Immunopathology of Interleukin (IL) 2-deficient Mice: Thymus Dependence and Suppression by Thymus-dependent Cells with an Intact IL-2 Gene

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Summary

Interleukin (IL) 2-deficient mice develop a fatal immunopathology characterized by lymphadenopathy, splenomegaly, T cell infiltration of the bone marrow, loss of B cells, anemia, and inflammation of the gut. The thymus dependence of these disease symptoms was tested by introducing the IL-2 mutation into athymic mice. With the exception of an increase in CD8+ intrahepatic α/β T cells, IL-2 deficiency had no detectable effect on leukocyte composition or health of athymic mice, indicating a key role for thymus-derived T cells in the initiation of disease and demonstrating that B cell development and survival are independent of IL-2. In adoptive transfer studies, lymph node and spleen cells from euthymic IL-2-deficient mice induced disease in athymic mice with an intact IL-2 gene, suggesting that thymus-independent IL-2-expressing cells are unable to control the development of immune pathology. Both IL-2+ and IL-2−/− bone marrow cells repopulated the thymus and the peripheral T cell compartment of the recombination activator gene 2−/− deficient recipients, and chimeras that had received IL-2-deficient bone marrow developed immune pathology. Disease development was, however, fully or at least partially prevented when 30% of the bone marrow inoculum was derived from mice able to express IL-2. These results demonstrate that the IL-2 deficiency syndrome depends on the intrathymic differentiation of T cells carrying the IL-2 mutation, and that the abnormal activation of IL-2-deficient lymphocytes can be controlled by thymus-derived but not thymus-independent lymphocytes.

IL-2, originally described as T cell growth factor, has pleiotropic effects on the proliferation and differentiation of T, B, and NK cells. In addition, inhibition of T cell development by antibodies to the α chain of the IL-2 receptor (IL-2R) reported by some (1, 2) but not by other investigators (3) had suggested an essential role for IL-2 in intrathymic T cell maturation. Unexpectedly, however, mainstream T cell development in mice with a disrupted IL-2 gene is normal (4), and after 3–4 wk of age, peripheral T cells are even increased in number and display an activated phenotype. This marks the onset of a lethal syndrome characterized by splenomegaly and lymphadenopathy, an early burst of B cell activation followed by their virtual disappearance, T cell infiltration of the bone marrow, anemia, autoantibody production, and a severe colitis preceded by an infiltration of the gut epithelium with CD4 and CD8 α/β T cells (5–8). Hyperactivation of T cells and development of autoimmunity have also been observed in mice lacking the IL-2Rβ chain (9), supporting the concept that IL-2R-mediated signals are required for the containment of T cell activation in vivo. Among the mechanisms that could lead to the development of the symptoms listed above are an incomplete deletion of autoreactive clones in T cell ontogeny, unchecked lymphocyte activation caused by a disturbance of the cytokine network, physical or functional absence of specialized suppressor cells, and a requirement for IL-2 in limiting clonal growth by predisposing mature activated T cells toward apoptotic death (10). In addition, breakdown of the B cell compartment could either result from T cell-mediated destruction or from an impaired replenishment of the peripheral pool from precursors expanded by IL-2, a possibility suggested by the expression of the IL-2Rα chain on pro-B cells (11). So far, attempts at identifying defects in T cell repertoire selection have yielded negative results (12). With regard to a breakdown in peripheral regulation, the delayed onset of immunopathology in germ-free IL-2−/− mice suggests that an antigen trigger is required to drive the abnormal lymphoproliferation observed (6). Since the oligoclonal repertoire of athymic mice allows alloreactive but not self-MHC–restricted T cell responses to environmental antigens (13–16), the IL-2 mutation was introduced into nu/nu mice to ask whether thymus-processed T cells (with a diverse repertoire) are required to initiate disease, including the destruction of B cells and bone marrow cells. Furthermore, it remained to be formally proven that lack of IL-2 expression in bone marrow-derived cells suffices for the development of the IL-2 deficiency syndrome. Therefore, recombination activator gene...
(RAG) 1-2-deficient mice repopulated with H-2-matched bone marrow from IL-2-/- mice were investigated for signs of disease. Finally, the simultaneous transfer of IL-2+ and IL-2- bone marrow into RAG-2-deficient recipients was used to address the question of whether autocrine production of IL-2 is required for the control of clonal expansion or whether IL-2-producing or -dependent bystander cells could interfere.

