Challenging Cytokine Redundancy: Inflammatory Cell Movement and Clinical Course of Experimental Autoimmune Encephalomyelitis Are Normal in Lymphotoxin-deficient, but Not Tumor Necrosis Factor-deficient, Mice

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Summary

Lymphotoxin (LT) is widely regarded as a proinflammatory cytokine with activities equivalent to tumor necrosis factor (TNF). The contribution of LT to experimental autoimmune encephalomyelitis (EAE) was examined using TNF/LTα−/− mice, TNF−/− mice, and a new LTα−/− line described here. All mice were generated directly in the C57BL/6 strain and used for the preparation of radiation bone marrow chimeras to reconstitute peripheral lymphoid organs and restore immunocompetence. This approach overcame the problems related to the lack of lymph nodes that results from LTα gene targeting. We show here that when LT is absent but TNF is present, EAE progresses normally. In contrast, when TNF is absent but LT is present, EAE is delayed in onset and inflammatory leukocytes fail to move normally into the central nervous system parenchyma, even at the peak of disease. In the absence of both cytokines, the clinical and histological picture is identical to that seen when TNF alone is deficient, including demyelination. Furthermore, the therapeutic inhibition of TNF and LTα with soluble TNF receptor in unmanipulated wild-type or TNF−/− mice exactly reproduces these outcomes. We conclude from these studies that TNF and LT are functionally distinct cytokines in vivo, and despite sharing common receptors, show no redundancy of function nor mutual compensation.

Lymphotoxin (LT), like TNF, is considered to be a proinflammatory, cytotoxic cytokine (1) and critical mediator of lymphocyte-dependent autoimmune pathologies such as multiple sclerosis (MS) and the MS animal model, experimental autoimmune encephalomyelitis (EAE; for review see reference 2). Evidence for a key role of LT in MS and EAE comes from four types of experiments. First, LT is identified within the lesions of MS (3, 4) and EAE (5). Second, the encephalitogenicity of T cells is associated with their ability to synthesize LT (6–8), although this association is not absolute (9). Third, LT is toxic to oligodendrocytes in culture (10). Fourth, LT and TNF blockade prevents or ameliorates disease (11–13).

LT in its secreted form (LTα3) is thought to contribute to pathologies of this kind by its ability to bind to TNFR–IgG (14) and induce nitric oxide synthase; LT; lymphotoxin; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; RAG, recombinase activation gene; RT–PCR, reverse transcriptase PCR; TNFR–IgG, TNFR–human IgG fusion protein; WT, wild type. Radiation bone marrow chimeras: WT→WT, WT bone marrow transplanted into WT recipients; WT→RAG, WT bone marrow transplanted into RAG-1−/− recipients; LT→RAG, LTα−/− bone marrow transplanted into RAG-1−/− recipients; TNF→RAG, TNF−− bone marrow transplanted into RAG-1−/− recipients; TNF→TNF, TNF−− bone marrow transplanted into TNF−− recipients; TNF/LT→TNF, TNF/LTα−/− bone marrow transplanted into TNF−− recipients.

Abbreviations used in this paper: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ES, embryonic stem; iNOS, inducible nitric oxide synthase; LT, lymphotoxin; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; RAG, recombinase activation gene; RT–PCR, reverse transcriptase PCR; TNFR–IgG, TNFR–human IgG fusion protein; WT, wild type. Radiation bone marrow chimeras: WT→WT, WT bone marrow transplanted into WT recipients; WT→RAG, WT bone marrow transplanted into RAG-1−/− recipients; LT→RAG, LTα−/− bone marrow transplanted into RAG-1−/− recipients; TNF→RAG, TNF−− bone marrow transplanted into RAG-1−/− recipients; TNF→TNF, TNF−− bone marrow transplanted into TNF−− recipients; TNF/LT→TNF, TNF/LTα−/− bone marrow transplanted into TNF−− recipients.
question. In the latter two studies using the EAE disease model, opposite conclusions have been drawn about the role of LT. In the first of these (24), it was argued that neither LT nor TNF play any role in EAE, and in the second (25), that secreted LT α is critical in the pathogenesis of this disease. The direct use of LT or LT β gene-deleted mice for studies of immune pathology, however, is compromised by the immune deficiencies that follow, for example, the absence of peripheral lymph nodes in these mice (20, 26). The fact that control animals in these two studies had normal immune systems necessarily implies that any differences observed in experimental outcomes between wild-type (WT) and LT-negative mice cannot be attributed to the activities of the cytokine alone. The difficulty in interpretation of these experiments is compounded by the fact that the TNF, LT α, and LT β genes are located within the MHC (27). The backcrossing of 129 strain gene-deleted mice onto EAE-susceptible strains, such as SJL or C57BL/6, would create partial chromosome 17 congenics that differ from WT controls in this fundamentally important disease susceptibility locus (28). For these reasons, we regard the question of the relative contribution of LT to EAE pathogenesis to be unresolved.

