD8 T-cells? It is our hope that answers to these and many more questions regarding the physiological roles of the cytokines will be prompted by these studies.
In closing, we felt a need for a single volume that consolidated much of the cytokine knockout data. The chapters here treat some of the most important experiments conducted in cytokine research, and much is to be learned from reading, comparing, and reflecting on their results. Finally, many thanks to Paul Dolgert of Humana Press for facilitating the efforts that have led to this volume.

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Chapter 1

The IL-2 Deficiency Syndrome

A Lethal Disease Caused by Abnormal Lymphocyte Survival

Thomas Hünig and Anneliese Schimpl

Introduction

Interleukin-2 (IL-2), a cytokine with pleiotropic effects on T-, B- and NK-cells (1), binds to cell surface receptors consisting of at least three subunits (for review, see ref. 2): an IL-2 specific α-chain with low binding affinity and no signal-transducing capacity; a β-chain that is shared with the IL-15 receptor (3,4) and participates in signaling through its cytoplasmic tail; and the “common” γ-chain (γc) shared by receptors for IL-4, -7, -9, and -15 that also takes part in signal transduction (3,5–9, for a review see ref. 10). In line with the sharing of β- and γ-chains by IL-2 and IL-15 receptors, a number of overlapping biological activities have been reported for both cytokines such as the induction of T- and NK-cell proliferation and, at least in humans, the promotion of B-cell growth and Ig production (4,11,12). However, additional IL-2 or IL-15-specific biological responses can so far not be excluded, and the different sources of the two lymphokines, i.e., mainly activated T-cells for IL-2 versus a wide variety of non-lymphocytic cells for IL-15 (4) predict that in vivo, the two cytokines will play quite distinct roles in the regulation of lymphocyte activation. Therefore, it will be important to keep in mind the possible contributions of IL-15 to the development and functioning of the immune system in the IL-2 deficient mice.
that are the object of this chapter, as compared to mice lacking components of the IL-2 receptor system.

**Lymphopoiesis in IL-2 and IL-2R-Deficient Mice**

The three chains of the interleukin-2 receptor (IL-2R) are differentially expressed on developing lymphocytes: the common \( \gamma_c \) is already found on early developmental stages, where it forms part of the IL-7 receptor through which stromal-cell derived IL-7 promotes the expansion of B-cell precursors (13) and early thymocytes (14). The IL-2R\( \alpha \)-chain is transiently expressed on pro-B-cells (15) as well as on a subset of CD3\(^{-}\)CD4\(^{+}\)CD8\(^{-}\) immature thymocytes before their entry into the CD4\(^{+}\)CD8\(^{-}\) compartment (16–18). Finally, IL-2R\( \beta \) expression in primary lymphoid organs of mice is restricted to fetal and adult \( \gamma \delta \) thymocytes including the precursors of dendritic epidermal \( \gamma \delta \) T-cells (DETC) (19), and to a small subset of phenotypically mature \( \alpha \beta \) T-cells in the postnatal thymus (20) that apparently express autoreactive receptors (21). Mice with an inactivated \( \gamma_c \) gene display an early although incomplete block in mainstream T- and B-cell development (22,23), and a complete lack of \( \gamma \delta \) T-cells and NK cells (22). Although the more pronounced defect in lymphopoiesis observed in IL-7R defective mice (24) is beyond the scope of the present discussion, the importance of IL-7 and its receptor for early lymphoid development preclude an assessment of a missing IL-2 signal to this phenotype (that, however, could be responsible for the abnormal activation of those CD4 T-cells generated in the absence of a functional \( \gamma_c \) gene, see below).

In mice deficient for IL-2 (25) or for the \( \alpha \)- (26) or \( \beta \)-chains (27) of its receptor, mainstream development of both T- and B-cells is apparently normal, excluding an essential role for IL-2 or IL-15 in T- and B-cell lymphopoiesis. More detailed analyses regarding minor subsets of thymocytes and repertoire selection have so far been only reported for IL-2 deficient mice. These studies demonstrated a normal representation of \( \gamma \delta \) T-cells in the thymus (28) and of DETC in the skin (29), indicating that these subsets, too, do not depend on IL-2 for their development, but not excluding a requirement for IL-15. No difference between wild-type and IL-2 deficient mice was detected with regard to positive
and negative repertoire selection, using mice with major histocompatibility complex (MHC) I (30) and MHC II (Wolf, M., Schimpl, A., Hünig, T., unpublished) restricted transgenic T-cell receptor (TCR) as well as mtv-mediated deletion of thymocytes expressing particular TCR Vβ-segments (Krämer, S., Hünig, T., unpublished). Interestingly, the only abnormality in thymocyte composition of IL-2 deficient mice has, so far, been detected in a minute subset defined on the basis of IL-2R expression: among the 1% IL-2Rαβ+ TCRαβ+ thymocytes, the major CD4−8− subset was reduced whereas the frequency of CD4−8+ cells was increased threefold (21). If this effect is caused by the direct action of IL-2, a transient expression of the IL-2Rα-chain must be postulated in the generation of these cells, because in mice, IL-2R lacking this chain fail to bind IL-2 (31).

In peripheral lymphoid organs of IL-2 deficient mice, the representation of B-cells, T-cell subsets, and of TCR-NK1.1+(NK) cells is initially normal (32). In IL-2Rα (26) and IL-2Rβ (27) deficient mice, T- and B-cells also seed the periphery in normal numbers, confirming that IL-2 is not required for their development or export, and extending this finding to IL-15.

The IL-2 Deficiency Syndrome

**Immunopathology**

Contrary to all expectations, IL-2−/− mice were found not to suffer from immunodeficiency as a result of a failure to generate (25,30) or activate (33,34) T-lymphocytes but rather from a lethal inflammatory disease affecting multiple organs (35,36). The kinetics with which these pathological alterations develop, depend on the genetic background into which the IL-2 mutation was crossed, with most C57BL/6 mice remaining relatively normal for about a month (25,35), whereas about half of Balb/c mice do not survive past three weeks of age and the rest succumb to full blown immunopathology during the following few weeks (37).

The first abnormalities detectable are an increase in CD4 T-cells expressing activation markers such as high levels of CD44, and a relative reduction in CD4 cells of the naive phenotype (CD45RBlow, Mel-14high), followed by massive activation of B-cells and, to some extent, CD8 T-cells (32,35). At this stage, spleens and
lymph nodes are enlarged and hypercellular, and eventually lose their normal architecture. In the further course of the disease, some animals die from anemia as a result of autoantibody production and/or destruction of bone marrow cells by infiltrating T-cells (35–37). In parallel, massive invasion of the gut epithelium by CD4 and CD8αβ T-cells as well as dramatically enhanced local production of antibodies is observed, culminating in a severe colitis with lethal outcome (35). Although anemia and colitis appear to be the primary causes for death, the IL-2 deficiency syndrome is a generalized inflammatory disease with mononuclear infiltrations and lesions observed in pancreas, lungs, heart, blood vessels, and liver, in which a generalized amyloidosis was frequently observed, and the production of autoantibodies with multiple organ specificities (35,36). As already mentioned, the kinetics and frequencies with which these various manifestations of the IL-2 deficiency syndrome are observed depend on the genetic background: The most fulminating and rapid development of disease is observed in Balb/c mice, whereas C57BL/6 mice are slow progressors. Recently, Horak and coworkers (36) reported that disease manifestations are further modulated by the additional inactivation of the IL-4 gene: On the more resistant C57BL/6 background, such “double knockouts” (38) displayed reduced colitis but more pronounced inflammation of the other organs listed above.

Among mice targeted for the three chains of the IL-2R, those lacking the α-chain exhibit pathological alterations most similar to the IL-2 deficiency syndrome. Thus, the hallmarks of this disease, i.e. accumulation of activated lymphocytes, anemia and inflammatory bowel disease (IBD) have all been observed in these animals (26). The similar phenotype of IL-2−/− and IL-2Rα−/− mice fits well with the recent finding that the murine IL-2Rβγ fails to bind IL-2 (31), resulting in a loss of IL-2 responsiveness but a maintenance of IL-15 reactivity in IL-2Rα−/− mice. Hyperplasia of peripheral lymphoid organs, hyperactivation of CD4 T-cells and B-cells followed by B-cell destruction, loss of splenic architecture, anemia, and production of auto-antibodies have also been observed in IL-2Rβ−/− mice (27). However, IBD characterized by massive lymphocytic infiltration, which is a main cause of lethality in IL2−/− and IL-2Rα−/− lines, was absent from the II-2Rβ deficient animals although granulocyte infiltration of the intestine was observed (27). Given the
strong influences of genetic background and of antigenic load of the gut on the onset and severity of the IBD in IL-2−/− mice, it seems premature to conclude that the additional loss of IL-15 reactivity in IL-2Rβ−/− as compared to IL-2−/− and IL-2Rα−/− mice has an ameliorating effect on disease development.

In view of the strongly impaired T-cell development in γc-deficient mice (22,23), it is not surprising that the generalized inflammation that characterizes immunopathology of the other mutant strains is not observed in these animals. However, in spite of the absence of gut associated lymphoid tissue and intestinal intraepithelial lymphocytes, a different form of colitis with mononuclear infiltrates of the lamina propria has been reported (10,22), further adding to the heterogeneity of immunologic dysfunctions leading to IBD that had already emerged from the analysis of other cytokine- and TCR-deficient strains (39,40). Interestingly, γc−/− mice with age also accumulate activated CD4 T-cells in their spleens (22), supporting the concept of a role of the IL-2R in the containment of their clonal expansion (see Induction and Termination of CD4 T-Cell Responses).

Thymus and Antigen Dependence

Although the early appearance of activated CD4 T-cells in IL-2 deficient mice suggested that they are central to the development of the IL-2 deficiency syndrome, this has only recently been formally shown by the following series of experiments (Tables 1 and 2):

1. IL-2 deficient mice also lacking recombination activating gene (RAG)-2 are spared from immunopathology (41), whereas transfer of IL-2−/− bone marrow into these animals results in the development of disease (42), demonstrating that, as expected, the IL-2 deficiency syndrome is mediated by cells of the hematopoietic system.

2. Nu/nu mice lacking a functional IL-2 gene do not develop disease symptoms and have a normal B-cell compartment (42), indicating that thymus-derived T-cells are required for the initiation of disease and that the decline of B-cells is secondary to the action of thymus-derived T-cells.

3. IBD, but not hemolytic anemia, is observed in B-cell deficient IL-2-mutant mice, showing that an intact T-cell compartment suffices for inflammation and that autoantibody production by B-cells, “helped” by activated T-cells, is the primary cause of anemia (41).
Table 1
Effect of Genetic Background on the Manifestation of the IL-2 Deficiency Syndrome

| Genetic background of IL-2<sup>−</sup> mice | Contribution of genetic background | Effect on disease development |
|--------------------------------------------|-----------------------------------|------------------------------|
| C57BL/6                                   | Unknown                           | Slow progression            |
| C3H/J                                     | Unknown                           | Intermediate progression    |
| Balb/c                                    | Unknown                           | Rapid progression           |
| nnu/nu                                    | Athymic                           | No disease                  |
| RAG<sup>−</sup>                            | No T, no B-cells                  | No disease                  |
| JH<sup>−</sup>                             | No B-cells                        | IBD but no anemia           |
| IL-4<sup>−</sup>                           | No IL-4                           | Reduced IBD, delayed but more generalized organ manifestations |
| TCR-transgenic                            | Limited TCR repertoire            | Greatly delayed disease     |

*aFor details and references, see text.*

Table 2
Prevention and Control of the IL-2 Deficiency Syndrome

| Experiment                                  | Result                          | Conclusion                                           |
|---------------------------------------------|--------------------------------|------------------------------------------------------|
| Reduction of antigenic load                 | Delayed disease                 | Environmental antigens promote inflammation          |
| Anti-CD40L treatment                        | Delayed disease, no autoantibodies| CD4 activation is key event                          |
| Transfer-experiments:                       |                                |                                                      |
| IL-2<sup>−</sup> lymphocytes into nnu/nu    | Disease                        | No control by thymus-independent cells               |
| IL-2<sup>−</sup> BM into RAG-2<sup>−</sup>   | Disease                        | No control by non-B, non-T                             |
| IL-2<sup>−</sup> plus IL-2<sup>+</sup> BM into RAG-2<sup>−</sup> | Greatly reduced disease | Control by thymus-dependent cells                      |

*aFor details and references, see text.*

4. Treatment of IL-2 deficient Balb/c mice (the strain most strongly afflicted by the IL-2 deficiency syndrome) with antibodies to the CD40 ligand inhibited the activation of B-cells and CD4 (but not of CD8) T-cells, and delayed disease development, indicating that abnormal activation is initially restricted to CD4 cells that then
recruit the other lymphocyte subsets \( (37) \). In a similar approach, treatment of IL-2Rβ\(^{-/-}\) mice with CD4-specific depleting antibodies prevented development of immunopathology in these animals \( (27) \).

As a basis for unchecked CD4 T-cell hyperactivation, both a disturbance of lymphocyte homeostasis independent of antigenic stimulation and a defect in the control of clonal expansion in response to environmental and autoantigens had to be considered. The following observations have demonstrated that the latter mechanism is responsible for the development of immune pathology and have excluded alternative mechanisms (Tables 1 and 2).

1. Germ free mice do not develop IBD \( (35) \).
2. Introduction of a rearranged MHC II restricted T-cell receptor into IL-2 deficient Balb/c mice extends their life span from an average of 3 wk to over 6 mo (Wolf, M., Schimpl, A., Hünig, T., unpublished). In this situation, CD4 T-cells are generated and seed the periphery in normal numbers, but their transgenic receptor fails to see environmental or auto-antigens. Preliminary evidence suggests that full protection in this model is not achieved because T-cells utilizing endogenous TCRα chains eventually undergo unchecked activation after antigenic exposure.

In summary, these results indicate that the development of the IL-2 deficiency syndrome requires the presence of a diverse T-cell receptor repertoire on CD4 T-cells, and stimulation by self- and/or auto-antigens. Moreover, they document that disease is not consequent to an impaired immune response to pathogenic microorganisms, which would be aggravated in IL-2 deficient mice lacking a thymus or expressing a monoclonal T-cell repertoire. The delay in disease progression in germ free and specified pathogen free (SPF) mice suggest that autoreactive cells are recruited after a persistent inflammatory response to exogenous antigens provides costimulatory signals. Once activated, such autoreactive T-cell clones would then also display abnormal survival.

**Function of Lymphocyte Subsets in IL-2-Deficient Mice**

In view of the pleiotropic effects of IL-2 on T-, B- and NK-cell activation the function of these lymphocyte subsets was analyzed both in vivo and in vitro.
**Humoral Immune Responses**

Within the first several weeks when B-cell representation in the periphery is still normal, serum immunoglobulins are markedly elevated in IL-2 deficient mice. This is particularly noticeable with respect to IgG1 and IgE that were 20–100-fold increased. IgG2a, IgG2b and, with time, IgA were also higher than in age-matched controls, whereas IgM was largely unaffected (25,35). The isotype distribution at first suggested a skewing towards a TH2 response in the IL-2−/− mice. However, ex vivo analyses of cytokines by reverse transcriptase polymerase chain reaction (RT-PCR) showed an overall increase in the TH1 and TH2 cytokines tested (Berberich, I., Schimpl, A., unpublished), including γIFN, in keeping with high cytokine production by CD44hi Mel14lo cells (reviewed in ref. 43). However, the increase in IgG1 is unlikely to greatly contribute to disease since mice lacking both IL-2 and IL-4 did not overproduce IgG1, as expected, but still got severely ill (38). It is at present unclear whether this difference in isotype distribution is the basis for the altered disease manifestations observed in these double-deficient mice (36).

Increases in serum Ig levels, with a similar isotype distribution as found in IL-2−/− mice were also reported for mice with an inactivated IL-2Rα gene (26). Similarly, IL-2Rβ deficient mice exhibited 10–100-fold higher IgG1 and IgE levels in the serum, the other isotypes being unchanged (27). In mice with an inactivated γc gene serum IgM levels were normal (22) or about threefold reduced (23) inspite of dramatically diminished numbers of conventional B-cells. All other isotypes were diminished in γc mice >5 wk (22).

The humoral immune response to T-dependent antigens or after virus infection was also investigated in IL-2- and IL-2Rβ-deficient mice. Hapten-specific IgM and IgG responses to hapten-carrier conjugates were normal or even elevated in the absence of IL-2 (28). The mice could also mount a neutralizing anti-vesicular stomatitis virus (VSV) response that was of the same magnitude with respect to IgM; the IgG response was slightly lower and delayed (33). In contrast, no formation of neutralizing IgG or IgM antibodies to VSV were detected in IL-2Rβ deficient mice (27). One possible explanation for the discrepancy between the antiviral humoral response in IL-2- and IL-2Rβ-deficient mice could be co-utilization of the IL-2Rβ chain by IL-15 that has been shown, at
least in the human system, to influence B-cell growth and differ­
entiation (12).

**Cell-Mediated Immunity**

*In Vivo Activation of CD8 T-Cells: Different Answers From Different Systems*

To date, four different models of CD8 T-cell activation in vivo have been investigated: virus infection (33), transplant rejection and induction of alloreactive cytotoxic T-lymphocytes (CTL) to P815 tumor cells (44), immunization of mice with a transgenic TCR that recognizes an influenza nucleoprotein peptide presented by H-2-D\(^b\) (30) and activation of CD8 cells by superantigens (SAg) (34).

1. CD8 T-cell responses against vaccinia virus and lymphocytic chorio-
meningitis virus (LCMV). Kündig et al. (33) showed a close to normal primary in vivo CTL response of IL-2 deficient mice against vaccinia virus and an only slightly reduced anti-LCMV response. LCMV specific foot pad swelling early after infection—which in wild-type mice depends on activated CD8 cells—was also normal. The infected animals could clear the virus from spleen and liver. Based on their previous experience with normal mice the authors concluded that massive proliferation of virus-specific CD8 T-cells must have occurred and that both proliferation and functional maturation were independent of IL-2 in vivo. In IL-2RB-deficient mice, neither early (CD8 dependent) nor late (CD4 dependent) foot pad swelling was observed (27), again confirming the more severe phe­
notype of IL-2RB\(^{-/-}\) mice in which IL-15 cannot replace IL-2.

2. Skin allografts transplanted onto IL-2-deficient mice were rejected although with a delay of about 10 d (44). Alloreactive CTL were also induced by the injection of P815 tumor cells into C57BL/6 IL-2\(^{-/-}\), although the titration of CTL activity showed a >90% reduction as compared to IL-2 wild-type mice (44). Thus there seems to be a dif­
ference between the mild effects of IL-2 deficiency on the genera­
tion of CTL after vaccinia virus and LCMV infection and the very distinct effect when tumor cells were used as antigens. This discrep­
ancy could be caused by differences in the disease states between the animals analyzed. A more interesting possibility would be that the local and/or systemic cytokine milieus established after virus infec­
tion versus immunization with alloantigens are distinct.

3. Positive and negative selection of CD8 T-cells in mice expressing the D\(^b\) + Influenza nucleoprotein (NP)-specific F5 TCR proceeded
normally (30, see Lymphopoiesis in IL-2 and IL-2R-Deficient Mice). Proliferation of CD8 cells in IL-2−/− mice was also unimpaired after short time peptide injection as shown by BrdU incorporation. CTL activity, however, was only observed ex vivo in TCR transgenic mice which could produce IL-2. This result obtained with a viral peptide is in marked contrast to normal virus specific CTL responses described above.

4. Injection of the bacterial superantigens (SAg) staphylococcal enterotoxin A (SEA) or staphylococcal enterotoxin B (SEB) induced short time expansion of SAg reactive CD8 and CD4 cells carrying the respective VB segments in IL-2−/− mice (34). No increase in cytotoxic activity following SAg injection could be demonstrated (Herrmann, T., Kneitz, B., Schimpl, A., unpublished), contrary to results obtained for CD8 cells induced by SAg in IL-2-producing mice (45). Subsequent to the near normal expansion observed even in the absence of IL-2 there was a drastic loss of CD8 SAg reactive cells from the peripheral lymphoid organs and a concomittant increase in the liver (34). CD8 cells are greatly overrepresented in livers of untreated IL-2−/− mice (42). Possibly, this is due to CD8 cells that migrate to the liver after activation but are not eliminated there as quickly as in normal mice (46).

Comparison of the various systems used to activate CD8 T-cells in vivo show that proliferation seems to be independent of IL-2. How many rounds of division the cells can undergo is, however, unclear, since the prolonged life span of T-cells activated in the absence of IL-2 makes it difficult to assess the number of divisions necessary to reach a particular population size. CTL activity itself is compromised to some degree in all situations. The mildest effect is observed following virus infection and skin grafting across a full H-2 difference. In both cases a local inflammatory response may lead to the local production of IL-15 and induce additional cytokines in concomittantly activated CD4 cells or other cell types. Induction of CTL following injection of P815 or peptide injection into TCR transgenic mice seem to be most severely affected by the absence of IL-2. In both cases CD4 cells will not be directly addressed and the alternate cytokines may become limiting.

In Vitro Requirement for IL-2 in CD8 Responses

The initial description of IL-2 as an important in vitro growth and differentiation factor for CD8 T-cells was fully born out by
experiments using cells from IL-2−/− mice. Thus, a strict dependence on exogenous IL-2 was observed for the induction of CTL activity in vitro both in secondary antiviral responses (33) and the generation of peptide-specific CTL from F5 TCR transgenic mice (30). However, transient DNA synthesis was induced in TCR transgenic IL-2−/− T-cells stimulated with the antigenic peptide pointing again at a more stringent requirement for IL-2 in the induction of cytolytic activity as compared to proliferation in this peptide driven system. CTL generation after polyclonal activation in vitro with anti-CD3 or alloantigen was also defective in the absence of IL-2 (47).

**Natural Killer Cells**

As mentioned, the spleens of young (3–4 wk old) IL-2-deficient mice contain normal numbers of TCR-NK1.1+(NK) cells. Whereas in such young animals, NK activity even of wild-type mice is very low, it becomes detectable after injection of poly I:C (29) or after viral infection (33). In both systems, IL-2-deficient mice do respond, although NK activity is generally lower and more variable than in wild-type mice.

**Induction and Termination of CD4 T-Cell Responses**

The first phenotypic change detectable in IL-2 deficient mice is an increase in CD44hi Mel-14lo cells (32), particularly within the CD4 subpopulation. A higher than normal proportion of T-cells also expresses CD69 (35), an early activation marker and incorporates BrdU (38). With the exception of IL-5, most cytokines tested (IL-4, IL-6, IL-10, γIFN) are overproduced. Together, these phenotypic changes are indicative of uncontained in vivo activation rather than a defect in the initial steps of CD4 T-cell responses. Consistent with this, in vivo priming for in vitro proliferation with protein antigens such as KLH is near normal and T-helper function for B-cell isotype switching is elevated.

When unseparated lymph node cells from IL-2 deficient mice were stimulated in vitro with ConA (25), anti-CD3 (28), or allogeneic spleen cells, proliferation was reduced compared to control cells, but usually only by a factor of 2–3. There were two notable features of such in vitro cultures: cells from IL-2−/− mice retained higher viability with time, even though incorpo-
rating less thymidine; and they invariably became enriched for CD4 cells.

The combined data clearly show that CD4 cells can be activated both in vivo and in vitro in the absence of IL-2, indicating that lymphokines other than IL-2 or cytokine-independent signals mediated through cell interaction molecules can give the second signal required for cell cycle progression. CD28 is a likely candidate to take over that function both in vivo and in vitro. This is based on the following observations:

1. CD4 cells isolated from IL-2−/− mice respond to plate-bound anti-CD3 in the presence of anti-CD28 with short-term proliferation (32,35). This reaction is also independent of IL-4 since CD4 cells from IL-2/IL-4 “double knockout” mice also respond. Long-term thymidine incorporation (>4–5 d) in vitro, is, however, IL-2 dependent (unpublished).

2. Thymocytes from γc−/− mice respond to anti-CD3 plus anti-CD28 stimulation (22), further illustrating that costimulation through CD28 does not necessarily act via an autocrine IL-2 or IL-4 loop.

**In Vivo and In Vitro Responses to Superantigens**

One of the best suited systems to follow expansion and contraction of defined cell populations is the response of T-cells using particular VB segments reactive to SAg. The in vivo reaction is characterized by an early deletion of a fraction of SAg reactive cells within the first 10–20 h, followed by expansion of SAg reactive clones over 2–3 d and deletion and/or anergy thereafter (48,49). C57BL/6 and C3H IL-2−/− mice injected with SEA or SEB, respectively, showed both the early deletion and subsequent expansion phase in CD4 and CD8 cells (34). The late deletion of CD4 cells was, however, diminished in IL-2−/− mice. In addition, cells taken from the lymph nodes of IL-2−/− mice at the late phases of the in vivo response and restimulated in vitro with the homologous superantigen gave proliferative responses equal or superior to those found with cells from unimmunized mice, whereas in IL-2 producing animals, SAg reactive T-cells had become anergic (or died) after restimulation in vitro. Analogous results were obtained when both primary and secondary stimulation were carried out in vitro (34). In IL-2Rα−/− mice, late deletion of SEB reactive VB8+ cells was also substantially impaired (26).
Defective CD95-Mediated Cell Death: 
Basis of Uncontained Activation?

Defective deletion of SAg expanded CD4 cells and accumulation of T-cells with an activated phenotype in untreated IL-2−/− mice suggested a defect in peripheral T-cell death following activation. One of the mechanisms of clonal contraction of CD4 cells involves CD95 and its ligand, both of which are expressed on activated T-cells (for review, see ref. 50). This is illustrated by MRL-lpr/lpr mice which have a defective CD95 gene, accumulate activated T-cells and are prone to autoantibody formation (for review, see ref. 51). In analogy, it seemed conceivable that activation in the absence of IL-2 either leads to a defect in CD95 expression, a defect in CD95L expression or defective execution of the death program.

Cell surface staining of SAg or anti-CD3 activated CD4 cells from IL-2−/− mice clearly showed that CD95 was expressed in vivo and in vitro even when activation proceeded in the absence of IL-2 (34). CD95 was also observed on the surface of cells from IL-2Rα−/− mice (26). Defective deletion of activated CD4 cells from IL-2−/− mice is also not owing to a lack of CD95L expression, since the frequency of CD95L expressing T-cell blasts even exceeds that obtained with wild-type cells. However, when CD95 expressing CD4 T-cell blasts from IL-2−/− mice were cultured on plate-bound anti-CD95 antibodies, cells failed to die whereas T-cell blasts from control animals readily underwent apoptosis. When IL-2 was present during blast generation in vitro, many though not all cells from IL-2 deficient mice again became sensitive to CD95 mediated cell death.

The data so far indicate that the intracellular programs leading to surface expression of CD95 and CD95 ligand are intact in T-cell blasts from IL-2 deficient mice, but that the execution of the death program is defective. Several groups have previously shown that CD95 ligation at early times during T-cell activation does not lead to cell death, presumably because the necessary machinery to transmit the apoptotic signal is not yet in place. This machinery involves membrane proximal components such as ceramide and membrane distal proteins such as the ICE- and the bcl-2 families, individual members of which have been shown to either protect from or enhance apoptosis (for review, see ref. 52). It remains to be shown which of these molecules in the CD95 pathway are affected when T-cells are activated in the absence of IL-2.
Prevention of the IL-2 Deficiency Syndrome: A Model to Study T-Cell Homeostasis

As summarized in sections on Thymus and Antigen Dependence and Induction and Termination of CD4 T-Cell Responses, the pathology of IL-2-deficient mice is the result of defective “clonal contraction” after antigen-driven expansion of CD4 T-cells, and this abnormal persistence of activated CD4 cells may at least in part be explained by a failure to undergo CD95-triggered apoptosis. An important question arising from these observations is whether clonal contraction is normally mediated via a direct effect of auto- or paracrine IL-2 which sets up the apoptotic response to CD95 stimulation, or whether additional regulatory cells that depend on IL-2 for their generation or functional maturation are involved.

In order to establish whether the CD4 T-cells themselves have to be able to produce IL-2 in order to contain their clonal expansion, two types of experiments have been performed so far (Table 2):

1. Peripheral lymphocytes from apparently healthy young IL-2−/− mice were transferred into IL-2+ athymic recipients that are devoid of thymus-derived T-cells but do contain thymus-independent T-cell populations. The development of several manifestations of the IL-2 deficiency syndrome in these animals indicated that extrathymic IL-2+ T-cells and other thymus-independent populations of hematopoietic origin such as NK cells are unable to control accumulation of activated IL-2−/− CD4 T-cells (42).

2. In contrast, cotransfer of bone marrow cells from IL-2−/− and IL-2+ donors into RAG-2−/− mice and analysis of their Ly5-marked progeny showed that in the presence of IL-2+ thymus-derived T-cells, IL-2−/− CD4 cells behave normally. Thus, there is no requirement for autocrine IL-2 in maintaining CD4 T-cell homeostasis (42).

From these analyses of mixed chimeras with a diverse TCR repertoire and multiple T-cell subsets, it can, however, not be decided whether the control of immune pathology was mediated by paracrine IL-2 derived from the wild-type CD4 cells or by IL-2 dependent regulatory cells. If, as observed in vitro, exogenous IL-2 restores sensitivity to CD95-triggered apoptosis also in vivo, paracrine delivery of IL-2 would be the most likely mechanism. In this case, we would predict that IL-2-producing CD4 T-cells specific for the antigen also recognized by the IL-2-deficient CD4
T-cells would be most efficient in delivering IL-2 and protecting from immunopathology since they may localize in the same cluster around an antigen-presenting cell. On the other hand, a role of CD8 T-cells in downregulating clonal expansion of CD4 cells in vivo has been described in the response to bacterial superantigens (53). Since at least in the response to peptide antigens, induction of CD8+ CTL is defective in IL-2−/− mice (see In Vivo Activation of CD8 T-Cells), the protective effect of IL-2+ lymphocytes in mixed chimeras containing IL-2+ and IL-2−/− T-cells could also be mediated by CD8 T-cells. These possibilities are currently addressed in adoptive transfer experiments using TCR-transgenic IL-2−/− CD4 T-cells and populations of candidate regulatory T-cells.

On a final note, the regulatory mechanisms operative in the artificially constructed chimeras containing both IL-2+ and IL-2− T-cells may also be of relevance to the maintenance of T-cell homeostasis in normal animals, where CD4 T-cells, e.g., by differentiating into the TH2 phenotype no longer express IL-2 and may be controlled by IL-2-producing TH1 cells via paracrine delivery of IL-2 or by promoting the maturation of counterregulatory CD8 T-cells.

In summary, the abnormal survival of activated CD4 T-cells that is at the center of the IL-2 deficiency syndrome, confirms that costimulatory signals leading to T-cell activation are redundant, whereas IL-2 has a quite specific and unexpected function in the downregulation of immune responses and in maintaining T-cell homeostasis.

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Chapter 2

IL-7 Knockout Mice
and the Generation of Lymphocytes

Ursula von Freeden-Jeffry, Thomas A. Moore, Albert Zlotnik, and Richard Murray

Introduction

A common theme in the molecular interaction between cytokines and their receptors is that an individual cytokine binds a receptor complex. In general, this requires a cytokine-specific receptor subunit and a “common” receptor subunit. The common subunit is, by definition, shared among numerous cytokines. The result is a grouping of cytokines into families that all require a particular common subunit for high-affinity binding and signal transduction. This is well exemplified by the cytokine grouping of interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15 (1–7). Each of these cytokines binds, at minimum, a cytokine-specific subunit as well as a common subunit, referred to in this chapter as the common gamma (γc) chain. Subsequent to the binding of a cytokine to its corresponding receptor complex, signal transduction events, including the Jak/STAT pathway, deliver a signal for a biological response of the cell. Since IL-7 has been extensively reviewed elsewhere (8,9), this chapter is not an exhaustive description of IL-7 from cloning to present, focusing instead on IL-7 related biology learned from the gene knockout model and how these observations
integrate into other gene knockout studies of cytokines and receptors defined by the \( \gamma c \) chain receptor. We summarize published work as well as refer to and provide brief descriptions of current experiments.

The cloning of IL-7 (10), and production of recombinant protein, revealed a molecule capable of potent stimulation of lymphocytes in culture. In particular, many biological activities were attributed to IL-7 in the stimulation of early lymphocytes (11–14). One notable feature was the ability of recombinant IL-7 (rIL-7) to maintain the viability of early thymocytes in culture (15–17). Thymocytes maintained in IL-7 were capable of repopulation when introduced back into lymphocyte depleted fetal thymic organ culture. Another unique feature of this molecule was that purified protein alone, with no additional costimulation, mediated these biological events. Thus, the results from many independent analyses using rIL-7 showed activities that were similar to other cytokines in a general sense, but also exhibited unique and intriguing features.

A number of standard approaches have been used to evaluate the role and activity of IL-7 in vivo. These include use of monoclonal antibody (MAb) reagents to block the biological activity of IL-7 (18), overproduction of IL-7 in transgenic mice (19–21), and the production of a null mutation in the IL-7 gene, by gene targeting techniques (22). As mentioned, this chapter focuses mainly on gene knockout work and comparison of gene knockout studies centered on the biology of the cytokines and receptors in the \( \gamma c \) system.

The IL-7 gene was deleted from the mouse germline by standard techniques, and the resulting mutant mice maintained in a pathogen-free animal facility. The mice were healthy and fertile under these conditions. Analysis of the blood gave the first indication of severe lymphopenia in the mutant mice. Animals from different age groups ranging from 5–14 wk had an average reduction from the 70% lymphocytes in normal controls to approx 10% lymphocytes in the mutant animals. The cellularity of the spleen was reduced approx 10-fold. Even more striking was the approx 20-fold reduction of cellularity in the thymus. The cellularity of the bone marrow (BM) was unchanged. More specific analysis of the B- and T-cell phenotype of these animals are described in the following sections.
Phenotypic Analysis of Lymphoid Cells in IL-7−/− Mice

**B-Cell Development**

To evaluate if B-cell ontogeny was blocked at a particular stage of development, we used the characterization scheme of Hardy et al. (23). BM cells were stained with MAbs in a four-color fluorescence-activated cell sorter (FACS) analysis that allowed the discrimination of particular B-cell subsets. IL-7−/− mice lacked most of the B220+/IgM+ subset in the BM, the mature subset of B-cells. However, IL-7−/− mice retained a significant proportion B220+/IgM− cells, a subset of B-cells that contains more immature cell types. These B220+/IgM− cells were then simultaneously evaluated for S7 (CD 43) and HSA (CD 24) expression. This scheme allowed identification of fraction A cells (S7+/HSA−), fraction B-C (S7+/HSA+), and fraction D (S7−/HSA+). IL-7−/− mice showed a clear block at the transition to population D. This represents a decrease in pre-B-cells. It should be pointed out that our FACS staining strategy did not allow for the separation of fraction B from fraction C.

B-cells from the spleen were reduced in number, but some of the remaining cells appeared normal by B220 and IgM staining. An abnormal population of B220+/IgM− cells was also detected. This population may be indicative of premature migration of cells from the BM, or compensatory lymphopoiesis in the spleen of the adult animal. Curiously, CD5+ B-cells from the peritoneal cavity are present in normal to slightly expanded numbers, although the conventional B-cell population (CD5− IgM+) was reduced similar to the periphery (U. von Freeden-Jeffry et al., unpublished observations).

**T-Cell Development**

IL-7−/− mice displayed an equally lymphopenic phenotype in their T-cell compartment. Thymus cellularities were reduced 20-fold whereas splenic T-cells displayed a 10-fold reduction. Interestingly, the remaining T-cells retained a normal distribution of CD3, CD4, and CD8 molecules. As described in the preceding section, BM B-cell lymphopoiesis is blocked during the transition of the pro- to pre-B cell stage. When a similar detailed phenotypic analysis of early thymic T-cell differentiation was performed, no obvious block in maturation was observed, although this is still an
active area of pursuit. This lack of a defined block in thymic differ­
entiation suggests three possible scenarios:

1. First, IL-7−/− mice may be deficient in BM progenitors which contin­
ually enter the blood stream and seed the thymus.
2. These primitive blood-borne, BM-derived progenitor cells may exist in normal frequencies but may be unable to efficiently home and/or seed the thymic microenvironment.
3. Thymic maturation may be less critically dependent on IL-7 for differ­
entiation owing to as of yet undefined overlapping compensa­tory cytokines, whereas expansion and/or maintenance of thymic subsets is more critically dependent on the activities of IL-7. These other signals may be known cytokines, such as thymic stromal­
derived lymphopoietin (TSLP) as discussed in sections to follow, or may be the result of uncharacterized signals in early lymphocyte development.

As mentioned, thymic populations, as defined by CD3, CD4, and CD8, were found at normal frequencies, but severely reduced in number, in these mice. The one notable exception to this was the extreme paucity of γδ-T-cell receptor (TCR) T-cells. The majority of thymic γδ-T-cells are CD4−CD8− (DN), comprising 10–15% of CD4−8− double negative (DN) cells. IL-7−/− DN thymocytes contained ~1% γδ-T-cells and these few remaining cells expressed lower levels of γδ-TCR, suggesting an immature phenotype. To determine if the lack of γδ-T-cells extended beyond the thymus in these mice, we investigated whether γδ-T cells were present in the intestinal lining of IL-7−/− mice.

T-cells can be found along the mucosa of the small and large intestine; the best characterized of these cells are those found between the epithelial cell lining of the small intestine, termed intraepithelial lymphocytes (IEL) (for review see refs. 24–26). More than of 50% of IEL cells can be γδ-T cells, depending on the strain of mouse examined. The vast majority of these γδ-T cells express the CD8αα homodimer and lack CD5 and Thy-1 expression. These phenotypic characteristics have suggested an extrathymic maturational origin for these cells. Although this is a topic beyond the scope of this chapter, there is evidence that the intestinal epithelium can support T-cell development independent of any thymic influences (27). The remaining IEL T-cells are αβ-T-cells, either conventional CD8αβ heterodimer or of CD8αα phenotype and variable levels of CD4. As with γδ-T-cells, evidence
suggests both thymic and extrathymic origins for the IEL αβ-T-cells. IL-7−/− IEL were examined for γδ-T- and αβ-T cells, and a striking difference was seen when compared to +/+ controls. Although IL-7−/− mice contained normal-to-slightly increased percentages of αβ-T-cells, they completely lacked γδ-T-cells (27a). The normal or increased numbers of IEL αβ-T-cells indicate that IL-7 does not play a crucial role in TCR rearrangement as suggested (28), at least in this particular population of T-cells.

The data discussed to date clearly indicate a critical role for IL-7 in the generation of γδ-T-cells. In order to extend these findings, we examined the dependence on IL-7 for the generation of the first wave of γδ-T-cells seen during early murine ontogeny. In fetal thymic development, the first wave of T-cells produced occurs at d 15 of gestation. These early γδ-T-cells express an invariant TCR complex of Vγ3 and Vd1 (for review, see ref. 29). Detailed phenotypic analysis of the maturation of these Vγ3 T-cells has been recently elucidated (30). Briefly, Vγ3 expression is low while CD24 expression is high. As differentiation occurs during fetal ontogeny, Vγ3 levels are upregulated with a concomitant decrease in CD24, resulting in mature Vγ3hi CD24lo T-cells. These mature cells then seed the skin epithelium, generating dendritic epidermal T-cells. To determine if Vγ3 fetal T-cells were as dependent on IL-7 as adult thymic and intestinal T-cells, we examined d 17 and d 18 fetal thymocytes from IL-7−/− and IL-7+/+ control mice for their expression of Vγ3 and CD24. Both gestational ages in +/+ mice contained distinct populations of immature Vγ3lo CD24hi and mature Vγ3hi CD24lo T-cells. In sharp contrast, IL-7−/− mice displayed a strong reduction in Vγ3hi CD24lo mature T-cells while still containing normal to slightly decreased frequencies of immature Vγ3lo CD24hi cells (27a). These data show that IL-7 plays a critical role in the survival and/or expansion of immature γδ-T-cells, but may be less mandatory for the initial steps of TCRγ chain rearrangement, as has been suggested (31,32). However IL-7 receptor deficient mice display an even more pronounced difference in TCRγ chain rearrangement (32a, see γc Receptor-Independent Stimuli).

Collectively, the data on the remaining populations of cells in IL-7−/− mice show a profound reduction in numbers of certain lymphocyte subsets. It is curious that certain branches of lymphoid development, such as IEL CD8 αβ T-cells and CD5+ B-cells in the peritoneal cavity, are present in normal numbers. This implies a
strong degree of selectivity in the types of lymphoid expansion dependent on IL-7. It will be of interest to evaluate if the unaffected lymphoid populations represent cells that define a separate branch of cells based on growth requirements, as well as what signals are important for these cells in contrast to the lymphoid populations that are heavily dependent on IL-7.

**Functional Analysis of Remaining Lymphocyte Populations in IL-7−/− Mice**

IL-7 was cloned, and has been described, as a stromal product (10). The inability of stimulated leukocytes to produce IL-7 implies that this mutation should be a defect in the lymphoid microenvironment. This is currently being evaluated by BM transfer from mutant-to-normal and from normal-to-mutant animals. Nevertheless, we would not expect a cell autonomous defect in the lymphocytes themselves. Our initial analysis of the remaining lymphocytes in IL-7−/− mice showed this to be true, at least in a general sense. Thymocytes and splenocytes were stimulated by polyclonal activating agents, LPS for B-cells, and ConA for T-cells or thymocytes. On a per cell basis, the remaining T- and B-cells from IL-7−/− mice were as responsive as cells from control animals. However, it still remains to be determined if the cells from IL-7−/− mice function normally under situations of more realistic immunological stimuli. These types of experiments, such as with administration of infectious agents, are currently underway.

We have investigated the levels of serum immunoglobulin from adult IL-7−/− mice. The remaining B-cells are capable of producing normal serum levels of IgM, IgG1, IgG2a, IgG2b and IgE (unpublished observations).

**Comparison of IL-7, γc, and IL-7 Receptor (IL-7R) Knockout Mice**

Figure 1 shows a schematic representation of the IL-2, IL-4, IL-7, IL-9, and IL-15 system of individual cytokines binding to the γc receptor chain. Each cytokine requires additional component(s) to confer specificity. Gene knockout mice have been produced for a number of the molecules depicted. We compare some of these mutant animals to understand a more global picture of the
molecules involved in lymphocyte stimulation. Gene targeting experiments in 1991 indicated that IL-2 and IL-4 (33,34) did not play crucial roles in the development of lymphocytes. Although these animals displayed other phenotypes associated with the immune system, IL-2 and IL-4 were clearly not involved in the production of lymphocytes. In addition, IL-2 mutant mice were crossed with IL-4 mutant mice, again without lymphoid developmental abnormalities (35).

A fundamental discovery was made when the γc receptor chain was shown to be the gene mutated in human X-linked severe combined immunodeficiency disease (SCID) (36). Chemical crosslinking experiments performed with numerous cytokines revealed that the γc was a functional part of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptor complex (2–7). The implication from this work was, since numerous cytokine molecules interacted with this receptor, the collective elimination of these cytokines had profound effects on human lymphocyte development and function.
It is reasonable to mention that many cytokine gene knockout studies produced phenotypic changes in animals that were much more subtle than originally expected. Thus, a picture was beginning to emerge where “redundancy in function” was part of most discussions involving the role of cytokines. As the \( \gamma_c \) mutation is a collective elimination of cytokines, perhaps any of the individual cytokines that bind with the \( \gamma_c \) chain could be dispensed with and cause no lymphoid developmental abnormalities. However, as we have summarized, the production of IL-7 knockout mice showed a phenotype in lymphoid development very analogous to the early lymphoid abnormalities to the \( \gamma_c \) receptor gene knockout studies (37,38). Thus IL-7 was the crucial signal in early T- and B-cell ontogeny that functions through the \( \gamma_c \) receptor. It is also useful here to compare the gene knockout mice of IL-7 R, the \( \alpha \) subunit that specifies binding of IL-7 to the \( \gamma_c \) chain (39).

Table 1 (pp. 30–31) shows a comparison of the IL-7, \( \gamma_c \), and IL-7R mutant mice. In general, all three knockout mice showed similar phenotypes, although there are some important differences. First, it is apparent that the IL-7 ligand is playing a dominant role in the stimulation and expansion of lymphocytes, since the phenotype shows striking similarities to the \( \gamma_c \) receptor mutation. The IL-7 and \( \gamma_c \) mutant mice showed a block in B-cell lymphopoiesis at the pro-B-cell stage, whereas the IL-7R mutant animals showed a block at an earlier stage of development, the pre-pro B-cell stage. This difference implies a role for another developmentally important molecule that functions through the IL-7R but not the \( \gamma_c \) receptor. In the spleen of the IL-7 mutant mice, a small population of B220+ IgM− B-cells were detected, but these cells were apparently not detected in either of the other two mutant mice. The significance of this is unclear. One striking difference between the IL-7 mutant and \( \gamma_c \) mutant mice was in the natural killer (NK) lineage. IL-7/− animals had normal populations of NK cells (27a) whereas the \( \gamma_c \) mutant mice did not have detectable NK cells, measured by phenotype and by function. The description of the thymic abnormalities were almost identical between IL-7/− and \( \gamma_c \) chain mutant mice. However, the IL-7R/− mice were reported to have a split phenotype, one identical to the other two mice, and another that resulted in a more severe reduction of cellularity (>99% and a block at the pro-T-cell stage).
These results are difficult to interpret in that the same genotype presents two distinct phenotypes, but again this may imply the role of another cytokine interacting through the IL-7R but not the γc receptor. IELs showed a very similar pattern in the IL-7 and γc mutant mice. Both IL-7 and γc mutations did not affect the development of CD5+ B-cells.

Functional responses of the remaining lymphocytes show a predictable difference between IL-7 and γc mutant animals. Stimulation of IL-7/− cells with mitogens showed a normal response on a per cell basis. In contrast, mitogen responses from γc mutant cells were decreased or absent, a finding attributable to the presence of a cell-autonomous mutation on these lymphocytes.

**Summary and Discussion**

**Receptor Expression Versus Signal Transduction**

It is interesting to speculate on whether the unique role of IL-7 in early development is mediated by specificity of receptor expression. This would explain the reason for the importance of the IL-7 signal as simply the result of the expression of IL-7R on the early lymphoid populations (40).

Alternatively, the mechanism of signal transduction into the lymphocytes may contribute to the importance of the IL-7 stimulus. This would imply that the molecules downstream of receptor ligand engagement would be able to discriminate an IL-7 versus, for example, an IL-2 signal. This seems unlikely given our current understanding of how signal transduction mechanisms operate. Additionally it is now known that Jak1, Jak3, and STAT 5 (41,42) seem to be used similarly for IL-2, IL-7, and IL-15. However, specificity dictated by other molecules in the signal transduction cascade cannot be ruled out.

**NK Cell Development**

As mentioned, an interesting developmental difference between the γc and the IL-7 knockout mice exists in the NK cell lineage. A potential candidate for this difference may be IL-15, as this cytokine should not be able to stimulate cells from the γc mutant mice but would be predicted to stimulate cells from IL-7/− mice. IL-15 has been shown
| Cells            | IL-7                                      | γc                                        | IL-7R                                    |
|------------------|-------------------------------------------|-------------------------------------------|------------------------------------------|
| Bone marrow      | Block in pro-B to pre-B lymphopoiesis (fr. B/C to D) | Block in pro-B to pre-B lymphopoiesis (fr. B/C to D) | Block in pre-pro-B to pro-B lymphopoiesis (fr. A to B/C) |
| Spleen-cellularity | 90% reduction                             | 90% reduction                             | 90% reduction                            |
| B-cells          | Mature (B220+ IgM+) B-cells present, increase in % of immature (B220+ IgM-) B-cells | Mature (B220+ IgM+) B-cells present, no increase in % of immature (B220+ IgM-) B-cells | ND                                       |
| Thymus-cellularity | Mature T-cells present, increase in CD4+8⁻:CD4⁻8⁺ ratio | Mature T-cells present, increase in CD4+8⁻:CD4⁻8⁺ ratio | Mature T-cells present                    |
| CD4/8            | Normal ratios, slight increase in CD4+8⁻ | Normal ratios, slight increase in CD4+8⁻ | 95 to >99% reduction                     |
| Lymphocyte function | IL-7 mutant mice | yc mutant mice | IL-7R mutant mice |
|---------------------|------------------|----------------|------------------|
| Thymocytes          | Normal ConA response | No ConA response | ND               |
| Splenic T-cells     | Normal ConA response | No ConA response | ND               |
| Splenic B-cells     | Normal LPS response | Decreased LPS response | ND               |

*Comparative summary of the phenotype of IL-7, yc, and IL-7R mutant mice. Summarized information for IL-7 mutant mice includes published work (22,27a), as well as experiments in recently submitted manuscripts or from manuscripts in preparation. Summarized information for yc mutant mice is combined from two independent reports (37,38), and information from IL-7R mutant mice was summarized from published work (32a,39,45).*
to have stimulatory activity for NK cells (43). It is unclear at the moment if this stimulatory activity will be of physiological significance to NK cells in vivo. It will also be of interest to investigate potential infectious disease susceptibility differences between IL-7 and \( \gamma c \) mutant mice that might be attributable to the loss of the NK lineage.

**Stromal Cytokines Versus Immune Stimulation Cytokines**

An interesting comparison among the cytokines that interact with the \( \gamma c \) chain is to consider the source of cytokine production and how that may relate to mutant phenotypes in vivo. Like IL-7, IL-15 has been shown to be produced by stromal cells (44). Although gene knockouts for IL-15 have not been reported at the time of this chapter, it is tempting to speculate that molecules produced by stromal cells may play a more developmental role in lymphocyte biology. This would be consistent with the idea presented above for a role for IL-15 in NK development. In mechanistic terms, a low but continual production of a molecule in a particular microenvironment is consistent with the ongoing developmental process of lymphopoiesis. In contrast, molecules that are produced by active immune stimulation may be more important in the functional operation of the immune system (i.e., when cells have already fully matured and need to respond). IL-2 and IL-4 would fit more into this profile. The production of these molecules would more likely impact on the need for a particular response after an insult to the immune system, as opposed to a continual need for these molecules during an ongoing developmental process. The phenotypes of the IL-2 and IL-4 knockout mice would fit with this concept.

**\( \gamma c \) Receptor-Independent Stimuli**

As the IL-7 and \( \gamma c \) mutant mice still have residual lymphocyte development, it is of interest to consider what other stimuli or receptors may be responsible for these cells. An interesting point raised by Peschon et al. (39), is that TSLP may be responsible for part of the more severe lymphopenic phenotype in IL-7R\(^{-} \) mice which include more pronounced effects on B- and T-cells, including TCR\( \gamma \) chain rearrangement (see Table 1 and refs. 27a and 32a). This means an IL-7R/ligand interaction independent of the \( \gamma c \) receptor. Of course other molecules may also be involved. The remaining lymphocytes in IL-7\(^{-} \) mice appear to be more of a “leak” of typical populations, rather
than the result an inappropriate selective process or unusual subset of cells, as no autoimmunity or pathology (other than lymphopenia) is present in these animals to date (unpublished observations).

**Linking Cytokine Stimulation to New Patterns of Gene Expression**

The continuing approach of genetic analysis of cellular stimulation in the immune system will likely shed new light on how cytokine engagement with their appropriate receptors transmit signals, resulting in new gene expression and increased or decreased levels of activation of cells. The production of knockout mice for signal transduction molecules and transcription factors that appear to be downstream of cytokine receptors will provide interesting comparisons in the understanding of specificity or redundancy in the mechanisms of cell activation. Thus a more complete picture of cellular activation in the immune system, in realistic physiological settings, is likely to emerge from the comparative analysis of in vivo gene mutations.

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Introduction

The stages of B- and T-cell progenitor development have been thoroughly defined on the basis of cell surface marker expression, antigen receptor gene rearrangement, and cytokine responsiveness. For example, interleukin-7 (IL-7) was originally identified as a factor produced by bone marrow stromal cells that could support the proliferation of B-cell precursors (1,2). The IL-7 responsive B-lineage cells were clearly defined in vitro using sorted B-cell precursor populations and shown to express B220, CD43 heat stable antigen (HSA), and to carry heavy-chain gene rearrangements (3). IL-7 responsiveness is lost following light-chain gene rearrangement and acquisition of the pre-B-cell phenotype (4). IL-7 is also a potent mitogen for thymocytes (5). In a similar fashion, it has been established that the CD44\(^+\)CD25\(^-\) fraction of CD4\(^-\)CD8\(^-\) thymocytes, comprising <1% of the total thymocyte population, is IL-7 responsive, whereas the predominant CD4\(^+\)CD8\(^+\) thymocyte subpopulation is not (6).

Cytokine responsiveness in vitro does not necessarily reflect cytokine dependence in vivo. Two general approaches have been used to examine the specific roles of IL-7 in vivo. First, administra-
tion of neutralizing antibodies to either IL-7 or IL-7R in vivo results in impaired lymphopoiesis at the specific developmental stages predicted from in vitro work (7–9). Second, mice rendered genetically deficient in IL-7, IL-7R, or IL-2Rγ by gene targeting in embryonic stem cells all display defects in lymphoid development (10–13). The populations of B- and T-cell progenitors affected in IL-7R-deficient mice are precisely those that respond to IL-7 in vitro. The phenotypes of mice harboring mutations affecting the biological activity of IL-7 thus generally correlate well with activities previously ascribed to IL-7 on lymphoid precursors in vitro and underscore the importance of this cytokine in the development of both B- and T-cell lineages.

In this chapter, we summarize the phenotype of IL-7Rs deficient mice. We highlight the important phenotypic differences among mice lacking IL-7, IL-7R, and IL-2Rγ, and the implications these observations have on the biology of IL-7, and related cytokines, in both mouse and humans.

**Generation of IL-7R-Deficient Mice**

Mice genetically deficient in IL-7R were generated by homologous recombination in a 129-derived embryonic stem (ES) cell line. A neo-cassette was inserted into exon 3 at approximately amino acid 90 of the 180 amino acid IL-7R extracellular domain (11). Chimeras carrying targeted ES cells were crossed to both C57BL/6 and 129/J mice. The resulting heterozygotes (IL-7R+/−) were intercrossed to generate mice homozygous for the IL-7R mutation (IL-7R−/−) on both 129 and random C57BL/6 × 129 hybrid backgrounds. Additionally, the IL-7R mutation was backcrossed onto C57BL/6 for five generations (B6N5IL-7R−/−). We have not observed any obvious phenotypic differences between these various IL-7R deficient strains. The hybrid and B6N5IL-7R deficient strains have been deposited in the Jackson Laboratory Induced Mutant Resource program.

**Biochemical Characterization of IL-7R−/− Mice**

Several criteria were used to establish that IL-7 binding and bioactivity are ablated in IL-7R−/− mice. According to Scatchard analyses, IL-7R−/− cells derived from various lymphoid organs fail
Phenotype of IL-7R-Dicient Mice

Fig. 1. Flow cytometric analyses of biotinylated human IL-7 binding to bone marrow cells. Unfractionated bone marrow from IL-7R+/− and IL-7R−/− mice was harvested and incubated with biotinylated human IL-7 and streptavidin-conjugated phycoerythrin (SAPE) in the presence (thin line) or absence (thick line) of a 500-fold excess of unbiotinylated human IL-7 as described previously (14) and analyzed by flow cytometry. Significant shifts are not observed with IL-7R+/− or IL-7R−/− cells incubated with SAPE alone (data not shown).

to bind IL-7 with high affinity, whereas binding to +/+ cells is readily evident (11). Thus, high-affinity IL-7 binding is abolished in cells derived from IL-7R deficient mice. A very low-affinity IL-7 binding moiety, distinct from high affinity IL-7R, is expressed on a variety of primary and established human hematopoietic cells at 10^4–10^5 sites per cell as determined by flow cytometry using biotinylated IL-7 (14). In comparison, IL-7R is present at 10^3–10^4 sites per cell and binds IL-7 with an affinity 100- to 1000-fold higher (15). In contrast to the results obtained by Scatchard analyses, the binding profiles of IL-7R+/− and IL-7R−/− bone marrow cells using biotinylated IL-7 in conjunction with flow cytometry are identical (Fig. 1). Thus, IL-7R−/− cells fail to bind IL-7 with high affinity, whereas binding to the low-affinity IL-7 binding site, as detected by flow cytometry, is not affected by the IL-7R mutation. The physiological significance of this low-affinity IL-7 binding moiety remains obscure.

IL-7 was originally identified as a cytokine critical for B-lymphopoiesis in bone marrow Whitlock Witte (W/W) cultures (1,2). It was therefore of interest to determine if such cultures could be established from IL-7R−/− bone marrow. Repeated attempts at
establishing W/W cultures from IL-7R<sup>−/−</sup> bone marrow have failed (Peschon, J. J. and Namen, T., unpublished data), suggesting that IL-7R is required for B-lymphopoiesis under these conditions. Control fetal liver W/W cultures established in the presence of IL-7 or steel ligand factor (SLF) support the development of nonadherent B-lymphoid or myeloid cells, respectively (Fig. 2). In contrast, IL-7R<sup>−/−</sup> cultures fail to generate significant numbers of nonadherent cells in response to IL-7, whereas cultures established in the presence of SLF are indistinguishable from control cultures (Fig. 2). Thus, IL-7R deficient cells fail to bind IL-7 with high affinity and do not respond to IL-7 in vitro.
**Gross Characterization of IL-7R-Deficient Mice**

IL-7R<sup>−/−</sup> mice are generated from IL-7R<sup>+/−</sup> intercrosses at the expected Mendelian frequency, breed normally, and do not display any obvious external phenotypic anomalies on either 129 or C57BL/6 backgrounds. We have not observed any defects outside of the lymphoid system in IL-7R deficient mice. The IL-7 and IL-7R genes are both expressed in the brain, and IL-7 influences neuronal differentiation in vitro (17,18). Possible deficits in neural activity and function are currently being examined in IL-7R deficient mice (Kessler, J., personal communication).

Within the lymphoid compartment, the consequences of the IL-7R mutation are dramatic. Splenic and lymph node cellularity are reduced approx 10-fold relative to age- and strain-matched controls (11). This reduction in cellularity is owing to diminished levels of CD3<sup>+</sup> and B220<sup>+</sup> cells. Myeloid lineage cells are not affected. Thymic cellularity is variably reduced 10- to 10,000-fold relative to age- and strain-matched controls (11). The mechanism underlying the variability in thymic phenotype remains unclear. In our specific pathogen-free colony, approx 65% of the 129 derived IL-7R<sup>−/−</sup> mice have thymi composed of 1% of the number of cells of control thymi. Thymic cellularity in the remaining 35% of IL-7R<sup>−/−</sup> mice is 1–10% that of controls. We have considered the possibility that although thymopoiesis is critically dependent on IL-7R, other cytokine receptors may be triggered to partially rescue the severe IL-7R<sup>−/−</sup> phenotype. Potential candidates for such receptors are those also utilizing the IL-2Rγ chain. To address this issue, we are in the process of analyzing cohorts of mice lacking both IL-2Rγ and IL-7R (Peschon, J. J. and Muller, W., unpublished observations). In this context, it is interesting to note that a greater arrest in thymocyte development in vitro is achieved using a combination of neutralizing antibodies to IL-7R and IL-2Rγ than using either antibody alone (19). Finally, the possibility that environmental factors might contribute to the variable thymic phenotype warrants further attention. For example, the observation that both tumor necrosis factor (TNF) and IL-1 can promote thymocyte proliferation (20) suggests that conditions inducing the synthesis of these cytokines may influence the severity of the IL-7R mutation in this organ.
B- and T-Cell Progenitors in IL-7R-Deficient Mice

To determine which stages of B- and T-lymphopoiesis are affected in IL-7R-deficient mice, four-color flow cytometry was performed on bone marrow and thymus, respectively. The stages of B-lymphopoiesis have elegantly been dissected using a panel of cell-surface markers (3). Additionally, the growth factor requirements of these B-cell progenitor fractions and the rearrangement status of their heavy- and light-chain gene segments have been determined in vitro (3). The observation that late pro-B-cells and their descendents, as defined by the expression of HSA and CD43, are dramatically underrepresented in IL-7R-deficient mice (11 and Fig. 3) corroborates the conclusions drawn from in vitro work and indicates that IL-7R serves a critical, nonredundant role in driving the expansion of B-lineage cells undergoing heavy-chain gene rearrangement.

Because of the observation that thymopoiesis is variably affected in IL-7R−/− mice, determining which stages in thymocyte development are affected by the mutation is complicated (11). In severely affected IL-7R−/− mice, thymii are composed almost exclusively of CD4+CD8− (DN) cells (Fig. 4). This population of cells is further fractionated on the basis of CD25 and CD44 expression, because these markers define key subpopulations within the DN fraction (21–23). It is evident from these analyses that IL-7R-deficient CD4+/CD8− thymocytes do not acquire CD25 expression (11 and Fig. 4), placing the developmental arrest at the first major phase of thymocyte expansion occurring prior to the rearrangement of T-cell receptor (TCR) β-chain genes. These data thus define IL-7 as a critical cytokine that drives expansion of early thymocytes. IL-7 is posited to promote β-chain gene rearrangement (24). However, it is unlikely that the arrest observed is a direct consequence of a failure to rearrange β-gene segments because IL-7R-deficient mice display an earlier thymic arrest than that observed in mice harboring mutations affecting TCR α/β rearrangement (25). IL-7R may be required to maintain thymocytes in a state of competence to receive other signals directing β-chain rearrangement.

In contrast, thymii derived from IL-7R−/− mice of the less severe phenotype display a CD4/CD8 thymocyte profile that does not significantly differ from that of controls, although total thymic cellularity remains significantly reduced (Fig. 4). Interestingly, the CD25
Phenotype of IL-7R-Dificient Mice

Fig. 3. Flow cytometric analysis of IL-7R deficient bone marrow sub-populations. The B220+ (CD45R+)IgM- fraction of bone marrow from age-matched 129-derived IL-7R-/- and control mice (gated area boxed) was further fractionated on the basis of CD43 (S7) and CD24 (HSA) expression as described (3).

(Fig. 4) and CD25/CD44 profiles (unpublished) of DN cells from these thymii still resemble those observed in the severely affected thymii. These data suggest that a normal CD4/CD8 thymocyte profile can be restored in a fraction of IL-7R deficient mice despite a fixed early block at the CD44+/CD25- stage of thymocyte development.

**Functional Consequences of IL-7R Deficiency**

It is clear that both B- and T-lymphopoiesis are affected in IL-7R-deficient mice and that IL-7R is a critical molecule driving the expansion of these lineages. However, mature B- and T-cells are
Fig. 4. Flow cytometric analyses of IL-7R-deficient thymic subpopulations. The CD4 and CD8 profiles of thymic subpopulations from 129-derived IL-7R<sup>−/−</sup> mice composed of either <1% or >1% the cellularity of age-matched controls were analyzed by flow cytometry. The CD4<sup>+</sup>CD8<sup>+</sup> fractions were further fractionated on the basis of CD25 (IL-2Rα) expression.

... present in IL-7R<sup>−/−</sup> mice. A remaining question is whether or not these lymphocytes are functional. In the mouse, mature B-cells apparently do not express IL-7R and do not respond to IL-7. Thus, any defects in T-independent B-cell responses would be the result of intrinsic B-cell defects acquired as a consequence of development in the absence of an IL-7R signal. Antibody production in IL-7R<sup>−/−</sup> mice following administration of the T-independent antigen, trinitrophenol (TNP)-ficoll, is not significantly different than that observed in strain matched controls (Maliszewski, C. R., unpublished observation). Thus, B-cells developing in the absence of an IL-7R signal are capable of Ig secretion following direct stimulation. At this time,
**Phenotype of IL-7R-Dificient Mice**

Fig. 5. Allogeneic tumor cell challenge in IL-7R-deficient mice. C57BL/6 (n = 5), DBA/2J (n = 5), and B6N5IL-7R−/− (n = 5) adult female mice were injected ip with 5000 live P815 tumor cells and monitored for survival.

we cannot rule out the possibility, however, that the frequency of B-cells responding to TNP-ficoll is affected by the mutation.

T-cells express IL-7R and respond to IL-7 (26). Thus, defects in T-cell function in IL-7R−/− mice could either be caused by abnormal development or a failure to appropriately respond to IL-7 following peripheral stimulation. IL-7R−/− T-cells display dramatic defects in both survival and proliferation following a variety of in vitro activation regimens (27). These defects are apparent under conditions that are IL-7 and IL-7R independent, suggesting that T-cells developing in the absence of an IL-7R signal are compromised. These results complicate an analysis of the requirement for IL-7 during peripheral T-cell activation. We have analyzed two T-dependent immune respones in vivo in IL-7R deficient mice. Because IL-7 stimulates T-cell proliferation and promotes the survival of tumor-specific cytotoxic T-lymphocyte (CTL) (28), we examined the ability of IL-7R deficient mice to reject allogenic tumor cells. B6N5IL-7R−/−, C57BL/6, and DBA/2 mice were injected with a DBA/2-derived mastocytoma cell line P815. As expected, DBA/2 mice succumbed to tumor challenge and C57BL/6 mice were resistant. Remarkably, 80% of IL-7R−/− mice succumbed to allogeneic tumor challenge and displayed peritoneal tumor cell infiltrates comparable to those of injected DBA/2 mice (Fig. 5). Thus, cell-mediated immunity against allo-
geneic tumor cells is clearly compromised in IL-7R-deficient mice. Limiting dilution analyses reveal that while IL-7R−/− CTL are functional, the frequency at which they are generated is greatly reduced relative to that observed in control mice (Maraskovsky, E., unpublished observations). Additionally, antibody responses to the T-dependent antigen TNP keyhole limpet hemocyanin (KLH) are compromised in IL-7R-deficient mice (Maliszewski, C., unpublished observation). Thus, it is apparent that the T-cells generated in IL-7R-deficient mice are defective with respect to survival, proliferation, and function.

The observation that IL-7R-deficient T-cells are also hyporesponsive to receptor independent stimuli, such as phorbol myristate acetate (PMA) and ionomycin (27), further suggests that T-cells developing in the absence of an IL-7R signal are functionally impaired. The mechanism underlying this phenomenon is unclear. It is possible that the establishment of a normal repertoire of signaling molecules within T-cells requires that T-cell precursors either undergo an IL-7-driven phase of expansion or directly receive a signal delivered by IL-7R. The Jak3 tyrosine kinase associates with, and is activated by, the IL-7R-IL2Rγ receptor complex (29). Jak3-deficient mice display defects in lymphoid development comparable to those observed in IL-7, IL-7R, and IL-2Rγ-deficient mice (30,31). It is particularly intriguing to note that T-cells from Jak3-deficient mice fail to respond to both Jak3-dependent and Jak3-independent stimuli. Thus, a theme potentially emerges that IL-7R signaling, either directly or indirectly, is required during T-cell development at a quantitative level, by driving expansion of precursor pools, and at a qualitative level by promoting the expression of various gene products required for subsequent activation.

IL-7R is expressed by macrophages and IL-7 stimulates proinflammatory cytokine secretion by these cells (32,33). Additionally, IL-7 has been demonstrated to increase macrophage microbicidal activity against Leishmania major (34). Thus, defective macrophage activation and cytokine release in vivo as a consequence of IL-7R deficiency may result in impaired resistance to intracellular pathogens or increased resistance to lipopolysaccharide (LPS)-induced endotoxemia. However, IL-7R deficient mice are able to overcome a primary infection by the intracellular pathogen Listeria monocytogenes (Peschon, J. J., unpublished observation). Additionally, IL-7R−/−
remain sensitive to lethal endotoxemia induced by LPS administration (Peschon, J. J., unpublished observation). Although we have not specifically assessed cytokine release by activated IL-7R<sup>−/−</sup> macrophages, gross defects in macrophage function following in vivo challenge by LPS or Listeria are not apparent in IL-7R<sup>−/−</sup> mice.

The IL-7R Mutation in Context

The activity of IL-7 is dependent on both IL-7R and IL-2Rγ (35,36). Thus, phenotypic similarities between IL-7<sup>−/−</sup>, IL-7R<sup>−/−</sup> and IL-2Rγ<sup>−/−</sup> mice and humans are expected. We and others have proposed that a critical phenotype displayed by IL-2Rγ deficient patients, namely defective T-cell development, can largely be explained by the effects of this mutation on the activity of IL-7R (11,35–37). However, specific phenotypic differences are observed in these models that warrant further investigation because they suggest the presence of novel cytokine and cytokine receptor interactions in the development of B- and T-cell lineages.

Thymopoiesis in IL-7- and IL-2Rγ-deficient mice is comparably affected; thymocyte levels are reduced approx 10-fold whereas the characteristic distribution of the CD4 and CD8 subpopulations is maintained (10,12,13). This too is observed in those IL-7R<sup>−/−</sup> mice displaying the less severe thymic phenotype (Fig. 4). In marked contrast is the thymic phenotype displayed by the majority of IL-7R deficient mice. In these mice, thymocyte levels are 10- to 10,000-fold less than those of controls and the thymi are composed predominantly of immature CD4<sup>−</sup>/CD8<sup>−</sup> cells (11). One explanation for this data invokes the existence of a cytokine that utilizes the IL-7R, but not IL-2Rγ, and displays overlapping activity with IL-7. In fact, a cytokine with some of these characteristics has been recently identified and cloned. Thymic stroma derived lymphopoietin (TSLP) shares many activities in common with IL-7 and has an obligate requirement for IL-7R in addition to TSLP-R (38; Park, L. S., manuscript in preparation; Williams, D. E., manuscript in preparation). Thus TSLP may partially alleviate the effects of IL-7 deficiency. The IL-7R mutation is proposed to represent the functional inactivation of both IL-7 and TSLP. An additional prediction, which we are currently examining, is that TSLP does not require IL-2Rγ for function.
Peripheral B- and T-cell levels in the three mouse mutants are uniformly reduced approx 10-fold. Additionally, these three strains display similar phenotypes with respect to B-cell development in the bone marrow. It is, however, interesting to note that the arrest in B-lymphopoiesis occurs earlier in IL-7R\(^{-/-}\) mice than in IL-7\(^{-/-}\) mice. As mentioned earlier and previously noted (10), this apparent discrepancy may be due to the functional inactivation of both IL-7 and TSLP in IL-7R-deficient mice.

B-cell development in IL-7, IL-7R, and IL-2R\(\gamma\) deficient mice is clearly affected and supports a large body of evidence that IL-7 is a critical cytokine in early B-cell development in the mouse. However, B-lymphopoiesis is apparently unaffected in IL-2R\(\gamma\)-deficient humans (39,40). It will be of interest to examine whether IL-7R-dependent, but IL-2R\(\gamma\)-independent cytokines, are important factors for B-cell development in humans. Additionally, signals for appropriate B-cell progenitor development may be delivered by cytokine receptors other than IL-7R and IL-2R\(\gamma\). Such receptors may play an important role in human B-lymphopoiesis and may also account for the residual B-cell development that occurs in the absence of IL-7R or IL-2R\(\gamma\) in the mouse. In this context it is particularly interesting that IL-3 can functionally substitute for IL-7 in promoting the proliferation of murine B-cell progenitors under specific in vitro conditions (41).

An issue that frequently arises in the analysis of cytokine and cytokine receptor-deficient mice is the extent to which cytokine redundancy, either forced or physiological, affects the phenotype observed. Faced with the genetic absence of a critical molecule, alternative developmental pathways may be adopted to bypass a given mutation. Such pathways may only be uncovered following genetic deficiency. In fact, one explanation for the existence of two phenotypes in IL-7R deficient mice is that the less severely affected mice have partially overcome the lack of IL-7R by adopting a novel developmental strategy. In this regard, it is interesting to note that long-term treatment of mice with neutralizing antibodies to IL-7 produces a phenotype that is more severe than that of genetic IL-7 or IL-2R\(\gamma\) deficiency and is comparable to that observed in the more severely affected IL-7R\(^{-/-}\) mice (7,8). Antibody-mediated IL-7 ablation is clearly effective. However, it may be “leaky” enough to mask the ability of an IL-7R-dependent cell to adopt a novel and
potentially redundant pathway of development. These pathways may reveal the existence of either novel cytokines, or novel functions for known cytokines, in the development and function of lymphoid lineages.

Summary

IL-7 stimulates the proliferation of B- and T-cell progenitors, is mitogenic for mature T-cells, and induces proinflammatory cytokine release by monocytes. IL-7 acts through a heteromeric receptor complex composed of IL-7R and IL-2Rγ subunits, both members of the hematopoietin receptor superfamily. Mice lacking IL-7R display a severe reduction in lymphoid cellularity owing to defined arrests at specific stages of B- and T-lymphopoiesis. The thymic defect in IL-7R⁻⁻ mice resembles that observed in X-linked severe combined immunodeficiency patients carrying null mutations in IL-2Rγ. Together, these observations implicate both receptor subunits in the development of B- and T-cell lineages. Mice lacking IL-7 or IL-2Rγ are less severely affected than mice lacking IL-7R, providing genetic evidence that IL-7R is a required subunit for at least one other cytokine receptor and that IL-7R is a component of both IL-2Rγ-dependent and independent receptor systems.

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Chapter 4

CD40 Ligand Knockout Mice

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Introduction

CD40 is a 50-kDa glycoprotein expressed on B-cells, monocytes, dendritic cells, follicular dendritic cells, thymic epithelial cells, and certain carcinomas (for review, see ref. 1). Its counterstructure, CD40 ligand (CD40L), is a 33-kDa glycoprotein expressed primarily on activated CD4+ T-cells, but also found on CD8+ T-cells, mast cells, basophils (1), and eosinophils (2). CD40L belongs to the tumor necrosis factor (TNF) superfamily (3), members of which stimulate a broad range of biological functions including proliferation, induction of cytokine secretion, and apoptosis (For review, see ref. 4).

