Uncoupling the Proinflammatory from the Immunosuppressive Properties of Tumor Necrosis Factor (TNF) at the p55 TNF Receptor Level: Implications for Pathogenesis and Therapy of Autoimmune Demyelination

By George Kassiotis and George Kollias

From the Laboratory of Molecular Genetics, Hellenic Pasteur Institute, Athens 115-21, Greece; and the Institute of Immunology, Biomedical Sciences Research Center "Alexander Fleming," Vari 166-72, Greece

Abstract

Multiple sclerosis (MS) is a disabling inflammatory demyelinating disease of the central nervous system, considered to result from self-reactivity to myelin antigens. Tumor necrosis factor (TNF) and the p55 TNF receptor (TNFR) have been strongly implicated in MS pathogenesis. We reveal in this study a dual role for TNF in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS. In addition to its well-established proinflammatory effects, TNF exhibits potent immunosuppressive properties, providing one possible explanation for the immune and disease activating effect of anti-TNF treatment of MS. We show that in TNF-deficient mice, myelin-specific T cell reactivity fails to regress and expansion of activated/memory T cells is abnormally prolonged, leading to exacerbated EAE. Strikingly, immnosuppression by TNF and protection against EAE does not require the p55 TNFR, whereas the same receptor is necessary for the detrimental effects of TNF during the acute phase of the disease. Thus, blocking the function of the p55 TNFR in autoimmune demyelination may inhibit the noxious proinflammatory activities of TNF without compromising its immunosuppressive properties.

Key words: autoimmunity • cytokines • encephalomyelitis/multiple sclerosis • T lymphocytes • transgenic/knockout

Introduction

Multiple sclerosis (MS)[1] is a chronic relapsing inflammatory demyelinating disease of the central nervous system (CNS), generally considered to result from aberrant T cell reactivity to myelin antigens (1). No definite treatment for MS yet exists and the role of certain suspected mediators in animal models is often contradictory to what is seen when tried experimentally in MS (2). A possible flaw in attempts to rationalize therapeutic interventions in MS, based on the activity of target molecules in animal models, may stem from the fact that many such animal models, including experimental autoimmune encephalomyelitis (EAE), are often used to reproduce the acute phase of the disease, providing a partial representation of the chronicity of pathogenic mechanisms operating in MS (2). This becomes crucially important especially when target molecules show context-dependent, pleiotropic, and often contrasting biological activities. One such molecule is TNF, an established mediator of inflammation, but also a molecule implicated in the suppression or the termination of T cell responses (3, 4).

An interesting feature discovered recently for both MS and EAE is that primary myelin reactivity associated with onset of disease invariably regresses with time and becomes undetectable during consequent phases of the disease (5). In contrast, secondary self-reactivity to spreading determinants is consistently associated with disease progression (5, 6). Although the physiological significance of spontaneous regression of myelin reactivity remains unclear, it is likely that this functional property of the immune system serves to protect against potential autoimmune complications after exposure to self-antigens. Conversely, delay or failure to suppress self-reactivity may lead to self-aggressiveness and target tissue damage. Understanding the molecular details of this important process is eagerly awaited.

Address correspondence to George Kollias, Institute of Immunology, Biomedical Sciences Research Center "Al. Fleming," Vari 166-72, Greece. Phone: 301-9656507; Fax: 301-9656563; E-mail: giorgos_kollias@hol.gr

[1]*Abbreviations used in this paper:* CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PTx, pertussis toxin.

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TNF has been suggested as an important mediator of MS pathogenesis (7) and its detrimental role has been strongly supported in several animal models for MS (8–11). Surprisingly, however, systemic blockade of TNF in progressive MS patients led to immune activation and increased disease activity (12, 13). Although an antiinflammatory effect of TNF in the initiation of EAE has been described (14), it has not been associated with excessive myelin-directed T cell reactivity. Furthermore, the bulk of evidence (including this study) suggests that TNF/p55 TNFR signaling, in at least the initiation phase of EAE, is detrimental rather than antiinflammatory (8, 9, 15), and in all cases examined, primary T cell responses in $TNF^{-/-}$ mice have been comparable to those in control wild-type mice for up to 5 wk after immunization (8, 9, 14). Therefore, although TNF shows a clear proinflammatory activity in EAE and MS, it has remained uncertain whether this molecule may also be involved in the regulation of myelin-directed autoimmunity.