We have disrupted the TNF and LT α genes directly in C57BL/6 mice (29), a strain that is highly susceptible to EAE induced by immunization with the 35–55 peptide of myelin oligodendrocyte glycoprotein (M O G). This avoids the problem of genetic heterogeneity introduced by backcrossing. In the first series of experiments using TNF−/− mice (23), direct immunization was possible because peripheral immunity in these mice was essentially intact. The clinical course and pathological changes of EAE in TNF−/− mice were remarkable, revealing key activities for this cytokine in the initiation of inflammatory lesions and the control of leukocyte movement within the central nervous system (CNS).

To extend these experiments to the role of LT in EAE, the model of immunocompetence had to be accounted for. Unlike TNF, LT is predominantly, if not exclusively, a cytokine in the initiation of inflammatory lesions and the control of leukocyte movement within the CNS autoimmune inflammatory lesions (6, 8). Bone marrow cells derived from LT−/− mice have the capacity to repopulate lymph nodes in irradiated recipient animals (31). Therefore, chimeras may be generated that are LT deficient, but with a reconstituted functional immune system.

In a second series of experiments reported here, bone marrow cells from a newly generated LT−/− C57BL/6 strain (see Results), in combination with previously described TNF−/− and TNF/LT−/− C57BL/6 mice (29), were used to reconstitute peripheral lymphoid structures in lethally irradiated recipient animals. This enabled the application of a standard EAE induction protocol to all mice and the study of the activities of LT both in the presence and absence of TNF. Furthermore, inhibition of TNF and LT by a soluble TNF-human IgG fusion protein (TNF−IgG) in WT and TNF−/− mice represents the first direct comparison of the effects of gene targeting and therapeutic inhibition in a single disease model. Using these experimental systems, we reveal the lack of any unique contribution by LT to clinical manifestations of disease, CNS inflammation, or demyelination. In addition, we demonstrate that the profound role of TNF in the control of normal inflammatory cell movement within the CNS (23) is not shared by LT.

Materials and Methods

Animals. C57BL/6.TNF−/−, C57BL/6.TNF/LT−/− (29), and C57BL/6.LT−/− mice were generated and bred in-house under specific pathogen-free conditions (Centenary Institute, Sydney, Australia). WT C57BL/6 (Ly5.2), C57BL/6.Ly5.1, and C57BL/6.6R AG-1−/− (RAG, recombine activation gene) mice (32) were obtained from Animal Resource Centre (Perth, Australia). Adult (> 6 wk old) female mice were used in all experiments. All animal procedures were approved by the Animal Care and Ethics Committee of the University of Sydney (Sydney, Australia).

Generation of C57BL/6 Strain LT−/− Mice. A Dra I fragment encompassing the genes encoding for LT α and TNF was derived from a C57BL/6 genomic DNA clone (Fig. 1 A) comprising TNF, LT α, and LT β (33; obtained from C. V. Jongeneel, Ludwig Institute for Cancer Research, Lausanne, Switzerland), and cloned into the Smal site of pUC 19 (GIBCO BRL, Paisley, UK). A neo-ycin resistance cassette was inserted in sense direction into an unique Apal site in exon 2 of the LT α gene to create the targeting vector (Fig. 1 A), with total length of homology of 6,580 bases (1,549 bases 5’ homology; 5,031 bases 3’ homology). The C57BL/6 embryonic stem (ES) cell line Bruce 4 (provided by F. Köntgen, Walter and Eliza Hall Institute, Melbourne, Australia; reference 34) was cultured on neomycin resistance embryonic fibroblasts (35), and transfected, cloned, and screened by nested set PCR following standard procedures (36). LT α−/− C57BL/6 ES cells were injected into recipient BALB/c blastocysts matured in vitro (37), and embryos were reimplanted into pseudopregnant (C57BL/6 × BALB/c) F1 foster mothers. All chimeric animals were mated to C57BL/6 partners and germ line transmission was detected by coat color. The presence of the targeted allele was confirmed by Southern blot (Fig. 1 B). The establishment of LT−/− mouse line was maintained on a pure C57BL/6 background.

