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

By D. Sean Riminton,* Heinrich Körner,* Deborah H. Strickland,* Frances A. Lemckert,* John D. Pollard,‡ and Jonathon D. Sedgwick*

*From the *Centenary Institute of Cancer Medicine and Cell Biology, Sydney, New South Wales 2050, Australia; and ‡Department of Medicine, University of Sydney, Sydney, New South Wales 2006, Australia*

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)[1], 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 ($LT\alpha_3$) is thought to contribute to pathologies of this kind by its ability to bind to TNFR1 and TNFR2 (1, 14), resulting in the promotion of inflammation by the upregulation of endothelial adhesion molecules (15) and the delivery of cytotoxic signals to target cells (16). Furthermore, LTα in association with the related cell surface molecule LTβ ($LT\alpha_1\beta_2$; references 17, 18), binds the LTβ receptor (19) in a system found to be essential for normal peripheral lymphoid development (20, 21), and is capable of delivering a cytotoxic signal to target cells (22).

Until recently, the means to test the relative contributions of LT and TNF to inflammatory processes have not been available. TNF (23), TNF/LTα (24), LTα, and LTβ (25) gene-targeted mice have now been used to address this

[1]*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.

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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 $LT\alpha_3$ is critical in the pathogenesis of this disease. The direct use of LTα or LTβ 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, LTα, and LTβ 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 LTα 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 (MOG). 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 problem of immunocompetence had to be accounted for. Unlike TNF, LT is predominantly, if not exclusively, a product of leukocytes (14) and particularly, Th1 T cells (30). It is LT produced from these hemopoietically derived sources that is thought to be crucial in CNS autoimmune inflammatory lesions (6, 8). Bone marrow cells derived from $LT\alpha^{-/-}$ 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 $LT\alpha^{-/-}$ C57BL/6 strain (see Results), in combination with previously described $TNF^{-/-}$ and $TNF/LT\alpha^{-/-}$ 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 $LT\alpha_3$ by a soluble TNFR–human IgG fusion protein (TNFR–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.$TNF/LT\alpha^{-/-}$ (29), and C57BL/6.$LT\alpha^{-/-}$ 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.RAG-$1^{-/-}$ (RAG, recombinase 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 $LT\alpha^{-/-}$ Mice. A DraI fragment encompassing the genes encoding for LTα and TNF was derived from a C57BL/6 genomic DNA clone (Fig. 1 *A*) comprising TNF, LTα, and LTβ (33; obtained from C.V. Jongeneel, Ludwig Institute for Cancer Research, Lausanne, Switzerland), and cloned into the SmaI site of pUC 19 (GIBCO BRL, Paisley, UK). A neomycin resistance cassette was inserted in sense direction into an unique ApaI site in exon 2 of the LTα 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). $LT\alpha^{+/-}$ 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 established $LT\alpha^{-/-}$ mouse line was maintained on a pure C57BL/6 background.

Southern Blot, Nested 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 CCT TCC CGC TTC AGT GAC AAC GTC; 20 cycles) followed by another round of amplification with an inner primer pair (5′ sense: CAG TTC CTC ATG TTG TGG TGA CCC; 3′ antisense: CCG ACT GCA TCT GCG TGT TCG A; 35 cycles). The annealing temperature was 60°C for both primer pairs. The Southern blot strategy used the introduction of an additional PstI 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 PMA-stimulated splenocytes (5 ng/ml for 12 h) or whole unperfused 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 tail base injection of 50 μg of MOG peptide (35-MEVGWYR-SPFSRVVHLYRNGK-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 MOG peptide dose-ranging experiments. Animals were observed daily and neurological deficits were quantified on an arbitrary clinical scale: 1+, flaccid tail; 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 deaggregated through 70 μm 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 (2×10^7 cells/recipient) were injected intravenously on day 0. Cage water was supplemented with trimethoprim (50 μg/ml)–sulphamethoxazole (0.25 mg/ml) between weeks 2 and 3 after transplantation. As a means of tracking engraftment, reciprocal transplantations between $TNF^{-/-}$ (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-α/β, and isotype-matched control antibodies are detailed elsewhere (23). Other mAbs were rat anti–I-A^b (M5/114.15.2; 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 Ly5.1 (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-α/β (T cells), anti–Mac-1 (monocytes, granulocytes, and NK 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-α/β–FITC antibodies, or appropriate controls. Flow cytometric data was acquired on a FACScan® (Becton Dickinson, San Jose, CA) and analyzed using CELL Quest™ (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 μm) were stained with various mAbs via the immunoperoxidase technique, and counterstained with hemotoxylin (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 $LT\alpha_3$ 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 LTα (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 μg/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 LTα was confirmed by RT-PCR. LTα-specific mRNA was not detectable in PMA-activated splenocytes (Fig. 1 *C*), whereas expression of TNF mRNA was readily detected. By contrast, LTα transcripts were detected in stimulated cells from $TNF^{-/-}$ mice, but neither TNF nor LTα 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 $LT\alpha_3$ and membrane $LT\alpha_1\beta_2$ forms of LT because functional surface expression of LTβ depends on the presence of LTα (17).