In an attempt to reconcile the pathogenic and immunosuppressive properties of TNF in autoimmune demyelination, we examined the long-term effects of TNF deficiency on the induction and regression of a myelin-specific T cell response. We show here that in the absence of TNF, myelin-specific T cell reactivity fails to regress and expansion of activated/memory T cells is abnormally prolonged. Surprisingly, failure to control myelin-specific autoimmunity in TNF-deficient mice results in the exacerbation of autoimmune demyelination in both resistant and susceptible backgrounds. Our results suggest that TNF deficiency prolongs and intensifies pathogenic autoimmunity, providing one possible explanation for the immune activating effect of anti-TNF treatment of MS. Intriguingly, immunosuppression by TNF and protection from chronic demyelinating disease occurs independent of the presence of the p55 TNFR. Thus, uncoupling of the proinflammatory from the immunosuppressive properties of TNF at the p55 TNFR level becomes crucially important for immune modulation. By specifically blocking the function of the p55 TNFR in autoimmune demyelination, it may be possible to inhibit the detrimental proinflammatory contributions of TNF without compromising its immunosuppressive properties.

Materials and Methods

Mice. $TNF^{-/-}$ mice, generated in-house (16), and control animals were maintained in a random C57BL/6 × 129/Sv ($H\text{-}2^b$) genetic background (referred to as B6,129). Mice originated from C57BL/6 × 129/Sv F_2 littermates, homozygous for the wild-type or the mutant TNF allele, and were subsequently kept as individual lines. Additionally, $TNF^{-/-}$ mice were backcrossed to C57BL/6 mice for at least six generations (referred to as inbred B6.$TNF^{-/-}$). B6,129.$p55^{-/-}$ mice (17) were provided by Dr. H. Bluethmann (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Inbred B6.$p55^{-/-}$ mice (18) were obtained from The Jackson Laboratory. B6,129.$p55p75^{-/-}$ mice were generated by crossing B6,129.$p55^{-/-}$ mice with B6.$p75^{-/-}$ mice (19), provided from Dr. M.W. Moore (Genentech, Inc., South San Francisco, CA). Specifically for myelin oligodendrocyte glycoprotein (MOG)-induced EAE (see Fig. 4 b), B6,129.$p55p75^{-/-}$ mice and B6,129 F_2 control mice were obtained from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions.

Immunizations. Antigens were emulsified in CFA (Sigma-Aldrich) containing 1 mg/ml *Mycobacterium tuberculosis* H37Ra. T cell priming was assayed in popliteal LNs isolated from mice (7–11 wk of age) 9 d after footpad immunization with 30 μg bovine myelin basic protein (MBP; Sigma-Aldrich) in CFA containing a total of 50 μg H37Ra per mouse. T cell "memory" was assayed in the spleen of mice at indicated time points after tail-base immunization with 30 μg MBP or 50 μg OVA (Sigma-Aldrich) in CFA containing a total of 100 μg H37Ra per mouse. Alternatively, T cell "memory" was examined in popliteal LNs 5 d after secondary footpad immunization with 30 μg MBP in IFA, 11–15 wk after the primary tail-base immunization.

T Cell Proliferation Assay. T cell proliferative responses were assessed at indicated time points in total LN or spleen cells. After erythrocyte lysis, cells were cultured in flat-bottomed 96-well plates at a density of 7×10^5 per well. Culture medium consisted of DMEM (Seromed) supplemented with 5% FCS, L-glutamine, and antibiotics. Triplicate cultures were incubated at 37°C in the presence or in the absence of indicated concentrations of the antigens. After 52 h, cultures were pulsed with 1 μCi of [methyl-^{3}H]TdR (Amersham Pharmacia Biotech) for 18 h. Cells were then harvested on glass fiber filters and incorporation of [methyl-^{3}H]TdR was measured by liquid scintillation counting. Results are expressed as stimulation indices (cpm of antigen-stimulated cultures/cpm of culture with medium alone).

Induction and Evaluation of EAE. EAE was induced in female B6.$TNF^{-/-}$, B6.$p55^{-/-}$, and control C57BL/6 mice or B6,129p55$p75^{-/-}$ and B6,129 F_2 mice after immunization with a rat MOG peptide (MOGp35–55, MEVGWYRSPFSRVVHLYRNGK; Sigma-Aldrich). Mice received in the two flanks 150 μg MOGp35–55 emulsified with a total of 1 mg H37Ra (Difco Laboratories) per mouse. Indicated doses of pertussis toxin (PTx; Sigma-Aldrich) were injected intraperitoneally at the time of immunization and 48 h later. Mice were monitored regularly for clinical signs and were scored on a scale of 0–5: 0, no clinical disease; 1, tail paralysis; 2, paraparesis (incomplete paralysis of one or two hindlimbs); 3, paraplegia (complete paralysis of one or two hindlimbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund or dead animals. For histological evidence of EAE, brains and spinal cords were removed, immersion fixed in ice-cold 4% phosphate-buffered paraformaldehyde for 3 h, and embedded in paraffin. 5-μm thick sections from the brain and spinal cord were prepared. The degree of demyelination and inflammation was assessed by luxol fast blue staining and nuclear fast red counterstaining. All sections were examined using standard bright-field optics and images were collected with a digitized camera (3CCD PowerHAD; Sony).