Southern Blot. Nucleated set PCR, and Reverse Transcriptase PCR. Genomic DNA was purified from ES cells according to standard procedures (38) or from white blood cells using a genomic DNA kit (Promega Corp., Madison, WI). To detect homologous recombination, nested set PCR was performed with an outer primer pair (5’ sense: CTA GGA CAG GGT TCT CAA CCT TCC T; 3’ antisense: CCA GTC CTC CTT CCC GGC TTC AGT GAC AAC GTC; 20 cycles) followed by another round of amplification with an inner primer pair (5’ sense: CAG TTT CTG ATG TTT TGG TGA CCC; 3’ antisense: CCG ACT GCA TCT GCC GTG TGC A; 35 cycles). The annealing temperature was 60°C for both primer pairs. The Southern blot strategy used the introduction of an additional PsI site into the genome within the neomycin resistance cassette (29). Successfully targeted alleles showed a fragment length of 6.4 kb (WT 9.4 kb; Fig. 1 B). For analysis of cytokine mRNA transcripts, total RNA was extracted (39) from PMN-stimulated splenocytes (5 ng/ml for 12 h) or whole unfused brain and spinal cord. oligo(dT) 12–18 (Boehringer Mannheim GmbH, Mannheim, Germany) primed cDNA was syn-
thesized and analyzed by reverse transcriptase PCR (RT-PCR) using primers and methods as previously described (29). The PCR products were size fractionated by electrophoresis on 1.2% agarose gel containing ethidium bromide.

Induction of EAE. EAE was induced actively by subcutaneous tal base injection of 50 \( \mu \)g of M OG peptide (35-M EVGWY RSPSR VHVLY RNK-55) in CFA containing 1 mg of heat-inactivated Mycobacterium tuberculosis (H37RA; DIFCO Labs., Detroit, MI). 200 ng of pertussis toxin (LIST Biological Labs., Inc., Campbell, CA) was injected intravenously on days 0 and 2. This disease induction protocol was optimized in WT C57BL/6 mice by pertussis toxin and M OG peptide dose-ranging experiments. Animals were observed daily and neurological deficits were quantified on an arbitrary clinical scale: 1, normal; 2, hind limb weakness or abnormal gait; 3+, severe hind limb weakness with loss of ability to right from supine; 4+, hind quarter paralysis; 5+, forelimb weakness or moribund; 6+, death. Supplementary food and water were provided on the cage floor for disabled animals.

Generation of Radiation Bone Marrow Chimeras. Bone marrow cells, harvested from the long bones of matched donor mice by flushing with cold PBS, were gently disaggregated through 70 \( \mu \)m nylon cell strainers (Becton Dickinson, Franklin Lakes, NJ), washed, and counted. Recipient animals were preconditioned with 5.5 Gy gamma radiation on day 2, and again on day 0. Bone marrow cells (2 \( \times \) 10\(^6\) cells/recipient) were injected intravenously on day 0. Cage water was supplemented with trimethoprim (50 \( \mu \)g/ml)–sulphamethoxazole (0.25 mg/ml) between weeks 2 and 3 after transplantation. As a means of tracking engraftment, reciprocal transplantations between TNF\(^{a/2}\) (C57BL/6.Ly5.2) and C57BL/6.Ly5.1 mice were performed. Peripheral blood was drawn from recipients after transplantation and analyzed by flow cytometry for the presence or absence of the Ly5.1 (CD45.1) congenic marker on leukocytes. Engraftment was considered satisfactory when in excess of 95% of circulating leukocytes expressed the CD45 marker of donor type.

Antibodies. The origins of mAbs specific for mouse CD4, Mac-1 (CD11b), vascular cell adhesion molecule 1 (VCAM-1), CD45, TCR-\(\alpha\)/\(\beta\), and isotype-matched control antibodies are detailed elsewhere (23). Other mAbs were rat anti-\(I^A\)-\(\alpha\) (M 5/114.15.2; reference 40) provided by G. Halliday (University of Sydney, Sydney, Australia), rat anti-mouse B220-PE (RA3-6B2; Caltag Labs., South San Francisco, CA), and biotinylated mouse anti-mouse Lys.1 (A20; reference 41) detected by Streptavidin-FITC (Pharmingen, San Diego, CA). Polyclonal rabbit anti-inducible nitric oxide synthase (anti-iNOS; Upstate Biotechnology, Inc., Lake Placid, NY) and rabbit IgG control (Vector Labs., Burlingame, CA) antibodies were used in immunohistochemical studies.

MOG-specific IgG determination. Relative serum IgG anti-MOG 35-55 responses were quantified by ELISA as described (23).