The Course of EAE in WT C57BL/6, $TNF^{-/-}$, and $TNF/LT\alpha^{-/-}$ Mice. Subcutaneous immunization of WT C57BL/6 mice with 50 μg 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

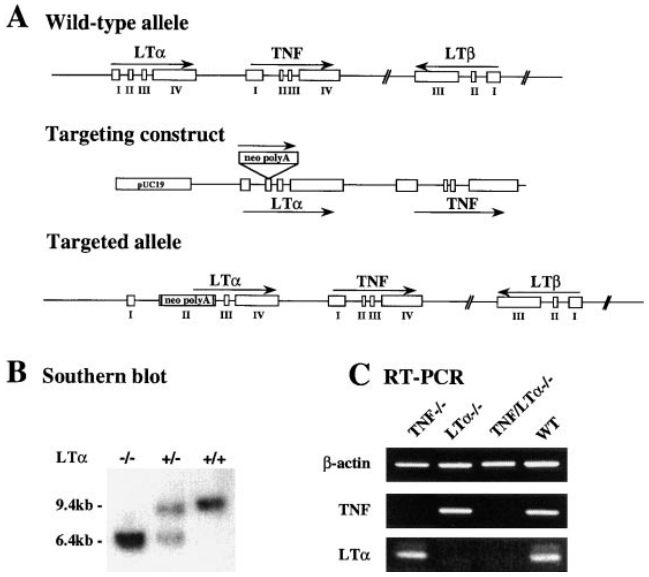

Figure 1. Generation of C57BL/6 LTα$^{-/-}$ mice. (*A*) LTα gene targeting strategies. The LTα gene is indicated in relation to TNF and LTβ genes. All are within the murine H-2 locus on chromosome 17. Transcription direction is indicated by arrows. A neomycin resistance cassette was inserted in sense direction into a unique ApaI site in exon 2 of the LTα gene to produce the targeting construct that was transfected in Bruce4 C57BL/6 ES cells. The predicted targeted allele is illustrated. (*B*) Southern blot of genomic DNA extracted from peripheral blood of homozygous, heterozygous, or WT mice confirming homozygous mutations of the LTα gene. A PstI site introduced by the neomycin resistance cassette was used to differentiate between mutated and WT alleles. (*C*) RT-PCR confirms absence of LTα transcripts. RNA was derived from PMA-stimulated splenocytes from WT, LTα$^{-/-}$, TNF$^{-/-}$, or TNF/LTα$^{-/-}$ mice (29), and analysis by RT-PCR for TNF, LTα, and β-actin transcripts. β-actin PCR product, 304 bases; TNF PCR product, 256 bases; LTα PCR product, 229 bases.