The biological importance of CD40L:CD40 signaling was first elucidated in studies on T-cell:B-cell interactions (for review, see refs. 5 and 6). Activated CD4+ T-cells can stimulate resting B-cells to proliferate and differentiate into antibody-secreting cells (7,8). This cascade of events requires two signals, one from the T-cell surface and the other from secreted cytokines. The T-cell surface requirement for B-cell stimulation, as seen in studies using membranes from activated and fixed CD4+ T-cells (9–11), can be replaced by immobilized anti-CD40 monoclonal antibody (MAb) (12–14) and, as seen in subsequent studies, by recombinant CD40L
CD40L can also trigger monocyte functional activity, including cytokine secretion (17–19) and tumoricidal activity (17), through interactions with CD40. The stimulatory effect that activated T-cells provide to CD40-expressing cells is part of a dynamic, bidirectional process. For example, CD40L upregulates B7 molecule expression on B-cells (20,21) that can in turn bind to and stimulate T-cells via cell-surface CD28 (22). This process is likely to occur between T-cells and other antigen-presenting cells (APC) as well, and the consequences of such interactions could have a significant impact not only on immune effector functions, but also on tolerance induction and the development of autoimmune disease.

A role for CD40L in antibody secretion in vivo has been clearly demonstrated. Administration of a neutralizing anti-CD40L MAb (anti-gp39) to normal mice blocks antibody responses to thymus-dependent (TD) antigens (23). In addition, these mice fail to form germinal centers, and memory B-cells are undetectable (24). CD40L also appears to play an important role in cell-mediated immune responses. Anti-gp39 treatment prevents autoimmune disease development in a murine collagen-induced arthritis model (25) in which CD4+ T-cells are known to have a clear involvement (26). Anti-gp39 treatment also blocks acute graft-versus-host disease, which is associated with allospecific cytotoxic T-lymphocyte activity (27). Other in vivo studies have shown that anti-gp39, when administered with allogeneic APC, can induce a state of allospecific unresponsiveness (28) and enhance the survival of pancreatic islet allografts (29).

The human gene for CD40L and the hyper-IgM syndrome (HIGM) phenotype both map to the same region of the X chromosome (Xqs6.3–27.1) (for review see ref. 30). Because patients with HIGM fail to express functionally active CD40L they have a markedly decreased ability to secrete antibodies of “switched” isotypes, i.e., IgG, IgA, and IgE. Although B-cells from HIGM patients are functionally normal, the defect in humoral immune responsiveness can be easily explained by an absent CD40L trigger. HIGM patients also exhibit an increased susceptibility to Pneumocystis carinii pneumonia and cryptosporidial diarrhea, diseases often associated with T-cell deficiencies (31). Wiley and Harmsen (32) described a mouse model for this phenomenon by demonstrating that severe combined immunodeficiency disease (SCID) mice
reconstituted with spleen cells from normal mice resolve *P. carinii* pneumonia, whereas mice treated with anti-CD40L MAb do not. In this model, CD40L appears to regulate both cell-mediated and humoral immune responses.

Many of these results have been confirmed or extended using CD40 knockout (CD40KO) and CD40L knockout (CD40LKO) mouse models. This report is a compilation of experimental results from our CD40LKO mouse model (33) and data from the CD40LKO of Xu et al. (34) and the CD40KO of Kawabe et al. (35).

**Experimental Observations**

**In Vitro Biology**

In our laboratory, mice deficient in CD40L expression were generated by standard procedures using a targeting vector designed to delete exons 3 and 4 of the CD40L gene on the X chromosome (33). The resultant recombination excised a portion of the coding sequence for the CD40L extracellular region. Mice were generated on a C57Bl/6 × 129/J genetic background and housed under specific pathogen-free (SPF) conditions. There were no viability or fertility defects in male hemizygous and female homozygous offspring, and segregation of the mutant allele followed a typical Mendelian pattern.

**Leukocyte Subpopulations: Phenotype and Function**

Lymphoid tissues were examined for alterations in cell number or subpopulation skewing that might result from absence of functional CD40L. The total number of cells recovered from lymph nodes (LN) was two- to threefold lower in the CD40LKO mice than in littermate controls. However, cell-surface marker analysis indicated that the percentages of B-cells, T-cells, and macrophages were unchanged in the LN and spleens of CD40LKO mice. One notable but predictable difference in cell-surface marker expression was the absence of CD40L on anti-T-cell receptor (TCR) activated, CD4+ T-cells. Xu et al. (34) and Kawabe et al. (35) also reported no major lymphoid subset alterations in the CD40LKO and CD40KO models, respectively. However, when mice were housed in a conventional (vs. SPF) facility, significantly lower numbers of granulocytes were recovered from the CD40KO mice relative to controls (35). This finding correlates with the observation that HIGM patients are often
neutropenic (31) and suggests that CD40L:CD40 interactions are required for induced, but not constitutive, granulopoiesis.

Functionally, CD4+ LN cells from CD40LKO mice proliferate normally in response to treatment with polyclonal activators (34). B-cells from CD40LKO mice display normal proliferative responses to treatment in vitro with LPS, anti-IgM, or recombinant CD40L (33). CD40LKO-derived B-cells are also capable of undergoing Ig class switching and secretion on treatment with cytokines plus LPS or recombinant CD40L. Thus, the absence of CD40L does not appear to have a gross effect on T- or B-cell development.

To further assess T-cell function in vitro, we generated alloreactive CD4+ clones from CD40LKO mice (36). CD40LKO or C57Bl/6 splenocytes were stimulated for 10 d with irradiated DBA/2 splenocytes and cloned by limiting dilution in the presence of allogeneic splenocytes and IL-2. Clones were exposed to subsequent rounds of allostimulation in the presence of IL-2 followed by expansion and maintenance in cytokine alone. The KO3 (CD40LKO-derived) and C3G9 (control) clones exhibited Th1-like cytokine secretion profiles in response to stimulation with anti-CD3. Unlike the C3G9 clone, the KO3 clone exhibited only a weak alloreactive proliferative response when cultured in the absence of exogenous IL-2 or IL-12. As described below, this deficiency may be explained, at least in part, by an inability of the CD40L-deficient clone to stimulate IL-12 production from the APC component of the irradiated, allogeneic splenocytes (see T-cell:Macrophage Interactions).

**T-Cell:Macrophage Interactions**

Activated T-cells stimulate macrophage cytokine production and this activity is mediated through CD40L:CD40 interactions (17–19). To assess whether T-cells from CD40LKO mice are capable of stimulating murine macrophage IL-12 production, T-cell clones were activated by in vitro culture with allogeneic Balb/c peritoneal macrophages and IL-12 levels in culture supernatants were measured by bioassay (36). The C3G9 clone induced IL-12 production, and this activity was inhibited by inclusion of anti-CD40L MAb in the cultures. The KO3 clone was unable to induce IL-12 production. This defect was not the result of an inability to be alloactivated by the peritoneal macrophages because the KO3 cells were induced to secrete normal levels of IL-2 and interferon-gamma
In another set of experiments, unfractionated splenocytes from CD40LKO and control mice were stimulated with immobilized anti-CD3, and IL-12 production was measured. In this model, anti-CD3 activated T-cells from control mice stimulated the APC component of the splenocyte culture to secrete IL-12, and this effect could be blocked with anti-CD40L MAb. By contrast, IL-12 was undetectable in the CD40LKO splenocyte cultures. This inability to induce IL-12 production was not a defect at the APC level because CD40LKO-derived macrophages produced IL-12 in response to stimulation with recombinant CD40L and cytokines. Thus, the absence of CD40L expression by activated T-cells renders them incapable of inducing IL-12 secretion by APC.

**T-Cell:B-Cell Interactions**

The absence of functional CD40L renders T-cells incapable of providing help to B-cells. Anti-CD3 activated and irradiated CD4+ T-cells from CD40LKO mice cannot stimulate B-cell polyclonal IgG1 secretion in the presence of cytokines; however, polyclonal IgM secretion is similar to controls (34). In order to assess antigen-specific Ig secretion, KO3 or QH5 (wild-type-derived) Th clones were activated with anti-CD3 MAb and added to cultures containing normal B cells, sheep red blood cells (SRBC) as antigen, IL-2 and IL-5 (37). Induction of an anti-SRBC IgM response was measured in a plaque-forming cell (PFC) assay. Activated, fixed KO3 cells induced the generation of anti-SRBC-PFC, but at levels approx 30-fold lower than was seen with QH5 cells as stimulators. Over 50% of the residual anti-SRBC response observed in the KO3-containing cultures was blocked by recombinant CD30.Fc, indicating that another member of the TNF superfamily, CD30L (38), can provide some helper activity. Flow cytometric analysis of anti-CD3 activated KO3 cells revealed the presence of normal levels of CD30L but no CD40L. The molecular mechanism underlying the remaining T-cell help, not attributable to either CD40L or CD30L, has yet to be determined.

**Humoral Immune Responses**

***Serum Nonimmune Immunoglobulin***

CD40LKO mice, like HIGM patients, exhibit a selective deficiency in Ig isotype production (33). Serum from CD40LKO mice contains no detectable IgE, whereas IgG1, IgG2b and IgA levels are...
significantly reduced, particularly in older mice. A similar deficiency in switched Ig isotypes is seen in the CD40KO mice (35), although IgG3 levels are somewhat elevated. Interestingly, IgM levels in CD40LKO (33,34) and CD40KO (35) mice are normal, which contrasts with the human hyper-IgM syndrome phenotype. One possible explanation for this difference is that the knockout mice were housed under SPF conditions, and are therefore not exposed to typical environmental antigens such as bacterial polysaccharides, which are capable of stimulating IgM production (39,40).

**TD Antibody Responses**

The generation of antibody responses to TD antigens requires CD40L:CD40 engagement between activated T- and B-cells. Anti-gp39 treatment of normal mice severely diminishes antibody responses to TD antigens but leaves responses to thymus-independent (TI) antigens intact (23). CD40LKO mice exhibit a similar phenotype. For example, mice injected with SRBC fail to mount a detectable primary anti-SRBC IgM response (Campbell, K. and Maliszewski, C., unpublished data), which is considered to be a TD response. Xu et al. (34) reported similar findings.

We also investigated whether CD40LKO mice are capable of mounting a secondary response to a protein antigen. Mice were immunized with trinitrophenylated-keyhole limpet hemocyanin (TNP-KLH) hapten-carrier conjugate and boosted 3 wk later. Anti-TNP levels in secondary immune sera were measured by TNP-specific enzyme-linked immunosorbent assay (ELISA). The CD40LKO mice generated a weak but detectable secondary IgM anti-TNP antibody response relative to control mice. However, striking differences were observed in anti-TNP responses of the other isotypes. Whereas control mice secreted high levels of anti-TNP IgA, IgE, IgG1, IgG2a, IgG2b, and IgG3, the CD40LKO exhibited a complete absence of these isotypes. Similar antigen-specific isotype profiles were observed in CD40LKO mice challenged with KLH (34) and in CD40KO mice challenged with dinitrophenylated (DNP)-ovalbumin (35). These findings contrasted with results from the anti-gp39 MAb-treated mice in which secondary, IgG antibody responses against the TD antigens KLH and human Ig were markedly diminished but detectable (23). It is possible that the qualitative difference between the knockout and the antibody-treated mouse studies is owing to
variations in experimental protocols or to incomplete neutralization of CD40L with the anti-gp39 MAb. Nevertheless, the results show that the complete absence of CD40L from embryonic development through adulthood renders these mice incapable of mounting switched-isotype, secondary antibody responses.

The lack of a compensatory mechanism for secondary immune responsiveness in the CD40LKO mice raises an interesting issue regarding the potential role of other molecules expressed on the surface of activated T-cells, particularly members of the TNF family, in the generation of humoral immune responses. More specifically, despite the ability of murine CD30L to stimulate polyclonal Ig secretion and a suboptimal anti-SRBC response in vitro (37), this signal apparently cannot substitute for an absent CD40L signal in vivo. The results also suggest that membrane-bound TNF, which has been implicated as a costimulator of polyclonal Ig secretion by human B-cells in vitro (41), cannot stimulate a detectable TD antigen-specific antibody response in the absence of CD40L expression.

Affinity maturation and isotype switching in response to TD antigens occurs in the context of germinal centers (GC). GC are completely absent from TNP-KLH challenged CD40LKO mice, whereas prominent GC are observed in spleens and LN of immunized control mice (33). Similar results have been reported for the CD40KO mouse (35) and in TD antigen-immunized normal mice treated with anti-gp39 MAb (23). These observations are in apparent contradiction of those by Gray et al. (42) who show that recombinant CD40-Ig administration blocks memory B-cell development but has no effect upon GC formation in response to TD antigens. One interpretation of the latter study is that the CD40L requirement in GC formation comes in the form of a trigger molecule on the activated T-cell surface. In such a scenario, CD40-Ig binding to CD40L would result in reverse signaling via the intracellular region of CD40L (43). The absence of either CD40L or CD40 in the knockout and anti-gp39 MAb models would render this putative signaling pathway nonfunctional.

**TI Antibody Responses**

Unlike protein antigens, prototypical TI antigens such as bacterial polysaccharides contain highly repetitive epitopes that can crosslink specific B-cell Ig receptors for these epitopes. This signal,
in combination with cytokines, may be sufficient to induce a potent primary antibody response \((44,45)\), although molecular triggers like CD40L may contribute to this process. In order to measure responses to a TI-2 antigen, mice were immunized with DNP-Ficoll, bled 10 d later, and anti-DNP antibody levels were measured by ELISA \((33)\). The anti-DNP responses in CD40LKO and control mice are equivalent at the level of the two dominant isotypes, IgM and IgG3. Similarly, IgM and IgG3 responses to TNP-Ficoll and to a TI-1 antigen, TNP-LPS, are normal in CD40KO mice \((35)\). The ability to respond to TI antigens, but not to TD antigens, can at least in part be explained by structural differences between the two types of antigens. What is clear is that CD40L is not a necessary component of the response to TI antigens.

An additional and particularly compelling finding emerged from the DNP-Ficoll immunized CD40LKO mice. These mice secrete antigen-specific IgA, IgG1 and IgG2b at levels that are as high as or higher than those in immunized control mice. Moreover, antigen-specific isotype switching is observed in CD40KO mice immunized with either a TI-1 or TI-2 antigen \((35)\). Thus, the requirement for CD40L in Ig class switching does not extend to TI antigen responses. Rather, an additional set of signals, perhaps acting on the CD5\(^+\) B-cell subset \((46,47)\), must be involved in this TI-antigen directed, Ig class-switching phenomenon.

**Cell-Mediated Immune Responses**

**Susceptibility to Experimental Murine Leishmaniasis**

The protozoan parasite *Leishmania major* infects and replicates within host macrophages, and susceptible mice die. Resolution of infection is mouse strain specific and is associated with the ability to mount a cell-mediated immune response (for review see ref. 48). CD4\(^+\) T-cell subsets derived from resistant strains like C3H/HeN and C57BL/6 mice are skewed toward a Th1-like, IFN-\(\gamma\) dominated, cytokine secretion profile, whereas susceptible strains like Balb/c exhibit a Th2-like, IL-4 dominant response. The experimental leishmaniasis model has proven useful in assessing the involvement of cytokines and cell-surface signaling molecules in CD4\(^+\) T-cell differentiation and in the development of protective cell-mediated immune responses. We therefore applied this model to the CD40LKO
mouse (49). Mice were injected with *L. major* promastigotes, and the course of infection was measured by footpad swelling over a 7-wk period. The resistant strains C57Bl/6, 129/J, and C57Bl/6 × 129/J all display self-limiting increases in footpad size. The CD40LKO mice, like the Balb/c susceptible strain, develop ulcerating lesions and fail to resolve the infection. Thus, the absence of CD40L renders otherwise genetically resistant (C57Bl/6 × 129/J) mice susceptible to severe cutaneous leishmaniasis.

We tested the hypothesis that this susceptible phenotype is the result of skewed CD4+ T-cell differentiation by examining cytokine secretion profiles of LN cells derived from infected mice. Draining popliteal LN cells from CD40LKO and control mice were stimulated in vitro with soluble leishmania antigen (SLA), and IFN-γ and IL-4 levels were determined by ELISA. Compared to (C57Bl/6 × 129/J)F1 controls, SLA-stimulated, CD40LKO-derived cultures contain considerably less IFN-γ; moreover, IL-4 is easily detected in CD40LKO cultures but below levels of detectability in the C57Bl/6 × 129/J cultures. Balb/c derived LN cultures contain intermediate levels of IFN-γ relative to CD40LKO and C57Bl/6 × 129/J, and contain generally higher levels of IL-4 than CD40LKO cultures. Thus, the absence of CD40L appears to shift the cytokine secretion profile away from the Th1-like phenotype in response to infection with *L. major*.

**IL-12 and CD40LKO Susceptibility to Leishmaniasis**

T-cell dependent regulation of IL-12 production is also altered in the CD40LKO mice (49). Spleen cell cultures (i.e., containing both anti-CD3 activated T-cells as stimulators and APC as potential IL-12 secretors) from *L. major* infected CD40LKO mice contain 12- and 16-fold less IL-12 than C57Bl/6 × 129/J and Balb/c cultures, respectively. The working assumption from these results is that T-cells in CD40LKO mice are incapable of stimulating macrophage IL-12 production. This deficiency might therefore be responsible for the skewed IL-4/IFN-γ secretion profile observed in CD40LKO mice because IL-12 can drive CD4+ T-cell differentiation toward a Th-1 like phenotype (50,51).

A direct role for IL-12 in antileishmanial responses has been established (reviewed in ref. 52). In particular, recombinant IL-12 administration cures Balb/c mice of infection (53), whereas anti-
IL-12 MAb exacerbates disease in resistant C57Bl/6 mice (54). Attempts have been made to induce a protective antileishmanial response in CD40LKO mice by administering recombinant IL-12. Intraperitoneal injection of IL-12 at 0.5 μg/d for the first 7 d of infection provides partial protection. IL-12 at 1 μg/d restores the wild-type resistant phenotype, as manifested by limited footpad swelling and dramatically reduced parasite burden. The resistant phenotype appears to be associated with CD4+ T-cell differentiation toward a Th1-like phenotype, as anti-CD3 or SLA-activated LN cells from IL-12 treated CD40LKO mice secrete levels of IFN-γ that are roughly equivalent to those seen in C57Bl/6 × 129/J derived LN cultures. Collectively, these results suggest that the susceptibility of CD40LKO mice to *L. major* infection stems from an inability to stimulate IL-12 production in a CD40L-dependent manner, which is consistent with the in vitro studies showing a direct effect of soluble CD40L on macrophage IL-12 production (36). Alternatively, CD40L:CD40 interactions may play a more direct, but as yet undefined, role in the induction of IFN-γ production. It is clear from other studies that IFN-γ is also required for protective anti-leishmanial responses (55).

**Conclusions**

Results from the CD40LKO mouse studies have verified that multiple arms of the immune response require functional CD40L expression. One notable exception is the retention of humoral immunity to TI antigens, which could have some relevance to certain antimicrobial responses. An intriguing implication from these studies, obviously requiring further experimentation, is that CD40L can in some way direct CD4+ T-cell differentiation. Thus, CD40L is likely to play a central determinative role in the spectrum of potential immune responses mediated by various Th subsets. Several other issues should be addressed using the CD40LKO model, including the functional relevance of CD40L expression on non-T-cells such as mast cells, basophils, and eosinophils; the importance of CD40L stimulation of other CD40+ cells, particularly dendritic cells and follicular dendritic cells; and the role of CD40L in responses to infectious agents such as viruses, fungi, and other intracellular pathogens.
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Chapter 5

Physiological, Immunological, and Pathological Functions of Tumor Necrosis Factor (TNF) Revealed by TNF Receptor-Deficient Mice

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Introduction

Tumor necrosis factor (TNF) has a long history that goes back well into the last century. It is now a hundred years ago that the surgeon William Coley observed a remission of inoperable tumors in some patients after infusion of bacterial toxins (1,2). Unacceptable side effects, however, and a lack of understanding of how these toxins induced hemorrhages in tumors, prevented the further development and application of this treatment. It was more than 60 years later when O’Malley et al. (3) realized that bacterial toxins acted indirectly by inducing an endogenous factor in the host that caused hemorrhagic necrosis in tumors and was hence later called TNF (4,5). Since then, TNF has attracted a great deal of attention and was found to participate in a vast variety of cellular activities that collectively make it the most pleiotropic cytokine identified so far.
Structural Features of the TNF/TNFR Family

TNF and lymphotoxin (LT) were the first in a rapidly growing family of structurally related molecules, grouped together in the TNF/TNFR family, which is now comprised of 10 ligands and 10 receptor molecules (6–9). The ligands of the TNF/TNFR family are predominantly type II transmembrane molecules that are believed to be trimeric proteins in their biologically active form and to induce aggregation, i.e. dimerization and trimerization, of their respective receptors on target cells (10, 11).

Not only the ligands, the receptors of the TNF/TNFR family also share structural features. They are all type I transmembrane proteins containing characteristic cysteine-rich consensus repeats in their extracellular domains that form structural units as determined by X-ray crystallography. Their cytoplasmic domains, however, are unrelated and may trigger very distinct, even opposite effects, such as proliferation and cell death. Despite this dissimilarity, some receptors of this family show a remarkable functional overlap with respect to apoptosis, i.e., the TNFR1, the Fas receptor, and the CD40 receptor. These three receptors carry in their cytoplasmic domain a consensus sequence of about 80 amino acids near the C-terminus that was dubbed the death domain (11–13). Whereas ligand binding of TNFR1 and Fas activates the death domain (11–16), ligand binding to the CD40 receptor induces proliferation (17) and postpones cell apoptosis (18).

Within this family there is a closely related group of three ligands, i.e., TNF, LTα, and LTβ, and of three receptors, i.e., TNFR1 p55, TNFR2 p75, and LTβR. The TNF family shows characteristic features that are unique: TNF is synthesized by the cell in a transmembrane form (19) and as such is thought to trigger preferentially TNFR2 (20). In addition to this strictly localized activity during cell–cell contact, membrane-bound TNF can be released by metalloproteinases from the cell surface into the circulation (21–23) and also trigger, as a soluble homotrimer, responses in distal target cells that may eventually be fatal for the organism if the response becomes generalized. The soluble TNF homotrimer binds both TNF receptors with similar affinities, albeit slightly better to TNFR2 than to TNFR1, with $K_d$ values of approximately 100 and 500 pM respectively (24–26). Although the on-rates are similar, the off-rates are very different: Soluble TNF dissociates from TNFR2
much faster than from TNFR1 (half times of dissociation 10 and 180 min, respectively \(27,28\)). TNF is synthesized mainly by activated monocytes and different tissue-specific macrophages, e.g., by Kupffer cells in the liver and by astrocytes in the brain, also by activated T-cells in immune and inflammatory responses and by nonhematopoietic cells including fibroblasts \(29\).

In contrast to this quite broad spectrum of cells that can produce TNF, the synthesis of LT\(\alpha\) is restricted to activated T-cells and natural killer (NK) cells \(30\). LT\(\alpha\) is synthesized in vivo in a secreted form and, like soluble TNF, binds as a homotrimer to both TNF receptors with similar affinities, thus inducing functions largely overlapping with TNF. In addition, LT\(\alpha\) can complex during biosynthesis with two subunits of the type II transmembrane ligand LT\(\beta\) \(31\). This cell surface \((LT\alpha)_1(LT\beta)_2\) heterotrimer binds to the recently discovered LT\(\beta\) specific receptor \(32\) inducing novel physiological functions during fetal development, e.g., the formation of lymph nodes and a follicular architecture in the spleen \(33,34\). The extracellular domain of LT\(\beta\)R contains the typical fourfold repeated cysteine-rich motifs. The two distal repeats are structurally related to those of TNFR1, although the two proximal ones resemble those of TNFR2 \(32\). The cytoplasmic domain, however, is distinct from either of the two TNF receptors and may therefore be connected to a different signaling pathway.

Both TNF receptors are co-expressed on most cells in vivo, but differ in their level and inducibility of expression \(35,36\). Whereas TNFR1 is constitutively expressed at a rather low level in the range of 100–1000 molecules per cell, the expression of TNFR2 can be strongly induced in the course of immune and inflammatory responses to about 10,000 molecules per cell. The cytoplasmic domains of both receptors are entirely unrelated and may therefore induce very different intracellular pathways. Both receptors can be shed from the cell surface and may bind free TNF in the circulation in an ongoing immune response, thus acting as physiological antagonists as well as storage molecules for TNF in the plasma.

**Physiological Roles of TNF**

Expression of TNF has been detected in mouse embryos \(37\), and in the adult mouse by immunocytochemistry in hepatocytes, kidney tubule epithelial cells, and in activated NK cells in the
pregnant uterus (37–39). These observations suggest participation of TNF in various physiological processes. To assess the different functions of TNF mediated by both TNF receptors in vivo, TNFR1-deficient mice (16,40,41) and TNFR2-deficient mice (16,42) were generated, and mice deficient for both receptors were obtained by cross breeding. Homozygous Tnfr1°- and Tnfr2°-mutant mice develop and breed normally with normal litter sizes, demonstrating that lack of TNFR1 or TNFR2 does not cause overt alterations in embryonic development and organogenesis. In addition, no obvious anomalies were observed in Tnfr1°, Tnfr2° double mutant mice. Constitutive expression of TNF mRNA can be detected in thymus and spleen of normal unchallenged mice (43,44), and in addition, TNF has been shown by in vitro experiments to be a growth factor for thymocytes (45). T-cells in Tnfr1°- and Tnfr2°-mutant mice, however, develop normally with respect to cell number, expression of major differentiation markers, and clonal deletion of potentially autoreactive T-cells, indicating that neither TNFRI nor TNFR2 is essential for T-cell differentiation in vivo (40,42). A recent study employing specific antibodies has shown that either TNF or IL1α can support thymocyte commitment and differentiation in an in vitro thymocyte reconstitution assay (46). Targeted mutant mice that are deficient in TNF and IL1α signaling pathways will show whether these cytokines are indeed crucial for thymocyte development in vivo.

A detailed analysis of the bone marrow revealed that TNFR1-deficient mice have significantly increased proportions of both primitive hematopoietic stem cells and more committed progenitor cells (47). It was demonstrated that signaling of TNF through TNFR1 is antiproliferative by blocking the cell cycle of hematopoietic stem and progenitor cells, suggesting an important physiological role for TNF in hematopoiesis. Indeed, pretreatment of mice with TNF resulted in enhanced hematopoietic recovery following administration of cell cycle specific chemotherapy, as shown previously (48). This protective effect of TNF may be owing to its antiproliferative activity, thus preventing hematopoietic cell populations from cell cycle dependent death.

The importance of LTα signaling for the morphological and functional differentiation of the spleen was recently detected in TNF/LTα- and LTα-deficient mice (33,34). Both targeted mutant
strains lack defined follicles in the spleen and are completely devoid of lymph nodes and Peyer’s patches in addition to defective Ig isotype switch. Although Tnfr1°-mice displayed a normal splenic microarchitecture and mounted an antigen-specific IgM and IgG response following immunization with sheep red blood cells, the IgG production was minimal, with titers leveling off 6 d after immunization (49). Immunofluorescent analysis revealed that Tnfr1°-mice lack a follicular dendritic network and fail to develop germinal centers (49,50). Tnfr2° mice, however, displayed a normal splenic microarchitecture and react with normal germinal center formation. Transplantation of normal bone marrow restored the ability in LTα- and TNF/LTα-deficient mice to form germinal centers, indicating that the required LTα-secreting cells are bone marrow derived (50,51). Collectively, these data suggest that normal splenic architecture, normal lymph node development, and isotype switch require signaling of LTα through the LTβ receptor, whereas differentiation of follicular dendritic cells, development of germinal centers, and a full IgG response depend on signaling via TNFR1.

Although TNFR2-deficient mice develop without obvious phenotypic alterations (42), further analysis may be needed to reveal the physiological relevance of TNFR2-signaling by soluble or transmembrane TNF.

**TNF in Host Defense**

**Defense Against Viral Infection**

Host defense to viral infection was assessed in Tnfr1°-mice using different viruses that induce strong humoral or cellular immune responses in wild-type mice. Infection with vesicular stomatitis virus (VSV) is controlled in normal animals by virus-neutralizing antibodies of the IgM and IgG isotypes and was not defective in Tnfr1°-mice (40). Lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (VV) induce strong cellular immune responses in wild-type mice. Infection is limited in the early phase of the immune response by activated macrophages, before virus-specific cytotoxic T-lymphocytes (CTL) finally control the infection. Again, no defects in antiviral CTL responses were detected in Tnfr1°-mice and virus replication was controlled in Tnfr1°-mutant mice as in wild-type mice. Also for Tnfr2°-mice, no defect in anti-viral responses
was reported (42). This shows that signaling through TNFR1 or TNFR2 is essential neither for the induction of a normal CTL response nor for the effector phase of the response.

**Defense Against Bacterial and Parasite Infections**

Host defense of mutant mice against bacterial infection has been analyzed with *Listeria monocytogenes*. *L. monocytogenes* is a gram-positive facultative intracellular bacterium, and host defense depends on cell-mediated immune responses (for review, see ref. 52). In an early phase of the immune response, the infection is limited by a T-cell independent, natural immunity represented by blood monocytes and resident tissue macrophages. Bacteria that manage to invade organs give rise to infectious foci. In the further course of the infection, antigen-specific immune T-cells are generated that cooperate with macrophages in infectious foci in the final resolution of the infection. Once established, antigen-specific T-cells confer a high degree of acquired resistance to a subsequent challenge with *L. monocytogenes*. It has been shown by several groups that neutralizing antibodies to TNF blocked antilisterial defense in mice by inhibiting activation of macrophages (53–55). The same defect in host defense against *L. monocytogenes* could be induced in mice with neutralizing antibodies to IFNγ (56,57). These experiments established the critical role of TNF and IFNγ in antilisterial responses.

*Tnfr1*°-mutant mice were challenged with sublethal doses of *L. monocytogenes*, i.e., $4 \times 10^3$ cfu, which is about 1/10 of the lethal dose for wild-type mice. In *Tnfr1*°-mutant mice, early defense against *L. monocytogenes* was completely abrogated (40,41). Already after 4 d, bacterial titers in infected organs (spleen and liver) were $10^3$ to $10^5$ times higher than in controls. Histological sections of the liver showed large necrotized infected areas that spread over the entire organ and led eventually to the death of the animal. Even a dose of only 250 *L. monocytogenes* progressed uncontrolled in *Tnfr1*°-mice and killed all mutant mice by d 7, but none of the controls. IFNγR-deficient mice were also severely impaired in their defense against *L. monocytogenes* (58), although the increase of bacterial load in infected organs was less dramatic than in *Tnfr1*°-mice ($10^2$- to $10^3$-fold compared to wild-type controls). Additionally, a protective immune response in mice
Function of TNF in TNFR-Deficient Mice

against *Mycobacterium tuberculosis* was shown to require signaling of TNF through TNFR1 (59). Again, IFNγR-deficient mice showed a similar defect in their defense against *M. bovis* BCG (60). Moreover, the defense against the parasite *Toxoplasma gondii* was shown to crucially depend on functional TNFR1 and IFNγR molecules (61). All *Tnfrl*^0^ and *Ifgr*^0^ mice, but not control mice, died very rapidly after infection. Significantly, virtually all *Ifgr*^0^-mice died before there were any deaths in *Tnfrl*^0^-mice, indicating that a possible sequential importance in the production of these cytokines exists. Collectively, these results document that signaling through TNF/TNFR1 and IFNγ/IFNγR are both essential for the initial, nonspecific defense against intracellular pathogens and that these pathways are not redundant.

The reduced antimicrobial activity in the analyzed mutant mice could be owing, in part, to a failure to induce synthesis of nitric oxide (NO) intermediates in mutant macrophages, which has been shown to be the toxic principle in defense against intracellular bacteria (for review see ref. 62). When stimulated in vitro with a combination of IFNγ and TNF, peritoneal macrophages from wild-type mice, but not from *Tnfrl*^0^-mutant mice, responded with a burst of NO synthesis (63). Thus, macrophages from *Tnfrl*^0^- mice lack an important effector molecule for the elimination of intracellular pathogens. A similar defect in NO synthesis and defense against *M. bovis* BCG was detected in IFNγR-deficient mice (64). In addition, TNF may no longer induce cytotoxic signals in TNFR1-deficient, infected target cells and therefore fail to eliminate the pathogen. Moreover, not only the effector phase, but also the activation of macrophages is severely impaired in *Tnfrl*^0^-mutant mice, similar to *Ifgr*^0^ mutant mice.

It has been shown that IL-12 plays a decisive role in the generation of a protective Th1 response in host defense against various pathogens (for review see ref. 65). Macrophages react to an infection by a pathogen with the release and synthesis of TNF and IL-12 (66–68) that activates NK cells to produce IFNγ (69). This IFNγ supplied by NK cells in the early phase of an immune response (70) not only activates macrophages to become microbicidal but also initiates the differentiation of Th1 cells. Activated macrophages produce increased levels of TNF and other cytokines involved in the local defense against the infection. This points to a crucial role of
NK cells for the amplification of an initially limited and weak response of an infected macrophage, leading to the full-blown response by large numbers of activated macrophages attracted to the site of infection.

**TNF-Mediated Toxicity**

If the immune response of the host, however, fails to localize and eradicate the pathogen, the infection may spread and then release large amounts of toxins into the circulation, inducing TNF systemically together with other cytokines, like IL-1 (71–74). The critical role of systemic TNF in the pathology associated with the systemic inflammatory response syndrome (SIRS) can be directly analyzed by the iv injection of TNF in wild-type and TNF receptor mutant mice. It was shown that a dose of 10 μg TNF is lethal for wild-type mice but leaves TNFR1-deficient mice completely unaffected (40). At this dose TNFR2-deficient mice are already severely reduced in their mobility but not lethally affected (42). The lethal dose for *Tnfr2*° mice is 15 μg TNF administered iv, whereas *Tnfr1*° mice resist even 100 μg TNF, iv.

It was shown that IL-1 strongly potentiates the lethal effect of TNF (75) so that a mixture of 1 μg of each cytokine is already lethal for wild-type mice. Again, *Tnfr1*° mice resist this challenge without any symptoms of illness (40). These results show that the lethal activity of systemically applied soluble TNF is signaled by TNFR1, whereby TNFR2 seems to sensitize TNFR1, but is not sufficient on its own to cause lethal complications in the mouse. Indeed, *Tnfr2*°-mice showed a decreased sensitivity to tissue necrosis induced by repeated subcutaneous injections of soluble murine TNF, indicating that this receptor plays a sensitizing role in the necrotic effects of locally applied TNF (42).

The systemic toxic activity of TNF can be induced indirectly in animals by the addition of bacterial toxins like lipopolysaccharide (LPS), which induces a massive release and synthesis of TNF and other proinflammatory cytokines in blood monocytes and tissue macrophages. Galanos et al. (76) showed that D-galactosamine dramatically sensitizes animals to the toxic effect of LPS, so that nanogram amounts already induce a fulminant liver toxicity that is lethal for wild-type animals in a period of 6–7 h. *Tnfr1*°-mice, however, are unaffected by this treatment and resist even a 100-
fold higher dose of LPS in the presence of D-galactosamine (40). *Tnfr*2° mice, on the other hand, show no resistance under these conditions and are as sensitive as wild-type mice (42). This suggests that the toxic activity of endogenously induced TNF is also mediated by signaling through TNFR1. Although the transmembrane form of TNF was suggested to trigger preferentially TNFR2 (20), lack of TNFR2 does not lead to an increased resistance to TNF in this in vivo model. Instead, an inhibitor of TNF processing, which prevented its release from the cellular membrane, protected D-galactosamine-sensitized mice from a lethal dose of endotoxin-induced TNF (21), similar to the protection provided by inactivation of TNFR1. This shows that soluble TNF, but not membrane-bound TNF, can trigger lethal cytotoxic events in vivo. It also demonstrates that soluble LTα, the synthesis of which was not affected by the metalloproteinase inhibitor, does not signal toxicity on its own in this animal model.

The increased susceptibility of rodents to TNF-induced lethality after pretreatment with D-galactosamine was shown to be largely owing to a relatively selective liver failure (77,78). D-galactosamine acts as a specific transcriptional inhibitor for hepatocytes which have a unique metabolic pathway, adding uridine nucleotides to D-galactosamine until the free pool of the nucleotide is exhausted and transcription ceases (79). Transcriptional arrest then strongly sensitizes primary hepatocytes to the cytotoxic action of TNF and induces death by apoptosis (80). The same effect can be shown with the more general transcriptional inhibitor actinomycin D which strongly sensitizes not only hepatocytes but also other cells, like fibroblasts, to TNF-induced apoptosis. Inhibition of translation, however, has no sensitizing effect, nor is TNF on its own very toxic to primary hepatocytes. Transcriptionally arrested primary hepatocytes from *Tnfr*1° mice were found to be protected from TNF-induced apoptosis (81). In addition, no symptoms of hepatic failure were detected in *Tnfr*1° mice treated with TNF plus D-galactosamine. These results demonstrate that lack of TNFR1-signaling is a necessary but also sufficient condition that protects transcriptionally arrested hepatocytes from TNF-induced apoptosis. The authors hypothesize that TNF may induce in hepatocytes two opposing effects that balance each other under normal conditions, i.e., a protective pathway that depends on *de novo* RNA synthesis from inducible genes and an apoptotic path-
way employing already available messenger RNA (80). In this model, translational inhibitors block both pathways, whereas transcriptional inhibitors interfere only with TNF-mediated apoptosis, thus explaining the aforementioned findings.

If LPS is given to mice in the absence of a sensitizing agent like d-galactosamine, the lethal dose for wild-type animals increases by five orders of magnitude to more than 600 μg instead of a few nanograms (40). Under these conditions, Tnfr1° mice are not protected, whereas Tnfr2° mice show a slightly reduced sensitivity compared to wild-type controls (42). It seems that high-dose LPS lethality is mediated by very different processes compared to TNF-mediated cytotoxicity. In fact, other gene-knockout mice have been found to be resistant to LPS-induced septic shock, such as IL-1β-converting enzyme (ICE)-deficient mice (82), which are defective in the production of mature IL-1β and in the release of IL-1α. Although ICE-deficient mice demonstrated signs of endotoximia after LPS treatment, these symptoms were milder than those observed in wild-type and could perhaps be attributed to the effects of TNF in these animals. In addition, intercellular adhesion molecule (ICAM-1) deficient mice also resist high doses of LPS (83). Interestingly, these mice are not protected from lethality when challenged with LPS in the presence of d-galactosamine and thus show a complementary phenotype to TNFR1-deficient mice. Thus, in the presence of d-galactosamine, LPS-induced and TNF-mediated hepatoxocity is lethal but suppressed by inhibitors of TNF processing or blocked in Tnfr1° mice. High doses of LPS, however, upregulate ICAM-1 expression on vascular endothelial cells which leads to reperfusion injury as the main pathological event, characterized by aggregation and extravasation of neutrophils following transient ischemia.

**Activation-Induced Cell Death Mediated by TNFR1 and Fas**

Fas, another receptor of the TNFR family, shares homology with TNFR1 in a cytoplasmic death domain (12,13) and also induces apoptosis on ligand binding. Similar to TNF, the Fas ligand is a type II transmembrane molecule expressed on activated T-cells, induces aggregation of the Fas receptor on target cells, and can be released by a protease from the cellular membrane into the circula-
tion where it is active as a homotrimer. Fas and TNFR1, however, utilize different intracellular signaling pathways to induce cell death (11,14,15,84). Both signal transduction pathways operate independently from each other, as analyzed in Tnfr10 mice and Fas-deficient lpr mice (85). The combination of both mutations in double mutant Tnfr10, lpr mice, however, revealed that both pathways can partially compensate for each other in activation-induced cell death (AICD) (86). Inactivation of both pathways in Tnfr10, lpr mice accelerated most features of the autoimmune disease characteristic for C57BL/6-lpr mice. Thus, Tnfr10, lpr mice exhibited high mortality accompanied by greatly accelerated lymphadenopathy and autoantibody production. Histological analysis revealed massive mononuclear cell infiltrates in liver, kidney, lung, and knee joints in these mice. In addition, Tnfr10, lpr mice suffered from a high incidence of arthritis, characterized by synovial cell hyperplasia and mononuclear cell aggregates in the periarticular tissue, whereas no arthritis occurred in Tnfr10 or C57BL/6-lpr mice. Interestingly, arthritis in Tnfr10, lpr mice was accompanied by only minor cartilage destruction analyzed at 20 wk of age. These results suggest that during the early phase of an autoimmune disease, signaling by TNFR1 may be beneficial in mediating AICD in mononuclear cells, and thereby preventing tissue infiltration. At a later stage, deleterious effects may dominate, such as induction of collagenase and stromelysin resulting in tissue damage (87,88).

**Ligand Passing Versus Cooperative Signaling Model**

To account for the different biological functions of both TNF receptors so far identified, Tartaglia et al. (28,89) proposed a ligand passing model, in which TNFR2 is especially capable of transiently binding TNF at low concentration because of its higher binding affinity and much faster binding kinetics compared to TNFR1. This enhances the local concentrations of TNF on the cell surface to a level required for binding to neighboring TNFR1 molecules that are usually coexpressed on the cell surface. TNFR1 binds TNF trimers with much lower off-rates compared to TNFR2 and undergoes a conformational change upon binding that brings its cytoplasmic chains in contact. Depending on which additional factors are present in the cell (90–92), different signal transduction pathways may be
induced, ranging from proliferation in fibroblasts to cytotoxicity in transcriptionally arrested hepatocytes.

An alternative model for TNF receptor signaling by the transmembrane form of TNF was recently proposed by Grell et al. (20). They noted that agonistic antibodies to human TNFR2 were much more potent in inducing responses in various human cell lines than soluble human TNF, the natural ligand. They related this difference to the different binding kinetics of both ligands to TNFR2, which are fast for soluble TNF and slow for the agonistic antibody, and suggested that the latter mirrors the in vivo activity of transmembrane TNF as the prime activating ligand for TNFR2 during intercellular contacts. Instead of using agonistic antibodies, Decoster et al. (93) utilized an engineered noncleavable transmembrane form of TNF to study the interaction of membrane-bound TNF to both TNF receptors. They also found that membrane-bound TNF, but not soluble TNF, is capable of inducing signaling by TNFR2. However, no preference of membrane-bound TNF for TNFR2 was noted, contrary to the finding of Grell et al. (20) using agonistic antibodies. Nevertheless, the binding kinetics of membrane-bound TNF to its receptors on neighboring cells is determined by the length of the intercellular contact, and not by the dissociation constant for soluble TNF. Thus, prolonged interaction of membrane-bound TNF may trigger signals via TNFR2 which are not inducible by soluble TNF. In addition, cooperative signaling through both receptors may create novel responses in target cells (20). It seems that the ligand-passing and the cooperative signaling model are not mutually exclusive and that both may have physiological relevance in vivo.

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Chapter 6

Cooperation Between the TNF Receptors Demonstrated by TNF Receptor Knockout Mice

Eve Shinbrot and Mark Moore

Tumor necrosis factor (TNF) is a potent cytokine that mediates many biological events, including proliferation of fibroblasts and T-cells, induction of NF-κB, cytotoxicity, tumor necrosis as well as antiviral, inflammatory, and immunoregulatory responses. TNF has been implicated as a central mediator of septic shock as well as graft-vs-host disease, arthritis, and several autoimmune disorders. There are two related TNF molecules, TNFα (tumor necrosis factor or cachectin) and TNFβ (lymphotoxin). TNFα is produced mainly by T-cells, macrophages, and mast cells, whereas TNFβ is produced by activated lymphocytes. TNFα and β mediate their actions by binding to two distinct cell surface receptors, TNF-R1 (55 kDa also known as TNFRβ and TNF-R55) and TNF-R2 (75 kDa, also known as TNFRα, and TNFR-75). Both receptors are found on most cell types.

Both TNF receptors are members of a receptor superfamily whose members include the type 1 membrane proteins CD30, CD40, NGFR, OX40, 4-1BB, PV-T2, TNFR-RP, CD27, and Fas (1). They are activated by ligand binding leading to receptor oligomerization, similar to tyrosine kinases (2). Their extracellular domains consist of highly conserved cysteine-rich pseudorepeats.
The highly conserved placement of the cysteine residues suggests a common structural pattern. The cytoplasmic domains are usually small with little sequence homology among members. Consistent with their family members, TNF-R1 and TNF-R2 are most homologous in their extracellular domains, made up of highly conserved cysteine-rich domains. In contrast, their intracellular domains do not show extensive sequence homology suggesting that the receptors may activate different signaling pathways (2). Although the cytoplasmic domains of TNF-R1 and R2 are not homologous, the cytoplasmic domain of TNF-R1 is homologous to another family member, Fas (reviewed in ref. 1). TNF-R1 and Fas share homology in a 65-residue region, known as the death domain, that has been shown to be important in the process of apoptosis (programmed cell death). The death domain of TNF-R1 has been identified as an 80 amino acid domain within the intracellular region (3). Mutations within this region not only alter cell death signaling, but also interfere with the antiviral activity of TNF-R1 and are thought to be important in mediating many of the cellular responses induced by activation of TNF-R1.

Several studies have sought to elucidate the individual roles of each receptor. Studies using antibodies have indicated that TNFR-1 is involved in cell death, antiviral activity, and cytokine production, whereas TNFR-2 is involved in T-cell development and the proliferation of cytotoxic lymphocytes (3-6). Although only TNF-R1 contains a death domain, some reports have suggested both receptors are involved in the process of apoptosis (7). However, the precise function of the two receptor types is largely unknown. Since the intracellular domains of TNF-R1 and R2 are not homologous, it is predicted that they activate different signaling pathways. This is supported by recent reports identifying specific intracellular proteins that bind to either TNF-R1 or R2. One such protein, TNF-R1-associated death domain protein (TRADD) has been shown to interact specifically with an intracellular domain of TNF-R1, but not TNF-R2 (8). Overexpression of TRADD induces cellular responses attributed to TNF-R1, such as apoptosis and NF-κB activation. Proteins that specifically interact with TNF-R2 have also been found. Two related proteins, TNF receptor associated factor 1 and 2 (TRAF1 and TRAF2) have been shown to associate with sequences in the cytoplasmic domain of TNF-R2 (9). The characterization of
proteins that bind specifically with TNF-R1 or TNF-R2 supports the idea that the two receptors activate different signaling cascades leading to different responses.

Although some studies have implied distinct functions for TNF-R1 and R2, their actual roles have not yet been resolved. One approach to determining the individual roles of TNF-R2 and R1 is targeted disruption of each of the genes. Our lab generated mice deficient in TNF-R2 (10), whereas others have generated TNF-R1 (11,12) deficient mice. A comparison of the phenotype of mice deficient in TNF-R1 and TNF-R2 may help distinguish their individual roles.

**Development of the Immune Response**

TNF and its receptors have been implicated in the process of thymocyte, T- and B-cell development. Since TNF-R2 is expressed on lymphocytes and is thought to be involved in T-cell development, we examined the TNF-R2 mice for abnormalities in T-cell development (10). Surprisingly, T-cells develop normally in these mice (Fig. 1). A similar result was seen with the TNF-R1-deficient mice (11,12). They did not show any difference in thymocyte development or clonal deletion of potentially self-reactive T-cells. The TNF-R2 homozygous mice were examined for any differences in their T-and B-cell populations using cell-surface markers and cytofluorometric analysis (10). No differences were seen in the homozygous mice compared to wild-type mice (Fig. 2). Next, our lab studied T-cell activity by mixed-lymphocyte response, alloctotoxic response, and protein-antigen-specific responses. No differences were seen in the TNF-R2 homozygous mice compared to wild-type mice. The response of B-cells to lipopolysaccharide (LPS) challenge was also analyzed. The B-cells were still responsive to LPS challenge. This is similar to the result seen in the TNF-R1-deficient mice (11,12). Normal populations of T- and B-cells were seen in lymphatic organs as evaluated by cytofluorometric analysis. These results indicate that the simultaneous presence of both TNF-R1 and R2 is not required for normal lymphocyte development. One possibility is that either TNF-R1 or TNF-R2 can compensate for the loss of the other in the deficient mice. However, in the absence of both TNF receptors, lymphocytes may develop abnormally. This possibility can be addressed by the analysis of mice deficient in both TNF-R1 and TNF-R2. Recently, our lab has
Fig. 1. Thymocyte analysis in TNF-R2+/+ mice. The expression patterns of the indicated cell-surface markers were analysed in TNF-R2+/+ and TNF-R2−/− mice. Methods: Single-cell suspensions were prepared from the thymus of 6-week-old mice and stained with FITC-conjugated antibodies specific for either CD8, or CD8 and CD4 (Becton-Dickenson, San Jose, CA) and PE-conjugated anti-CD4 (Becton-Dickinson), αβ TCR (Boehringer-Mannheim, Indianapolis, IN), γδ TCR (Boehringer-Mannheim, Indianapolis, IN), or HSA (Pharmingen, San Diego, CA). Cells were fixed in 1% paraformaldehyde prior to analysis on a FACScan (Becton-Dickinson).
Fig. 2. T-cell receptor selection in TNF-R2<sup>−/−</sup> mice. Thymocytes were analyzed for the expression of V<sub>β</sub>5 (which should be deleted on the Balb/c background) and V<sub>β</sub>8 (which should undergo positive selection).

bred mice deficient in both TNFR genes (manuscript in preparation). The double-receptor ablated mice were examined for differences in thymocyte development and lymphocytic populations using cytofluorometric analysis. Surprisingly, their T- and B-cell populations appear normal, with no major differences detected. This indicates that another molecule compensates for the loss of either TNF-R1 or TNF-R2 in the receptor-deficient mice. One candidate is lymphotoxin receptor (LT-βR). When this receptor is disrupted, the mice display abnormal peripheral lymphocyte development (13).
Splenic Morphology and Antibody Response

Recently, the TNF receptor knockout mice have been used to determine the role of TNF in antibody responses. TNFR1 was shown to be important in the morphological and functional differentiation of splenic B-cells. The TNF R1-deficient mice failed to develop germinal centers in response to immunization with sheep red blood cells, whereas the TNF R2-deficient mice had morphologically normal germinal centers (14,15). TNF R1 was also shown to be involved in the differentiation of follicular dendritic cells and T-cell-dependent antibody responses (15).

Sepsis

TNF has been shown to be involved in tissue damage during sepsis. Furthermore, antibodies against TNF have been shown to protect against LPS-induced lethality (5,16). To determine the role of TNF in LPS-induced lethality and tissue necrosis, we challenged the TNF-R2-deficient mice with LPS. The TNF-R2-deficient mice showed decreased sensitivity to LPS in the midrange dose, but no difference in higher doses (Table 1). Next they were challenged with both LPS and D-galactosamine, which enhances LPS sensitivity. No significant difference in sensitivity was seen between the wild-type and TNF-R2-deficient mice. This is in contrast to the TNF-R1-deficient mice (12). The TNF-R1-deficient mice were resistant to the lethal effect of low doses of LPS after sensitization with D-galactosamine, but did remain sensitive to higher doses of LPS (12). Taken together, these results indicate that TNF-R1 may play a greater role than TNF-R2 in mediating the toxic effects of LPS. However, TNF-R1-deficient mice were not insensitive to high doses of LPS. Therefore, either both receptors contribute to LPS-induced shock, or other cytokines are also involved, such as IFNγ and IL-6.

Tissue Necrosis

TNF has been shown to be involved in the process of tissue necrosis. Therefore, we analyzed the TNF-R2−/− mice for their response to TNF-induced tissue necrosis (10). When TNF was injected under the skin of TNF-R2-deficient homozygous mice, a
Cooperation Between TNF Receptors

Table 1
The Effects of LPS and TNF-α on TNF-R2-/- Mice

| LPS, μg | D-Galactosamine, mg\(^a\) | TNF-α\(^b\), μg | TNF-R2\(^{+/+}\) | TNF-R2\(-/-\) |
|---------|-----------------------------|-----------------|-----------------|----------------|
| 800     | —                           | —               | 5/5             | 5/5            |
| 600     | —                           | —               | 9/10            | 4/10           |
| 500     | —                           | —               | 5/5             | 2/5            |
| 300     | —                           | —               | 3/4             | 1/4            |
| 100     |                             | —               | 0/4             | 0/4            |
| 10      | 20                          | —               | 5/5             | 5/5            |
| 1.0     | 20                          | —               | 5/5             | 5/5            |
| 0.1     | 20                          | —               | 9/10            | 9/10           |
| 0.01    | 20                          | —               | 6/7             | 6/7            |
| 0.001   | 20                          | —               | 1/5             | 2/5            |
| 10      |                             | 10/10           | 1/11            |
| 15      |                             | 4/4             | 4/4             |

\(^a\)Mice were injected ip with the indicated amount of LPS (Salmonella abortus equi, Sigma Cat. no. L-5886) with or without D-galactosamine (20 mg/mouse) in 0.2 mL Hank’s Balanced Salt Solution.

\(^b\)Recombinant murine TNF-α (Genentech) was injected intravenously.

drastic reduction in tissue necrosis was seen compared to wild-type mice. Both visual inspection and histological analysis revealed that the TNF-R2-/- mice were only mildly affected, whereas the wild-type mice displayed ulcerated areas that extended deep into the dermis (Fig. 3). Although the TNF-R2-/- mice are less sensitive to TNF induced necrosis than wild-type mice, they were not resistant to iv injections of TNF. The TNF-R2-deficient mice survived a 10-μg injection of TNF, a lethal dose for wild-type mice, but died in response to high doses of TNF. It is possible that TNF-R1 mediates the lethality induced by high concentrations of TNF in the TNF-R2-deficient mice. The TNF-R1-deficient mice appear to be very resistant to TNF, but the double-receptor-ablated mice have not yet been analyzed for their response to TNF. It will be interesting to see if the double-receptor-ablated mice are more resistant to TNF than either single-receptor-deficient mouse. The comparison of those results will help unravel the role of each receptor in the toxic effects of tissue necrosis and system lethality.
Signal Transduction

The signaling pathways of TNF-R1 and TNF-R2 have not yet been identified. It has been proposed that TNF-R1 is primarily responsible for cytotoxicity, manganese superoxide dismutase induction, and NF-κB induction, whereas TNF-R2 is responsible for thymocyte and T-cell proliferation (6). The differences in cellular responses to TNF-R1 and R2 may be explained by the ligand passing model (17). In this model, the role of TNF-R2 is primarily to concentrate TNF and pass it to TNF-R1. TNF-R2 is not involved in intracellular signaling, just ligand passing, whereas TNF-R1 is important for signal transduction. According to this model, at low concentrations, TNF preferentially binds TNF-R2 and is passed to TNF-R1. However, at high TNF concentrations, TNF will bind
TNF-R1 directly (17). This model is directly supported by our analysis of the TNF-R2-deficient mice (10). The TNF-R2-deficient mice are resistant to doses of TNF that induce necrosis in wild-type mice. However, when the concentration of TNF is significantly increased, the TNF-R2-deficient mice are no longer resistant. It is possible that in the absence of TNF-R2, low concentrations of TNF are unable to bind to TNF-R1, protecting the TNF-R2-deficient mice from tissue necrosis. At high concentrations, TNF can bind TNF-R1, which then mediates its toxic effects. The ligand-passing model was further supported in a study that compared fibroblasts isolated from TNF-R1-deficient mice to wild-type mice (18). They found that TNF-R1 had an essential role in many cellular functions, such as upregulation of ICAM-1, VCAM-1, CD44, and MHC class 1 expression, fibroblast proliferation, and NF-κB activation. When they stimulated fibroblasts isolated from TNF-R1-deficient mice with TNFα, they were unable to elicit a proliferative response, activate cell adhesion, IL-6 or GM-CSF release, or activation of NF-κB. It was also shown that in the absence of TNF-R1, TNF-R2 was unable to mediate responses attributed to TNF receptors, indicating TNF-R2 plays an accessory role. In the TNF-R1-deficient mice, TNF is bound by TNF-R2, yet the absence of TNF-R1 prevents cellular responses to TNF from being generated, which is consistent with the ligand-passing model. The same report further supported the ligand-passing model by examining the TNF response of wild-type fibroblasts (18). They found that human TNFα (only binds TNF-R1) gave a weaker cellular response than that caused by mouse TNFα (binds both mouse TNF-R1 and TNF-R2), which is consistent with the knockout mouse data. The availability of mice deficient in TNF-R2 and double-receptor-ablated mice provides valuable tools for further testing of this model.

Conclusions

The generation of mice deficient in TNF-R1 and TNF-R2 has helped to elucidate the role of these receptors in several cellular processes. However, many questions remain unanswered. In a surprising result, many systems in which TNF and its receptors were thought to be essential are normal in the receptor-deficient mice.
These unexpected results have complicated the proposed roles of these receptors.

Analysis of TNF-R1- and TNF-R2-deficient mice has confirmed a role for each receptor in host defenses. Evaluation of mice deficient in TNF-R1 indicates that receptor is involved in non-specific immunity against microorganisms, whereas TNF-R2 does not play a major role (11,12). Examination of mice deficient in TNF-R2 indicates that receptor is important in TNF-induced tissue necrosis (10).

Previous studies have indicated TNF is involved in the process of thymocyte development. Constitutive expression of TNFα has been described in the mouse thymus (19). Furthermore, stimulation of TNF-R2 has been shown to induce thymocyte proliferation (6). TNF-R1 was thought to be involved in T-cell selection based on studies that used antagonistic (blocking) antibodies to inhibit TNF-induced apoptosis (16). Therefore, it was expected that the absence of either TNF-R1 or TNF-R2 would affect thymocyte development or selection. Surprisingly, this was not the case. Lymphocyte populations were normal in both TNF-R1- and TNF-R2-deficient mice (10,11,20). This argues that neither receptor is essential for thymocyte selection or T-cell development. One explanation is that TNF and its receptors do not influence T-cell development, yet other cytokines and their receptors are more crucial. Alternatively, other cytokines and receptors may be compensating for the loss of TNF receptors in the deficient mice. This is consistent with the redundancy seen in the biological effects of TNF and other cytokines. Many effects of TNF are shared by other cytokine-receptor systems. This redundancy of functions is biologically advantageous, whereby an organism can survive the loss of one or more genes by compensating with genes that have overlapping functions.

Analysis of the TNF-R1- and TNF-R2-deficient mice has supported the ligand-passing model of TNF signal transduction (17). The response of TNF-R2-deficient mice to TNF lends support to this model (10). At high TNF concentrations, TNF-R1 is able to bind TNF and mediate its cellular responses. However, in the absence of TNF-R2, low concentrations of TNF are unable to affect any cellular changes. This indicates TNF-R2 is necessary to concentrate and bind TNF and then pass it to TNF-R1, which mediates its cellular responses. Fibroblasts isolated from the TNF-R1-
deficient mice also support the ligand-passing model (18). In the absence of TNF-R1, TNF-R2 is unable to mediate any cellular responses to TNF. This model may be further tested by comparing the cellular responses to TNF in the double-receptor-deficient mice. It would be expected that in the absence of both TNF receptors, the mice would be resistant to all concentrations of TNF. If the mice still show TNF sensitivity at high TNF concentrations, it would indicate there is another unidentified receptor that is able to bind TNF and mediate its cellular responses.

The receptor-deficient mice have supported some theories on the role of TNF-R1 and TNF-R2, whereas others have become less clear. However, many questions remain. The role of TNF receptors in an organism’s defense to a variety of viral and bacterial pathogens still remains to be explored. Comparison of the response of TNF-R1, TNFR-R2, and double-receptor-deficient mice to various pathogens (both bacterial and viral) will help identify the role of each receptor in host immunity. The results of these studies may help determine potential interventions to use in defense against various pathogens.

The availability of TNF-R1, TNF-R2, and double-receptor-deficient mice provides materials for in vitro analysis of the function of TNF receptors. Cells may be harvested from each of the receptor-deficient mice, and the role of each receptor in response to various stimuli compared. Most interestingly, various domains of a receptor can be transfected back into cell lines made from mice lacking that receptor. In this way, the function of that domain may be analyzed. The signaling pathways utilized by each receptor can be examined using chimeric proteins consisting of labeled cytoplasmic domains of either receptor transfected into cell lines isolated from the double-receptor-deficient mice. Cell lines derived from the double-receptor-deficient mice can also be used to find other unidentified members of the TNF receptor family.

Many questions remain regarding the individual roles of TNF-R1 and TNF-R2. The availability of mice deficient in each of these receptors along with double-receptor-deficient mice allows for both in vivo and in vitro experiments to determine the specific function of each receptor. The results of these experiments might explain the redundancy in the TNF system and possibly point to new therapeutics in the management of TNF-related pathologies.
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Chapter 7

Immunodeficiency of Tumor Necrosis Factor and Lymphotoxin-α Double-Deficient Mice

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Introduction

Tumor necrosis factor (TNF) is a proinflammatory cytokine involved in host defense and pathogenesis of various diseases (1,2). Soluble trimeric TNF ligands bind to TNF receptor 1 (TNFR1) and TNFR2, leading to receptor homotrimer formation (3) and consecutive triggering of various biological responses such as proliferation, cytotoxicity and apoptosis in target cells. Lymphotoxin-α (LTα), which like TNF belongs to the family of TNF-like ligands (4), can bind to the same receptors as TNF. Consequently, administration of TNF and LTα leads to similar biological responses in vivo and in vitro. In mice, TNF is mainly expressed in macrophages and T-cells, whereas LTα expression is confined to T- and B-cells. The redundancy in receptor binding and the overlapping expression pattern of TNF and LTα renders a clearcut dissection of their function rather difficult. Therefore, the importance of both ligands to TNF receptor-mediated effects tends to be obscured in single gene knockout mice, because the loss of one ligand may be compensated by the remaining one. Since both genes...
are very closely linked and map within the major histo compatibility complex (MHC), single mutations cannot be crossed together. Rather, mice deficient for both ligands have to be generated by simultaneous targeting of both genes. Prominent functions of TNF include central roles in endotoxic shock, host defence, and tumor—or parasite—induced cachexia. In addition TNF plays an important, yet undefined role in the pathogenesis of autoimmune diseases (5–9). LTα deficiency has been shown to lead to aberrant development of the spleen and absence of lymph nodes (LN) (10–11). Recently a third ligand, LTβ, was cloned and characterized as present on the cell surface as a LTα/2LTβ heterotrimer, able to bind to the newly described LTβ specific receptor (12–14).

To investigate the roles of TNF and LTα in the development and function of the immune system, the Tnf and Lta genes were simultaneously inactivated in mice by homologous recombination (15). The mutant mice develop normally and are fertile, but have no LN and Peyer’s patches (pp), reduced mucosal IgA producing plasma cells, and an undifferentiated splenic structure. The alypophlasia is accompanied by a marked B-lymphocytosis and hypogammaglobulinemia. Whereas the antibody response to sheep red blood cells (SRBC) does not show a IgM/IgG class switch, the humoral response to vesicular stomatitis virus (VSV) infection is normal. The ex vivo cytotoxic T-cell responses are normal against vaccinia virus (VV), however reduced against lymphocytic choriomeningitis virus isolates (LCMV-ARM) and LCMV-WE. The host defense of mutant mice against Listeria monocytogenes is severely impaired and infected mice succumb to massive bacteremia. In conclusion, the combined inactivation of Tnf and Lta affects the immune system at the structural as well as the functional level.

**Combined Genetic Inactivation of Tnf and Lta**

Simultaneous inactivation of Tnf and Lta genes was achieved in GS1 embryonic stem (ES) cells (15) with a linearized replacement vector encompassing essential parts of both genes (16). The null mutation of both genes was introduced into the mouse germ line by blastocyst injection using established methods. Inactivation of the Tnf and Lta loci was shown at the level of the corresponding
Immunodeficiency of TNF and LTα transcripts. In contrast to control mice, no transcripts for TNF and LTα could be demonstrated from concanavalin A (Con A)-stimulated splenocytes. The generated TNF/LTα double-deficient mice represent the first mice devoid of TNF- and LTα-dependent signaling. Homozygous mutant mice are viable, develop normally, and are fertile. The macroscopic phenotype of TNF/LTα double-deficient mice consists in a slightly reduced birth weight and reduced body weight in adult mice and absence of LNs.

**Resistance to Endotoxic Shock**

TNF has been shown to be a central mediator of endotoxemia and neutralizing antibodies to TNF could protect mice from endotoxic shock (17). We therefore assessed our TNF/LTα double-deficient mice in the lipopolysaccharide/d-galactosamine (LPS/d-GalN) model. Although wild-type mice succumbed to cardiovascular shock and acute hepatic failure at 1 μg LPS, in the presence of 20 mg of d-GalN, TNF/LTα double-deficient mice survived a 100 times higher dose of LPS (Table 1). Histology confirmed a complete protection in mutant mice, whereas wild-type mice showed distinct centrilobular liver necrosis (not shown). No toxicity was observed after administration of d-GalN alone. Genetic inactivation of TNFR1 (18,19), but not TNFR2 (20), provides resistance toward LPS-induced endotoxic shock after sensitization with d-GalN. The fact that T-cells represent the main source of LTα in adult mice and that SCID mice, devoid of T- and B-cells, are responsive in the LPS/d-GalN model of shock (21), precludes an important role of LTα in the pathogenesis of LPS-induced endotoxic shock. Our data, which show complete protection of TNF/LTα-deficient mice from LPS/d-GalN induced hepatic failure and endotoxic shock, confirm the central role of TNF in this endotoxic shock model.

**Alymphoplasia and Absence of Lymphoid Follicles in the Spleen**

It has been shown that administration of TNF antibodies caused atrophy of thymus and lymph nodes (22). We therefore investigated whether these alterations also occurred in our TNF/LTα-deficient mice. The size and microscopic structure of mutant thymi, however, were normal. In addition the percentage of single CD4+, CD8+, and
CD4/8 double-positive thymocytes was not altered in mutant mice (not shown). On the other hand, mutant mice were completely devoid of LNs and PPs. Microscopic analysis of the tissues at the sites of LNs revealed the presence of lymphatic vessels, but no analog of lymphoid organs were detectable (not shown). Hence, the only peripheral lymphoid organ present in mutant mice is the spleen, which has a normal weight, size and cellularity, but exhibits an altered lymphocyte composition and microarchitecture visualized by the expression of the leukocyte endothelial cell adhesion molecule (LECAM) (Fig. 1). The altered splenic microarchitecture is accompanied by a loss of clearly defined T- and B-cell zones and a marked reduction of adhesion molecule expression such as, mucosal vascular addressin (MAdCAM-1), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1) and Mac-1 in the marginal zone and red pulp area of mutant mice (data not shown). LTα-deficient mice revealed similar alterations in spleen microarchitecture and absence of LNs (11). As TNFR1- and TNFR2-deficient mice develop lymph nodes, signaling through the newly discovered LTβ-receptor is likely to be responsible for morphogenesis of LNs. Together, this defines the LTα/LTβ-receptor interaction as the cognate ligand-receptor signaling necessary for proper spleen and LN development. Systemic absence of LNs and PPs and presence of ill-defined splenic follicles have also been reported for the aly mouse (23). These overlapping phenotypic traits suggest that the aly gene product is involved in the LTα/LTβ-receptor signaling pathway.

Table 1
Mortality of Wild-Type, Heterozygous, and Homozygous Mutant Mice After ip Administration of LPS/D-GaIN

| Treatment | Mortality, dead |
|-----------|----------------|
| D-GaIN, mg/mouse | LPS, µg/mouse | ++/+ | +/− | −/− |
| 20         | 100           | 4 of 4 | 4 of 4 | 0 of 4 |
| 20         | 10            | 4 of 4 | 4 of 4 | 0 of 4 |
| 20         | 1             | 6 of 6 | 6 of 6 | 0 of 6 |
| 20         | 0.1           | 3 of 6 | 2 of 6 | 0 of 6 |
| 20         | 0             | 0 of 3 | 0 of 3 | 0 of 3 |
B-Lymphocytosis

We further asked, whether the leukocyte counts in peripheral circulation were increased in the context of alymphoplasia, altered splenic microarchitecture and reduced adhesion molecule expression. The hemogram showed indeed a fourfold increase of total leukocyte counts (Table 2). Cytofluorometric analysis revealed 65 ±
4% B220-positive B-cells in mutant mice compared to 44 ± 2% in wild-type mice, and a relative decrease of CD3-positive T-cells from 45 ± 3% in wild-type to 25 ± 3% in mutant mice (not shown). Considering the absolute lymphocyte counts, mutant mice have significantly increased peripheral lymphocyte counts with a sixfold increase of B-cells and a twofold increase in T-cells. The distinct lymphocytosis observed in TNF/LTα-deficient mice is also found in the LTα-deficient mice (11), but not in aly mice (23). Therefore the lack of LNs and PPs cannot be invoked as the explanation for this observation, as all three mutants are devoid of these peripheral lymphatic organs. Adhesion molecules play a critical role in leukocyte emigration (24) and it has been shown that TNF can induce MAdCAM-1, ICAM-1 and VCAM-1 (25). The complete absence of splenic MAdCAM-1 expression combined with the marked reduction of ICAM-1, VCAM-1 and Mac-1 expression of mutant mice might be responsible for the reduced margination of lymphocytes and, as a consequence, for the observed lymphocytosis.

**IgA Deficiency**

PPs represent the adequate environment for differentiation of precursors of IgA positive plasma cells (26). Immunohistochemical analysis revealed dramatically reduced numbers of IgA positive plasma cells in ileum (Fig. 2A,B) and duodenum (not shown) of the mutants. A similar mucosal IgA deficiency as in the TNF/LTα-deficient mouse was also seen in IL-6 deficient and in aly mice. In aly and the TNF/LTα-deficient mice, this deficiency is likely owing to the absence of PPs and might lead to decreased mucosal immu-
Fig. 2. Reduced intestinal IgA producing plasma cells in mutant mice. Ileum from a wild-type mouse (A) showing intensely IgA-positive plasma cells in the lamina propria in contrast to the mutant mouse (B). The epithelial cells are weakly IgA positive (400x).

IgA deficiency is the most common form of immundeficiency in man and its incidence is increased in patients with allergies, autoimmune, and gastrointestinal tract diseases (29). Whether the absence of these pathologies in the mutant mice is the result of the specific-pathogen free housing conditions is currently under investigation.
Hypoimmunoglobulinemia and Antibody Response

We then asked to what extent the basal immunoglobulin synthesis and the humoral immune response was affected in the context of alymphoplasia, disorganized spleen, and B-lymphocytosis. We found a distinct reduction of IgM and all IgG isotypes; and in accordance with the strong reduction of mucosal IgA producing plasma cells, very low IgA levels in the serum. The antibody response was measured after immunization with VSV or SRBC. Neutralizing immunoglobulin titers against VSV were determined at different time points, on d 4 for the T-cell-independent IgM response and on d 8 and 12 for the strictly T-cell-dependent IgG response. Surprisingly, no differences between mutant and wild type neutralizing IgM and IgG titers against VSV were detectable and the antibody response correlated with the clearance of VSV (Fig. 3C,D). However, the SRBC-specific antibody response was markedly altered (Fig. 3A,B). Mutant mice were unable to mount a primary IgG1 response against SRBC.

The general reduction of basal immunoglobulin levels is likely caused by the absence of LNs and PPs. The reduction in the basal immunoglobulin levels and the SRBC specific deficiency in IgG1 class switch is reminiscent of similar defects seen in aly, CD40, and CD40 ligand-deficient mice (23,20,31), and suggests a common defect in all three mutant mice provoked by different mutations in the same or convergent signaling pathways of isotype switching. The fact that CD40 ligation has been shown to induce surface LTα on human B-cells (32) and that TNF/LTα-deficient mice show an isotype switch deficiency to the T-cell-dependent antigen SRBC, strongly suggests a costimulatory function of LTα in T/B-cell interaction necessary for efficient T-cell dependent isotype switching. The discrepancy in T-cell dependent immuneresponses against SRBC and VSV suggests alternative costimulatory pathways in T/B-cell interaction which might be TNF/LTα-dependent for the SRBC, but TNF/LTα independent for the VSV response.

Susceptibility to Viral Infections

TNF has been shown to exert variable effects on antiviral activity in vivo and in vitro (33–38). Wild-type and mutant mice were therefore tested for their cytotoxic T-lymphocytes (CTL) responses against VV-WR, LCMV-ARM and LCMV-WE. The primary in vivo CTL
response from mutant mice infected intravenously with VV was slightly reduced (not shown) but strongly reduced in the case of LCMV-ARM and LCMV-WE (Fig. 4A,B). Nevertheless, secondary CTL responses (d 8) against LCMV-ARM and LCMV-WE are present, although still reduced compared to control mice (Fig. 4C,D). These present CTLs were able to clear the virus from spleen and liver by d 20 (not shown). If mice were infected into the foot pad (3000 PFU), secondary CTLs were present with LCMV-ARM, but not with LCMV-WE (Fig. 4E,F) demonstrating the lack of LCMV-WE specific
Fig. 4. Primary and secondary CTL responses against LCMV-ARM and LCMV-WE. Each bar represents the mean from two mice. All data points are mean values of duplicate assays. Primary CTL responses (d 8) after iv priming with (A) $2 \times 10^3$ PFU LCMV-ARM and (B) $2 \times 10^2$ PFU LCMV-WE. Secondary CTL responses (d 8) after iv priming with (C) $2 \times 10^3$ PFU LCMV-ARM and (D) $2 \times 10^2$ PFU LCMV-WE. Secondary CTL responses after footpad injection of (E) $3 \times 10^3$ PFU LCMV-ARM and (F) $3 \times 10^3$ PFU LCMV-WE. Spontaneous lysis of all used target cells in absence of effector cells was <15%. Unspecific lysis of uninfected target cells was <20% in all assays.

memory CTLs. These different results may be the result of the different growth kinetics of both virus strains and the difference is accentuated if the viruses are injected into the hind foot pads. In the latter case, the faster replicating LCMV-WE might have led to CTL exhaustion owing to faster appearance and increased virus load in the spleen com-
pared to the wild-type mouse. This faster appearance might be due to the lack of peripheral LNs allowing efficient virus translocation to the spleen. Therefore, mice lacking peripheral LNs might be prone to CTL exhaustion and become viral carriers.