Flow Cytometry. Total spleen cells (10^6) were stained with each antibody for 20 min at 4°C in phosphate-buffered saline containing 0.2% BSA, in 100 μl total volume. CD4-PE, CD44-FITC, and CD45RB-FITC antibodies were all obtained from BD PharMingen. 10,000 cells/sample were analyzed on a FACSCalibur™ (Becton Dickinson) using CELLQuest™ analysis software (Becton Dickinson). Dead cells were excluded according to forward and side scatter parameters.

Results

Abnormally Prolonged Myelin-directed T Cell Reactivity in $TNF^{-/-}$ Mice. To examine whether TNF is involved in the regression of autoimmune T cell responses, B6,129 and

B6,129.TNF$^{-/-}$ mice were immunized with MBP in CFA containing 50 μg H37Ra, and their responses were monitored for 15 wk. Immunization of SJL.TNF$^{-/-}$ and SJL.H-2^{b} congenic mice with MBP in CFA containing 50 μg H37Ra results in comparable T cell priming in both groups of mice (9). Priming to MBP in B6,129 mice was, however, at least threefold lower than in SJL/J mice, with reproducibly lower stimulation indices in B6,129.TNF$^{-/-}$ than in B6,129 mice 9 d after immunization (Fig. 1 a). Defective priming to MBP (with the protocol employed) in B6,129.TNF$^{-/-}$ mice, compared with B6,129 mice, was due to reduced innate responsiveness of the former and could be restored by supplementary H37Ra in the emulsion (our unpublished observation). Consistent with the notion of spontaneous regression of primary myelin-specific T cell reactivity (5), virtually no "memory" was induced in B6,129 mice, as spleen cells from these mice responded poorly or did not respond at all to MBP at later time points (Fig. 1 a). Immunization with the control antigen, OVA, confirmed that inability to elicit "memory" T cell responses in B6,129 mice is a property of myelin-derived but not of foreign antigens (data not shown), in agreement with previous reports (5). Surprisingly, despite defective MBP-specific T cell priming in B6,129.TNF$^{-/-}$ mice and in sharp contrast to B6,129 mice, MBP-specific T cells accumulated in the spleen of B6,129.TNF$^{-/-}$ mice where strong proliferative responses to MBP were detected 49 d after immunization (Fig. 1 a). At 77 d after immunization, MBP-specific T cell responses in B6,129.TNF$^{-/-}$ mice were considerably reduced but still detectable, compared with B6,129 mice (Fig. 1 a). These results reveal that regression or inactivation of the MBP-specific T cell response in B6,129 mice is mediated, at least in part by TNF, and that in the absence of TNF MBP-specific T cell "memory" is induced.

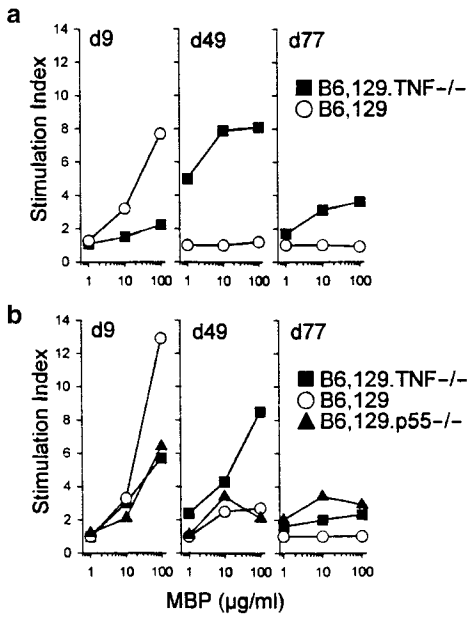

Figure 1. Abnormally prolonged T cell reactivity to MBP in B6,129.TNF$^{-/-}$ mice. T cell priming was examined in popliteal LN isolated from B6,129.TNF$^{-/-}$ and B6,129 (a) or B6,129.TNF$^{-/-}$, B6,129, and B6,129.p55$^{-/-}$ mice (b) 9 d after footpad immunization with 30 μg MBP in CFA. MBP-specific "memory" responses were tested in the spleen of B6,129.TNF$^{-/-}$ and B6,129 (a) or B6,129.TNF$^{-/-}$, B6,129, and B6,129.p55$^{-/-}$ mice (b) 49 and 77 d after tail-base immunization with MBP. Each curve represents the stimulation index (cpm of antigen-stimulated cultures/cpm of cultures with medium alone) of two mice of each genotype per time point pooled together. One representative set of experiments (out of three performed) is shown.