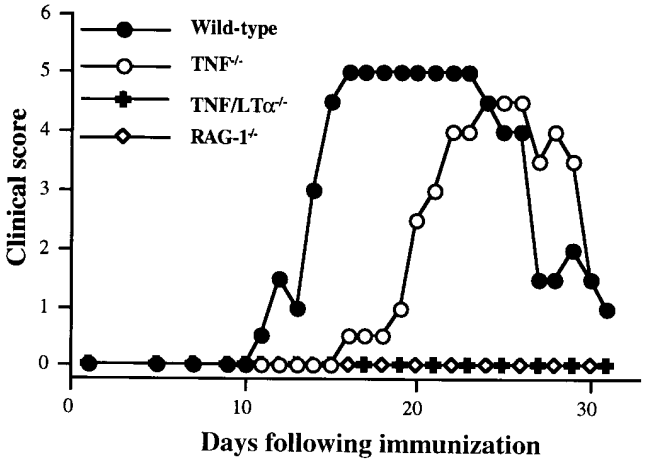

Figure 2. Natural history of MOG 35–55 peptide-induced EAE in C57BL/6 strain mice. Shown are disease courses of individual mice that represent groups of WT ($n > 20$), TNF$^{-/-}$ ($n > 20$), TNF/LTα$^{-/-}$ ($n = 5$), and RAG-1$^{-/-}$ ($n = 3$) mice immunized on day 0. See text for explanation of the clinical disease score.

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 RAG 1$^{-/-}$ 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.

Characteristics of Radiation Bone Marrow Chimeras. Chimeras were generated using a split-dose irradiation protocol and large bone marrow inoculum to ensure complete conversion of the recipient hemopoietic compartment to donor type. 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 is important in the development of demyelination (46). To determine whether antibody responses in chimeric mice had been normalized, serum samples were col-

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	$TNF/LT\alpha^{-/-}$	–	WT	LN absent, Ig deficient; references 20, 29, 45	Absence of LTα and TNF	Not susceptible to EAE‖	2
C	$RAG\text{-}1^{-/-}$	–	WT	LN anlage only, Ig deficient; reference 32	No mature T or B cells	Not susceptible to EAE‖	2
D	Chimera: LT→RAG	–	WT→RAG	Reconstituted	LT deficiency in TNF-competent mice, with reconstituted immunity	Unaltered	3 C
E	Chimera: TNF/LT→TNF	–	WT→WT or TNF→TNF	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 $LT\alpha_3$	Delayed	6 A
G	$TNF^{-/-}$	TNFR–IgG	$TNF^{-/-}$ treated with PBS	Intact	Inhibition of $LT\alpha_3$ in the absence of TNF	Unaltered	6 C

*$TNF^{-/-}$, $TNF/LT\alpha^{-/-}$, and $LT\alpha^{-/-}$ mice were generated by gene targeting directly in C57BL/6 ES cells.
‡Lymph node status.
§Serum immunoglobulin status. See also Fig. 3 B.
‖Not susceptible to MOG 35-55–induced EAE using our standard disease induction protocol, optimized in WT C57BL/6 mice.

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.