Materials and Methods

Mice. IL-2+/- mice from a mixed C57BL/6/H129OLA background (4) were back-crossed for four generations to C57BL/6 mice. After mating with C57BL/6 nu/nu mice, IL-2-/- mice were identified by PCR among the nude offspring of the F2 generation. Euthymic IL-2+/- and IL-2+/- mice (which are phenotypically identical and will be referred to as IL-2+/-) were used as controls. RAG-2+/- H-2b mice were originally generated by Shinkai and colleagues (17). These and B6 Ly5.2-congenic mice (following the original nomenclature used for derivation of congenic strains and mAbs [18]) were kindly provided by Dr. H. R. Rodewald, Basel Institute for Immunology, Basel, Switzerland. All animals were bred in a barrier-sustained facility and were free of common pathogens.

Preparation and Transfer of Cells. Cells from thymus, lymph nodes, and spleen were prepared by grinding organs through a stainless steel sieve into ice-cold balanced salt solution (BSS). To prepare intraepithelial lymphocytes, the small intestine was opened longitudinally in PBS, and Peyer’s patches were removed. Small pieces of the residual tissue were treated with Ca/Mg-free BSS/1 mM dithiothreitol for 20 min and with RPMI-1640/10% FCS for 40 min at 37°C. Filtered cells were enriched for lymphocytes by Percoll-gradient centrifugation. Hepatic mononuclear cells were prepared by injecting PBS into the portal vein to remove circulating cells from the liver before dissecting the animal. The tissue was then crushed and washed twice with BSS. Lymphocytes were enriched by Ficoll-gradient centrifugation. Bone marrow was ejected from femur and tibia of hind legs by injecting BSS. Bone marrow cells were washed twice with BSS. In the case of euthymic bone marrow donors, cells were further depleted of Thy-1+ cells by a magnetism-activated cell sorter (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). To reconstitute RAG-2-deficient mice, 2 × 108 BM cells, and for transfer into nu/nu mice, 5 × 107 lymph node and spleen cells (1:1), were injected intravenously.

Antibodies and Flow Cytometry. The following antibodies were used: PE-conjugated anti-CD4 (clone H129.19; Becton Dickinson & Co., Mountain View, CA), biotinylated or FITC-conjugated anti-CD8a (clone 53-6.7; Boehringer Mannheim GmbH, Mannheim, Germany), PE-conjugated anti-B220 (clone RA3-6B2; Serva, Heidelberg, Germany), and FITC-conjugated anti-αβ TcR (clone H57-597; PharMingen, San Diego, CA). FITC-conjugated anti-Ly5.1 (clone 104.2.1), and biotinylated anti-Ly5.2 (clone A20-1.7) (18) were kindly provided by Dr. H. R. Rodewald.

For two- or three-color flow cytometry, 2 × 10^5 cells in 0.1 ml PBS/0.02% sodium azide were stained with the biotinylated antibody for 15 min on ice, washed, and incubated for 15 min on ice with streptavidin red670 (Life Technologies GmbH, Eggenstein, Germany), the FITC-labeled mAb, and/or the PE-conjugated mAb. All antibodies were used at saturating concentrations. Flow cytometry was performed with a FACScan® flow cytometer using Lysys II software (Becton Dickinson & Co.). Results are shown as log-log dot plots or as histograms.

Results

Thymus Dependence of the IL-2 Deficiency Syndrome. Both the nu and the IL-2- mutation were bred to homozygosity into the C57BL/6 background. Athymic IL-2-deficient mice appeared healthy and, in contrast to their euthymic littermates, showed no increased mortality. Even at >6 mo of age, a time point where few euthymic IL-2-/- mice survive, none of the pathological alterations regularly observed in IL-2-/- euthymic mice were found.