**Susceptibility to Bacterial Infections**

TNF previously has been shown to play an important role in the defence against intracellular bacteria such as *L. monocytogenes* and *Mycobacterium bovis* (18,19,38). Therefore, mutant and wild-type mice were infected with low titers of *L. monocytogenes*. In contrast to wild-type mice, mutant mice could not control this dose of *L. monocytogenes* and died from listeriosis characterized by strongly increased titers of *L. monocytogenes* in liver and spleen and large necrotic liver lesions with boundaries of heavily infected hepatocytes (Fig. 5). Infection with the Gram-positive, facultative intracellular bacterium *L. monocytogenes* has been shown to induce cell-mediated immunity and efficient clearance of this pathogen critically depends on proper T-cell and macrophage function (39,40). Genetic inactivation of T-cell receptor (TCRα), TCRβ, MHC class I and II, INFγR, TNFR1 and IL-6 (for a review, see ref. 41) have defined a critical network of molecules that play a crucial role in macrophage activation, extravasation, and granuloma formation. The sensitivity toward low titer infection with *L. monocytogenes* seen in the TNF/LTα-deficient mice is comparable to that of TNFR1-deficient mice and confirms a crucial role for TNF in the defense against *L. monocytogenes* infections. The fact that perforin (42) and MHC class I deficiency (43) does not lead to a drastic increase in sensitivity to primary *L. monocytogenes* infection shows that other cell populations than T-cells, like neutrophils and macrophages (39,44) are critical for an efficient primary host defence against *L. monocytogenes*. The antilisterial activity of mutant macrophages might be impaired at least at two levels. First, the lack of TNF, which is an important costimulator of IL-12 for INFγ production by natural killer (NK) cells, might lead to a decreased activation of macrophages and a reduced development of an efficient TH1 response (45,46). Second, phagocytosed *L. monocytogenes* might not be killed efficiently. The second assumption is supported by the
Fig. 5. Liver section after infection with *L. monocytogenes*. Liver sections (H&E) of wild-type (A) and mutant mice (B) 6d after *L. monocytogenes* infection. In the wild-type section, small granuloma like infiltrates are present. In the section of mutant mice extensive and confluent necrosis is present (400x). Small arrows indicate infected hepatocytes. Big dark arrowhead indicates a bile duct and a perivascular infiltrate characteristic for TNF/LTα deficient mice, independent of infection. PV, portal vein.

The fact that INFγ-stimulated bone marrow-derived macrophages (BMDM) from mutant mice showed reduced in vitro killing of phagocytosed *L. monocytogenes* compared to control macrophages (not shown).
Conclusions and Perspectives

Simultaneous deletion of the closely linked and homologous genes coding for TNF and LTα resulted in viable and fertile homozygous mutant animals which allowed us to investigate the specific phenotypic alterations in the immune system caused by the lack of the TNF and LTα. The combined genetic inactivation of TNF and LTα leads to distinct morphological and functional defects of the peripheral immune system whereby absence of TNF and LTα markedly affects innate and acquired immunity, respectively. The described phenotypic features render these mice interesting tools to investigate in more detail the roles of both ligands for the development and functions of the immune system. The interchangeability of TNF and LTα still leaves open questions about the functions of the soluble form of LTα that might be answered if a TNF knockout mouse would become available.

Outlook

In order to obtain definitive answers on the roles of each gene, the generation of transgenic mice from double knockout oocytes expressing either TNF or LTα is ongoing. Investigations of TNF-related pathologies are planned in several areas. A key role in the pathophysiology of pulmonary fibrosis by toxicants, in allergic and irritant skin reactions, parasitic disease, and many other systemic toxic and immunological responses is assumed and presently assessed using the mutant mouse.

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Chapter 8

Characterization of Lymphotoxin-α-Deficient Mice

Theresa A. Banks

Introduction

The tumor necrosis factor (TNF) family of proteins consists of a rapidly growing collection of ligands and receptors, many of which appear to be critically involved in the regulation of the immune system. To date, a large number of studies have focused on the structural and functional relationships of three cytokines in particular, tumor necrosis factor-α (TNFα), lymphotoxin-α (LTα), and lymphotoxin-β (LTβ). Whereas TNFα and LTα were one of the first members of the TNF family to be studied at the genetic level (1), the organization of the LTβ locus has only recently been defined (2). All three genes are located in close proximity to one another in the major histocompatibility complex (MHC) class III region of human chromosome 6 and murine chromosome 17 (1).

Structurally related to one another, both TNFα and LTα mediate various inflammatory and immunoregulatory responses (3–7). Indeed, the range of their activities appears diverse, including tumor cytotoxicity, cell proliferation and differentiation, antiviral responses, and the activation of numerous cellular genes and kinases (8–11). Moreover, the fact that secreted forms of TNFα and LTα both compete for the same two cell surface receptors (TNFRp55 and...
TNFRp75), has further confounded efforts to define individual roles for each of these cytokines (12). However, the discovery that LTα also exists as a nonsecreted, membrane-associated protein when complexed with LTβ (13) and that this complex is in turn recognized by a receptor (14) distinct from the TNFRp55 and TNFRp75 receptors, has provided the strongest indication so far that LTα and LTβ possess unique functions separate from those of TNFα.

Gene targeting remains a powerful technique for deciphering gene function and this approach has been or is currently being used to generate mice lacking one or more of these members of the TNF gene family: TNFRp55, TNFRp75, TNFα, LTα, LTβ, and the LTβ-specific receptor. Analysis of these mutant mouse strains will continue to greatly facilitate our understanding of the complex functions and interactions of this gene family.

The goal of this chapter is to specifically feature the phenotype of mice deficient in LTα (LTα−/−). Gene targeting was used successfully by two independent groups of investigators, DeTogni et al. (15) in 1994 and Banks et al. (16) in 1995 to generate LTα−/− mice. Although the later report by Banks et al. largely serves to confirm the work of DeTogni et al., some phenotypic differences were observed that are highlighted in the sections that follow. It should also be noted that since the main focus of this chapter is to provide a phenotypic analysis of the LTα−/− mice, readers seeking details of the targeting techniques used to disrupt the LTα gene as well as other specifics are referred to the original articles (15,16).

**Phenotypic Analysis of LTα-Deficient Mice**

**Anatomical Findings: Abnormal Development of Secondary Lymphoid Organs**

As reported by both DeTogni et al. and Banks et al. LTα−/− mutant mice were born at the expected Mendelian frequency and were grossly indistinguishable from their wild-type and heterozygous littermates. Moreover, LTα−/− mice maintained in conventional animal facilities (as opposed to germ-free) did not, at any time, appear overtly immunocompromised. However, mutants necropsied and analyzed at approx 4–9 wk of age demonstrated a striking phenotype characterized by a lack of peripheral lymph nodes (e.g., popliteal, inguinal, para-aortic, axillary, and cervical),
Peyer’s patches, and a significant disruption of splenic architecture. Specifically, targeted mutagenesis of the LTα locus appears to dramatically affect the normal development of secondary lymphoid tissues. Interestingly, although both the Banks and DeTogni groups observed the same overall phenotype, some differences were found. Perhaps most notably was the finding by Banks et al. that some of their LTα−/− mutant mice (4/14) did, in fact, possess abnormal lymph node-like structures grossly visible within the mesenteric fat. Moreover, in one particular mutant, abnormal nodes were histologically identified in both the mesenteric and submandibular fat. In all cases these lymphoreticular structures contained medullary cords and sinuses but the capsule was either incomplete or absent. Furthermore, the corticomedullary architecture was disorganized and follicles were absent. It remains unclear as to why these abnormal lymph nodes were observed in only some of the mutants examined, and why all but one of these nodes was found in the mesentery as opposed to other lymph node sites (e.g., inguinal, cervical, and so on). One possibility is that although the mice, in which the abnormal nodes were detected, were naive and had not been subject to any type of deliberate immunization, an undetected infection could perhaps correlate with the appearance of these nodes. Banks et al. suggested that this issue may be resolvable by housing the mice in both conventional and germ-free environments.

Further histological analysis of LTα−/− mice performed by both research groups revealed that the thymus and the bone marrow appeared normal in all mice examined. The thymuses of LTα−/− mice display normal cortical and medullary regions and the bone marrow from these mutants appears similar to that of bone marrow from wild-type and heterozygous littermates; all marrows examined appeared very cellular and contained appropriate myeloid and erythroid cells exhibiting complete and sequential maturation; normal numbers of megakaryocytes were also seen.

However, in contrast to the thymus and bone marrow, a variety of abnormalities was observed when the spleens from LTα−/− mice were examined. Both Banks et al. and DeTogni et al. reported that in all mice examined, the splenic white pulp was reduced and disorganized and lacked marginal zones. However, Banks et al. also observed splenomegaly in 5 of 14 LTα−/− mice examined. Furthermore, some but not all of the mutants that exhibited splenomegaly also had
increased white blood cell counts. Whether these findings reflect an underlying infection in these particular animals was not determined, but certainly should be examined in the future. However, other hematological parameters (e.g., hemoglobin, hematocrit, red blood cell count, and platelet estimate), appeared within expected normal limits and were very similar to results derived from wild-type littermates.

With regard to the levels of B-cells in the spleen and peripheral blood of LTα−/− mutant mice, De Togni et al. reported a 30% increase in the number of IgM+ B220+ B-cells in LTα−/− spleens compared to control mice. Similarly, examination of the peripheral blood of their LTα−/− mutant mice revealed a nearly fourfold increase in B-cells as compared to controls. In contrast to their findings, Banks et al. were unable to consistently detect increased levels of B-cells in either the spleen or peripheral blood of their LTα−/− mice. Although it is true that some of their mutant LTα−/− mice demonstrated increased white blood cell counts and/or splenomegaly, these cases were rare and the majority of their LTα−/− mice did not exhibit increased B-cell counts. The reason for these discrepancies is not known at this point in time but may become clearer as more mutants are analyzed.

Although there was good agreement between the two laboratories regarding most aspects of the histopathologic analysis of the LTα−/− mice, Banks et al. observed an interesting lung and liver pathology not reported by the DeTogni group. All mutant mice in which liver and lung sections were examined histologically (n = 8) demonstrated accumulations of small, homogeneous lymphocytes in the periportal and perivascular regions of the liver and lung, respectively. Moreover, the consistent absence of vasculitis suggests that these lymphocytes more than likely represent an accumulation of cells rather than an active inflammatory infiltrate. Indeed, Banks et al. speculated that lymphocytes from LT-α−/− mutants, unable to home to “non-existent” lymph nodes and Peyer’s patches, may accumulate instead in the liver and lung.

**Immunological Findings: Flow Cytometric Analyses**

Cytofluorimetric analysis performed by both research groups on the thymocytes, splenocytes, and peripheral blood lymphocytes (PBL) from LT-α−/− mice revealed normal ratios (approx 2:1) of CD4+CD8− to CD4−CD8+ T-cells. In addition, Banks et al. also
reported that splenocytes, PBL, and thymocytes from LTα−/− mutant mice analysed for the expression of the lymphocyte homing marker L-selectin (17) expressed levels comparable to those of their LTα+/+ and LTα+/− littermates. Thus it appears that, phenotypically at least, lymphocytes from LTα−/− mutant mice are normal.

Bone Marrow Reconstitution of SCID Mice

Since LTα−/− mutant mice lack functional lymph nodes but their lymphocytes appear phenotypically normal, an interesting question is whether LTα−/− lymphocytes are functionally capable of engaging in proper cell trafficking. To address this question, Banks et al. designed reconstitution experiments in which bone marrow from LTα−/− mutants was transferred into recipient SCID mice. SCID mice were chosen since they lack functionally mature B- and T-cells of their own but do possess rudimentary lymph node structures. Moreover, successful reconstitution of irradiated SCIDS has been reported (18,19). In these experiments, the presence of LTα−/− cells in the lymph nodes and spleens of the SCID hosts was confirmed by both PCR and by histological examination. In addition, flow cytometric analysis of PBL and splenocytes from the reconstituted SCID hosts demonstrated that the donor cells, whether from LTα−/− or LTα+/+ mice, expressed normal levels of L-selectin, CD4, CD8, Thy-1, and B220. Overall, the results of these bone marrow reconstitution experiments revealed that LTα−/− bone marrow could reconstitute SCID spleens and lymph nodes to nearly the same degree as bone marrow from wild-type controls, indicating that the homing capabilities of LTα−/− lymphocytes appear to be intact.

Immunoglobulin Levels in Serum and Feces

When Banks et al. examined immunoglobulin levels in naive, unimmunized LTα−/− mice, they found essentially similar levels of both total serum IgG and IgM in the mutants, as compared to their littermate controls. In contrast, the levels of both serum and fecal IgA were dramatically decreased in naive LTα−/− mice compared with their wildtype littermates. The mutants demonstrated serum and fecal IgA levels that were in all cases essentially undetectable, whereas their littermate controls demonstrated levels of both serum and fecal IgA that were many fold higher.
Antigen-Specific Antibody Responses to Cholera Toxin, Herpes Simplex Virus, and Keyhole Limpet Hemocyanin

In light of the decreased levels of total serum and fecal IgA observed in naive, unimmunized LTα−/− mice, Banks et al. went on to test the ability of immunized LTα−/− mice to mount antigen-specific responses. LTα−/− mice immunized orally on three occasions with cholera toxin (CT), were able to mount both CT-specific serum IgG and IgA responses. However, in all cases, the levels of antibodies induced in the mutant mice were lower than the levels induced in littermate controls. In particular, the levels of CT-specific serum IgA were barely detectable in the LTα−/− mutants. In other experiments, control mice immunized subcutaneously with ultraviolet-inactivated herpes simplex virus-type 1 (HSV-1) were able to generate HSV-1-specific serum IgG antibodies, whereas such antibodies could not be detected in the serum of HSV-1-immunized LTα−/− mutant mice. Similar results were also observed when mutant and control mice were immunized subcutaneously with the antigen keyhole limpet hemocyanin (KLH). Whereas littermate control mice were able to mount KLH-specific serum IgG responses, such responses were not detected in LTα−/− mutant mice. Thus it appears that LTα−/− mutant mice are markedly deficient in their ability to respond humorally to immunization with three different antigens, administered either via mucosal or systemic routes.

Cell-Mediated Immune Responses

DeTogni et al. were able to show that both CD8+ and CD4+ lymphocytes appear functionally intact in LTα−/− mutant mice. For example, following immunization with β-galactosidase in complete Freund’s adjuvant, the in vitro proliferative response of splenic T-cells from LTα−/− mutants was similar to responses produced from positive controls. Splenic T-cells from LTα−/− mice were also shown capable of demonstrating both MHC class I and class II-restricted allocytotoxic responses. Indeed, it is tempting to speculate that the ability of LTα−/− mice to generate normal cell-mediated immune responses may in part explain why LTα−/− mice appear healthy in spite of their abnormal secondary lymphoid organs and impaired humoral responses.
Summary

Mice lacking LTα/− exhibit a phenotype dominated by defects in secondary lymphoid organ development. In particular, LTα/− mice lack normal lymph nodes and Peyer's patches, and possess spleens in which the usual architecture is disrupted. Surprisingly however, abnormal lymph node-like structures can be occasionally observed mainly within the mesenteric fat of some mutant mice, a finding that to date remains difficult to explain. The LTα/− mutant phenotype is also characterized by the accumulation of abnormal clusters of lymphocytes in the periportal and perivascular regions of the liver and lung suggesting perhaps that lymphocytes that may normally home to peripheral lymph nodes accumulate in these sites instead. And yet in general, lymphocytes from LTα/− mice appear remarkably normal phenotypically, expressing the expected ratios of B- and T-cell surface antigens as well as the lymphocyte homing marker, L-selectin. In addition, bone marrow cells from LTα/− mice appear functionally intact, capable of successfully reconstituting the lymphoid organs of SCID mice. Finally, whereas LTα/− mutant mice examined for humoral immune responsiveness appear impaired in their ability to respond to several different antigens administered either systemically or mucosally, their ability to mount cell-mediated immune responses appears intact.

In conclusion, the LTα/− mouse offers a model system for the study of secondary lymphoid organogenesis and its role in immune responsiveness. In particular, this model should prove particularly useful for exploring all aspects of mucosal immune responsiveness. Interestingly, mice deficient in either of the two receptors (TNFRP55 and TNFRP75), with which LTα is known to bind, do not exhibit the same phenotype as LTα/− mice (20,21). This suggests that LTαs interaction with LTβ and/or with the LTβ-specific receptor may be the critical one in terms of lymphoid organogenesis. For this reason, mice targeted in the LTβ and/or LTβ-specific receptor genes are eagerly anticipated.

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Chapter 9

Interleukin-1β-Deficient Mice

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Introduction

There are three members of the interleukin-1 (IL-1) gene family: IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1Ra). IL-1α and IL-1β are agonists and IL-1Ra is a specific receptor antagonist. IL-1α and IL-β are synthesized as precursor molecules, without leader sequences. The molecular weight of each precursor is 31 kDa. Processing of IL-1α or IL-1β to “mature” forms of 17 kDa requires specific cellular proteases.

IL-1 (IL-1α and IL-1β) is the prototypic “multifunctional” cytokine (for review, see ref. 1). IL-1 affects nearly every cell type, often in concert with other cytokines or small mediator molecules. There is growing evidence that the production of IL-1, particularly IL-1β, is a highly regulated event.

There are two IL-1Rs, the type I receptor (IL-1RI) transduces a signal, whereas the type II receptor (IL-1RII) binds IL-1 but does not transduce a signal. In fact, IL-1RII acts as a sink for IL-1β and has been termed a “decoy” receptor (2). When IL-1 binds to IL-1RI a complex is formed that then binds to the IL-1R accessory protein (IL-1R-AcP) resulting in high affinity binding (3). The extracellular or “soluble” portions of the IL-1RI (IL-1sRI) and IL-1RII (IL-1sRII) circulate in health and disease functioning as natural “buffers,” binding IL-1α, IL-1β, or IL-1Ra. In addition, several cytokines and hor-
mones exert a negative influence on both the production and activity of IL-1.

Although several similarities between IL-1α and IL-1β exist, in many respects IL-1β is a different molecule from IL-1α. The mature forms of the two agonists are comprised of similar three-dimensional structures of all beta sheets (4,5), both molecules are translated in the cytosol associated with cytoskeletal rather than endoplasmic reticulum structures (6,7) and both precursors undergo myristoylation on lysine residues in their respective pro-pieces. However, differences between these two cytokines are remarkable when examining regulation of gene expression, mRNA stability, translation, processing, and secretion. In addition, the affinities of pro and mature IL-1β binding to surface and soluble forms of the receptors are different from those of IL-1α. Once released from cells, mature IL-1β encounters two antagonistic molecules: IL-1sRII, which tightly binds IL-1β, and sIL-1Ra, which competes with IL-1β for cell surface receptor occupancy. IL-1β appears to be a systemic, hormone-like mediator intended to be released from cells, whereas IL-1α is primarily a regulator of intracellular events.

**Gene Expression of IL-1β**

Unlike the promoter of IL-1α, the promoter region for IL-1β contains a TATA box, a typical motif of inducible genes. The half-life of IL-1β mRNA depends upon the cell type and the conditions of stimulation. Endotoxin (e.g., lipopolysaccharide [LPS]) triggers transient transcription and steady state levels of IL-1β mRNA, which accumulate for 4 h, followed by a rapid fall due to synthesis of a transcriptional repressor (8). Using IL-1 itself as a stimulant of its own gene expression, IL-1β mRNA levels are sustained for over 24 h (9).

Unlike most cytokine promoters, IL-1β regulatory regions can be found distributed over several thousand basepairs upstream and a few basepairs downstream from the transcriptional start site. The topic of IL-1β gene regulation has recently been reviewed in detail, concluding that IL-1β gene expression is regulated at different levels (10).
Inducers of IL-1

Nearly all microbes and microbial products induce production of the three IL-1 proteins, but stimulants of nonmicrobial origin can also stimulate transcription and in many cases synthesis of the IL-1 family.

Stimulants such as the complement component C5a, hypoxia, adherence to surfaces, or clotting of blood induce the synthesis of large amounts of IL-1β mRNA in monocytic cells without significant translation into the IL-1β protein. This dissociation between transcription and translation is characteristic of IL-1β, but also of tumor necrosis factor alpha (TNFα) (11). Without translation, most of the IL-1β mRNA is degraded. However, adding bacterial endotoxin or IL-1 itself to cells with high levels of steady-state IL-1β mRNA results in augmented translation (11). One explanation is that stabilization of the AU-rich 3’ untranslated region takes place in cells stimulated with LPS. The stabilization of mRNA by microbial products may explain why low concentrations of LPS or a few bacteria or Borrelia organisms per cell induce the translation of large amounts of IL-1β. Another explanation is that IL-1 stabilizes its own mRNA (9) by preventing deadenylation.

Processing and Secretion

Following synthesis, pro-IL-1β remains primarily cytosolic until it is cleaved and transported out of the cell (Fig. 1). Unlike IL-1α, proIL-1β has no membrane form and is only marginally active (12). Some IL-1β is found in lysosomes or associated with microtubules and either localization may play a role in the secretion of IL-1β. In mononuclear phagocytes, a small amount of pro-IL-1β is secreted from intact cells (13) but the pathway for this secretion remains unknown. On the other hand, release of mature IL-1β appears to be linked to processing at aspartic acid-alanine 116–117 by the IL-1β converting enzyme (ICE) (14).

Although well-controlled in the setting of laboratory cell culture, death and rupture of inflammatory cells is not an unusual occurrence in vivo. There are several sites in the N-terminal 16-kDa part of pro-IL-1β that are vulnerable to cleavage by enzymes in the
Fig. 1. A human blood monocyte producing IL-1β. Pro-IL-1β remains cytosolic until it is cleaved by ICE. ICE is translated as an inactive precursor (pro-ICE), remains cytosolic and requires two cleavage steps to form the enzymatically active heterodimer. Two heterodimers form a tetramer in association with two molecules of pro-IL-1β, and cleavage occurs. After cleavage, 17 kDa IL-1β is released from the cell. The 16-kDa pro-piece can be found either inside or outside the cell compartment. A small amount of pro-IL-1β can be released from intact cells.

vicinity of alanine 117. These are trypsin, elastase, chymotrypsin, a mast cell chymase, and a variety of other extracellular proteases that are commonly found in inflammatory fluids. The role that these proteases play in the in vivo conversion of pro-IL-1β to mature forms is uncertain but, in each case, a biologically active IL-1β species is produced.

**IL-1 Blockade**

Blocking IL-1 receptors with IL-1Ra, anti-IL-1RI antibodies and, more recently, soluble IL-1 receptors has increased our understanding of IL-1 as a mediator of disease. However, the use of IL-1 receptor blockade does not allow a distinction between the specific roles played by IL-1α or IL-1β. For this purpose, antibodies directed either against IL-1α or IL-1β have been used. In Table 1, we summarize the in vivo and in vitro results obtained with the use of anti-IL-1β antibodies. However, the generation of IL-1β-deficient
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Table 1
In vivo and in vitro Inhibitory Effects of Anti-IL-1β Antibodies

In vivo
LPS-induced fever in rats (15).
Collagen-induced arthritis in mice (16,17).
Nephrotoxic nephritis in rats (18).
Zymosan-induced neutrophil infiltration in mice (19).
Glucan-induced chemokine production and granuloma formation in rats (20).
Immune complex-induced alveolitis in rats (21).
Haemophilus influenzae-induced meningitis in rabbits (22).
Δ⁹-Tetrahydrocannabinol-induced catalepsy in mice (23).
Increase in nonrapid eye movements sleep after sleep deprivation in rabbits and rats (24,25).

In vitro
Acute myelogenous leukemia blasts spontaneous proliferation (26).
Spontaneous IL-6 and granulocyte-macrophage-colony-stimulating factor production from acute myeloblastic leukemia blasts (27,28).
Synovial cells growth promoting activity of adult T-cell leukemia cells (29).
Ubenimex-induced increased in cytotoxic activity of mononuclear cells from cancer patients (30).
Accessory cell-dependent stimulation of murine primitive pluripotential hematopoietic cells by platelet-derived growth factor (31).
Human thyroid carcinoma cell line NIM 1 growth (32).
TNF-α-induced fibronectin production in smooth muscle cells (33).
Increased fibronectin production in donor coronary artery smooth muscle cells after cardiac transplantation in piglets (34).
Production of MCP-1 from pulmonary fibroblasts following stimulation with conditioned media from LPS-stimulated alveolar macrophages (35).
Nitric oxide-dependent delayed increase in ciliary motility from bovine bronchial epithelial cells incubated with alveolar macrophage-conditioned medium (36).

(IL-1β⁻ⁿ) mice has provided a powerful tool for the investigation of the specific role played by this cytokine.

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Generation of the IL-1β null mutation

The generation of IL-1β⁻⁻ mice has been described in detail (37,38). To date, two different groups have independently generated IL-1β⁻⁻ mice.
Phenotype

Reproduction

A role for IL-1β in the control of embryonic development, implantation, and birth has been suggested by the demonstration that IL-1β, along with other cytokines, is expressed in the reproductive organs, in particular during the peri-implantation period (39,40) and in the blastocyst (41). However, no abnormalities in ovulation, fertilization, implantation, or parturition have been observed in IL-1β−/− mice (37,38), suggesting that either IL-1β does not play a role in the regulation of the reproductive function or other cytokines can compensate for its absence. It is interesting to note that no reproductive abnormalities have been observed in IL-1RI-deficient mice (M. Labow, personal communication), thus ruling out the possibility that IL-1α might compensate for the lack of IL-1β.

Homeostasis

IL-1β−/− mice are healthy and no differences in the life span have been observed to date (37,38). No abnormalities at tissue or organ levels have been revealed by histopathological examination. Hematopoiesis appears to be normal, T- and B- cell lineages are not affected by the lack of the IL-1β gene (37). These data support earlier findings demonstrating that administration of IL-1Ra to humans is not associated with hematological changes (42). Furthermore, no differences in mitogen-induced lymphocyte proliferation have been observed between IL-1β−/− and wild-type mice (our unpublished observations).

IL-1 is an endogenous pyrogen and its administration induces anorexia (43–45). However, the circadian variation of body temperature recorded at basal conditions does not differ between IL-1β−/− mice and their wild-type counterparts (46), indicating that IL-1β does not play a role in the regulation of the normal circadian temperature rhythm. Furthermore, no differences in basal food and water intake and body weight have been observed between IL-1β−/− and wild-type mice (46,47), again indicating that IL-1β is not involved in the regulation of feeding behavior under basal conditions.

A reduced basal locomotor activity is observed in IL-1β−/− mice (37,46). Constitutive and inducible expression of IL-1β and IL-1 receptors in different areas of the central nervous system
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IL-1 (CNS) has been extensively demonstrated (for review see ref. 48). The lower activity observed in IL-1β−/− mice thus suggests a role for constitutive IL-1β in the regulation of locomotor behavior.

IL-1 is a potent activator of the hypothalamus–pituitary–adrenal (HPA) axis and its administration induces a dysregulation of glucose metabolism (48). Under basal conditions, glucose metabolism appears to be normal in IL-1β−/− mice. Fasted and nonfasted glycemia, circulating insulin levels, and the glycemic response to a glucose-tolerance test are normal in IL-1β−/− mice when measured under basal conditions (47, and our unpublished observations). The circadian rhythm of corticosterone production is also unaffected by the lack of the IL-1β gene. In fact, basal morning and afternoon serum corticosterone levels do not differ between IL-1β−/− and wild-type mice (47, and our unpublished observations). Although not consistent, in some experiments we observed higher basal serum glucose and corticosterone levels in IL-1β−/− mice. IL-1β is expressed in the adrenal glands (49) and corticotropin-releasing factor administration induces IL-1β gene expression in the spleen (50). Our observations, although sporadic, might suggest a role for IL-1β in the regulation of the stress response.

In summary, with the notable exception of locomotor activity, IL-1β does not seem to play a major role in maintaining homeostasis under basal conditions.

Disease Models

Lethality

IL-1 is a highly toxic cytokine and its administration can induce death, especially when a chronic treatment is used (51). Various reports have demonstrated a protective effect of IL-1Ra in different models of infection or inflammation. In particular, the protective role of IL-1RI blockade has been demonstrated in experimental models of endotoxemia (52), of TNFα-induced lethality (53), and following injection of either Escherichia coli or Klebsiella pneumoniae (54,55).

To date, the effect of IL-1β gene deletion on lethality has been studied in five different models (see Table 2). These are: administration of high doses of LPS, administration of low doses of LPS after treatment with a sensitizing agent, injection with high doses of
Table 2
The Response of IL-1β−/− Mice in Different Experimental Models of Lethality

| Experimental model                              | Response                              |
|------------------------------------------------|---------------------------------------|
| High-dose LPS                                   | As susceptible as wild-type mice      |
| Sensitization/low-dose LPS                      | As susceptible as wild-type mice      |
| High-dose zymosan                               | Reduced mortality                     |
| Infection with *L. monocytogenes*               | As resistant as wild-type mice        |
| Infection with influenza virus                   | Earlier and increased mortality       |

zymosan, infection with *Listeria monocytogenes*; and infection with influenza virus.

1. IL-1β−/− mice are susceptible to the lethal effect of high doses of LPS (38,47). These data appear to contrast with the protective effect of IL-1RI blockade on LPS-induced lethality and with the observation that ICE-deficient mice are resistant to high doses of LPS (52,56,57). However, IL-1RI blockade inhibit both IL-1α and IL-1β effects, whereas ICE-deficient mice show a deficient secretion of both IL-1α and IL-1β (57,58). On the other hand, IL-1α production is not altered in IL-1β−/− mice (see Effects on Cytokine Production). This important difference probably accounts for the discrepancy in the results and suggests the necessity of blocking both IL-1α and IL-1β in order to obtain protection in this model.

2. A pretreatment with a sensitizing agent (D-galactosamine, actinomycin-D, or bacillus Calmette-Guerin [BCG]) followed by challenge with a low dose of LPS induces lethality. The toxic effect of LPS in this model is usually rapid and accompanied by hepatic damage, with death occurring in the first 24 h after challenge. IL-1β−/− mice are susceptible to the lethal effect of a low dose of LPS administered after sensitization with D-galactosamine (38,47). Mice deficient for the TNF p55 receptor, or for the interferon gamma (IFNy) receptor (59–61) and transgenic mice overexpressing the soluble p55 receptor for TNF (62) are resistant in this same model, thus indicating that TNFα and IFNy, not IL-1β, play the essential role.

3. The administration of a high dose of zymosan is a well-characterized model for complement-mediated multiple organ failure in the absence of sepsis (63). Reduced lethality is observed in zymosan-injected IL-1β−/− mice compared with wild-type controls (64). In this model, the administration of anti-TNFα antibodies also affords
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protection (65). The reduced cytokine levels observed in IL-1β−/− mice treated with zymosan (see Effects on Cytokine Production), are probably responsible for the decreased mortality observed in this model in the absence of IL-1β expression.

4. *L. monocytogenes* is a Gram-positive, obligatory intracellular bacterium that requires a functional cellular immune response to be eliminated (66). The use of knockout mice has demonstrated that the expression of IL-6, TNFα, and IFNγ is necessary to mount an efficient response to *Listeria* (59,60,67–69). However, this is not the case for IL-1β. In fact, despite the observation that IL-1 is involved in the development of anti-*Listeria* immunity (70), IL-1β−/− mice do not show any defect in host defense against this type of bacteria (37).

5. Infection with influenza virus in mice induces pneumonitis and death (71). When IL-1β−/− mice are infected with influenza virus, an earlier and increased lethality occurs, with an associated diminished hypothermic response to the virus (46). Whether the lack of IL-1β expression worsens the outcome after infection with other kinds of viruses remains to be investigated.

**Fever**

Different cytokines, namely IL-1α, IL-1β, TNFα, TNFβ, IFNα, IL-6, and ciliary neurotrophic factor (CNTF) can directly induce fever when injected in humans or animals. IL-1 was the first endogenous pyrogen to be purified and characterized.

The administration of either IL-1α or IL-1β induces a hyper-responsive febrile reaction in IL-1β−/− mice compared to wild-type controls (72). The exaggerated febrile response of IL-1β−/− mice to IL-1 cannot be attributed to an increase in the number of IL-1 receptors. In fact, the binding of IL-1 to its receptors in the hypothalamus does not differ between wild-type and IL-1β−/− mice and steady-state mRNA level for either type I or type II IL-1 receptor in the different brain areas are not increased in these mice. A possible hypothesis for the observed effect is that in the absence of IL-1β, the ability of the IL-1 receptors responsible for the regulation of body temperature to transduce the IL-1-mediated signal is increased.

Studies using anti-IL-1β antibodies have shown that LPS-induced fever is dependent on IL-1β (15). It appears that LPS-induced intracerebroventricular levels of IL-6 are due to IL-1β (15).
The use of IL-6-deficient mice supported the concept that CNS induction of IL-6 is responsible for fever after LPS administration. In fact, IL-6-deficient mice are completely resistant to LPS-induced fever (73). However, contrasting results have been obtained by using endotoxemia in IL-1β−/− mice. There is either a slightly reduced (46) or a sharply increased (72) febrile response to LPS in IL-1β−/− mice. At the present time, no clear explanation for this discrepancy has been proposed, other than that the type of LPS used by the two groups was different. The demonstration that IL-1RI-deficient mice also develop a normal febrile response to LPS rules out the possibility that IL-1α might be the involved mediator (74). One can hypothesize that LPS directly induces IL-6, without the need of an intermediate production of IL-1β. The observation that IL-1β−/− mice are not deficient in LPS-induced IL-6 production (see Effects of Cytokine Production) (25) is in agreement with this hypothesis.

However, the situation is different when the febrile response to the sterile inflammation induced by turpentine is studied. In this model, IL-1β−/− mice, as well as IL-1RI-deficient mice, do not develop fever (37,74). Also in this case, IL-6 is the most likely candidate for the role of an endogenous pyrogen. In fact, IL-1β−/− mice do not produce IL-6 in response to turpentine (see Effects of Cytokine Production) (37).

Local inflammation would thus induce IL-1β which in turn will induce fever via production of IL-6. On the other hand, following LPS administration, the intermediate production of IL-1β is not required for the induction of IL-6 and of the febrile response (Fig. 2).

**Anorexia and Sickness Behavior**

Anorexia and increased protein breakdown lead to the cachectic state observed during inflammation, infection, or cancer. IL-1 is an important modulator of the synthesis of various neuropeptides involved in the regulation of food intake, as for example corticotropin-releasing hormone (CRH) (48). Furthermore, a role for IL-1 in the induction of leptin, a protein involved in the regulation of food intake, has recently been demonstrated (75).

In agreement with data showing inhibition of turpentine-induced anorexia with anti-IL-1RI antibodies (76), IL-1β−/− mice do not develop anorexia when injected with turpentine (37). Interestingly,
Fig. 2. The febrile response following LPS administration or induction of sterile inflammation. Both in the LPS and in the turpentine models, IL-6 is the pyrogenic cytokine. However, LPS directly induces the production of IL-6, without an intermediate role for IL-1β or TNFα. On the contrary, the induction of IL-6 following sterile inflammation is completely dependent on IL-1β production. In both cases, the presence of IL-6 in the central nervous system is a prerequisite for the febrile response to take place.

the resistance of IL-1β⁻/⁻ mice to turpentine-induced anorexia is associated with a lack of induction of leptin mRNA in the adipose tissue (our unpublished observations)

No difference in decreased food and water intake has been observed between IL-1β⁻/⁻ and wild-type mice injected with either LPS (46,47) or zymosan (64), or in mice infected with influenza virus (46). These data are in agreement with previous reports indicating that IL-1Ra does not prevent LPS-induced anorexia in rats (77).
The administration of IL-1 induces a sickness behavior, characterized by lethargy and decreased locomotor activity. These responses are typical of different infectious or inflammatory conditions, and IL-1 might thus be an important mediator involved in the development of sickness behavior. In IL-1β⁻/⁻ mice, the decrease in locomotor activity follows the same pattern observed for the anorexic response. In fact, as for anorexia, IL-1β⁻/⁻ mice are resistant when injected with turpentine (37) but not when treated with LPS (46).

**HPA Axis Activation**

IL-1 is a potent activator of the HPA axis, acting at both the hypothalamic and the pituitary level (48). The activation of the HPA axis results in a marked increase in glucocorticoid production. The rise in glucocorticoid level, in concert with cytokines using gp130 as a receptor subunit, is an important modulator for the development of the subsequent acute phase response. In addition, glucocorticoids downregulate the inflammatory response, in part via an inhibition of cytokine production (78).

IL-1β⁻/⁻ mice have a normal response to LPS in terms of activation of the HPA axis (47). The role of IL-1 in the LPS-induced activation of the HPA axis is controversial. IL-1Ra administration did not decrease HPA axis activation in human volunteers or in mice injected with LPS (79,80). On the other hand, anti-IL-1RI antibodies were effective in reducing LPS-induced adrenocorticotropic hormone (ACTH) increase in mice (81). The results obtained in IL-1β⁻/⁻ mice show that either IL-1β is not required for LPS to activate the HPA axis, or its role may be fulfilled by other cytokines.

In a model of zymosan-induced peritonitis, only a modest reduction in the increase of serum corticosterone levels has been observed in IL-1β⁻/⁻ mice (64). In addition, when IL-1β⁻/⁻ mice were injected with high-dose IL-2, the increase in serum corticosterone levels was not impaired (our unpublished observations).

Despite the fact the IL-1β⁻/⁻ mice do not develop fever nor anorexia following the administration of turpentine, the early activation of the HPA axis is comparable to that observed in wild-type mice (82). These data are in agreement with previous observations obtained in mice treated with anti-IL-1R I antibodies (76). A direct activation of neural afferents at the site of turpentine injection has
been associated with the activation of the HPA axis in this model (83), and cytokines do not appear to be involved in the early corticosterone and ACTH increase.

From the data obtained to date, it appears that the activation of the HPA axis, an important anti-inflammatory mechanism, is preserved in the absence of IL-1β, even when other responses are inhibited, as in the case of turpentine-induced sterile inflammation. As demonstrated by studies using adrenalectomized animals, the glucocorticoid increase is a fundamental mechanism in determining the outcome after inflammation (84). Thus, it is likely that such an important response would not be dependent on a single mediator.

**Hypoglycemia**

Administration of IL-1 alters glucose metabolism. In mice, IL-1 administration induces a transient, but profound, hypoglycemia, associated with increased insulin and glucagon secretion. IL-1 increases glucose uptake and utilization, both in vivo and in vitro (for review, see ref. 85).

IL-1β−/− mice have a normal hypoglycemic response to LPS (47) and to zymosan (64). These findings are in agreement with previous data showing that IL-1Ra administration does not reverse the alterations in glucose metabolism observed in baboons injected with a sub lethal dose of endotoxin or live *E. coli*, despite the fact that, in the latter model, IL-1Ra improved survival (54).

**Production of Acute Phase Proteins**

The liver is one of the principal targets of inflammatory mediators, which alters its biosynthetic properties. In particular, the synthesis of a family of proteins, collectively known as acute phase proteins (APP), is dramatically increased during inflammatory responses. Fibrinogen, C-reactive protein, serum amyloid A and P, and various proteinase inhibitors are part of the APP family. The synthesis of other proteins, such as albumin and cytochromes P-450, is decreased during the course of an inflammatory response. These proteins are known as negative APP. Cytokines, in particular IL-1, IL-6 (and IL-6-like cytokines), as well as TNF-α are major regulators of APP, acting in concert with glucocorticoids. IL-1 can induce the synthesis of APP either directly or through the induction of IL-6.
In fact, APP can be classified into two major categories, the first being IL-1- and TNF-α-inducible, the second being IL-6-dependent (for review, see refs. 86 and 87).

The induction of acute phase proteins by LPS is not altered in IL-1β−/− mice (37,47). This result is in sharp contrast with the lack of induction of acute phase proteins in IL-1β+/− mice in a model of turpentine-induced sterile inflammation (37,88). These observations suggest that the role of IL-1β in the turpentine-mediated induction of APP is mainly mediated through the induction of IL-6. In fact, in turpentine-induced inflammation, both APP and IL-6 responses are impaired, whereas following LPS administration the induction of both APP and IL-6 is normal (see Effects of Cytokine Production) (37,47). This hypothesis is also supported by the results obtained using IL-6-deficient mice, which parallel the data obtained in IL-1β−/− mice. IL-6-deficient mice, in fact, do not produce APP in response to turpentine, but have a nearly normal APP induction following LPS (67,89).

IL-2 administration induces an acute phase response, which is mediated through the induction of different pro-inflammatory cytokines (90,91). Treatment with IL-1Ra partly inhibits the induction of serum amyloid A (SAA) by IL-2 (92) However, IL-1β−/− mice respond to high-dose IL-2 with a production of SAA comparable to that observed in wild-type mice (our unpublished observations).

Models of Nonspecific Resistance

It is well established that a pretreatment with a low dose of LPS protects against a subsequent challenge with the same agent, a phenomenon known as LPS tolerance. The mechanism for this protection is not completely understood, but appears to involve a downregulation of the production of proinflammatory cytokines (for review, see ref. 93). A role for IL-1 in the development of LPS tolerance has been demonstrated with the use of IL-1Ra, that partially reverses the reduced production of colony-stimulating factors observed during LPS tolerance (94). However, when pretreated with LPS, IL-1β−/− mice are fully resistant to a subsequent lethal challenge with LPS (47), thus indicating that IL-1β is dispensable for the development of LPS tolerance.
Contact Hypersensitivity (CH)

In the epidermis, IL-1β is expressed both in keratinocytes and in Langherans cells, where it plays an important role for the development of CH (95,96). The development of CH in IL-1β−/− mice is normal when the mice are challenged with oxazolone, but not when trinitrochlorobenzene (TNCB) is used (37,38). In the latter case, IL-1β−/− mice do not develop CH when low doses of TNCB are used for sensitization. However, higher doses of antigen can overcome the impaired CH response. The lower TNCB-induced CH response observed in IL-1β−/− mice is characterized by a decreased inflammatory cell infiltrate and by the absence of a specific proliferative response to the antigen in lymphocytes obtained from sensitized animals (38). The mechanism for the different susceptibility of IL-1β−/− mice to oxazolone- vs TNCB-induced CH still need to be clarified.

Effects on Cytokine Production

The balance between pro- and anti-inflammatory cytokines is a critical factor in determining the outcome following infection or inflammation. When mice are pretreated with the sensitizing agent Propionibacterium acnes, circulating levels for IL-1α, TNFα and IL-6 are as elevated in IL-1β−/− mice as they are in wild-type controls (37). Even naive IL-1β−/− mice injected with LPS, either at low or high doses, manifest the expected high levels in circulating cytokines (47). These results are consistent with the findings that, with the exception of colony-stimulating factors (94,97), the administration of IL-1Ra did not decrease cytokine levels in animal models of endotoxemia or in LPS-injected volunteers (52,79,97).

Conflicting data exist for the in vitro LPS-induced production of IL-1α from thioglycollate-elicited peritoneal macrophages obtained from IL-1β−/− mice. In fact, the in vitro LPS-induced levels of IL-1α have been reported to be either unaffected (38) or reduced by 50% (47). Differences in the macrophage culture might well account for the discrepancy in the results. In fact, in one case (38), cells were allowed to adhere overnight before a 4-h stimulation with 1 μg/mL of LPS. In the other report (47), cells were stimulated with LPS immediately after plating, and the stimulation was continued for 24 h. It is well established that adherence to surfaces provides a strong priming effect for the synthesis of IL-1 (11).
It is thus possible that the prolonged period of adherence used by Shornick et al. (38) might have rendered the production of IL-1α less dependent on IL-1β synthesis. No differences between IL-1β−/− and wild-type mice have been observed for the in vitro LPS-induced production of either IL-6 or TNFα (47).

In keeping with the observations that IL-1Ra treatment does not decrease IL-2-induced IL-6 production (92), no differences in serum IL-6 levels following administration of high-dose IL-2 were present between wild-type and IL-1β−/− mice (our unpublished observations).

In turpentine-treated IL-1β−/− mice, IL-6 is not detectable in the circulation, whereas high IL-6 levels are present in wild-type mice (37). The likely mechanism for the loss of systemic responses to turpentine observed in IL-1β−/− mice (see Production of Acute Phase Proteins) is indeed the failure to induce IL-6. In fact, IL-6-deficient mice are as resistant to turpentine as the IL-1β−/− mice (67,89). The results obtained with the turpentine-model of sterile inflammation are in sharp contrast with the data acquired by studying the response of IL-1β−/− mice to LPS. In fact, following induction of sterile inflammation, IL-6 production is completely under the control of IL-1β. On the other hand, TNF-α is a major regulator of IL-6 production when LPS is used as a stimulus (60). In the turpentine model, a specific induction of IL-1β followed by IL-6 is observed, with no evidence of significant TNFα or IL-1α production. This pattern of cytokine production might be typical of sterile inflammation, since high levels of IL-6, have been observed in patients with isolated head injury, but no TNFα or IL-1α were detectable (98).

In IL-1β−/− mice, the production of circulating IL-1α and IL-6 following zymosan-induced peritonitis is reduced. Peritoneal lavage fluid concentrations of these cytokines are also reduced (64). However, no differences in the levels for TNF-α were observed between wild-type and IL-1β−/− mice. This was expected, because TNFα production precedes, and is probably partly responsible for, the induction of IL-1β. The lower cytokine production observed in IL-1β−/− mice is associated with a 50% decrease in the leukocyte infiltration in the peritoneal cavity. When splenocytes from IL-1β−/− mice are stimulated in vitro with zymosan, a 50% reduction of IL-6, but not of TNFα, production was observed (64). It is interesting that IL-1α does not seem to play a role in zymosan-induced IL-6
Fig. 3. The role of IL-1β in the in vivo production of pro-inflammatory cytokines following LPS, zymosan, or turpentine administration. 

(A) When administered in vivo, LPS directly induces the synthesis of IL-1α, IL-6, and TNFα, without the requirement for an intermediate production of IL-1β. IL-6 and TNFα reciprocally regulate each other synthesis, IL-6 inhibiting TNFα and TNFα potentiating LPS-induced IL-6 levels. (B) Zymosan can directly induce the synthesis of IL-1α, IL-6, and TNFα. However, the presence of IL-1β provides a strong potentiating signal for the induction of IL-1α and IL-6. (C) Following induction of sterile inflammation, a specific production of IL-1β is observed, without evidence for IL-1α or TNFα induction. In this model, the production of IL-6 is totally dependent on IL-1β.
production. In fact, no further reduction of IL-6 levels is observed when cells obtained from IL-1β−/− mice are stimulated in vitro with zymosan in the presence of IL-1Ra (64).

The data obtained from IL-1β−/− mice on the role of IL-1β in the in vivo production of cytokines following different stimuli are summarized in Fig. 3.

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Chapter 10

Functional Phenotype of Mice Deficient in the IL-1R Type I Gene

A Comparison with Other Genetically Modified Mice

Philip J. Morrissey, Moira Glaccum, Charles R. Maliszewski, and Jacques Peschon

Introduction

Interleukin 1 (IL-1) is a cytokine that is involved in the mechanism of host resistance and immune function (for review, see ref. 1). Evidence indicates that IL-1 functions as a proinflammatory cytokine with significant and well-documented effects on innate resistance and the inflammatory response (2). The biological effects of IL-1 also influence the development of the specific immune response. IL-1 has been long studied as a costimulator of both B-cells and T-cells and the adjuvant effect of IL-1 is well documented (3–5). Thus, it is believed that IL-1 plays a central role in both innate resistance mechanisms and specific immune responses.

The biological activity of IL-1 is mediated by its binding to specific cell surface receptors (IL-1R) (for review, see ref. 6). The IL-1/IL-1R system is somewhat unique physiologically in that there are two related forms of IL-1 (IL-1α and IL-1β) that are encoded by separate, but linked genes (7). Both IL-1α and IL-1β are initially produced as 30–35 kDa precursor proteins. In this state, IL-1α has biological activity, however IL-1β does not. The
35 kDa precursor proteins are proteolytically cleaved to 17 kDa mature forms that are both biologically active. There is evidence to suggest that cleavage of precursor to mature IL-1α and IL-1β utilizes distinct processing enzymes. Precursor IL-1β is known to be cleaved by a cysteine protease termed ICE (IL-1 converting enzyme) (8). Precursor IL-1α is thought to be processed by a calpain-like converting enzyme (9,10).

There are two distinct but related cell surface receptors for IL-1 (the type I and type II receptors [IL-1RI and IL-1RII]) (11,12). These are products of distinct, but linked genes. Both IL-1RI and IL-1RII can bind either form of IL-1 although the affinities with which they do so differ (13). Evidence indicates that the type I and type II receptors are functionally separate cell-surface binding sites and are not subunits of a composite receptor. The extracellular portions of the two receptors are homologous and consist of approx 325 amino acids each. The receptors differ significantly in the size of the intracytoplasmic region. The intracytoplasmic portion of the type I receptor is 215 amino acids in length whereas the intracytoplasmic portion of the type II receptor consists of only 29 amino acids. Studies have shown that only the type I receptor is capable of mediating a biological signal (14,15). It is thought that the type II receptor could function as a "decoy" receptor that binds and neutralizes excess IL-1 or somehow regulates the level of soluble, biologically active IL-1 (16). Soluble IL-1RII has been detected in pathological states (17). In addition, there is also a IL-1R antagonist (IL-1RA) that binds predominately to IL-1RI and blocks binding and signaling by IL-1 (18). Thus, it is apparent that the precise regulation of IL-1-mediated signaling is important physiologically.

In order to understand the physiological role of IL-1, we have generated mice genetically deficient in the IL-1RI through targeted gene mutation (19). In this chapter we review the findings on the physiological consequences of IL-1RI deficiency. We also compare these results with published reports on the phenotypes of other cytokine or cytokine receptor knockout animals, focusing on alterations in genes encoding pro-inflammatory or inflammatory cytokines and their receptors. It should be understood that the comparative phenotypes discussed may not be entirely a function of the deleted gene but might well be influenced by subtle differences in
reagents, experimental procedures, genetic purity of the mice and their housing environments.

**Results and Discussion**

**Generation of IL-1RI<sup>−/−</sup> Mice**

A clone encoding a portion of the murine IL-1RI gene was isolated from a 129 genomic library. The clone was mapped by a combination of restriction, polymerase chain reaction (PCR) and sequence analyses. A targeting vector was constructed by replacing a 2.4-kb EcoRI-PstI fragment containing two exons encoding amino acids 4–146 of the mature IL-1RI protein with a PGK-neo cassette. In addition, a thymidine kinase cassette (MC-TK) was inserted into the 3′ end of the vector. The targeting vector was electroporated into AB1 embryonic stem (ES) cells. Of 2000 G418 and ganciclovir resistant clones screened, one carried a disrupted IL-1R allele (as determined by PCR and genomic southern blot analysis). The clone was injected into blastocysts derived from C57BL/6J mice. Male chimeras were bred to C57BL/6 females and screened for germ line transmission of the disrupted IL-1R allele by PCR. Mice homozygous for the IL-1R mutation were generated at the expected mendelian frequency from crosses of the heterozygotes. The homozygous IL-1RI knockout mice used throughout these studies are random C57BL/6 × 129/SvJ hybrids unless otherwise indicated. Sex and age matched C57BL/6J, 129/SvJ, and (C57BL/6J × 129/J)F<sub>1</sub> mice were used as controls in these studies. Mice were maintained in a specific pathogen free facility. The IL-1RI<sup>−/−</sup> mice are undergoing backcross breeding to C57BL/6J mice to provide a better defined genetic model. These mice will be submitted to the Induced Mutant Resource Program instituted and developed by The Jackson Laboratory (Bar Harbor, ME).

Initial studies of IL-1RI<sup>−/−</sup> mice demonstrated normal fertility, viability, and vigor. Assessment of the composition of the lymphoid and hematopoietic system revealed no abnormalities relative to wild-type mice. Cellularity of bone marrow, thymus, spleen, and selected lymph nodes were within normal parameters when assessed in young adults of both sexes. The phenotypic composition of these organs determined by multiparameter flow cytometry was within normal limits for the following markers: CD4, CD8, B220, slgM, TCRα/β, TCRγ/δ, Gr1, and Mac1.
Table 1

Proliferative Response of B-cells from IL-1RI⁻/⁻ Mice to Anti-Ig Plus IL-1 or IL-4

| Strain | C57BL/6 | 129/SvJ | (C57BL/6 × 129)F₁ | IL-1RI⁻/⁻ |
|--------|---------|---------|--------------------|-----------|
| Anti-Ig | 0.8     | 2.5     | 2.3                | 3.6       |
| Anti-Ig + IL-4 | 8.2     | 5.7     | 8.3                | 14.1      |
| Anti-Ig + IL-1 | 12.5    | 11.0    | 10.1               | 3.5       |

*The results are the average ³H-TdR uptake of a typical experiment in which purified splenic B-cells were cultured in microtiter plates for 3 d with the described stimuli.*

**Evidence that the Targeted Mutation of the IL-1RI Gene Results in the Absence of IL-1-Mediated Signaling**

The ability to assess the deletion of IL-1RI by radioligand binding studies was not attempted because of the presence of IL-1RII. Thus, the initial evidence that the targeted mutation of the IL-1R resulted in a lack of IL-1-mediated signaling came from in vitro studies of B-cell function. IL-1 has long been known to stimulate a proliferative response by B-cells that were activated by crosslinking of slg (3). In these experiments, purified splenic B-cells were cultured with affinity-purified goat anti-mouse IgM and either IL-1β or IL-4. A representative experiment is shown in Table 1. The proliferative response of anti-IgM activated B-cells from IL-1RI⁻/⁻ mice was not increased by the addition of IL-1 to the cultures, but was by the addition of IL-4. The proliferative response of anti-IgM activated B-cells from wild-type mice was increased by the addition of IL-1, as well as IL-4. Anti-IgM activated B-cells from the IL-1RI⁻/⁻ mice proliferated in response to recombinant derived CD40L as well (data not shown). These results indicate that IL-1 signaling is defective in B-cells isolated from IL-1RI⁻/⁻ mice although these cells remain responsive to other stimuli. We next assessed whether IL-1 induced changes when injected into IL-1RI⁻/⁻ mice. Administration of IL-1 to normal mice induces a broad spectrum of responses amongst which are increased Ia expression on B-cells and thymic hypoplasia (22,23). After 4 d of IL-1 or mouse serum albu-
Table 2

| Mouse     | Treatment | Cellularity<sup>b</sup> | %B220<sup>+</sup>/Ia<sup>+</sup> | MFI  |
|-----------|-----------|-------------------------|---------------------------------|------|
| C57BL/6   | MSA       | 146                     | 56.5                            | 140.2|
|           | IL-1      | 139                     | 55.1                            | 220.7|
| IL-1<sup>-/-</sup> | MSA   | 153                     | 59.0                            | 88.7 |
|           | IL-1      | 137                     | 54.2                            | 83.5 |

<sup>a</sup>Mice were injected with 2 µg of either MSA or huIL-1β ip daily for 4 d. On d 5, the mice were sacrificed and Ia expression determined on spleen cells by immunofluorescent staining and flow cytometric analysis. The data represent the arithmetic average of the values obtained from three individually analyzed mice/group. The IL-1<sup>-/-</sup> mice used in this experiment had been backcrossed four times to C57BL/6J mice.

<sup>b</sup>Cells × 10<sup>-6</sup>.

min (MSA) injection, spleen cells were isolated from wild-type and IL-1<sup>-/-</sup> mice and Ia expression on B-cells assessed by immunofluorescent staining and flow cytometric analysis. As can be seen in Table 2, the mean fluorescent intensity (MFI) of B-cells from wild-type mice injected with IL-1 was increased compared to wild-type mice injected with MSA, however the MFI of the B-cells from IL-1 injected IL-1<sup>-/-</sup> mice was no different from that of B-cells from MSA injected IL-1<sup>-/-</sup> mice. Interestingly, the MFI of the class II MHC expression on B-cells from IL-1<sup>-/-</sup> mice injected with MSA was less than that of similarly treated wild-type mice, suggesting that IL-1-mediated signaling may be involved in maintaining homeostatic levels of Ia expression. Thymic cellularity was assessed after 7 d of injections and it can be seen in Table 3 that thymic cellularity from IL-1<sup>-/-</sup> mice was unchanged after IL-1 administration whereas thymic cellularity from wild-type mice treated with IL-1 was drastically reduced compared to MSA injected wild type mice. These experimental data generated in vivo support the lack of IL-1-mediated signaling in IL-1<sup>-/-</sup> mice.

**Serum Ig Levels and Antigen-Specific Antibody Response in IL-1<sup>-/-</sup> Mice**

Since freshly isolated splenic B-cells from IL-1<sup>-/-</sup> mice did not respond to anti-slgM and IL-1 in vitro, it was of interest to determine the in vivo innate serum Ig levels as well as the antigen-
Table 3
Effect of IL-1 Administration on Thymic Cellularity

| Mouse     | Treatment | Thymic cellularity | Thymic cellularityb |
|-----------|-----------|--------------------|---------------------|
| C57BL/6   | MSA       | 274.6              |                     |
|           | IL-1      | 62.6               |                     |
| IL-1RI⁻/⁻| MSA       | 266.3              |                     |
|           | IL-1      | 280.3              |                     |

* aMice were injected with 2 μg of either MSA or huIL-1β ip daily for 7 d. On day 8, the mice were sacrificed and thymic cellularity determined. The data represent the arithmetic average of the values obtained from three mice/group. The IL-1RI⁻/⁻ mice used in this experiment had been backcrossed four times to C57BL/6J mice.

bCells x 10⁻⁶.

specific antibody response. Sera were isolated from young adult mice (10–16 wk of age) and the Ig subclass concentrations determined by subclass-specific enzyme-linked immunosorbent assay (ELISA). There was no significant difference in the concentrations of IgM, any of the IgG subclasses, IgA, or IgE between control and IL-1RI⁻/⁻ mice (19).

The antigen-specific antibody response was assessed by determining the serum antibody response to the T-independent type II antigen, DNP-Ficoll as well as the T-dependent antigen, TNP-KLH. The primary antibody response was assessed by immunizing mice with 10 μg of DNP-Ficoll ip and bleeding these mice on d 10. Antigen-specific serum IgM was assessed by a TNP-specific ELISA assay. There was no difference between the groups of mice in the anti-DNP-Ficoll response (19). To assess secondary, T-dependent antibody responses, mice were immunized with alum-precipitated TNP-KLH ip on d 0 and d 21. Mice were bled on d 26 and the hapten-specific antibody response determined by Ig-subclass specific ELISA. The secondary TNP-response to this antigen is predominated by the IgG₁ and IgE subclasses and IL-1RI⁻/⁻ mice had similar levels of anti-TNP-specific IgG₁ and IgE (19). Thus, in the absence of a functional IL-1RI, these mice have normal levels of serum Ig and are still able to mount an antigen-specific primary IgM response to a T-independent type II antigen and a secondary IgG and IgE response to a classical T-dependent antigen.
Resistance to LPS-Induced Endotoxic Shock

Significant experimental evidence has highlighted the role of IL-1 and TNFα in the induction of endotoxic shock (for review, see refs. 24,25). For example, LPS administration to experimental animals induces significant production of both IL-1 and TNFα, injection of either or both of these cytokines to experimental animals results in the induction of symptoms that mimic those seen in septic shock and antagonists to either of these cytokines results in the reduction of pathology seen post-LPS injection. Thus, it was of interest to assess the deficiency of IL-1-mediated signaling on survival of mice to a challenge with a lethal dose of LPS. Wild-type and IL-1RI⁻/⁻ mice were injected ip with 50 μg of LPS/g of body weight. Administration of this dose of LPS resulted in death of 78% of wild-type mice and 80% of IL-1RI⁻/⁻ mice (19). There was also no difference in mean time to death between the groups of mice (data not shown). Thus, the inability to signal through IL-1RI does not increase resistance to LPS-mediated endotoxic shock. Interestingly, results from other gene knockout models provide additional insight as to the role of cytokines in the pathogenesis of endotoxic shock. These results are summarized in Table 4. ICE⁻/⁻ mice show greater resistance (decreased lethality) to high-dose LPS challenge compared to wild-type mice (29). It is not immediately apparent why these differences exist between IL-1RI⁻/⁻ and ICE⁻/⁻ mice. The resistance of ICE⁻/⁻ mice to high-dose LPS challenge is surprising since...
ICE was thought to be largely responsible for processing only IL-1β and not IL-1α. Thus, it might have been expected that IL-1α production in response to LPS would be able to mediate the pathological consequences of LPS injection. However, it has been shown that in response to LPS stimulation in vitro, macrophages from ICE−/− mice are deficient not only in the production of IL-1β, but also show reduced production of IL-1α, TNFα, and IL-6 (31). Thus, ICE may have broader activities important in proper cellular function that are in addition to IL-1 processing. The disruption of these functions may contribute to the enhanced resistance of ICE−/− mice to LPS.

TNFα as well as IL-1-mediated signaling is thought to be important in the pathogenesis of septic shock. Thus, the results of challenging TNFR−/− mice with LPS are of significant interest. It has been reported that TNFRI(p55)−/− mice do not differ in susceptibility compared to wild-type mice and TNFRII(p75)−/− show a tendency toward increased resistance (26,27). The reported resistance of TNFRII(p75)−/− to high-dose LPS is not absolute since a portion of the mice succumb at each dose tested. We have independently derived TNFRII(p75)−/− mice and in our hands (and in, perhaps more importantly, our animal colony) these mice do not differ from wild-type control mice in their resistance to high-dose LPS challenge (Peschon, J., unpublished). The difference between these experimental results remains to be determined. The analysis of the phenotypic mice bred to express multiple gene deletions (for instance, IFNγR−/−/TNFRI−/− mice) will be of great interest.

A slightly different phenotypic pattern emerges from another model of septic shock which involves co-administration of the hepatic toxin, D-gal, with low-dose LPS. In this model, mice succumb within days as a consequence of acute liver failure. Coadministration of D-gal plus LPS resulted in the death of 87% of wild-type mice and 84% of IL-1RI−/− mice (19). Thus, deficient IL-1 signaling does not increase the resistance of mice to LPS plus D-gal challenge. The results from other gene knock out animals injected with D-gal and LPS are also summarized in Table 4. The susceptibility of ICE−/− or IL-1β−/− mice to D-gal plus LPS has not been reported. TNFRII(p55)−/− as well as IFNγR−/− mice are more resistant than wild-type to challenge with D-gal and LPS (27,28,30). TNFRII(p75)−/− are as susceptible as wild-type animals to D-gal plus LPS (29). These findings implicate signaling through TNFRI and
IFNγR, but not TNFRII or IL-1RI as being involved in the pathogenesis of acute liver failure as a consequence of D-gal and LPS administration.

**Assessment of the Acute Phase Response**

The early physiological response to significant tissue injury or infection (known as the acute-phase response) involves dramatic alterations in temperature regulation, metabolism, cytokine production, hematopoiesis, and hepatic protein synthesis. Experimental induction of the acute-phase response has generally used two methodologies; one is the systemic administration of LPS which mimics severe endotoxemia, the second is the local injection of turpentine in a subcutaneous site which induces localized tissue destruction and inflammation.

Hepatic synthesis of acute-phase proteins (APP) is an important component of these inflammatory stimuli (for review, see ref. 32). As with many other aspects of the inflammatory response, APP synthesis can be induced by cytokines such as IL-1, TNFα and IL-6. Thus, it was of interest to assess ability of LPS to induce APP in IL-1RI−/− mice. Wild-type and IL-1RI−/− mice were injected ip with 50 μg of LPS and 24 h later, the mice were sacrificed. Total RNA was isolated from livers and subjected to Northern blot analysis using probes specific for serum amyloid A (SAA-1), serum amyloid P (SAP), and α1 acid glycoprotein (AGP). IL-1RI−/− mice injected with PBS did not express detectable APP. In response to LPS injection, there were significant levels of mRNA detected for SAA-1, SAP, and AGP from the livers of IL-1RI−/− mice. The levels were not significantly different from those induced in wild-type mice (19). Similar analysis of APP induction in response to LPS has been reported for other gene knockout animals (summarized in Table 5). APP induction in IL-6−/− and IL-1β−/− mice was not significantly different from wild-type indicating that the induction of AAP in response to LPS does not require IL-1 or IL-6 (20,33,34).

Others have investigated different parameters of the acute phase response such as serum cytokine production, fever, anorexia, or weight loss in gene knockout animals. For instance, IL-1β−/− mice exhibit a diminished, but not absent, fever response to LPS (35). LPS or LPS plus D-gal administration induced less of an acute phase response in IFNγR−/− mice compared to wild-type as deter-
Table 5
Summary of the Effects of Cytokine or Cytokine Receptor Gene Deletions on the Induction of the Acute Phase Response

| Gene knockout animal | Response (relative to wildtype) | LPS | Turpentine* |
|----------------------|---------------------------------|-----|-------------|
| IL-1RI−/−            | Equivalent fever, equivalent anorexia, equivalent serum TNFα (33) | No fever, no anorexia (33) | |
| IL-1β−/−             | Less severe fever, equivalent APP (34) | No fever, lower APP (35) | |
| ICE−/−               | Less serum IL-6, TNFα, no serum IL-1α, IL-1β (28,29) | ND* | |
| IL-6−/−              | Equivalent APP (20,33) equivalent weight loss, equivalent anorexia (33) | Lower APP (20,33) no weight loss, mild anorexia (33) | |
| IFNγR−/−             | No body weight loss, decreased serum transaminase, and TNFα (31) | ND | |

*ND, Not determined.

mined by lack of body weight loss, decreased serum TNFα and serum transaminase levels (31). These findings are consistent with the greater resistance of these mice to a lethal challenge with LPS or LPS plus d-gal (31).

As mentioned, sc injection of turpentine into mice induces local tissue injury and inflammation resulting in an acute phase response (fever, weight loss, APP synthesis). Importantly, this insult is localized and thus differs dramatically from the systemic injection of LPS. Subcutaneous injection of turpentine induced fever, lethargy, anorexia, or body weight loss in wild-type, but not in IL-1RI−/− mice (35). In this study, injection of LPS produced similar changes in the parameters in both types of mice (35). Again in response to turpentine injection, IL-6−/− mice had a dramatically reduced induction of APP, no weight loss, and only mild anorexia and hypoglycemia (33). IL-1β−/− mice were completely resistant to fever development and anorexia in response to turpentine (34).
Thus, in contrast to LPS administration where little difference is seen in the response of wild-type and IL-6\(-/-\) or IL-1R\(-/-\) mice, the induction of localized damage results in a diminished acute phase response in these gene knockout animals which indicates a role of IL-6- and IL-1 mediated signaling in the response to localized injury. Interestingly, IL-6\(-/-\) mice develop severe anorexia and weight loss 5 d after turpentine injection when wild-type mice are recovering. Thus, although these mice do not develop an acute phase response early, it appears that they are impaired in their ability to resolve the lesion. Observations similar to this have not been reported for other knockout mice to date.

**Susceptibility of IL-1R\(-/-\) Mice to Infection with *Listeria Monocytogenes***

Experimental infection of mice with *L. monocytogenes* is a widely studied model of host response to bacterial infection (37). On introduction into the host, *L. monocytogenes* are readily phagocytosed by macrophages, but the bacteria can avoid immediate destruction because of their ability to escape the hostile environment of the phagolysosome and replicate in the cytoplasm of the cell. The initial response of the host involves interactions among natural killer (NK) cells, γδ T-cells, and results in macrophage activation. This process is mediated at least in part via the elaboration of cytokines. The results of published studies highlight the importance of IL-1, TNFα, IL-6, IL-12, and IFNγ in stimulating the process of early host resistance (38–44). Sterilizing immunity, which is T-cell dependent, occurs between 8 and 12 d postinfection.

It should be noted that the analysis of host resistance to *L. monocytogenes* in gene knockout mice is complicated owing to genetic differences in the resistance of the strains most commonly used for these studies. Because of the widespread use of embryonic stem cell lines derived from the 129 strain of mice and the fusion with blastocysts from C57BL/6 mice, the initial analysis of knockout mice often utilizes mice that genetically should be considered random C57BL/6 × 129 hybrids. However, C57BL/6 and 129 strains of mice differ genetically in their susceptibility to *L. monocytogenes* which has been mapped to a single locus (*Lsr1*) on chromosome 2. C57BL/6 mice are resistant, 129 mice are susceptible, and resistance is dominant (45). Thus, the results of studies on the resistance of...
gene knockout mice to *L. monocytogenes* that utilize random hybrids may produce misleading results, especially if small numbers of mice are used. For instance, in random C57BL/6 × 129 hybrids, 25% of the mice would be genetically susceptible. Indeed in our initial assessment of host resistance to *L. monocytogenes* in IL-1RI<sup>−/−</sup> mice which were mixed hybrids, increased susceptibility was observed. In these studies, the survival of IL-1RI<sup>−/−</sup> mice was significantly, but not dramatically, less than that of control mice. The LD<sub>50</sub> for IL-1RI<sup>−/−</sup> mice was roughly three- to fivefold less than that for wild-type control mice which included (C57BL/6 × 129)<sub>F1</sub> mice. However, after five backcross generations to C57BL/6 mice, the increased susceptibility of the IL-1RI<sup>−/−</sup> mice to *L. monocytogenes* was no longer observed. This highlights the need for caution in interpreting the results of experiments on resistance of mice on a mixed 129/C57BL/6 background to *L. monocytogenes*. Thus, our conclusion at this time is that lack of a functional IL-1RI does not compromise host resistance to *L. monocytogenes*.

The results of the deletion of other cytokine or cytokine receptor genes on host resistance to *L. monocytogenes* is summarized in Table 6. Here it can be seen that the functional deletion of genes encoding IL-6, TNFRI(p55), or the IFNγR resulted in diminished host resistance (20,21,26,30). Interestingly, IL-1β<sup>−/−</sup> mice did not show diminished resistance to challenge with *L. monocytogenes* as assessed by bacterial numbers in spleen and liver 4 d after infection (34). It has been reported that TNFRII(p75)<sup>−/−</sup> mice have diminished resistance to *L. monocytogenes* as well (27), however, in the TNFRII(p75)<sup>−/−</sup> mice
generated here, there was no evidence of diminished resistance to infection with *L. monocytogenes* (Peschon, J., unpublished). Again the difference between these sets of observations remains to be clarified and the contributions of genetic background analyzed. However, we have also independently derived TNFRI(p55)^-/- mice and these do show increased susceptibility to *L. monocytogenes* infection (Peschon, J., unpublished). Thus, a more complete understanding of the role of cytokines in host resistance awaits both the generation of knockout mice on defined inbred genetic backgrounds as well as the assessment of resistance to other infectious agents.

**Conclusion**

Cytokines play an important role in both the specific and non-specific resistance of the host to infectious agents. Because of the functional redundancy of the cytokine family, it has been difficult to precisely assess the role of a single cytokine in the process of inflammation and host resistance to infectious agents. The generation and availability of mice lacking cytokines or cytokine receptors through targeted gene mutation allows for a more exact determination of the role of a particular cytokine in physiological homeostasis and the pathogenesis of disease states.

In this communication we have described the phenotype of mice lacking IL-1RI. Cells from IL-1RI^-/- mice or the mice themselves fail to respond to IL-1 in a number of systems:

1. Purified B-cells activated by anti-Ig did not proliferate to IL-1 in vitro;
2. Splenic B-cells from mice injected with IL-1 did not have increased Ia expression;
3. Mice injected with IL-1 did not have diminished thymic cellularity;
4. There was no serum TNFα in response to IL-1 injection (19).

The functional impact of the lack of IL-1-mediated signaling described thus far is an absence of a fever and anorectic response to tissue damage induced by sc turpentine injection (36). Interestingly, there was not a dramatic effect of the absence of IL-1-mediated signaling on immune system development or function in our preliminary assessment. However, many more detailed studies are required before a valid conclusion can be made concerning the role of signaling through IL-1RI in immune function.
The further analysis of these mice as well as other genetically modified mice such as the IL-1β−/− and ICE−/− mice will allow for a better understanding of the physiological and pathological role of the IL-1/IL-1R cytokine system.