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

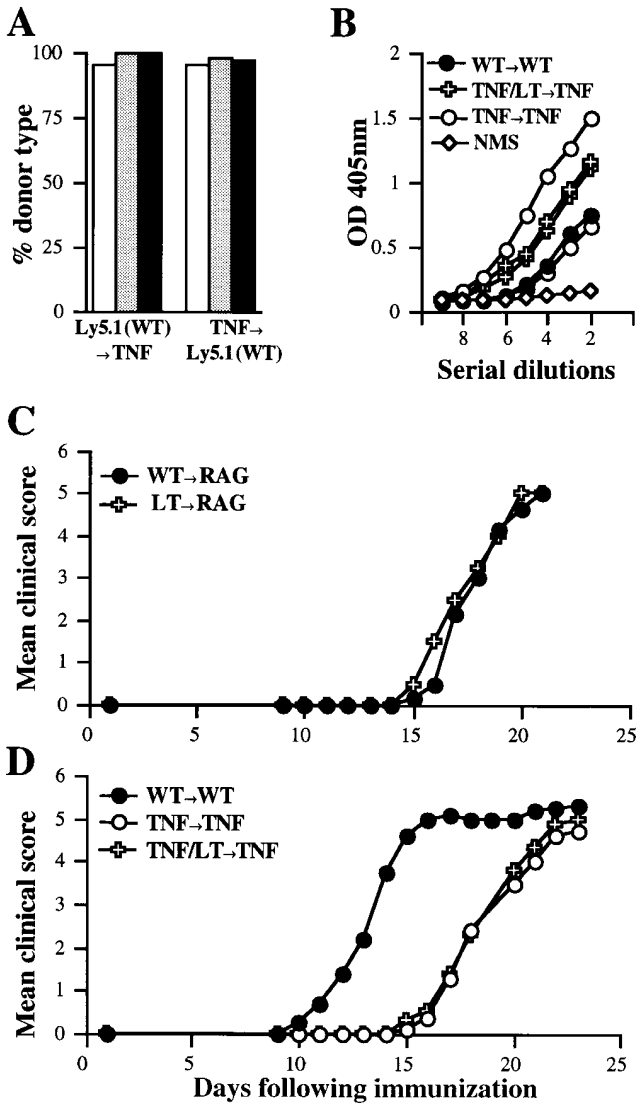

Figure 3. EAE in LTα- and TNF/LTα-deficient radiation bone marrow chimeric mice. (*A*) Reconstitution analysis showing the proportion of peripheral blood leukocytes bearing the CD45-congenic marker of donor type at 14 wk after bone marrow transplantation. T cell reconstitution was slower than B cells or Mac-1$^+$ cells, so this period of time was allowed for complete reconstitution in all compartments. *White bars*, α/β T cells; *gray bars*, B cells; *black bars*, Mac-1$^+$ cells. (*B*) Representative serum MOG 35–55 peptide–specific IgG responses in individual EAE-affected chimeras, compared with normal mouse serum (*NMS*) 23 d after standard MOG/CFA/pertussis immunization. Optical density at 405 nm for serial serum dilutions of individual animals is shown. (*C*) Mean EAE clinical score in WT→RAG (n = 3) and LT→RAG (n = 4) radiation bone marrow chimeras on days after immunization. (*D*) Mean EAE clinical score in WT→WT (n = 8), TNF→TNF (n = 10), and TNF/LT→TNF (n = 15) radiation bone marrow chimeras on days after immunization.

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.

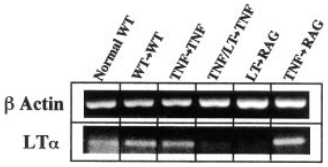

Figure 4. LTα mRNA expression in the inflamed CNS of radiation bone marrow chimeric mice. RNA was extracted from the unperfused whole CNS of a normal unimmunized C57BL/6 mouse (*Normal WT*) and from various chimeras, all EAE-affected and matched for disease severity (clinical score of 5). See Materials and Methods for PCR primers used.