As shown in Fig. 1 A, cellularity of spleens remained normal in athymic IL-2-/- mice, whereas their euthymic littermates developed severe lymphadenopathy (not shown) and splenomegaly, as previously described (5, 6). Moreover, the loss of splenic B cells (Fig. 1 B) and mature B cells from the bone marrow (Fig. 2), which follows initial polyclonal T and B cell activation in euthymic IL-2-/- mice, was not observed in athymic IL-2-deficient mice, suggest-

![Figure 1](image-url)
ing that uncontrolled activation of thymus-derived T cells is crucial for B cell depletion. Similarly, infiltration of the bone marrow with mature T cells, which is likely to play a role in the loss of B cells and development of anemia in euthymic IL-2−/− mice, did not occur in IL-2-deficient athymic mice (Fig. 2). Importantly, adoptive transfer of peripheral lymphocytes from IL-2−/− but not from IL-2+ C57BL/6 mice into athymic IL-2+ C57BL/6 mice, caused T cell infiltration of the bone marrow (Fig. 2) and loss of B cells from bone marrow and spleen (not shown).

One of the key symptoms of the IL-2 deficiency syndrome is inflammatory bowel disease with fatal outcome (6). The onset of colitis is preceded by an infiltration of the gut epithelium with CD4 T cells, which represent a minority among normal intestinal intraepithelial lymphocytes (IEL). None of the athymic IL-2−/− mice developed overt colitis, and even at the more sensitive level of T cell subset composition, no significant effects of the IL-2 mutation were observed (Fig. 3). As shown above for the bone marrow, transfer of peripheral lymphocytes from euthymic IL-2−/− but not from IL-2+ mice resulted in infiltration of the gut epithelium with CD4 T cells (Fig. 3).

In summary, development of the IL-2 deficiency syndrome depends on an intact thymus, indicating that destruction of B cells and bone marrow cells as well as development of colitis are initiated by thymus-derived T cells, and that thymus-independent T cell lineages do not mediate immune pathology if deprived of IL-2. Furthermore, thymus-independent cells expressing an intact IL-2 gene are unable to control the abnormal activation of thymus-derived IL-2−/− T cells, at least under the conditions of adoptive transfer used here.

**Intrahepatic T Cells in Athymic IL-2−/− Mice.** Aging athymic mice contain variable numbers of T cells in their peripheral lymphoid organs, probably because of the expansion of few T cell clones from a small number of precursors (13–16). Based on phenotypic similarities such as a reduced level of TCR expression between hepatic T cells of euthymic mice (19) and peripheral T cells found in athymic mice (20, 21), the liver has been proposed as a site of extrathymic T cell differentiation (19, 22). On the other hand, the absence of RAG transcripts in the liver and influx of pre-apoptotic activated peripheral T cells that acquire the phenotype of liver T cells has argued against this view and has suggested a role for the liver in the elimination of activated T cells (23, 24). When T cell subsets were compared in spleens and lymph nodes of IL-2+ and IL-2−/− C57BL/6 nu/nu mice, no differences beyond the individual variability also found in IL-2+ athymic mice were observed (data not shown). Analyses of mononuclear cells from the livers of athymic mice, however, revealed a clear-cut effect of IL-2

![Figure 2](image-url)

**Figure 2.** T cell infiltration and B cell destruction in the bone marrow of IL-2−/− deficient mice are thymus dependent. Flow cytometric analysis of bone marrow cells from IL-2+ and IL-2−/− euthymic (top) and athymic (center) C57BL/6 mice, and from IL-2+ athymic mice injected 6 wk previously with 5 × 10^7 peripheral lymphocytes from 4-wk-old IL-2+ or IL-2−/− C57BL/6 mice.
Thymus-dependent Initiation and Control of IL-2 Deficiency Syndrome

Figure 3. Thymus dependence of CD4 T cell infiltration in the epithelium of the small intestine of IL-2-deficient mice. Flow cytometric analysis of intestinal IEL from IL-2+ and IL-2/- euthymic (top) and athymic (center) C57BL/6 mice, and from IL-2+ athymic mice injected 6 wk previously with 5 x 10^7 peripheral lymphocytes from 4-wk-old IL-2+ or IL-2- C57BL/6 mice.

Table 1. Number of Intrahepatic Mononuclear Cells Isolated from the Livers of IL-2+ and IL-2- Euthymic and Athymic Mice

| Mouse          | Age | Mononuclear cells/ liver* |
|----------------|-----|---------------------------|
| C57BL/6 IL-2+  | 3   | 1.0 x 10^5                |
| C57BL/6 IL-2+  | 3   | 0.7 x 10^5                |
| C57BL/6 IL-2/- | 3   | 1.2 x 10^6                |
| C57BL/6 IL-2/- | 3   | 2.6 x 10^6                |
| C57BL/6 nu/nu IL-2+ | 22  | 1.4 x 10^5                |
| C57BL/6 nu/nu IL-2+ | 30  | 2.0 x 10^5                |
| C57BL/6 nu/nu IL-2/- | 22  | 6.0 x 10^5                |
| C57BL/6 nu/nu IL-2/- | 30  | 7.2 x 10^5                |