Flow Cytometric Analysis. Flow cytometric analysis of bone marrow engraftment in chimeras was performed on peripheral blood leukocytes after red blood cell lysis. Cells were incubated with biotinylated anti-Ly5.1 mAbs and one of the following directly PE-conjugated mAbs anti-B220 (B cells), anti-TCR-\(\alpha\)/\(\beta\) (T cells), anti-Mac-1 (monocytes, granulocytes, and N K cells), or their appropriate controls. Ly5.1+ cells were identified using a second step incubation with Streptavidin-FITC. CNS-associated leukocytes were isolated from collagenase/DNase-digested whole CNS tissue after heparin-saline perfusion of animals, and Percoll gradient density purification steps were performed as described (42). Purified cells were incubated with directly conjugated anti-CD45-PE and anti-TCR-\(\alpha\)/\(\beta\)-FITC antibodies, or appropriate controls. Flow cytometric data was acquired on a FACScan (Becton Dickinson, San Jose, CA) and analyzed using CELLQuest software (Becton Dickinson, San Jose, CA) software.

Immunohistology and Neuropathology. Dissected specimens of nonperfused whole brain were embedded in OCT compound (Tissue Tek, Miles, Inc., Elkhart, IN), frozen in liquid nitrogen vapor, and stored at –70°C until use. Serial cryostat sections (6 \( \mu \)m) were stained with various mAbs via the immunoperoxidase technique, and counterstained with hematoxylin (23). Sections were photographed using transmitted light interference contrast optics. Demyelination studies were performed on CNS tissue obtained from animals perfused with warm PBS followed by 4% paraformaldehyde and 2.5% glutaraldehyde in PBS, and processed as previously described (23). Sections were examined and photographed using standard bright-field optics.

Treatment of Mice with TNFR-IgG. The activities of LT\(\alpha\) and TNF were inhibited in vivo by injection of a recombinant fusion protein consisting of the mouse p55 TNFR–IgG (provided by J. Ghrayeb and B. Scallon, Inc., Centocor, Malvern, PA). Molecules of this type have been shown to bind and inhibit mouse secreted LT\(\alpha\) (43) and TNF (44). TNFR-IgG was delivered at various time points throughout the course of EAE by intraperitoneal injection at a dose of 500–1,000 \( \mu \)g/injection. PBS or equivalent amounts of human IgG (protein A affinity purified from normal serum) were used as controls.

Results

Generation of C57BL/6 LT\(\alpha^{-/-}\) Mice. The injection of one targeted C57BL/6 ES cell clone into blastocysts of the BALB/c strain resulted in the birth of chimeric mice, all of which transmitted through the germ line after mating with C57BL/6 mice. Founder heterozygous mice were chosen, interbred, and screened by Southern blot. LT\(\alpha^{-/-}\) mice were born in the expected Mendelian ratio of 1:2:1 and were sustained as a homozygous line on the C57BL/6 background (Fig. 1 B). The successful disruption of the gene encoding LT\(\alpha\) was confirmed by RT-PCR. LT\(\alpha\)-specific mRNA was not detectable in PMA-activated splenocytes (Fig. 1 C), whereas expression of TNF mRNA was readily detected. By contrast, LT\(\alpha\) transcripts were detected in stimulated cells from TNF\(^{-/-}\) mice, but neither TNF nor LT\(\alpha\) transcripts from TNF/LT\(\alpha^{-/-}\) mice (Fig. 1 C). LT\(\alpha^{-/-}\) mice bred well and were grossly normal. Closer inspection of the lymphoid organs showed the expected absence of peripheral lymph nodes and Peyer’s patches, a disturbed splenic architecture, and peripheral blood lymphocytosis (20, 21, data not shown). LT\(\alpha^{-/-}\) mice lack both secreted LT\(\alpha\) and membrane LT\(\alpha\)\(\beta\) forms of LT because functional surface expression of LT\(\beta\) depends on the presence of LT\(\alpha\) (17).

The Course of EAE in WT C57BL/6, TNF\(^{-/-}\), and TNF/LT\(\alpha^{-/-}\) Mice. Subcutaneous immunization of WT C57BL/6 mice with 50 \( \mu \)g of MOG 35–55 in CFA, and pertussis toxin intravenously on days 0 and 2, resulted in a highly reproducible severe acute meningoencephalomyelitis, with clinical signs of rapidly progressing, ascending symmetrical motor deficits, appearing on or around day 10. Clinical disease was present for ~15 d and spontaneously...
PMA-stimulated splenocytes from WT, LTα2/2. RT-PCR confirms absence of LTα version of the recipient hemopoietic compartment to do-and large bone marrow inoculum to ensure complete con-

Table 1, remitted (Fig. 2) to leave a mild, chronic, nonrelapsing deficit (approximately disease score 1). Disease in TNF/−/− mice (Fig. 2) was delayed in onset by ~6 d. Once established, however, clinical deficits in these mice progressed in a parallel course to an equivalent mean peak severity, but were of a reduced overall duration when compared with WT, as we have previously described (23).