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

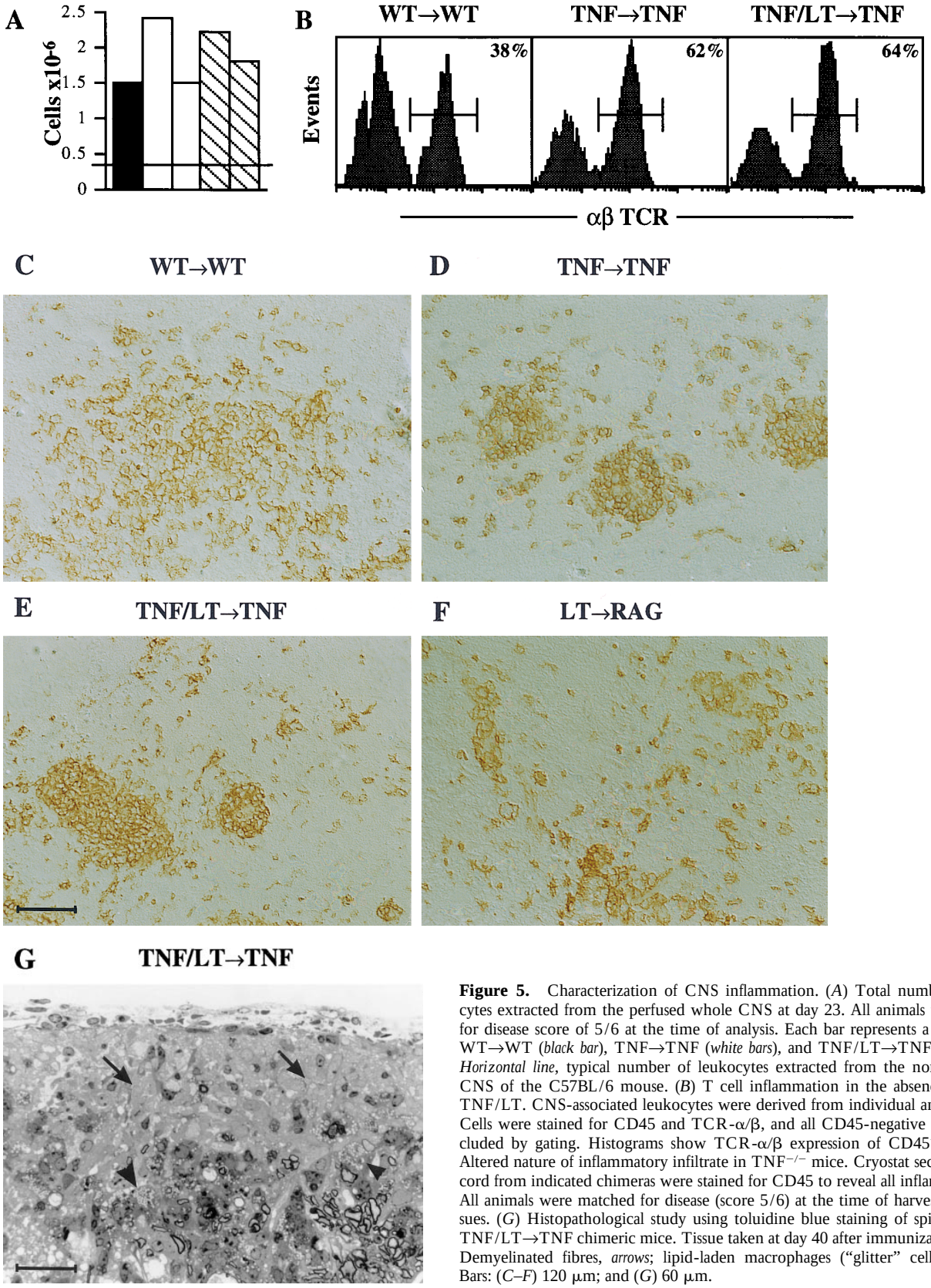

Figure 5. Characterization of CNS inflammation. (*A*) Total numbers of leukocytes extracted from the perfused whole CNS at day 23. All animals were matched for disease score of 5/6 at the time of analysis. Each bar represents a single animal. WT→WT (*black bar*), TNF→TNF (*white bars*), and TNF/LT→TNF (*hatched 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 $TNF^{-/-}$ 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.