*Mononuclear cells were isolated as described in Materials and Methods, and viable cells were counted by trypan blue exclusion.
Discussion

The hallmark of the inflammatory and autoimmune manifestations of the mouse IL-2 deficiency syndrome is a hyperactivation of T cells, which precedes all other symptoms described above and, according to the present results, is likely to be the key event in the development of immune pathology. Thus, athymic mice carrying the IL-2 mutation remain free of bone marrow atrophy and red cell destruction, maintain normal B cell numbers, and do not develop colitis. Consequently, a direct effect of IL-2 deficiency on the survival of bone marrow stem cells and on the generation and maintenance of their B cell and erythrocyte progeny can be excluded. Furthermore, the thymus-independent T cell lineages originating in the gut epithelium do not mediate the inflammatory response found at that site in euthymic IL-2-deficient animals. In contrast, peripheral T cells transferred into nude mice from IL-2−/− donors infiltrate the gut and the bone marrow, supporting the conclusion that thymus-derived T cells are responsible for disease development.

The only difference detected in IL-2-deficient athymic mice compared with their IL-2+ littermates was a numeric increase in hepatic T cells that displayed the TCRint phenotype found on the majority of hepatic T cells in normal mice and on T cells in both lymphoid organs and liver of athymic mice. This increase was mainly caused by CD4−8+ cells, which were also severalfold expanded in the liver of euthymic IL-2-deficient mice compared with IL-2+ controls. We have previously reported that among the small subset of α/βTCRintIL-2RB+ thymocytes, which in normal mice are predominantly CD4−8+, IL-2 deficiency also causes a marked skewing toward the CD4−8+ phenotype (25). Such α/βTCRintIL-2RB+ T cells in the thymus and

Figure 4. Increase of TCRint CD4−8+ intrahepatic lymphocytes in both euthymic and athymic IL-2−/− mice. (A) TCR expression on mononuclear cells isolated from the lymph nodes (top) and liver (center) of 3-wk-old euthymic IL-2+ and IL-2−/− mice, and on intrahepatic mononuclear cells from 22-wk-old athymic IL-2+ and IL-2−/− mice (bottom). (B) Expression of CD4 and/or CD8 on TCR int intrahepatic lymphocytes. (C) CD4/CD8 distribution of all intrahepatic mononuclear cells.
Table 2. Phenotype of RAG-2<sup>−/−</sup> Mice Reconstituted with IL-2<sup>+</sup> and/or IL-2<sup>−/−</sup> Bone Marrow Cells

| Intact IL-2 gene in bone marrow | Splenomegaly/lymphadenopathy<sup>*</sup> | Red cell destruction<sup>‡</sup> | % T cells in bone marrow<sup>$</sup> | % CD4 in IEL<sup>$</sup> | Other |
|--------------------------------|--------------------------------|-------------------------------|----------------------|-----------------|-------|
| +                              | -                              | -                            | 3.1                  | 10.5            | -     |
| -                              | -                              | +                            | 9.3                  | 7.3             | -     |
| +                              | -                              | +                            | 6.2                  | 8.4             | -     |
| -                              | +                              | -                            | 3.0                  | 20.1            | Diarrhea |
| +                              | +                              | +                            | 36.2                 | 20.7            | -     |
| -                              | +                              | +                            | 30.0                 | 16.4            | -     |
| -                              | +                              | +                            | 52.9                 | 12.6            | -     |
| -                              | +                              | +                            | 18.3                 | 19.3            | -     |
| -                              | ?                              | ?                            | ?                    | ?               | Dead  |
| +,- (3:7)                      | -                              | -                            | 3.8                  | 8.4             | -     |
| +,- (3:7)                      | +                              | -                            | 4.8                  | 24.0            | -     |
| +,- (3:7)                      | -                              | +                            | 8.3                  | 18.4            | -     |
| +,- (3:7)                      | -                              | +                            | 5.0                  | 8.8             | -     |

RAG-2<sup>−/−</sup> mice were injected intravenously with a total of 2 × 10<sup>7</sup> bone marrow cells (for details see legend to Fig. 5) and analyzed 6 wk after transfer.