C57BL/6 TNF/LTα−/− mice, which lack peripheral lymph nodes and exhibit a range of immune deficiencies (29, 45), were entirely resistant to clinical disease induction by our standard immunization protocol (Fig. 2). Similarly, immunization of RAG1−/− mice, which lack mature lymphocytes, also resulted in no clinical syndrome (Fig. 2 and Table 1, A–C). Thus, disease resistance in TNF/LTα−/− mice could indicate either a critical role for LT, or the consequences of a broadly deficient immune system as exemplified by the RAG-1−/− mice. Determination of the role of LT in EAE required the reconstitution of immunocompetent peripheral lymphoid organs by other means.

Two types of radiation bone marrow chimeras were generated. The first involved the transfer of WT or LTα−/− bone marrow to RAG-1−/− mice (WT→RAG, LT→RAG). This chimeric system tested whether the absence of both forms of LT, but the presence of TNF, affected susceptibility to EAE. The absence of lymphocytes in RAG-1−/− mice guaranteed that any T or B cells in these chimeras were LT negative. These mice were used for EAE studies 8 wk after bone marrow reconstitution. A further chimera, generated by the transfer of TNF−/− bone marrow to RAG-1−/− recipients (TNF→RAG), was used as a control for the CNS cytokine mRNA expression analysis (see below).

The second series of chimeras involved the transfer of TNF/LTα−/− bone marrow (completely deficient in both TNF and LT) to TNF−/− recipient mice (TNF/LT→TNF) or appropriate controls (WT→WT, TNF→TNF). This arrangement tested how the absence of both TNF and LT affected susceptibility to EAE and, in particular, if LT was responsible for the EAE observed in TNF−/− mice (23). Each type of radiation bone marrow chimera was examined for the presence of lymphoid tissue after experimental use. As expected, lymph nodes were repopulated in all mice (not shown).

The efficacy of the irradiation and reconstitution protocol for engraftment was examined in parallel using a CD45 congenic marker (Ly5.1.WT→TNF, and TNF→Ly5.1.WT) and serial flow cytometric analyses of peripheral blood leukocytes (Fig. 3 A). At the time of immunization, in excess of 95% of circulating α/β-TCR +, B220 +, and CD11b + (Mac-1) cells were of donor type. Importantly, engraftment proceeded irrespective of the TNF status of the donor or recipient (Fig. 3 A).

Antibody responses in chimeric mice have been measured, serum samples were col-
lected from WT → WT, TNF→TNF, and TNF/LT→TNF mice at day 23 after immunization with MOG 35–55/ CFA/pertussis toxin and were assayed by ELISA. Peptide-specific IgG responses were observed in all mice (Fig. 3 B) with responses in TNF/LT-deficient mice at least as great as in WT mice.

EAE Course in Mice Lacking LT Alone. WT → RAG and LT → RAG mice were immunized according to the standard protocol. Disease onset, rate of progression, and peak severity were equivalent in both groups (Fig. 3 C and Table 1 D) indicating no unique role for LT in the clinical neurological deficit. Disease course is illustrated up to day 22 only, due to the ethical requirement to kill severely affected animals. The day 15 onset of disease in both groups (rather than day 10 in unmanipulated animals; see Fig. 2) is probably due to the use of these mice only 8 wk after transplantation when T cell reconstitution to normal levels may not have occurred (see Fig. 3 legend). Irrespective of the time of use, all lymphocytes in these chimeras were of donor type.

EAE in Chimeras Lacking Both LT and TNF. To explore the possibility that the activity of LT in EAE was obscured by the compensatory influence of normal TNF expression in Fig. 3 C, the role of LT was examined in a TNF-deficient system (TNF/LT→TNF). The clinical course of disease in WT→WT and TNF→TNF mice followed that predicted by previous experiments (Fig. 3 D and Table 1 E), reproducing the delay in disease onset seen in Fig. 2 and validating this experimental approach. Remarkably, TNF/LT→TNF mice followed a course of disease that was indistinguishable from TNF→TNF mice, with no augmentation of the delay in onset, normal slope of the disease progression curve, and normal peak severity of disease (Fig. 3 D). Of those mice allowed to continue on to recovery, a normal resolution of clinical deficits occurred (data not shown). This result excluded the possibility that an important activity of LT in the course of EAE in the LT→RAG experiment (Fig. 3 C) was obscured by the compensations of TNF.