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Chapter 11

Mice Deficient in Interleukin-1\(\beta\) Converting Enzyme

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Introduction

Interleukin-1 (IL-1) is a cytokine that has been implicated in the pathogenesis of acute and chronic inflammatory diseases \(1\). IL-1 has two isoforms, IL-1\(\alpha\) and IL-1\(\beta\) of which IL-1\(\beta\) is the predominant form released by human monocytes in culture. IL-1\(\beta\) is synthesized as a 31-kDa precursor devoid of a conventional signal sequence and is processed to its active 17-kDa form by an enzyme called IL-1\(\beta\) converting enzyme (ICE) which has an unique cleavage specificity for Asp-X \(2-4\). The purification and cloning of this enzyme have been described \(5,6\). ICE is synthesized primarily as an inactive 45-kDa precursor and auto-processed to 20- and 10-kDa subunits. The crystal structure of ICE reveals that the active enzyme is composed of two 20-kDa and two 10-kDa subunits to form a tetramer \(7,8\).

The recent discovery by Yuan and colleagues \(9,10\) has shed light on the function of ICE in programmed cell death or apoptosis. They cloned the \textit{ced-3} gene which controls cell death during development of the nematode, \textit{Caenorhabditis elegans}, together with another gene, \textit{ced-4} \(9\). They found that the \textit{ced-3} protein shared
28% identity with ICE (10). Moreover the amino acid residues that are important for substrate recognition are all conserved between ced-3 and ICE (7,8). Although mutations in ced-3 genes abrogates cell death, overexpression of ICE in the rat fibroblast cell line Rat-1 was shown to induce apoptosis. Furthermore a mutation in the catalytic Cys residue of ICE abolished its ability to induce apoptosis (11). This effect was reduced when ICE was coexpressed with bcl-2, a mammalian proto-oncogene, which is homologous to ced-9 in the nematode (11). The function of ced-9 has been shown to protect cells from undergoing apoptosis (12). bcl-2 is able to substitute for ced-9 in blocking cell death in a mutant lacking the endogenous ced-9 activity (13). Further crmA protein, a serpin-like inhibitor of ICE (14), protected cells from apoptosis that was induced by various stimuli (15,16).

Extensive search for another homolog of ICE revealed that ICE is a member of a new family of related cysteine proteases. In humans it includes ICH-1L, CPP32, Mch2, Tx (ICErel-II or ICH-2), and ICErel-III (17–21). All the members have pro-apoptotic activity. The role of the ICE protease family both in apoptosis and in cytokine secretion is of great interest. In order to investigate the physiological functions of ICE, we therefore generated gene-targeted mice with a nonfunctional ICE gene (22).

The Generation of ICE Knockout Mice

We disrupted the murine ICE gene in D3 embryonic stem (ES) cells by replacing part of exon 6 and 7 (22) with a neomycin resistance cassette (23). Exon 6 contains a critical pentapeptide motif which is conserved in the ICE protease family (24). The construct was introduced in D3 ES cells by electroporation. Sixty-three ES cell clones were screened by polymerase chain reaction (PCR) using an exon 10 primer and a neocassette-specific primer. One clone was found to be targeted correctly and this event was confirmed by Southern blot analysis. Chimeric mice were generated by injection of mutant ES cells into C57BL/6 blastocysts. Chimeric male mice were mated with C57BL/6 female mice to obtain heterozygous mice for the mutated ICE gene. According to the Southern blot analysis of the ICE gene, interbreeding of the heterozygous mice generated the expected mendelian 1:2:1 ratio of wild-type (ICE+/−), heterozygous
(ICE<sup>+</sup>) and homozygous (ICE<sup>−/−</sup>) mutant mice. The absence of ICE mRNA was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) analysis (22). The ICE<sup>−/−</sup> were healthy and fertile, and did not show any gross abnormality in appearance, body weight, or organ size up to 6 mo. This result is consistent with the report from Li and colleagues (25). They further examined histopathologically various organs including spleen, lung, kidney, liver, adrenal gland, brain, gastrointestinal tract, pancreas, salivary gland, thymus, and testis at 8 wk old. ICE<sup>−/−</sup> did not show any abnormalities. Numbers of leukocytes, erythrocytes, and platelets in the peripheral blood were also normal in ICE<sup>−/−</sup> mice. Since the immune system undergoes apoptosis to exclude self-reactivity even in adult mice, we checked T-cell populations in thymus and B-cell populations both in spleen and in bone marrow (Fig. 1). There were no significant differences among T-cell populations in thymus (CD4<sup>+</sup>CD8<sup>−</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>−</sup>, and CD4<sup>+</sup>CD8<sup>+</sup>) and lymph nodes (CD4<sup>+</sup>CD8<sup>−</sup> and CD4<sup>+</sup>CD8<sup>+</sup>). B-cell populations were normal as compared with those of wild-type mice. Although several lines of evidence show that ICE is involved in apoptosis, these results suggest that ICE is dispensable for development.

**The Role of ICE in Apoptosis**

Overexpression of ICE or its homologs is able to induce apoptosis in cultured cells (11,17–21,26). To determine whether intracellular expression of ICE under normal physiologic conditions mediates apoptosis, thymocytes from ICE<sup>−/−</sup> mice were treated with three apoptotic inducers: glucocorticisone (27), ionizing radiation (28), and anti-Fas antibody (29,30). Thymocytes isolated from ICE<sup>+/+</sup> and ICE<sup>−/−</sup> mice were sensitive to both dexamethasone- or radiation-induced apoptosis as evaluated by cell viability and DNA fragmentation (Figs. 2 and 3, p. 180). A monoclonal antibody against Fas antigen-induced apoptosis in ICE<sup>+/+</sup> thymocytes in a dose-dependent manner, but did not trigger apoptosis in ICE<sup>−/−</sup> thymocytes (Fig. 4). To exclude the possibility that expression of Fas antigen was affected by the ICE gene mutation, we examined surface expression of Fas antigen on ICE<sup>+/+</sup> and ICE<sup>−/−</sup> thymocytes by fluorescence-activated cell sorting (FACS). There was no difference in expression of Fas antigen between ICE<sup>+/+</sup> and ICE<sup>−/−</sup> (Fig. 5, p. 181). We also
Fig. 1. Lymphocyte populations in ICE<sup>+/+</sup> and ICE<sup>−/−</sup> mice. (A) Flow cytometric analysis of T-cell populations in thymus and lymph node. (B) Flow cytometric analysis of B-cell populations in spleen and bone marrow.
observed that a potent ICE inhibitor, Cbz-Val-Ala-Asp-(OEthyl)-[2,6-dichlorobenzoyl]oxy]methyl-ketone, prevented Fas-mediated apoptosis of ICE+/+ thymocytes in a dose-dependent manner (data not shown). The Fas antigen is a cell surface molecule and belongs to the tumor necrosis factor (TNF)-α (TNFα) receptor family (31). Fas can mediate apoptosis in various cells and is encoded by the gene responsible for a lymphoproliferative disorder (lpr) in mice.
Fig. 3. Agarose gel electrophoresis of total DNA from thymocytes. Thymocytes were treated with medium alone (N), dexamethason (D) or radiation (R). The doses of these stimuli were the same as those indicated in Fig. 2. Genomic DNA was isolated from $10^6$ thymocytes after 10 h and subjected to electrophoresis.

Fig. 4. Fas-induced cell death in thymocytes from ICE$^{+/+}$ and ICE$^{-/-}$. Thymocytes were incubated for 24 h with anti-Fas antibody at the concentration indicated. Cell viability was assessed as described in Fig. 2.
Fig. 5. Expression of Fas on thymocytes from ICE^+/+ and ICE^-/. Cells were stained with anti-Fas antibody followed by a fluorescein isothiocyanate-conjugated antibody against hamster immunoglobulin G. Shaded areas represent staining by the second antibody only.

Disruption of Fas expression or function in lpr mutant mice results in a progressive lymphoadenopathy and systemic autoimmune disease (32). Although Fas-mediated apoptosis is abrogated in ICE^-/- thymocytes, ICE^-/- mice do not develop autoimmune symptoms within 6 mo. Li et al. (25) also showed that thymocytes from ICE^-/- underwent apoptosis in response to being treated with ionizing radiation or dexamethasone. Apoptosis in macrophages from ICE^-/- mice was induced by adenosine triphosphate (ATP) treatment. ATP-induced DNA fragmentation was detected both in ICE^+/+ and in ICE^-/- mice macrophages (25). ICE does not seem to be rate limiting for apoptosis in macrophages.

It has since been shown that an ICE-like protease is involved in Fas-mediated apoptosis (33,34). Overexpression of ICE in a mouse fibrosarcoma cell line expressing Fas enhanced Fas-mediated apoptosis and transfection of antisense-ICE construct into the cell line
resulted in reducing Fas-mediated apoptosis (34). These data support
our finding that Fas-mediated apoptosis is deficient in thymocytes
from ICE\textsuperscript{−/−} mice. Thymocytes treated with a glucocorticoid or radia-
tion may either undergo apoptosis through different pathways that
may involve another protease-like intracellular serine proteases
(35,36) or alternatively these stimuli may enter a single apoptotic
pathway downstream from ICE. The existence of multiple apoptotic
pathways in T-cells has been implicated in transgenic study of bcl-2,
a proto-oncogene that blocks apoptosis. Gene disruption of the
murine bcl-2 gene resulted in an increased sensitivity to glucocorticoid-
and radiation-induced cell death (23,37). Stimulation with anti-CD3 antibody, however, rescued T-cells from apoptosis (23).
Targeted expression of human bcl-2 in lymphoid tissues prevents
thymocytes from undergoing apoptosis induced by glucocorticoids,
radiation and anti-CD3 antibodies, but does not block negative selec-
tion in the thymus (38,39). Similarly, p53 is involved in apoptosis
induced by radiation and etoposide, an inhibitor of topoisomerase II,
but is not required for apoptosis induced by glucocorticoid and com-
pounds that mimic T-cell receptor (TCR)-mediated signals (40,41).

Unlike lpr or gld mice, which lacks normal expression of Fas
and Fas ligand respectively, ICE\textsuperscript{−/−} mice did not develop auto-
immune symptoms such as lymphadenopathy, splenomegaly, hyper-
globulinemia, autoantibody production, glomerulonephritis, and
arthritis (22; our unpublished observation) that are seen in the former
mutants (42). However homozygosity for either lpr or gld results in
deficiency of TCR-mediated cell death in mature T-cells (43–45).
mRNA expression of Fas ligand was not detected in thymic stromal
cell lines (46). Autoimmunity in these mice may be caused by a fail-
ure of cell death in peripheral, rather than a defect in negative selec-
tion in thymus (47). Given that interaction of Fas and its ligand
induces apoptosis after T-cell activation (48–50), deletion of mature
T-cells might be dependent on ICE homologs and their functions.

**Cytokine Secretion In Vitro**
and **In Vivo in ICE Knockout Mice**

Several serine proteases can process the IL-1\textbeta precursor to
bioactive forms but ICE is the only protease to generate a mature
17-kDa cytokine with naturally occurring Ala118 N-terminus
A tetrapeptide inhibitor of ICE [Ac-Try-Val-Ala-Asp-CHO; (Ki) = 0.7 nM] blocks processing and secretion of IL-1β from stimulated human whole blood monocytes or murine leukocytes [IC50 (the amount required to inhibit activity by 50%) ∼ 1.5 μM] (51,52,54). To elucidate the role of ICE in cytokine secretion, we challenged monocytes from ICE+/+ and ICE−/− mice with lipopolysaccharide (LPS) and LPS plus nigericin. LPS can induce secretion of several cytokines such as IL-1β, IL-1α, TNFα, and IL-6. Nigericin is a K⁺-H⁺ ionophore that alters K⁺ homeostasis and activates a plasma membrane adenosine triphosphatase (ATPase). Nigericin treatment after LPS stimulation enhances processing of the IL-1β precursor and export of the mature 17-kDa IL-1β from human and murine monocytes (55,56). LPS stimulated IL-1β secretion (39.1 ± 28 pg/mL) and LPS/nigericin treatment significantly enhanced IL-1β secretion by ICE+/+ monocytes (140 ± 72 pg/mL, p < 0.02). In contrast, ICE−/− monocytes did not secrete any detectable IL-1β after LPS stimulation or LPS/nigericin treatment. No processed IL-1β is present in cell lysates from these cultures as compared to ICE+/+ monocytes (Fig. 6A). Similar results were obtained by Li and colleagues (25).

IL-1α binds to the same receptor as IL-1β and is also synthesized as a precursor (1), but the IL-1α precursor is not cleaved by ICE (2–4). LPS and LPS/nigericin treatment also enhance IL-1α secretion by ICE+/+ monocytes. Surprisingly, ICE−/− monocytes fail to secrete IL-1α after LPS/nigericin treatment despite significant intracellular levels of IL-1α in the LPS/nigericin-treated ICE−/− monocytes (115 ± 38.5 pg/mL) (Fig. 6B). This result implicates that ICE mediates secretion of IL-1α from monocytes. We also observed decreased secretion levels of TNFα and IL-6 in the cultures of ICE−/− monocytes (Fig. 7).

IL-1 is thought to be a mediator of endotoxin-induced shock and bacterial sepsis. Administration of IL-1β alone can induce shock (57) and a natural IL-1 receptor antagonist (IL-1RA) reduces mortality from endotoxin shock in animal models (58,59). Therefore we challenged ICE+/+ and ICE−/− mice by injecting a high dose of LPS (140 mg/kg) intraperitoneally. ICE−/− mice showed a more severe hypothermic response and earlier mortality compared to ICE+/+ mice (Table 1). Serum level of IL-1β in LPS-challenged ICE−/− mice was significantly lower than that in ICE+/+ mice
Fig. 6. Cytokine secretion by adherent monocytes from ICE^{++} and ICE^{--} mice. Cytokines were quantitated in supernatant or cell lysates by the enzyme-linked immunosorbent assay (ELISA) specific for murine IL-1β (A) or IL-1α (B). The IL-1β ELISA is highly specific for mature IL-1β and shows <0.2% cross-reactivity against the murine IL-1β precursor. The IL-1α ELISA can detect both precursor and mature forms of IL-1α.

Fig. 7. Secretion of IL-6 and TNFα by adherent monocytes from ICE^{++} and ICE^{--} mice. Cytokines were assayed by specific ELISAs for IL-6 and TNFα.
**IL-1β-Deficient Mice**

Table 1

| Cytokine/acute phase protein | Control       | ICE+/+        | ICE−/−        |
|-----------------------------|---------------|---------------|---------------|
| IL-1β, pg/mL                | 19.8 ± 25.7   | 138 ± 51      | 56 ± 15       |
| IL-1α, pg/mL                | 26.4 ± 32     | 1587 ± 671    | 2165 ± 532    |
| TNFα, ng/mL                 | 0             | 10.1 ± 5.0    | 5.8 ± 2.1     |
| IL-6, ng/mL                 | 3.0 ± 1.2     | 13.7 ± 2.0    | 15.7 ± 2.5    |
| Serum amyloid A, μg/mL      | 320 ± 62      | 596 ± 23      | 513 ± 3       |

*Male and female ICE+/+(n = 6M and 6F) or ICE−/− mice (n = 1M and 3F) were challenged with 140 mg/kg LPS injected interaperitoneally. Blood samples were collected and cytokines were quantitated in sera using specific ELISAs. Body temperature was also monitored at 2, 7, 12, 17, and 22 h after LPS challenge. It was changed from 34.7°C to 33.4, 30.2, 25.8, 24.9, and 26.2°C at each time point in ICE+/+ mice. For ICE−/− mice body temperature was dropped from 37.4°C to 33.2, 29.3, 23.8, 23.9 and 22.7 at the same time points after LPS challenge. All the ICE−/− mice died within 23 hours while ICE+/+ died between 48 and 72 h after mice were injected with LPS.*

Injected with LPS (56 ± 15 vs 138 ± 51 pg/mL, respectively). The diminished serum level of IL-1β in LPS-challenged ICE−/− mice demonstrates the dependence of IL-1β processing and secretion and suggests that IL-1β is not a required mediator of sepsis induced by high dose of LPS. Enhanced survival of ICE−/− mice at lower doses of LPS is conceivable. In fact, ICE−/− mice becomes resistant to LPS-induced shock when injected with around one third the amount of LPS that we used (25). Neither IL-1α nor IL-1β was detected in serum of ICE−/− mice 4 h after LPS injection. The levels of TNF-α and IL-6 were also decreased in ICE−/− mice. In contrast to these data obtained after low dose LPS challenge, we observed comparable amount of IL-1α, TNF-α, and IL-6 in serum of ICE−/− mice when we used high doses of LPS (Table 1). This result contrasts the observation that both IL-1β and IL-1α were not secreted from ICE−/− monocytes. It is possible that IL-1 is released by other cells in vivo or by an ICE-independent secretion pathway if high dose of LPS is used. Furthermore, the pathophysiological response of ICE−/− mice to high doses of LPS may be attributable in part to IL-1α or other cytokines, which may override any IL-1β dependency.
**Summary**

ICE cleaves the inactive precursor of IL-1β to generate mature IL-1β. ICE is a member of a family of proteases which have been recently implicated in apoptosis. ICE deficient mice do not show any anatomical or developmental abnormality. Thymocytes from ICE−/− mice were sensitive to apoptosis induced by glucocorticoid and ionizing radiation. Fas-induced apoptosis, however, is defective in ICE−/− thymocytes. Adherent monocytes from ICE−/− mice did not export either IL-1β or IL-1α after stimulation with LPS or LPS plus nigericin. ICE−/− mice challenged with LPS at a high dose had a reduced level of IL-1β in serum but the pathophysiological response of ICE−/− mice to LPS-induced sepsis was not altered. This demonstrates that IL-1β is not an exclusive mediator of high-dose LPS-induced sepsis.

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Chapter 12

Neutrophilia and B-Cell Plasmacytosis in Mice Lacking the Murine IL-8 Receptor Homolog

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Introduction

Interleukin-8 (IL-8) is a member of a family of proinflammatory cytokines containing four conserved cysteine residues that are related by a C-X-C motif, and is a major factor in acute inflammation, being responsible for the activation and chemotaxis of neutrophils to the site of acute injury (1–5). Neutrophils provide the first line of defense in fighting infection by destroying bacteria with phagocytosis and the release of super oxides and peroxides. The response is rapid and is neither acquired nor antigen specific (6,7). With sepsis or trauma, this usually beneficial response can result in death since an excess of activated neutrophils can produce extensive organ and tissue damage. IL-8 is produced by a large variety of cell types in vitro and has been implicated in neutrophil migration and, to a lesser extent, T-cell migration, to sites of IL-8 injection (8–10). Despite rapid advances in the chemokine field, there has been some frustration in developing small animal models of IL-8 mediated inflammation as neither mouse nor rat IL-8 has been identified (11). Reports that anti-human IL-8 antibodies inhibit lung inflammation in rats (12) suggest the presence of a similar
molecule in rodents. Because of the tremendous importance of this molecule in humans, dogs, and rabbits, it is likely that if there is not a murine equivalent of IL-8 then other factor(s) must mediate similar physiological events.

The search for the human IL-8 receptor resulted in the cloning of two high affinity receptors that share 77% amino acid sequence identity and are well characterized (13–16). They are members of the superfamily of seven transmembrane domain receptors that are coupled to guanosine triphosphate (GTP)-binding proteins. IL-8 binds receptors A and B with nearly identical affinity ($K_d = 1.7$ and 1.5 nM, respectively). Melanoma growth-stimulating activity (MGSA), another chemokine of the C-X-C family, also binds to both receptors but with 100-fold lower affinity for receptor A than receptor B ([A] $K_d = 450$ nM and [B] $K_d = 4$ nM) (15,16). We have approached the role of chemokine-mediated inflammation from the aspect of the receptor and have cloned a murine homologue of the human IL-8 receptor. To determine this receptor’s role in inflammation, we used homologous recombination in embryonic stem (ES) cells to generate a mouse strain with a deletion of this gene.

### Genomic Cloning of the Murine IL-8 Receptor and Deletion by Homologous Recombination in ES Cells

A murine IL-8 receptor gene was isolated using cDNA probes from both human IL-8 receptors and screening a mouse genomic library at reduced stringency (17–20). DNA sequencing shows that the mouse receptor is encoded by a single exon as are the two human receptors. The murine exon contains a 350 amino acid open reading frame that has 68 and 71% amino acid identity with human IL-8 receptors A and B (Fig. 1A). The degree of similarity indicates that this molecule is also a member of the receptor family containing seven transmembrane domains that signals through GTP-binding proteins. To determine whether a second receptor is present in mice as in humans, we screened mouse genomic DNA blots under low stringency conditions with the complete mouse gene fragment as a probe. Using several different restriction enzymes, we detected a single band suggesting that unlike humans, mice contain a single gene for the putative IL-8 receptor (IL-8R) (Fig. 1B). Others (18) have reported the existence of a second IL-8 receptor (IL-8R) gene
Fig. 1. Sequence of a murine homolog of the human IL-8R. (A) Nucleotide sequence alignments of the mIL-8R and human IL-8R-A and IL-8R-B. Boxed sequences indicate amino acid similarity. The seven predicted transmembrane segments are numbered and indicated by a solid line. The closed circles indicate potential glycosylation sites. A mouse 129 genomic library was screened with cDNA probes from both human IL-8 receptors. Two λ phage clones were isolated that contained a 14- and 12-kb DNA fragment, respectively. A 1.9-kb EcoRI fragment containing the coding region was subcloned into pUC118 and sequenced by Sanger dideoxy polymerase chain elongation (25). Sequence data confirmed homology to the two human IL-8R. (B) Southern blot analysis of mouse 129 genomic DNA cut with (A) BamHI, (B) BglII, (C) BstEII, (D) EcoRI, (E) HindIII and hybridized at low stringency with a fragment containing the entire mIL-8R gene.
based on DNA hybridization, but we have been unable to clone a second highly related gene.

A gene targeting vector was constructed by deleting the single exon containing the open reading frame of the mIL-8R and replacing it with an expression cassette containing the neomycin resistance gene (Neo). This ensures the complete elimination of the gene following homologous recombination (19). Individual ES clones were screened by genomic blot hybridization; 7 clones (of 814 clones tested) were identified as having undergone homologous recombination and three of these were used to generate germline mice. Two of these lines were selected to interbreed to produce mice homozygous for the deletion of the mIL-8R (19). The deletion of the entire mIL-8R gene was confirmed by using the complete exon as a probe (data not shown).

**Phenotype of mIL-8R Deficient Mice**

The homozygous receptor deleted mice were indistinguishable from their wild-type and heterozygous litter mates in size and health. However, necropsy showed obvious morphologic changes. In all mIL-8R/ mice examined from two ES cell clones, the spleens were greatly enlarged from two to four times normal size and cervical lymph nodes were 3- to 10-fold enlarged. Other lymph nodes, including the parathymic, mesenteric, brachial, axillary, and lumbar, were enlarged but the degree of enlargement varied among animals (data not shown), whereas inguinal and popliteal lymph nodes were grossly normal. Comparative histopathology (Fig. 2) of mIL-8R+/+ and / mice demonstrated that the splenomegaly in mIL-8R/ mice was the result of expansion of the splenic white pulp by marked proliferation of myeloid elements (metamyelocytes, bands, and neutrophils) and megakaryocytes. In lymph nodes, the medullary cords were expanded by abundant foci of myelopoiesis, Russel bodies, and plasma cells, and compressed the adjacent medullary sinuses. Longitudinal sections of the femur and tibia demonstrated grossly white marrow in mIL-8R/ mice compared to the normal red appearance in mIL-8R+/+ mice. Histologically, there was a great increase in bone marrow cellularity composed of the normal myeloid maturation series (myelocytes, metamyelocytes, bands, and neutrophils) in these mice. The erythroid series was unaffected.
Fig. 2. Comparative histopathology of mIL-8R\(^{-/-}\) and mIL-8R\(^{+/+}\) mice. Spleen: Marked expansion of the splenic white pulp in mIL-8R\(^{-/-}\) mice characterized by increased numbers of megakaryocytes (1), myeloid hyperplasia (2), and numerous mitotic figures (3). Lymph nodes: In mIL-8R\(^{-/-}\) mice, peripheral lymph nodes were enlarged the result of pronounced expansion of the medullary cords (4), low magnification. Lymphatic sinusoids (5) were compressed by the hypercellular medullary cords containing numerous plasma cells (6), Russel bodies (7) and hypersegmented neutrophils (8). Bone marrow: Compared to mIL-8R\(^{+/+}\) mice, there was increased granulocyte production in the bone marrow of \(^{-/-}\) mice. The normal neutrophilic maturation series (metamyelocytes, bands, and segmented neutrophils) was observed in mIL-8R\(^{-/-}\) mice (original magnifications 16x and 256x). Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 \(\mu\)m, and stained with hematoxylin-eosin using standard techniques.
Approximately 25% of the mIL-8R−/− animals analyzed demonstrated metamyelocyte, band, and hypersegmented neutrophils in the periportal region of the liver, an indication of multiple foci of granulopoiesis (data not shown). However, there was no indication of inflammation or hepatic damage, and no apparent infiltration into the parenchyma. The extramedullary myelopoiesis occurring in the liver, lymph node, and spleen, is excessive in light of the concurrent production of myeloid elements by the bone marrow.

Blood analysis and differential cell counts, demonstrated that despite the myeloid hyperplasia of the marrow, the mIL-8R−/− mice were not anemic and had normal levels of red blood cells, hemoglobin, and hematocrit. The number of circulating neutrophils increased approx 12-fold in the mIL-8R−/− mice. The increased cellularity and alteration of granulocyte to lymphocyte ratios was evident on FACS analysis both by forward and side scatter, and detection of the granulocyte marker Gr-1 (19, and Fig. 3). The thymus and all other organs appeared normal (data not shown). T-cell populations and T- to B-cell ratios were normal. We have not observed greater changes in these phenotypes with advancing age up to 52 wk. Heterozygous mice (mIL-8R+/−) did not display any overt phenotype.

**Neutrophil Response to Acute Inflammatory Challenge**

The major role of neutrophils is to rapidly migrate to sites of injury and infection and destroy bacteria as part of an acute immune response. Injection of thioglycollate into the peritoneum of mice leads to the recruitment of neutrophils to the site of injection within 3 h. We demonstrated that the number of neutrophils from the mIL-8R−/− mice that migrated to the peritoneum after injection of thioglycollate was fivefold lower than mIL-8R+/+ mice, showing that the acute migration ability of neutrophils had been compromised (19, and Fig. 4).

To determine whether the neutrophils had matured to a functional state and if other aspects of neutrophil function other than migration have been affected, we tested purified bone marrow neutrophils for their ability to kill bacteria and demonstrated that both mIL-8R−/− and mIL-8R+/+ neutrophils are effective at both intracellular and extracellular killing of bacteria (19, and Table 1).
Identification of Ligands of the mIL-8R

The binding of ligand to the IL-8R that ultimately leads to activation of the neutrophil, initiates a series of cellular events including a transient rise in intracellular Ca$^{2+}$ concentration. As shown in Fig. 5, neutrophils from mIL-8R$^{+/+}$ mice show a highly significant Ca$^{2+}$ response to both human IL-8 as well as to mouse MIP-2 (a member of the C-X-C chemokine family, 21), while no response to these ligands was found for the mIL-8R$^{-/-}$ derived neutrophils. These data show that the mIL-8R can function as an IL-8 receptor and that it will respond to at least one murine-derived chemokine, MIP-2. Both wild-type and mIL-8R$^{-/-}$ mouse neutrophils respond to the C-C chemokine MIP-1α (for reviews, see ref. 5) and the unrelated peptide formyl-methionyl-leucyl-phenylalanine (fMLP), showing that neutrophil activation is mediated by different receptors for these ligands. The dose dependence of the mouse neutrophil Ca$^{2+}$ response to the chemokines, IL-8, MIP-2, and KC (another member of the
Fig. 4. Thioglycollate induced migration of neutrophils: mL-8R+/+ and −/− mice were injected intraperitoneally with 3 mL of thioglycollate. After 3 h, peritoneal exudate cells were harvested by peritoneal lavage. Total cell numbers were determined using a hemocytometer. Percent neutrophils was determined by differential cell counts from cytopsin slide preparations. Number of neutrophils was calculated by multiplying percent neutrophils by total cell counts. Each bar represents individual mice.

Table 1
Bacterial Killing by Neutrophils

| mL-8R  | Total live | Phagocytosed bacteria | Number of intracellular bacteria |
|--------|------------|-----------------------|---------------------------------|
|        |            |                       | Live | Killed    |
| +/+    | 1.9        | 68.3                  | 0.31 | 68.0      |
| +/+    | 2.7        | 67.5                  | 0.21 | 67.3      |
| −/−    | 2.9        | 67.2                  | 0.18 | 67.0      |
| −/−    | 2.4        | 67.8                  | 0.21 | 67.6      |

*Bone marrow neutrophils from mL-8R+/+ and −/− mice were tested for their ability to kill Staphylococcus aureus (24). The initial inoculum was 70 × 10⁶ bacteria and 2 × 10⁶ neutrophils. All values are × 10⁶.
Fig. 5. Ca\(^{2+}\) flux analysis of mouse neutrophils: Neutrophils were purified from bone marrow by Percoll density gradients of mIL-8R\(^{++/+}\) and \(+/−\) mice and loaded with 2 mM Indo-1 AM (24). Cells were analyzed by flow cytometry before and immediately after addition of 500 nm of each chemokine. On activation, a transient rise in intracellular Ca\(^{2+}\) causes a detectable shift in fluorescence seen as a sharp, sudden peak in the histogram shortly after addition of chemokine (blank space in histogram). Cell profiles (y-axis) were followed over 3-min time (x-axis) on an EPICS 752 flow cytometer.
Fig. 6. Dose dependence of Ca\textsuperscript{2+} flux intracellular Ca\textsuperscript{2+} response of wild-type mouse neutrophils. The chemokine concentration (nM) is indicated in the upper right corner of each graph.
C-X-C chemokine family (22), was determined. A Ca²⁺ response is detected at 0.5 nM IL-8, at 0.005 nM MIP-2, and at 0.05 nM KC (Fig. 6). Thus, the potency of Ca²⁺ response is MIP-2 > KC > IL-8; hIL-8 is ~100-fold less potent than mMIP-2, whereas mKC is ~10-fold less potent (20).

The dose dependence of the in vitro migration response for the three C-X-C chemokines (human IL-8 and mouse MIP-2 and KC) has been reported (20). Wild-type neutrophils migrate in response to all three ligands with a potency ranking of MIP-2 > KC > IL-8: exactly the rank order observed in Ca²⁺ responses (Fig. 7). No migration was found for neutrophils isolated from mIL-8R⁻/⁻ mice demonstrating that the mouse IL-8 receptor is required for signaling by these three chemokines. Migration induced by the unrelated C5a peptide was unaffected in the mIL-8R⁻/⁻ mice, indicating that the neutrophil loco-
motor function is not impaired. Thus, the mIL-8R is able to respond to concentrations of C-X-C chemokines that could be physiologically relevant. From these studies one might conclude that this receptor is specific for MIP-2 and KC. It is possible that there is no IL-8 in mice and that the MIP-2 molecule and the mIL-8R have co-evolved to serve a similar role in mice as IL-8 and IL-8R serve in humans.

To establish that MIP-2 and IL-8 are ligands for the mIL-8R, binding experiments were performed (20). Radiolabeled MIP-2 specifically binds to mIL-8R<sup>+/+</sup> neutrophils, whereas no binding was detected to neutrophils from mIL-8R<sup>−/−</sup> mice. Competitive binding experiments show that MIP-2 binds with high affinity ($K_d = 2.0$ nM) to about 1000 sites/cell on wild-type neutrophils. Similar results were obtained using 293 cells expressing the mIL-8R, with a $K_d = 1.3$ nM. Competition binding with unlabeled IL-8 shows that human IL-8 binds with a much lower affinity ($K_d = 63$ nM). Competition binding with MGSA shows an intermediate binding affinity ($K_d = 13$ nM). Thus, the 50-fold lower binding affinity of human IL-8 for the mIL-8R as compared to the affinity of MIP-2 parallels the ~100-fold difference in Ca<sup>2+</sup> and in vitro migration responses found for these two ligands (20).

Whereas we anticipated effects on the inflammatory response of neutrophils in the mIL-8R<sup>−/−</sup> mice, the major increase in myelopoiesis was quite unexpected. We have shown that the mIL-8R<sup>−/−</sup> neutrophils display greatly decreased migration in response to thioglycollate injected into the peritoneum of mice. Bacterial killing studies demonstrated that the mIL-8R<sup>−/−</sup> neutrophils were as effective at in vitro bacterial killing as mIL-8R<sup>+/+</sup> neutrophils, therefore the neutrophils appear to be functional but have lost their ability to migrate properly. These data are even more impressive when one considers that the total number of neutrophils in the mIL-8R<sup>−/−</sup> animals has greatly increased, providing these animals a much greater potential pool of neutrophils than wild-type animals. Whereas other chemokines have been implicated in neutrophil migration, we have clearly demonstrated that the loss of this receptor virtually eliminates their ability to migrate to the very potent challenge of thiolglycollate. These in vivo data lend support to efforts to block neutrophil damage in inflammation by either removing IL-8 or blocking its receptor. Such agents could be of great therapeutic benefit.
Several pieces of evidence suggest that two closely related IL-8 receptor genes may not exist in mice as is the case for humans and rabbits. The two human and two rabbit IL-8 receptors are 77 and 76% identical, respectively, in protein sequence and both contain multiple DNA regions with \( >90 \) bp of exact match. Therefore, crosshybridization of these pairs of sequences is readily detectable. The results shown in Fig. 1B for the mouse genome where only one band is observed, except for enzymes that cut within the probe region, suggest a single gene for the IL-8R in mouse. We have attempted to isolate genes related to the IL-8 receptor by low stringency screening (data not shown) and have not been able to isolate a second closely related IL-8 receptor in mice as is the case for humans and rabbits.

The targeted deletion of the one identified mouse IL-8 receptor shows that it is required for neutrophil migration and for the activation of an intracellular \( \text{Ca}^{2+} \) response (Fig. 5). If two independent IL-8 receptors were present in mice, one would expect that the deletion of one of receptor would not abolish the neutrophil migration and \( \text{Ca}^{2+} \) activation, as either human IL-8 receptor can independently mediate both of these effects (20). Thus, on a functionality basis as well as molecular hybridization, only one IL-8 receptor appears to be present in mice.

**Conclusions**

We were quite surprised by the consistent expansion of lymph nodes and plasmacytosis, as well as the marked neutrophilia in the mIL-8R\(^{-/-}\). There are clearly regulatory systems to signal increased neutrophil production, such as in humans with depleted bone marrow. Perhaps the normal neutrophil regulatory system detects the mIL-8R\(^{-/-}\) neutrophils as nonfunctional and continues to stimulate neutrophil production in an attempt to replace the defective neutrophils. Alternatively, the expansion of the neutrophil lineage could indicate that this receptor is directly involved in the negative regulation of neutrophil production. A third possibility is that a disruption of a cytokine’s function could have profound effects on a number of different cell types and immunological functions. The immune system could be compensating for the loss of properly functioning neutrophils by boosting the humoral arm of the immune response, or
Fig. 8. Peripheral blood neutrophils from mIL8R^{++} and ^{-/-} mice derived under specific pathogen-free (SPF) and germ-free (GF) conditions. Blood was collected by retro-orbital venous puncture and was analyzed in a Serono-Baker Diagnostics System 9000 Diff Model Hematology Analyzer. Blood smears were stained with hematoxylin-eosin for differential cell counts performed microscopically. The total number of neutrophils was determined by multiplying the percentage of neutrophils by total white cell counts. Bars represent individual mice.

this could be an example of a cytokine cascade. There is a report that in humans IL-8 negatively affects IL-4 expression in tonsilar and circulating B-cells (23). It has been reported (24) that the MIP-1α chemokine can inhibit hematopoietic stem cell proliferation. These studies, together with our results, raise the very interesting possibil-
ity that chemokines may be playing a much more complex role than solely affecting leukocyte trafficking, and that they may also be involved in the regulation of hematopoiesis and myelopoiesis.

Recently (25), we directly addressed some aspects of these questions by rederiving the mIL-8R+/− mice in a germ-free setting. As opposed to mice reared in conventional animal breeding facilities that are still subject to bacterial and fungal exposure, germfree mice show a completely naive immune system and are negative for cultured flora. Quite dramatically, this simple procedure ameliorates the neurophilia normally observed in mIL-8R+/− mice. Upon reintroduction into a conventional animal breeding facility, the phenotype of neutrophilia returns (Fig. 8). Likewise the plasmacytosis is pathogen dependent and is absent in germfree mice (Cacalano et al., manuscript in preparation). Thus the unexpected aspects of the mIL-8R−/− mice phenotype can be explained as immunocompensation for poorly migrating neutrophils. Such a resolution to the phenotype was most easily manifested by rederivation into a germfree environment. Considering the potential for similar compensations caused by environmental stimuli, it may be worth making this step a common procedure for mice with hyperimmunological responses.

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Chapter 13

Knockouts of Interferons, Interferon Receptors and Interferon Signaling Components

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Introduction

The interferons are arguably the oldest known cytokines (1). For many years after their original discovery, the only recognized biological function of interferons was the inhibition of virus replication. Only much later did it become apparent that, as is characteristic of cytokines in general, interferons have many pleiotropic activities, including modulating effects on the rate of cell growth and a variety of actions on functions of the immune system (reviewed in refs. 2 and 3). The generation of knockout mice with null mutations in the interferon genes or in the genes encoding interferon receptors or signaling components essential for interferon actions has provided the means to define better the biological functions of interferons in the intact organism. Specifically, the availability of these knockout mice has made it possible to determine the importance of the interferons in the resistance to infections and in some specific functions of the immune system. These investigations, to be briefly reviewed in this chapter, led to some unexpected findings.

A very brief introduction to the field of interferon and interferon signaling is in order. There are two major families of interferon genes...
and proteins: the IFN-α/β or type I IFN superfamily and IFN-γ or type II IFN (2,3). The IFN-α/β superfamily is subdivided into four subfamilies, termed IFN-α, IFN-β, IFN-ω, and IFN-τ. All genes and proteins of the IFN-α/β superfamily are closely related to each other structurally. The genes form a cluster located on a single chromosome (the short arm of chromosome 9 in humans). IFN-γ or type II IFN is represented by a single gene that shows no significant structural homology to the IFN-α/β genes. (The interferon nomenclature was established before gene and protein sequences had been elucidated, and was based mainly on the shared ability of interferons to inhibit virus multiplication and on the antigenic properties of the different interferon proteins.) All IFN-α/β proteins interact with the same cell-surface receptor (see below). IFN-γ binds to its own cell surface receptor that is completely distinct from the receptor for IFN-α/β.

Much progress has been made in recent years in the elucidation of intracellular signaling by the IFN-α/β and IFN-γ receptors (4,5). Two separate chains have been identified in each of the two receptors (6–10). Ligand binding sets in motion signaling cascades whose components are partly shared by IFN-γ and IFN-α/β (4,5). This overlap in signaling cascades explains why the two structurally distinct interferon families have many similar biological actions. The binding of interferons to their receptors first leads to the activation of the receptor-associated Janus or “Jak” tyrosine kinases (Jak1 and Tyk2 in the case of the IFN-α/β receptor; Jak1 and Jak2 in the case of the IFN-γ receptor). These Jak kinases contribute to their own activation by autophosphorylation, and they phosphorylate specific tyrosine residues on the cytoplasmic domains of interferon receptor chains, thus creating docking sites for SH2 domain-containing cytoplasmic proteins termed “signal transducers and activators of transcription” (Stat). On their docking onto these sites within the IFN receptors, the Stat proteins become tyrosine-phosphorylated by the Jak kinases. (Ligation of the IFN-α/β receptor leads to the docking and phosphorylation of Stat1 and Stat2 proteins, IFN-γ recruits only Stat1.) Once they are phosphorylated, Stat proteins are released from the receptor. On their release, the phosphorylated Stat proteins form homo-and heterocomplexes that translocate to the nucleus, bind to DNA recognition sites present in interferon-inducible genes (termed ISRE and GAS), and thereby activate their transcription (4,5,11,12).
One protein that becomes part of a trimeric complex with phosphorylated Stat1 and Stat2 (or alternatively with Stat1 alone) is a protein termed p48 (11–13). p48 is structurally related to IRF-1 and IRF-2, and several other members of the IRF family of transcription factors (14). The IRF-1 and IRF-2 proteins act as positive and negative regulators, respectively, of some interferon-inducible genes, and under some conditions, they also play a role in the regulation of expression of the IFN-α/β genes themselves. The products of genes whose targeted deletions are reviewed in this chapter are listed in Table 1.

**IFN-γ and IFN-γ Receptor Gene Knockouts**

The earliest generated knockout animals in the interferon field were mice with a targeted disruption of the structural gene for IFN-γ (15) or the gene encoding the ligand binding chain (α-chain) of the IFN-γR (16). These mice develop normally, but they exhibit a decreased resistance to *Mycobacterium bovis* (BCG strain), *Listeria monocytogenes*, and vaccinia virus, despite apparently normal CTL and helper T-cell responses. Although the mutant mice showed no decrease in overall IgG levels, a moderate decrease in trinitrophenyl-specific IgG2a and IgG3 concentrations was seen in the IFN-γR−/− mice (16). Mutant mice showed no decrease in the constitutive levels of expression of MHC class I and class II antigens, but the increase in class II antigen expression seen after infection with BCG was impaired in IFN-γR−/− mice (17). Likewise, the activation of macrophages resulting in the enhanced production of nitric oxide (NO) during BCG infection was impaired in IFN-γR−/− mice and the latter defect, together with an impaired production of several macrophage-derived cytokines (TNF, IL-1, and IL-6), is likely to be one of the reasons why mice unable to produce or respond to IFN-γ show a decreased resistance to Mycobacteria, Listeria, and other intracellular infections (18–21). Surprisingly, IL-12 production by macrophages from BCG-infected IFN-γR−/− mice was also strongly impaired, indicating that early IFN-γ (as well as TNF) production is required for the stimulation of IL-12 synthesis (which in turn is needed for more IFN-γ production), and that IL-12 is not the earliest cytokine produced by macrophages exposed to BCG (22).
Table 1
Interferons and Interferon-Related Genes
and Their Products Reviewed in this Chapter

| Designation                                      | Abbreviation | Description                                                                 | Original reference(s) describing generation of knockout mice |
|------------------------------------------------|--------------|----------------------------------------------------------------------------|---------------------------------------------------------------|
| Interferon-γ                                    | IFN-γ        | Cytokine produced mainly by Th1, Tc, and NK cells                         | Dalton et al., 1993 (15)                                      |
| Interferon-γ receptor                           | IFN-γR       | Part of receptor for IFN-γ essential for IFN-γ action                     | Huang et al., 1993 (16)                                      |
| Interferon-α/β receptor (IFNAR1 chain)          | IFN-α/βR     | Part of receptor for all known IFN-α/β proteins, essential for IFN-α/β actions | Müller et al., 1994 (31)                                     |
| Signal transducer and activator of transcription 1 | Stat1        | Component of transcription factor complexes; important in IFN-α/β and IFN-γ actions | Hwang et al., 1995 (35)                                     |
| p48                                             | p48          | Component of transcription factors complexes important in IFN-α/β and IFN-γ actions | Meraz et al., 1996 (36)                                     |
| Interferon regulatory factor 1                  | IRF-1        | Transcription factor whose synthesis is inducible by IFN-γ and IFN-α/β    | Durbin et al., 1996 (37)                                     |
| Interferon regulatory factor 2                  | IRF-2        | Transcriptional inhibitor; acts as negative regulator of IFN-α/β and IFN-γ actions, counteracts IRF-1 | Kimura et al., 1996 (48)                                     |
| Double-stranded RNA-dependent kinase            | PKR          | Serine-threonine kinase, inducible by IFN-α/β and IFN-γ; requires double-standard RNA for activation | Matsuyama et al., 1993 (38)                                   |
|                                                 |              |                                                                           | Reis et al., 1994 (39)                                      |
|                                                 |              |                                                                           | Matsuyama et al., 1993 (38)                                   |
|                                                 |              |                                                                           | Yang et al., 1995 (56)                                      |
The absence of functional IFN-γ also led to an increased susceptibility of mice to infection with *Leishmania major* (23,24). Whereas IFN-γR<sup>-/-</sup> mice (on the 129 genetic background) after *L. major* infection appeared capable of developing a characteristic Th1 response (23), mice deficient in the structural gene for IFN-γ (on the C57BL/6 background) showed an increased Th2 response after infection with *L. major* (24). This difference in the development of polarized Th1/Th2 responses is likely caused by the different genetic backgrounds of the mice. Only a modest difference was seen in the course of parasitemia between IFN-γR<sup>-/-</sup> and wild-type mice after inoculation with sporozoites or blood forms of murine malaria parasites (25). In contrast to their wild-type counterparts, IFN-γR<sup>-/-</sup> mice failed to develop protective antimalaria immunity in response to a single injection with *Plasmodium yoelli* sporozoites (25). However, when multiple immunizations were given, both wild-type and mutant mice developed protective immunity. On infection with pseudorabies virus, IFN-γR<sup>-/-</sup> mice developed a Th1-type antiviral response, but the production of protective antibodies was impaired (26).

Other new information derived from the study of the IFN-γR<sup>-/-</sup> mice includes an analysis of the impact of the absence of a functional IFN-γ system on the susceptibility of mice to the cytokine release syndrome, induced by the administration of toxic doses of anti-CD3 (27). Unexpectedly, IFN-γR<sup>-/-</sup> mice were shown to be more sensitive to the toxic action of anti-CD3, and the authors ascribed the latter finding to a decreased production of NO in the mutant mice. The mechanism whereby NO may protect animals from the toxic action of cytokines produced in response to anti-CD3 is not clear. This apparent protective effect of IFN-γ contrasts with the finding that IFN-γR<sup>-/-</sup> mice are significantly less sensitive to the toxic action of LPS and development of endotoxic shock (18,28).

In a model of lung inflammation elicited by aerosol challenge of immunized mice with ovalbumin, IFN-γR<sup>-/-</sup> mice showed an increased generation of IgG1 and IgE, with decreased levels of IgG2a (29). The mutant mice also showed a prolonged infiltration of the lungs with eosinophils, indicative of a more sustained Th2 response in the absence of functional IFN-γ. Finally, two groups reported that the absence of the IFN-γ receptor can lead to significantly increased levels of IFN-γ in the serum, either after infection
with BCG (21) or after the administration of anti-CD3 antibody (27), suggesting that in wild-type mice, the IFN-γR contributes to the clearance of its ligand from the circulation.

IFN-γ has been implicated in the regulation of MHC class I and class II genes. IFN-γ was also shown to induce TAP-1 and TAP-2 transporters and proteasome subunits LMP2 and LMP7, and antigenic peptides presented by MHC Class I molecules in the presence of IFN-γ differ from those presented in the absence of IFN-γ (30). Therefore, mice that do not make or do not respond to IFN-γ would be expected to have a different repertoire of antigenic peptides presented by class I-expressing cells. Thus, IFN-γ−/− and IFN-γR−/− mice should have defined defects in CTL as well as in helper T-cell responses. This hypothesis has not yet been fully confirmed, but the data available to date suggest a role for IFN-γ in the generation of at least some specific immune responses.

**IFN-α/β Receptor Gene Knockouts**

The existence of a multitude of related IFN-α/β genes encoding proteins with identical or closely related functions precludes the generation of mice with a deletion of all functional type I interferon genes. However, since all IFN-α/β proteins are thought to bind to the same cell-surface receptor, it was expected that targeted disruption of the gene encoding one of the essential chains of the IFN-α/β receptor would lead to the generation of animals in which all IFN-α/β-mediated functions would be absent. This strategy proved to be successful.

Müller et al. (31) generated mice with a targeted deletion of the gene encoding the "IFNAR" chain (also termed IFNAR-1) of the IFN-α/β receptor. IFNAR is the first identified component of the IFN-α/β receptor and is known to be essential for its capacity to signal (6). The IFN-α/βR−/− mice generated were indeed completely unresponsive to different IFN-α/β proteins, confirming that the IFNAR chain is essential for signaling. IFN-α/βR−/− mice were more susceptible than wild-type mice to all experimental virus infections examined (31–34). There was an especially dramatic increase in the sensitivity of these mice to vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV). Resistance to vaccinia virus and to Theiler’s virus (the latter is a murine picornavirus
producing a chronic infection of the nervous system accompanied by demyelination) was also significantly decreased. In contrast, no differences have yet been reported in the susceptibility of IFN-\(\alpha/\beta R^{-/-}\) mice to nonviral infectious agents. The isotype pattern of LCMV-specific antibodies generated in the IFN-\(\alpha/\beta R^{-/-}\) mice was not significantly altered, but studies in double-knockout mice that lack the IFN-\(\alpha/\beta R\) as well as IFN-\(\gamma R\) indicate that when both IFN-\(\alpha/\beta\) and IFN-\(\gamma\) are rendered nonfunctional the generation of IgG2a and IgG2b antibodies is severely impaired (33,34). Analysis of these double-knockout mice for their susceptibility to infection with different viruses demonstrated the nonredundant functions of the IFN-\(\alpha/\beta\) and IFN-\(\gamma\) systems in host resistance to viruses. These studies were recently reviewed (33).

IFN-\(\alpha/\beta R^{-/-}\) mice were also generated by Hwang et al. (35). In addition to being unresponsive to IFN-\(\alpha/\beta\), macrophages from these mice were found to have reduced responses to CSF-1 and LPS. The authors have also detected elevated levels of myeloid lineage cells in peripheral blood and bone marrow.

### STAT1 Gene Knockouts

Mice with a targeted disruption of the Stat1 gene were recently generated by Meraz et al. (36) and Durbin et al. (37). Stat1\(^{-/-}\) mice showed no abnormalities during embryonic development or postnatally. The only phenotype demonstrated in these mice so far is a complete absence of responses to both IFN-\(\alpha/\beta\) and IFN-\(\gamma\), corroborating the essential role of Stat1 in all known actions of interferons on gene activation. (Whether Stat1 is also essential for the inhibition of gene expression by interferons is not yet known.) These mice also show an increased susceptibility to virus infections (VSV and mouse hepatitis virus were examined) and to infection with \(L.\ monocytophages\). Since in addition to IFN-\(\alpha\) and IFN-\(\gamma\) several other growth factors and cytokines have been shown to produce Stat1 activation, it was of interest to determine whether tissues from the Stat1\(^{-/-}\) mice retain responsiveness to these agents. The results obtained to date show that Stat1\(^{-/-}\) mice respond normally to growth hormone, EGF, and IL-10, all of which were previously shown to activate Stat1. Thus, the only known obligate function of Stat1 remains to be its role in IFN actions.
IRF-1 Gene Knockouts

IRF-1−/− mice were generated by Matsuyama et al. (38) and Reis et al. (39). The mutant animals showed no overt abnormalities during embryonic development or postnatally. One of the main reasons for generating IRF-1 knockout mice was to determine whether IRF-1 played an essential role in the generation of IFN-α/β. Although embryo fibroblasts from the mutant mouse showed a decreased capacity to produce type I IFN in response to double-stranded RNA, induction of this IFN in response to infection with Newcastle disease virus was not significantly impaired (38). Moreover, in intact IRF-1−/− mice, type I IFN production was unimpaired either in response to double-stranded RNA or after inoculation with virus (39).

Macrophages from IRF-1−/− mice have a significantly impaired capacity to produce NO in response to stimulation with IFN-γ and LPS or other inducing treatments (40). This defect was explained by the requirement for IRF-1 in the transcriptional activation of the murine inducible NO synthase (iNOS) gene. IRF-1−/− mice showed an increased susceptibility to infection with the BCG strain of M. bovis, probably because of their reduced capacity to produce NO. Subsequent studies showed that guanylate binding protein (GBP) mRNA synthesis was also greatly diminished in cells from IRF-1−/− mice in response to stimulation with IFN-γ, indicating the requirement of IRF-1 for the efficient transcriptional activation of the GBP gene (41,42). Another IFN-inducible gene whose activation was shown to be impaired in IRF-1−/− cell is the lysyl oxidase gene, earlier implicated as an IFN-inducible antioncogene (42a). Thus availability of IRF-1−/− mice helped to identify a subset of IFN-inducible (especially IFN-γ-inducible) genes that require IRF-1 for their transcriptional activation.

IRF-1−/− mice also showed a moderately accelerated mortality after infection with encephalomyocarditis virus (EMCV), and embryonic fibroblasts from IRF-1−/− mice were less readily protected from infection with EMCV by treatment with IFN-α and especially with IFN-γ (41). However, the antiviral effects of these interferons against two other viruses were essentially unaltered in IRF-1−/− fibroblasts. An unexpected finding in IRF-1−/− mice was an effect on radiosensitivity of mature T-lymphocytes (43). Immature T-cells were known to be killed by ionizing radiation because of apoptotic
events triggered by p53. An analogous induction of apoptosis in mature T-cells proved to be via IRF-1. This role in detecting DNA damage further implicates IRF-1, like p53, in tumor suppression.

Studies with embryonic fibroblasts from IRF-1−/− mice also suggested a role for IRF-1 in the protection of cells from malignant transformation and apoptosis (44). IRF-1−/− fibroblasts, but not wild-type fibroblasts, could be transformed by transfection with the c-Ha-ras oncogene. In addition, expression of c-Ha-ras (in combination with other stimuli) induced apoptosis in wild-type fibroblasts, but not in fibroblasts from IRF-1−/− mice.

Another phenotype of IRF-1-deficient animals was an unexpected lack of CD8+ single-positive cells, especially in the periphery. FACS analysis of splenocytes revealed a reduction of up to 90% of CD8+/CD4− cells, whereas the number of CD4+ single-positive cells was comparable with that of wild-type animals (38,39). Interestingly, the level of MHC class I molecules on the surface of splenocytes was also reduced in mutant mice, as determined by a two- to threefold decrease in the mean fluorescence value (39). Only a slight reduction of CD8+ single-positive cells was seen in the thymus, whereas the number of double-positive CD8+/CD4+ cells in the thymus of IRF-1−/− mice was slightly increased (44a). This latter result suggests that the expression of CD8 itself is not dependent on IRF-1. One possible explanation for the decrease in CD8+ T-cells in mutant mice would be that the reduced level of MHC class I results in a defect in the positive selection process. However, the reduction in class I expression was small, and it is unlikely that it would account for the dramatic reduction of CD8+ single-positive cells. Thus, there could be additional reasons why proper antigen presentation and positive selection are impaired in the IRF-1−/− mice.

The process of antigen processing and antigen presentation is known to be modulated by IFN-γ (30). Since IFN-γ is a potent inducer of IRF-1, it is possible that it is IRF-1 induction by IFN-γ which is necessary for the proper expression of proteins involved in positive selection. This hypothesis would also be in agreement with the increased number of double-positive CD4+/CD8+ cells in the thymus of IRF-1−/− animals. Recent work by White and coworkers (44a) revealed that the expression of TAP1 and LMP2 is dependent on IRF-1. These authors demonstrated the presence of IRF-1 binding sites within the promoters of these genes and
showed that IRF-1<sup>−/−</sup> mice indeed have a reduced expression of TAP1 and LMP2 in the spleen and thymus. It is noteworthy that mice deficient in the IFN-γ gene or the IFN-γ receptor gene have normal levels of CD8<sup>+</sup> single-positive cells (15, 16), suggesting that in vivo stimuli other than IFN-γ can bring about IRF-1 expression at a level adequate enough to ensure antigen processing and antigen presentation sufficient for the normal functioning of the positive selection process.

**IRF-2 Gene Knockouts**

IRF-2<sup>−/−</sup> mice were generated by Matsuyama et al. (38). No phenotypic changes in their development, reproductive ability, or behavior were observed. However, IRF-2<sup>−/−</sup> mice show a significantly higher mortality than wild-type mice, the causes of which have not yet been fully elucidated. Compared to wild-type mice, embryonic fibroblasts from IRF-2<sup>−/−</sup> animals showed a slightly increased production of IFN-α and IFN-β, consistent with a role for IRF-2 as a negative regulator of IFN-α/β gene expression (14). However, no significant differences in the level of expression of IFN-inducible genes have been observed (38). Numerous changes indicative of altered hematopoiesis in the bone marrow have been noted, including decreases in bone marrow Thy-1<sup>+</sup> cells, secretory IgM<sup>+</sup> cells, and in cells responsive to B-cell mitogens. Unlike IRF-1-deficient mice, IRF-2<sup>−/−</sup> mice have normal numbers of CD8<sup>+</sup> cells in the blood, lymph nodes, and spleen. When adult IRF-2<sup>−/−</sup> mice were infected with LCMV, all animals succumbed to the infection within 4 wks, unlike wild-type or IRF-1 mice, which showed no signs of disease after the same dose of virus. The reason for this exquisite susceptibility of IRF-2 mice to LCMV has not yet been explained.

Mice lacking both IRF-1 and IRF-2 have been generated by interbreeding of the two types of knockout animals (44). Such mice are viable, indicating the dispensability of both transcription factors during embryo development.

**p48 Gene Knockouts**

As mentioned in the Introduction, p48 is structurally related to IRF-1 and IRF-2. It was known that p48 can form a heterocomplex
with Stat1 alone, as well as become part of a heterotrimer with Stat1 and Stat2 (11,12). These p48-containing complexes bind to ISRE sequences in the promoters of IFN-inducible genes, thereby stimulating their transcription. The only type of direct gene activation by interferons that is not known to be dependent on p48 is activation mediated by Stat1 homodimers that bind to GAS sites; the latter pathway is more important in IFN-γ-mediated gene activation than in the actions of IFN-α/β. Activation of the IRF-1 gene by interferons is mediated through a GAS site and, therefore, would not be expected to depend on p48 (44). Thus, IRF-1-mediated interferon actions would be expected to proceed even in the absence of p48.

These predictions were largely confirmed by the recently published experiments of Kimura et al. (46) in which interferon actions were examined in embryonic fibroblasts from mice with a targeted disruption of the p48 gene. The induction of ISRE-containing genes (2′-5′-oligoadenylate synthetase and PKR genes) by both IFN-α and IFN-γ was completely absent in the p48−/− cells. On the other hand, induction of IRF-1 mRNA in response to either IFN-α or IFN-γ was completely unimpaired in fibroblasts from p48−/− mice. Induction of the GBP gene (which contains both an ISRE and a GAS site) was abolished in p48−/− cells in response to IFN-α, but normal in response to IFN-γ. The latter result, together with data obtained in fibroblasts from IRF-1−/− mice, suggests that GBP gene induction by IFN-α is completely dependent on p48 and largely IRF-1-independent, whereas induction of the same gene by IFN-γ is largely p48-independent, but virtually abolished in the absence of IRF-1. The authors also showed that the residual induction of small amounts of GBP mRNA seen with IFN-γ in IRF-1−/− fibroblasts is abolished in cells derived from mice lacking both the IRF-1 and p48 genes (obtained by mating IRF-1−/− and p48−/− mice with one another). What these results further suggest is that in wild-type cells, GBP gene activation in response to IFN-γ proceeds largely via IRF-1 binding to the ISRE site, with a minor contribution of Stat1 homodimer binding to the GAS site, whereas induction by IFN-α proceeds exclusively via the ISRE site through p48-containing complexes that either do or do not also contain IRF-1. (This is the only, admittedly indirect, evidence, suggesting the existence of a transcriptional activator complex containing both p48 and IRF-1.)
Kimura et al. (46) have also examined embryonic fibroblasts from p48−/− mice for their ability to develop an antiviral state in response to IFN-α and IFN-γ. Development of an antiviral state against EMC virus was abolished in response to IFN-α and very severely impaired in response to IFN-γ in the p48−/− cells. In contrast, antiviral actions against VSV and herpes simplex virus were decreased, but not abolished in p48−/− cells in response to both IFN-α or IFN-γ, suggesting that both p48-dependent and p48-independent mechanisms operate in the antiviral actions against the two latter viruses. Another interesting new finding derived from the study of p48−/− mice is that these mice have a decreased capacity to produce IFN-α/β, suggesting for the first time a role for p48 in the control of IFN synthesis (46a).

**PKR Gene Knockouts**

The double-stranded RNA-dependent protein kinase, PKR, is a serine-threonine kinase whose synthesis is potently induced by IFNs. In the presence of Mg^{2+} and ATP the enzyme is autophosphorylated, leading to its activation. One of the best-known substrates for activated PKR is the eukaryotic initiation factor eIF-2. Phosphorylated eIF-2 cannot be recycled from eIF-2-GDP to eIF2-GTP, and thus, protein synthesis is inhibited. This inhibition of protein synthesis by PKR was for long postulated to be one of the important mechanisms by which IFNs inhibit viral replication. It was reported that overexpression of human PKR in cell lines leads to inhibition of replication of vaccinia virus (47) and EMCV (48). PKR was also implicated in the induction of the IFN-β gene (49,50) as well as in the induction of some IFN-inducible genes (51).

Recently, a new role for PKR in regulating cell growth was proposed by several groups. First, it was shown that overexpression of human PKR in yeast leads to phosphorylation of eIF-2, and probably as a consequence of inhibition of protein synthesis, these cells have a reduced growth rate (52,53). Another possible role for PKR in the regulation of cell growth and tumor suppression was revealed in NIH3T3 cells stably transfected with a plasmid encoding a mutant, dominant-negative form of the human PKR. The resulting cell clones featured a transformed phenotype, showing growth in soft agar and tumor formation in nude mice (54,55).
In order to ascertain the physiological functions of PKR within the immune system as well as its role in controlling cell growth, Yang et al. (56) have generated mice deficient for the PKR. When infected with EMC or treated with poly(I)·poly(C), PKR-deficient mice showed levels of IFN-β and IFN-α gene expression in the spleen, liver, and lungs comparable to wild-type counterparts, suggesting that in vivo PKR is dispensable for normal induction of the type I IFN genes. Just as in the case of IRF-1, mouse embryonic fibroblasts deficient for PKR showed a diminished induction of both IFN-β and IFN-α genes when exposed to poly(I)·poly(C); this induction became normal when cells were treated with IFN (primed) prior to induction. Induction of type I IFN genes by Newcastle disease virus in embryonic fibroblasts was completely unaffected by the lack of PKR. These results suggest that induction of type I IFN genes by poly(I)·poly(C) or by virus involves different mechanisms. Interestingly, poly(I)·poly(C) failed to activate the transcription factor NF-κB in PKR-deficient cells, and this defect was also corrected if cells were primed with IFNs. On the other hand, activation of NF-κB by TNF was not altered in PKR-deficient mice. It would be important to identify the mechanism whereby priming can restore NF-κB activation by poly(I)·poly(C) in PKR⁻/⁻ cells.

The induction of several IFN-α-stimulated genes was equivalent in wild-type and PKR⁻/⁻ mice treated with IFN-α, suggesting that PKR is not involved in the signal cascade triggered by IFN-α. The ability of IFN-α to protect mice against infection by EMCV was also not affected. Interestingly, the ability of poly(I)·poly(C) or IFN-γ to protect mice from infection by EMCV was impaired in the PKR⁻/⁻ mice. Taken together, the conclusion from these experiments is that PKR apparently plays an important role in the signaling events triggered by IFN-γ rather than in type I IFN signaling.

Finally, studies in PKR⁻/⁻ mice provided no evidence supporting the role of PKR as a tumor suppressor. Cell lines derived from PKR-deficient primary fibroblasts (primary cells, Ras-transfected, or 3T3-like cell lines) showed similar growth rates to wild-type cell lines, and their injection into nude mice did not result in tumor formation. Also, the fact that PKR-deficient mice developed normally argues against a critical role of this enzyme in growth control. These findings suggest that the earlier observed transforming effect of a
dominant-negative PKR construct in 3T3 cells (54,55) may have been caused by some indirect mechanism or that, unlike other cells, 3T3 cells lack some system that can functionally replace PKR.

Conclusions

It has been said that the laborious generation of gene knockout mice is no guarantee of interesting results. Some knockouts merely confirm earlier established notions about the functions of a gene and its product, whereas others are lethal for the embryo and therefore of limited value. How much new information has been derived from the knockout of the interferon-related genes briefly reviewed in this chapter? The surprises came often in the form of an absence of expected phenotypic changes. For about 25 years, investigators have been accumulating evidence on a large array of IFN-α/β-mediated actions that suggested functions separate from its antiviral activities. However, the major clear defect demonstrated in the IFN-α/βR−/− mice was a decreased resistance to viruses, suggesting that the founding fathers of the interferon field were correct in assigning to it a major role in virus infections. Similarly, although Stat1 is activated by several different growth factors and cytokines, the study of Stat1−/− mice so far has failed to reveal the absence of any functions not related to interferon action. There were also findings of the unexpected presence of phenotypic changes, including a defect in the resistance to viruses (vaccinia virus and Theiler’s virus) in the IFN-γR−/− mice and the decrease in CD8+ cells in the IRF-1−/− mice, or the inability of the latter mice to generate NO via the iNOS pathway. Perhaps most interesting was the demonstration of the nonredundant, complementary roles of the type I and type II IFN system in resistance to some virus infections, as demonstrated with the aid of double-knockout mice lacking both the IFN-α/β and IFN-γ receptors.

Work with the different genetically altered mice lacking components of the interferon system continues. When all the dividends derived from the investment in these mice are tallied a few years hence, the profit is likely to be very handsome indeed.

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Chapter 14

IL-6 Gene Knockout Mice

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Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by a variety of cells, including macrophages, T-cells, B-cells, fibroblasts, and endothelial cells, in response to a wide range of stimuli and is thought to play regulatory roles in the immune system, the hematopoietic system, and the nervous system (1). This factor has been given at least nine different names each describing a single biological activity. It was originally identified as a T-cell factor which induced the terminal maturation of B-cells into antibody-producing plasma cells (2), but has also been shown to stimulate the differentiation of cytotoxic T-cells (3), the differentiation and/or proliferation of cells belonging to different hematopoietic lineages (4), and to be the major regulator of acute phase protein synthesis during the inflammatory response (5). In vitro, factors such as IL-1, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF) display overlapping activities with IL-6 that may be explained by a common receptor signal transducing component (gp130). In order to clarify the unique functions of IL-6 in vivo, we have disrupted the murine IL-6 gene in the second exon by insertion of a neo<sup>r</sup> cassette and derived mice homozygous for this mutation (6). Although IL-6 may be expressed as early as the eight-cell stage of embryonic development (7), the FI
was generated in the expected Mendelian pattern. Interbreeding resulted in normal numbers of pups with no obvious defects, suggesting that IL-6 is not crucial for embryonic development. In this chapter, we present data describing the roles of IL-6 in B- and T-cell function, hematopoiesis, and acute phase inflammatory responses obtained in studies using these IL-6$^{-/-}$ mice.

**IL-6 Regulates Progenitor Cells of the Hematopoietic System**

IL-6 has been reported to have several effects on hematopoietic stem and progenitor cells in vitro. In combination with IL-3, IL-6 promotes the formation of multilineage blast cell colonies and the proliferation of stem cells (8). Committed progenitor cells of the megakaryocyte, granulocyte-macrophage and erythrocyte lineages also respond to IL-6 (9). In IL-6$^{-/-}$ mice, numbers of primitive clonal progenitors in the bone marrow and spleen were found to be markedly reduced compared to controls and the function of the long-term repopulating stem cell compartment was compromised, suggesting a role for IL-6 in the survival and/or self-renewal of hematopoietic stem cells and their early progenitors (10). Furthermore, although the capacity of these cells to differentiate into committed progenitors is unaffected in IL-6$^{-/-}$ mice, the activity of this factor appears to be important in regulating the proliferation and final maturation of the granulocyte-macrophage and erythrocyte lineages (10).

**IL-6 and T-Cell Function**

IL-6 has been reported to mediate the induction of T-cell proliferation in vitro (11,12) and, indeed, numbers of thymocytes and peripheral T-cells are consistently reduced by 20–40% in IL-6$^{-/-}$ mice compared to controls (6). However, bulk splenic cultures from IL-6$^{-/-}$ and control mice show similar levels and kinetics of expression of IL-2 receptor after stimulation with the T-cell mitogen, concanavalin A, suggesting that there is no requirement for IL-6 in T-cell activation in the presence of accessory cells. In addition, T-cells from IL-6$^{-/-}$ mice show normal patterns of expression of T-cell receptor $\alpha$, $\beta$, $\gamma$, and $\delta$ chains and CD4, CD8, CD44 (Pgp1), and CD24 (HSA) markers.
In order to assess T-cell function in vivo in the absence of IL-6, IL-6<sup>−/−</sup> mice were infected with vaccinia virus (VV), strain Western Reserve (WR), or lymphocytic choriomeningitis virus (LCMV) (6). IL-6 has been reported to induce the development of classical antiviral cytotoxic T-cells (CTL) in vitro (3). The generation of CTL and their lytic activity were reduced 3- to 10-fold in IL-6<sup>−/−</sup> mice infected with VV-WR, and these animals could not control the infection efficiently, with titers of virus recovered from ovaries and lungs ranging from 10- to 1000-fold higher than in controls at 6 d postinfection. Interestingly, specific lytic activity mediated by CTL from IL-6<sup>−/−</sup> mice infected with an attenuated recombinant VV (rVV) or LCMV was not impaired, suggesting that the reduction in activity against wild-type VV-WR may have been a secondary effect owing to uncontrolled spread of the virus. There is now clear evidence that antiviral cytokines, particularly the type I and type II interferons (IFN) and tumor necrosis factor alpha (TNF-α), also mediate important antiviral activities in vivo and may be crucial for virus clearance, at least in the case of some virus infections (13,14). It is possible that one of the functions of IL-6 in antiviral immunity may be to promote the production of these factors by T-cells and other effectors.

**IL-6 and B-Cell Development**

**B-Cell Terminal Differentiation**

Numbers of B-cells in the bone marrow and spleen are normal in IL-6<sup>−/−</sup> mice and their expression of markers such as B220, IgM, IgD, and CD23 is also within normal ranges (6). In addition, natural serum antibody levels in young adult mice did not differ between mutants and controls (6). When considered together with the aforementioned T-cell data, these findings suggest that lymphoid cell development is not seriously impaired in the absence of IL-6. IL-6 was originally described as a late acting factor in the differentiation of B-cells into antibody-producing cells (2,4), however the stage at which this factor acts and how it affects the differentiating B-cell has been a matter for speculation.

B-cells are activated in T-cell zones in secondary lymphoid organs and colonize the follicles to form germinal centers where B-cell blasts undergo massive clonal expansion and activate a site-specific somatic mutation mechanism (15). It is thought that switch-
ing of immunoglobulin isotypes occurs during cell cycling. Some of these cells apparently leave the germinal center as memory cell clones whereas others continue their differentiation towards antibody secretion and leave the follicles as plasmablasts. Those cells leaving the spleen and lymph nodes are largely committed to IgG production, whereas those from small intestinal Peyer’s patches (PP) are destined to produce IgA. Committed plasmablasts continue to differentiate into plasma cells in sites such as the bone marrow and the lamina propria of gut surfaces which are, respectively, where antigen-specific IgG and IgA antibodies are largely produced.

Immunization with benzylpenicillinoyl-keyhole limpet hemocyanin, a T-cell dependent antigen, resulted in significantly reduced IgG levels in IL-6−/− mice compared to controls at d 14 (16). Serum levels of specific IgG1, IgG2a, and IgG2b antibodies were up to 10-fold lower in mutants, even after secondary immunization, although IgM levels were comparable. Similarly, infection with vesicular stomatitis virus, which is thought to be controlled by T-independent IgM and T-dependent IgG antibodies, resulted in serum antibody levels of neutralizing IgG (d 14) that were 10-fold lower in mutants, whereas IgM levels (d 4) were similar in mutants and controls (6,16). These findings suggest that B-cell development to the stage of IgM production is unaltered in the absence of IL-6, although this factor may be important for the expansion of IgG plasmablasts. Immunohistochemical examination of germinal centers using peanut agglutinin (PNA)-binding B-cells as a marker revealed that germinal centers in IL-6−/− mice were reduced in size with impaired formation of dark and light zones in the centers (M. Kosco-Vilbois, personal communication). The low IgG levels as seen in the mutant mice may therefore be partially owing to an inability to form the germinal center microenvironment in which B-cells receive signals for differentiation and expansion. Indeed, the stimulation of proliferation of plasmablasts by IL-6 has already been demonstrated in humans (17) and in rodents (18).

IL-6 was originally identified as a factor-stimulating growth of plasmacytoma and myeloma cells (4). In addition, there is ample evidence of a role for IL-6 in murine plasma cell neoplasia and in multiple myeloma in humans (19). Other factors, such as LIF, OSM, M, IL-11, and CNTFS may also stimulate the growth of some myeloma cells (19,20). However no plasmacytomas were found in
Balb/c IL-6⁻/⁻ mutants treated with a myc/raf expressing retrovirus (J3V1) in combination with pristane oil (a protocol that rapidly induces myeloid and plasma cell tumors in wild-type Balb/c mice), suggesting that IL-6 may be a crucial factor for the development of plasma cell tumors (21). By contrast, myeloid tumors may be IL-6 independent, since tumors of this type were found at comparable frequencies in IL-6⁻/⁻ and control mice (21).

**Mucosal Immunity**

Mucosal surfaces, including the oral and nasal cavities and the respiratory, gastrointestinal, and reproductive tracts, represent the most frequent portal of entry for pathogens. These surfaces are protected by an immune system that appears to function, at least partially, independent of systemic immunity. Organized lymphoid tissues or nodules are found associated with most mucosal surfaces, the most prominent being the gut-associated lymphoid tissue (GALT), comprising the PP and smaller lymphoid nodules of the small intestine and the bronchus-associated lymphoid tissue (BALT) of the respiratory tract, where plasmablasts are generated after exposure to antigen and leave the germinal centers to populate the surrounding mucosae (22). Antigen stimulated B- and T-cells migrate from mucosal lymphoid tissues in efferent lymphatics and circulate to mucosal effector sites such as lamina propria and bronchi, where the B-cells eventually differentiate to IgA-secreting plasma cells.