<sup>*</sup>More than twice the size of organs in age-matched C57BL/6 mice.<br>
<sup>‡</sup>Only white cells in bone marrow.<br>
<sup>$</sup>As determined by flow cytometry using fluorescent TCR- and CD4-specific mAbs.

α/βTCR<sup>+</sup> cells in the liver could either represent a distinct lineage or the end stage of clonally expanded cells before apoptosis. In the former scenario, the development of the CD4<sup>−</sup>8<sup>+</sup> subset of this lineage might be favored by the absence of IL-2; in the latter, IL-2 deficiency may retard the process of elimination of clonally expanded CD4<sup>−</sup>8<sup>+</sup> cells more severely than that of other T cells. This view is compatible with recent evidence showing that the CD4<sup>−</sup>8<sup>+</sup> α/β T cells present in the thymus (26) and those accumulating in Fas-deficient lpr/lpr mice are predominantly MHC class I dependent and, by implication, may be derived from the CD8 lineage (27-29). Furthermore, activated TCR-trans-
genic CD8 T cells downregulate CD8 before undergoing apoptosis in the liver (24). Thus, IL-2 deficiency may retard the conversion of activated CD8 T cells to CD4+/CD8+ T cells and their subsequent elimination by apoptosis, resulting in an accumulation of the intermediate CD8+/CD4+ TCRαβ phenotype in the liver and a shift from CD4+/CD8+ to CD4+CD8+ cells among IL-2Rαβ+ thymocytes.

The resemblance of the IL-2 deficiency syndrome to some features of GVHD has prompted an earlier search for a defect in negative repertoire selection in these animals (12). Neither central deletion of VB-defined T cell subsets by endogenous superantigens nor its acute induction by peptide administration in an MHC class I-restricted TCR-transgenic model, however, revealed any obvious effects of the IL-2 mutation. We thus favor the view that disease is initiated by an uncontrolled peripheral activation of T cells with a "normal" repertoire, rather than an increased escape of autoreactive T cells from intrathymic deletion. This uncontrolled activation is most likely driven by environmental antigens since athymic mice, which do contain functional T cells, albeit with a very restricted repertoire (13-16), do not develop disease. This conclusion is further supported by the delayed onset of inflammatory bowel disease in mice with a reduced antigenic environment (6).

As shown by cotransfer of IL-2−/− and IL-2+ bone marrow into RAG-2−/− mice, the simultaneous presence of lymphocytes with an intact IL-2 gene can greatly attenuate the development of the IL-2 deficiency syndrome. Therefore, IL-2−/− deficient mice either lack a specialized immunoregulatory cell population that depends on IL-2 for its development or function, or IL-2 provided in an autocrine or paracrine fashion is required for the termination of clonal expansion by apoptosis, as has been suggested by Lenardo (10). Indeed, recent results indicate that activated T cells from IL-2−/− deficient mice are more resistant to Fas-induced apoptosis than those from IL-2 wild-type mice (30). On the other hand, IL-2 could also be required for the induction of cytotoxic activity in CD8 T cells, which, as suggested by recent experiments by Jiang and colleagues (31), might eliminate clonally expanded CD4 T cells (which are the main population that expands in peripheral lymphoid organs of IL-2−/− deficient mice). Although antiviral CTL responses have been observed in IL-2−/− deficient mice (32), this seems to be a viable explanation since in a TCR-transgenic model, peptide immunization has revealed a stringent requirement for IL-2 in the induction of CTL effector function (12), raising the possibility that the proposed TCR-specific CTL responses required for the containment of CD4 T cell expansion are suboptimal in IL-2−/− deficient mice.

Whatever the mechanism by which IL-2R-mediated signals limit T cell activation in vivo, it seems unlikely from the present experiments that thymus-independent leukocyte populations are, by themselves, able to control the excessive activation of IL-2−/− deficient thymus-dependent T cells. Thus, despite the presence of NK cells and extrathymically derived T cells in athymic mice, IL-2−/− T cells initiated the characteristic symptoms of the IL-2 deficiency syndrome upon cell transfer. Further experiments will be aimed at identifying the subset of thymus-derived T cells with the most "curative" effect and to elucidate whether they contain T cell hyperactivation via paracrine IL-2 delivery or through the action of a specialized suppressor cell.

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