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*J Immunol* 2011; 187:5660-5670; Prepublished online 4 November 2011;
doi: 10.4049/jimmunol.1100663
http://www.jimmunol.org/content/187/11/5660

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/11/01/jimmunol.1100663.DC1

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Pathogenic and Protective Functions of TNF in Neuroinflammation Are Defined by Its Expression in T Lymphocytes and Myeloid Cells

Andrey A. Kruglov,*†‡ Vicky Lampropoulou,* Simon Fillatreau,* and Sergei A. Nedospasov*,†‡

TNF displays pathogenic activities in many autoimmune disorders. However, anti-TNF therapy in multiple sclerosis patients failed because of poorly understood reasons. We used a panel of gene-targeted mice that allowed cell-type specific ablation of TNF to uncover pathogenic and protective contributions of this cytokine during autoimmune disease of the CNS. T cells and myeloid cells were found to be critical cellular sources of TNF during experimental autoimmune encephalomyelitis (EAE). TNF produced by myeloid cells accelerated the onset of disease by regulation of chemokine expression in the CNS, driving the recruitment of inflammatory cells into the target organ. TNF produced by T cells exacerbated the damage to the CNS during EAE by regulating infiltration of inflammatory myeloid cells into the CNS. In secondary lymphoid organs, TNF expressed by myeloid cells and T cells acted in synergy to dampen IL-12p40 and IL-6 production by APCs, subsequently inhibiting the development of encephalitogenic T cell responses of Th1 and Th17 types. This dual role of TNF during EAE (protective in lymphoid organs and pathogenic in CNS) suggests that global TNF blockade might be inefficient in multiple sclerosis patients because augmented autoreactive T cell development in lymphoid tissues might overwhelm the beneficial effects resulting from TNF inhibition in the CNS. The Journal of Immunology, 2011, 187: 5660–5670.

Tumor necrosis factor is a pleiotropic cytokine with many essential functions during immune responses. For example, TNF facilitates host defense against intracellular pathogens and may mediate inflammation during sepsis (1, 2). Elevated TNF levels are associated with pathology in many autoimmune disorders, and TNF blockade is an approved treatment in several diseases, such as rheumatoid arthritis, autoimmune psoriasis, and Crohn’s disease (3). In contrast, the role of TNF in multiple sclerosis (MS) remains controversial. Initial reports claimed that in vivo TNF blockade in mice and rats resulted in experimental autoimmune encephalomyelitis (EAE) amelioration (4, 5). Further studies using TNF-deficient mice identified TNF as a critical pathogenic cytokine acting during the onset of EAE to induce chemokine expression in the CNS (6, 7). On the contrary, therapeutic administration of TNF was reported to provide protection from EAE (8). TNF was also shown to promote proliferation of oligodendrocyte precursors and remyelination of the CNS (9). Additionally, TNF ablation in mice resulted in prolonged retention of activated T cells in the secondary lymphoid organs, leading to exacerbated disease (10). Finally, clinical trials of lenercept, the soluble TNFR1-Ig, in MS patients were terminated as a result of disease exacerbation (11), and several cases of demyelinating disease were reported in subgroups of rheumatoid arthritis patients treated with TNF inhibitors, with symptoms disappearing after anti-TNF therapy interruption (12). These data suggested a protective effect of TNF in MS and possibly in other demyelinating diseases.

TNF can be expressed by multiple cell types, such as macrophages, dendritic cells, NK cells, neutrophils, and T and B cells, as well as by some nonhematopoietic cells (1, 13, 14). TNF from these various cell types can be produced with different kinetics and magnitude during inflammation. In MS patients, TNF expression was detected in situ both in inflammatory macrophages from spinal cord biopsies (15) and in T cell clones generated from cerebrospinal fluid (16). However, it remained unresolved whether TNF produced by different cell types plays distinct roles in MS and how TNF blockade may lead to MS exacerbation.

In the current study, using cell type-specific gene targeting, we addressed the role of distinct cellular sources of TNF during development and progression of EAE. We found that the phenotype of complete TNF-deficient mice could be recapitulated by concomitant ablation of TNF in myeloid cells and in T cells, highlighting T cells and myeloid cells as the critical sources of this cytokine during EAE. However, both of these cellular sources exhibited distinct functions in CNS inflammation: T cell-derived TNF exacerbated the severity of EAE by regulating accumulation of inflammatory myeloid cells in the CNS, whereas TNF produced by myeloid cells accelerated EAE onset by controlling the early expression of chemokines in the CNS. In addition to these pathogenic functions of TNF in the CNS, TNF produced by myeloid cells and T cells acted additively to limit autoreactive T cell development in secondary lymphoid organs by restricting IL-12p40 and IL-6 production from APCs. Our findings link protective and pathogenic effects of TNF in CNS autoimmunity to specific cellular functions of TNF in multiple sclerosis patients because augmented autoreactive T cell development in lymphoid tissues might overwhelm the beneficial effects resulting from TNF inhibition in the CNS. The Journal of Immunology, 2011, 187: 5660–5670.

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sources and suggest that the balance between the effects of TNF in the target organ and peripheral lymphoid tissues is crucial for the final outcome of the disease.