Table 1. Summary of Experimental Outcomes

| Label | Mice (all C57BL/6*) | Treatment | Control | LN and Ig status | Purpose | Disease course compared to control | Figure |
|-------|---------------------|-----------|---------|------------------|---------|----------------------------------|--------|
| A     | TNF−/−              | −         | WT      | Intact           | Absent TNF | Delayed                          | 2      |
| B     | TNF/LTα−/−          | −         | WT      | LN absent, Ig deficient; references 20, 29, 45 | Absence of LTα and TNF | Not susceptible to EAEi | 2      |
| C     | RAG-1−/−            | −         | WT      | LN anlage only, Ig deficient; reference 32 | No mature T or B cells | Not susceptible to EAEi | 2      |
| D     | Chimera: LT→RAG     | −         | WT→RAG | R reconstituted | LT deficiency in TNF-competent mice, with reconstituted immunity | Unaltered | 3 C |
| E     | Chimera: TNF/LT→TNF | −         | WT→WT or TNF→TNF | R reconstituted | Deficiency of both LT and TNF, with reconstituted immunity | Delayed, equivalent to the absence of TNF alone | 3 D |
| F     | WT                  | TNFR−IgG | WT treated with PBS or human IgG | Intact | Inhibition of TNF and LTα3 | Unaltered | 6 A |
| G     | TNF−/−              | TNFR−IgG | TNF−/− treated with PBS | Intact | Inhibition of LTα3 in the absence of TNF | Unaltered | 6 C |

†TNF−/−, TNF/LTα−/−, and LTα−/− mice were generated by gene targeting directly in C57BL/6 ES cells.
‡Lymph node status.
§Serum immunoglobulin status. See also Fig. 3 B.

iNot susceptible to MOG 35-55-induced EAE using our standard disease induction protocol, optimized in WT C57BL/6 mice.

CNS LT and TNF mRNA Expression at the Peak of Disease Severity. The use of the Ly5 markers established the validity of this approach for effective reconstitution of the hemopoietic compartment in recipients. The most rigorous determination of cytokine expression within the CNS itself is RT-PCR analysis, and this was applied to mRNA extracted from inflamed CNS tissue (Fig. 4). LTα message was abundantly expressed in the CNS of all EAE-affected animals with the exception of the TNF/LT→TNF and LT→RAG chimeras, consistent with the hemopoietic origin of this cytokine. Likewise, LTα expression was seen in...
the unperfused normal WT CNS (Fig. 4), but not in perfused WT tissue (data not shown). TNF mRNA expression was clearly evident in the CNS of all experimental groups, with the exception of the TNF→TNF and TNF/LT→TNF chimeras (data not shown). As expected, abundant expression of LTα was found in the TNF→RAG chimera (Fig. 4), but also abundant TNF message (data not shown), consistent with TNF production by nonhemopoietic, radiation-resistant CNS structures, such as microglia and astrocytes (47). Similarly, a trace amount of LTα message was sometimes observed in the CNS from TNF/LT→TNF and LT→RAG chimeras at the peak of disease (Fig. 4), most likely due to low level LTα production by activated glia (47). It is improbable that LTα expression of this nature is significant within the terms of these experiments, an assertion supported by further studies using TNFR–IgG (see below).

Inflammatory Cell Recruitment to the CNS in the Absence of TNF and LT. The influence of individual cytokines on the number of inflammatory cells recruited to the entire CNS was examined by flow cytometric analysis of leukocytes extracted from the whole perfused CNS of chimeras (Fig. 5, A and B). The magnitude of inflammatory cell recruitment was comparable in WT→WT, TNF→TNF, and TNF/LT→TNF chimeras (Fig. 5 A). Immunophenotyping was then performed to evaluate the possibility that TNF or LT influenced the relative proportions of individual inflammatory cell subsets. Fig. 5 B shows that, in the absence of TNF, there was a relative increase in the proportion of α/β+ TCR+ cells as a percentage of total CD45+ cells, comprising populations of monocyte–macrophages, T cells, and microglia (42). Most importantly, differences were not observed between the TNF→TNF and TNF/LT→TNF chimeras (Fig. 5 B, middle and right) indicating no additional alteration of the infiltrate imposed by the absence of LT.