A large number of studies have supported a major role for Th2-type cytokines in the development of mucosal IgA immunity. Type 2 T-cells are present at high frequency in mucosal tissues (23,24), whereas cells expressing mRNA for Th2 cytokines predominate in the murine small bowel, with IL-4 and IL-5 numerous in PP and these factors, along with IL-6, also frequently expressed in the lamina propria where IgA production occurs (25). It is now accepted that IL-6 markedly and selectively enhances IgA production in vitro by isotype-committed B-cells but not sIgA⁻ B-cells (26). In this respect, IL-6 appears to be a significantly more potent factor than IL-5, a factor with which it may also synergize in IgA production (27). The in vivo relevance of these findings had not been determined, but the presence in mucosal tissues of T-cells, macrophages and other cells capable of IL-6 production in vitro (28,29), and the broad distribution of cells containing IL-6 mRNA in intestinal
mucosa (25), are consistent with the idea that this factor is important in regulating the effector stage of IgA responses.

Studies in IL-6−/− mice have indicated that IL-6-deficiency results in a marked reduction in numbers of IgA-producing cells at mucosae and reduced mucosal responses to conventional B-cell antigens (30). In the absence of deliberate immunization, IL-6−/− mice had substantially fewer IgA plasma cells in their small intestines, lungs, and mesenteric lymph nodes compared to wild-type mice. In addition, IgA-positive cells stained much less brightly in the mutants. The presence of relatively low numbers of IgA plasma cells in IL-6−/− mice compared to wild-type mice was consistent with the distribution of IL-6 mRNA in intestinal tissues, in that whereas IL-6 mRNA was broadly distributed throughout the lamina propria of the latter, no signal was detected in IL-6−/− mice (30).

Further studies suggested that IL-6 is less important for the development of IgA-producing B1 cells, which largely express the CD5 antigen and may respond to a different set of antigens than conventional B2 cells, and which are present in greater numbers in IL-6−/− mice than in controls (31). Small intestinal tissues of IL-6−/− mice had about 50% fewer IgA-staining cells than wild-type mice and the majority of these cells stained only diffusely in the former. This difference may be explained by the observation that over 40% of murine intestinal IgA cells (50% in humans) may be B1 cells, deriving from the peritoneal cavity rather than mucosa-associated lymphoid tissue (MALT) (32). It is now clear that there is a population of IgA precursors originating in the peritoneal cavity in wild-type mice which do not respond to IL-6 by secreting IgA (31). It also appears that small intestinal IgA cells in IL-6−/− mice display a higher level of CD5 expression than in wild-type mice (31). Together, these data suggest that there may be a subset of intestinal IgA plasma cells in normal mice derived from peritoneal cavity precursors that does not require IL-6 for IgA production and that this subset may account for many of the IgA+ cells found in IL-6−/− mice.

When immunized intraduodenally with the conventional B-cell antigen ovalbumin, IL-6−/− mice mounted markedly deficient specific intestinal IgA responses (30), whereas normal IgA responses were found in mutants given oral inocula of this antigen together with the mucosal adjuvant, cholera toxin (33). Recombinant vaccinia viruses (rVV) were used to study mucosal responses to virus infec-
tion in the lung. These constructs encoded the gene for the hemagglutinin (HA) glycoprotein of influenza virus (VV-HA-TK) and local numbers of HA-specific antibody secreting cells (ASC) were monitored following intranasal immunization. Wild-type mice given VV-HA-TK mounted strong, specific IgA and IgG responses, however IL-6-/- mice did not develop significant numbers of ASC (30).

The ability of IL-6-/- mice to mount sustained mucosal antibody responses was fully restored following local administration of rVV expressing IL-6 (30). This was not the case when the mutants were given rVV encoding IL-4 or IL-5, despite the in vitro evidence that these factors also promote mucosal antibody responses. Recombinant VV constructs encoding genes for foreign proteins produce these factors in a highly localized manner at sites and levels determined by the extent of virus replication and have proven to be useful vehicles for gene delivery in vivo (13,34). Whereas vector-encoded IL-6 promoted the development of mucosal IgA precursor cells, it clearly also provided proliferative signals for plasma cell precursors entering the lung from systemic immune sites, as shown by the restoration of IgG responses in IL-6/- mice (30). These findings provide strong evidence that IL-6 plays a role in the development of mucosal antibody responses to virus infection. Deficient-specific mucosal IgA responses have also been found in IL-6-/- mice infected with Candida albicans (35), but not in those given Helicobacter felis (33).

**IL-6 and Acute Phase Responses**

The acute phase response (APR) is a reaction to tissue injury characterized by fever, leukocytosis, and the synthesis of acute phase proteins (APP) by hepatocytes and is apparently mediated by several factors including IL-1, IL-6, TNF, and LIF (36). To clarify the role of IL-6 in the APR, IL-6/- or control mice were given turpentine subcutaneously or bacterial lipopolysaccharide (LPS) intravenously, or were infected with the gram-positive bacterium, Listeria monocytogenes. Striking increases in liver mRNA for the APP, haptoglobin (HP), α-1 acid glycoprotein (AGP), and serum amyloid A (SAA) were found in control mice challenged with turpentine or L. monocytogenes, whereas IL-6-/- mice responded only weakly to these stimuli (6). Serum levels of HP, AGP, and SAA reflected these differences (6,16). In contrast, treatment with LPS led to similar levels
of liver mRNA for these APP in both wild-type and mutant mice, suggesting that IL-6 is an important mediator of the APR after infection with gram-positive intracellular bacteria, but not gram-negative bacteria (16). The latter may stimulate the release of inflammatory mediators from a wide variety of cell types, thus the APR to LPS may be less dependent on IL-6 than other stimuli.

Conclusions

The absence of IL-6 in vivo affects the survival of hematopoietic stem cells and their early progenitors, the regulation of proliferation and maturation of the granulocyte-macrophage and erythrocyte lineages, the number of T-cells and their function, B-cell responses in systemic and mucosal tissues and plasma cell tumor formation, and the APR mediated by hepatocytes. Although optimal responses to infection and injury appear to be mediated only in the presence of IL-6, its relative importance varies in each case, possibly the result of a redundancy of factors with overlapping activities.

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Chapter 15

IL-6 Unique Functions in Inflammation, Bone Metabolism, and B-Cell Neoplasias Revealed by Studies on IL-6-Deficient Mice

Valeria Poli

Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine regulating various aspects of immune response, hemopoiesis, and inflammation. It induces the maturation of B-lymphocytes into antibody-producing plasma cells and the activation of T-cells, and it stimulates proliferation and differentiation of hematopoietic stem cells and thymocytes (for review see ref. 1). IL-6 is also a central mediator of several host responses to acute inflammation, including the acute phase reaction (APR) in the liver (2). Circulating IL-6 levels are normally very low; however, they are rapidly increased by a number of stimuli such as bacterial or viral infection, tissue damage-induced inflammation, and different kinds of traumas. IL-6 disregulated production has been implicated in the pathogenesis of several diseases including autoimmune disorders, plasma-cell discrasias, and postmenopausal osteoporosis.

IL-6 signaling occurs through the assembly of a receptor complex composed of two subunits, the ligand binding IL-6Rα and the
signal transducing gp130. gp130 also acts as a signaling subunit for a family of cytokines structurally related to IL-6: leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), interleukin-11 (IL-11), and cardiotrophin-1 (CT-1; for review, see ref. 3). In contrast with IL-6 and IL-11, which trigger gp130 homodimerization, LIF, OSM, and CNTF assemble a heterodimeric complex between gp130 and a second signaling molecule, the LIFR. The common sharing of gp130 and the differential involvement of the LIFR are likely explanations for the overlapping and diverging functions displayed by these cytokines. Interestingly, since the IL-6Rα intracytoplasmic domain is not required for signaling, soluble IL-6Rα forms (sIL-6Rα) can act as agonists, conferring IL-6 responsiveness on cells that only harbor gp130 (4,5). Although the physiological role of sIL-6R has not yet been demonstrated, circulating forms of IL-6Rα are naturally present in both humans and mice and their levels increase in a variety of pathological conditions (6–8). Moreover, simultaneous expression of IL-6 and sIL-6R in transgenic mice triggers continuous activation of gp130 signaling (9).

The interaction of IL-6 with its receptor complex is known to trigger the activation of two different pathways: the mitogen-activated protein (MAP) kinase-dependent activation of members of the C/EBP family of transcription factors (10), and the activation through tyrosine phosphorylation of members of the Jak family of nonreceptor tyrosine kinases and of transcription factors belonging to the signal transducers and activators of transcription (STAT) family (11). Originally characterized as signaling components of the interferon (IFN)-α and -γ receptor complexes, Jak kinases and STAT proteins have been more recently shown to be important players in the signaling through many cytokines and growth factors receptors (12). All members of the IL-6 cytokine family were shown to be able to activate STAT3 and, to a lesser extent, STAT1.

The complex functional interplay between different cytokines, that influence one another’s synthesis and whose effects are different, or sometimes overlapping depending on the tissue, has made the unequivocal identification of the specific in vivo role of these molecules often evasive. The development of mutant mice lacking a specific gene product has recently provided the opportunity to unambiguously characterize which functions are “unique” to certain cytokines, and which are “redundant” and can be functionally com-
implemented by other molecules. With this goal in mind, we and other laboratories have generated IL-6-deficient (IL-6−/−) mice by gene targeting (13–15). Mice lacking IL-6 were found to be viable and fertile, providing a valid model to study the pathophysiological functions of this cytokine. In particular, we have used these mice to characterize the role of IL-6 in several pathological conditions, including the onset of postmenopausal osteoporosis, inflammatory responses to both localized and systemic inflammation, and the development of plasma-cell tumors. Moreover, we have also analyzed the role played by this cytokine in the immune responses to infection with the yeast *Candida albicans*.

**Systemic Responses to Localized Inflammation Are Strictly Dependent on IL-6 Production**

Systemic and localized acute inflammation elicit a number of host responses including fever, anorexia, loss of body weight, hypoglycemia, and changes in the serum concentration of several plasma proteins produced by the hepatocytes, the so-called liver APR (for review, see ref. 77). The inflammatory response can be triggered by different stimuli such as bacterial infection, endotoxemia, and sterile tissue damage (burns, surgical traumas). Experimentally, systemic inflammation is commonly reproduced with intraperitoneal injection of bacterial endotoxin (lipopolysaccharide [LPS]), a component of the Gram-negative bacterial wall. Conversely, subcutaneous injection of turpentine causes the formation of sterile abscesses as in peripheral tissue damage (16). Whatever the treatment, the host responses to inflammation have been found to be regulated by circulating mediators, the main players being cytokines and corticosteroid hormones.

IL-1, TNFα, and IL-6 are the principal cytokines implied in this process. Production of both IL-1 and IL-6 is induced in systemic as well as in localized inflammation, and when their activity is inhibited with neutralizing antibodies a number of systemic reactions to inflammation are reduced or blocked (17–19). TNFα is rapidly and strongly induced by LPS but not by turpentine, and it plays a major role in lethality following endotoxic shock (20,21). Finally, the IL-6-related cytokines LIF, OSM, IL-11, and CNTF all induce the same spectrum of acute phase genes as IL-6 in hepatic cells (22), suggesting functional redundancy. However, little is
known about their production and presence in the circulation or in hepatic tissue during acute inflammation.

Both LPS and turpentine rapidly induce corticosterone production through the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the release of adrenocorticotropic hormone (ACTH) (23,24). IL-1 is a major mediator of this induction, as its blockage inhibits ACTH and corticosterone induction by LPS (25,26). IL-6 is not able to induce the HPA axis by itself, but it has been proposed as synergizing with IL-1 to generate maximal response (27). The role of corticosteroid hormones in the inflammatory response is dual: On one hand they synergize with cytokines to induce most acute phase proteins and are therefore required for their optimal induction, on the other hand they inhibit cytokine production, hence playing an important role in the termination of the inflammatory responses (28–30).

The comparison of a variety of responses to either localized or systemic inflammation in IL-6-/- and +/+ mice showed that IL-6 is an essential mediator of host responses only to localized (turpentine oil-induced) inflammation, although it is mostly dispensable in systemic (LPS-induced) inflammation (31). This conclusion rests on a number of observations:

1. Induction of several acute phase mRNAs was almost completely abolished in turpentine-treated IL-6-/- mice (Fig. 1A). In contrast, with the sole exception of the α2M mRNA, the same mRNAs were induced normally by LPS injection (Fig. 1B).

2. Symptoms of cachexia such as anorexia, loss of body weight, and hypoglycemia are common clinical manifestations of acute inflammation. Again, IL-6-/- mice did not lose body weight in the first 3 d after turpentine oil treatment, and their food intake decreased much less than in the wild-type mice (Fig. 2A), whereas the same responses were equivalent in the two kinds of mice following LPS injection (Fig. 2B).

3. Corticosterone production was normal in IL-6-/- mice treated with LPS, turpentine, or IL-1β (Fig. 3), indicating that IL-6 is not required to activate the HPA axis and that the inadequate inflammatory responses of the IL-6-/- mice to turpentine are not a secondary effect caused by defective CS production.

4. LPS-triggered tumor necrosis factor (TNF)α induction was about threefold stronger in the IL-6-/- mice (31), in agreement with previous reports suggesting that IL-6 is a physiological inhibitor of TNFα production. Interestingly, this inhibitory effect seems to be confined to the periphery, as brain TNFα production is not inhibited by IL-6.
**IL-6 Functions IL-6-Deficient Mice**

| Treatment | Turpentine oil | LPS |
|-----------|----------------|-----|
| Genotype  | IL-6 +/-       | IL-6 +/- |
| Hours     | 0 2 18 24     | 0 2 18 24 |

- **A**
  - Hp
  - Hpx
  - AGP
  - SAP
  - α2M
  - GAPDH

- **B**
  - Hp
  - Hpx
  - AGP
  - SAP
  - α2M
  - GAPDH

Fig. 1. Northern blots showing liver acute phase mRNA in IL-6+/- and +/- mice following treatment with turpentine oil (A) or LPS (B). Total liver RNA was extracted at 0, 2, 18, and 24 h after the treatment and hybridized with the indicated cDNAs. GAPDH, glyceraldehyde-3-phosphate-dehydrogenase (internal control); Hp, haptoglobin; Hpx, hemopexin; AGP, α1-acid glycoprotein; SAP, serum amyloid P; α2M, α2-macroglobulin. Reproduced from ref. 31 with permission.

and accordingly is normal in IL-6+/- mice. In contrast to TNFα production, IL-1α induction in response to both turpentine oil and LPS was normal in the IL-6-/- mice (31).

Taken together, these results show that IL-6 plays a central role in responses to localized inflammation, and that its functions cannot be compensated for by other cytokines. Moreover, the finding that IL-1, although produced at normal levels in the IL-6-/- mice, is not sufficient to induce cachexia or acute phase mRNAs on turpentine treatment, strongly suggests that its functions in this system are normally exerted through IL-6 synthesis rather than autonomously. In line with this idea, it was recently reported that in response to turpentine oil, IL-1β-deficient mice show a defective production of acute phase mRNAs that correlates with defective IL-6 induction (32). In contrast, IL-6 is dispensable in the host responses to systemic inflammation, suggesting that in this case other cytokines are produced that can compensate for IL-6 function. Good candidates are other members of the gp130 family and especially LIF, which is
Fig. 2. Changes in body weight and cumulative food intake following treatment of IL-6−/− and +/+ mice (6 mice/group) with turpentine oil (A) or LPS (B). Body weight and food intake were measured every 24 h at the same time of the day starting from the day of treatment (d 0); the mean values are indicated. Daily food intake: The mean values for individual animals were derived from cumulative food intake within the groups. *, turpentine treated IL-6−/− versus +/+ mice, p < 0.01. (■), IL-6 +/+ mice, (○), IL-6−/− mice. Reproduced from ref. 31 with permission.

Fig. 3. Serum CS induction in IL-6−/− and +/+ mice. Mice were treated with turpentine (TURP., A, n = 4), LPS (B, n = 7), rIL-1β (C, n = 5), or with sterile saline solution as control. CS levels were measured in the serum 1.5 h after treatment. Black bars and open bars represent IL-6 +/+ and −/− mice, respectively. Data are shown as mean values ± SD.
known to be induced by LPS. In addition, the abnormally high amounts of TNFα produced by the IL-6−/− mice after LPS injection suggest it may also play a compensatory role. Interestingly, it was reported that IL-6 deficient mice are neither more susceptible to, nor protected from the lethal effects of TNFα (33), indicating that IL-6 is not involved in protection against TNF-induced shock.

IL-6 and several other cytokines and growth hormones are able to rapidly trigger phosphorylation and activation of the transcription factor STAT3 (12). We have recently observed that, like the acute inflammation responses, also activation of STAT3 is abolished in turpentine oil-treated IL-6−/− mice (Alonzi, T., Fattori, E., Cappelleti, M., Ciliberto, G., and Poli, V., submitted). This finding establishes a direct correlation between the two phenomena, and indicates that no other STAT3-inducing cytokine is produced at bioactive levels in response to localized tissue damage. As expected however, STAT3 activation is almost normal in response to LPS treatment in the IL-6−/− mice, with only a delay in the appearance of the STAT3 DNA-binding activity. This delay indicates that, although LPS induces cytokines other than IL-6 able to activate STAT3, IL-6 is normally the first to be synthesized in order of time.

The Development of a Fever Response Requires IL-6 Production in the Central Nervous System

Fever is a systemic response that can be elicited by a multitude of exogenous stimuli including inflammation and infection. It consists of a rise in body temperature apparently caused by the upregulation of the thermostatic set point in the preoptic area of the brain (34,35). The same cytokines involved in mediating the other host responses to inflammation (namely IL-1α and β, TNFα and IL-6) have also been identified as putative endogenous pyrogens, since they can evoke fever when applied systematically or centrally (36–38). In addition, cytokine production is increased in the brain during LPS-induced fever response, and cytokine receptors are present in the preoptic area of the hypothalamus (21,35,39–42). However, as in the case of other inflammatory reactions, the relative role of the different cytokines has not yet been established.

IL-6−/− mice were found to have normal circadian variations of the body temperature (43). Surprisingly however, they were
totally unable to mount a fever response to peripheral injection of either LPS, a strong exogenous pyrogen, or recombinant IL-1β, a most efficient endogenous pyrogen (Fig. 4). Peripheral injection of recombinant hIL-6 was not able to elicit a fever response in either IL-6−/− or +/+ mice, and even when injected in conjunction with LPS it could only evoke a small and atypical fever response in the IL-6−/− mice, possibly reflecting some IL-6 leakage to the brain as suggested by its slow onset (43). In contrast, when injected centrally (in the cerebroventriculi) recombinant hIL-6 was able to cause a rapid and long lasting fever response in both the wild-type and the IL-6-deficient mice, whereas central injection of recombinant mIL-1β was only able to elicit fever in the wild-type mice (Fig. 5).

Thus, IL-6 is an essential mediator of the fever response, and it acts downstream of other known endogenous pyrogens such as IL-1.
**IL-6 Functions IL-6-Deficient Mice**

Fig. 5. Effect of intracerebroventricular injection of rhIL-6 (500 ng/mouse, A and C) or of rmIL-1β (100 ng/mouse, B and D) on the core body temperature of IL-6−/− (C,D) and +/+ (A,B) mice. Data are shown as mean values ± SE *p < 0.05; **p < 0.01. Reproduced from ref. 43 with permission.

and TNFα, both strong inducers of IL-6 synthesis. Accordingly, IL-1β-deficient mice were found to be only partly deficient in mounting a fever response to LPS although totally defective in response to turpentine oil, and the entity of the fever response correlated with partially or totally defective induction of IL-6 (32). The observation that IL-6−/− mice are resistant to IL-1β-induced fever, and that rIL-6 can elicit a fever response solely when injected in the central nervous system suggests that IL-6 is not only essential, but also required centrally rather than peripherally. It also indicates that the IL-6R-gp130 complex in the brain of the IL-6−/− mice is functional, and that their overall ability to mount a fever response remains intact. Interestingly, although both LIF and CNTF are produced in the CNS, and CNTF was proposed as being an endogenous pyrogen, none of these gp130 cytokines is able to elicit the fever response in the absence of IL-6.
IL-6-Deficient Mice Are Protected from Bone Loss Following Estrogen Depletion

The skeleton is a dynamic organ, where bone is continuously remodeled through the resorption of old mineralized bone by osteoclasts and the deposition of new bone by osteoblasts. These two events, normally tightly coupled and controlled, allow the skeleton to undergo renewal while maintaining integrity (44). The equilibrium between bone resorption and deposition is known to be regulated by systemic hormones such as parathyroid hormone and sex steroids, as alterations in their production lead to abnormal bone metabolism (for review, see ref. 45). In particular, estrogen deficiency caused by either natural or surgical menopause results in an imbalance between bone resorption and formation leading to bone loss and ultimately to osteoporosis, a disabling condition affecting about one third of caucasian women in postmenopausal age (reviewed in refs. 45, 46). Although the molecular mechanisms for the protective action of estrogens on the skeleton are not fully understood, the hormones are thought to act directly on bone cells and to regulate in the bone microenvironment the production of cytokines, which in turn act as regulators of osteoclasts formation and function (reviewed in ref. 47). IL-1β, TNFα, IL-11, and IL-6 all influence osteoclast development and function, but require the presence of stromal or osteoblastic cells, suggesting that their osteoclastogenic activity is exerted indirectly through the stimulation of other functions in these cells (for review see ref. 48). In accordance with this, it has been recently shown that IL-6-mediated osteoclast differentiation in cocultures of mouse bone marrow and osteoblastic cells requires expression of the IL-6Rα chain on the osteoblasts, but not on the osteoclast progenitors (49). There is compelling evidence that IL-6 expression in the bone microenvironment is directly under the control of sex steroids. Indeed, IL-6 promoter activity is inhibited by 17β-estradiol in a variety of cell types, and estrogen loss causes IL-6 production to increase in bone marrow cell cultures (50,51). Moreover, the increase in osteoclastogenesis caused by ovariectomy in mice can be prevented by administrating either 17β-estradiol or anti-IL-6 neutralizing antibodies (52).

The importance of IL-6 in mediating the effects of estrogen loss on bone metabolism was unequivocally demonstrated through utilizing the IL-6-deficient mice (14). In the wild-type mice,
removal of the ovarian function causes marked trabecular bone loss. As calculated by histomorphometric analysis, trabecular bone volume in the femoral metaphysis decreases by about 50% (Fig. 6A). At the same time, the extent of trabecular bone surface lined with osteoclasts or with osteoblasts is significantly increased (Fig. 6B, C), as well as several parameters of bone formation (14). Bone resorption leading to bone loss is in fact known to be coupled to increased bone formation, probably an attempt on the part of the organism to maintain the bone mass (reviewed in ref. 53). None of these changes occurred after ovariectomy in the IL-6−/− mice: their bone volume did not decrease and both osteoblast and osteoclast surfaces remained constant (Fig. 6A–C). Interestingly, whereas in the wild-type mice ovariectomy caused an increase in the number of granulocyte-macrophage colony forming units (CFU), the osteoclast precursors, the CFU-GM number remained unchanged in the IL-6−/− mice (Fig. 6D), suggesting that IL-6 function is already important at the stage of precursor recruitment. These results clearly demonstrate that, at least in mice, IL-6 is required to generate those changes in bone metabolism that ultimately lead to bone loss and to increased bone turnover in response to estrogen depletion. Interestingly, the group of S. Manolagas has recently reported that androgen-depleted male IL-6−/− mice are also protected from bone loss and increased osteoclastogenesis (54), indicating that the pathogenic role played by IL-6 in bone loss caused by estrogen or by androgen deficiency is equivalent. IL-6 may also play a more general role in triggering bone loss. Bone destruction and osteoporosis are in fact commonly associated to pathological conditions such as multiple myeloma, rheumatoid arthritis, and Paget’s disease, all characterized by an overproduction of IL-6 (55–57).

The idea that IL-6 is a causative factor in bone loss, especially following loss of gonadal function in both sexes, does not imply that other cytokines may not be involved. Indeed, IL-1, TNFα, and IL-11 are essential for osteoclast development because their blockade abrogates osteoclast formation in bone marrow cultures (58–60). However, in contrast to IL-6, this effect occurs both in the estrogen-depleted and in the estrogen-repleted state (48), indicating that these cytokines are probably basal essential factors for osteoclast development and function, regardless of the estrogen state, whereas IL-6 may represent the triggering signal to cause increased osteoclastogenesis and finally bone loss.
Fig. 6. Analysis of histomorphometric parameters and of colony forming units in the femora and bone marrow from ovariectomized (OVX) and non ovariectomized (N-OVX) IL-6+/+ (black bars) and IL-6−/− (open bars) mice. Data are shown as mean values ± SEM. The asterisks indicate a statistically significant difference from IL-6+/+ N-OVX; *p < 0.05; **p < 0.01; ***p < 0.005. (A) Cancellous Bone Volume (BV/TV, %), expressed as the percentage of the metaphyseal area occupied by cancellous bone excluding the cortices. (B) Osteoblast Surface, expressed as a percentage of the total trabecular surface (ObS/BS, %). (C) Osteoclast Surface, expressed per unit of mineralized trabecular bone surface (Ocs/MdBS, %). (D) Bone marrow granulocyte-macrophage colony forming units (number of CFU/tibia).

**IL-6-Deficient Mice Are Highly Susceptible to *Candida Albicans* Infection**

IL-6 is thought to play important roles in the regulation of both innate and acquired immune functions. IL-6-deficient mice have been reported as more susceptible to certain viral and bacterial infection, with decreased IgG production and impaired cytotoxic responses and neutrophil activation (13,15).
The role played by IL-6 during infection with the fungal pathogen *C. albicans* is controversial. The observation that IL-6 is rapidly detected in the serum during the course of infection and that its levels directly correlate with fungal dose and are higher in non-healer mice \((61,62)\), would point toward a detrimental role of this cytokine in the outcome of infection. However, administrating recombinant IL-6 to healer mice during the early phases of infection decreases the fungal load \((62)\), suggesting that early production of IL-6 may be important mechanism in limiting candidal infection.

The IL-6\(-/-\) mice were found to be highly susceptible to infection with both the virulent CA-6 or the live vaccine PCA-2 *C. albicans* strains \((62)\). IL-6-deficient mice were killed by a low inoculum \((10^5\text{ cells})\) of the CA-6 strain, and even by a high inoculum of the attenuated PCA-2 strain. Important to note is that only the IL-6\(+/-\) mice were able to raise a protective immunity and to survive a secondary infection (Table 1). In agreement with susceptibility to infection, a higher number of viable yeast cells was recovered from the kidneys, livers, and in particular brains of infected IL-6\(-/-\) mice \((62)\).

The balance between humoral and cellular immunity is an important factor in determining susceptibility or resistance to infection for a variety of bacterial, fungal and viral pathogens, and is known to be associated with the activation of different CD4+ T-helper cell subsets, characterized by a differential production of cytokines (for review, see ref. 63). The development of protective immunity in *C. albicans* infection is associated with the prevalence of Th-1 mediated cellular responses, whereas a prevailing Th-2 response correlates with high susceptibility to infection (reviewed in ref. 64). Accordingly, the non healer IL-6\(-/-\) mice produced lower amounts of IL-12 and higher amounts of IL-10 as compared to their wild type counterparts (Fig. 7). Surprisingly, both types of mice produced relatively low levels of IL-4, indicating that the susceptibility of IL-6\(-/-\) mice to candidiasis is associated with the activation of CD4+ Th2 cells and a preponderant production of IL-10, rather than IL-4. Interestingly, IL-10 neutralization significantly increased survival of CA-6-infected IL-6\(-/-\) mice, resulting at the same time in increased serum IL-12 levels and improved nitric oxide release by macrophages \((62)\).

Cells mediating innate immunity such as antigen presenting cells and phagocytic cells are known to play an important role in
Table 1
Susceptibility of IL-6−/− and +/+ Mice to *C. albicans* Infection

| Genotype  | PCA-2 Inocula | MST | D/T | CA-6 Inocula | MST | D/T |
|-----------|---------------|-----|-----|--------------|-----|-----|
| IL-6−/−   | —             | —   | —   | 10^6         | 5   | 8/8 |
| IL-6+/+   | —             | —   | —   | 10^6         | 8   | 8/8 |
| IL-6−/−   | —             | —   | —   | 10^5         | 8   | 8/8 |
| IL-6+/+   | —             | —   | —   | 10^5         | >60 | 0/8 |
| IL-6−/−   | 10^6          | 18  | 6/8 |              |     |     |
| IL-6+/+   | 10^6          | >60 | 0/8 |              |     |     |
| IL-6−/−   | 10^5          | >60 | 0/8 | 10^6         | 3   | 8/8 |
| IL-6+/+   | 10^5          | >60 | 0/8 | 10^6         | >60 | 0/8 |

*Mice were infected with different inocula of the PCA-2 or the CA-6 strain. Mice surviving to the 10^5 inoculum with PCA-2 were rechallenged with 10^6 CA-6 cells. MST, median survival time (days); D/T, dead mice over total mice injected.

Fig. 7. Serum cytokine levels in *C. albicans*-infected IL-6−/− and +/+ mice. Mice were injected iv with 10^5 CA-6 or 10^6 PCA-2 cells, and cytokines assessed 6 d after infection; −, uninfected control mice. White bars, IL-6−/− mice; black bars, IL-6+/+ mice. *, below the assay detection limit.

shaping the T-helper response, mainly through modification of the cytokine milieu (reviewed in ref. 65). Interestingly, increased IL-10 production is a common finding in *C. albicans* infected neutropenic mice, that exhibit high susceptibility to infection associated to Th2 responses. Indeed, the number of neutrophils was normal in the blood of IL-6−/− mice, but failed to increase following *C. albicans* infection (Fig. 8). Interestingly, both neutrophilia and survival were significantly improved in the IL-6−/− mice by administering recombinant IL-6, but the treatment was effective only in
Fig. 8. IL-6 administration induces peripheral blood neutrophilia and increases resistance in *C. albicans*-infected IL-6<sup>−/−</sup> mice. Mice were injected iv with $10^6$ PCA-2 cells. Antigranulocyte monoclonal antibodies (affinity-purified RB6-8C5 monoclonal antibody, 30 μg/mouse ip) was given 4 h prior to infection. rhIL-6 (30 μg/mouse sc) was given 4 h before and 1 d after infection. Peripheral blood neutrophils were measured at 2 d after infection. MST, median survival time, days. D/T, dead mice over total mice infected.

the presence of neutrophils, since neutrophil-depleted IL-6<sup>−/−</sup> mice failed to be rescued (Fig. 8).

In conclusion, IL-6 seems to play an important role in the responses of the immune system to *C. albicans* infection, at least in part by stimulating blood neutrophilia that in turn is involved in shaping the Th response. The effects of impaired neutrophil stimulation in conjunction with the increased production of IL-10, that is known to inhibit IL-12 production and nitric oxide release by macrophages, are enough to explain the inability of the IL-6-deficient mice to mount protective Th1 responses in candidiasis. However, the limited IL-6 production found in conventional mice with a healing yeast infection and the correlation of high IL-6 levels with a nonhealing disease would suggest that its production needs to be strictly regulated in order to be beneficial.
IL-6 Plays an Important Role in the Pathogenesis of Myeloma–Plasmacytoma

IL-6 is known to be an important growth factor for a number of neoplastic cell types, and in particular for myeloma–plasmacytoma cells. Human patients with multiple myeloma have high circulating levels of this cytokine \((66,67)\), and both primary myeloma explants and myeloma-derived cell lines are dependent on IL-6 for their growth \((68–70)\). The high levels of IL-6 found in myeloma patients are also thought to play an important role in the development of the kidney and bone damage associated with the disease, and clinical studies have recently shown that treatment with IL-6-neutralizing monoclonal antibodies can improve several disease parameters in late-stage patients \((55)\). In mouse, intraperitoneal injections with pristane oil cause the formation of granulomas and gave rise in the Balb/c strain to the development of plasmacytomas, a form of plasma-cell tumor analogous to the multiple myeloma \((71)\). Also in mice, IL-6 is an essential factor for the in vitro growth of primary plasmacytoma cells and of plasmacytoma cell lines \((72–74)\). Moreover, IL-6 neutralization inhibits tumor growth in mice transplanted with IL-6-dependent plasma-cytoma cell lines \((75)\).

We found that IL-6-deficient mice in the Balb/c genetic background are completely resistant to the development of pristane oil-induced plasmacytomas, although granuloma formation and B-cell activation in the lymphnodes was apparently unchanged (Lattanzio, G., Libert, C., Aguilina, M., Cappelletti, M., Ciliberto, G., Musiani, P., and Poli, V., submitted). This finding highlights the crucial pathogenetic role played by IL-6 in the onset of B-cell neoplasias, and was independently described by the group of S. Rudikoff making use of a modified tumor induction system based on the infection with the myc/raf-expressing retrovirus J3V1 \((76)\).

Conclusions

The availability of mice deficient for IL-6 production has allowed the pathophysiological role played by this cytokine to be analyzed in detail. We found that IL-6 is a key molecule in triggering the onset and development of diseases such as osteoporosis and B-cell neoplasias, and is the main inducer of disease-associated manifestations like localized inflammatory responses and fever. On the other
hand, it would seem that IL-6 is required to raise an efficient immune response to infectious diseases such as systemic *C. albicans* infection. Therefore, IL-6 production can to be “good” or “bad,” depending on the site and time of production and the amount of cytokine produced: disruption of the physiological control of IL-6 regulation and chronic production of the cytokine may lead to disease development, although controlled IL-6 release may be beneficial to the organism.

Further studies on the IL-6-deficient mice will help to elucidate the role played by this cytokine in other pathological conditions associated to IL-6 overproduction, such as systemic lupus erythematosus and rheumatoid arthritis, and to define more precisely this cytokine’s mechanisms of action. Moreover, the availability of mice defective in the production of other cytokines opens up the possibility of generating mice in which more than one cytokine gene has been inactivated. Double or triple mutant mice in which IL-6 together with other related or unrelated cytokines have been inactivated will allow the functional interplay of IL-6 with other cytokines exhibiting overlapping functions to be analyzed in detail and characterized.

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Chapter 16

Myocardial, Hematological, and Placental Disorders Caused by Targeted Disruption of gp130, A Common Signal Transducer for IL-6 Family of Cytokines

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Introduction

Growth and differentiation of cells during development of organs are precisely coordinated by various membrane-anchored and soluble factors, the latter of which include so-called cytokines. Cytokine signals are mediated through specific receptor complexes expressed on target cells. Most of the cytokine receptor components, in particular those involved in hematopoietic cell regulation, belong to a large group of proteins called the cytokine receptor family (1). A notable finding is that receptor complexes from this family are usually composed of a ligand-specific receptor chain.
and a signal transducer common to multiple cytokines (2,3). gp130 was initially identified as a signal transducing receptor component that associates with the interleukin-6 receptor (IL-6R) when the receptor binds with IL-6 (4,5). It has been shown that gp130 is also utilized as a critical signal transducing component in the receptor complexes for IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (6–10). The discovery of this shared signal transducer, gp130, helps to explain how these different cytokines can mediate overlapping biological functions (11). This system of utilizing multichain components with a shared signal transducer is not confined to the IL-6-family of cytokines, but is also applicable to two other families of cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and IL-5, all of which share a common chain referred to as βc (12); and IL-2, IL-4, IL-7, IL-9, and IL-15 which share the IL-2Rγ chain, γc (13–15). A general first step in the process of signaling by members of the cytokine receptor family is believed to be the ligand-induced dimerization of receptor components. In the case of the IL-6-family of cytokines, IL-6-binding to IL-6R induces homodimerization of gp130 (16), whereas stimulation by LIF, OSM, CNTF, and CT-1 leads to heterodimerization of gp130 with a closely related protein, LIFR (7,10,17). OSM is suggested to signal also through a different type of heterodimer composed of OSM-specific receptor and gp130 (18,19). From the close structural similarity of IL-6R and IL-11R, the gp130 homodimer could be a candidate complex for IL-11 signaling (20). Homo- or heterodimerization of gp130 triggers the activation of JAK1, JAK2, and TYK2, all of which are in the JAK-family of cytoplasmic tyrosine kinases and are associated with gp130 (21–23). This leads to the subsequent tyrosine-phosphorylation and acquisition of DNA-binding capability of a latent cytoplasmic transcription factor, APRF/STAT3 (for acute phase response factor or signal transducer and activator of transcription 3) (24–26). It has recently been shown that phosphorylation of a serine residue in STAT3 is important for the full activation of STAT3 (27–29). The Ras/MAPK cascade has been revealed to be activated following gp130-stimulation (30–32). One of the targets of MAPK is NF-IL6, which was demonstrated to be activated on threonine phosphorylation by MAPK (33). A precise mechanism
which links the gp130-dimerization and MAPK activation remains to be elucidated.

gp130 is ubiquitously expressed in almost all organs and cell lines examined, including heart, spleen, kidney, lung, liver, placenta, and brain (5,34). In contrast, expression of the ligand-binding receptor chains for the IL-6-family of cytokines shows somewhat restricted distribution and does not necessarily parallel that of gp130. From the developmental point of view, gp130 is expressed at relatively high levels in embryos and placentas. Its expression is observed even in embryonic stem (ES) cells (34). The normal physiological role of gp130 expressed in this broad range of organs has not fully been elucidated, since gp130 has been studied primarily in cultured cell lines. In addition, despite its obvious importance in immune, hematopoietic, and nerve cell regulation as well as in other systems, no diseases have been identified for which an abnormality in the gp130-signaling pathway has been shown to be responsible. To examine the developmental and physiological roles of gp130 and to understand the pathological consequences resulting from the lack of this common signal transducer, we have created mice deficient for gp130.

**Phenotypic Changes Induced by gp130 Deficiency**

*Targeted Disruption of the gp130 Gene Leads to Embryonic Lethality*

The gp130 gene contains 18 exons, of which exon 2 contains a translational initiation codon (35). To construct a targeting vector, we used an approx 5-kbp DNA fragment of the gp130 locus including exon 1 and exon 2. The pMC1neo-poly(A) cassette was inserted 3' proximal to the translation initiation site to facilitate positive selection of integration events, and the herpes simplex virus thymidine kinase (HSV-tk) gene was inserted into the 5' end of the targeting vector to facilitate negative selection of nonhomologous recombination events. This targeting vector was introduced into E14.1 ES cells by electroporation, and transfectants were selected with G418 and gancyclovir (GANC) on feeder layers of irradiated STO fibroblasts. Double resistant ES cell colonies were screened by the polymerase chain reaction (PCR) assay and the positive clones were subjected to Southern blot analysis to further
confirm that homologous recombination had taken place. The targeted clones were injected into C57BL/6 blastocysts and the chimeric mice obtained were crossed with normal C57BL/6 mice. Three lines of mice from independent ES cell clones were found to transmit the mutation through the germ line (35). Heterozygous mutant (gp130+/−) mice did not show any apparent phenotype.

In order to obtain mice homozygous for the gp130 gene mutation (gp130−/−), heterozygotes were intercrossed. Of 203 offspring from the heterozygous matings, no gp130−/− mice were observed when genotyped at 4–6 wk of age. Among these offspring, gp130+/− mice appeared at a frequency of 64%, which is close to the theoretical value, 67%, based on Mendelian laws, indicating the lethal phenotype of the null mutation. No significant phenotypic difference was observed among the three independent ES clone-derived lines. To determine the time of death, embryos in utero at 11.5–18.5 d postcoitum (pc) and newborn pups derived from the heterozygous intercrosses were analyzed for their gp130 genotypes. As summarized in Fig. 1, gp130−/− embryos on 11.5 d pc were found at a frequency of nearly 25%, which follows the Mendelian distribution. Thereafter recovery of live homozygous mutant embryos decreased. Accordingly, in stages later than 12.5 d pc, a number of gp130−/− embryos that were already dead on inspection became apparent. At 18.5 d pc, live homozygous mutant embryos were observed at a frequency of only 2.7% of the total live embryos, and eventually in the newborns, no live null-mutants were found. Among the live gp130−/− embryos in particular after 13.5 d pc, approximately half were smaller in size (mostly by about 10%; in a few severe cases by up to 20%) than their wild-type and heterozygous littermates, gp130−/− embryos displayed no obvious malformations in surface appearance at any stage examined. Disappearance of the gp130 protein in the live mutant embryos was confirmed by immunoprecipitation followed by immunoblotting using antibodies to this protein.

**Heart Abnormality in the gp130−/− Embryos**

Histological analysis revealed an extreme hypoplastic development of the myocardium in gp130−/− embryos, apparent at 16.5 d pc and later. As shown in Fig. 2, the ventricular walls of the gp130−/− heart at 16.5 d pc were abnormally thin, showing a minimum thickness of one cell layer. This type of extreme abnormality in the
Fig. 1. Recovery of gp130^-/- fetuses from intercrossings of gp130^+/^- mice. Genotypes of fetuses at the indicated stages (in d pc) were determined by Southern blotting. The number of live gp130-null mutant fetuses was divided by the total number of live fetuses. \( n \) (total live fetuses) = 106, 209, 254, 211, 112, 192, 140, and 112 on each day from 11.5 and 18.5 d pc.

Fig. 2. Histological analysis of the control and gp130 null mutant embryonic hearts. Sagittal sections from control and gp130^-/- littermates at 16.5 d pc were stained by hematoxylin and eosin. Bars, 200 \( \mu \)m.
myocardium was observed in all the 16.5 d pc (n = 5) and 17.5 d pc (n = 1) gp130\(^{-/-}\) embryos examined histologically. In addition, the mutant heart was somewhat swollen and displayed globular appearance with a round apex, probably owing to the thinner ventricular walls. Despite the fact that a compact layer of the ventricle was extremely thin, trabeculation inside the ventricle chamber occurred normally in the homozygous mutant hearts. In all the aforementioned six gp130\(^{-/-}\) cases at 16.5 and 17.5 d pc, no ventricular septal defect was detected by examination of serial sections encompassing the entire ventricle. On the contrary, the ventricular thickness of the gp130 null embryos appeared normal relative to the control littermates at 14.5 d pc. Among the four histologically examined 15.5 d pc gp130\(^{-/-}\) embryos, no extremely thin ventricular walls were detected. In two such cases, however, slightly hypoplastic development (moderate reduction in the wall thickness at the site where the thinning was most obvious) of the ventricular myocardium was observed. In the remaining two cases of the gp130\(^{-/-}\) hearts, their histologies appeared normal and indistinguishable from those of gp130\(^{+/+}\) and gp130\(^{+/1}\) littermates’ hearts throughout the sections in terms of their ventricular size, shape, and wall thickness.

In order to examine whether the subcellular ultrastructures of heart muscle cells were affected in gp130\(^{-/-}\) embryos, electron microscopic examination was carried out. At 17.5 d pc, when the extreme thinning of the compact layer in the gp130\(^{-/-}\) heart was observed, the presence and shape of subcellular structures such as nuclei, mitochondria, myofibrils, sarcomeric Z bands, and intercalated discs in the cardiomyocytes of the gp130\(^{-/-}\) compact layer were indistinguishable from those of the control (35). In normal mouse embryos, it has been reported that the compact layer cells of the 14.5 d pc heart are less differentiated and possessed poorly organized myofibrils (36). They become more differentiated, having well-organized myofibrils with clear Z bands at 16.5 d pc and later. In contrast, trabecular cells are already well differentiated at 14.5 d pc, as reported previously. We considered that if precocious differentiation of the compact layer cells occurred in the gp130\(^{-/-}\) heart, this could possibly lead to abolishment of the maintenance of normal compact layer cells. We thus examined the ultrastructures of the gp130\(^{-/-}\) heart at 14.5 d pc. The presence and shape of subcellular structures in the cells of the 14.5 d pc gp130\(^{-/-}\) compact
layer and trabeculae showed no difference from those in the wild-type controls. The scarce appearance of organized myofibrils in the compact layer cells (in both control and gp130-deficient hearts) is consistent with a previous report showing that cells at this stage are ordinary less differentiated than trabecular cells. Trabecular cells of both genotypes showed well-organized myofibrils as expected. Our data thus indicate that the differentiation status of the myocardium in terms of ultrastructural organization was not altered in the gp130$^{-/-}$ heart.

In order to investigate whether the cardiac abnormalities in the gp130-deficient embryos might reflect a maturational disturbance in the ventricular muscle cell lineages which could not be detected by structural examination alone, we next analyzed the expression of cardiac chamber-specific proteins by reverse transcription (RT)-PCR. As a ventricular marker, we examined the myosin light chain (MLC)-2v gene which is expressed exclusively in the ventricular chamber and whose expression precedes the development of distinct cardiac chambers (37–39). Another ventricular marker, MLC-1v, was examined as well (39,40). We also analyzed atrial natriuretic peptide (ANP), whose expression is observed in both atria and ventricles during embryogenesis (41,42). As shown in Fig. 3, there was no significant alteration in the expression of these cardiac chamber-
specific genes in the 17.5 d pc homozygous mutant ventricles. Furthermore, α and β myosin heavy chains (α- and β-MHC), expressed in cardiac muscle cells (40,43), were normal in their expression in the 17.5 d pc gp130-deficient ventricular cells. At 14.5 d pc when the myocardium appeared normal in the mutant heart, the expression of these markers in the mutant heart was indistinguishable from that in the control. These ultrastructural and gene expression studies indicate that the extremely thin ventricular walls in the gp130⁻/⁻ heart may not be the result of a maturational alteration in cardiomyocytes.

We then examined whether the hypoplastic development of myocardium in the gp130⁻/⁻ heart was caused by the lack of proliferative signals transmitted from gp130 in cardiomyocytes. Since a combination of IL-6 and an extracellular soluble form of IL-6 receptor (sIL-6R) is known to interact with gp130 and induce its homodimerization to trigger cytoplasmic signaling (44,45), this combination was added to the cultured cardiomyocytes derived from 16.5 d pc normal ICR embryos. Stimulation of gp130 by the IL-6/sIL-6R complex led to approximate 2.5-fold increase in DNA synthesis in comparison with the medium control. Either IL-6 or sIL-6R alone showed no effect. There appeared to be undetectable contamination of fibroblasts in this experiment, as a fibroblast growth promoting factor, platelet derived growth factor B (PDGF-B), did not induce any proliferative response in the cardiomyocyte preparation but did induce proliferation of embryonic fibroblasts derived from the same embryo.

**Hematopoietic Abnormality in gp130⁻/⁻ Embryos**

The total number of mononuclear cells in the 13.5 dpc fetal liver was dramatically reduced in the null mutant embryos. The colony forming unit in spleen (CFU-S; a total count per liver), as measured by injecting the fetal liver mononuclear cells into lethally irradiated mice (2 × 10⁵ cells/recipient), was also greatly reduced in the homozygous mutant embryos as compared with wild-type littermates (Fig. 4A). CFU-S counts in the heterozygous livers were intermediate between those in the wild-type and homozygous livers. The result indicated that gp130 plays a critical role in the development of the pluripotent stem cell pool in the fetal liver. We then examined whether erythroid progenitors (burst-forming unit for erythroid [BFU-E]) and granulocyte-macrophage progenitors (CFU-
Effects of gp130 Targeted Disruption

Fig. 4. Reduction of pluripotential and committed hematopoietic progenitors in 13.5 dpc gp130 null-mutant fetal livers. (A) The number of spleen colonies (CFU-S). Colonies formed in the recipient spleen 11 d after inoculation of $2 \times 10^5$ mononuclear cells from gp130$^{+/+}$ ($n = 4$) and gp130$^{-/-}$ ($n = 4$) fetal livers were counted. (B) The number of erythroid progenitors (BFU-E). gp130$^{+/+}$ ($n = 5$) and gp130$^{-/-}$ ($n = 6$) fetal liver mononuclear cells were subjected to semi-solid in vitro colony assay. (C) The number of CFU-GM. gp130$^{+/+}$ ($n = 6$) and gp130$^{-/-}$ ($n = 5$) fetal liver mononuclear cells were analyzed as in (B). In all of these assays, each dot represents the value derived from an individual fetal liver, and horizontal bars represent the mean of each group.
GM) detectable in semi-solid in vitro assays were affected by the lack of gp130. As shown in Fig. 4B,C, although both types of committed progenitors were present in the gp130 deficient fetal livers, their numbers were very much reduced.

Although BFU-E numbers were reduced in the gp130-/- fetal livers, most of the mutant fetuses did not appear significantly anemic. However, about 20% of gp130-/- embryos at 15.5 through 18.5 d pc exhibited paleness. In these anemic mutant embryos, there were no signs of hemorrhage. The pale appearance of the gp130-/- embryos led us to examine the development of erythrocytes in these mutants. Liver sections from a 16.5 d pc wild-type embryo and an obviously anemic littermate (gp130-/-) were stained with hematoxylin and eosin, and peripherally existing red blood cells found in the blood vessels as well as erythroid lineage cell pools developing in the liver were inspected microscopically. A larger number of nucleated erythrocytes (and thus a smaller number of enucleated erythrocytes) were found in the blood vessels of the gp130-/- embryo than in the wild-type littermate. Furthermore, in the normal 16.5 d pc liver (Fig. 5), obvious pools of reddish enucleated reticulocytes and erythrocytes in addition to proerythroblasts and erythroblasts, the latter two of which both contain dark purple nuclei, are usually seen. However, in the gp130-/- littermate...
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... liver, the number of such erythroid lineage pools and the average pool size were smaller. Smaller numbers of enucleated reticulocytes and erythrocytes, and larger proportions of nucleated proerythroblasts and erythroblasts were observed in the gp130⁻/⁻ fetal livers. These results suggested that gp130 deficiency led to impaired proliferation and maturation of erythroid lineage cells. Megakaryocytes were detected in the hematoxylin-eosin-stained fetal liver sections from both gp130⁻/⁻ and control littermates at a roughly comparable frequency. Considering the smaller size of the gp130⁻/⁻ fetal liver, this suggests that the lack of gp130 results in a decrease in the total number of megakaryocytes per liver, as was observed for mononuclear cells.

The thymus was readily visible in 16.5 d pc gp130-deficient embryos, although its size was smaller (less than half that of the wild-type control). To examine the effect of the lack of gp130 on T-cell development, 16.5 d pc thymocytes of the gp130⁺/⁺ or gp130⁻/⁻ genotype were analyzed by flow cytometry for the expression of Thy1 and CD8. Thy1⁺/CD2⁻ and Thy1⁺/CD2⁺ are cell surface markers indicative of differentiating thymocytes. The proportion of thymocytes showing either of these two profiles were similar in gp130⁺/⁺ and gp130⁻/⁻ embryos, although the absolute number of thymocytes was considerably smaller in the latter (35). From these results, we conclude that the number of T lineage cells were severely reduced by the lack of gp130, probably because of the extremely reduced stem cell pool, but that cells of these lineages may undergo normal development giving rise to at least pre-B and immature T-cells, respectively, in the absence of signals from gp130.

Reduced Numbers of Primordial Germ Cells in gp130⁻/⁻ Embryonic Gonads

Primordial germ cells (PGCs) resemble ES cells in that both cell types exhibit the same morphology in culture, show pluripotentiality, and undergo self-renewal (46,47). In addition to the inner cell mass of the blastocyst, PGCs can give rise to pluripotential stem cells. LIF has been shown to induce proliferation of PGCs taken from mice embryonic gonads and cultured in vitro (48). We and others have shown that the IL-6-family of cytokines, including LIF, OSM, CNTF, and the complex of IL-6 and sIL-6R can support...
Fig. 6. Reduction in primordial germ cells in gp130\(^{-/-}\) gonads. The abdominal part of the control (left) or gp130\(^{-/-}\) (right) littermate (11.5 dpc) was stained for alkaline-phosphatase (ALP) activity as described in Experimental Procedures. Bar, 500 \(\mu\)m.

the proliferation and maintenance of the pluripotential phenotype of PGCs and ES cells \((46,47,49,50)\). These results suggest that gp130 may play an important role in the development of PGCs in vivo. We therefore investigated PGCs in the gp130-deficient embryos by staining for alkaline-phosphatase activity, a marker for this cell type. PGCs are known to emerge at 7 d pc in the posterior of the primitive streak as a cluster of a few cells \((51)\). The cells continue to proliferate from this day on and, at the same time, migrate along the hind gut, reaching the gonadal ridges by around 11.5–12.5 d pc. As shown in Fig. 6, alkaline-phosphatase positive cells were significantly reduced in the gonadal ridges of 11.5 d pc gp130\(^{-/-}\) embryos. Alkaline-phosphatase positive cells were not frequently visible along the hind gut at this stage in either gp130\(^{+/+}\) or gp130\(^{-/-}\) embryos.

**gp130\(^{-/-}\) Placentas Were Smaller**

*in Size and Showed Histological Abnormalities*

In gp130\(^{-/-}\) placentas, chorio-allantoic circulation was obviously established and appeared normal. The external appearance and histologically examined structures of gp130\(^{-/-}\) placentas on and before 12.5 d pc looked normal with regard to the number and shape of trophoblast giant cells, spongiotrophoblasts and labyrinthine tro-
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Fig. 7. Reduced thickness in 14.5 d pc gp130<sup>−/−</sup> placentas. Sagittal sections of the medial part of the control and gp130<sup>−/−</sup> placentas were stained by hematoxylin-eosin and used for measuring the average thickness around the central part of the placenta (excluding the maternal decidual part). Each dot represents the value for the individual placenta. The horizontal line indicates the mean of each group.

phoblasts. However, at 14.5 d pc obviously smallersized placentas were found (approx 25% reduction in size). As shown in Fig. 7, the average thickness of the placentas of gp130<sup>−/−</sup> embryos measured in the sagittal section under the microscope, was 76.2 ± 8.7% of that of the wild-type littermate at 14.5 d pc. Histological examination revealed that this reduction was largely owing to a thinner spongiotrophoblast layer and labyrinthine zone in the gp130<sup>−/−</sup> placentas (Fig. 8). Hematoxylin-eosin-stained histological specimens did not reveal any dramatic changes in the cell populations composing these thinned compartments. In some small areas in the labyrinthine zone of the gp130<sup>−/−</sup> placentas, a slight dilatation and congestion of blood vessels were observed. These blood vessels were of maternal origin, since they contained almost exclusively enucleated mature erythrocytes. As shown in Fig. 8, at 17.5 d pc the dilatation and congestion of maternal blood vessels deteriorated, and the formation of thrombus of maternal blood was observed in approximately one fifth of the total number of examined placentas from the gp130<sup>−/−</sup> embryos. This change in the gp130<sup>−/−</sup> placenta was considered to result in poorer maternal blood circulation in the placenta. As for the gp130<sup>−/−</sup> embryonic blood vessels, in which a larger proportion of nucleated erythrocytes were observed, they looked normal. In order to investigate potential functional changes in the gp130<sup>−/−</sup> placenta,
Fig. 8. Histological changes in 14.5 and 17.5 d pc gp130⁻⁻ placentas. (A,B) Histologies of the 14.5 d pc control and gp130⁻⁻ placentas. Note the thinner spongiotrophoblast layer (Sp) and labyrinthine zone (La) in the latter section. De, decidua. (C,D) Sagittal sections of 17.5 d pc control and gp130⁻⁻ placentas. In the latter section, thrombus (T) of maternal blood was observed. Bar, 200 μm. (see color insert)

the maternofetal transport rate was examined at 13.0–13.5 d pc by administrating radiolabeled immunoglobulin into the pregnant mother. As shown in Fig. 9, the transfer of the labeled immunoglobulin to the gp130⁻⁻ fetus was significantly lower than that to the
Fig. 9. Functional changes in 13.0–13.5 d pc gp130\(^{-/-}\) placentas. Maternofoetal transport of radiolabeled IgG was measured. \(^{125}\)I-IgG was intravenously injected into a pregnant mother from a heterozygous intercrossing (at 13.0 d pc). Radioactivities of \(^{125}\)I-IgG transferred to the fetuses were measured 12 h later (at 13.5 d pc). Each dot represents the ratio of TCA precipitable \(^{125}\)I-IgG detected in each fetus to that in maternal serum.

Discussion and Comparison With Related Mutant Mice

One of the most striking observations in the gp130 knockout mice is the extreme hypoplastic development of ventricular myocardium. In these mice, ventricular myocardial development was indistinguishable from that in the controls until 14.5 d pc. However, from 16.5 d pc on an extreme thinning of the ventricular compact layer, without any accompanying septal defect, became apparent. Electronmicroscopic examination of the cardiomyocytes and RT-PCR analysis of marker protein expression in the ventricles of gp130\(^{-/-}\) hearts revealed that the lack of gp130 did not cause any detectable disturbance in their differentiation status; no signs of precocious differentiation at 14.5 d pc or maturational arrest at 17.5 d pc were observed. Developmental cardiac abnormalities have also been observed in mice disrupted for some other genes. Mice homozygous for an RXR\(\alpha\) mutation were nonviable and the compact layer of the ventricular walls was found to be considerably thinner than that of control littermates, similar to as was observed in the gp130 knockout study (52,53). However, the abnormality in the RXR\(\alpha^{-/-}\) hearts was apparent as early as 13.5 d pc and was accom-
panied by a defect in ventricular septation. Furthermore, precocious differentiation of the compact layer cells in these mice was obvious at 14.5 d pc, in which stage the ultrastructures of such cells were already of mature type (53). From these points, the heart abnormalities caused by the gp130 null mutation were different from that caused by the RXRα deficiency, suggesting that these two mutations affected different points in cardiomyocyte development. A homozygous mutation in the transcriptional enhancer factor 1 (TEF-1) gene also led to the development of abnormally thin ventricular walls, but this phenotype was apparent even earlier (at 11.5 and 12.5 d pc) than observed with the RXRα null mutation, and was accompanied by much poorer trabeculation, unlike in the case of gp130 or RXRα deficiency (54). The right ventricle of PDGF-B deficient embryos was, again, abnormally thin-walled. However, this was observed on 17.5 and 18.5 d pc, which was definitely later than the stage when gp130 deficiency-induced hypoplastic myocardium appears (55). Considering the variety of the phenotypes, each of these mutants presumably affects heart development at different time points in different manners. In other words, development of the heart is regulated by different types of signals at different stages.

The number of cardiomyocytes in the thin-walled compact layer of the gp130−/− ventricle was very much reduced at 16.5 d pc or later. These cells, however, possessed normal ultrastructural components and expressed ventricle-specific markers comparable to the wild-type cardiomyocytes. Taken together with the result that stimulation of gp130 by the IL-6/sIL-6R complex induced proliferation of 16.5 d pc cardiomyocytes, this suggests that although gp130 signaling plays a role in the growth of cardiomyocytes it does not influence their differentiation, at least at around 16.5 d pc. Our findings suggest the possible existence of a new member of the IL-6-family that regulates heart muscle cell growth. Myocardial proliferation caused by IL-6/sIL-6R-complex stimulation may be mimicking the function of such a cytokine. Indeed, a novel cytokine called CT-1 has recently been cloned which acts on neonatal cardiomyocytes to cause hypertrophy, and whose structure is closely related to, e.g., LIF and CNTF (56). CT-1 is suggested to act through the LIFR/gp130 heterodimer, since CT-1 and LIF cross-compete for binding to their target cells and CT-1-binding to these cells can be inhibited by anti-gp130 antibody (10,56). A role of gp130 in inducing hypertrophy has also been
shown by transgenic mice which overexpress both IL-6 and IL-6R and which exhibit pathological ventricular hypertrophic change in adults (57). The effect of CT-1 on embryonic cardiomyocytes has so far not been examined. Because the size of each cardiomyocyte in the gp130−/− embryonic heart appeared to be comparable to that observed in the wild-type heart, the function of CT-1, assuming it to be mediated during embryogenesis by gp130, might be to maintain (or increase) the cell number rather than hypertrophy. If the lack of CT-1 signaling is responsible for the extreme thinning of the compact layer of the gp130 deficiency, LIFR may not be the exclusive dimerizing partner of gp130 in the functional receptor for CT-1. This is because the phenotype in the cardiac development in gp130-deficient mice was not observed in the LIFR-deficient one (35,58).

Since approximately one fifth of the gp130−/− embryos were anemic at 15.5 through 18.5 d pc, one could argue that poor oxygenation might have led to the hypoplastic development of myocardium in these embryos. However, considering that all the remaining (i.e., approx 80% of the total) gp130−/− embryos did not show anemic paleness whereas 100% of the null mutant embryos showed heart abnormalities at 16.5 d pc, this explanation does not appear to be sufficient. Several mutant mice have been previously reported which show severe anemia during embryogenesis (dominant white spotting, W; steel, sl; and PU-1 mutant mice), but none of them reportedly exhibit the extreme thinning of the ventricular walls (59,60).

By examining hematopoietic progenitor cells in fetal livers, we demonstrated that the lack of gp130 dramatically decreased the numbers of pluripotential stem cells; the number of CFU-S per liver was reduced by 88% (based on the average CFU-S values in Fig. 4). It should be noted that colony forming efficiency of gp130−/− progenitors in the recipient spleen was not much impaired by the lack of gp130. In fact, the average colony number in the spleen of a recipient mouse inoculated with $2 \times 10^5$ gp130−/− fetal liver-derived mononuclear cells was only slightly reduced compared to that observed with the control cells ($6.2 \pm 2.9$ for gp130−/−, $8.7 \pm 3.0$ for gp130+/+; n = 4 for each). As for committed hematopoietic progenitors, the numbers of erythroid progenitors and granulocyte-macrophage progenitors as measured by in vitro semi-solid cultures were also reduced (by 86% for BFU-E and by 66% for CFU-GM as calculated...
from Fig. 4). The decrease in the committed progenitors might be the result of the greatly reduced numbers of pluripotential hematopoietic stem cells. Similar to CFU-S, the seeding efficiency in these semi-solid cultures seemed to be less affected by the lack of gp130. Regarding the more differentiated hematopoietic lineage cells, at least immature T, and megakaryocytes were detected in the thymus or liver of the gp130<sup>−/−</sup> mutant fetus, but their cell numbers were, again, much smaller than those of the controls. Taken together, the gp130-mediated signaling was shown in this study to be essential for the generation of pluripotential hematopoietic stem cell pools in mouse embryos. The extreme reduction in such pools led to a similar decrease in the number of committed progenitors. Although few in numbers, some pluripotent hematopoietic progenitors did emerge in the absence of gp130 signaling, and once emerged, they appeared to remain multi-potent and capable of differentiating into at least the aforementioned lineages. gp130 was thus required for the early stages of hematopoiesis to make progenitor pools but did not seem to be required strictly for later stages of hematopoiesis, i.e., differentiation procedures. In erythroid lineage differentiation, however, finding of anemic gp130<sup>−/−</sup> embryos suggested a presence of a gp130-dependent mechanism also at later stages.

It may be worth noting that gp130<sup>+/−</sup> embryos showed intermediate phenotypes in terms of the numbers of total mononuclear cells and CFU-S in the liver. This suggests that the generation of the hematopoietic progenitor pool responds to the dose of gp130 signaling provided. This is not the case in cardiomyocyte development, since gp130<sup>+/−</sup> embryos did not exhibit any of the defects in the ventricular myocardium observed in gp130<sup>−/−</sup> embryos. There was variation in the numbers of fetal liver mononuclear cells and CFU-S among differentiated fetuses of the same genotype (see Fig. 4). In addition, among the gp130<sup>−/−</sup> embryos, the severity of anemic paleness varied considerably (only 20% showed significant anemia). One explanation for these fluctuations might be that the genetic background of the embryos was not uniform, but rather a mixture of 129 and C57BL/6. We are in the process of generating congenic gp130 mutant mice by repetitive crossing to an inbred strain.

Mouse PGCs originate at 7 d pc in the embryo at the posterior of the primitive streak (detectable as a cluster of a few cells) (51). They continue to proliferate up to approx 25,000 cells while migrat-
ing through the hind gut until about 11.5–12.5 d pc when they have settled in the gonads (61). In the gp130-deficient embryos, the numbers of PGCs in the gonadal ridges were reduced but the extent of reduction varied among embryos (from approximately one fourth to two thirds), possibly because of a nonuniform genetic background as discussed. It is important to note that although their numbers in the gp130−/− gonads were reduced, the alkaline-phosphatase positive PGCs that remained in the hind gut were also not so obviously detected. Thus, gp130-signaling may not be required for the migration of PGCs but rather for their proliferation or survival. Previous studies have shown that stem cell growth factor (SCF) and LIF synergistically promote the proliferation of cultured mouse PGCs (49). This combination together with basic fibroblast growth factor (FGF) act on PGCs to keep them undifferentiated and pluripotent, and to form ES cell-like colonies (46,47). These PGC-derived stem cells can contribute to the development of differentiated tissues including germ cells in chimeras following blastocyst injection, just as ES cells. Mutations at the W and sI loci in mouse, the former of which encodes c-Kit (62,63) and the latter c-Kit ligand or SCF (64,65), severely affect the proliferation and/or migration of PGCs and the development of hematopoietic precursors for multiple lineages (59). This, together with the observation that the numbers of pluripotent and committed hematopoietic stem cells were reduced in gp130-deficient embryos, suggests that gp130 signaling plays an important role in the self-renewal processes of various stem cell systems and that the SCF signals through c-Kit cooperate in these processes. In fact, Sui et al. (66) found that a complex of IL-6 and sIL-6R synergized with SCF in the ex vivo expansion of primitive hematopoietic progenitor cells from human cord blood. Sui et al. (66) also found that gp130 and c-Kit signals synergized in the proliferation and terminal maturation of human erythroid lineage cells, which is reminiscent of the anemia observed in some of the gp130 null mutant embryos (Sui et al., unpublished data).

From studies with LIF-deficient mice (67,68), LIF has been shown to be critical for blastocyst implantation: Adult female mice deficient for LIF are infertile as a result of a failure of the embryo to implant. This phenotype arises as a consequence of the lack of LIF production in the maternal host, rather than in the embryo, since LIF−/− embryos develop normally in a wild-type host mother but
LIF<sup>+/+</sup> embryos do not in a LIF<sup>−/−</sup> mother. Whether LIF acts on the blastocysts or on the endometrium of the uterus in a paracrine manner was not clarified in this LIF knockout study. Since embryos deficient for gp130, the critical component of functional LIF receptor, survived and developed through the implantation stage, LIF is believed to promote implantation via a paracrine effect on the endometrium and not on the blastocyst. After placentation, smaller sized placentas in gp130<sup>−/−</sup> fetuses became apparent from 13.5 d pc. Up until this stage, gp130 deficient placentas seem to have developed normally at least in their size and macroscopic structure. The placenta expresses a relatively high level of gp130, and several reports have demonstrated that stimulation of gp130 in placental trophoblasts regulates the expression and/or secretion of placental hormones such as human chorionic gonadotropin (69) and mouse placental lactogen-II (70). It would be of interest to examine whether production and secretion of these hormones are affected by the lack of gp130. At this moment, it is not clear how the gp130 deficiency caused the thinning of the spongiotrophoblast layer and labyrinthine zone observed in the present study. As for possible functional defects in gp130-deficient placentas, at least one aspect of the maternal-fetal transport, i.e., the transport conducted by immunoglobulin-specific transporters, was demonstrated to be impaired. Other forms of maternal-fetal transport, such as passive transport, remain to be examined. Although we observed a sign that might result in poorer maternal blood circulation in the placenta or that would possibly lead to preterm detachment of the gp130<sup>−/−</sup> placenta (histologically observed thrombus of maternal blood in the 17.5 d pc placenta), the precise mechanism of its occurrence is not known yet.

The shared usage of gp130 may explain why mice lacking IL-6, LIF, or CNTF do not exhibit overt developmental abnormality and why their phenotypes are much less severe than those expected from the known pleiotropic functions of each cytokine. One explanation is functional compensation by remaining gp130-stimulatory cytokines. However, functions specific to a cytokine is severely affected by its absence. For instance, although mice deficient for IL-6 develop normally, (71, 72), they exhibit severe impairment in antibody production following viral infection and acute-phase protein production after mineral oil administration (71). In normal mice, bone loss is observed after ovariectomy caused by the elevated
expression of IL-6. The ovariectomy-induced bone loss does not occur in IL-6-deficient mice (72). CNTF knockout mice develop and behave normally, but they show motor neuron degeneration in adulthood (though the extent is small) (73). In contrast to these cytokine knockout cases, the deletion of the gp130 or LIFR gene is developmentally lethal or perinatally fatal. As described, from the heterozygous matings, no live gp130−/− mice are born and gp130-deficient fetuses exhibit severe hypoplastic development of the heart ventricular walls, hematopoietic progenitors, and placentas. From intercrossings of mice heterozygous for the LIFR mutation, recovery of LIFR−/− fetuses is smaller than expected from the Mendelian laws (approximately half of the theoretical value), but they continue to develop to term in contrast to the case in the gp130-deficient mice (58). LIFR-deficient mice, however, die very shortly after birth. No hypoplastic development of the ventricular walls of the heart has been reported for the LIFR null mutation. Poor development of the placenta by the lack of LIFR is obvious, but fetal hematopoiesis is relatively normal even in the absence of LIFR−/−. Astrocyte numbers are reduced in the spinal cord and brain stem in LIFR-deficient newborns. Because of the considerably limited recovery of gp130−/− fetuses in particular at the late stages of gestation, histological analysis of the brain has not been completed. Precise comparison of the abnormalities in gp130−/− and LIFR−/− fetuses may be necessary.

In conclusion, the gp130 deficiency was lethal and affected ventricular myocardial development, hematopoiesis, primordial germ cell proliferation, and placental development. Other organs in the gp130+/− embryos appeared normal at least by examining hematoxylineosin-stained histological specimens. However, since gp130 is expressed in many organs examined, it could be possible that even the apparently normally developed organs in the gp130+/− fetuses would exhibit abnormalities if the fetuses were to continue to develop. Tissue-specific targeting of the gp130 gene, for instance in the neural system, would clarify the role of gp130 in various organs in more detail.

Summary

gp130 is a ubiquitously expressed signal transducing receptor component shared by several cytokines including IL-6, IL-11, LIF, OSM, CNTF, and CT-1. To investigate the physiological roles of gp130 in detail and to understand the pathological consequences of
a lack of gp130, a targeted disruption of the mouse gp130 gene has been performed. Homozygous mutant embryos deficient for gp130 progressively die between 12.5 d pc and term. They exhibit hypoplastic development of the ventricular myocardium, accompanying no defect in septum formation and trabeculation. The mutant embryos have greatly reduced numbers of pluripotential and committed hematopoietic progenitors in the liver, and some show severe anemia owing to impaired proliferation and maturation of erythroid lineage cells. gp130 null mutant placentas are smaller than controls, showing a thinner spongiotrophoblast layer and labyrinthine zone. These results indicate that gp130 plays a crucial role in myocardial development, hematopoiesis, and placental development during embryogenesis.

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Chapter 17

NF-IL6 Knockout Mice

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NF-IL6

Structure and Expression of NF-IL6

Nuclear factor for IL-6 expression (NF-IL6) was originally identified as a nuclear factor binding to a 14-bp palindromic sequence (ACATTGCACAATCT) within an interleukin (IL)-1 responsive element in the human IL-6 gene (1). The cloning of the cDNA encoding human NF-IL6 revealed that it shows a high degree of homology with C/EBP in the carboxy-terminal basic and leucine zipper domains, responsible for DNA binding and dimerization, respectively (2). NF-IL6 recognizes the same nucleotide sequences as C/EBP. Both proteins bind to a variety of the divergent nucleotide sequences with different affinity, the consensus sequence is T(T/G)NNGNNAAT(G). The NF-IL6 gene is intronless, and produces two proteins, liver-enriched transcriptional activator protein (LAP, equivalent to NF-IL6) and liver inhibitory protein (LIP) by alternative usage of two AUG initiation codons within the same open reading frame (3). LIP contains the DNA binding and dimerization domains but is devoid of the N terminal transcriptional activation domain, and therefore behaves as an antagonist of LAP-induced transcription.

NF-IL6 is expressed at an undetectable or a minor level in all normal tissues, but it is drastically induced by stimulation of
lipopolysaccharide (LPS), IL-1, tumor necrosis factor (TNF), or IL-6. Accumulating evidence have implied that NF-IL6 may be responsible for the regulation of genes encoding many acute phase proteins and cytokines (4). Meanwhile, NF-IL6 was cloned as a transcriptional activator of a variety of genes and is also known as LAP, AGP/EBP, IL-6DBP, C/EBPβ, or NF-M (5–10). NF-IL6 expression is dramatically induced during macrophage differentiation (11,12). NF-IL6 binding motifs are also found in the functional regulatory regions of genes specifically induced in activated macrophages, such as IL-6, IL-1α, IL-8, TNFα, granulocyte colony-stimulating factor (G-CSF), nitric oxide (NO) synthase, and lysozyme genes (11,13–15).

**Activation of NF-IL6 Though Phosphorylation**

NF-IL6 is a phosphoprotein. NF-IL6 is activated through phosphorylation of Thr-235 by a ras-dependent mitogen-activated protein (MAP) kinase cascade (16) NF-IL6 is a repressed transcription factor whose transactivation domains are concealed with the inhibitory domains, and phosphorylation of the MAP kinase site within the inhibitory domains of NF-IL6 eradicates the inhibition by exposing its transactivating function (17). NF-IL6 is also phosphorylated within the leucine zipper in response to increased intracellular calcium concentrations via the activation of a calcium–calmodulin-dependent kinase (18). In addition, cAMP-mediated phosphorylation of NF-IL6 is shown to be associated with nuclear translocation and transcriptional activation (19). Recently NF-IL6 has been shown to be activated through phosphorylation of the N-terminal domain by protein kinase C (PKC) (20). Thus, NF-IL6 is activated via multiple signaling pathways.