Regression of Myelin-specific T Cell Responses Is Independent of p55 TNFR Function. Inactivation of autoimmune T cells is thought to take place in splenic germinal centers (20). To examine whether regression of autoimmunity by TNF depends on the presence of the p55 TNFR and/or correct splenic architecture, we employed p55 TNFR-deficient mice, which like TNF$^{-/-}$ mice (16) are also defective in germinal center formation (21). MBP-immunized B6,129.p55$^{-/-}$ mice showed defective priming to MBP (Fig. 1 b). Surprisingly, however, and in sharp contrast to B6,129.TNF$^{-/-}$ mice, MBP-specific T cell responses were effectively controlled in B6,129.p55$^{-/-}$ mice and were similar to B6,129 mice 49 d after immunization (Fig. 1 b). However, at 77 d after immunization, MBP-specific T cell responses in B6,129.p55$^{-/-}$ mice were overall low but consistently higher than those in B6,129.TNF$^{-/-}$ (Fig. 1 b). TNFR dependence of MBP-specific T cell regression was confirmed in B6,129.p55p75$^{-/-}$ mice, which, similarly to B6,129.TNF$^{-/-}$ mice, also failed to inactivate the MBP-specific T cell response examined 49 d after immunization (data not shown). Together, these findings show that MBP-specific T cell inactivation by TNF is independent of germinal center formation or the function p55 TNFR in general, as it is not compromised in B6,129.p55$^{-/-}$ mice.

Differential Termination of MBP-specific T Cell Responses in p55$^{-/-}$ Versus TNF$^{-/-}$ Mice. The p55 TNFR has been implicated in the apoptotic removal of activated T cells during the termination of T cell responses (3). Abnormally prolonged MBP-specific T cell responses in the absence of TNF could be indicative of defective T cell apoptosis. However, after primary immunization, abnormally prolonged MBP-specific T cell reactivity is not observed in B6,129.p55$^{-/-}$ mice, and MBP-specific T cell responses eventually decline even in B6,129.TNF$^{-/-}$ mice. To examine the mechanism of termination of MBP reactivity in the absence of TNF or the p55 TNFR, mice were immunized with MBP in CFA and rechallenged with MBP in IFA, 11–15 wk later, when the primary response to MBP had subsided in all groups of mice (Fig. 1). We reasoned that if termination of the MBP-specific T cell response involved apoptotic removal of all activated MBP-specific T cells, then we would not be able to detect a T cell response to MBP after rechallenge. B6,129 mice showed strong MBP-specific reactivity 5 d after rechallenge (Fig. 2), indicating that antigen-specific T cells were not deleted in wild-type mice and that they were inactivated by a different mechanism, such as anergy or tolerance. B6,129.p55$^{-/-}$ and B6,129.p55p75$^{-/-}$ mice exhibited significantly increased MBP-specific T cell responses compared with

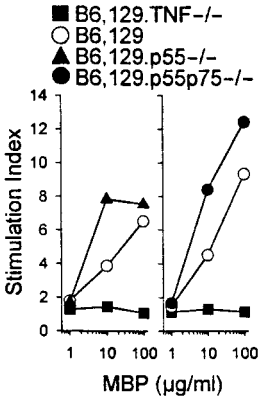

Figure 2. Differential mechanism of termination of MBP-specific T cell responses in B6,129.p55$^{-/-}$ and B6,129.p55p75$^{-/-}$ vs. B6,129.TNF$^{-/-}$ mice. Mice were immunized in the tailbase with MBP in CFA and 11–15 wk later, MBP-specific T cell responses were assayed in popliteal LN, isolated 5 d after secondary footpad immunization with MBP in IFA. Each curve represents the stimulation index of two mice of each genotype pooled together. One representative experiment of two performed is shown.

B6,129 mice (Fig. 2), indicative of lack of T cell apoptosis (note that T cell priming in mice lacking p55 TNFR signaling is severely compromised; Fig. 1), in agreement with the established function of the p55 TNFR (3). Similarly to B6,129 mice, mechanisms other than apoptosis might control primary MBP reactivity in mice lacking the p55 TNFR because at this time point, the primary T cell response is not readily detectable in these mice (Fig. 1), despite lack of MBP-specific T cell apoptosis (Fig. 2). Surprisingly, in contrast to all other groups of mice, rechallenge of B6,129.TNF$^{-/-}$ mice failed to induce any measurable T cell response in the draining LN (Fig. 2). These data imply a differential mechanism of termination of myelin-specific T cell responses in the absence of TNF or the p55 TNFR, and suggest a TNF-independent role for the p55 TNFR in this process.

Sustained MBP Reactivity in TNF$^{-/-}$ Mice Breaks Resistance of H-2^{b} Mice to MBP-induced EAE. Mice of the H-2^{b} haplotype are generally resistant to MBP-induced EAE. In agreement to that, immunization of B6,129 mice with 30 μg MBP in CFA containing 100 μg H37Ra per mouse without PTx coadministration failed to induce clinical or histological evidence of EAE (Fig. 3, c and d, and Table I).