Neuropathological Studies at Peak of Disease Severity. To examine the influence of TNF and LT on the formation of CNS inflammatory lesions, immunohistochemical studies using CD45 staining of cells within CNS tissue were performed (Fig. 5, C–F). In all specimens, large numbers of CD45+ cells could be identified at the peak of disease. Lesions within the CNS of WT→WT chimeras exhibited the normal appearance of cells moving freely from the vessel lumen into the perivascular space, and beyond into the CNS parenchyma (Fig. 5 C). In contrast, the perivascular cuffs of TNF→TNF and TNF/LT→TNF chimeras (Fig. 5 D and E) showed a striking perivascular congestion of cells. Significantly, the diffuse appearance of lesions within the CNS of LT→RAG chimeras (Fig. 5 F) clearly indicated that this role is not shared by LT. Moreover, despite abundant LT expression in the TNF→TNF mice (Fig. 4), normal cell movement within the CNS parenchyma failed...
Figure 5. Characterization of CNS inflammation. (A) Total numbers of leukocytes extracted from the perfused whole CNS at day 23. All animals were matched for disease score of 5/6 at the time of analysis. Each bar represents a single animal. WT → WT (black bar), TNF → TNF (white bars), and TNF/LT → TNF (hashed bars). Horizontal line, typical number of leukocytes extracted from the normal, perfused CNS of the C57BL/6 mouse. (B) T cell inflammation in the absence of TNF or TNF/LT. CNS-associated leukocytes were derived from individual animals as in A. Cells were stained for CD45 and TCR-α/β, and all CD45-negative cells were excluded by gating. Histograms show TCR-α/β expression of CD45+ cells. (C–F) Altered nature of inflammatory infiltrate in TNF→TNF mice. Cryostat sections of spinal cord from indicated chimeras were stained for CD45 to reveal all inflammatory cells. All animals were matched for disease (score 5/6) at the time of harvest of CNS tissues. (G) Histopathological study using toluidine blue staining of spinal cord from TNF/LT→TNF chimeric mice. Tissue taken at day 40 after immunization is shown. Demyelinated fibres, arrows; lipid-laden macrophages ("glitter" cells), arrowheads. Bars: (C–F) 120 μm; and (G) 60 μm.
to occur (Fig. 5D) in the absence of TNF. No alteration in the magnitude or distribution of cells expressing MHC class II, MAC-1 (CD11b), CD4, INOS, or vascular cell adhesion molecule 1 could be observed in the absence of LT (data not shown). The normal expression of INOS in the TNF/LT→TNF brain indicated that the activation of this critical macrophage effector was not dependent on the activities of either TNF or LT (not shown).

A neuropathological study of fixed tissues from TNF/LT→TNF CNS at day 40 demonstrated clear primary demyelination, despite the lack of both TNF and LT (Fig. 5G). Lipid-filled glitter cells (phagocytic macrophages), which are pathognomonic of inflammatory demyelinating disease, were also present.

Collectively, these findings confirm normal histopathological features of EAE, despite the lack of LT. The abnormal features described above are explained by the absence of TNF alone.

TNFR-IgG Alters the Course of EAE in WT, but Not TNF−/−, Mice. The effect of TNF and LT α3 blockade on EAE susceptibility in otherwise unmanipulated WT mice was examined. Very high doses of TNFR-IgG administered immediately before the onset of clinical neurological deficits resulted in a delay in the onset of disease when compared with animals treated with PBS (Fig. 6A and Table 1F), in a manner that was analogous to findings in the TNF−/− animals (Fig. 2). Similarly, the rate of disease progression and the eventual peak severity of disease was not affected by treatment, even when treatment was continued through the emergence of clinical signs. This effect depended exclusively on the TNFR component of the fusion protein (Fig. 6B).

As an alternative means of studying the activities of LT α3 in a TNF-deficient system, LT α3 was blocked in TNF−/− mice by administration of TNFR-IgG (Fig. 6C and Table 1G). Disease onset and disease severity in both human IgG control- and TNFR-IgG–treated mice was identical, and although some divergence of the disease curves was observed, there was no indication that LT α3 inhibition influenced its course. Again, most mice were killed at the peak of disease to conform with ethical requirements. Mice from both groups that were followed through to disease resolution progressed similarly.

Discussion

It is widely accepted that the secreted LT α3 or cell membrane (LT α3β2) forms of LT exhibit TNF-like properties, particularly since LT α3 can bind to the TNFRs (14). A feature common to gene-targeted mice lacking TNF (29, 48), LT α2 (20), or LT β (26), or of mice in which the action of these cytokines is blocked during gestation (49), is that all exhibit changes in lymphoid structures. Nevertheless, the magnitude and precise nature of anatomical changes in TNF−/− versus LT α−/− or LT β−/− mice are largely nonoverlapping, indicating distinct in vivo functions for these molecules. The results of this study, in which the roles of LT and TNF in autoimmune inflammation were compared, are entirely consistent with the view that TNF and LT are functionally distinct entities in vivo.