**C/EBP Family Members**

There are five members of the C/EBP family (Fig. 1). These include C/EBP, NF-IL6, Ig/EBP (also referred to as GPE-1-BP, C/EBPγ) (21,22), NF-IL6β(C/EBPδ) (8,23,24), and CHOP-10 (gadd153) (25). C/EBP is expressed in adipose, liver, and placental tissues that play a vital role in energy metabolism. C/EBP mRNA increases markedly during differentiation of 3T3-L1 preadipocytes to adipocytes (26). C/EBP can transactivate the promoters of the
NF-IL6 Knockout Mice

adipocyte-specific genes, 422/adipose P2 protein (422/aP2), Stearoyl-CoA desaturase 1 (SCD1), and insulin-responsive glucose transporter-4 (GLUT4) (27). Several lines of recent evidence have demonstrated that C/EBP is an important regulatory factor in adipocyte differentiation (28–33).

NF-IL6β is normally expressed at a minor level but drastically and rapidly induced in many tissues by LPS or several inflammatory cytokines including IL-1, TNF, and IL-6 as in the case of NF-IL6 (23). Ig/EBP was originally cloned as a nuclear factor that binds to the functionally important C/EBP binding sites in immunoglobulin gene enhancers and promoters (21). Ig/EBP alone has no transcriptional activity. Ig/EBP is ubiquitously expressed in normal adult tissues but most abundant in the early stage of B-lymphocyte (34). CHOP-10 was cloned as a protein interacting with the bZip domain of NF-IL6 (25). CHOP-10 is specifically induced under the condition of growth arrest and/or DNA damage.

Fig. 1. C/EBP family members. DNA, DNA-binding domain, P, Pro rich, G, Gly rich, S, Ser rich, E, Glu rich, D, Asp rich; T, Thr rich region.
Cooperative Interaction of NF-IL6 and NF-κB in Gene Expression

It has become increasingly evident that combinatorial effects of transcription factors are very important in gene regulation. Both NF-IL6 binding and NF-κB binding sites work synergistically in expression of the genes involved in inflammatory and immune responses. Those genes include IL-6, IL-8, the serum amyloid A1, serum amyloid A3, complement C3, α1-acid glycoprotein, angiotensinogen, and G-CSF genes (35–37). Interestingly a λgt11 expression library screening with radiolabeled NF-κB p50 as a probe has led to the isolation of NF-IL6 cDNA clones in addition to several clones of the NFκB/rel family members, which provided a first indication of a direct protein-protein interaction between NF-IL6 and NF-κB (38). Subsequent studies have provided evidence for functional and physical interaction between NF-IL6 and NF-κB (39–41).

NF-IL6 Knockout (KO) Mice

Markedly Increased Susceptibility of NF-IL6 KO Mice to Listeria Infection

To know the specific regulatory role of NF-IL6 in vivo, NF-IL6 KO mice were generated by gene targeting in embryonic stem (ES) cells. The NF-IL6 targeting vector was constructed by inserting a neomycin resistance gene into the bZip domain of the NF-IL6 gene (42). Two lines of mice carrying the mutation at the NF-IL6 locus were generated from independently isolated ES cell lines. Homozygous mutant mice were obtained from heterozygous parents below the expected Mendelian ratio, suggesting prenatal mortality, but newborn mice appeared normal, and there was no significant increase in mortality rate under specific pathogen-free (SPF) conditions. However, NF-IL6 KO mice presented a high susceptibility to Listeria infection (42). When NF-IL6 KO and control mice were infected intraperitoneally with $5 \times 10^2$ colony-forming units (CFU) (50% lethal dose of wild-mice is $1 \times 10^6$ CFU) of *L. monocytogenes*, all NF-IL6 KO mice died within 5 d after challenge with *Listeria* (Fig. 2A). Histopathological examination showed the multiple foci of microabscesses consisting of neutrophils and macrophages in the liver and spleen of NF-IL6 KO mice (Fig. 2B).
Fig. 2. *Listeria* infection in wild-type and NF-IL6 KO mice. (A) Survival of mice infected with *L. monocytogenes*. Wild-type and NF-IL6 KO mice were injected intraperitoneally with $5 \times 10^2$ CFU of *L. monocytogenes* ($n = 6$). Percent survival of wild-type ($\square$) and NF-IL6 KO mice ($\bullet$) is shown. (B) Histological analysis of liver and spleen sections after *L. monocytogenes* infection. Liver and spleen were removed from wild-type and NF-IL6 KO mice 4 d after ip injection of *L. monocytogenes* ($5 \times 10^2$ CFU). Tissues were fixed with 10% buffered formalin solution. Sections were stained with hematoxylin and eosin. (A,C), liver ($\times 40$); (B,D), Spleen ($\times 100$).
**Cellular Immunity Against Listeria Infection**

*L. monocytogenes* is a Gram-positive facultative intracellular bacterium. Infection of mice with *L. monocytogenes* has been widely used for the experimental analysis of immunity against intracellular bacteria. At the early phase, the infection is limited by a T-cell-independent natural immunity represented by neutrophil, natural killer (NK) cells, and monocytes/macrophages. Neutrophils accumulate at infectious foci during the first 24 h of infection, where they cause dissolution of infected host cells, thereby liberating *L. monocytogenes* into the extracellular environment for ingestion by neutrophils themselves and by macrophages. Macrophages infected with microorganism release IL-12 and TNFα. IL-12 from such macrophages induces interferon (IFN)-γ production in NK cells. In turn, IFN-γ produced by NK cells activates macrophages to endow enhanced microbicidal capabilities. TNFα acts alone or in synergy with IFN-γ to induce macrophage activation (43-45). Some IFNα induced effects are shown to be mediated by TNFα, probably via an autocrine loop. Although a considerable degree of innate resistance can be observed in the absence of T-cells (e.g., in nude and SCID mice), the complete elimination of *L. monocytogenes*, and the long-lasting protection against reinfection (immunologic memory) depends on a *L. monocytogenes*-specific T-cell response. During intracellular persistence, microbial proteins are processed and presented, thus initiating T-cell activation. In the further course of the infection, CD8+ cytotoxic T-lymphocytes (CTL) play an important role in the immune response to *Listeria* infection. CTLs lyse infected cells and also activate resident macrophages by producing IFNγ. Thus macrophages play a central role in both natural and acquired immune responses to *Listeria* infection. Microbicidal activity of macrophages is not expressed constitutively, but is acquired after exposure to cytokines such as IFNγ, IL-2, and TNF as well as bacteria products such as LPS. After phagocytosis by permissive resident macrophages, *L. monocytogenes* produces a hemolysin (listeriolysin O) that allows it to escape from the phagosomes and to enter the cytoplasm and freely replicate there. In addition, the organism uses a surface protein encoded by the *actA* gene to induce actin assembly and consequently becomes coated with host cell F-actin. As the infection progresses, the bacteria are motile within the cytoplasm and the actin coat rearranges to form a comet
Fig. 3. Schematic model of *Listeria* killing mechanism in macrophages. In resident macrophages, phagocytosed *Listeria* escapes from phagosome and replicates in the cytoplasm. In contrast, when macrophages are activated by IFNγ and LPS, iNOS is induced by the transcription factor IRF-1, which results in the increased production of NO. NO finally prevents phagosomal escape of *Listeria* and kills them.

tail. In contrast, in activated macrophages able to kill *Listeria*, the bacteria neither enter the cytoplasm nor associate with F-actin, but appear restricted to the phagosome (Fig. 3). Therefore the appearance of actin-coated *Listeria* in the cytoplasm after phagocytosis represents evidence of productive intracellular infection (46).

**Cytokine Induction in NF-IL6 KO Mice**

A number of cytokines such as IFN-γ, TNFα, IL-6, IL-1β, G-CSF, M-CSF, and GM-CSF have been shown to be involved in the host resistance to bacterial infections. The administration of rIFNγ, rTNFα, or rIL-1 resulted in increased resistance of mice to subsequent challenge, whereas treating mice infected with *L. monocytogenes* with neutralizing antibodies directed against certain lymphokines can cause exacerbation of infection (47–53). First, the induction of various cytokines was examined in NF-IL6 KO mice. RNA was prepared from several sources (resident peritoneal macrophages; proteose peptone-elicited peritoneal macrophages
with or without stimulation of LPS in vitro). Reverse transcribed cDNA was amplified by primers for nine cytokines (TNFα, IL-1β, IL-6, IL-10, IL-12, MIP1α, G-CSF, GM-CSF, and M-CSF). Induction of all cytokines in NF-IL6 KO mice was comparable to that observed in wild-type mice with the exception of G-CSF (Fig. 4A). It has been shown that IFNγ and TNFα are key cytokines for the induction of antimicrobial activity by macrophages. However, IFNγ and TNFα mRNAs and proteins were substantially induced during Listeria infection in NF-IL6 KO mice, ruling out the possibility of impaired induction of IFNγ or TNFα (Fig. 4B).

**Impaired G-CSF Induction in NF-IL6 KO Mice**

Since G-CSF mRNA expression was not detected in proteose peptone-elicited peritoneal macrophages treated for 1 h with LPS, the kinetics of G-CSF mRNA after LPS stimulation was examined. In the wild-type macrophages, expression of G-CSF mRNA was induced within 2 h, reached a peak level at 5–8 h, and then declined, whereas in the NF-IL6-deficient macrophages there was a small induction of G-CSF mRNA only detectable at 5–8 h (Fig. 5A). The impairment of G-CSF production by NF-IL6-deficient macrophages was verified by lower levels of biological G-CSF activity in the culture supernatants (Fig. 5B). Induction of G-CSF mRNA was also impaired in NF-IL6-deficient embryonic fibroblasts and bone marrow-derived fibroblasts. However, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA from the liver, spleen, and lung revealed a similar intensity of signals for G-CSF mRNA between wild-type and NF-IL6 KO mice. G-CSF activities in the spleen culture supernatants were also comparative between NF-IL6 KO and wild-type mice, suggesting that G-CSF was normally induced by other cell types in these organs from NF-IL6 KO mice (Fig. 5B). In fact, cultures enriched in endothelial cells from NF-IL6 KO mice expressed G-CSF mRNA as much as those from wild-type mice (Fig. 5A). As expected, NF-IL6 KO mice had a normal circulating neutrophil level, and developed a significant neutrophilia during L. monocytogenes infection. Taken together, these results demonstrate that NF-IL6 is essential for the induction of G-CSF in macrophage and fibroblasts. This observation is consistent with the recent finding that both antisense oligonucleotides and ribozyme-mediated specific elimination for
Plate 1 (Fig. 3; see full caption on p. 96 and discussion in Chapter 6). Tissue necrosis in response to TNF injections. Upper, skin patches from the site of injection. Lower, histological sections at 200× magnification of H&E-stained tissues.

Plate 2 (Fig. 5; see full caption on p. 268 and discussion in Chapter 16). Erthroid lineage cell abnormality in the 16.5 d pc gp130 null mutant.
Plate 3 (Fig. 8; see full caption on p. 272 and discussion in Chapter 16). (A,B) Histologies of the 14.5 d pc control and gp130\(^{-/-}\) placentas. (C,D) Sagittal sections of 17.5 d pc control and gp130\(^{-/-}\) placentas.
Plate 4 (Fig. 2; see full caption on p. 426 and discussion in Chapter 22). (A) Intra-alveolar substance accumulated in 2-yr-old mutant mice, along with areas of lymphocytic infiltration. (B) Similar but more extensive pathology is seen in 6-mo-old mice. (C) Extensive intra-alveolar material resulting in a blockage of a significant portion of airway space in 1-yr-old mutant mice.
Fig. 4. Cytokine expression and induction. (A) RT-PCR analysis of cytokine mRNA expression. Total RNA was isolated from resident macrophage (R), proteose peptone-elicited peritoneal macrophages (P) with or without 1 h stimulation of LPS (100 ng/mL) and subjected to RT-PCR using the cytokine-specific primers. (B) Induction of IFNγ and TNFα mRNA by Listeria infection. Total RNA was extracted from spleen and peritoneal exudate cells (PEC) of NF-IL6 KO mice before and 4 d after *L. monocytogenes* (5 × 10^2 CFU, intraperitoneally) infection, and subjected to RT-PCR.
Fig. 5. (A) RT-PCR analysis of G-CSF mRNA expression. (Top) Proteose peptone-elicited peritoneal macrophages (MP) were incubated with or without LPS (100 ng/mL) for 2, 5, 8, and 12 h. Embryonic fibroblast (EF), bone marrow fibroblast (BM-F), and endothelial cell-enriched culture (EC) were incubated with or without LPS (100 ng/mL) for 6 h. Total RNA was isolated from cells at each time and subjected to RT-PCR. (Botom) Total RNA was isolated from liver, spleen, and lung before and 1 or 4 h after ip injection of LPS (50 µg) and subjected to RT-PCR. (B) G-CSF bioassay. Culture supernatant of peritoneal macrophages, embryonic fibroblasts, and spleen cells were collected. G-CSF activity was measured using a G-CSF-dependent cell line, NSF-60.
NF-IL6 transcripts abolished TNFα-induced synthesis of G-CSF in human fibroblasts (54).

**Escape of Listeria from the Phagosome to the Cytoplasm in NF-IL6-Deficient Macrophages**

Activated macrophages are the primary host cells mediating immunity to *L. monocytogenes*. Immigration of macrophage to the site of listerial replication, phagocytosis, and the intracellular killing by macrophages represent three major processes for eradication of *L. monocytogenes*. Among them, the immigration of macrophages to the peritoneal cavity and the phagocytic activity of NF-IL6-deficient macrophages were shown to be comparable with that of wild-type macrophage, respectively (Fig. 6A,B). However, the intracellular killing of *Listeria* by NF-IL6-deficient macrophage turned out to be severely impaired. Proteose peptone-elicited macrophage from wild-type and NF-IL6 KO mice were treated for 20 h in vitro with IFNγ plus LPS. Activated peritoneal macrophage were infected with *L. monocytogenes* for 4 h, then fixed, sectioned, and examined by electron microscopy (Fig. 6C). In the IFNγ plus LPS-treated proteose peptone-elicited macrophage from wild-type littermates, the vast majority of the bacteria (87%) were in vacuoles, although 13% were free in the cytoplasm (Table 1). In contrast, in NF-IL6-deficient macrophages 84% of the bacteria were found in the cytoplasm. The majority of these bacteria (79%) were surrounded by actin filaments and some were in the process of division.

Furthermore, FACS analyses of several surface markers specific for activated macrophage, such as major histocompatibility (MHC) class II molecules, FcyR, Mac 1, and lymphocyte function-associated antigen (LFA)-1 showed that these surface activation markers were normally induced in NF-IL6-deficient macrophages when treated with LPS plus IFN-γ (Fig. 7). Taken together these results indicate that NF-IL6-deficient macrophages cannot kill *Listeria* even when maximally activated.

As described previously, NF-IL6-deficient macrophages have impairment of G-CSF production. However, neither intraperitoneal injection of G-CSF improved the susceptibility to *Listeria* infection in vivo nor could addition of G-CSF improve the efficacy of *Listeria* killing by NF-IL6-deficient macrophages in vitro. These results indicate that impairment of G-CSF may
Fig. 6. (A) Immigration of macrophages to the peritoneal cavity. The number of peritoneal exudate macrophages was counted 4 d after ip injection of 2 mL of 10% proteose peptone. (B) Phagocytic activity of macrophages. Proteose peptone-elicited peritoneal macrophages were mixed with FITC-labeled latex particles (polyscience) and incubated for 2 h. After washing the cells with PBS, phagocytosed FITC-latex particles were measured by FACScan. (C) Electron micrographs of peritoneal macrophages infected with L. monocytogenes. (A–C) wild-type macrophages; L. monocytogenes present in a phagosome (A, ×9100; B, ×26,000; C, ×36,000). (D–F) NF-IL6-deficient macrophages; (D) L. monocytogenes found in the cytoplasm (×9100). (E) L. monocytogenes in the process of division is surrounded by actin filaments (×26,000). (F) L. monocytogenes in the cytoplasm has an asymmetric tail of actin filaments which requires for spreading to neighboring cells (×26,000). Adapted from ref. 42.
Table 1
Intracellular Location of *L. monocytogenes* in Peritoneal Macrophages from Wild-Type and NF-IL6 KO Mice

| Mice | Bacteriaa | Bacteria in endosome (%) | Bacteria in cytoplasm (%) | Bacteria in cytoplasm and actin (%) |
|------|-----------|--------------------------|---------------------------|-----------------------------------|
| +/+  | 75        | 65 (87)                  | 7 (9)                     | 3 (4)                             |
| −/−  | 75        | 12 (16)                  | 13 (17)                   | 50 (67)                           |

*aThe total number of intact bacteria observed by electron microscopy in one section.

not play a role in the *Listeria* killing defect observed in NF-IL6 KO mice.

**NO and Antimicrobial Activity**

The production of NO and its reactive nitrogen intermediates by macrophages has been suggested to be an important mechanism
Fig. 7. FACS analysis for macrophage activation markers. Resident peritoneal macrophages (R) and proteose peptone-elicited peritoneal macrophages cultured for 48 h in the presence (P-I) or absence (P-N) of IFNγ (100 U/mL) were stained with indicated antibodies, and analyzed by FACScan. The vertical axis represents relative cell number, and the horizontal axis is fluorescence intensity.
for elimination of intracellular bacteria and parasites (55). NO in macrophages is generated by the enzyme L-arginine-dependent nitric oxide synthase (NOS) that is itself inducible by cytokines like IFNγ and/or microbial cell wall products such as LPS. Once induced, the enzyme is active and does not require exogenous Ca2+/calmodulin, unlike constitutive NOS in endothelial cells and neurons, that is Ca2+- and calmodulin-dependent. Interestingly, NF-IL6 binding sites are present in the region responsible for LPS induction of the mouse macrophage NOS gene (15).

NO production was compared between peritoneal macrophages from wild-type and NF-IL6 KO mice, respectively. Proteose peptone-elicited macrophages were cultured in the presence of IFN-γ and LPS for 48 h, and the NO2− concentrations in the culture supernatants were determined. NO2− production was detectable in NF-IL6-deficient macrophage cultures to the same extent as in wild-type macrophage cultures (Fig. 8A). NOS mRNA was also induced at a similar extent in NF-IL6-deficient and wild-type macrophages within 2 h after IFNγ and LPS stimulation as well as at 4 d after Listeria challenge (Fig. 8B). These results indicate that NO production is not impaired in NF-IL6-deficient macrophages. However our NF-IL6 KO mice study supported a role for NO in Listeria killing since the microbial growth was enhanced when N/G monomethyl-L-arginine (L-NMMA), an inhibitor of NOS function, was added to wild-type macrophages. Taken together, these results indicate that both NO production and NF-IL6 function are essential for Listeria killing, and loss of either completely abolishes the bactericidal activity.

Recently, inducible nitric oxide synthase (iNOS) KO mice have been generated (56). As expected, the mice succumbed to inocula of Listeria at least 10-fold lower than those lethal to wild-type mice, in association with -100-fold greater bacterial burdens in the liver and spleen when mice were challenged intraperitoneally with \(4 \times 10^5\) CFU of Listeria and sacrificed 3 d later. However, the increase of bacterial load in infected organs and the severity of Listeria infection seem to be less dramatic than in NF-IL6 KO mice. This indicates that NF-IL6 is a critical factor involved in Listeria killing and supports our finding that macrophage-mediated cytotoxicity consists of two different mechanisms, one unidentified but depending on NF-IL6 and the other involving iNOS (Fig. 9).
Fig. 8. Production of NO and oxygen intermediates by macrophages.

(A) NO$_2^-$ release in peritoneal macrophages from wild-type and NF-IL6 KO mice. Proteose peptone-elicited peritoneal macrophages were cultured for 48 h in the presence or absence of IFNγ (100 U/mL) and LPS (100 ng/mL). NO$_2^-$ release into the culture medium was determined.

(B) RT-PCR analysis of NOS messenger in peritoneal macrophages from NF-IL6 KO mice. (Left) Total RNA was isolated from proteose peptone-elicited peritoneal macrophages (MP) with or without stimulation by IFNγ (100 U/mL) and LPS (100 ng/mL), or peritoneal exudate cells (PEC) before and 4 d after infection with *L. monocytogenes* (5 × 10$^2$ CFU), and subjected to RT-PCR.
There is evidence to suggest that reactive oxygen intermediates are also important in the killing of microorganisms. The production of reactive oxygen intermediates by macrophages was measured. Production of superoxide and hydrogen peroxide was significantly suppressed in NF-IL6-deficient macrophages in comparison to wild-type macrophages (Fig. 10). However, neither catalase (which converts hydrogen peroxide to water and oxygen) nor superoxide dismutase (which converts superoxide anion to hydrogen peroxide) had any effect on the inhibition of growth of *L. monocytogenes*. Furthermore, the reactive oxygen intermediate production from neutrophils was comparable between wild-type and NF-IL6 KO mice. These results seem to indicate that the respiratory burst is not directly involved in growth restriction of *L. monocytogenes*.
Listeria Infection Experiments in Other KO Mice

Recently the involvement of several cytokines in anti-listerial defense has been ascertained using mice with a targeted mutation of the respective cytokine gene or cytokine receptor gene.

TNFRp55 KO mice were severely impaired to clear *L. monocytogenes* and readily succumbed to infection (57,58). In the case of TNFRp55 KO mice reported by Rothe et al. (57), 4.0 × 10³ CFU of *L. monocytogenes* were infected intravenously. Four days after infection mutant mice exhibited 5 × 10³ to 5 × 10⁵-fold increased bacterial titers in spleen and liver when compared with control mice. Even a challenge with only 250 CFU *L. monocytogenes* killed all mutant mice by d 6 after infection.

IFN-γR KO mice were also severely impaired in the defense against *L. monocytogenes* (59). When mutant and control mice were infected intravenously with 10⁴ CFU of *L. monocytogenes*, three of five mutant mice died before d 5. The number of bacteria recovered from spleens and livers on d 5 increased approximately 100-fold in the livers and about 10-fold in the spleens of mutant mice in comparison to that of controls.

G-CSF KO mice have been generated and have a chronic neutropenia (60). G-CSF KO mice had a markedly impaired ability to
NF-IL6 Knockout Mice

control infection with *L. monocytogenes*, with diminished neutrophil and delayed monocyte increases in the peripheral blood and reduced infection-driven granulopoiesis. When a dose of $5 \times 10^3$ CFU was inoculated intravenously (LD$_{50}$ was $2 \times 10^5$), the cumulative mortality from infection of G-CSF KO mice by d 5 was 50% (8/16), whereas no deaths occurred in control mice over the same period.

IL-6 also contributes significantly to the defense against *L. monocytogenes*. IL-6 KO mice were shown to be substantially compromised in their anti-listerial response (61,62). The IL-6 KO mice displayed a 50–60% mortality rate within a week of IV inoculation of $10^4$ bacteria. Five days after infection, mutant mice had $10^2$ times higher bacteria titers in infected organs than wild-type control mice. It has been shown that IL-6 KO mice are unable to induce peripheral blood neutrophilia in response to listeriosis, which is prominent in the control animals.

NF-κB is a heterodimeric transcription factor composed of p50 and p65 subunits. NK-κB can be activated in many cell types and is thought to regulate a wide variety of genes involved in immune function and inflammatory reaction (63). Recently mice lacking NF-κBp50 have been generated (64). When infected with *L. monocytogenes*, p50 KO mice were unable to clear the bacteria effectively. At 6 d after injection of $5 \times 10^3$ bacteria, control mice had few or no splenic or peritoneal bacteria whereas p50 KO mice had several thousand splenic, but no peritoneal bacteria. This defective clearance likely reflects a defect in macrophage activation because both resting peritoneal macrophages and blood neutrophils from p50 KO mice were present in normal numbers and had normal phagocytic activity. However, the defect in control of *L. monocytogenes* in NF-κBp50 KO mice seems to be less dramatic than that in NF-IL6 KO mice.

**Impairment of Tumor Cytotoxicity in NF-IL6-Deficient Macrophage**

The tumor cytotoxic activity was also examined in NF-IL6-deficient macrophages. Tumoricidal and tumoristatic activities were assayed by measuring released and incorporated radioactivity in co-cultures of proteose peptone-elicited macrophages and P815 tumor target cells, respectively. The tumoricidal and tumoristatic activities of NF-IL6$^{-/-}$ macrophages against P815 were extremely low (Fig. 11). Furthermore, the ability of wild-type macrophages to kill or inhibit the cell growth of P815 was markedly inhibited by
Macrophage/target ratio

Fig. 11. Tumor cytotoxic assay of activated macrophages. Proteose peptone-elicited peritoneal macrophages from wild-type and NF-IL6 KO mice were stimulated with IFNγ (100 U/mL) and LPS (100 ng/mL) for 48 h, and cytolytic and cytostatic activity against the P815 mastocytoma cell line was assayed in the presence or absence of 1 mM NMMA. Adapted from ref. 42.

L-NMMA, and this inhibition was accompanied by loss of NO production. In contrast the tumoricidal and tumoristatic abilities of NF-IL6-deficient macrophages were severely impaired irrespective of the amounts of NO production. Taken together these results demonstrate the existence of a mechanism of tumoricidal and tumoristatic activities that is independent of NO and is mediated by NF-IL6, although it is clear that NO otherwise plays a central role in mediating these activities in activated macrophages.

Mutlicentric Castleman’s Disease in NF-IL6 KO Mice

Dr. Poli and her colleagues (65) also generated the NF-IL6 KO mice. They demonstrated that the NF-IL6 KO mice developed skin lesions, swellings in the mucosal regions, splenomegaly, and lymphadenopathy with age, although morphological and histological analysis of young KO mice (6–12 wk) did not show abnormalities. We always utilized the young KO mice (under 10 wk) for the experiments described, however, we also noted similar abnormalities in older KO mice. Histological features of lymph nodes showed
diffuse plasmacytosis and prominent germinal centers with preservation of nodal architecture, which was remarkably similar to that observed in patients affected by multicentric Castleman’s disease. Systemic alterations seen in Castleman’s disease such as increased serum IL-6 levels, polyclonal hypergammaglobulinemia, transient neutrophilia, a high number of cells of the granulocytic series, and glomerulonephritis were also found in the NF-IL6 KO mice. These pathogenic alterations found in NF-IL6 KO mice were strikingly similar to those observed in mice overexpressing IL-6 \((66)\). In fact, the pathogenesis of Castleman’s disease has been linked to deregulated IL-6 production \((67)\). Furthermore, humoral, innate, and cellular immunity were also profoundly distorted, as shown by the defective activation of splenic macrophages, the strong impairment of IL-12 production, the increased susceptibility to *Candida albicans* infection, and the altered T-helper function. Considering the data obtained from Poli’s NF-IL6 KO mice and ours, these abnormalities in humoral, innate, and cellular immunities may have been caused by dysregulation of IL-6 production and defective macrophage function. We have demonstrated that NF-IL6-deficient macrophages have severe defects in bactericidal and tumoricidal activities. The results from Poli and colleagues \((65)\) may also suggest the impairment of macrophage function in T-helper response.

Contradictory to what was suggested based on tissue culture experiments, NF-IL6 KO mice can overexpress IL-6, indicating that this transcription factor is not essential for IL-6 gene transcription. Recent NF-κBp50 KO mice experiment has demonstrated that p50-deficient macrophages stimulated with LPS, TNFα, and IL-1α release were normal, but IL-6 release was decreased several-fold relative to control macrophage, indicating that NF-κBp50 is actually involved in induction of IL-6 from macrophage.

What is the mechanism of deregulated IL-6 expression in NF-IL6 KO mice? One possibility is that NF-IL6 might act both as a positive and as a negative factor in vivo because NF-IL6 mRNA is shown to generate two proteins with opposite functions, LAP acting as a transactivator and LIP that does not have a transactivating domain and act as a repressor of all C/EBP factor activity. In the NF-IL6 KO mice, both LAP and LIP proteins are not produced. Therefore the disruption of the NF-IL6 gene might manifest only the impairment of inhibition of IL-6 production because IL-6 induction
could be fully compensated for by other NF-IL6 family members such as NF-IL6β (C/EBPδ), C/EBP, and Ig/EBP as well as other different transcription factors. Another possibility is that IL-6 overproduction may be caused by persistent opportunistic infection owing to impairment of macrophage microbicidal activity. In this case, abnormality in IL-6 production may be secondary. Thus, at present the mechanism underlying the IL-6 deregulation in NF-IL6 KO mice remains to be determined.

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Chapter 18

Disruption of the LIF Receptor

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Introduction

The interleukin-6 (IL-6) family of cytokines and their receptors all require dimerization of one receptor subunit with gp130 to transduce an intracellular signal (for reviews see refs. 1 and 2). Leukemia inhibitory factor (LIFR) binds LIF that is converted to high affinity binding by heterodimerization with gp130. Other ligands that use the LIFR are oncostatin M (OSM) and cardiotrophin-1 (CT-1). CT-1 utilizes LIFR although all the receptor components for CT-1 have not been elucidated (3), whereas OSM binds directly to gp130 and can use either LIFR (4) or another receptor (alternate OSMR; B. Mosley, personal communication) for dimerization and signal transduction. Ciliary neurotrophic factor (CNTF) also requires LIFR for biological activity, but first binds CNTF receptor (CNTFR) and this complex then interacts with LIFR and gp130 (5). Therefore, of the known ligands, the absence of LIFR would completely disrupt activity of LIF, CNTF, and CT-1, and would affect some OSM activity.

Other members of the IL-6 family of cytokines include IL-6 and interleukin-11 (IL-11). In both cases, these cytokines bind their specific receptor (6,7). These complexes bear similarities to the CNTF/CNTFR complex, including short (IL-6R, IL-11R) or absent (CNTFR) cytoplasmic domains (6,7). However, it is the homodimerization of two gp130 molecules, rather than LIFR heterodimer-
ization with gp130, that allows functional signaling to occur following IL-6 binding to IL-6R (6) while the dimerization partner with gp130 has not been described for IL-11R.

To date, null mutants of two ligands that utilize LIFR, LIF, and CNTF, have been made. Regardless of embryo genotype, embryos are unable to implant in the LIF null uterus (8,9). This defect can be overcome by embryo transfer into pseudopregnant mice that have at least one functional LIF gene (8). LIF null postnatal mice are growth retarded. The hematological consequences include impaired thymic T-cell activation and reduced myeloid progenitor cellularity in the spleen. However, LIF null spleen or marrow cells can sustain long-term host survival. This illustrates the importance of LIF in the microenvironmental influence on hematopoietic cells (9). CNTF null mice are normal until adulthood, when they display weakness that involves motor neuron loss (10). This neural degeneration does not become lifethreatening. The CNTF null mutation has no apparent effects outside the nervous system.

Both the CNTFR (11) and gp130 (see Chapter 13) have been functionally disrupted. CNTFR null animals die of starvation within 21 h of birth owing to a motor neuron deficit that precludes suckling, although their movements appear normal. The gp130 null animals show a broad range of defects and die in utero from d 12.5 postcoitum to term. Among the defects in evidence are cardiac (hypoplasia of the ventricular myocardium), hematopoietic (reduced cellularity of pluripotent progenitors in the fetal liver, with a subsequent reduction of cellularity of committed progenitors and a defect in the maturation and proliferation of the erythroid compartment), and reproductive (reduction in placental size involving the spongiotrophoblast and labyrinthine zone, impaired materno-fetal immunoglobulin transport and a reduction in the number of primordial germ cells present in the genital ridge).

The LIFR null animals would be expected to have an intermediate phenotype between the CNTFR and gp130 null animals. Our results agree with this expectation (12). However, because the LIFR animals can successfully reach term, defects in bone development, neural development, and fetal liver glycogen storage could be identified in the LIFR null animals that would presumably be identified in the gp130 null animals surviving to term. The hematopoietic and primordial germ cell compartments were apparently normal in the LIFR null animals, unlike the gp130 null animals.
Phenotypic Changes Induced by the LIFR Null Mutation

Perinatal Lethal

A null mutation in the LIFR gene was created by replacing LIFR coding sequences with a promoterless lacZ/neomycin resistance fusion cassette (lacZ/neo) by homologous recombination in embryonic stem (ES) cells (12). Thus, in correctly targeted ES clones, expression of the lacZ/neo fusion gene is under the control of endogenous LIFR transcriptional regulatory sequences. Chimeras were mated to 129J females to retain a pure strain background. However, LIFR+/- x +/- matings in 129J animals were unproductive. Thus, mice heterozygous for the LIFR mutation were maintained on a random C57BL/6 x 129 background. No LIFR-/- mice were observed 4 wk after birth; otherwise, LIFR+/- and +/- pups were observed in close to expected ratios (36 vs 64%, respectively; see ref. 12 and Table 1). Pups found dead when newborn litters were first observed were analyzed by Southern blot analysis. Approximately half were found to be LIFR-/- . Only rare live pups were detected. Therefore, pregnancies generated from LIFR+/- x +/- matings were disrupted from embryonic d 3.5 (E3.5, where E0.5 is the day of plug) to E19.5. The number of LIFR-/- animals detected throughout gestation was 15%, 40% below the Mendelian expectation. Wild-type and heterozygous animals were represented in the expected numbers. LIFR null pups born by Cesarian section on E18.5 either succumbed within 2 h of delivery or else sometime during the night after being placed with a foster mother. Although littermates varied in size, the overall impression was that LIFR null animals were smaller (Ware and Willis, unpublished observation). The number of primordial germ cells in the LIFR null animals
Genotype ratios of the preimplantation (E3.5) embryos mirrored numbers seen throughout gestation, suggesting a role for LIFR prior to embryo implantation.

LIF null mothers can no longer implant embryos (8,9). This study does not allow a direct comparison of data because LIFR null mothers are unobtainable. However, LIFR null embryos have no obvious implantation impairment, which suggests that LIF acts within the uterine endometrium and that implantation is not mediated through a maternal LIF embryonic LIFR interaction. It can be predicted that were LIFR null mothers obtainable, the same implantation disruption observed in the LIF null mothers would occur.

The pups derived in another study of targeted disruption of the LIFR gene resulted in live born that died during the first day following birth (13) and, although the gp130 null phenotype is expected to be more severe than the LIFR null phenotype, a Mendelian ratio for the gp130 mutation was seen on E11.5 (see chapter 13). In both these studies the mice were outcrossed from an ES cell 129 background directly from the chimeras. The mice in this study were not exposed to the influence of C57BL/6 until a colony of 129 heterozygotes was established. One possible explanation of our conflicting results is that the mice in this study had a stronger influence from the 129 background. This explanation is supported by the unsuccessful attempt in our hands to keep this mutation on a pure 129 background by LIFR+/- x +/- matings. The differences seen in genotype ratios between the LIFR and gp130 null mice may also indicate that there is a signal transducing molecule other than gp130 that can interact with LIFR. This may be particularly relevant in light of measureable serum levels of soluble LIFR (sLIFR) in the mouse (14). The LIF/sLIFR complex is thought to act as an antagonist of LIF-induced signaling through gp130. However, these data suggest that it may also have a signal transducing function that has not yet been identified.

**Disrupted Placental Architecture**

The LIFR mutant placentas displayed a number of deficiencies. Overall the LIFR null placentas appeared smaller than those associated with their littermates (Hunt, unpublished observation). On periodic acid-Schiff (PAS) staining, E13.5 and 15.5 placentas from LIFR-deficient embryos demonstrated architectural distortion...
Fig. 1. Distribution and morphological characteristics of NK cells in E13.5 metrial glands. (A) LIFR<sup>+/+</sup> fetus <> LIFR<sup>+/−</sup> mother. Large, vacuolated cells containing prominent intracellular granules (arrows) are randomly distributed through the metrial gland. (B) LIFR<sup>−/−</sup> fetus <> LIFR<sup>+/−</sup> mother. The distribution and morphological appearance of the NK cells in the metrial glands of LIFR deficient mice (arrows) were similar. Placentas were stained with periodic acid Schiff’s reagent. Original magnification, 400×.

and lacked organization into distinct spongiosotrophoblast and labyrinthine zones (12). The placentas were edematous and contained multiple large fluid-filled spaces. Accumulations of PAS+ material, possibly glycogen or proteoglycans, were scattered throughout the spongiosotrophoblast and labyrinthine zone. Unusual pale grey structures resembling multinucleated cells were present. These did not stain with PAS, had indistinct borders, and contained aggregates of cell nuclei. Some of these structures also contained apparently intact PAS+ cells that could have been either spongiosotrophoblast cells or maternal natural killer (NK)-like cells. Otherwise, natural killer-like cells (granulated metrial gland cells) normally resident in the decidua did not appear to be influenced by the LIFR mutation (Fig. 1). By E17.5, multinucleated structures and fluid filled spaces were uncommon, although the placentas remained noticeably disorganized. The labyrinthine region lacked the lacy appearance of a normal placenta due to thickening of the trophoblast or its underlying mesenchyme (12).

All of the implants gestated in the LIFR<sup>+/−</sup> mothers contained more fibrin than expected. Karyorrhexis was evident at the decidual/placenta interface, where bands of fibrin frequently contained polymorphonuclear leukocytes and nuclear fragments from degen-
erated cells. Because the LIFR-deficient mice did not successfully survive natural delivery, it is speculated that fibrin accumulation caused premature detachment from the mother. Then possibly, only LIFR\(^{-/-}\) fetuses that were first in birth order would be spared succumbing to hypoxia, which would account for the rare LIFR null survivor of natural birth. The few LIFR\(^{-/-}\) mice that were born alive following natural delivery did not thrive, and died shortly after birth (Ware and Willis, unpublished observation).

The presence of vast maternal blood spaces could be seen macroscopically by E17.5. On closer examination, the blood pools were associated with fibrin and margined leukocytes were detected. The blood pools often extended from the spongiotrophoblast down to the chorioallantoic plate (12). The placentas of mice and humans perform the same basic functions and contain the same cellular elements but there are important structural differences. Therefore, it is difficult to draw parallels between aberrations in mouse and human placentas. However, large maternal chorionic blood spaces are seen in placentas from mothers with metabolic diseases, such as diabetes, and have also been observed in severe pre-eclampsia (W. P. Faulk, personal communication).

Although fetal erythrocytes were present in all of the placentas, fetal vessels appeared less numerous and nucleated fetal cells were more difficult to locate in LIFR-deficient than normal placentas; whereas, maternal vessels were apparent (Fig. 2). Staining for \(\beta\)-galactosidase activity in placentas to determine LIFR expression patterns highlighted strong staining surrounding maternal vessels in the spongiotrophoblast (Fig. 3). Staining within the labyrinthine zone was not as pronounced. LIFR expression was not evident in the area surrounding the fetal vessels. The staining density in LIFR null spongiotrophoblasts was greater than twofold than that seen in LIFR\(^{+/+}\) placentas (data not shown), suggesting that differential staining density was not owing solely to a gene dose effect. Keeping in mind that the spongiotrophoblast and labyrinthine zones are of fetal origin, these data suggest a LIFR-mediated communication between fetus and mother to determine appropriate vessel interactions.

Disrupted maternofetal transport of radioactive immunoglobulin was described in the gp130 null mice (see Chapter 13). This is presumably caused by a defect in the yolk sac splanchno-
Fig. 2. Evaluation of maternal and fetal blood cells in E13.5 labyrinthine placentas. (A) LIFR⁺/⁺ fetus <> LIFR⁺⁻ mother. Maternal erythrocytes (small arrows) and nucleated fetal erythrocytes (large arrows) are abundant in the labyrinthine region, which has a normal lacy appearance. (B) LIFR⁻⁻ fetus <> LIFR⁺⁻ mother. Maternal erythrocytes (small arrows) are abundant whereas, fetal erythrocytes (large arrow) are uncommon in the labyrinthine region of LIFR deficient placentas. The trophoblastic layers are thickened and lack the normal lacy appearance. Placentas were stained with periodic acid Schiff’s reagent. Original magnification, 400×.

Fig. 3. Distribution of LIFR in E11.5 placentas. β-glactosidase expression patterns were analyzed by X-gal staining. (A) LIFR⁻⁻ fetus <> LIFR⁺⁻ mother. Trophoblast cells lining maternal blood spaces in the spongiotrophoblast zone (sz) are strongly stained and trophoblast cells in the labyrinthine zone (lz) are weakly stained. (B) LIFR⁺/⁺ fetus <> LIFR⁺⁻ mother. The placenta is not stained. Weak staining is present in the maternal (LIFR⁺⁻) decidua at the top of the frame. Original magnification, 100×.

pleure, the placental tissue that mediates maternal immunoglobulin transfer from the mother in rodents (for review, see ref. 15). It will be interesting to see if this defect is also associated with the LIFR mutation.
Table 2
Histomorphometric Analysis of Bone in Relation to LIFR Genotype

|                        | +/+ (n = 5) | +/- (n = 7) | --/-- (n = 9) |
|------------------------|------------|------------|--------------|
| Bone volume (%)        | 13.4 ± 0.9 | 15.2 ± 0.2 | 4.8 ± 0.6b   |
| Osteoid volume (%)     | 2.2 ± 0.7  | 2.5 ± 0.4  | 1.4 ± 0.4    |
| Osteoid surface (%)    | 3.5 ± 1.1  | 4.1 ± 0.5  | 2.6 ± 0.7    |
| Osteoid thickness (µm) | 1.7 ± 0.1  | 1.6 ± 0.1  | 1.5 ± 0.2    |
| Osteoclast surface (%) | 0.6 ± 0.3  | 0.7 ± 0.2  | 4.1 ± 0.6b   |
| Osteoclasts/mm²        | 0.3 ± 0.2  | 0.4 ± 0.1  | 1.8 ± 0.2b   |

aData are presented as mean ± standard errors.

bIndicates significance at \( p < 0.001 \) as determined by ANOVA.

**Decreased Bone Volume and Increased Osteoclast Numbers**

Alizarin red staining of E18.5 LIFR null fetuses to detect mineralized bone revealed reduced bone density at the proximal and distal ends of long bones and metacarpi and reduced density overall in skull, vertebrae, and ribs, relative to control littermates. Histomorphometric measurements were made on a fixed region just below the growth plate (primary spongiosa) in tibiae and femora of E17.5–19.5 fetuses (see ref. 12 and Table 2). Bones from wild-type and heterozygous littermates were normal for this stage of development and indistinguishable from one another. LIFR null skeletons exhibited a two-thirds reduction in bone volume in the areas investigated. The number of bone spicules in the primary spongiosa was reduced and there were few well-formed trabeculae resulting in architectural disruption (see ref. 12 and Fig. 4). There was a sixfold increase in osteoclast numbers and a sevenfold increase in osteoclast surfaces (see ref. 13 and Table 2). Numerous osteoclasts were observed attached to the bone spicules that remained in the primary spongiosa and at the interface with the mineralized cartilage (see ref. 12 and Fig. 4B). Curiously, chondroclasts, a cell type rarely detected, were frequently seen in association with the LIFR null primary spongiosa (Horowitz, unpublished observation; Fig. 4B).

Osteoblasts cannot be definitively identified at the fetal stages measured, but osteoid, the mineralizing substrate laid down by osteoblasts, can be measured. Osteoid volume was reduced by
Fig. 4. Disruption of architecture in the primary spongiosa of LIFR null mice. The primary spongiosa in E18.5 fetuses stained with toluidine blue. The hypertrophic region is immediately outside the field to the left. (A) LIFR+/+ fetus. Note the darkly staining trabeculae (arrows) and the absence of osteoclasts. (B) LIFR−/− fetus. Note the reduction in trabecular structures and the presence of chondroclasts (large arrows) and osteoclasts (small arrows). Magnification 240×.

~33% in LIFR null animals (see ref. 12 and Table 2). Although not statistically significant, this decrease would contribute to loss of bone mass. There was no observed difference attributable to LIFR genotype in osteoid thickness once bone formation had commenced. Cortical bone development appeared normal, although there was less mineralized bone radiating from midshaft to the epiphysis.

A dynamic balance exists between osteoclasts, the bone resorbing cells and osteoblasts, the bone forming cells, where osteoblasts regulate osteoclast development and action. Therefore, a
crosstalk mechanism is assumed to exist between the two cell types. Osteoblasts both secrete LIF and display LIF receptors while no direct LIF associated proteins have been identified in osteoclasts (16). LIF function is implied during bone development by its ability to potentiate or inhibit the expression of alkaline phosphatase and type I collagen synthesis (17) and by LIF-induced inhibition of bone resorption in E16.5 mouse metacarpal cultures, presumably by disruption of osteoclast migration from the periosteum into mineralized bone (18). IL-6 has been implicated as an inducer of osteoclast activity (19,20). It has been speculated that levels of IL-6 family members play a role in the regulation of other members of the family (7). It may be that absence of LIFR causes a disregulation of levels or function resulting in the observed increase in osteoclasts. Furthermore, deregulation may be mediated along a hormonal avenue (21). Bone defects have not been reported in relation to the LIF or CNTF mutant animals. Therefore, another ligand(s) such as CT-1 or OSM may be responsible in the maintenance of balance. OSM is an intriguing cytokine in this regard due to its ability to use either LIFR or an alternate receptor to mediate signaling. Biological consequences following signaling through the alternative receptor complexes (22) could be responsible for changes in bone balance throughout life.

**Reduction in Sensory Neuron Survival, Astrocyte Deficit, and Spinal Cord Architectural Disruption**

Because LIF acts as a survival factor for sensory neurons (23–25), an assay to determine the ability of sensory neurons derived from E19.5–20.5 dorsal root ganglia (DRG) to survive in vitro was explored. Neurons were cultured in medium containing serum alone or in the presence of IL-6, as a negative control for survival; nerve growth factor (NGF), as a positive control for survival; or CNTF, LIF, or OSM to determine survival relative to the status of LIFR-mediated signal transduction. Regardless of genotype, none of the cultures survived in medium with no cytokine supplementation or containing IL-6. All of the cultures survived in medium supplemented with NGF. The wild-type and heterozygous LIFR cultures were able to survive in the presence of CNTF, LIF, and OSM, whereas none of the LIFR null DRG cultures survived in the presence of the cytokines that require LIFR for signal transduction (12).
Hematoxylin and eosin stained sections of E17.5–18.5 fetuses did not reveal any overt central nervous system (CNS) disruption. On specific staining for glial fibrillary acidic protein (GFAP), the wild-type and LIFR\textsuperscript{+/-} animals displayed normal glial development, in which staining increased from E17.5–E18.5 and was comprised of strongest staining along the outer edge of the marginal layer with scattered staining toward the center of the mantle layer (see ref. 12 and Fig. 5A). However, the glial compartment was noticeably reduced the entire length of the spinal cord extending into the brainstem in LIFR null animals (see ref. 12 and Fig. 5B). The window of time investigated reflects the time of astrocyte emergence in the fetal CNS; therefore, the GFAP\textsuperscript{+} cells are presumably astrocytes.

No GFAP staining was detected on or before E15.5, regardless of genotype. The observation that astrocyte outgrowth was <1\% in LIFR null cultures when compared to littermate neuroepithelial outgrowths (Koblar, personal communication) is further evidence for disruption of the glial compartment in the absence of LIFR. The LIFR null reduction in the glial compartment was accompanied by architectural disruption, apparently caused by neuronal distress. The LIFR null spinal neurons contained cytoplasmic vacuoles and condensed pyknotic nuclei were scattered throughout the spinal cord. The processes appeared thickened and tangled, as highlighted by microtubule-associated protein (MAP-2) staining (see ref. 13 and Fig. 6). Astrocytes represent a substantial neural population; although their precise function remains unclear. A widely held theory on astrocyte function suggests that this glial compartment has a supportive role for neuronal survival. Normal neuronal morphogenesis and tissue architecture are seen when astrocytes do not make GFAP, indicating that GFAP is not the functional protein involved in these astrocyte effects (26). Consequently, it will be interesting to determine how much the neuronal defects are a cause of glial defects.

LIF and CNTF have common activities as neurotrophins owing to shared signal transduction component requirements (for review, see ref. 27). Functional disruption of the gene for CNTF results in motor neuron degeneration in adulthood (10) whereas no severe neuronal defect has been described following the disruption of LIF. Disruption of the CNTFR resulted in motor neuron deficits in the
Fig. 5. Astrocyte deficiency in LIFR null mice. Sagittal sections of E18.5 lumbar spinal cords were stained for GFAP. (A) LIFR\(^{+/+}\) fetus. Positively stained cells are indicated by arrows. (B) LIFR\(^{-/-}\) fetus. No positively stained cells are present. Magnification 290\(\times\).

newborn brainstem motor neuron nuclei and lumbar spinal cord (11). It is expected that LIFR dysfunction would have neuronal consequences at least as severe as CNTFR dysfunction. This has proven to be the case. Motor neuron deficit has also been described in the absence of LIFR, where counts detected fewer motor neurons
Fig. 6. Disruption of lumbar spinal cord architecture in LIFR null mice. Transverse sections of E18.5 lumbar spinal cords were stained for MAP-2. (A) LIFR\(^{+/+}\) fetus. The neuronal bodies are evenly stained and the processes form an evenly stained background. (B) LIFR\(^{-/-}\) fetus. A swollen neuron containing cytoplasmic vacuoles is indicated by the arrow. Neuron processes are thickened and tangled relative to those seen in the wild-type littermate. Magnification 290×.

in the facial motor nuclei and spinal cord, similar to the CNTFR null mice (13). A motor neuron deficit in the nucleus ambiguus was also determined in the LIFR null animals (13). The facial motor nuclei
and spinal cord defects resulted in an inability to suckle in the CNTFR and LIFR null mice and is the suspected cause of the perinatal lethality of both mutations, but an inability to right themselves was described only for LIFR null mice. The data described for glial deficit has not been noted in CNTFR null mice. Taken together these data suggest that there is a ligand(s) other than CNTF for the CNTFR of vital neurological significance. The more extreme phenotype of the LIFR null mice suggests that there is another ligand(s) that acts via LIFR regardless of CNTFR status. OSM and CT-1 are two candidate ligands.

**Elevated Late Gestation Fetal Liver Glycogen**

PAS staining of whole animal sections highlighted a dramatic increase in glycogen stored in the LIFR null fetal livers beginning on E17.5 (12). Wild-type and heterozygous littermates also began storing glycogen at this time, but at greatly reduced levels. The glycogen level remained elevated in LIFR null livers until either natural or Cesarian-assisted birth, at which point the glycogen levels dropped rapidly so that no glycogen could be detected an hour following birth, thus ruling out a glycogen storage dysfunction. Rapid glycogen removal from the newborn liver was also noted in LIFR+/- littermates. Staining of late gestation fetuses for pancreatic insulin and glucagon did not reveal any obvious differences between LIFR null and controls (Ware and Liggitt, unpublished observation).

LIF is an inhibitor of lipoprotein lipase (LPL; 28). LPL is a modifying enzyme that breaks down the fats in extracellular lipoproteins, which ultimately participate in the formation of fatty tissue. Elevated in vivo levels of LIF result in loss of body fat and cachexia (29). The leptin receptor (OB-R) is most closely related to gp130, LIFR and granulocyte colony-stimulating factor (G-CSF) receptor and is implicated in body weight homeostasis. Developing OB-R was isolated from an expression library made from murine choroid plexus (30). LIFR is expressed in the choroid imagination of E11.5 fetuses as detected by staining for β-galactosidase (Ware, unpublished observation). The glycogen storage alteration described above is evidence of metabolic function being mediated through the LIFR, which is of particular interest given the precedents of LIF-mediated LPL inhibition and OB-R biological function.
Hematopoiesis is Unaffected

The effects of the LIFR mutation on three aspects of hematopoiesis were explored:

1. The ability of the pluripotent fetal liver stem cells to engraft a host as determined by spleen colony forming units on d 14 (CFU-s14) following intravenous transfer;
2. The effect on erythroid maturation as evidence of in utero malnutrition and
3. The effect on fetal liver stromal ability to support hematopoiesis (13).

Engraftment of wild-type hosts by LIFR null fetal liver cells was equivalent to engraftment ability of the littermate fetal liver cells. There was no effect on the ability of the erythroid compartment to expand from these pluripotential progenitors as evidenced by normal numbers of erythroid burst forming units (BFU-e) following culture in methylcellulose. There was no statistically significant evidence that erythroid maturation was affected. Consequently, the placental disruptions seen in LIFR null animals did not overtly affect the ability of the mother to maintain nutritional balance in the fetus, as evidenced by normal erythroid maturation. Outgrowth of wild-type cells on LIFR null fetal liver stromal layers relative to wild-type stroma was unaffected as determined by measuring macrophage, granulocyte and B-cell production. However, the stromal support assay did reveal a sixfold increase in overall outgrowth of wild-type cells when LIFR null stromal cells were used as a feeder layer, relative to all other genotype combinations tested. The reason for this was not explored but may tie in to mutual inter-regulation of IL-6 family members (7).

There were profound hematopoietic progenitor deficits noted in the gp130 null animals (see Chapter 13). The pluripotent progenitors responsible for giving rise to CFU-s14 were reduced in gp130 null animals, with an intermediate effect noted in fetal liver cells derived from heterozygous littermates. Deficits were also noted in the in vitro outgrowth of granulocyte-macrophage (CFU-GM) cells and erythroid cells (BFU-e). Anemia was noted in some gp130 null mice and this appeared to be associated with a defect in erythroid maturation. The thymus was smaller in gp130 null animals (see Chapter 13), a defect not seen in LIFR null animals (McKenna, unpublished observation). Because these defects were not observed
in the LIFR null mice there must be another ligand(s) that functions through gp130 responsible for the effects seen on progenitor cells. OSM would be a candidate ligand because the use of an alternate receptor gives it the ability to signal in the absence of LIFR.

**Conclusions**

There are probably other systems that have been affected by the null mutation of LIFR. For example, when the fetuses were cleared, during the process of alizarin red staining for bone mineralization, the skin of LIFR null fetuses did not appear to be a barrier; thus, LIFR⁻/⁻ pups cleared noticeably faster and more thoroughly than their littermates (12). This suggests a defect in the LIFR null skin. There are no explanations to date concerning the cause or impact of fetal liver glycogen level alterations detected in the LIFR null animals. Preliminary data suggests that a cardiac defect does exist in LIFR null mice, which involves thinning of the right, but not left, ventricular wall (Liggitt, unpublished observation). All of the effects described are developmentally involved, but the ligands for LIFR are known to have an impact in the adult. Therefore, it is assumed that the longer the LIFR null pups live, the more the range of effects will be revealed. Indeed, protraction of gestation using progesterone allowed the studies on DRG sensory neuron survival described.

It is interesting to note the profound, multisystemic and lethal consequences of null mutations to receptor members of the IL-6 subfamily that utilize LIFR in relation to the effects seen with null mutations of the ligands. There are either many undiscovered ligands within this subfamily that together trigger the assortment of biological activities that require these receptors—or the activities of CT-1 and OSM, the two known ligands for which gene disruption data are not yet reported, may be far more crucial than suspected. It is gratifying to note that among these IL-6 subfamily mutations the order of phenotype severity follows the prediction, that is, gp130 null animals have a more severe phenotype than the LIFR null animals and the LIFR null phenotype is more severe than the CNTFR null phenotype. Although loss of gp130 and LIFR have multisystemic effects, CNTFR does not appear to have apparent nor unique effects outside the nervous system during development. Comparison of the pheno-
types of the CNTFR and CNTF null mutations is compelling evidence for at least one undiscovered ligand of CNTFR. The only candidate ligand to effect the observed severity of the gp130 null phenotype is OSM functioning through the alternate receptor, while both OSM and CT-1 are candidate ligands for the LIFR null phenotype.

It is surprising that there is so little redundancy in LIFR function. The presence of an alternate OSM receptor could be an evolutionary means of lessening the impact of LIFR. Because the signaling cascade mediated following OSM binding is both overlapping and different from LIF, the alternate OSM receptor is not directly equivalent to the LIFR (22). This suggests the relative importance of OSM as a ligand. The fact that four known ligands require LIFR either indirectly or directly to mediate signaling is also suggestive of essential LIFR function that has been modified in the past by ligand mutation and would very likely continue to change during future evolution. The null mutants generated within the IL-6 family are unraveling an intriguing story of interactions and coordination which is likely to become more complex and fascinating as new animal models are made.

**Summary**

The low-affinity receptor for leukemia inhibitory factor (LIFR) interacts with gp130 by heterodimerization to mediate signal transduction. The events following signal transduction are implicated in the biology of diverse systems. LIFR was disrupted by targeted mutagenesis. Fewer LIFR null animals than expected were present throughout gestation and LIFR null animals did not survive beyond the day of birth. A disruption of placental architecture including an altered interrelation between maternal and fetal vasculature was seen in the absence of LIFR. Fibrin accumulation in late gestation LIFR\(^{-/-}\) placentas may have led to death upon natural delivery. Bone volume in LIFR null animals was reduced threefold and osteoclast numbers were increased sixfold resulting in osteopenia of perinatal bone. The LIFR mutation resulted in a reduction of glial cells, disruption of architecture in the spinal cord and brainstem and a disruption of sensory neuron survival. Elevated glycogen levels were detected in the LIFR null late gestation fetal livers. No severe
defects were observed in the hematologic or primordial germ cell compartments.

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Chapter 19

Phenotypes of TGFβ Knockout Mice

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Introduction

The transforming growth factor-β (TGFβ) family consists of structurally related polypeptides that mediate important physiological processes. Significant advances have been made recently in understanding the functions of these molecules in vivo by the targeted ablation of various members of the super gene family. The family members include the TGFβs, activins, inhibins, bone morphogenetic proteins, Müllerian-inhibiting substance, nodal, dorsalin, and the products of the Drosophila decapentaplegic, and Xenopus Vg-1 genes (for review see refs. 1–4). Recently, the growth differentiation factor (GDF) family of proteins, a new member of the TGFβ family, has been described (5–7). TGFβ1 was first isolated from human platelets (8) and the cDNA sequence was later determined (9). TGFβ1 is a disulfide-linked homo-dimer of two 112 amino acid chains. Each chain is synthesized as the C-terminal domain of a 390 amino acid precursor. It has the characteristics of a secretory polypeptide, contains a hydrophobic signal sequence for translocation across the endoplasmic reticulum, and is glycosylated. The precursor cleavage site is a sequence of four basic amino acids immediately preceding the bioactive domain (9). TGFβ signals by binding directly to receptor type II, which is a constitutively active
kinase. Receptor type I recognizes receptor II-bound TGFβ, is recruited into the complex, and becomes phosphorylated by receptor II. Phosphorylation allows receptor I to propagate the signal to downstream substrates \((10)\). The type III receptor, a polymorphic membrane-anchored proteoglycan (betaglycan) binds with high affinity to TGFβ. Although the type III receptor is not directly involved in propagating the signal cascade, it probably modulates the presentation of the ligand, especially TGFβ2 \((11)\) to the type II receptor \((12)\).

TGFβs are believed to play an important regulatory role in a variety of developmental and homeostatic processes in several tissues. These include reproduction and development \((13–15)\), inflammation and immune function \((16,17)\), osteogenesis \((18–20)\), hematopoiesis \((21,22)\), angiogenesis \((23)\), tissue repair, and remodeling \((24,25)\). At a cellular level, TGFβ1 mediates these actions by controlling cell growth and differentiation \((4,26)\), cell adhesion, and the synthesis of extracellular matrix \((27)\).

To study the in vivo function of TGFβ1, we disrupted the TGFβ1 and TGFβ3 genes by homologous recombination \((28,29)\). In both cases, we used a targeting vector designed to disrupt the gene in exon 6, the first full exon encoding the mature TGFβ peptide, and to leave the prodomain intact \((28,29)\). Kulkarni et al. \((30)\) disrupted the open reading frame in exon 1 (prodomain region) of the TGFβ1 gene. Kaartinen et al. \((31)\) also disrupted exon 6 of the TGFβ3 gene. In animals with knockouts for the TGFβ1 and TGFβ3 genes, wild-type mRNA and mature protein expression were eliminated \((28–30)\). The phenotypes resulting from the two TGFβ1 knockout strategies are remarkably similar with minor differences. These differences are likely to be due to strain differences. In the following sections we discuss the phenotypes resulting from TGFβ1 and TGFβ3 gene knockouts.

**Phenotypes of TGFβ1 Null Mutant Mice**

Interbreeding of animals heterozygous for the TGFβ1 null mutation resulted in the birth of only about 50% of the expected homozygous null mutant pups. However, live born animals homozygous for the mutated TGFβ1 allele showed no gross developmental abnormalities. By about 20 d after birth they succumbed
Fig. 1. Inflammatory lesions in TGFβ1 deficient heart: Sections through hearts of mice of weaning age (around 3 wk). (A) Wild-type (+/+) heart, (B) TGFβ1 null (−/−) heart showing wide spread inflammation. m, myocardium. Arrow points to inflammatory cells invading the myocardium. Bar = 0.13 mm.

to a wasting syndrome accompanied by a multifocal, mixed inflammatory cell response, and tissue necrosis, leading to organ failure and death (28,30) (Fig. 1). Table 1 depicts the various phenotypes
Table 1
Investigational Studies Using TGFβ1 Knockout Mice

| Phenotype                                                                 | Refs. |
|--------------------------------------------------------------------------|-------|
| **Inflammation**                                                         |       |
| Increased leukocyte adherence                                            | 32    |
| Elevated MHC class I and II, and ICAM-1 levels                           | 33,34 |
| Elevated autoantibodies, cytokines                                       | 28,35 |
| Activated thymic lymphocytes.                                            | 36,37 |
| In vitro anergy of T-lymphocytes                                         | 36    |
| Rescue by anti-LFA-1 antibody therapy.                                   | 33    |
| Rescue by placing TGF-β1 null mutation on a SCID background              | 33    |
| **Development**                                                          | Unpublished |
| Embryonic lethality different phenotypes on different background strains |       |
| Impaired yolk sac hematopoiesis and angiogenesis                         | 38    |
| **Reproduction**                                                         | Unpublished |
| Impaired male and female fertility                                       |       |
| Impaired growth, failure to thrive, wasting                              | 28,30 |
| Impaired myocardial β-adrenergic responsiveness (cytokine effect)       | 39    |
| Increased leukocytes, platelets in peripheral blood                      | 28,40 |
| and increased megakaryocytes in bone marrow                             |       |
| Impaired wound healing after postnatal d 10 (cytokine effect)            | 41    |
| Peripheral nerve: normal proliferation and myelination                    | 42    |

seen in the null mutant mice. We discuss each of these phenotypes in detail.

**Inflammation**

TGFβ1 is produced by nearly all cell types of the immune system and is an important immunomodulatory molecule (reviewed in refs. 16,17,43–46). In vitro studies have shown TGFβ1 to possess both proinflammatory and anti-inflammatory actions. TGFβ has chemotactic activity for neutrophils (47), monocytes (48), mast cells (49), and T-lymphocytes (50). This chemotactic activity for leukocytes might be mediated in part by modulating expression of extracellular matrix molecules like fibronectin (51). TGFβ modulates integrin expression and increases monocyte-matrix adhesion (48). At picomolar concentrations, TGFβ increases resting mono-
cyte mRNA levels for interleukin-1 (IL-1) (48,52), TNF-α (53), TGF-α (52), and IL-6 (54), cytokines that can then further trigger an inflammatory cascade. TGFβ also induces FcγRIII (CD16) on newly recruited monocytes (55).

The anti-inflammatory actions of TGFβ1 consist of direct growth arrest of susceptible T-lymphocytes by inhibition of G1-S cell cycle transition, inhibition of lymphokine-mediated clonal T-cell expansion (56,57), and modulation of CD4+CD8+ lymphocyte production in the thymus (58). TGFβ inhibits hematopoietic precursor cell proliferation (21,22). It inhibits IL-1 receptor expression (59), and induces IL-1 receptor antagonist (60). TGFβ also inhibits B-lymphocyte proliferation and immunoglobulin production (61).

Given the regulation of many immune cells by TGFβ, it was not surprising that the TGFβ1 null mutants exhibited a multi-organ inflammatory disorder that ultimately led to organ failure and death. Inflammatory lesions were first observed at 5 d of age in the hearts and lungs (37). They became more prevalent at 7 d of age and involved the salivary gland, liver, and pancreas. At 10 d of age, lesions appeared in the diaphragm and mesentery. The infiltrate consisted primarily of lymphocytes and occasional neutrophils. By d 14, gastric lesions appeared, consisting of lymphocytic infiltrate primarily in the submucosa of the nonglandular region at the glandular/nonglandular border. Lesion severity increased with age. At weaning age (21 d), the inflammation was most severe in the heart, diaphragm, pancreas, stomach, mesentery, lungs, skeletal muscle, salivary glands, conjunctiva, and lachrymal glands. In contrast to the neonatal inflammatory lesions, the infiltrate in these moribund weanling mice was mixed, including lymphocytes, neutrophils, plasma cells, and fibrocytes.

Components of the cardiac inflammatory pathology seen in these mice were endocarditis, myocarditis and pericarditis (Fig. 1). Hypercellularity of the cardiac myocytes as evidenced by BrdU labeling was observed (33). Postinflammatory changes such as myocardial cell necrosis and marked fibrosis were seen in some animals. Ultrastructural studies revealed disrupted myofibrillar arrangement and swollen mitochondria. Changes consistent with congestive heart failure and cardiomyopathy were present and were a frequent cause of death (37). Pulmonary pathology consisted mainly of inflammatory perivascular cuffing, and at a later stage,
parenchymal interstitial inflammation. Pleomorphic periductal salivary adenitis was apparent. In the pancreas, diffuse lymphocytic inflammation and moderate duct hyperplasia with moderate edema separating the lobules was evident (37,62). In the liver, multifocal extramedullary myelopoiesis was seen in the mutant mice. This lesion then progressed to a lymphocytic infiltration with bile duct hyperplasia (37). Kidney and brain were relatively spared from the inflammatory process (37,62).

The lymph nodes were enlarged and hypercellular. However, there was reduced thymic size and depletion of thymic cortex cellularity (62). A preponderance of mature thymocytes (CD3hi, CD4+, CD8+ single-positive cells) and a relative depletion of the more primitive double-positive CD4+CD8+ cells were observed (36). Spleen was less affected and the splenic size corrected for body weight was similar to controls although increased number of actively dividing B-cells with increased immunoglobulin were seen. The bone marrow was histologically normal. Several cytokine levels were elevated in the mutant mice including interferon-γ, TNF-α, IL-1β, and macrophage inflammatory protein-1β (28). Serum from mutant mice also showed elevated antibody levels to dsDNA, ssDNA, and Sm ribonucleoprotein (35). Aberrant expression of major histocompatibility complex (MHC) class I and class II expression was observed in these mutant mice (34).

The overall inflammatory pathology was one of initial lymphocytic infiltration followed by other inflammatory cell types and elevation of cytokines in multiple organs. The primary involvement of lymphocytes, elevation of autoantibodies, and high immunoglobulin production by spleen and lymph nodes suggest an autoimmune mechanism for the inflammation. Indeed, aspects of the TGFβ1 null phenotypes are similar to Sjögren’s syndrome, graft-vs-host disease, polymyositis and systemic lupus erythematosis in humans (28,35). An understanding of the mechanisms underlying the inflammatory process requires examination of the early events before the establishment of inflammation. Also, since inflammation is widespread and severe, it is reasonable to assume that inflammatory cells and their cytokines may be the primary causative agent (combined with the absence of TGFβ1) underlying some of the pathological processes rather than the absence of TGFβ1 alone. In the following section, we review some of the studies that attempt to address these issues.
Mechanisms of Inflammation in the TGFβ1 Null Mutant Mouse

The TGFβ1 null inflammatory phenotype occurs in the absence of any detectable pathogen and therefore appears to involve autoimmune disease mechanisms (28,37). MHC class I and class II mRNA and protein expression in tissues of the TGFβ1 null mouse was elevated compared with controls at around 6 d of age. Consistent with this observation, downregulation of MHC class I expression by TGFβ1 was demonstrated in vitro in fibroblasts isolated from TGFβ1 null mice. These findings suggest that TGFβ1 regulates MHC expression that in turn could trigger the inflammatory cascade (34). However, the latter interpretation must be made with caution since:

1. Inflammatory changes were observed as early as 5 d of age in some null mutant hearts (37);
2. Elevated levels of MHC did not correlate with inflammation in some tissues such as the kidney (34); and
3. The MHC levels were similar to controls in the immunocompromised TGFβ1 null mice that were devoid of inflammatory lesions (33).

Monocytes isolated from spleen, thymus, and peripheral blood of symptomatic, 7-8-d old TGFβ1-deficient mice exhibited increased adhesion to extracellular matrix proteins and to endothelial cells in vitro compared with controls of the same age. Incubation of these monocytes with selected synthetic peptides corresponding to cell and heparin-binding sequences of fibronectin (FN) significantly attenuated adhesion of these cells not only to FN but also to endothelial cells in vitro. Furthermore, synthetic FN peptide treatment initiated on d 8 in the null mice not only blocked tissue infiltration but also moderated the lethal wasting syndrome (32). These observations implicate altered leukocyte-endothelial adhesion as a contributory mechanism for tissue inflammation. However, it has not yet been determined whether aberrant adhesion initiates inflammation.

One question regarding the TGFβ1 null phenotypes is whether myocardial hyperplasia is a primary result of the absence of TGFβ1 or is secondary to inflammation. Experiments from our laboratory suggest that myocardial hyperplasia is secondary to inflammation. Intercellular adhesion molecule-1 (ICAM-1) was elevated in the TGFβ1 null cardiac cells (33). It was therefore not surprising that
chronic treatment of neonatal TGFβ1 null mice with antibody against leukocyte function-associated antigen-1 (LFA-1), which is the αLβ2 integrin receptor for ICAM-1, reduced the inflammation and nearly tripled the longevity of these mice (33). Surprisingly, cardiac myocyte proliferation was also delayed as judged by BrdU labeling (33). Further confirmation of this observation came from experiments in which the TGFβ1 mutation was transferred to severe combined immunodeficiency disease (SCID) mice lacking T- and B-lymphocytes. These immunodeficient TGFβ1 null mice had approximately fivefold increased longevity, no inflammation, normal cardiac cell proliferation, and normal MHC levels (33). These results indicate that lymphocytes are essential for the inflammatory response and for cardiac myocyte proliferation in the mutant mice. It also suggests an important role for TGFβ1 in modulation of lymphocyte function, maintenance of self tolerance and initiation of the inflammatory cascade.

The role for TGFβ1 in regulating T-lymphocyte immune function is also supported by experiments demonstrating elevated levels of constitutive IL-2 mRNA in the thymus of TGFβ1 null mutants (36). Also both IL-2 and IL-2R mRNA were increased in the lymph nodes of these mice, consistent with a hyperproliferative response (36). In contrast, mitogen challenge of TGFβ1 deficient lymphoid cells in vitro revealed suppressed proliferation that was associated with a defect in inducible IL-2 mRNA expression and IL-2 secretion (36). Moreover, the addition of rIL-2 restored the deficient mitogen-induced proliferation. Although it is unclear whether this mechanism that leads to T-cell anergy is secondary to inflammation or results from the deficiency of TGFβ1, these data confirm the essential role for TGFβ1 in maintaining normal immune function (36).

**TGFβ and Development**

TGFβs may be important regulators of several developmental processes (for review, see refs. 13–15,63). TGFβs have been detected immunocytochemically in one-cell embryos, presumably translated from maternal mRNA (64). TGFβ expression was barely detectable in two-cell embryos, but intense expression was detected from four-cell embryo to blastocyst stage (64,65). Similar results have been observed using reverse transcriptase-polymerase chain reaction RT-PCR (66). Specific binding of radiolabeled TGFβ1 and
TGFβ2 was detected in the eight-cell stage embryo to the blastocyst stage embryo suggesting that these embryos could specifically respond to the TGFβ signal (64). In the postimplantation embryo, TGFβ is expressed in a variety of different cell types and appears to modulate a number of different developmental processes. The first appearance of high levels of TGFβ1 mRNA in the postimplantation embryo was detected at 7 d pc in the blood islands of the yolk sac and later in the liver. This is temporally correlated with the appearance of hematopoietic stem cells in the liver suggesting a role for TGFβ1 in the regulation of embryonic hematopoiesis (67). TGFβ1 expression was also seen prominently in areas undergoing vascularization and angiogenesis (67). In the lungs, TGFβ2 was expressed in the cuboidal epithelium and alveolar epithelium whereas TGFβ3 was expressed submucosally in the trachea and proximal bronchi (68). Enchondral bones such as the developing skull and mandibular areas express TGFβ1 (69). In the central nervous system of the embryo, TGFβ1 protein expression is mainly seen in the meninges whereas TGFβ2 and TGFβ3 are present in the differentiating neurons suggesting a possible role for these in neuronal differentiation (70).

In developing tissues containing epithelial and mesenchymal components, including whisker follicles, teeth, salivary glands and secondary palate, greater expression of TGFβ1 is observed in the epithelial cells (67,71–75). The presence of TGFβs in areas of epithelial–mesenchymal interaction make them ideal candidates for involvement in epithelial–mesenchymal transitions. Examples include atrio-ventricular cushion formation in the developing heart (76,77) and prostate morphogenesis (78).