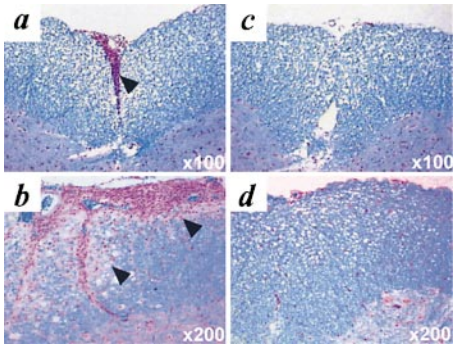

Figure 3. Histological evidence of inflammation and demyelination in MBP-immunized B6,129.TNF$^{-/-}$ mice. B6,129.TNF$^{-/-}$ (a and b) and B6,129 mice (c and d) were immunized with MBP in CFA and tissues were collected 9–15 wk later. Inflammation and demyelination were visualized by nuclear fast red and luxol fast blue staining, respectively. Arrowhead in the top panel indicates inflammation; arrowheads in the bottom panel indicate additionally demyelination; numbers within the microphotographs indicate original magnification.

Table I. *Histopathological Assessment of EAE in MBP-immunized B6,129 Mice*

Genotype	Mean week (range)	Inflammation (%)	Demyelination (%)
B6,129.TNF$^{-/-}$	11.6 (9–15)	7/10 (70)	2/10 (20)
B6,129	11.7 (9–15)	0/8 (0)	0/8 (0)
B6,129.p55$^{-/-}$	11.3 (9–12)	0/9 (0)	0/9 (0)
B6,129.p55p75$^{-/-}$	12.5 (9–15)	2/6 (33)	1/6 (17)

Sections throughout the spinal cord of the immunized mice were stained with luxol fast blue and nuclear fast red. The presence of inflammation and demyelination was assayed qualitatively in 30–50 sections per mouse.

Surprisingly, however, starting from 4–5 wk after immunization almost half of B6,129.TNF$^{-/-}$ mice exhibited clinical evidence of EAE, which progressed into a chronic nonremitting disease (10/23, maximal clinical score 2), whereas B6,129 and B6,129.p55$^{-/-}$ remained healthy (data not shown). Histological examination throughout the spinal cords of MBP-immunized B6,129.TNF$^{-/-}$ mice revealed the presence of extensive inflammation and demyelination (Fig. 3, a and b). Inflammation was evident histologically even in B6,129.TNF$^{-/-}$ and B6,129.p55p75$^{-/-}$ mice that showed no clinical disease and affected a significant percentage of the immunized animals, whereas fewer mice exhibited clear demyelination (Table I). In contrast, no histological evidence of inflammation or demyelination were observed in B6,129 or B6,129.p55$^{-/-}$ mice (Table I). These experiments indicate that inability to control or inactivate an MBP-specific T cell response due to TNF (but not p55 TNFR) deficiency effectively unmasks the resistance of B6,129 mice to MBP-induced EAE.

TNF Protects MOG-susceptible Backgrounds against Chronicity of MOG-induced EAE. To address the effect of TNF deficiency on the autoimmune disease resulting from MOG immunization of susceptible backgrounds, TNF$^{-/-}$ mice were backcrossed to C57BL/6 mice for at least six generations and EAE was induced by MOGp35–55 immunization and PTx coadministration. In agreement with previous reports (7, 8), onset of clinical symptoms was delayed by several days in B6.TNF$^{-/-}$ mice compared with C57BL/6 mice (Fig. 4 a). Surprisingly, however, despite delayed onset and overall reduced disease, after recovery from the acute episode of disease, B6.TNF$^{-/-}$ mice developed a chronic-progressive form of EAE (Fig. 4 a), in contrast to C57BL/6 mice, which retained only a mild deficit (Fig. 4 a). In separate experiments, EAE was induced by MOGp35–55 immunization without PTx coadministration, a protocol that induces EAE only in C57BL/6 but not in B6.TNF$^{-/-}$ mice (see below, Fig. 7). Although B6.TNF$^{-/-}$ mice were not susceptible to the acute episode of disease, they developed the late chronic-progressive form, as seen in Fig. 4 a (data not shown). Similarly, p55p75$^{-/-}$ mice on a MOG-resistant B6,129 background

(resistance conferred by the 129 genetic background [13]), although fully refractory to the acute episode of EAE (Fig. 4 b), developed the late chronic-progressive form of EAE, in contrast to B6,129 F_2 control mice (Fig. 4 b). Together, these experiments demonstrate that despite being beneficial during the onset of disease, TNF deficiency is associated with late complication of myelin-directed autoimmune disease.