In these studies, the clinical and pathological course of EAE was influenced in a striking way by the absence of TNF (Fig. 2 and Table 1A), an outcome that appears to be
explained by the abnormal movement of inflammatory cells within the target tissue early in the clinical course (23). This phenotype is not due to the failure of inflammatory cell recruitment per se, because normal numbers of CD45+ cells can be extracted from the perfused whole CNS of mice lacking TNF at any given time point after immunization (Fig. 5 A; reference 23). TNF-dependent cell adhesion events are not, therefore, a satisfactory or complete explanation. The importance of TNF in the control of cell movement within the tissue is supported by the observation that even when disease is established in TNF-deficient mice, leukocytes fail to move freely from a perivascular location into the CNS parenchyma (compare Fig. 5 C [WT→WT] with Fig. 5 D [TNF→TNF]). This consolidates the impression that the normal formation of the inflammatory lesion depends upon an activity of TNF on the abluminal side of the CNS vascular endothelium as suggested previously (23). Possible mechanisms to explain this effect include a role for TNF in the generation of chemotactic gradients (50), in the digestion of the extracellular matrix (51, 52), or in the final effector phase activation of the recruited inflammatory cells. The relative reduction in the proportion of non-T inflammatory cells in mice lacking TNF (Fig. 5 B) or in rats treated with TNFR-IgG (12) may also indicate that the T cell is less dependent on TNF for normal tissue infiltration. Irrespective of these considerations, primary demyelination, which is the characteristic end point of the pathological process and which depends upon many of the coordinated processes of acquired immunity including antibody production, proceeds without inhibition in the absence of TNF (Fig. 5 G; reference 23).

Clearly, LTα could not compensate for the abnormal course of disease in TNF−/− mice (Fig. 2), although it was possible that the emergence of disease, when it occurred, was indeed a LT-dependent process. To pursue this further, we adopted a combination of approaches, the results of which are summarized in Table 1. The use of direct immunization in LT-deficient mice was precluded by the failure of these mice to respond to a standard disease induction protocol (Fig. 2 and Table 1 B), an explanation being the absence of peripheral lymphoid organs. Thus, we sought to examine the role of LT in this model by the use of gene-targeted radiation bone marrow chimERIC mice.

The first set of experiments (LT→RAG; Fig. 3 C and Table 1 D) demonstrated that LT has no unique role in the clinical manifestations of disease. It was possible, however, that TNF expression compensated for the absence of LT in this experiment. It would follow, therefore, that in a TNF-deficient mouse, the role of LT might become apparent, although such an activity would then be regarded as fully redundant to TNF. Thus, a second set of studies was performed using TNF/LT→TNF chimeras (Fig. 3 D and Table 1 E). These experiments showed that the absence of LT had no additional influence on the clinical course of EAE in TNF-deficient mice. The concept that TNF and LT have redundant functions in autoimmunity therefore fails to be supported by these observations, which have been made despite the knowledge that both cytokines are abundantly expressed in the inflammatory lesion (Fig. 4 and text) and share common receptor binding (14). Although LT(β2-LTβR) interactions do seem to elicit a cytotoxic signal (22), it seems likely that the importance of the LTα3–TNF interaction in cytotoxicity in the mouse has been overestimated (43). The use of LT(−/−) mice in this study, which lack both the secreted and membrane forms of LT, indicates that neither plays an indispensable role in the processes leading to clinical manifestations of EAE.

The ability of therapeutic inhibition of TNF and LT to modulate, and in some cases ablate, the clinical manifestations of EAE is well described (11–13) and has represented the most persuasive source of experimental evidence for a key role for these molecules in the pathogenesis of CNS autoimmune inflammation. In the Lewis rat, inhibition of TNF and LTα3 resulted in abrogation of disease, but did not impede T lymphocyte accumulation within the CNS (12, 53). The results obtained after the administration of TNFR-IgG to WT C57BL/6 mice (Fig. 6 A and Table 1 F) precisely reproduced the clinical outcome observed in TNF−/− mice (Fig. 2 and Fig. 3 D). Differences in the extent of disease inhibition by TNFR-IgG between other models and MOG 35-55-induced EAE in C57BL/6 mice may be due to the severity of the clinical disease and particularly the development of substantial primary demyelination in the MOG model. These findings reinforce the need for a reevaluation of the influences of TNF inhibition in other autoimmune disease models, particularly with respect to the nature of the inflammatory process.

In summary, once factors such as immune competence and genetic heterogeneity are accounted for, EAE in the absence of LT progresses in a manner that is indistinguishable from controls, as measured by the clinical manifestations of disease, the generation of inflammatory lesions, and specific target damage in the form of demyelination. On the other hand, the lack of TNF alone results in profound changes in the movement of leukocytes within the CNS (Fig. 5) and LT appears unable to compensate for its absence (Fig. 4 and Fig. 5 D). These findings do not support the concept that LT is an independent mediator of acute autoimmune inflammatory processes. Therefore, the purpose of abundant LT expression within these lesions is unexplained. The capacity of transgenic overexpression of LTα to generate organized ectopic lymphoid structures within tissues (54), raises the possibility that LT establishes the cellular architecture of chronic inflammatory lesions. Further studies investigating the role of LT in the late phases of autoimmune inflammation are warranted.

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