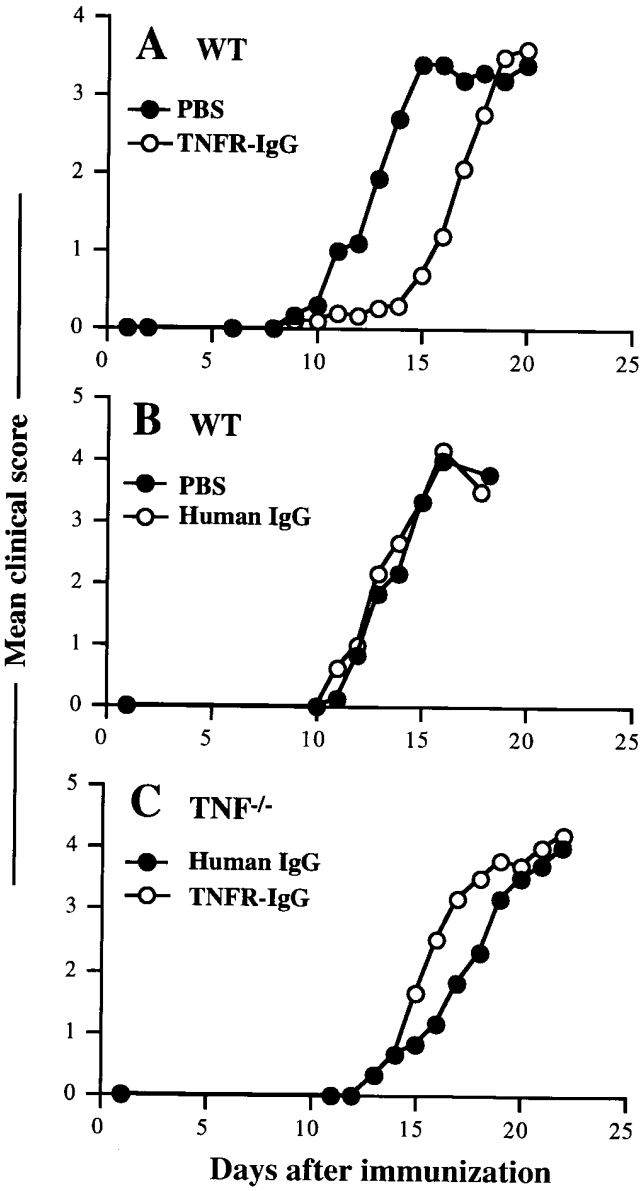

Figure 6. Influence of TNFR–IgG treatment on the clinical course of EAE in WT and $TNF^{-/-}$ C57BL/6 mice. (*A*) Mean EAE clinical score of MOG 35-55–induced EAE in WT mice, each treated by intraperitoneal injection with high dose TNFR–IgG ($n = 10$) on days 8 (500 μg), 11 (1 mg), and 14 (1 mg) after immunization (day 0) or an equivalent volume of PBS on these days ($n = 10$). (*B*) Mean EAE clinical score of MOG 35-55–induced EAE in WT mice treated by intraperitoneal injection with affinity purified human IgG ($n = 4$, 500 μg, days 11 and 14) or an equivalent volume of PBS on days 11 and 14 ($n = 3$). (*C*) Mean EAE clinical score of MOG 35-55–induced EAE in $TNF^{-/-}$ mice treated by intraperitoneal injection with TNFR–IgG ($n = 4$) or affinity purified human IgG ($n = 3$). Mice received 500 μg TNFR–IgG or human IgG immediately after the onset of clinical signs and again 4 d later.

to occur (Fig. 5 *D*) 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. 5 *G*). 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 $LT\alpha_3$ 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. 6 *A* and Table 1 *F*), 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. 6 *B*).

As an alternative means of studying the activities of $LT\alpha_3$ in a TNF-deficient system, $LT\alpha_3$ was blocked in $TNF^{-/-}$ mice by administration of TNFR-IgG (Fig. 6 *C* and Table 1 *G*). 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 $LT\alpha_3$ 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 ($LT\alpha_3$) or cell membrane ($LT\alpha_1\beta_2$) forms of LT exhibit TNF-like properties, particularly since $LT\alpha_3$ can bind to the TNFRs (14). A feature common to gene-targeted mice lacking TNF (29, 48), LTα (20), or LTβ (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 $LT\alpha^{-/-}$ or $LT\beta^{-/-}$ 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 1 *A*), 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 chemoattractant 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 $LT\alpha_1\beta_2$–LTβR interactions do seem to elicit a cytotoxic signal (22), it seems likely that the importance of the $LT\alpha_3$–TNFR interaction in cytotoxicity in the mouse has been overestimated (43). The use of $LT\alpha^{-/-}$ 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 $LT\alpha_3$ 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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Address correspondence to Jonathon D. Sedgwick, Centenary Institute of Cancer Medicine and Cell Biology, Bldg. 93, Royal Prince Alfred Hospital, Missenden Rd., Camperdown, Sydney, NSW 2050, Australia. Phone: 61-2-9565-6116; Fax: 61-2-9565-6103; E-mail: j.sedgwick@centenary.usyd.edu.au

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