Enhanced MOG-specific T Cell "Memory" Correlates with Higher Numbers of Activated/Memory CD4$^+$ T Cells in TNF$^{-/-}$ Mice. To examine whether susceptibility to the late chronic form of EAE in MOGp35-55–immunized B6.TNF$^{-/-}$ mice is associated with enhanced MOG reactivity, "memory" T cell responses to MOGp35–55 were measured 9–11 wk after EAE induction. Although spleen cells from C57BL/6 mice retained a substantial degree of MOG reactivity even after 11 wk after EAE induction, MOGp35-55–specific T cell "memory" responses were consistently higher in B6.TNF$^{-/-}$ mice (Fig. 5 a), and this was particularly evident in B6.TNF$^{-/-}$ that did not receive PTx (Fig. 5 b). To test whether enhanced MOG reactivity in B6.TNF$^{-/-}$ mice is related to uncontrolled expansion of MOGp35-55–specific T cells, the numbers of

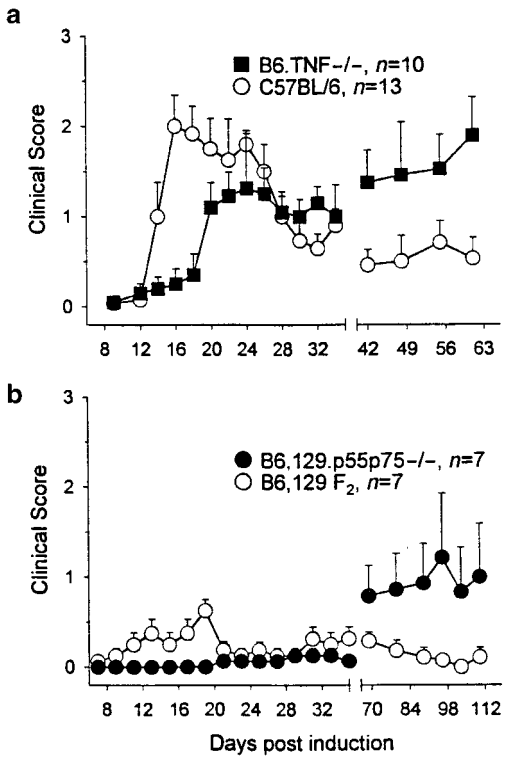

Figure 4. B6.TNF$^{-/-}$ and B6,129.p55p75$^{-/-}$ mice develop a late, chronic form of EAE after immunization with MOGp35–55. EAE was induced in mice by MOGp35–55 immunization in CFA containing 1 mg H37Ra per mouse. On the day of immunization and 48 h later, (a) 400 ng PTx (in the case of B6.TNF$^{-/-}$ and C57BL/6 mice) or (b) 200 ng PTx (in the case of B6,129.p55p75$^{-/-}$ and B6,129 F_2 mice) were coadministered intraperitoneally. Each curve represents the mean clinical score ± SD of each group. Note that intervals in the first or the acute phase are in alternate days, whereas the second or chronic phase are in weeks.

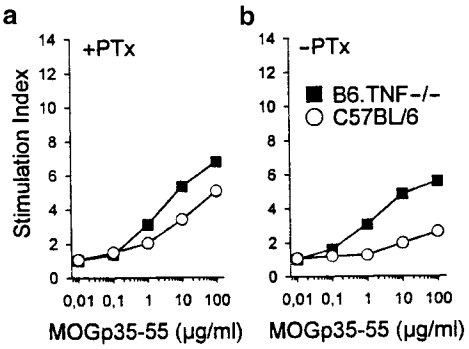

Figure 5. Enhanced MOGp35-55–specific T cell responses in B6.TNF$^{-/-}$ 9–11 wk after immunization. EAE was induced in B6.TNF$^{-/-}$ and C57BL/6 mice by MOGp35–55 immunization with (a) or without PTx coadministration (b), and T cell responses to MOGp35–55 were tested in total spleen cells 9–11 wk later. Each curve represents the stimulation index of two to five mice of each genotype pooled together. One representative experiment of three performed is shown.

CD44^{hi}CD45RB^{lo} CD4$^+$ T cells were quantified in the spleens of B6.TNF$^{-/-}$ and C57BL/6 mice 9–11 wk after EAE induction (Fig. 6). Notably, CD44^{hi}CD45RB^{lo} CD4$^+$ T cell numbers were elevated in nonimmunized B6.TNF$^{-/-}$ mice compared with control C57BL/6 mice (Fig. 6). More importantly, the numbers of CD44^{hi} CD45RB^{lo} CD4$^+$ T cells were considerably increased in MOGp35-55–immunized B6.TNF$^{-/-}$ mice, but more evidently without PTx coadministration (Fig. 6). In contrast, CD44^{hi}CD45RB^{lo} CD4$^+$ T cell numbers were constant or even reduced in MOGp35-55–immunized C57BL/6 mice (Fig. 6). Elevated CD44^{hi}CD45RB^{lo} CD4$^+$ T cell numbers were observed also in the spleens of MOGp35-55–