Although mRNA and protein expression often overlap in the developing embryo, there are several instances where they are also distinct, suggesting an autocrine and paracrine mode of action (79). Numerous in vitro studies suggest that many processes critical to morphogenesis such as cell proliferation, cell migration, cell differentiation, and extracellular matrix production are controlled by TGFβ (for review, see refs. 1,46). In light of this, we expected the TGFβ1 null animals to have abnormal developmental phenotypes. Although nearly half of the mutants have no gross developmental defects, the other half die during embryogenesis. In the next section, we review data concerning the embryonic lethality.
Intercrossing of mice heterozygous for the targeted TGFβ1 mutation yielded less than the expected number of homozygous mutant pups. Of 806 live offsprings, 280 were homozygous wild-type, 413 were heterozygous, and 113 were homozygous mutants. If the wild-type pups are normalized to 1, the ratio of the three genotypes of pups is 1:1.5:0.4 that is a significant deviation from the expected mendelian ratio of 1:2:1. Crosses between wild-type and heterozygous animals of both sexes revealed no underrepresentation of heterozygote pups regardless of which sex contributed the knockout allele (unpublished observations). Therefore the reduced number of homozygote mutants is caused by a partially penetrant embryonic lethality. Considerable strain dependency exists in the penetrance of the partial embryonic lethality. The ratio of homozygous mutant pups born to heterozygous parents can vary from 1–23% (unpublished data). In the C57BL/6J x 129/Sv x NIH/Olac strain, about 50% of the embryos die at around 10.5 d pc. Primary defects were found in the yolk sac vasculature and hematopoietic precursors (38). This correlated with previous studies showing high levels of TGFβ1 expression in the yolk sac hemangioblast cells, the likely precursors of endothelial and hematopoietic cells (80). In vitro data has shown a predominantly inhibitory action of TGFβ1 on endothelial cells (81,82). TGFβ1 has also been shown to be inhibitory for hematopoietic cell growth, both in vitro (83–85) and in vivo (86–89). On the basis of these results, one would have expected the TGFβ1 deficient animals to have exuberant growth of endothelial and hematopoietic cells rather than the hematopoietic and vascular deficiency reported by Dickson et al. (38). It is noteworthy that the in vitro and in vivo observations of hematopoietic suppression by the addition of TGFβ1 do not necessarily translate into the opposite in vivo phenotype in the absence of TGFβ1.

We had originally suggested that maternal transfer of TGFβ1 transplacentally and later in the milk might rescue TGFβ1 deficient animals from embryonic and postnatal lethalities (28). It has since been shown that radiolabeled TGFβ1 injected maternally can be detected in several tissues of the TGFβ1 null pups (90). It is not known how much, if any, of the latent TGFβ1 can cross the placenta, although small amounts of materno-fetal hemorrhage and leakage of leukocytes and red blood cells can occur during pregnancy (91). Although the TGFβ1 null females rarely carry pregnan-
cies to term, three pregnancies have been reported \((33, 38, 90)\). The male in all cases was heterozygous for the targeted TGFβ1 allele. In the first two cases, the pregnant females were sick, presumably caused by an inflammatory pathology. In one case the embryos had to be delivered at 12 d pc by cesarean section \((38)\). In the other, the female had to be given dexamethasone to keep her alive through parturition \((90)\). In both instances, there were severe developmental defects in all TGFβ1 null pups but not in the heterozygous pups. The developmental defects consisted of poorly formed ventricular lumina, disorganized ventricular muscle and valves, delayed hepatic development, and neural tube defects \((38, 90)\). Based on these studies, the investigators suggested that maternal TGFβ1 rescued the mutant embryos from lethal developmental defects. Contrary to these observations, in one pregnancy involving a healthy immunodeficient (SCID) TGFβ1 null female, neither homozygous mutant nor heterozygote pups had any evidence of cardiac or gross congenital malformations when killed and analyzed at 18 d after birth \((33)\). This study suggested that maternal transfer of TGFβ1 is not necessary for normal embryonic and fetal development of TGFβ1 deficient mice. In this case the TGFβ1 null female was of a C3H × 129 × CF-1 strain on a SCID background. In the two experiments in which developmental defects were found, the null female mother belonged to another mixture of strains. There are several possible explanations for these seemingly contradictory results:

1. All three experiments were done on mice with different genetic background strains;
2. Maternal inflammatory cytokines could lead to teratogenic effects in embryos deficient in TGFβ1; and
3. Dexamethasone could lead to teratogenic effects in embryos deficient in TGFβ1.

**Reproduction and TGFβ**

Multiple potential roles of the TGFβs in regulation of reproductive function have been reviewed recently \((13, 92)\). As briefly summarized in this section, the spatial and temporal patterns of TGFβ expression in reproductive organs and the activities of TGFβ in cell culture systems using cells from reproductive tissues, suggest that the TGFβs are important regulators of ovarian, uterine, and testicular function.
Immunohistochemical, Northern, and RT-PCR analyses have revealed that mRNA transcripts and/or proteins corresponding to TGFβ1, TGFβ2, and TGFβ3 are present in mammalian ovary. The three isoforms exhibit differences in cell type-specific patterns of expression during various stages of the ovarian cycle (93–95). Functionally, TGFβ may affect both the proliferative and differentiative functions of ovarian follicle cells and may influence maturation of the oocyte. In in vitro studies, TGFβ has been shown to influence granulosa cell proliferation, to modulate steroid production by granulosa, thecal, and luteal cells; and to regulate expression of leutinizing hormone (LH) and epidermal growth factor (EGF) receptors on granulosa cells (96–110). In an in vitro study of oocyte maturation, TGFβ1 partially suppressed LH-induced maturation (germinal vesicle breakdown) of explanted follicle-enclosed rat oocytes, but had no effect on spontaneous maturation (111). In contrast, in another study, although TGFβ inhibited EGF-induced maturation of rat follicle-enclosed oocytes or cumulus-oocyte complexes, it stimulated the spontaneous maturation of these oocytes (112). The latter result is intriguing since TGFβ1 has been shown to inhibit the histone H1 kinase activity of p34cdc2 (113), an essential step in the progression of oocytes from prophase to metaphase of meiosis I (114,115). Patterns of TGFβ isoform expression in the ovary and in vitro modulation of ovarian cell function by TGFβ suggest that the TGFβs may function as autocrine/paracrine regulators of ovarian function in response to pituitary gonadotropins, particularly by influencing growth and development of the follicle, maturation of the oocyte–cumulus complex, and function of the corpus luteum (see references aforementioned).

The TGFβs may also play a role in uterine functions relating to implantation and placentation (116–121). During the preimplantation period, uterine TGFβ1 protein is synthesized primarily by the luminal and glandular epithelium and accumulates in the extracellular matrix of the stroma, peaking in intensity around the time of implantation (117). TGFβ2 is also detected in the uterine epithelia as well as in the myometrium and uterine vascular smooth muscle during this time period (119). After initiation of implantation, TGFβ1 protein appears to be synthesized primarily in the primary decidual zone and accumulate in the secondary decidual zone and decidua.
capsularis, whereas TGFβ2 is detected in the epithelia as well as in the decidua (117,119). During the entire peri-implantation period, TGFβ3 protein appears to be limited to the myometrium and vascular smooth muscle (119). TGFβ1 protein expression has been detected in the syncitiotrophoblast layer of the placenta (118), and may mediate trophoblastic invasion of the uterine epithelium, possibly by induction of tissue inhibitor of metalloproteinases (122). Thus expression of the TGFβs in the peri-implantation uterus and in the placenta along with the established roles of these molecules in the regulation of processes important for implantation such as control of cell proliferation, differentiation, cell migration, tissue remodeling, extracellular matrix formation, and angiogenesis, support a regulatory role for the TGFβs in implantation and placentation (117,118). Furthermore, TGFβs may promote maternal tolerance and prevent maternal rejection of the allogenic conceptus (116,123,124).

TGFβ may be an important regulator of testicular function (13,125,126). TGFβ mRNA and/or protein has been detected in the testis from the fetal period through adulthood. Cellular localization of the TGFβ isoforms reflects age-related changes during development (127,128). For example, TGFβ isoform mRNAs, particularly in Sertoli and peritubular cells, exhibit distinct temporal patterns of expression during prepubertal, midpubertal, and late pubertal periods of testicular development (128). In male germ cells, a unique TGFβ1 transcript (1.8 kb instead of the usual 2.4 kb form) is selectively and constitutively expressed throughout all spermatogenic stages examined (129). In in vitro studies, TGFβ can influence testicular cell function. TGFβ causes a reduction of hCG receptor expression on Leydig cells, modulates steroidogenesis in Leydig cells, and influences the migration and contractility of peritubular cells (130–134). Together, these studies suggest that TGFβ plays an important autocrine/paracrine role in the regulation of testicular function and differentiation during development (127–129,135).

Since the TGFβ1 null mutants died at around weaning age, the reproductive functions in the male and female null mutants could not be tested. As described, we have been successful in increasing their longevity by immunosuppression therapy with antibodies or by combining the TGFβ1 and SCID mutations (33). Although these immunosuppressed mice live well into reproductive age, males and
females are very poor breeders. Both the females and the males breed poorly, although exceptions clearly occur (unpublished observations). The cause(s) of poor reproductive performance remain unclear, but emphasize the importance of TGFβ1 in the regulation of reproductive functions.

**Growth and TGFβ1**

The birth weights of newborn TGFβ1 null animals are similar to those of their littermates until 7–10 d of age when the rate of weight gain in the mutants decreases significantly compared to that of their wild-type littermates. By weaning age (21 d) the mutants weigh only about 75% of controls (37,62). Given that inflammatory lesions can occur as early as 5 d after birth (37), this failure to thrive is not surprising. However, inflammation-free immunosuppressed TGFβ1 mutant SCID mice which live 5–10 times longer than their immunocompetent counterparts, also fail to thrive and by weaning age, their weights are only 75–85% that of their nonmutant littermates (33). The wasting syndrome is reminiscent of cachexia associated with chronic inflammation or malignancy. It is interesting that targeted disruption of inhibin, a member of the TGFβ superfamily, causes gonadal tumors and cancer-cachexia syndrome (136). Several hypotheses may explain the weight loss:

1. TNF-α and IL-1 are known to cause anorexia and cachexia (137,138) and these cytokines are elevated in immunocompetent TGFβ1 mutants (28). Since these cytokines are also produced by nonlymphocytic inflammatory cells, they may be elevated in the TGFβ1 mutant SCID animals.
2. Alternatively, TGFβ1 has been shown to have bone growth promoting activity by regulating osteoblast and osteoclast cell proliferation (for review see ref. 20).
3. Finally, TGFβ1 may affect cellular cholesterol trafficking by regulating the low density lipoprotein receptor-mediated cholesterol metabolism (139). To our knowledge, bone growth, endocrine function, or cellular metabolism in these animals has not yet been investigated.

**Hematopoietic System and TGFβ1**

A number of studies have demonstrated a predominantly growth inhibitory activity of TGFβ1 on hematopoietic cells and their precursors (21,22,83–89). In the immunocompetent TGFβ1 null animal, an
increase in peripheral blood leukocytes is observed (28). This increase becomes more pronounced when the animal is treated with anti-LFA-1 antibody, presumably the result of decreased leukocyte margination into tissues (33) (Table 2). However, in TGFβ1-deficient SCID animals that lack lymphocytes and tissue inflammation, leukocyte counts are normal (33) (Table 2). This suggests that the leukocytosis seen in the immunocompetent mutants does not directly result from a TGFβ1 deficiency, but rather from inflammatory lymphocytes that become activated in the absence of TGFβ1. Platelet and megakaryocyte counts are significantly increased in TGFβ1 null animals (peripheral platelets: 662,000 vs 152,000/mm³) (28,40), and this persists in the TGFβ1 null SCID animals, suggesting a potentially important role for TGFβ1 in the regulation of megakaryopoiesis. This phenotype is reminiscent of Kawasaki disease, an acute febrile illness in children characterized by lymphadenopathy, increased platelet counts, skin rash, conjunctivitis, and occasional coronary arteritis (140). In this disorder, cytokines such as TNFα, IL-6 and ICAM-1 are elevated (141,142). Again, the in vitro observations of suppression of hematopoiesis by TGFβ1 do not necessarily translate into an in vivo phenotype in the absence of TGFβ1.

Table 2

Hematological Profile of Anti-LFA-1 Antibody-Treated TGFβ1-Null Immunocompetent and TGFβ1-Null SCID Mice

|                      | Anti-LFA-1 antibody treated immunocompetent TGFβ1 null | SCID TGFβ1 null |
|----------------------|--------------------------------------------------------|----------------|
|                      | Control      | Mutant    | Control | Mutant |
| Total WBC           | 2089         | 4644b     | 2257    | 2114   |
| Lymphocytes         | 1481         | 3042b     | 586     | 495    |
| Total Neutrophils   | 525          | 1352c     | 1603    | 1566   |
| Immature Neutrophils| 44           | 230c      | 51      | 49     |
| Monocytes           | 72           | 251b      | 52      | 51     |

aData are presented as the mean absolute number of cells/mm³ of blood. Control refers to wild-type or heterozygous animals, whereas mutant refers to mice homozygous for the TGFβ1-null allele.

* Significant differences of \( p < 0.01 \) and \( p < 0.05 \) were determined by t-test of paired comparisons between age-, parent-, and, whenever possible, sex-matched mutant and control animals within anti-LFA-1 antibody (9 pairs) or SCID (7 pairs). WBC, white blood cell. Adapted from ref. 52 with permission.
Cardiac Physiology and TGFβ1

We have performed physiological analyses on myocardial tissue derived from TGFβ1 null mutants. Contractility measurements on cardiac muscle strips from immunocompetent TGFβ1-deficient animals revealed depressed contractility and diminished β-adrenergic responsiveness (39). However, in these animals, TNF-α and IL-1 levels are elevated, and these cytokines have been demonstrated to decrease myocardial responsiveness to β-adrenergic stimulation and to decrease myocardial contractility (143). Contractility measurements on TGFβ1 null SCID animals should determine whether the aberrant contractility in TGFβ1-deficient animals is caused directly by the absence of TGFβ1 or is secondary to inflammatory cytokines. Studies involving susceptibility of TGFβ1-deficient cardiac tissue to stress-induced damage should also be done on these animals since there is evidence that TGFβ1 mediates cardioprotection to cytokines such as TNF-α and IL-1 and to superoxide anions (144,145), as well as resistance to ischemia/reperfusion injury (146). Such experiments lend credence to the notion that in the adult a major role of TGFβ1 is to mediate tissue protection to stress response.

Wound Healing and TGFβ1

Since there is evidence that TGFβ1 is important for wound healing and tissue repair (24,25), we performed wound healing experiments on TGFβ1 null mice (41). Standardized full thickness wound incisions were created on the backs of mice, and serial wound measurements and wound closure was recorded over a period of time. The results revealed no significant differences in wound healing in animals 10 d of age or younger. In older mutant mice there was a significant decrease in wound epithelialization. However, this decrease was associated with increased expression of inflammatory cytokines such as TNF-α, IL-1β and IL-6 suggesting that the absence of TGFβ1 does not directly impair wound healing (41). Rather, it appears that the persistence of inflammatory cytokines in the TGFβ1 null mutant mice retards wound closure.

Peripheral Nerve Development

Several in vitro studies have demonstrated TGFβ to be mitogenic for neural-crest derived Schwann cells (147–150). TGFβ1 and
TGFβ2 are mitogenic for Schwann cells in the absence of neurons, but in the presence of neurons, TGFβ1 or antibody to TGFβ1 does not influence Schwann cell proliferation (42). The proliferation and myelination of neurite associated Schwann cells derived from TGFβ1 null embryos was indistinguishable from that of their wild-type-counterparts (42). These findings suggest that TGFβ does not function as a part of the mitogenic mechanism presented by neurons to Schwann cells. However, after peripheral nerve injury, TGFβ in the cellular environment might regulate Schwann cell proliferation and nerve regeneration.

**Strain Specificity in the Expression of TGFβ1 Null Phenotype**

A considerable strain-dependent variation exists in the TGFβ1 null phenotypes. In the 129 × CF-1 strain, significant inflammatory lesions were seen in the liver, stomach, and striated muscle, but not in the colon (28,37). In the 129 × C57BL/6J strain, TGFβ1 null mice have much less stomach, liver, and striated muscle inflammation when compared with 129 × CF-1 mutants (30,62). However the 129 × C57BL/J TGFβ1 null mice have colonic inflammation which is not seen in the 129 × CF-1 strain (28,30,37,62). Depending on the strain, TGFβ1 null pups comprise 1–23% of the liveborn pups derived from heterozygous matings (unpublished observations). Thus the embryonic lethal phenotype varies considerably in different strains. In the hybrid C57BL/6J × 129/Sv × NIH/Olac strain, about 50% of the embryos died at around 10.5 dpc owing to impaired yolk sac vasculature and decreased hematopoietic precursors in the yolk sac (38). Preliminary results from our laboratory indicate that all embryonic lethality in 129 × CF-1 mouse has already occurred before yolk sac development. Consequently, not only do TGFβ1 null phenotypes vary between backgrounds, but there are differences in penetrance between animals with similar genetic backgrounds suggesting the presence of important modifiers of TGFβ1 activity. Dramatic strain dependency in the mutant phenotype was also seen in the EGF-receptor and bone morphogenetic protein-4 (BMP-4) knockout mice (151–153). In EGF-receptor knockout mice, depending on the background strain, the cause of lethality was either a peri-implantation defect, placental defect, or postnatal death because of multiorgan failure (151).
Ablation of BMP-4, a member of TGFβ superfamily, causes variability in the type of embryonic lethality even within the same litter (153). These examples emphasize the importance of interpreting the phenotype of a knockout mouse in the context of the background strain, especially in the case of multifunctional proteins such as TGFβ.

**Phenotypes of TGFβ3 Deficient Mice**

TGFβ3 cDNAs were first cloned and sequenced from human and porcine tissue (154,155). Analysis of these cDNAs and genes indicated strong sequence similarity to TGFβ1 and TGFβ2 as well as similar intron–exon structure and secondary structure (154,155). This similarity suggested that TGFβ3 would also have similar functions. Like TGFβ1 and TGFβ2, TGFβ3 expression is detected in pre-implantation embryos by immunocytochemical methods (64). In the postimplantation embryo, high levels of TGFβ3 expression are detected in the intervertebral disc anlagen from embryonic d 11.5 until embryonic d 14.5 (156). TGFβ3 is predominantly expressed in the mesenchymal and epithelial component of developing tissues. Mesenchymal expression includes tissue surrounding the tooth bud, liver capsule, mesenchymally derived tissue of trachea and esophagus, mesenchyme surrounding umbilical arteries, and other large vessels and the fetal portion of the placenta (156). Epithelial expression is high in the medial edge of the secondary palate (72,75) and epithelial lining of major airways (156). In the palate, TGFβ3 expression is most intense prior to fusion of the palatal shelf, after which time it is barely detectable (72,75). As complex morphogenetic differentiation events take place, TGFβ isoform expression is seen in overlapping as well as differential patterns. For example, in the heart TGFβ3 expression is limited to mesenchymal condensations at the base of heart valves, whereas TGFβ2 expression is seen in the atrio-ventricular cushion and myocardium in the atrio-ventricular and outflow tract area (69). High expression of TGFβ2 is seen restricted to the basement epithelia of sense organs, however TGFβ3 expression is more diffuse in the cochlea of the d 13.5 embryo (79). In the central nervous system, TGFβ3 expression is most intense in areas of neuronal differentiation and not in the actively proliferating areas (157). Thus TGFβ3 expression is seen in an array of organs and tissues undergoing embryonic
morphogenesis. To study the specific function(s) of TGFβ3 during embryogenesis, we disrupted the TGFβ3 gene using homologous recombination (29).

Intercrossing of mice heterozygous for TGFβ3, yielded a 1:2:1 ratio of homozygous wild-type:heterozygous:homozygous offspring in three different genetic backgrounds indicating that no embryonic lethality existed. However, the TGFβ3 null pups began gasping, became cyanotic, could not productively suckle, became dehydrated, and died within 24 h of birth. All the TGFβ3 null pups had cleft palate without cleft lip (29,31) (Fig. 2). The extent of cleft palate varied with the genetic background strain. In the 129/Ola × CF-1 mice, about 90% of the TGFβ3 null pups had posterior cleft palate, 8% anterior cleft palate, and 2% complete cleft palate. In the 129 × C57BL/6 mutants, about half exhibited a complete cleft palate and the rest had only a posterior cleft palate (29). The mechanisms underlying the palate defect were interpreted to be poor adhesion after contact of the medial edge epithelia followed by a persistent epithelial seam (29). The latter is thought to result either from a continued deficiency in adhesion leading to an inability of the epithelial seam cells to accumulate at the nasal and oral epithelial boundaries (29), or from a transdifferentiation process by which the epithelial seam cells would transform to mesenchyme (31). Cranio-facial and other skeletal development was normal, ruling out a causative role for gross structural abnormalities. TGFβ3 mutant lungs exhibited an atelectetic pseudoglandular histology with alveolar hypoplasia, lacked alveolar septal formation and showed mesenchymal thickening and hypercellularity (31).

**Knockouts of Other Members of the TGFβ Superfamily**

Because of the intense interest in understanding developmental regulation by TGFβ superfamily genes, a number of these genes have been ablated by gene targeting. Table 3 describes the published knockout phenotypes of these genes. As is clear from Table 3, the phenotypes resulting from TGFβ superfamily gene knockouts are nonoverlapping. This does not rule out areas of functional compensation by other TGFβ superfamily members. Combined knockout strains will shed more light on possible functional redundancy within the TGFβ superfamily.
Fig. 2. Cleft palate in TGFβ3 deficient mice: Coronal sections through heads of the mid-posterior region of the secondary palate (embryonic d 15.5 mouse). (A) Wild-type (+/+ ) embryonic head with fused palate. (B) TGFβ3 null (+/−) embryo with a cleft palate. n, nasopharynx; p, palatal shelves; t, tongue. Arrow points to the cleft palate. Bar = 69 μm.

Clinical Usefulness of the TGFβ Knockout Mice

One of the goals of making knockout mice is to create animal models for human diseases. TGFβ1 null mice have already proved useful for various investigational studies (Table 1). We have been
| Gene                        | Phenotypes                                                                 | Refs.  |
|-----------------------------|-----------------------------------------------------------------------------|--------|
| TGFβ1                       | Wasting syndrome, multifocal multi-organ inflammation, growth failure, ~50% embryonic lethality, impaired intrauterine yolk sac hematopoiesis and vasculature | 28,30,38 |
| TGFβ3                       | Cleft palate and defective lung development leading to perinatal death       | 29,31  |
| Activin/                    | Eyelid fusion failure, Impaired female reproductive capability, Delayed labor and decreased nursing ability | 158    |
| inhibin βB                 |                                                                             |        |
| Activin/                    | Impaired craniofacial development, lack of whisker hair follicle, impaired teeth formation, cleft palate in some, death within 24 hr of birth | 159    |
| inhibin βA                 |                                                                             |        |
| Activin receptor II         | Combination of phenotypes of Activin βA and βB knockouts                   | 159    |
| Inhibin-α                   | Gonadal stromal and adrenal cortical tumors, cancer cachexia syndrome       | 136,160|
| BMP-4                       | Defective gastrulation and mesoderm formation, death between 6.5 and 9.5d pc | 153    |
| BMP-5                       | Short ear and multiple skeletal defects (short ear mouse)                    | 161    |
| BMP-7                       | Impaired kidney development, eye development and skeletal patterning        | 162,163|
| GDF-5                       | Alterations in the length and number of limb bones (Brachypodism mouse)     | 5      |

successful in increasing the longevity of TGFβ1 mutant mice, thereby making them potentially useful as model systems for many autoimmune diseases. They could also increase our understanding of the immunology of transplant rejection. TGFβ1 deficient mice could also be useful for designing novel approaches for anti-inflammatory therapy. Given the role of TGFβ1 in the regulation of cell-cycle, carcinogenesis can be evaluated in these animals by assessing the susceptibility to various genotoxic and mutagenic agents, even though these animals do not spontaneously develop frank tumors. It is estimated that a third of all cancer worldwide results from chronic infection (164), however the relative contributions of the infectious
agent versus the chronic inflammation in carcinogenesis is unclear. TGFβ1-deficient mice, which have inflammation without an infectious agent, will be useful in determining the role played by inflammatory cells and their cytokines in carcinogenesis. Various stress tests can be performed to evaluate the role of TGFβ1 in ischemia/reperfusion injury or in myocardial suppression secondary to cytokines. Studying the mechanisms of the cleft palate of TGFβ3 knockout mice may provide insights into human cleft palate that is one of the most common human congenital disorders. Were we able to rescue the TGFβ3 knockout animals from their developmental defects to generate adult animals, we could then study the roles of TGFβ3 in adult conditions such as wound healing. As more knockout mice are generated, the prospect of understanding homeostatic mechanisms and the bases of various human diseases is brighter than ever before.

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Chapter 20

The Transforming Growth Factor-β1 Knockout Mouse

The Phenotype and Its Implications for TGFβ1 Function

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Introduction

Transforming growth factor-β (TGFβ) represents the prototype in a large superfamily of secreted signaling molecules (1). Sharing several conserved structural features, these molecules act to control important events during growth and development, and in some instances function to maintain normal homeostasis in the adult. Each of the over 20 members of this extended family, which includes the activins, the inhibins, Mullerian inhibiting substance (MIS), and the decapentaplegic peptide of Drosophila (DPP), has been found to regulate events as critical as sexual development, pituitary hormone production, and bone and cartilage formation (2).

In mammals, the immediate family is composed of three isoforms (TGFβ1, -β2, and -β3), each encoded by a distinct gene, and expressed in unique patterns in vivo (3,4). Deriving their name from the first member of the family to be isolated (TGFβ1), these cytokines were initially defined by their ability to induce normal fibroblasts to form colonies in soft agar (5). We now appreciate that this activity is a manifestation of the effects of TGFβ on the production of matrix proteins and other cytokines (6,7), and understand
that TGFβ functions as a complex regulatory molecule whose activities are truly “context-dependent” (8).

This concept appears to govern the many functions of TGFβ during normal growth and development. Each TGFβ isoform plays a crucial role in morphogenesis (9), and participates in many important physiological functions, including wound healing (10), normal maturation and differentiation of intestinal epithelium (11), regulation of hematopoiesis (12), thymocyte progression and differentiation (13), and regulation of immunological homeostasis (14,15). More recent investigations have also revealed important tumor suppressor functions of TGFβ, including several studies that have associated the loss of detectible cell surface TGFβ receptor proteins with susceptibility for malignant transformation in both hematopoietic (16,17) and epithelial tissues (18). These events were shown to associate with a loss of sensitivity to the growth inhibitory effects of TGFβ in cell lines derived from these tissues. Additional studies suggest a role for signaling receptors and binding proteins in modulating TGFβ activity (19), substantiating its function as a critical regulator of cellular proliferation and differentiation, and lending significance to the many effects exerted by TGFβ on key cell cycle regulatory proteins (20).

The active form of mammalian TGFβs consists principally of disulfide-linked homodimers, derived by the cleavage of the 112 carboxy-terminal amino acids from a large, inactive precursor molecule (21). Though encoded by different genes, each isoform shares highly conserved structural features, with over 75% amino acid sequence identity in the mature processed protein (1). Each TGFβ family member is secreted as a latent complex, consisting of a non-covalently associated 75-kDa glycoprotein (known as the latency-associated protein [LAP]), and a covalently bound 135-kDa binding protein (22). Activation of this latent complex represents an important level of regulation of the activity of TGFβ, and may occur through a number of physiological processes in a very cell- and context-dependent fashion.

This high degree of structural similarity, along with the many overlapping activities observed in vitro, have led to the suggestion that the three isoforms may be functionally redundant in vivo (23). Although it remains plausible that each isoform may substitute for another in a specific context, the possibility of complete redundancy
is diminished by the presence of distinct genes encoding for each, and by the unique patterns of expression that exist in vivo. Moreover, whereas the expression of both TGFβs 2 and 3 are more developmentally and hormonally regulated, the type 1 isoform is selectively induced in response to a variety of signals, including the products of immediate early genes and several oncogenes (9). TGFβ1 is also unique in its ability to induce its own expression through a process of autoinduction (24), and the exclusive production of this isoform by leukocytes suggests it is of primary importance in the processes of hematopoiesis and immunoregulation. TGFβ1 appears to be the predominant isoform underlying numerous pathologies, including carcinogenesis, fibroproliferative, parasitic, and autoimmune diseases (25–27).

With the development of gene targeting strategies, truly isoform-specific activities may now be more clearly defined through models in which the expression of each individual isoform has been disrupted (28–31). Indeed, although the phenotype of the TGFβ3-deficient mouse has defined this isoform as a critical participant in the processes of palatogenesis and pulmonary maturation during development, the initial evaluations of TGFβ1-deficient mice have substantiated the importance of this isoform in the maintenance of immunological homeostasis (28,29,32,33). The profound immune dysregulation that develops in the absence of TGFβ1 gene expression is associated with apparent alterations in the control of normal hematopoiesis, and each may be linked to the loss of cell cycle controls normally exerted by TGFβ1. In fact, these models clearly show that the isoforms are not functionally redundant in vivo, and that selective loss of each isoform yields a severely abnormal phenotype.

This chapter focuses on specific histopathological and functional analyses of the TGFβ1 knockout mouse. We emphasize where these data support previously defined functions of TGFβ1, specifically those determined by studies performed prior to the availability of gene-targeting strategies. We highlight the isoform specific activities that can now be definitively assigned to TGFβ1, emphasizing those that may contribute to the maintenance of normal immune function and affect the development of an autoimmune-like process. We also discuss the results of more recent investigations which have utilized the TGFβ1-deficient mouse to more clearly delineate the tumor suppressor functions of this factor. We consider the mecha-
nisms of activity defined for TGFβ1, and discuss why this functional repertoire will expand to include endocrine as well as paracrine and autocrine functions. Finally, we evaluate the new insights that have been gained from this approach, and describe how this model can both further our understanding of TGFβ1 function, and direct our efforts in the development of TGFβ for future clinical applications.

**TGFβ1-Null Mice: The Phenotype and its Implications**

**TGFβ1 Deficiency Leads to Severe Immune Dysregulation**

Although TGFβ1 is the principal isoform produced by leukocytes, the three isoforms appear functionally interchangeable when added to many in vitro assays of leukocyte function. In vivo, TGFβ exerts profound suppressive effects on immune cell function, inhibiting the proliferation and differentiation of both B- and T-lymphocytes and altering the differentiated functions of all classes of mature leukocytes \((14,15)\). Specific examples include the ability to modulate the production and antagonize the effects of inflammatory cytokines, including tumor necrosis factorα (TNFα), interferonγ (IFNγ), interleukin-1 (IL-1), IL-2, and IL-6 \((34,35)\), to block the production of superoxide and nitric oxide by macrophages \((36,37)\), to alter patterns of expression of adhesion molecules (interfering with the adhesion of neutrophils and lymphocytes to vascular endothelium) \((38–40)\), to inhibit immunoglobulin secretion of mature B-cells \((41)\), and to suppress natural and lymphokine-activated killing by large granular lymphocytes \((42,43)\).

Given these observations, it is significant that the predominant phenotype of the TGFβ1 knockout mouse suggested the loss of a critical regulator of immune function. The phenotype is best characterized as an excessive inflammatory response, with a massive infiltration of leukocytes (principally lymphocytes and macrophages) in several organs: heart, lung, liver, salivary gland, pancreas, stomach, and intestine are uniformly involved, with occasional involvement of brain, kidney, and skeletal muscle. The syndrome develops rapidly, beginning during the first week of life and resulting in severe wasting and death by the fourth week of life (Fig. 1) \((28,29,32)\).

The typical pattern of tissue infiltration in TGFβ1-deficient mice is perivascular in nature. It appears to evolve from the earliest lesions, which are primarily defined by the increasing adhesion of
Fig. 1. Progressive immune dysregulation of the TGF-β1 knockout mouse. Disruption of TGFβ1 gene expression is associated with some form of embryonic lethality in at least 50% of the affected animals. Intrauterine demise occurs at approximately d 8 gestation, and is associated with abnormalities in yolk sac vasculature, and erythropoiesis. Those that survive to birth appear normal and exhibit patterns of immunostaining that suggest they have acquired TGFβ1 protein transplacentally from the mother. As they approach weaning, the animals exhibit increasingly elevated expression of both classes of histocompatibility antigens (class I and class II MHC), with associated leukocyte adhesion to vascular endothelium and progressive perivascular accumulation of lymphocytes, granulocytes, and macrophages. The inflammatory process involves multiple organs, and is associated with a wasting phenotype and subsequent death between 3 and 4 wk of age.
exposure (systemic v. local), and on the state of differentiation of the target cell \((15,44)\). The relevance of this activity has been demonstrated in a feline model of cardiac ischemia-reperfusion injury, where the iv administration of TGFβ1 results in significant suppression of the adhesion of polymorphonuclear leukocytes to vascular endothelium \((45)\). In this model, a single iv administration of TGFβ1 prior to reperfusion reduced neutrophil adhesion and leukocyte infiltration into ischemic myocardium, and reduced the area of cardiac necrosis by 60%. Similarly, prophylactic exposure to TGFβ1 prior to the administration of streptococcal cell wall (SCW) fragments prevents the development of joint swelling and distortion in a rat model of acute and chronic arthritis \((46)\). Evidence for increased leukocyte–endothelial cell interactions in the genesis of the TGFβ1 knockout phenotype is provided by studies in which the repeated administration of synthetic fibronectin peptides reduced tissue infiltration and damage, while prolonging survival of the animals \((47)\). Similarly, the administration of anti-LFA-1 (leukocyte function-associated antigen-1) antibodies was shown to double the longevity of TGFβ1 knockout mice, while reducing the severity of cardiac inflammation \((48)\). Although the exact mechanisms underlying these effects remain to be elucidated, the outcome of these experiments support previous conclusions regarding the relevance of TGFβ in the regulation of adhesion molecule expression and leukocyte–endothelial cell interactions.

It is important to note that although TGFβ1 itself may serve as a potent chemoattractant for monocytes, neutrophils, and lymphocytes during the earliest stages of an inflammatory response, the phenotype of the TGFβ1 knockout mouse intimates that the ability to control and promote the resolution of such a process may be its most critical function. This “dual role” of TGFβ1, functioning as a chemoattractant and initiating specific inflammatory cytokine production during the recruitment phase of the process while suppressing both the production of and the response to these mediators during the resolution phase, is a unique feature of this molecule \((39,49)\). Chronic systemic exposure to other proinflammatory cytokines, including IFNγ, IL-1, and TNFα, can lead to a progressive wasting syndrome with associated tissue inflammation, quite similar to the phenotype of the TGFβ1 knockout \((50)\). In vitro demonstrations of the inhibition of IFNγ and TNFα production by
TGFβ1 are corroborated by the appearance of elevated levels of mRNA for these cytokines within tissues of the TGFβ1 knockout (29). Although the events precipitating this inflammatory process remain unclear, the dysregulated production of inflammatory mediators may either sustain it or contribute to its progression.

**TGFβ1-Deficient Mice as a Model of Autoimmunity**

*TGF-β1 and Mechanisms of Immunologic Tolerance*

In addition to its ability to regulate the activation and function of mature immune cell populations, TGFβ1 is also a key participant in the development of the normal immune repertoire and clearly has an established role in the setting of autoimmune disease. In many autoimmune-based pathologies in humans, endogenous TGFβ1 appears to function as a natural suppressor of the inflammatory process, and behaves similarly in animal models of these conditions. This has been demonstrated quite clearly in experimental autoimmune encephalomyelitis ([EAE], the murine model of multiple sclerosis) (51), and in both SCW- and collagen-induced models of rheumatoid arthritis (CIA); in each instance, systemic administration of TGFβ1 can either delay the onset or reduce the severity of the disease process (46,52). Immunohistochemical studies have demonstrated an absence of TGFβ1 immunoreactivity in brain during the acute inflammatory phase of EAE, while substantial levels are detectable at the onset of disease remission (53), intimating its participation in the participation in the recovery phase. Such a role for endogenous TGFβ1 in these experimental models is suggested by studies in which systemic administration of TGFβ1 antibodies increased the severity (54). More recent investigations into the mechanisms underlying the induction of tolerance to specific target antigens provide support to the argument that endogenous TGFβ1 functions as a natural suppressor of autoimmunity. Specifically, T-cells secreting activated TGFβ1 mediate the establishment of tolerance in either EAE or CIA following the oral administration of the particular autoantigen (myelin basic protein and collagen respectively) (55,56). Finally, just as the administration of anti-TGFβ1 antibodies can heighten the disease course, this induction of peripheral tolerance is also reversed by a single systemic administration of blocking antibody (56).
In addition to the beneficial effects it exerts on an autoimmune-based inflammatory process, the elaboration of endogenous TGFβ1 as part of a host response may have other important, and potentially adverse consequences. This is perhaps most clearly demonstrated by the MRL/lpr mouse, a model of spontaneous autoimmunity resembling systemic lupus erythematosus (SLE) in humans. MRL/lpr mice have elevated circulating levels of active TGFβ, which may represent a natural host response to an ongoing inflammatory process, but more importantly are clearly responsible for their susceptibility to gram-negative and gram-positive bacterial infections (57). Reversed by the administration of anti-TGFβ1 antibody, this susceptibility results from acquired defects in neutrophil function. Thus, an accumulation of the active form of this cytokine will introduce additional risks to the host. Considering the data provided by studies in these experimental systems, one might conclude that the absence of TGFβ1, as in the TGFβ1 null mice, might lead to an autoimmune process. Phenotypic features consistent with this interpretation are the presence of SLE-like serum autoantibodies to nuclear antigens, immune complex deposits in renal glomeruli, and a progressive lymphocytic tissue infiltration similar to that present in human autoimmune syndromes such as Sjögrens disease (58–60).

The timing and onset of the TGFβ1-null phenotype within the neonatal period, and in the absence of any identifiable pathogens, leaves a number of unanswered questions regarding the nature of those events precipitating the inflammatory process. This period represents a critical time in the development of immune function in mice, during which the establishment of tolerance and the maturation of immune function takes place. For example, there are important distinctions between adult and neonatal lymphocytes, including the absence of major histocompatibility complex (MHC) class II expression during fetal and neonatal B-lymphopoiesis (61). Class II expression ordinarily is absent from B-cells in the neonatal spleen but present as early as the pre-B-cell stage in adult bone marrow. Other cell surface molecules, such as complement receptor, MHC class I, and CD45R(B220) are also absent, or present at lower levels (62). At a functional level, neonatal B-cells are also intrinsically more sensitive to tolerance induction than adult B-cells, in the absence of extensive T-cell help (63). Alterations in this developmental programming may interfere with normal tolerance-induction mechanisms, and
potentially contribute to an autoimmune process such as that observed in the TGFβ1 knockout.

Another characteristic distinguishing the murine neonatal immune system is the relatively poor antigen-trapping capability of the neonatal lymph nodes. Ordinarily, antigens draining into a rodent node are localized on the surface of macrophages and dendritic cells, a process that does not occur very effectively in a neonatal node (63). Consequently, the presentation of antigens to B- and T-cells may occur either directly, or in the context of nonprofessional antigen-presenting cells, having important consequences for these lymphocytes as they again encounter such epitopes. Altered functional responses have, in fact, been observed in splenocytes of TGFβ1 knockout mice, for whom an enhanced in vivo proliferation of lymphocytes is contrasted by defective mitogen-induced proliferation in vitro (64). This may also suggest that the normal cytokine regulatory network is perturbed in the absence of TGFβ1, particularly since this relative anergy has been linked to alterations in IL-2 production in response to mitogen.

A feature of the TGFβ1 knockout mouse phenotype that may also contribute to autoimmunity is the appearance of enhanced expression of antigens of the MHC class I and class II genes, first detected during the neonatal period, within several peripheral tissues (65).

Susceptibility to several autoimmune diseases has been linked to MHC class II genes, as in insulin-dependent diabetes mellitus (IDDM) and pemphigus vulgaris in humans, and in the non-obese diabetic (NOD) mouse model of IDDM (66-68). Furthermore, pathogenic autoreactivity may in some instances result from the inappropriate or aberrant expression of MHC class II antigens on cells where they are not ordinarily expressed (67). TGF-β is known to regulate the transcription of class II MHC II antigen through conserved S and X₁ proximal promoter elements (69), and has been shown to strongly inhibit MHC class II antigen expression in both lymphoid and non-lymphoid cells (70). This includes the ability to reverse IFNγ-induced MHC class II expression on human astrocytoma cells (71), and to coordinately inhibit the antigen-presenting capacity of astrocytes (72). The relief of this repression in the TGFβ1-deficient mouse specifically implicates the type I isoform in the regulation of MHC antigen expression in vivo.
The significance of the enhanced expression of both classes of MHC antigens seen in the TGFβ1 knockout mouse remains indeterminate, and it is not clear if this is sufficient to elicit an autoimmune-like process. Enhanced expression of MHC II antigens is a feature of other murine models of autoimmune disease, as in EAE where enhanced expression of MHC class II molecules is observed within the central nervous system (73). In this model, the ability of TGFβ1 to ameliorate or inhibit the central nervous system inflammatory process coincides with a reduction in the local expression of MHC class II molecules as well. It appears that the absence of endogenous TGFβ1 either directly or indirectly results in a loss of the regulation of MHC antigen expression. This may in turn permit the aberrant expression of self-antigens at inappropriate times, and by cells that ordinarily do not perform this function. Preliminary results obtained by crossing TGFβ1 knockout mice onto either an MHC class I-deficient (Yoshida and Kulkarni, unpublished) or MHC class II-deficient (73a) background seem to support this hypothesis, as in the absence of either class of MHC antigen, TGFβ1 deficiency neither results in significant autoantibody production, nor in immune complex deposition.

The concept that the TGFβ1-deficient phenotype results somehow from a failure to either establish or maintain normal immunologic tolerance can be extended further, to encompass other known regulatory functions of this cytokine. The term tolerance describes the process that provides the immune system with the ability to discern self from nonself, and to prevent pathologic reactivities against self-antigens. Clonal deletion of T-cells strongly reactive with self-antigens is a major mechanism contributing to immune tolerance (74), and is established centrally in a process known as negative selection, through which many of these T-cells undergo apoptosis as they encounter self-antigens expressed in the thymus (63). Negative selection ordinarily takes place at a stage of thymocyte development known as the CD4+CD8+ “double-positive” stage, requiring the participation of CD4 and the recognition of MHC class II molecules (75). Although these CD4+CD8+ thymocytes represent the cell type in which most repertoire selection events occur, the differentiation of their immediate precursors (the CD4-CD8lo cells) can also be aborted by T-cell receptor engagement; this provides a means for eliminating early thymocytes
Fig. 2. TGFβ1 controls thymocyte progression. Representing an important step in the establishment of immunologic tolerance, negative selection occurs at a distinct stage during thymocyte development. Through a series of steps defined by cell surface expression of CD4/CD8 coreceptor molecules, mature, “self-tolerant” T-cells arise, while those which are strongly reactive with self antigens (expressed on thymic epithelium) are deleted through mechanisms involving apoptosis. The CD4−CD8+ cells are the first to enter this pathway, and presumably represent the stage at which negative selection occurs. The progression of these CD4−CD8+ cells to the CD4+/CD8− “double-positive” stage is also inhibited by TGFβ; its absence may result in a more rapid production of these double positive thymocytes, potentially allowing more autoreactive T-cells to escape the selection process.

expressing autoreactive T-cell receptors specific for self antigens (76). This same stage along the CD4/CD8 developmental pathway in the thymus is also regulated by TGFβ (Fig. 2). Specifically, TGFβ can regulate cell cycle progression and differentiation of CD4−CD8+ precursor cells into the CD4+CD8+ double-positive thymocytes (13). TGFβ proteins are expressed on subcapsular and cortical thymic epithelium, where they can contact CD4−CD8+ precursor cells and actively regulate the rate at which CD4+CD8+ thymocytes are generated. Control of the rate at which CD4+CD8+ thymocytes are produced might be important for subsequent developmental events in the thymus, such as selection of the T-cell repertoire. It is possible that in the absence of TGFβ1, CD4+CD8+
thymocytes are generated too rapidly for autoreactive cells to be appropriately removed in the thymus.

Although these mechanisms of clonal deletion are important, it is now very clear that many autoreactive T-cells can pass through thymic selection, and exist in the peripheral compartment. Thus, in healthy individuals tolerance is maintained in part by mechanisms which control or suppress these autoreactive T-cells in the periphery (77). In a process generally referred to as clonal anergy, these autoreactive T-cells are kept in check by other T-cells, via production of a regulatory network of cytokines that also includes TGFβ1 (55). Direct evidence for the role of TGFβ in mediating T-cell suppression comes from studies of oral tolerization in the EAE model of multiple sclerosis. In these studies the oral administration of target antigen induces peripheral tolerance by generating a regulatory population of Th2-like cells producing TGFβ1 (55,56).

It is thus conceivable that an imbalance in this relationship between autoreactive and suppressor T-cells could also contribute to the autoimmune process in TGFβ1 knockout mice. An example of such an imbalance is provided by a murine model of inflammatory bowel disease created by reconstituting C.B.-17 severe combined immunodeficiency disease (SCID) mice (lacking all lymphocytes) with low numbers of a subset of CD4+ T-cells designated as CD45RBhi, and characterized by their production of inflammatory mediators such as IFNγ and TNFα (78). Isolated from normal Balb/c mice, these “TH1-like” CD4+ T-cells produce intestinal inflammation and a wasting phenotype in SCID mice, which is completely prevented by co-injection of the reciprocal population of CD45RBlo T-(TH2) cells. It is interesting that TGFβ has a profound effect on the in vitro development of antigen activated T-helper cells, leading to the development of a CD45RBlo phenotype (79). Thus, the absence of endogenous TGFβ1 may disturb this delicate balance between Th1 and Th2 cells, and may by analogy lead to the progressive inflammatory and autoimmune-like process seen in the TGFβ1-deficient mice. The importance of CD4+ T-cell subsets in the pathogenesis of this process is supported by ongoing studies in our laboratory involving the in vivo administration of anti-CD4 monoclonal antibodies. The resultant depletion of CD4+ T-cells delays the onset of the autoimmune process in TGFβ1 null mice, similar to the results of this approach in murine models of SLE.
In addition to its effects on T-cell development and function, TGFβ is also known to have profound effects on B-cell differentiation and activation. One aspect of the TGFβ1 knockout phenotype which suggests such a defect in B-cell regulation is the presence of high-affinity SLE-like autoantibodies in their sera (59). These autoantibodies, detected by enzyme-linked immunosorbent assay (ELISA) and Western blot, exhibited specificities for dsDNA, ssDNA, and Sm ribonucleoprotein, similar to those observed in other models of autoimmune disease, such as the MRL/lpr mouse. The autoantibodies are predominantly IgG, and their pathogenicity is suggested by the presence of Ig glomerular deposits within kidneys of the knockout mice. Although this autoantibody response may be triggered through classical antigen-driven mechanisms, it may also reflect inherent abnormalities of B-lymphocytes which lack autocrine production of TGFβ1. We consider the relevance of autocrine vs paracrine effects of TGFβ1 later in this chapter.

In general, the effects of TGFβ on B-lymphocytes are suppressive. This includes the ability to inhibit proliferation (80), differentiation, and Ig secretion (81), both in normal human B-cells, and in B-cells isolated from individuals with autoimmune disease (82). TGF-β interrupts the transition from the pre-B stage to the mature antigen-responsive B-cell, by interfering with the acquisition of κ light chain, and altering the κ/λ ratio (83). Furthermore, in mature B-cells TGFβ interferes with the surface expression of IgM, IgD, κ and λ light chains, inhibits secretion of IgG and IgM by activated B-lymphocytes, and suppresses IL-2-dependent B-cell proliferation (14,81). Indeed, the TGFβ1 knockout appears to exhibit enhanced proliferation within the B-cell containing lymphoid follicles, as assessed by the abundance of proliferating cell nuclear antigen (PCNA) and cyclin-dependent kinase (CDK) (p34cdc2) expression (64). Thus, it appears that the loss of endogenous TGFβ1 may disrupt the normal process of B-cell development, with inappropriate activation and failure to eliminate autoreactive B-cells as a consequence of their rapid, continuous cycling (59).

As evident in this discussion of the immune defects present in the TGFβ1 knockout mouse, the precise mechanisms and course of events leading to these abnormalities and the appropriateness of the analogy to autoimmune disease require further definition. What we
and others have learned and presently describe truly seem to substantiate many of the known or presumed functions of TGFβ1 in the regulation of immune function. By affecting so many cell types and influencing so many processes, it appears to serve as the “belt and suspenders” of immune modulation. Understanding these activities and harnessing their potential for clinical application, particularly in the setting of autoimmune disease, will be an important goal for the future of this field.

**TGFβ1: Beyond the Autocrine and Paracrine Pathways**

*TGFβ1 in Development: The Function of Maternal TGFβ1*

As presented in the introduction, TGFβ belongs to a large family of proteins known to be important for normal growth and development (9). Indeed, the embryonic expression patterns of TGF-β1 mRNA and protein suggest that this protein has a vital role during murine embryogenesis (3,84). Thus, the fact that newborns with complete disruption of both TGFβ1 alleles (TGFβ1−/− mice) were clearly indistinguishable from littermates either homozygous +/+ or heterozygous +/− for the normal allele was quite surprising. In general, such absence of a developmental phenotype in these types of gene targeting studies can be attributed either to a redundancy with other isoforms of the disrupted gene product, or to the expendable role of such a protein during development (23).

Although it remains possible that a functional redundancy exists at some level for the three mammalian isoforms of TGFβ, several observations suggest alternative explanations for the absence of a developmental phenotype in liveborn, TGFβ1-deficient mice. In particular, the initial observation that only 50% of TGFβ1−/− offspring survive to birth intimates the existence of some form of embryonic or prenatal lethality. In fact, studies of embryonic development demonstrated that 50% of TGFβ1−/− and 25% of TGFβ1+/− conceptuses die at around d 10.5 p.c. (85). These embryos exhibit developmental retardation, edema and necrosis, all of which appear secondary to extraembryonic lesions, including impaired differentiation of yolk sac vasculature and reduced erythropoiesis within the yolk sac (85). Thus, one must assume that a mechanism exists that allows for the survival and normal development of the remaining TGFβ1−/− offspring.
Fig. 3. Transplacental transfer of matenal TGFβ1. TGFβ1 immunoreactivity found in TGFβ1−/− embryos and fetal tissues suggested the acquisition of TGFβ1 protein from maternal sources. Staining patterns were nearly indistinguishable between the TGFβ1−/− offspring and either wild type or heterozygous littermates. The absence of such staining in TGFβ1-null pups when born to a TGFβ1−/− female supports the conclusion that TGF-β1 can function in an endocrine mode, clearly derived from the maternal circulation and supporting development in the fetus.

We have recently demonstrated that the mechanism underlying the survival and normal development of the remaining TGFβ1 offspring involves the transfer of maternal TGFβ1 protein to the fetus (86). TGFβ1 immunostaining of TGFβ1−/−, +/-, and ++ embryos reveals little difference if they are progeny of a TGFβ1+/- female, suggesting these TGFβ1−/− offspring have acquired the protein from an alternate source (Fig. 3). The capacity of maternal sources to contribute to this pattern of immunostaining was supported by our ability to recover intact labeled TGFβ1 protein from various fetal tissues following its administration to pregnant mice; it was confirmed by demonstrating the ability of fetal tissues to incorporate such maternally derived protein in tissue-specific patterns, identical to that seen by immunohistochemical analysis of TGFβ1 expression. Lactational transfer of TGFβ1 is also supported by similar studies in which labeled protein was recovered from various tissues following oral administration to neonatal mice (86). Finally, and perhaps most important, maternal leukocytes with the capacity to
produce TGFβ1 both have access to the fetus through the placental circulation (87), and transit the neonatal gastric mucosa during suckling (88), with the latter representing an important part of the mucosal immunity of the neonate. Each represents an important mechanism through which maternal compensation of disrupted gene expression in the fetus can take place, both during gestation and throughout the neonatal period.

The significance of maternal contributions of TGFβ1 to normal development was suggested by studies of progeny born to a TGFβ1−/− female, sustained by the administration of dexamethasone to ameliorate the autoimmune process. Surviving to birth, the TGFβ1−/− offspring present in this litter displayed grossly abnormal cardiac development and did not exhibit TGFβ1 immunostaining as seen in TGFβ1−/− offspring of heterozygous females (86). It is possible that the cardiac phenotype of these offspring reflects a cooperation between the effects of gestational exposure to dexamethasone and the absence of TGFβ1 gene expression. However, these studies clearly demonstrate that maternal TGF-β1 contributes to the normal pattern of immunostaining seen in the developing fetus, and to normal fetal and postpartum development. Moreover, the normal development of both TGFβ1−/− liveborn offspring of TGFβ1+/− females, and of TGFβ1+/− offspring of the TGFβ1−/− female, suggests that either endogenous or maternally acquired TGFβ1 can function to assure normal cardiogenesis.

Although the question of functional redundancy still exists, targeted disruption of the other TGFβ isoforms already has demonstrated that this will not be the case for every developmental function (30,31). A more detailed analysis of the relevance of maternal TGFβ1 to normal development can be performed through breeding studies that place the TGFβ1−/− genotype onto background strains carrying immune system defects. This approach interferes with the development of autoimmunity, allowing the animals to live well into breeding age, and includes backcross experiments with strains such as the SCID mouse (48), and the RAG1 or RAG2 knockout mice, as well as the crosses with nude mice and MHC class I and class II knockout mice that we have performed. Each of these approaches promises to provide insight, not only into the issue of maternal compensation of TGFβ1, but also into the basis of autoimmunity in the TGFβ1 knockout mouse.
Adding Endocrine to Autocrine and Paracrine Functions

TGFβ1 Knockout Mouse Defines New Modes of TGFβ1 Activity

The recent descriptions of circulating levels of TGFβ1 in plasma suggest that there may be important endocrine functions for this molecule, in addition to the well-known autocrine and paracrine activities (89). Levels of plasma TGFβ1 have been shown to be important prognostic indicators of fibrotic complications following bone marrow transplantation (90), to correlate with metastatic prostatic carcinoma (91), to serve as a marker of vascular disease (92), and to be elevated in retroviral disease (93). Such endocrine activities appear to be specific to the type 1 isoform, as there is no evidence to date for circulating TGFβ2 or β3.

The demonstration of maternal transfer of TGFβ1 protein from TGFβ1+/− mothers to TGFβ1−/− pups clearly defines an endocrine-like mode of action for this particular isoform. The molecular complex in which TGFβ1 crosses the placental circulation may include both active and latent forms, but this mode of transport has not been fully characterized and may even occur with TGFβ bound to specific serum proteins, particularly IgG (94). Whatever the predominant mode of transfer, the immunohistochemical staining patterns for TGFβ1 within TGFβ1−/− fetuses and newborns suggests that the targeting is highly tissue-specific. Moreover, the localization of maternally administered, radiolabeled TGFβ1 within fetal tissues closely resembles the patterns of endogenous TGFβ1 immunolocalization, raising the possibility that maternal protein may in fact contribute to the TGFβ1 staining normally found in these tissues.

In contrast to such specific endocrine roles of TGFβ1, studies in TGFβ1 null mice also suggest that certain paracrine activities of this isoform could be replaced by the actions of TGFβs 2 and 3. This possibility is probably best demonstrated by recent wound healing studies in the TGFβ1-deficient mouse. The potent activity of TGFβ in wound repair results from an ability to orchestrate a series of events, including the chemotaxis of macrophages, fibroblasts, and granulocytes to the site of tissue injury, the activation of fibroblasts to elaborate extracellular matrix and collagen, and the induction of other cytokines important to the wound-healing process. TGFβ1 is the only isoform of TGFβ found in α-granules
of platelets, and it had been postulated that the release of this factor from degranulating platelets was critical to initiation of a repair response. Moreover, the autoinduction of TGFβ1, which is mediated principally through AP-1 sites in the TGFβ1 promoter (24), is thought to be important for enhancement and extension of its activity throughout the course of the healing process. Indeed, the type 1 isoform represents greater than 85% of all TGFβ in adult wound fluid (95).

Based on these isoform-specific roles for TGFβ1 in tissue repair, it was predicted that healing would be significantly impaired in TGFβ1−/− mice. Surprisingly, initial healing rates for excisional wounds are indistinguishable in wild type and TGF β1−/− mice, with significant differences only appearing at later times, when the inflammatory and wasting phenotype of the knockout mice overwhelms the healing process (96). In addition, we have noted that immunohistochemical detection of TGFβ1 protein is nearly identical in incisional wounds and in implanted polyvinyl alcohol sponges removed from either wild-type or TGFβ1−/− mice wounded at 10 d of age (Roberts and Letterio unpublished). In contrast, immunostaining for TGFβ2 and β3 in TGFβ−/− mice appears to be somewhat more intense in the newly formed tissue encapsulating implanted sponges and wounds when compared to wounded control littermates. Based on our observations of maternal transfer, we postulate that maternally transferred TGFβ1 protein may substitute for some of the actions of TGFβ1 typically released from platelets, or synthesized by cells participating in the repair process.

To address this hypothesis more directly, several treatments have been utilized to suppress the inflammatory process and increase the life span of the TGFβ1−/− mice. Of these, the immune suppressant rapamycin extends their lifetime beyond 8 wk, with a corresponding inhibition of tissue inflammation. We have now used rapamycin-treated 30-d-old TGFβ1−/− mice and similarly treated wild-type littermates to reinvestigate the effects of TGFβ1 deletion on wound healing. Tissues of these TGFβ1−/− mice exhibit no staining for TGFβ1, suggesting that maternal protein has been depleted. Contrary to expectations, preliminary results indicate that healing is not impaired, and is even slightly enhanced in the TGFβ1−/− mice, with evidence for more mature granulation tissue and faster re-epithelialization (Roberts and
TGFβ Knockout Mice

Letterio unpublished). In addition, there are substantial differences in the relative staining patterns of TGFβ1 and TGFβ3 in the healing wounds: TGFβ1 staining is strong in the granulation tissue of wild-type wounds, but absent in wounds of the TGFβ1-deficient mice, whereas the opposite pattern is observed for TGFβ3 staining. These data support the outcome of previous studies which showed that the use of blocking antibodies to TGFβs 1 and 2, or the addition of TGFβ3 to incisional wounds in rats, significantly reduced the scarring as characterized by a more normal dermal architecture at the wound site (97).

Thus, since exogenous application of TGFβ1 has a paracrine effect on wound healing, and since TGFβ1 released from platelets also acts in a paracrine fashion, it is possible that enhanced synthesis and secretion of TGFβs 2 and 3 in the TGFβ1-deficient mice may somehow be able to compensate for the absence of paracrine TGF-β1 in a process such as wound healing. In contrast, the profound dysregulation of immune cells present in TGFβ1−/− mice is not reversed by systemically injected TGFβ1 (A. Geiser and J. Letterio, unpublished), suggesting that immune cells, and perhaps other bone marrow-derived cells, rely solely on autocrine, or possibly even intracrine TGFβ1. Such autocrine functions are therefore truly isoform-specific and nonredundant. This hypothesis is now supported by bone marrow transplantation studies in which the reconstitution of lethally irradiated wild-type mice with marrow from TGFβ1−/− donors leads to an autoimmune process, closely resembling that of the TGFβ1 knockout mouse (60). Indeed, this transfer of the phenotype occurs despite the presence of TGFβ1 produced by nonmarrow-derived cells of the wild-type recipient. Overall, these results require a re-evaluation of the role of TGFβ1 released from platelets, and of the endogenous expression of TGFβs 2 and 3 in wound healing. Further investigation of the differences in these modes of TGFβ action and their implications for this model are needed.

Carcinogenesis: Tumor Suppressor Functions of TGF-β1

TGFβ has an established role in controlling cell proliferation, interacting with key cell cycle regulatory proteins to produce G1 growth arrest. These interactions include transcriptional repression
of c-myc in early G1, inhibition of retinoblastoma (Rb) protein phosphorylation in late G1 (20), and induction of several tumor suppressor proteins, including the CDK inhibitors p15^INK4 (98) and p21^CIP1/WAF1 (99,100). TGFβ also inhibits the expression of CDK4 (101), causes mobilization of the CDK inhibitor p27 (102), and influences the expression of cyclins A and D, as reviewed elsewhere (20).

The significance of these effects on cell cycle and cell proliferation has been demonstrated in many experimental systems, suggesting that TGFβ exerts important tumor suppressor functions. Such a link between TGFβ signaling and tumor progression has been observed in studies of squamous cell carcinoma, including recent studies utilizing the TGFβ1 knockout mouse (103,104). In normal epidermis, expression of the type 2 isoform of TGFβ is confined to suprabasal strata, whereas TGFβ1 is expressed in the basal cell compartment and can be induced suprabasally following a single exposure to the tumor promoting agent phorbol 12-myristate 13-acetate (PMA) (98,100,105,106). Loss of TGFβ1 protein expression is one of the earliest changes of premalignant progression specifically associated with benign tumors or papillomas exhibiting high rates of malignant conversion, and the majority of high-risk papillomas are devoid of both TGFβ1 and TGFβ2 at their inception (48,103). This loss of expression of TGFβ in skin leads to a hyperproliferative state, which also associates with a significantly increased risk for malignant conversion. Indeed, TGFβ1-deficient mice exhibit a hyperproliferative basal cell layer, whereas their epidermis maintains wild-type levels of TGFβ2 expression and remains without suprabasal proliferation (48,103).

Keratinocyte cell lines derived from TGFβ1 knockout mice provided a unique opportunity to study the contribution of TGFβ1 to this process of tumor progression. Combinations of v-ras^Ha retrovirus-initiated keratinocytes and dermal fibroblasts from either TGFβ1-deficient mice or wild type control animals have been used to reconstitute papillomas in a skin graft model system of multistage carcinogenesis. In this system, grafts of v-ras^Ha initiated TGFβ1-deficient keratinocytes progressed rapidly to multifocal squamous cell carcinomas within dysplastic papillomas, whereas the initiated wild-type keratinocytes formed well-differentiated papillomas
Fig. 4. Autocrine TGFβ1 prevents progression to squamous cell carcinoma. Keratinocytes established from TGFβ1-deficient mice were compared to wild-type keratinocytes in a model of multistage carcinogenesis. In this system, keratinocytes are initiated with the v-ras<sup>Ha</sup> oncogene in vitro, and their in vivo tumorigenic properties are determined by skin grafting the keratinocytes onto athymic nude mice with either wild-type or TGFβ1-null dermal fibroblasts. Whereas the initiated TGFβ1-null keratinocytes progressed rapidly to multifocal squamous cell carcinomas within dysplastic papillomas, regardless of the genotype of the fibroblasts, the control keratinocytes formed only well-differentiated papillomas.

(104). The initiated TGFβ1-deficient keratinocytes formed carcinomas even when grafted in the presence of wild-type fibroblasts (Fig. 4). Moreover, although nontransformed TGFβ1-null keratinocytes can sequester TGFβ1 derived from paracrine sources, and maintain a normal growth inhibitory response to TGFβ1, squamous tumor cells cannot sequester TGFβ1 from either host or stroma, and their proliferation is in fact enhanced in the presence of wild-type, TGFβ1 producing fibroblasts. These results clearly demonstrate that autocrine TGFβ1 suppresses malignant progression, and that loss of autocrine expression in tumor cells leads to an accelerated progression to squamous cell carcinoma that cannot be suppressed by paracrine sources of TGFβ1.

These studies of TGFβ1-deficient keratinocytes have recently been extended to demonstrate a strong link between the loss of autocrine TGFβ1 and genomic instability (107). Clonal cell lines isolated from either TGFβ1-deficient –/– or heterozygote +/– primary newborn keratinocyte cultures were used to study the frequency
of drug-induced gene amplification (an assay of genomic stability) (107). TGFβ1−/− keratinocytes produced colonies resistant to the antimetabolite drug N-(phosphonacetyl)-L-aspartate (PALA) at frequencies ranging from 10−3 to 10−5, whereas the frequency of resistance in TGFβ1+/− cell lines was below 10−6. As the development of PALA resistance is dependent on amplification of the CAD gene, these results imply that loss of autocrine expression of TGFβ1 results in decreased genomic stability with consequent gene amplification. Whereas inactivation or loss of the p53 tumor suppressor gene product similarly leads to increased frequencies of drug-induced gene amplification, the increased frequency observed in the TGFβ1−/− keratinocytes occurs in the presence of a wild-type p53 gene. Finally, the addition of exogenous TGFβ1 to TGFβ1−/− keratinocyte cultures suppresses gene amplification at less than 1/100 the concentration required to induce G1 growth arrest, indicating that suppression of genomic instability occurs through a pathway independent of that mediating effects on cell proliferation.

Taken together, these studies demonstrate that disruption of the TGFβ signaling pathway contributes to the process of carcinogenesis, not just by providing a proliferative advantage for neoplastic cells, but also by substantially increasing genomic instability. Whereas disruption of TGFβ signaling in these studies results directly from the loss of autocrine expression of ligand, the ability of either autocrine or paracrine TGFβ1 to suppress genomic instability is itself dependent on functional TGFβ receptors. In fact, defects in the TGFβ type 2 receptor have been identified in a form of familial colon carcinoma, where DNA repair defects (microsatellite instability) lead to mutational inactivation of this receptor, and subsequent loss of the growth inhibitory response to TGFβ (18). Mutational inactivation of this receptor has also been observed in a high percentage of gastric cancer cell lines (108), and may be responsible for the loss of cell surface receptors observed in human T-cell malignancies (17). It is likely that disruption of TGFβ signaling resulting from postreceptor defects, from alterations of TGFβ binding proteins, or from impaired activation of latent forms of ligand will also contribute to tumor progression and increased genomic instability. The TGFβ1-deficient mouse not only has provided a valuable system to evaluate the role of TGFβ1 in these
processes, but has also provided a unique mechanism to distinguish between the roles of autocrine and paracrine TGFβ1 in the process of carcinogenesis.

Conclusions

The establishment of model systems in which the expression of each of the individual TGFβ isoforms has been disrupted provides a tremendous opportunity to more clearly define the activities that are specific to each, and to determine those for which expression of any of the isoforms may be sufficient. In this overview of studies in the TGFβ1-deficient mouse, we have considered many aspects of the phenotype of these animals and how they relate to known or presumed functions for this molecule. Overall, this approach has substantiated the importance of the type 1 isoform of TGFβ, both in the development and regulation of immune function, and in the maintenance of immunological homeostasis. At the same time, the initial studies detailed here clearly have also raised questions regarding many previously held assumptions about the critical role of TGFβ1 in the normal wound healing process. Similarly, the predicted functions of TGFβ1 during development, mainly based on appreciated patterns of embryonic gene expression, require re-evaluation in light of the disparate development of TGFβ1−/− offspring and the potential for maternal compensation of disrupted gene expression in the fetus.

Finally, data collected from preclinical studies conducted over the past decade have defined a tremendous array of activities as orchestrated by members of the TGFβ family. Our ability to determine the mechanisms mediating these activities through in vitro studies of function has been limited both by the nature of these systems, and by the often overlapping isoform activities observed in them. The use of experimental models developed around the TGFβ1 knockout mouse has helped to distinguish some important paracrine and autocrine functions of TGFβ1, particularly in the processes of carcinogenesis and tumor suppression, wound healing, and in the regulation of leukocyte function. They have also allowed for the addition of endocrine activities to the ever growing list of TGFβ1 functions. Expanding these concepts through ongoing studies in each of the three TGFβ isoform knockouts will help to clarify the
physiologic and pathologic activities of TGFβ, and guide the development of these molecules for future clinical application.

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Chapter 21

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)-Deficient Mice

Ashley R. Dunn and Graham J. Lieschke

Introduction

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a secreted glycoprotein hemopoietic growth factor (124 amino acids in mouse and 127 amino acids in human) that in vitro stimulates the survival, proliferation, and function of myeloid cells (For review, see ref. 1). GM-CSF has a broad range of actions on target cells that include neutrophils, eosinophils, macrophages, erythroid progenitors, megakaryocyte progenitors, and antigen-presenting dendritic cells (For review, see ref. 2). The potency of GM-CSF in vivo has been demonstrated by generating GM-CSF transgenic mice (3) and by reconstituting mice with marrow cells previously infected with a recombinant retrovirus expressing GM-CSF (4). The hemopoietic system of mice exposed to excess levels of GM-CSF in these ways is profoundly affected with increases in both progenitor cells and granulocytes and macrophages. These animal models suggest that GM-CSF excess results in a myeloproliferative syndrome, but this does not lead to leukemia. However, preleukemic growth factor-dependent clones infected with recombinant retrovirus encoding GM-CSF are able to proliferate in the absence of exogenous...
GM-CSF and are uniformly leukemogenic in the syngeneic mouse (5). To establish the normal physiological role of GM-CSF we (6) and others (7) generated GM-CSF deficient mice by gene targeting in embryonic stem cells.

**Structure of Disrupted GM-CSF Alleles**

The murine GM-CSF gene is located on chromosome 11A5-B1 (8) and is tightly linked to IL-3 (8) from which it is separated by 15 kbp. The GM-CSF gene is contained within about 2.5 kbp, comprises four exons, and encodes a single transcript of 780 bp (9,10). In our own laboratory, a targeting vector was constructed by replacing exons 1 and 2 of the murine GM-CSF gene by the *Escherichia coli lacZ* gene, the human β-globin gene 3′-untranslated and poly(A) addition sequences and the phosphoglycerate kinase (PGK)-neo expression cassette (6). Inactivation of the disrupted GM-CSF allele(s) was monitored by polymerase chain reaction (PCR) and confirmed by Southern hybridization analysis. Inactivation of the GM-CSF gene was demonstrated by the lack of GM-CSF immunoreactivity and bioactivity in conditioned media prepared from concanavalin A-stimulated splenocytes from mice homozygous for the mutated allele. In a parallel study, Dranoff and colleagues (7) constructed a murine GM-CSF targeting vector that by homologous recombination deleted exons 3 and 4 as well as 3′-untranslated sequences of the GM-CSF gene. In this latter study, homologous recombination between the targeting vector and the GM-CSF gene was confirmed by Southern hybridization analysis. As expected, supernatant of concanavalin A-stimulated splenocytes from homozygous mutant mice lacked bioactive GM-CSF (7).

**Phenotype of GM-CSF Deficient Mice**

The phenotype of the GM-CSF deficient mice produced by Stanley and colleagues (6) and Dranoff and colleagues (7) is essentially identical. GM-CSF mice develop normally and appear healthy at birth. Adult GM-CSF deficient mice are fertile and can be inbred; litters resulting from matings between mice homozygous for the GM-CSF disruption are of normal size; pups develop normally and appear superficially healthy.
Hemopoiesis

The peripheral blood of 6–7-wk-old GM-CSF-deficient mice is not significantly different from that of wild-type mice. There is no consistent quantitative or qualitative difference in bone marrow progenitor cell numbers, although there is a three- to sixfold increase in the frequency of splenic progenitor cells and an absolute increase in numbers of splenic progenitor cells.

GM-CSF-deficient mice tend to have a greater variation in the numbers of neutrophils, but this probably reflects variation in the pulmonary pathology that characterizes GM-CSF deficiency (see below). GM-CSF-deficient mice have normal numbers of splenic dendritic cells (7) (Shortman and Lieschke, unpublished observations) that is particularly interesting since GM-CSF is a mandatory requirement for the development of dendritic cells from bone marrow cells in vitro (11). Taken together, these data suggest that GM-CSF is not required for the regulation of steady-state hemopoiesis. It is important to emphasise that this conclusion does not categorically rule out a role for GM-CSF in maintaining steady-state hemopoiesis; rather it may mean that other factors are capable of fully compensating for GM-CSF in its absence. It is also possible that GM-CSF plays an indispensable role in some aspect of an hemopoietic emergency. This latter notion can be tested by gauging the response of GM-CSF-deficient mice raised in a germ-free environment to a range of specific mouse pathogens; experiments of this type are currently underway in our laboratory.

Pulmonary Pathology

GM-CSF-deficient mice raised in a conventional animal facility show striking abnormalities of the lungs. There appear to be two distinct pathologies although it is likely that each is a manifestation of a common underlying defect.

Pulmonary Alveolar Proteinosis

Histological sections of the lungs of older GM-CSF-deficient mice often show a cobble-stoned appearance owing to aggregates of lipoproteinaceous material (Fig. 1). The alveoli of GM-CSF-deficient mice invariably contain granular eosinophilic periodic
Fig. 1. Alveolar proteinosis in GM-CSF-deficient mouse lung. (A) Cobble-stoned appearance typical of established alveolar proteinosis owing to aggregates of eosinophilic intra-alveolar lipoproteinaceous material (black arrows). (B) Lipoproteinaceous aggregates at higher magnification indicating highly refractile lipoid crystalline deposits within them (black arrows). (Sections from apparently healthy 24-wk-old GM-CSF-deficient mouse.)
GM-CSF Deficient Mice

acid/Schiff (PAS)-positive, diastase-resistant proteinaceous material (Fig. 2). Numerous type C lamellar bodies with their characteristic onion skin-like appearance are present within alveoli and alveolar macrophages (Fig. 2) (6,7). Electron microscopic analysis reveals the presence of type II surfactant producing alveolar cells containing characteristic intracytoplasmic lamellar bodies (6). A marked increase (approximately eightfold) in total protein content is found in broncho-alveolar lavage material from GM-CSF knock-out mice and this corresponds to elevated levels of surfactant proteins SP-A, -B, and -C (7); the phospholipid composition of alveolar material is the same in GM-CSF-deficient and control mice from the same genetic background (12). S1 nuclease protection studies indicate that the increase in surfactant protein is not a result of increased levels of the corresponding mRNAs (7) and thus, the abnormal accumulations of surfactant material appears to be related to impaired clearance, probably as a result of a defect in alveolar macrophage function.

**Human Alveolar Proteinosis**

The distribution of lipo-proteinaceous material, and the overall lung histology that characterises GM-CSF deficient mice is highly reminiscent of human alveolar proteinosis, a heterogeneous groups of congenital and acquired lung disorders characterized by the accumulation of surfactant protein and lipid within alveoli, and often complicated by infection (13). Patients with alveolar proteinosis have dyspnoea, malaise, hypoxaemia, and a restrictive lung defect. The pathological similarity between the lungs of GM-CSF-deficient mice and those of patients with certain forms of human alveolar proteinosis raises the possibility that there exists a common genetic and/or molecular basis. In its simplest form, it is possible that some forms of human alveolar proteinosis might result from either an absolute deficit, or diminished levels, of GM-CSF; this in turn might reflect a null mutation, or an expression impairment mutation, in the GM-CSF gene. Although some types of alveolar proteinosis have been reported in association with perturbed hemopoiesis (14,15), GM-CSF-deficient mice have normal hemopoiesis and therefore there would be no *a priori* reason to anticipate that such a mutation would necessarily result in disregulated steady-state hemopoiesis in individuals with alveolar
Fig. 2. Pulmonary pathology in GM-CSF-deficient mice. Comparison of panels A and B shows a typical mononuclear cell infiltrate in the peripheral bronchovascular angle of a GM-CSF-deficient mouse. Comparison of panels C and D shows that the alveolar space (alv) of GM-CSF deficient mice (D) contains excess lipoproteinaceous granular material (grey arrow) and numerous intra-alveolar macrophages (dark arrows). (E) An electron micrograph of an
proteinosis. Of course a mutation in either a component of the GM-CSF receptor or a downstream signaling molecule could result in the same clinical syndrome; this would have therapeutic implications since an absolute or relative shortfall of GM-CSF could potentially be overcome through therapeutic administration of GM-CSF, although a mutation in a signaling molecule would likely not be overcome through the provision of excess ligand. As a model of the potential utility of GM-CSF for the treatment of alveolar proteinosis, it would be interesting to assess if prophylactic GM-CSF administration to newborn mice prevents or ameliorates the severity of alveolar proteinosis and whether therapeutic GM-CSF administration can reverse the pathological changes of established alveolar proteinosis.

Probably because alveolar proteinosis is an uncommon condition, no reports of mutational analysis of either GM-CSF or GM-CSF receptor components have been published, although studies of this type are underway in our own and other laboratories.

**Pulmonary Infection**

Aggregates of mononuclear cells are typically present around intralobular vessels (Fig. 2B), but this mononuclear cell infiltrate does not extend into alveolar septa. Some mice have particularly extensive lymphoid infiltration around hilar vessels which occasionally assume a follicular organization. Between younger mice, there is variation in the degree of lymphoid infiltration. In older mice, the lymphoid infiltrate seems generally more extensive. Immunostaining of lungs from 12–16-wk-old GM-CSF-deficient mice, using a variety of cell type-specific markers, shows the mononuclear cells to be mostly B-lymphocytes with smaller number of T-lymphocytes (6). Subclinical bronchopneumonia is

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intra-alveolar pulmonary macrophage from a GM-CSF-deficient mouse lung. The alveolar space contains fibrillary debris (short black arrows), and several type C lamellar bodies (long grey arrow). The macrophage cytoplasm contains phagocytosed type C lamellar bodies (long black arrow). bv, blood vessel; br, bronchiole; alv, alveolar space. Bar in panel E indicate 1 μm. (Sections from: 10-wk-old wild-type mouse [A and C]; 11-wk-old GM-CSF-deficient mouse [B and D]; 24-wk-old GM-CSF-deficient mouse [E].)
prevalent, and these focal inflammatory areas contain numerous neutrophils and macrophages, indicating that despite the absence of GM-CSF, these mice can mount an acute inflammatory cellular response. Rarely, subclinical pulmonary abscesses are found indicating that GM-CSF is not mandatory for mounting a chronic inflammatory response, or for localizing and containing infection. A histological survey of lungs from GM-CSF-deficient mice for bacterial and fungal pathogens reveals micro-organisms in only a minority of cases; these included Gram positive and Gram negative bacteria and fungal elements that stained with PAS and the Grocott methenamine silver stains (6). Although lobar pneumonia is unusual in GM-CSF-deficient mice, mice have occasionally died with lobar consolidation; from one such case, microbiological cultures resulted in growth of the murine pathogen Pasteurella pneumotropica (6).

Lymphoid and phagocyte infiltration characterize the normal murine response to some acute pulmonary infections (16), and interestingly, the lymphoid infiltrate of GM-CSF-deficient lungs resembles that seen in Pneumocystis carinii-infected lungs of immunocompromised mice (17). The simultaneous presence of a lymphoid infiltrate along with polymorphonuclear and mononuclear phagocytic cells in the lungs of GM-CSF-deficient mice indicates that the capacity to mount a range of cellular responses to infection is not impaired in GM-CSF-deficient mice. However, it is unclear whether the persistence of these features in GM-CSF-deficient lungs represents a defect in the capacity of these phagocytic and immunological cells to clear various infections, or if it represents the cellular response to ongoing recurrent (but usually controlled) infections owing to a vulnerability to infection that defective surfactant clearance must impose.

Intriguingly, embryo-derived GM-CSF-deficient mice transferred to pseudopregnant-specific pathogen-free mothers and subsequently suckled in a protected environment, show no histological evidence of pulmonary infection at 3 wk of age (Lieschke and Grail, unpublished observations) (Fig. 3). However, the alveoli of these mice contain excessive amounts of eosinophilic material in the absence of histological evidence of infection. Taken together, the temporal sequence of events indicates that the primary defect in
Fig. 3. Impact of the environment on pulmonary pathology of GM-CSF-deficient mice. (A–D) Lungs of four individual 3-wk-old GM-CSF-deficient pups born to GM-CSF-deficient mothers in the conventional animal house environment. Black arrows indicate areas of consolidation that involve entire lobes in A–C. (At higher magnification, these areas contain extensive perivascular lymphoid infiltration, dense eosinophilic alveolar material, and focal areas of an acute inflammatory infiltrate.) (E–H) Lungs of four individual 3-wk-old GM-CSF-deficient mice born to specified-pathogen-free wild-type mothers after embryo transfer in early gestation, and maintained in a clean isolated environment during suckling. Note, the total absence of consolidated areas as seen in panels A–D. h, heart. Original magnification ×20.
GM-CSF-deficient mice is the accumulation of alveolar lipoproteinaceous material and that pulmonary infection is a secondary phenomenon. Further support for this hypothesis comes from the GM-CSF-deficient mice from Dranoff and colleagues (7) that develop a progressive accumulation of surfactant lipids and extensive lymphoid hyperplasia, but show no signs of underlying infection (7). This group proposed that the basis for the lymphoid hyperplasia is a response to innocuous inhaled antigens rather than to infectious agents (7).

**Mice Deficient in GM-CSF and Other Hemopoietic Growth Factors**

On the basis of the overlapping activities of various hemopoietic growth factors in vitro, it has been suggested that there may be redundancy amongst these factors in vivo. The most logical way to test the notion of redundancy is to generate mice simultaneously deficient in two (or more) factors and to compare the resulting phenotype with that of mice deficient in either factor alone. Interbreeding between mice deficient in particular hemopoietic growth factors has been undertaken; some of these studies are advanced although information from others is beginning to appear in preliminary form.

**GM-CSF and CSF-1-Deficient Mice**

In light of the overlapping actions of GM-CSF and CSF-1 (M-CSF) in regulating the proliferation and function of cells of the monocyte/macrophage lineage, Lieschke and colleagues (18) crossed GM-CSF knockout mice with op/op mutant mice. op/op mice, have a naturally occurring point mutation in the CSF-1 gene resulting in a truncation and inability to express bioactive CSF-1 (19,20). op/op mice are characterized by a severe deficiency of macrophages and osteoclasts resulting in excessive bone formation, occlusion of the marrow cavity, and reduced marrow hemopoietic activity (21, and references therein). Intriguingly, the osteopetrosis and hemopoietic deficiency are not permanent and op/op mice show an age-related systemic correction of osteopetrosis and the hemopoietic defect is eventually resolved (22–24). At the outset, it seemed possible that by studying mice simultaneously
deficient in GM-CSF and CSF-1, it may be possible to gauge the role played by GM-CSF and CSF-1 in the face of a deficiency of CSF-1 and GM-CSF respectively.

From interbreeding mice heterozygous at both GM-CSF and CSF-1 loci, the ratio of mice deficient in both GM-CSF and CSF-1 to other genotypes is in accordance with Mendelian patterns of inheritance, precluding the possibility of pre- or neonatal mortality. Longer term survival studies, however, reveal a significant mortality among all toothless pups (op/op) and mortality is significantly worse in mice simultaneously deficient in GM-CSF and CSF-1. The striking pathology that contributes to the death of most of these mice is severe lung disease and acute broncho- or lobar-pneumonia. The morphological and microbiological studies indicate that the lung pathology is more severe than in mice deficient in GM-CSF alone. In the majority of mice, gram-negative and/or gram-positive bacilli can be seen microscopically and in some cases the corresponding organisms have subsequently been cultured using standard microbiological techniques. Numerous pulmonary macrophages with copious foamy cytoplasm are present within alveolar spaces (Fig. 4). Histologic and microbiological analyses indicates that the bacterial pneumonia is more prevalent in GM-CSF/CSF-1 deficient lungs than is observed in the lungs of GM-CSF-deficient mice housed in the same conventional animal facility.

In addition to severe pulmonary infection, the presence of granular eosinophilic material within alveoli is an invariable feature of GM-CSF/CSF-1-deficient mice. Electron microscopy reveals the presence of aggregates of fibrillary material and numerous type-C lamellar bodies within alveoli. As with the infection-related pathology, the proteinosis-related disease is more marked by morphological criteria than in GM-CSF-deficient mice of the corresponding age.

The worse pulmonary pathology of GM-CSF/CSF-1-deficient mice compared to that of GM-CSF-deficient mice unequivocally implicates CSF-1 in ameliorating the pulmonary effects of GM-CSF deficiency. This conclusion is interesting considering that the lungs of op/op mice are themselves unremarkable. Thus, either CSF-1 plays a role in steady-state pulmonary physiology but other factors are fully able to compensate for its absence, or its function is primarily as an available reserve factor in pulmonary host defences.
Interestingly, mice simultaneously deficient in GM-CSF and CSF-1 still have circulating monocytes and tissue macrophages. This is perhaps surprising since, on the basis of a wealth of evidence arising from studies carried out using in vitro and in vivo systems, GM-CSF and CSF-1 are regarded as two of the major factors influencing the production and differentiation of monocytes and macrophages. Either these two factors do not normally serve as important regulators of monocyte/macrophage production in vivo or, if they do, in their absence, other factors can support the production of these cell types. Evidence in support of this latter conclusion would be the demonstration of elevated levels of candidate factors (e.g., IL-3 or stem cell factor [SCF]) in GM-CSF/CSF-1-deficient mice or more saliently, the generation and analysis of the hematopoietic system of mice with inactivating mutations in genes encod-
ing candidate growth factors superimposed on GM-CSF/CSF-1 deficiency. Of course a practical difficulty here would be that the successful simultaneous deletion of the full repertoire of genes that collectively regulate monocyte/macrophage production would likely result in embryonic lethality.

As indicated previously, the osteopetrosis and hemopoietic deficiencies that characterize young \textit{op/op} mice progressively correct with age (22–24). Although the mechanism underlying the resolution of these deficiencies remains unknown, previous studies have indicated that daily administration of CSF-1 or GM-CSF to newborn \textit{op/op} mice ameliorates some, or all of the pathology associated with \textit{op/op} mice (25–27). These observations coupled with the fact the levels of endogenous GM-CSF are elevated in at least some \textit{op/op} animals (19) lead to speculation that the age-related recovery observed in \textit{op/op} mice might reflect the compensatory actions of GM-CSF in the face of CSF-1 deficiency. The most definitive means of assessing this possibility would be to determine whether mice simultaneously deficient in CSF-1 and GM-CSF showed an age-related correction of the \textit{op/op} phenotype. Nilsson and colleagues (21) showed that young GM-CSF/CSF-1-deficient mice are osteopetrotic and have the same hemopoietic characteristics and deficiencies as previously reported for \textit{op/op} mice; moreover, the GM-CSF/CSF-1-deficient mice undergo a similar age-related correction as observed in \textit{op/op} mice. Thus, although a role for GM-CSF in the correction of hemopoietic deficiencies in \textit{op/op} mice cannot be ruled out, GM-CSF is not the essential means by which this is achieved (21).

\textbf{GM-CSF- and G-CSF-Deficient Mice}

GM-CSF-deficient mice have been interbred with mice deficient in G-CSF (carrying the disrupted G-CSF allele generated by Lieschke et al. [28]). Preliminary observations from mice deficient in GM-CSF and G-CSF have been reported and are discussed in Lieschke and Dunn (Chapter 23).

\textbf{GM-CSF-, G-CSF-, and CSF-1-Deficient Mice}

Mice deficient in GM-CSF, G-CSF (granulocyte colony stimulating factor), and CSF-1 have been generated by interbreeding but the phenotype of these mice has not been reported.
**GM-CSF- and IL-3-Deficient Mice**

Since the GM-CSF and IL-3 genes are located on the same chromosome separated by only 15 kbp of DNA, it is improbable that recombination between them would occur and this therefore, in a practical sense, precludes the possibility of generating mice simultaneously deficient in both factors simply by interbreeding mice deficient in either. The most straightforward way of establishing GM-CSF/IL-3-deficient mice would be to disrupt either the GM-CSF or IL-3 genes in embryonic stem cells in which the IL-3 or GM-CSF genes, respectively, had already been disrupted. This strategy has recently been adopted (G. Dranoff, unpublished data; V. Tybulewicz, personal communication). Young mice simultaneously deficient in GM-CSF and IL-3 have been generated and although the mice have not been analyzed in detail, they appear superficially healthy (V. Tybulewicz, personal communication). In the event that GM-CSF/IL-3 knockout mice are fertile, they could potentially be crossed with either or both G-CSF and CSF-1 (op/op) mice.

**Comparison of GM-CSF-Deficient and IL-3/GM-CSF/IL-5 \(\beta_C\) Receptor Knockout Mice**

The receptors for GM-CSF, IL-5, and IL-3 share a common \(\beta\)-subunit (\(\beta_C\)) (29). Mice carrying a null mutation in the \(\beta_C\) are unresponsive to GM-CSF and IL-5, although they still respond to IL-3 by virtue of a second \(\beta\) subunit (\(\beta_{IL3}\)). \(\beta_C\) knockout mice develop normally, survive to young adulthood, and are fertile. The hemopoietic system of \(\beta_C\) knockout mice is essentially normal except for reduced numbers of eosinophils in peripheral blood and bone marrow (30,31). The lungs of young \(\beta_C^{+/−}\) mice are very similar to those of GM-CSF-deficient mice. Focal peribronchovascular infiltrates of lymphoid cells are present in histological sections of lung and eosinophilic proteinaceous material with associated foamy macrophages is evident in intra-alveolar spaces (30,31). Since environmental factors such as exposure to pathogenic microorganisms influence the morphology of lungs of GM-CSF-deficient mice, comparisons of the pulmonary pathology of GM-CSF-deficient and \(\beta_C^{+/−}\) mice would best be made between matched mice housed under identical conditions.
Conclusion

Analysis of GM-CSF deficient mice has revealed an important and indispensable role for GM-CSF in the regulation of normal pulmonary physiology. Apart from its intrinsic interest, this particular unexpected discovery highlights the value in creating mice carrying null mutations in genes encoding molecules of presumed known function. The study has also pointed to the fact that steady-state hemopoiesis does not depend on the availability of GM-CSF, although it still remains possible that in a genetically uncompromised and healthy mouse, GM-CSF contributes as a preferred, if not nonessential factor, to the production of monocytes and macrophages.

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Chapter 22

The In Vivo Role of the Receptors for IL-3, GM-CSF, and IL-5 (βc and βIL3)

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Introduction

Interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are cytokines that exhibit a number of similar biological activities when tested on hematopoietic cells in vitro. IL-3 stimulates the development of multiple hematopoietic lineages in colony assays. GM-CSF was originally defined as a factor that stimulates colony formation of granulocytes and macrophages and has subsequently been shown to share many of the properties of IL-3 stimulation (1). IL-5 was cloned as a B-cell stimulation factor (2), but additionally exhibited eosinophil stimulatory activity (3). IL-3 and GM-CSF also share the property of stimulating eosinophils in vitro. The genes for IL-3, GM-CSF, and IL-5 are tightly linked on chromosome 11 in the mouse, and on chromosome 5 in the human.

The cloning of receptor components revealed that all three cytokine molecules bind a common receptor component (βc) as well as a cytokine specific α-chain component. In human, the βc-chain does not bind any of the three cytokines by itself, but forms a
Fig. 1. The assembly of high-affinity receptors for IL-3, GM-CSF, and IL-5 consists of the βc component as well as a cytokine specific α-chain receptors for both mouse and human. In addition, mice have a second β-chain receptor, βIL3, which also interacts with the IL-3 receptor α-chain to form high-affinity binding.

High-affinity complex for a ligand in the presence of an appropriate α-chain, as measured by transfection studies (4). The mouse has two highly homologous β-chains. Similar to human, mouse βc binds IL-3, GM-CSF, or IL-5 in the presence of an appropriate cytokine specific α chain. βIL3 is a second mouse β-chain, 91% homologous to βc at the amino acid level (5,6), but serves as a second β-chain only for IL-3 and the IL-3 specific α-chain. Figure 1 illustrates the generation of high-affinity receptor complexes for IL-3, GM-CSF, and IL-5 in both human and mouse systems.

High-affinity stimulation of these receptor complexes with any of the three cytokines induces activation of similar patterns of protein phosphorylation (7,8). The ras-raf- mitogen-activated protein (MAP) kinase signal transduction pathway has been shown to be activated on stimulation of these three ligands. One of the kinases
responsible for protein tyrosine phosphorylation has been identified as JAK 2 (9), and acts upstream of the STAT transcription factors. On IL-3/GM-CSF/IL-5 stimulation, STAT 5 has been shown to be tyrosine phosphorylated, translocated from cytoplasm to nucleus, and binds to specific DNA elements (10). At least two homologous STAT 5 molecules exist in the mouse.

It is clear that a genetics-based approach will be quite useful in understanding the physiological significance of stimulation of hematopoietic cells via IL-3, GM-CSF, and IL-5, their receptor system, and subsequent signal transduction events. Over the past year, comparison of mice with induced mutation in these cytokines and the appropriate receptor components has begun to shed a new light on the importance and subtleties of the in vivo function of this system of cellular stimulation.

**Gene Knockout Studies**

We produced and described gene knockout animals for both $\beta_c$ and $\beta_{IL3}$ receptors (11). Each gene was inactivated independently by standard embryonic stem cell technology and separate mouse lines were produced for each mutated gene. Both types of homozygous mice were born at the expected frequency and were apparently healthy and fertile when maintained in a pathogen-free animal facility.

Earlier work with the $\beta_c$, $\beta_{IL3}$, and cytokine-specific $\alpha$-chain receptor chains had predicted the nature of how the bone marrow (BM) of the respective mutant animals would respond to stimulation with IL-3, GM-CSF, and IL-5 (12). These predictions held true in that no colony-forming unit-culture (CFU-c) colonies could be formed with $\beta_c$ mutant BM stimulated by GM-CSF and IL-5. However, as the $\beta_{IL3}$ receptor was normal in the $\beta_c$ mutant mice, IL-3 was able to stimulate normal multilineage colony formation. Likewise, BM from $\beta_{IL3}$ mutant mice still retained the capacity to respond to IL-3 owing to the function of the normal $\beta_c$ receptor, and GM-CSF and IL-5 stimulation was unaffected. Therefore, the $\beta_c$ mutation represents at minimum, loss of GM-CSF and IL-5 stimulatory capacity. As $\beta_{IL3}$ mutant mice have not lost their entire capacity to respond to IL-3 and exhibit no effect on GM-CSF and IL-5 response, these mice have appeared normal in all facets of investigation. This chapter focuses mainly on the biology of the $\beta_c$ mutant
animals. We have summarized our published work on these animals, and discuss these results in the context of other relevant gene targeting work. Since IL-3, GM-CSF, and IL-5, and their receptor systems have been extensively reviewed elsewhere (12), we focused our effort in this chapter primarily on the biology investigated in the mutant animals.

Pathological Examination

A comprehensive pathological examination of the βc mutant animals revealed a lung disorder. The condition resembled a respiratory disorder found in humans, termed alveolar proteinosis. In mice, this disorder in βc mutant animals has been ascribed to GM-CSF and not other molecules functioning with the βc receptor because GM-CSF ligand mutant mice have a very similar lung condition (13) (14). The disorder in humans and these mutant mice is characterized by progressive accumulation of surfactant in the lungs, a complex mixture of lipids and proteins that normally functions in the lung to maintain low surface tension at the air–liquid interface (15). The continued description of this pathological condition, how this disease can be reversed in the mutant mice, and the relationship of murine disease and disease reversal with respect to the human disease condition is covered later in this chapter.

Hematopoiesis

Analysis of the BM from βc mutant mice showed little variation from control animals except for the lack of response to GM-CSF and IL-5. Thus the predicted role of GM-CSF as an important stimulatory signal for BM progenitor cells was not apparent. These data are in good agreement when compared to the hematopoietic analysis from GM-CSF ligand mutant mice. Peripheral blood analysis showed a reduced number of eosinophils in the βc mutant mice. Except for this finding, the steady state development of circulating blood cells was not severely affected by the βc mutation. When mutant BM, harboring the cell autonomous receptor mutation, was transferred into lethally irradiated but otherwise normal animals, the kinetics of white blood cell (WBC) recovery was reduced when compared to peripheral reconstitution in control animals, indicating an effect of
signals generated through the $\beta_c$ receptor for optimal total WBC recovery. These data can be viewed as an “emergency response” function of $\beta_c$-mediated signals during hematopoietic crises. The effect of slower reconstitution kinetics in the $\beta_c$ mutant mice is consistent with the therapeutic use of GM-CSF protein as a drug which accelerates WBC recovery in patients that have undergone cytoablation therapies for cancer (16–18). However signals via the $\beta_c$ receptor are not critical in all hematopoietic crises responses (18a).

**Immune Response to Infectious Agents**

IL-5 has been extensively studied for its effects on B-cells and eosinophils (19,20). The B-cell lineage, including the Ly-1 (CD5) B-cell lineage, in the $\beta_c$ mutant mice was unaffected in comparison to controls. Numerous B-cell lineage markers were appropriately expressed in the major lymphoid compartments. Additionally, serum Ig levels were not altered in adult $\beta_c$ mutant mice (unpublished observations). However, as mentioned, $\beta_c$ mutant mice contained a reduced number of circulating eosinophils. To examine an active eosinophil response, we used a parasitic infection model. Infection of mice with the nematode parasite *Nippostrongylus brasiliensis* is a well-studied immunological model that characteristically induces eosinophilia and IgE production. This type of immune response has been viewed as a Th2 response that, in relevance to this study, induces production of IL-5. Infection of mice with this organism results in a strong blood eosinophilia as well as an inflammatory eosinophilia in the lungs owing to the transient migration of the parasite to the lungs (21). $\beta_c$ mutant mice were infected with *N. brasiliensis* and did not mount any detectable blood eosinophilia, nor any eosinophilic granulomatous lesions in the lung. These findings were in good agreement with earlier work showing that anti-IL-5 antibodies blocked blood and lung eosinophilia (22). When comparing the lung pathology post-infection between the anti-IL-5 antibody study and the $\beta_c$ mouse, a more severe inflammatory state existed in the lungs of the mutant mice, perhaps brought on by the existing lung pathology or by the lack of GM-CSF during the immune response. The lack of eosinophil expansion and migration to the lung, because of the absence of IL-5 stimulation, is of significance to the study of allergy, and the search for therapeutic intervention in the allergic response.
Another part of the immune response to *N. brasiliensis* infection is driven by another Th2 cytokine, IL-4, and results in marked increases in serum IgE levels. The increase of the IgE level in the infected βc mutant mice was comparable to that of the infected control animals. These data indicated that βc mutant animals simply lacked IL-5 responsiveness, and that the overall Th2 response was not defective, as it is well established that IL-4 is of primary importance in regulating IgE production (23, 24).

One interesting point of discussion concerning the lack of eosinophilia and the dominant role of IL-5 in this process is a consideration of other molecules that are thought to be chemoattractive for eosinophils. A number of chemokines, such as RANTES, MIP-1α, and MCP-3 have been shown to attract eosinophils in vitro in chemotaxis assays (25). The suspected biological activities of these chemokines on eosinophils must be viewed in the context of the results indicating the crucial importance of IL-5 in this process. As mentioned, the βc mutant mice still retain detectable circulating eosinophils, but these cells are unresponsive to their normal expansion and migration into the lung, even in the context of a strong Th2 response. Therefore, it is interesting to speculate if IL-5 stimulation of eosinophils in vivo may result in an induced responsiveness to chemokines, or if an IL-5 expansion phase is crucial prior to migration. Numerous interesting mechanisms could be envisioned, such as the induction of chemokine receptors or possibly other adhesion molecules on the eosinophils after IL-5 stimulation. Alternatively, the clear role of IL-5 in this process, shown in independent studies with anti-IL-5 treatment and mutant animals, may have identified the signal of overriding importance for eosinophil expansion and subsequent migration. The resolution of these questions and their important implications for Th2-driven allergic conditions await further studies with mice such as we describe here, and of in vivo biology concerning chemokines.

Further studies with other infectious agents have been carried out with the βc mutant animals. *Listeria monocytogenes*, a facultative intracellular pathogen, is another well-established mouse infectious disease model inducing an immune response with the characteristics of Th1 involvement. However it should be noted that aside from adaptive immunity, the response to *L. monocytogenes* is heavily controlled by the innate immune system (26–28). Numerous
biological activities of GM-CSF (and hence the naming of this cytokine) on macrophages and granulocytes have been described (1,29). These two cell types, macrophages and granulocytes, form the cornerstone of innate immunity, so we suspected that the $\beta_c$ mutant mice might be more susceptible to infection by *L. monocytogenes*. However, neither rates of mortality nor accumulation of viable bacteria in the tissues of these mice were different from control animals after systemic infection. Thus, the operation of the innate immune system was unimpaired in these animals with respect to resistance to *L. monocytogenes* (18a).

**Pulmonary Alveolar Proteinosis-Like Disease in $\beta_c$ Mutant Animals**

The pulmonary alveolar proteinosis-like disease state is easily identified in lung sections of 2-mo-old $\beta_c$ mutant mice as an eosin positive proteinaceous substance in the alveoli. Alveolar macrophages exhibited abnormal morphology, and lymphocytic infiltration was prominent around the bronchi. The disease state was clearly progressive in nature, and by 6 mo of age the mutant mice had more extensive intra-alveolar material. At this age, a significant amount of the intra-alveolar debris was periodic acid-Schiff (PAS) positive, a hallmark of alveolar proteinosis. By 1 yr of age the disease continued to progress to extensive blockage of airway space. An example of the pathological status of the lungs of these animals over different age periods is shown in Fig. 2.

The accumulation of material in the alveolar spaces was analyzed by scanning electron microscopy and found to contain a lattice work type structure, characteristic to surfactant accumulation, and similar to the data reported in the analysis of GM-CSF ligand-deficient mice (13,14). Curiously, the lattice-like material and lamellar structure was identified within alveolar macrophages, a cell type thought to be involved in the resorption of surfactant in normal lung biology. This suggested that the surfactant material was being phagocyted into the alveolar macrophage, but that degradation of the material was not occurring properly. Phagocytosis does not appear to be impaired as the mutant macrophages are still capable of ingesting experimentally administered latex beads (unpublished observations).
Fig. 2. Representative progressive nature of mouse pulmonary alveolar proteinosis. (A) Intra-alveolar substance accumulated in 2-mo-old mutant mice, along with areas of lymphocytic infiltration. (B) Similar but more extensive pathology is seen in 6-mo-old mutant mice. (C) Extensive intra-alveolar material resulting in a blockage of a significant portion of airway space in 1-yr-old mutant mice (see color insert).
These data are then consistent with two hypotheses. First, that loss of signaling capacity through the βc receptor leads to excessive surfactant production by type II pneumocytes. In this case, the alveolar macrophage may be overwhelmed by excessive surfactant production. However, this does not seem to be the case based on data presented Dranoff et al. (13) in analyzing GM-CSF ligand mutant mice, where mRNA levels of surfactant proteins in total lungs were found to be normal and no accumulation of surfactant proteins was detected in type II pneumocytes. Thus, a second hypothesis seems more likely, where a functional defect in the normal process of surfactant resorption and/or degradation accounts for the accumulation of this material in the alveolar spaces.

It should be noted that the βc mutant mice are housed in a pathogen-free animal facility. Extensive and routine screening for opportunistic and typically endemic infectious agents is carried out quarterly. Intensive screening of the βc mutant mice did not reveal the presence of infectious agents, especially in the lung where organisms such as Pneumocystis carinii may influence the lung pathology.

Bone Marrow Transplantation (BMT) Reverses the Lung Disease State in the βc Mutant Mice

If the hypothesis of ineffective phagocytosis and/or degradation of surfactant material is true, then the disease could be viewed in one of two ways. Nonhematopoietic cells may be a primary cause in the lack of surfactant degradation, since type II pneumocytes have been implicated in surfactant resorption as well as production (15). Alternatively, the unusual macrophage morphology in the mutant mice may be an indication that the disease state is hematopoietic in nature. In an attempt to understand the basis of the disease state we reasoned that transfer of wild-type (WT) BM into lethally irradiated βc recipient animals might be able to improve the condition of the lungs after hematopoietic reconstitution. This assumes that the main hematopoietic cell type in the lung, alveolar macrophages, could be ablated in the mutant recipient animals and effectively repopulated by the donor bone marrow. Reconstitution of βc mutant animals with WT BM resulted in a striking improvement in the lungs of the mutant animals. The improvement in the lungs was seen as early as 8 wk after BMT and was clearly evident at 12 wk post-BMT. Long-term
improvement in the lungs was evident at 29 wk post-BMT. The transplantation experiment was designed in a way such that the WT donor cells could be followed by a congenic cell surface marker when compared to recipient cells. Immunohistochemical analysis from lung sections of transplanted animals showed clear evidence of donor-type macrophage engraftment in the lungs. Since we used 2–7-mo-old mice, which have significant lung disease at the time of transfer, repopulation and replacement with WT cells can prevent the progress and reverse the course of established disease. These data show that the lung disease is primarily hematopoietic in nature, and that defective macrophage function in the lung is amenable to correction by BMT (29a).

The nature of the “curative” process in the lungs should be discussed in more detail. The pathological evaluation of the lungs of βc mutant mice that received WT BM were still distinguishable from the lungs of normal control animals. Much of this is attributable to hypercellular thickened alveolar septa, residual fibrosis, and possibly residual resident recipient macrophages that were not ablated by the radiation treatment prior to transplant. This type of pathology could be viewed as part of a reparative process. The pathology associated with the disease, accumulation of intra-alveolar PAS positive material along with eosinophilic debris, was significantly reduced or absent in the “cured” animals.

Because the reversal of the disease was mediated by WT hematopoietic cells, it is reasonable to suggest the alveolar macrophage as a defective cell type in the disease, consistent with the apparent lack of surfactant degradation in these mutant cells, a process in which WT alveolar macrophages expressing a normal βc gene are fully competent. Thus, this mutation appears restricted to a “specialized” macrophage function. Other mutations in the mouse affect macrophages in specialized compartments, such as the naturally occurring M-CSF mutation (op/op) where homozygous animals develop osteopetrosis, a bone remodeling disorder caused by ineffective bone resorption by osteoclasts, a cell type that can be considered a specialized macrophage (30). It is tempting to speculate that other disease conditions in humans may be associated with malfunction of the macrophage lineage, and good genetic models may continue to be found in mice via gene targeting techniques. The role of macrophages in scavenging functions, as well as a potentially more active
role in tissue remodeling, represent an important biological function where dysfunction could lead to a wide range of specialized or generalized pathological disorders that are not necessarily disorders strictly associated with the immune system. As emphasized by our data summarized above, cell autonomous defects associated with macrophages are replaceable by BM transfer with donor cells containing precursors to WT macrophages. This point is worthy of emphasis, especially in situations where particular tissues, such as the lung, are not typically appreciated to be hematopoietic in nature. The BMT reversal of alveolar proteinosis has important implications to the human disease, as discussed in the following sections.

**Human Alveolar Proteinosis**

Human alveolar proteinosis is a complex and rare disease with adult, infantile, and congenital forms (31). The adult form of the disease is manageable clinically (32). Repetitive bronchial lavage, along with careful evaluation for the onset of infection is typically sufficient treatment. Interestingly, the disease has been associated with hematopoietic malignancy in some but not all case reports of alveolar proteinosis (33,34). In contrast and for unknown reasons, the infantile and congenital forms are much more severe, with dire prognosis (31,35,36). Even within this more restricted group of patients, there is clear heterogeneity in the causes but not necessarily the general pathology of the disease. Surfactant B protein has been found to be mutant in some congenital cases, resulting in an imbalance and accumulation of other types of surfactant protein (37,38). Currently there is no cure for the patients. The immediate relevance of the BM transfer reversal of alveolar proteinosis in the lungs of the \( \beta_c \) mutant mice becomes evident owing to the recent work of Dirksen et al. (manuscript submitted for publication). Eight congenital and infantile alveolar proteinosis patients have been analyzed to examine the possibility that the disease state in humans may be attributable to deficiencies in GM-CSF production or GM-CSF response. Four of the eight patients do not express the \( \beta_c \) receptor chain at the cell surface. Peripheral blood cells of the four patients show no response to GM-CSF nor to IL-3, a specific characteristic of the human \( \beta_c \) chain. The same blood cells, however, do respond normally to stimuli not dependent on \( \beta_c \) receptor, such as G-CSF or
IL-6 plus stem cell factor (SCF). Therefore, a similar molecular
defect may be causing the disease in a subset of human patients
when compared to the $\beta_c$ mutant mouse.

Many considerations would need to be addressed prior to BMT
of humans that harbor cell autonomous mutations in or linked to the
$\beta_c$ receptor. For example BMT would require a cytoablative regi­
men to deplete the resident BM-derived cells prior to transplant. At
least one of these patients is colonized by opportunistic bacteria,
creating a grave concern during the neutropenic phase postcytoabla­
tive treatment and prior to full reconstitution with donor BM.
Nevertheless, considering the dire prognosis for congenital and
infantile patients, a new therapeutic option exists because of the
mouse model of this disease.

The direct comparison of the disease states between the human
and the mouse is curious. Prior to the aforementioned information
about the congenital human cases, we would have expected the
mouse disease to be a closer model to the adult form in humans.
Although we can detect the signs of the mouse disease in young
animals they remain healthy to adulthood, even though the disease
is clearly progressive in nature. This is not unlike the human dis­
ease in adults where onset is typically in mid-life, and presents clin­
cical symptoms of infection or exercise intolerance (32). Therefore
mice following a similar course of disease would not be expected to
present clinical symptoms in a pathogen-free environment.

There are a number of possible explanations regarding a mole­
cular similarity and a disease severity difference between mouse and
congential and infantile human disease. First, infection and inhalant
exposure may exacerbate the disease. Perhaps a more likely expla­
nation is the influence of genetic background on the severity of dis­
ease. It is well established that the penetrance of a phenotype in
many murine single gene deficiencies is heavily influenced by
“modifying” genes specific to certain genetic backgrounds. An addi­
tional possibility is the difference between mouse and human in the
$\beta$-chain receptors, and that humans are simply more dependent on
signals through $\beta_c$. At first glance this is an attractive idea as the
mouse contains an additional $\beta_{IL3}$-chain not found in humans. Thus
a $\beta_c$ mutation in human, but not mouse, would render human cells
unresponsive to IL-3 in addition to unresponsiveness to GM-CSF
and IL-5. We have tested this idea in a preliminary way where $\beta_c$
mutant mice were bred to IL-3 ligand mutant mice. This was a simple breeding experiment as a β_c mutation crossed to an IL-3 ligand mutation represents intercrossing two unlinked genetic loci, as opposed to intercrossing the tightly linked β_c and the β_IL3 receptor mutations. In double homozygous β_c-IL-3 ligand mutant mice there is no significant change in the status of the lung disease (18a).

Although the differences and similarities of the disease state between mouse and the two general categories of human disease raise some interesting points to contemplate, this work emphasizes the unique perspective gene knockout models can contribute to understanding the molecular basis of human genetic disease. Not only can precise genetic models of disease in mice contribute to understanding the pathology of a similar disease in humans, but experimental approaches to curing disease can be tested in the animal model with a realistic chance of impacting treatment of the corresponding human genetic disease. As the pace of genomic approaches at identifying new expressed sequences continues, as is the rate of gene knockout models, it is likely that an increasing number of mutant mice will mimic human genetic diseases.

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Chapter 23

Granulocyte Colony-Stimulating Factor (G-CSF)-Deficient Mice

Graham J. Lieschke and Ashley R. Dunn

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein hemopoietic growth factor that regulates the survival, proliferation, differentiation, and function of neutrophils and their precursors (for a review, see ref. 1). Neutrophils are the most common granulocytic cell found in the blood and hemopoietic tissues. G-CSF was first purified from other granulopoietic activities in 1983 (2) and the human and murine G-CSF cDNAs and genes were cloned in 1986–1987 (3–7). G-CSF was implicated as a major regulator of murine granulopoiesis in vivo by studies of the effects of administering pharmacological doses of recombinant protein (8,9), and of the consequences of the reconstitution of lethally irradiated mice with marrow cells infected with a recombinant retrovirus expressing G-CSF (10).

Evidence implicating endogenously produced G-CSF in the dynamic regulation of neutrophil production includes measurements of G-CSF serum levels in humans that identified some clinical situations where reduced blood neutrophil numbers coexisted with increased serum G-CSF levels (11,12), and clinical infections where elevated blood neutrophil numbers coexisted with increased serum G-CSF levels (13). However, inferences about the physiolog-
ical role of G-CSF from such correlative observations must be qualified on at least two grounds: these studies primarily show an association between serum factor levels and neutrophil numbers rather than prove a causal relationship, and the mechanisms of G-CSF clearance from the circulation are still not understood and it is probable that to some extent, neutrophils are themselves involved in G-CSF clearance (14). G-CSF had been implicated in steady-state granulopoiesis more directly by the observation that dogs treated with human recombinant G-CSF eventually developed reduced blood neutrophil numbers, apparently owing to the development of antihuman G-CSF antibodies that crossreacted with endogenous canine G-CSF (15) (see section on G-CSF-Deficiency Models). G-CSF is not the only factor that influences granulopoiesis in vitro and in vivo. Other factors stimulating or enhancing neutrophil-containing colony formation in vitro include: granulocyte-macrophage colony-stimulating factor (GM-CSF) (16), stem cell factor (SCF) (17), interleukin-3 (IL-3) (18); IL-6 (19), IL-11 (20), and flt3/flk2 ligand (21–23). Therefore, there exists considerable scope for functional redundancy among granulopoietic regulators, and it is an intriguing challenge to define the precise physiological role of each of these factors, particularly their unique and redundant roles.

G-CSF-deficient mice were generated primarily to define its unique physiological roles. It was hypothesized that any uncompensated phenotypic abnormality of G-CSF-deficient mice would point to a unique physiological role for this factor. This rationale has been previously reviewed (24), and was supported by the demonstration that op/op mice were genetically deficient in M-CSF (also known as CSF-1) (25,26), and that various steel alleles resulted in partial or total SCF deficiency (27–31). In particular, it was anticipated that studies of G-CSF-deficient mice would provide direct insight into the in vivo role of G-CSF in steady-state baseline granulopoiesis, in emergency granulopoiesis, about its potential role in several nonhemopoietic tissues, and about the extent of redundancy between G-CSF and the other factors influencing granulopoiesis. There was also the possibility that a better knowledge of the precise physiological roles of hemopoietic growth factors such as G-CSF and GM-CSF might strengthen the rationale for and appropriateness of their clinical application (32).
Structure of the Disrupted G-CSF Allele

The murine G-CSF gene is on chromosome 11 (33), separated by 28 cM from the tightly linked GM-CSF and IL-3 genes (34,35). The disrupted G-CSF allele was created by gene targeting (36), and is characterized by a deletion of most of three of the five G-CSF exons, at which site is inserted the \( \text{lac-Z} \) gene (placed under control of the G-CSF promoter) followed by the 3' untranslated sequence from the human \( \beta \)-globin gene (to complete this transcription unit), and a hybrid neomycin phosphotransferase expression cassette derived from phosphoglycerate kinase (PGK)-neo and pMC1neopA. The inability of tissues from mice homozygous for the disrupted G-CSF allele (G-CSF\(^{-/-}\) mice) to make G-CSF was confirmed by bioassay of organ conditioned media (36) using a microwell proliferation assay based on BAF/3 cells transfected with the G-CSF receptor (36,37). The disrupted G-CSF allele can be readily tracked in breeding programs using a polymerase chain reaction (PCR)-based screen of genomic DNA (for technical details, see ref. 36).

Phenotype of G-CSF-Deficient Mice

Survival and Fertility of G-CSF-Deficient Mice

G-CSF-deficient mice are viable, develop normally, and are ostensibly indistinguishable from wild-type mice. G-CSF-deficient males and females can be interbred and litters of normal size result (G. Lieschke, D. Grail, and K. Fowler, unpublished observations). The original colony of G-CSF-deficient mice was established in a conventional animal house (Ludwig Institute for Cancer Research, Parkville, Victoria, Australia). However, it is recommended that G-CSF-deficient mice be reared in specified-pathogen-free facilities wherever practicable, since several breeding females in conventional animal houses have developed pyogenic infections of the female reproductive tract (G. Lieschke and D. Grail, unpublished observations).

Hemopoiesis in G-CSF-Deficient Mice

The peripheral blood of G-CSF-deficient mice shows leukopenia owing to a selective chronic neutropenia, with neutrophil numbers 20–35% of age-matched wild-type controls (36) (Fig. 1). No
consistent perturbations of peripheral blood monocyte, lymphocyte, eosinophil erythrocyte, or platelet numbers have been observed under baseline conditions.

Marrow granulopoiesis is reduced, although marrows from G-CSF-deficient mice have normal cellularity (36) (Fig. 2). There is no compensatory splenic granulopoiesis under baseline conditions. Since marrow and splenic granulocytes together are thought to represent at least 90% of total body neutrophil numbers (38), this represents a major reduction in the total neutrophil mass of G-CSF-deficient mice.

The reduction in morphologically identifiable marrow granulocytic precursors is reflected in a reduction of granulocytic progenitor cells, particularly those assayed in cultures stimulated by GM-CSF or SCF. Reduced numbers of macrophage progenitor cells were also observed in cultures stimulated by GM-CSF, IL-3, and GM-CSF plus M-CSF (36). These initial observations were based on the study of small groups of 6–8-wk-old mice, and it is possible that other consistent perturbations of progenitor cell types might be recognized in more extensive analyses, and in analyses of older mice.

To measure the number of neutrophils available for immediate mobilization from noncirculating reserves, G-CSF was administered

Fig. 1. Chronic neutropenia in G-CSF-deficient mice. Data are mean ± standard deviation for mice of the three ages shown. G-CSF genotypes are: +/+, homozygous wild-type; +/−, heterozygous; −/−, homozygous null. *p < 0.05 compared to wild-type mice. #p < 0.05 compared to heterozygous mice.
Fig. 2. Marrow granulopoiesis in G-CSF-deficient and control mice. (A) Cytospin preparations of marrow cells from wild-type (G+/+) and G-CSF-deficient (G−/−) mice. Note the presence of mature neutrophils: black arrow indicates mature segmented neutrophils and gray arrow band forms. (B) The relative cellularity of femoral marrow of wild-type (+/+) and G-CSF-deficient (−/−) mice. (C) The relative percentages of immature (myelocyte and promyelocyte) and mature (metamyelocyte and polymorph) neutrophil granulocytes in femoral marrow of wild-type (+/+) and G-CSF-deficient (−/−) mice. Data are mean ± standard deviation.

to G-CSF-deficient mice. This was done because 3 h after G-CSF is administered to wild-type mice, peripheral blood neutrophil levels are elevated. However, this does not reflect neutrophil production resulting from G-CSF-driven proliferation (39), but rather a redistribution of neutrophils into the blood from the marrow, from those
marginated in the blood vessels, and from other sites (39). In G-CSF-deficient mice, the 3-h increment in blood neutrophil numbers after a dose of G-CSF was only 21% that of G-CSF-treated wild-type mice (36), indicating that G-CSF-deficient mice had a smaller store of readily mobilizable neutrophils.

**Rescue of G-CSF-Deficient Granulopoiesis by G-CSF Administration**

To determine the degree to which therapeutic G-CSF administration could reverse the consequences of chronic G-CSF deficiency, G-CSF administration was continued for several days. Daily G-CSF administration “rescued” G-CSF-deficient granulopoiesis promptly (36). After 1 d of exposure to G-CSF, G-CSF-deficient animals attained blood neutrophil numbers equivalent to basal levels in wild-type mice, but marrow granulopoiesis was still reduced. After 4 d of G-CSF administration, marrow granulopoiesis exceeded basal levels in wild-type mice. An increase in splenic granulopoiesis was only evident after 4 d of G-CSF administration to G-CSF-deficient mice.

It is possible that the sensitivity of G-CSF-deficient mice to G-CSF may be altered by several mechanisms (e.g., reduced sensitivity because of a lack of prior priming of responding cells by endogenous G-CSF, or increased sensitivity to G-CSF mediated by upregulation of receptor numbers in the chronic absence of G-CSF). Although this has not been directly assessed in vivo or in vitro, it is evident that G-CSF-deficient mice clearly responded to the pharmacological dose of G-CSF used in these studies (5 µg/kg/d).

**Hematological Response of G-CSF-Deficient Mice to Infection**

To assess the hematological response of G-CSF-deficient mice to infection, the consequences of infection of G-CSF-deficient mice with *Listeria monocytogenes* were analyzed (36). Five days after *L. monocytogenes* inoculation, G-CSF-deficient mice had elevated bacterial loads in liver and spleen, indicating an impaired capacity to control this bacterial infection. One day after *L. monocytogenes* inoculation, the increase in peripheral blood neutrophils
in G-CSF-deficient mice was only 15% of that of wild-type mice. Whereas the neutrophilia was maintained in surviving G-CSF-deficient mice, neutrophil numbers had decreased to baseline levels on d 5 and there was an increase in the relative proportion of circulating immature forms. Interestingly, in G-CSF-deficient mice there was also a delay in the development of the monocytosis characteristic of *Listeria* infection in wild-type mice. These observations demonstrate that a G-CSF-driven marrow is necessary for the normal sustained granulopoietic response to *L. monocytogenes* infection, and support the notion that G-CSF is involved in this “emergency” hemopoietic response. They also indicate that the combined granulopoietic effect of the remaining factors produced in response to *L. monocytogenes* infection is less efficient at elevating peripheral blood neutrophil numbers and driving granulopoiesis than the pharmacological doses of G-CSF used above (which result in serum G-CSF levels comparable to those that accompany a range of infections in humans [13,40]). However, since the G-CSF-deficient mice had a deficit of mature neutrophils and their precursors at the time of infection, experiments of this design do not unequivocally indicate that G-CSF is specifically required as part of the endogenous emergency granulopoietic response during infection. This could be evaluated more directly by experiments that used G-CSF-deficient mice that had been pretreated with appropriate amounts of G-CSF that would elevate neutrophil numbers to those of healthy wild-type mice. The consequence of absent emergency G-CSF production alone on the subsequent course of infection and granulopoietic response could then be assessed.

**Lac-Z Activity in G-CSF-Deficient Mice**

In the disrupted G-CSF allele, the lac-Z reporter gene was placed under control of the intact endogenous G-CSF promoter taking advantage of an NcoI site spanning the translational initiation ATG codon of the murine G-CSF gene. This strategy provided a means of generating mice in which the sites of G-CSF production in vivo could be identified by histological staining for β-galactosidase activity. Preliminary studies indicated that β-galactosidase production can be induced from this allele in some cell types in vitro (G. Lieschke, unpublished observations). Initial attempts to examine histological
sections from mice heterozygous and homozygous for the mutant G-CSF allele were complicated by background endogenous β-galactosidase-like activity in some of the cell types that would be anticipated from in vitro studies to be sources of G-CSF such as macrophages (41) and lymphocytes, neutrophils, eosinophils, and platelets (42) (G. Lieschke, unpublished observations).

**Use of Tissues from G-CSF-Deficient Mice for In Vitro Studies**

The utility of tissues from hemopoietic growth factor-deficient mice in several in vitro systems has been demonstrated using stromal cell lines derived from op/op mice that are unable to make M-CSF (43). In particular, the durability of the genetically based lack of factor production by such cells and the certainty of absolute factor absence are advantages over other options for the elimination of factor activity such as antibody neutralization. Although no such studies using tissues from G-CSF-deficient mice are yet reported, the generation of primary and transformed cell lines using tissues from G-CSF-deficient mice is now feasible.

**Mice Deficient in G-CSF and Other Hemopoietic Growth Factors**

The interbreeding of mice deficient in G-CSF with mice deficient in other hemopoietic factors is a way of directly assessing in vivo whether one or other factor is compensating for the absence of the other factor, or is ameliorating the severity of the consequences of deficiency of the other factor. Compared with other experimental approaches that may be used to evaluate these possibilities (e.g., measurement of serum or tissue levels of remaining factors, or the ex vivo measurement of induced production of remaining factors by preparing and assaying organ conditioned medium), this genetic approach is a particularly definitive way of evaluating these possibilities directly in vivo.

**G-CSF- and GM-CSF-Deficient Mice**

G-CSF-deficient mice have been interbred with mice deficient in GM-CSF (carrying the disrupted GM-CSF allele generated by Stanley et al. [44]), and preliminary observations from mice defi-
cient in both G-CSF and GM-CSF have been reported (45). Mice deficient in both these factors are viable but have increased mortality. Males and females are fertile, but when interbred, litters of reduced size result. Although these mice have neutropenia, it is no more severe than that of age-matched G-CSF-deficient mice, and these mice have morphologically mature neutrophils in blood, marrow, and spleen. Marrow and splenic granulopoiesis are reduced compared to wild-type mice, but comparisons with mice deficient in either factor individually are not given in the preliminary reports. The increased mortality of G-CSF and GM-CSF-deficient mice appears to be the result of an exacerbation of the lung disease complicating GM-CSF deficiency, with multifocal pneumonia and pulmonary abscesses common at death.

**G-CSF-, GM-CSF-, and M-CSF-Deficient Mice**

Mice deficient in all three of these factors have been generated by interbreeding (45) but the phenotype of these mice has not yet been reported.

**G-CSF- and IL-3-Deficient Mice**

Interbreeding of G-CSF-deficient mice with IL-3 deficient mice (generated by Dr. G. Dranoff at the Whitehead Institute, Cambridge, MA) is underway (G. Lieschke and G. Dranoff, unpublished data).

**Other Models of G-CSF Deficiency**

No other genetic models of absolute G-CSF deficiency have been generated, but the effects of antibody-mediated neutralization of G-CSF in vivo have been reported. A canine model of G-CSF neutralization resulted from the treatment of dogs with human G-CSF. Dogs initially developed a G-CSF-driven neutrophilia, but subsequently neutropenia supervened (15). An immunological mechanism was implicated because the neutropenia subsided on cessation of human G-CSF administration, but then promptly recurred on further treatment with human G-CSF, because anti-G-CSF antibodies were demonstrable in the serum of animals exposed to human G-CSF, and because passive immunization with serum from immunized dogs resulted in neutropenia. Whereas this study implicated G-CSF in the
maintenance of baseline granulopoiesis, it did not resolve several issues. In particular, since neutrophil levels were slowly restored with time, G-CSF neutralization appeared incomplete, and so it was unclear if the residual granulocytes indicated a capacity of other factors to support granulopoiesis, or was an effect of residual nonneutralized G-CSF. This approach is not applicable to mice (a generally more practical experimental animal) because human G-CSF appears nonimmunogenic in mice. Although a polyclonal neutralizing antiserum to murine G-CSF has been used for G-CSF neutralization in vitro (46), no attempts to neutralize G-CSF by antibody administration to mice have been reported.

One study of G-CSF neutralization in rats has been briefly reported (47). A rabbit anti-G-CSF IgG antibody was used for passive immunization 2 h before a pulmonary challenge with Pseudomonas aeruginosa. Anti-G-CSF pretreatment reduced pulmonary neutrophil recruitment and intrapulmonary bactericidal activity suggesting that a local pulmonary G-CSF response had been impaired. Although a detailed characterization of the specificity and actions of the antibody used in these studies was not presented, these observations suggest a role for G-CSF in the immediate pulmonary response to infection.

In addition to their lack of G-CSF, genetically G-CSF-deficient mice offer several other advantages over in vivo antibody neutralization of G-CSF:

1. The G-CSF deficiency, being genetically based, is able to be perpetuated, and is not subject to the variability inherent in individually induced polyclonal immune responses;
2. The presence of G-CSF deficiency does not require an intact immune system and can be superimposed on other immunodeficient states by interbreeding; and
3. They are a source of G-CSF nonproducing cellular reagents suitable for in vitro studies.

**Implications for the Physiological Role of G-CSF**

The initial analyses of G-CSF-deficient mice indicates that G-CSF has unique physiological roles in granulopoiesis, the distribution of neutrophils within the body, and that the lack of G-CSF has functional consequences for host antimicrobial defences (Fig. 3).
Fig. 3. Summary of the phenotypic consequences of G-CSF deficiency.

Granulopoiesis may be divided into baseline and emergency granulopoiesis (discussed in refs. 1, 24, and 48). In baseline granulopoiesis, a steady-state balance exists between neutrophil production and clearance, one manifestation of which is the maintenance of blood neutrophil numbers within a narrow normal range. The chronic neutropenia of G-CSF-deficient mice unequivocally implicates G-CSF as a critical regulator in maintaining the quantitative balance of baseline granulopoiesis under the conditions prevailing in a conventional animal house. Peripheral blood neutrophil levels of wild-type mice are lower under germ-free animal house conditions (49), and the degree to which environmental factors are influencing the relative magnitude of this difference between G-CSF-deficient and wild-type mice has not been determined. However, under germ-free conditions the total number of marrow neutrophils and neutrophil precursors are not altered (49), supporting the notion that the marked reduction of these cell populations in G-CSF-deficient mice is intrinsically caused by the genetic deficiency of the critical regulator G-CSF, and not to different responses of wild-type and G-CSF-deficient mice to environmental circum-
stances. The impairment of defences against *L. monocytogenes* infection in G-CSF-deficient mice indicates that G-CSF is also necessary for an emergency granulopoietic response, although in the experiment reported (36) it is possible that this was either a consequence of a lack of G-CSF priming of granulopoiesis prior to the infective challenge or alternatively, a cumulative result of both the lack of a G-CSF-driven marrow at the onset of infection and a lack of an emergency G-CSF response to the infection itself.

The kinetic basis of the neutrophil depletion of G-CSF-deficient mice has not been determined. Administration of pharmacological doses of G-CSF to wild-type mice accelerates production of neutrophils by altering the kinetics of granulopoiesis: Estimates from tritiated-thymidine pulse-labeling of marrow indicated that G-CSF administration reduced the average cycle time of neutrophil precursors by 25–40%, although the half-life of peripheral neutrophils was not altered, hence requiring 3.8 extra amplification divisions in neutrophil production (39). A kinetic analysis of max 41 transgenic mice (which have a sustained 50–60-fold elevation of neutrophils in the peripheral blood) revealed only a four- to seven-fold increase in the much larger marrow and splenic pools of neutrophil progenitor cells and morphologically recognizable precursors, and a small egress of marrow and splenic neutrophils into the circulation from a much larger pool in hemopoietic organs (38). It would be interesting to quantify the kinetic parameters of neutrophil production in G-CSF-deficient mice along similar lines to these two studies.

It was also possible that G-CSF may have had important roles in nonhemopoietic tissues that would have been manifest in the phenotype of G-CSF-deficient mice. In particular, placental tissues are capable of synthesizing G-CSF ex vivo (50), and immunohistochemical and in situ hybridization studies demonstrated that G-CSF is widely expressed in murine placental decidua basalis and endometrial cells (51). Similarly, G-CSF receptor mRNA and protein were detected in uterine epithelial cells (51). These observations had contributed to speculation that G-CSF-deficient mice may have had a developmental or reproductive defect (24). However, the apparently normal fertility and fecundity resulting from interbreeding G-CSF-deficient mice indicates that this is not an indispensable role of G-CSF.
Comparison with Other Hemopoietic Growth Factor-Deficient Mice

Only one group has reported a disrupted G-CSF allele generated by gene targeting (36). No naturally occurring mutations in the murine G-CSF gene have been recognized. The gene for the single-component G-CSF receptor is on chromosome 4 (52) and no murine G-CSF receptor mutants have been reported. There have been genetic studies attempting to identify loci determining high- and low-leukocyte levels in mice, but a limitation of these studies was their failure to focus on the different subtypes of leukocytes, and it was likely that the predominant difference between mice with high and low blood leukocyte numbers was in lymphocytes (53,54).

Neutropenia occurs in several other strains of mice lacking hemopoietic growth factors. Mice deficient in SCF or its receptor c-kit indicate that SCF is indispensable for granulopoiesis. Although mice totally deficient in SCF or c-kit are nonviable, SI/SI<sup>d</sup> mice (carrying one null allele and one allele encoding only the soluble but not the membrane-bound form of SCF) and W/W<sup>o</sup> mice (carrying one null allele and one allele encoding a kinase-impaired c-kit receptor) have impaired marrow granulopoiesis and reduced peripheral blood granulocytes (55,56). However, unlike G-CSF-deficient mice, SCF-deficient mice have major perturbations in other hemopoietic lineages, most notably anaemia owing to impaired erythropoiesis (for review see ref. 57).

In marked contrast to G-CSF-deficient mice, GM-CSF-deficient mice do not have significant perturbation of baseline hemopoiesis (see Chapter 21). A reliable comparison between G-CSF-deficient and GM-CSF-deficient mice is possible because GM-CSF-deficient mice were also generated by our group (44) and housed together with the G-CSF-deficient mice. This indicates that G-CSF is indispensable for maintaining the quantitative balance of baseline granulopoiesis, but GM-CSF is not essential for this process. The pulmonary alveolar proteinosis characteristic of GM-CSF-deficient mice from soon after birth (44) does not develop in G-CSF-deficient mice up to 5 mo of age, indicating a further distinction between the physiological roles of these factors. It is possible that either factor is responsible for supporting haemopoiesis in the absence of the other;
studies investigating this possibility by generating mice deficient in both these factors are in progress.

G-CSF may be regarded as the hemopoietic regulator with actions most restricted to cells of the neutrophil granulocytic lineage. In this regard, it fulfills a role analogous to M-CSF in the monocyte/macrophage lineage, erythropoietin in the erythroid lineage, and IL-5 in the eosinophil lineage. Comparison of mice deficient in these various factors reveals several differences in the uniqueness and specificity of the roles they play as regulators of their respective hemopoietic lineages. Although M-CSF-deficient \( op/op \) mice have monocytopenia and macrophage deficiency in early life, M-CSF-deficiency also results in osteopetrosis (58), and impaired maternal fertility and lactation (58–60), ascribing indispensable nonhemopoietic roles to M-CSF. By contrast, no aspect of the phenotype of G-CSF-deficient mice has yet suggested an indispensable role for this regulator in any nonhemopoietic organ or physiological system. Erythropoietin-deficient mice die \textit{in utero} from a failure of erythropoiesis (61), indicating that life-sustaining red-cell development is totally dependent on erythropoietin. By contrast, there is a greater degree of regulator redundancy in granulocyte development, because G-CSF-deficient mice survive and still have mature neutrophils, indicating that other factors are able to support the production of adequate numbers of mature neutrophils and their precursors, albeit at reduced levels. IL-5-deficient mice have not yet been described, but mice lacking the \( \beta \)-subunit of the IL-5 receptor (common to the GM-CSF and IL-3 receptors) have low basal blood eosinophil levels and an impaired emergency eosinophil response (62,63), suggesting that IL-5 is critical for eosinophil production in vivo.

**Implications for Human Diseases**

The viability and phenotype of G-CSF-deficient mice suggests the possibility that a subgroup of autosomal recessive forms of human congenital neutropenia is caused by G-CSF-deficiency. However, among forms of congenital neutropenia described (64), none have been demonstrated to be the result of G-CSF-deficiency or to a mutation in the human G-CSF gene that is on chromosome 17 (65,66). Perhaps the best described and studied is Kostmann
syndrome, first recognized in 1956 (67). Since G-CSF-deficient mice do not show the granulocytic maturation arrest characteristic of Kostmann syndrome, they support the view that it is unlikely that G-CSF deficiency alone underlies this type of congenital neutropenia. Indeed, patients with severe congenital neutropenia studied to date have had normal or increased serum G-CSF levels and an intact capacity for stromal G-CSF production (12,68). Although severe congenital neutropenia is therefore generally not the result of an absolute failure of endogenous G-CSF production, blood neutrophil numbers are still elevated by G-CSF administration to these patients with substantial improvement in their quality of life owing to reduced morbidity from infective complications (69).

Recently, several patients with severe congenital neutropenia have been described with single allelic somatic mutations in the G-CSF receptor gene of hemopoietic cells (70,71). These mutations have all resulted in carboxyl truncations of the cytoplasmic domain of the G-CSF receptor, removing the distal domain important for transmitting the differentiation/maturation signal but leaving intact the more membrane-proximal domain transmitting the survival/proliferation signal (72–74). Two of the three patients ultimately developed acute myeloid leukemia (71). The association between a mutant G-CSF receptor and severe chronic neutropenia in these patients lends support to the hypothesis that G-CSF deficiency may be the basis of some human cases of severe congenital neutropenia. It should be noted that not all patients with severe congenital neutropenia have null or mutant G-CSF receptor genes, since others studies of congenital neutropenia patients have shown either normal numbers and affinity of receptors on neutrophils (75) or no genetic lesion in the G-CSF receptor including its cytoplasmic domain, as assessed by single-strand conformational polymorphism analysis (68).

**Conclusion**

The initial phenotypic description of G-CSF-deficient mice has resulted in significant insights into the nonredundant physiological importance of this granulopoietic regulator. Along with observations from mice deficient in other hemopoietic growth factors and their receptors, a refined understanding of the regulation of granulopoiesis in vivo is possible, and such reappraisals are now being
published (76). Further insights into the full range of possible roles of G-CSF in vivo can be expected from the analysis of mice deficient in both G-CSF and other granulopoietic factors.

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Chapter 24

Reduction of Platelets and Megakaryocytes in c-mpl-Deficient Mice

Mark W. Moore and Frederic J. de Sauvage

Introduction

The ability to treat various forms of cancer is often limited by the ability of patients to tolerate the dosing and frequency of chemotherapeutic agents. This is mostly caused by a loss of cells of hematopoietic origin, specifically red blood cells, neutrophils, and platelets. This results in anemia, increased susceptibility to infections, and the potential for severe bleeding episodes. Currently, there are cytokines that have the potential to address the loss of some of these cell types. Erythropoietin (EPO) is very effective in stimulating the production of red blood cells and granulocyte colony-stimulating factor (G-CSF) stimulates neutrophil production, thus it is possible to combat the anemia and neutropenia with cytokines that are approved for use in humans (although many of the precise indications related to cancer therapy are still in clinical trials). The treatment of the associated thrombocytopenia remains more problematic. To date, it can only be addressed by platelet transfusions, which has its own associated risks. For many years researchers have sought for a cytokine with the ability to specifically stimulate platelet production in a manner similar to EPO.
stimulation of red blood cells or G-CSF stimulation of white cells. Several cytokines, IL-3, IL-6, and IL-11 (1,2), have been shown to stimulate platelet production but the increase is relatively modest and there are potential side effects as these cytokines are not specific for platelets and stimulate a variety of responses. Clinical trials are underway with these cytokines to determine their benefit in treating thrombocytopenic patients.

A major breakthrough in the search for a more specific platelet-producing factor occurred with the discovery of a truncated cytokine-like receptor in the murine myeloproliferative leukemia virus (MPLV). The virus was shown to stimulate a wide variety of leukemias of hematopoietic origin. Most interestingly, anti-sense oligo nucleotides to the mammalian homolog (c-mpl) inhibited the in vitro production of colony forming unit-megakaryocyte (CFU-Meg) in colony assays. Furthermore, the expression of c-mpl was shown to be mostly restricted to stem cells and cells of the myeloid lineage (3–5). As megakaryocytes mature, each one produces and sheds thousands of platelets. Thus, c-mpl was speculated to be a receptor for the long sought factor thrombopoietin (TPO). To determine whether this was indeed the case, we initiated gene targeting of the c-mpl gene.

Results

The disruption of the c-mpl gene was achieved by insertion of a neo' cassette into the first coding exon (6). Targeting in embryonic stem (ES) cells was performed as previously described and occurred at a relatively high frequency of 1/80 clones screened. Four of the five clones selected for injection into blastocysts gave rise to germline transmitting male chimeras. Three of these were randomly selected for further study. All subsequent data reported herein has been confirmed in all three lines of mice. Despite initial concerns that if the c-mpl gene product indeed was the receptor for TPO then the phenotype may be lethal, we found live births of healthy mpt−/− animals. We did not observe any alterations in the gross appearance of any organs. Complete blood counts from these mice did reveal a dramatic 85% drop in platelets (Fig. 1). These initial findings confirmed that c-mpl was indeed extremely important in signaling platelet formation but not abso-
Fig. 1. Platelet and megakaryocyte counts in c-mpl<sup>++</sup>, +/-, and -/- mice. (A) Blood was collected by retro-orbital venous puncture and analyzed in a Serono-Baker Diagnostics System 9000 Diff Model Hematology Analyzer for platelet counts, <i>n</i> = 5 mice/group. (B) Animals were sacrificed for necropsy. Blood and bone marrow smears were stained with H&E for differential cell counts performed microscopically. Megakaryocytes were counted in both bone marrow smears and spleen 5 μm sections. The genotype of each group is indicated.

Absolutely required as some platelets are still present. This drop in platelet count does not affect either the long-term viability of the mice or their general health. In fact, as one compares different species, the normal platelet count in mice is about fivefold higher than most primates including humans. The mutation of the c-mpl gene lowers the mouse platelet count to the range of primates. The reason for these species differences is unclear.
Fig. 2. Ploidy analysis of megakaryocytes. A percoll step gradient (20, 40, 60, and 80%) was used to enrich for megakaryocytes in bone marrow from two to four mice. Cells from the 20/40% interface were incubated at 4°C with anti-CD61 (integrin β3 chain)-FITC (Pharmingen) and propidium iodide (PI), a DNA intercalating fluorescent dye. The DNA content in cells was quantified by two color flow cytometry on a Coulter Epics Elite. CD-61 positive cells were indicative of megakaryocytes.

While the overall number of platelets is decreased in the c-mpl−/− mice, it was important to determine at what step along the process of platelet formation does c-mpl exert its effect. First, we quantitated the number of megakaryocytes present in the bone marrow and spleen of the mice. There was an 85% reduction in megakaryocytes that was similar to the percentage drop in platelet counts (Fig. 1B). During megakaryocyte development, they continue to replicate their DNA without undergoing cell division and in mice megakaryocytes commonly have a mean ploidy of 16N and 32N as measured by labeling cells with propidium iodide and quantitating DNA content of individual cells. The megakaryocytes from c-mpl−/− mice have shifted their ploidy with 32N nearly absent, a great reduction in 16N, whereas 8N is increased (Fig. 2). Thus, it appears that c-mpl and its ligand, TPO, act both on the proliferation and the DNA content of megakaryocytes, as not only are the absolute number of megakaryocytes reduced but so is their ploidy.
Table 1

White Cell Counts in Blood of c-mpl-Deficient Mice

| c-mpl | +/+ | +/- | -/- | p values +/- vs. -/- |
|-------|-----|-----|-----|---------------------|
| Red blood cells | 8.86 ± 0.70 | 8.98 ± 1.11 | 9.90 ± 0.38 | 0.11 |
| Total white cells | 5.08 ± 1.61 | 5.52 ± 2.33 | 6.40 ± 2.41 | 0.33 |
| Lymphocytes | 3.86 ± 1.11 | 3.71 ± 1.00 | 4.19 ± 1.02 | 0.64 |
| Neutrophils | 0.67 ± 0.14 | 0.79 ± 0.32 | 0.85 ± 0.42 | 0.39 |
| Bands | 0.13 ± 0.16 | 0.09 ± 0.07 | 0.06 ± 0.07 | 0.35 |
| Eosinophils | 0.09 ± 0.07 | 0.16 ± 0.05 | 0.18 ± 0.22 | 0.31 |

Blood was collected by retro-orbital vp and analyzed in a Serono-Baker Diagnostics System 9000 Diff Model Hematology Analyzer, n = 4 mice/group. Blood smears were stained with H&E for differential cell counts. The total number of each indicated cell type was determined by multiplying the total white cell count by the percentage of each cell type determined by the differential counts. All values are in thousands per microliter, except for red blood cells which are at millions per microliter.

It has been reported that c-mpl is expressed on a very early hematopoietic stem cell population that is capable of bone marrow reconstitution (7,8). This could indicate an important role for c-mpl activation in a variety of cell types and could have serious implications for the potential use of TPO in clinical settings. Differential cell counts of peripheral blood did not reveal any significant alteration in the white blood cell lineages or red blood cells even after 1 yr of monitoring (Table 1). Analysis of lymphoid populations in spleen, thymus, and bone marrow using cell surface markers also did not show any alteration in the c-mpl<sup>-/-</sup> mice (Fig. 3). Thus, it would appear that the c-mpl signaling is specific for the platelet lineage; however one should consider that in analyzing the whole animal one is testing the steady state level of cell lineages and if there are more subtle changes in the kinetics of repopulation, those may not be detected.

We have tested bone marrow from the c-mpl<sup>-/-</sup> mice for progenitors in the CFU-Meg, CFU-GM, and BFU-E assays (9). We discovered that in all of these assays there is a 3–10-fold decrease in colony formation, which suggests that the loss of c-mpl or TPO has a broad effect on the myeloid progenitors. This is supportive of earlier work (7,8) showing that c-mpl is expressed in an early hematopoietic stem cell population. It is intriguing that this in vitro
Fig. 3. Flow cytometric analysis of c-mpl+/+ mice. Spleen, bone marrow, and thymus single cell suspensions were stained for T-cell- and B-cell-specific cell surface markers. No significant difference in cellularity between c-mpl+/+ and c-mpl−/− mice was detected in any of the indicated lymphoid organs. Samples were treated with the following antibodies: PE-conjugated anti-CD-4 (Becton-Dickinson), FITC-conjugated anti-CD-8 (Becton-Dickinson), FITC-conjugated anti-TCRαβ, and PE-conjugated anti-B220 (Boehringer-Mannheim, Indianapolis, IN) and analyzed on a FACScan (Becton-Dickinson, Mountainview, CA).
loss of progenitor activity is not reflected in adult mice on the level of differentiated circulating cells. It may be that in vivo other cytokines compensate for the loss of c-mpl activity or that given time the various cell populations can reach homeostasis and we are comparing the differences between a steady-state population in vivo with a short-term kinetic assay in vitro.

**Conclusions**

The gene targeting of the c-mpl gene confirmed the important role for c-mpl activation in production of megakaryocytes and platelets, because c-mpl\(^{-/-}\) mice had an 85% reduction in both populations (6). Indeed, as this project was in progress three separate groups (10–12) were successful in cloning the elusive factor TPO that indeed has been demonstrated to stimulate up to a 10-fold increase in platelets in normal animals. We used the c-mpl receptor itself in a purification scheme using plasma from irradiated pigs as a source of TPO. All three groups isolated the same gene that encodes a novel protein most closely related to EPO. TPO binds the c-mpl receptor with high affinity and stimulates its phosphorylation and the proliferation of c-mpl transfected cell lines. Both in vitro and in vivo experiments with recombinant TPO indicate that it can stimulate both the maturation and colony numbers of megakaryocytes (10–15). TPO stimulates the production of megakaryocytes and functional platelets from murine or human stem cell populations (7,8). The c-mpl\(^{-/-}\) mice not only confirm the role of this receptor ligand system, but also demonstrate the specificity of the system as other lineages are unaffected in the animal. This may indicate that treatment of individuals with TPO will lead to the desired increase in platelets with minimal or no side effects. These results also point to the power of ES cell/gene targeting technology as a research discovery tool. As more orphan receptors or ligands are isolated, it is often difficult and very time consuming to ascertain their function. The mutation of such genes in mice may not always demonstrate the function of a given gene. However, in some cases such as with the c-mpl gene, there may be a clear indication of the function of critical genes.

Recently, we have also generated TPO\(^{-/-}\) mice and find a near mirror image phenotype as with the c-mpl\(^{-/-}\) mice (16). There is also
a near 10-fold reduction in both megakaryocytes and platelets with other hematopoietic lineages unchanged. This strongly points to TPO and c-mpl being a single receptor ligand system that is the major yet not sole regulator of platelet production.

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