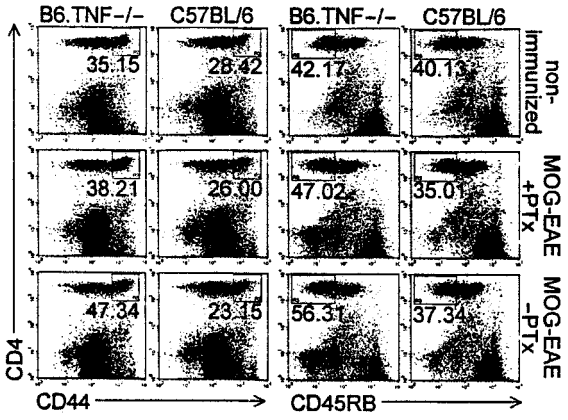

Figure 6. Elevated numbers of CD44^{hi}CD45RB^{lo} CD4$^+$ T cells in B6.TNF$^{-/-}$ mice. EAE was induced in B6.TNF$^{-/-}$ and C57BL/6 mice by MOGp35–55 immunization with or without PTx coadministration, and CD44 and CD45RB phenotypes of CD4$^+$ T cell were analyzed and compared with age-matched nonimmunized mice 9–11 wk later by flow cytometry. Each plot depicts CD4, CD44, and CD45RB phenotypes in total spleen cells pooled from four to five mice. Numbers underneath the regions represent the percentage of CD44^{hi} or CD45RB^{lo}CD4$^+$ T cells, expressed as percentage of total CD4$^+$ T cells. The percentage of total CD4$^+$ T cells in the spleens of the different groups did not vary significantly.

immunized B6,129.p55p75$^{-/-}$ mice (42% CD44^{hi}, 54% CD45RB^{lo} in B6,129.p55p75$^{-/-}$ mice versus 25% CD44^{hi}, 44% CD45RB^{lo} in B6,129 mice, of total CD4^{+} T cells at 18 wk after EAE induction). These data indicate that TNF influences the expansion or survival of activated/memory T cells.

Resistance to Reinduction of EAE Depends on TNF but Not on the p55 TNFR. Mice that have recovered from an acute episode of EAE are normally resistant to reinduction of the disease. To examine whether failure of B6.TNF$^{-/-}$ mice to control MOGp35-55–specific T cell responses is linked to inability to establish tolerance to MOGp35–55 and resistance to reinduction of disease, EAE was reinduced in mice 50 d after primary EAE induction. Moreover, to verify that the resulting disease after reinduction of EAE in B6.TNF$^{-/-}$ mice is due to enhanced MOGp35-55–specific T cell responses, we used a low dose of PTx, in light of the fact that TNF$^{-/-}$ mice, in contrast to control mice, are totally refractory to EAE induction if PTx is reduced or omitted (unpublished observations). Indeed, the EAE induction protocol employed induced the acute episode of EAE only in C57BL/6 but not in B6.TNF$^{-/-}$ or B6.p55$^{-/-}$ mice (Fig. 7). As expected, C57BL/6 mice recovered from the acute EAE and were totally refractory to reinduction of EAE (Fig. 7). In contrast, B6.TNF$^{-/-}$ mice were resistant to the initial episode of EAE but readily developed the disease after reinduction (Fig. 7). More importantly, B6.p55$^{-/-}$ mice, which were also resistant to the initial episode of EAE, were tolerant to EAE reinduction (Fig. 7). Thus, TNF but not the p55 TNFR is required for the induction of tolerance to the immunizing self-antigen and the establishment of resistance to EAE.

Discussion

Data presented in this study suggest that TNF plays at least two distinct roles during the progression of autoimmune encephalomyelitis. In agreement with previous reports (8, 9, 15), TNF appears to exert acute detrimental activities after initiation of a myelin-directed immune response. Interestingly, however, TNF appears to be additionally required for the spontaneous regression of this reactivity and for the subsequent remission of the clinical syndrome. In the absence of TNF, myelin-specific T cells that would otherwise be inactivated accumulate in the spleen of the immunized animals and a late chronic form of EAE develops. Our results demonstrate that TNF is essential in downregulating or inactivating a potentially detrimental autoimmune T cell response against myelin antigens, and that it can therefore protect against chronic EAE. This notion adds an extra level of complexity with respect to the role of TNF in MS pathogenesis. High TNF levels will ensure efficient inactivation of any potentially detrimental myelin-specific T cell response but will facilitate disease progression by accelerating onset of lesions and contributing to CNS demyelination. Conversely, low TNF levels will delay the onset of lesions and reduce the extent of CNS demyelination but will enable the mounting of an extremely unfavorable myelin-specific T cell response. These two extremes of many intermediate possible scenarios indicate that fine-tuning and precise control of TNF levels or function are required to minimize the risk of autoimmune disease development. A similar defect in suppressing the expansion of activated myelin-specific T cells, also leading to exacerbation of EAE, has recently been described in IFN-$\gamma^{-/-}$ mice (22). However, exacerbation of EAE in IFN-$\gamma^{-/-}$ mice appears to involve only the acute phase of the disease. Therefore, in contrast to IFN-γ, which shows early antiinflammatory properties, TNF is clearly proinflammatory during the acute phase of EAE, as well as dominantly immunosuppressive (and thus "antiinflammatory") during the consequent phases of the disease. This model is consistent with the immune activating and disease enhancing activities of anti-TNF treatment recently evidenced in MS patients (12, 13).

The precise mechanism by which TNF mediates regression of myelin-specific T cell responses is not yet clear. Both TNF receptors have been implicated in antigen-induced T cell apoptosis (2, 23). However, prolonged primary myelin reactivity and late onset EAE were not observed in p55$^{-/-}$ mice, indicating that defective p55 TNFR-mediated apoptosis cannot fully account for this phenomenon, and that the p75 TNFR may be sufficient to mediate apoptotic removal of activated T cells. Alternatively, TNF/p75 TNFR signals may influence the long-term survival or expansion of pathogenic activated/memory T cells through mechanisms other than apoptosis. Preliminary data with mice lacking the p75 TNFR suggested that although the p75 TNFR is sufficient to control myelin reactivity, it is not essential, as p75$^{-/-}$ mice, similarly to p55$^{-/-}$ mice, failed to develop the late autoimmune complications seen in TNF$^{-/-}$ or p55p75$^{-/-}$ mice. However, it remains unclear whether the same or an entirely different mechanism is responsible for the control of myelin reactivity in p55$^{-/-}$ and p75$^{-/-}$ mice, respectively. An additional

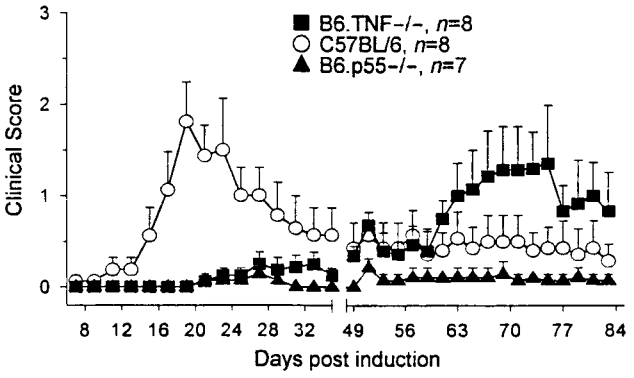

Figure 7. Establishment of resistance to MOG-induced EAE in B6.p55$^{-/-}$ and C57BL/6 mice but not in B6.TNF$^{-/-}$ after MOGp35–55 immunization. EAE was induced in mice by MOGp35–55 immunization in CFA containing 1 mg H37Ra per mouse. On the day of immunization and 48 h later, mice received 200 ng PTx intraperitoneally. 50 d later, EAE was reinduced using the same protocol. Each curve represents the mean clinical score ± SD of each group.

line of evidence against a role for p55-mediated T cell apoptosis in the control of autoimmune T cells by TNF may be the fact that lack of p55-mediated T cell removal, reflected by enhanced responses to secondary challenge in $p55^{-/-}$ and $p55p75^{-/-}$ mice, is not observed in $TNF^{-/-}$ mice, implying a TNF-independent role for the p55 TNFR in this process. Indeed, p55 TNFR triggering by ligands other than TNF, such as lymphotoxin (LT)-α, is also possible, given the potent T cell cytotoxic effect of LT-α (24). If p55-mediated T cell apoptotic pathways are largely unaffected in mice lacking TNF, it is conceivable that instead of, or in addition to, increasing the frequency of myelin-specific T cells, TNF deficiency allows autoimmune T cells to reach a disease-causing threshold of activation. Inactivation of autoimmune T cells by TNF could also explain the immune or disease suppressive effect of TNF in other organ specific (25, 26) or systemic autoimmune diseases (27, 28). However, it remains to be established whether a universal mechanism of suppression is employed in all cases.

Understanding the molecular and cellular mechanisms underlying the proinflammatory and immunosuppressive functions of TNF should allow more effective design of therapeutic approaches. The fact that the inactivation of myelin-specific autoimmunity by TNF displays different requirements for the p55 TNFR than the initiation of EAE becomes crucially important for immune modulation. An immunosuppressive role with clinical relevance for TNF is also supported by the systemic autoimmunity frequently associated with TNF blockade in human patients (29, 30). Interestingly, recent data suggest that systemic autoimmunity develops spontaneously in $TNF^{-/-}$ but not in $p55^{-/-}$ mice (31). Together, these results indicate that by specifically blocking the function of the p55 TNFR, it may be possible to inhibit the detrimental proinflammatory or cytotoxic contributions of TNF in demyelinating disease while retaining its beneficial involvement in the inactivation of the autoimmune response. The paradigm set in this study for autoimmune demyelination may also apply in a broad range of chronic inflammatory/autoimmune diseases, and may prove useful in the future design of rational therapies.

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