Materials and Methods

Animals

Mice with ablation of TNF in T cells (T-TNF knockout [KO]; TNFfl/fl; CD4-Cre), in myeloid cells (M-TNF KO; TNFfl/fl; MyIys-Cre), and in B cells (B-TNF KO; TNFfl/fl; CD19-Cre), as well as the TNF KO mice, used in this study were described elsewhere (14, 17). Concomitant ablation of TNF in both T cells and myeloid cells (MT-TNF KO; TNFfl/fl; MyIys-Cre; CD4-Cre) was achieved by crossing M-TNF KO and T-TNF KO mice. TNFfl/fl mice were used as wild-type (WT) controls for all experiments using cell type-specific TNF KO mice, unless otherwise stated. All cell type-specific TNF-deficient mice, as well as OT-II mice and OT-II on TNF-deficient background, were bred and kept in specific pathogen-free conditions. C57BL/6 mice were purchased from Charles River Laboratories. All animal procedures were carried out in accordance with German and Russian regulations for animal protection.

EAE induction

Mice were immunized s.c. with 50 mcg myelin oligodendrocyte protein (MOG)35-55 peptide emulsified in CFA (Mycobacterium tuberculosis concentration, 5 mg/ml). Pertussis toxin (220 ng/mouse) was administered i.v. on days 0 and 2. Mice were scored daily, as described elsewhere (18). Briefly, the following clinical scores were used: grade 0, no disease; grade 1, flaccid tail; grade 2, impaired gait/impaired righting reflex; grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis; grade 5, complete hind limb paralysis and partial front limb paralysis; and grade 6, moribund.

Histological analysis of spinal cord sections

Spinal cords were collected at the indicated time points and fixed overnight in 4% paraformaldehyde at 4°C. Samples were washed three times with ice-cold PBS for 30 min, incubated in 30% sucrose in PBS for 30 min, and snap-frozen in OCT compound (Sakura Teco). Seven-micrometer sections were cut, dried, and stored at −80°C. For demyelination analysis, sections were incubated in Luxol fast blue solution overnight at 37°C, washed in 0.005% lithium carbonate and 70% ethanol, and counterstained with cresyl violet. H&E staining was done, as described elsewhere (14). For quantification, inflammatory foci were identified as focal areas with the aggregation ≥10 cells. Immunofluorescent analysis was performed using anti–Mac-3 (eBioscience), followed by goat anti-rat IgG coupled with Alexa Fluor 488 (Invitrogen), anti–CD4–Alexa Fluor 546 (GK1.5; German Rheumatism Research Center [DRFZ]), anti–TNF-FITC (MP6-XT22; DRFZ), and anti–CD11b-Cy5 (M1/70.15.11; DRFZ); nuclei were counterstained with DAPI (Invitrogen). All of the images were acquired using a Karl Zeiss microscope (Axioplan).

Isolation of CNS-infiltrating cells

Mice were humanely sacrificed at the indicated time points and perfused with 15 ml PBS. Brain and spinal cord were cut, dried, and stored at −20°C. For FACS analysis, brains and spinal cords were incubated in 0.8 mg/ml DNase I (Sigma-Aldrich) for 12 min at 37°C. Tissues were passed through a 19G needle and further incubated for 12 min at 37°C. The suspension was passed through 19G and 25G needles for generation of a single-cell suspension. Cells were centrifuged, washed twice with RPMI 1640, resuspended in 30% Percoll, and overlayed on 70% Percoll. Gradient solutions were centrifuged at 2000 rpm for 20 min without braking, and the interface, containing mononuclear cells, was recovered. Cells were washed twice with RPMI 1640 and used for further manipulations.

Restimulation of cells in vitro

Spleen and lymph node cells were isolated at the indicated time points. Single-cell suspensions were made using a 70-μm cell strainer (BD Bioscience). For spleen, RBCs were lysed with erythrocytes lysis buffer. Finally, single-cell suspensions were counted and resuspended at 8 × 106 cells/ml and restimulated overnight with MOG35-55 peptide, in a final concentration of 40 μg/ml, or anti-CD3 (1 μg/ml)/anti-CD28 (1 μg/ml). For the analysis of cytokine-producing T cells, brefeldin A (5 μg/ml; Sigma-Aldrich) was added for the last 4 h of culture.

ELISA analysis

Protein levels of IL-12p40, IL-6, and TNF in supernatants were measured using ELISA Ready-Set-Go! kits (eBioscience), according to the manufacturer’s instructions.

In vitro T cell cultures

Naïve T cells (CD4+CD62L+CD25−) were isolated from OT-II mice and OT-II mice on a TNF-deficient background using a naïve T cell isolation kit (Miltenyi Biotec), according to the manufacturer’s instructions. The CD4+ fraction from OT-II mice on a TNF-deficient background was irradiated and used as APCs. T cells and APCs were mixed at a 1:5 ratio, and cytokine cocktails were added to the culture. For Th1 polarization, we used recombinant murine (rm)IL-12 (10 ng/ml; R&D systems), anti–IL-4AB (10 μg/ml; clone 1B11), and OVA peptide (1 μg/ml). For generation of Th17 cells, we used recombinant human TGF-β (1 ng/ml; R&D systems), rmIL-6 (20 ng/ml; R&D systems), anti–IL-4AB (10 μg/ml; clone 1B11), and anti–IFN-γ (10 μg/ml; clone AN18), together with OVA peptide (1 μg/ml).

Flow cytometry analysis and Abs

For flow cytometry, FeRs were blocked with Ab 2.4G2 (10 μg/ml), followed by staining with Abs against various surface markers. Abs for IL-17 PE (TC11-18H10), TNF-α alkalycyocyanin (MP6-XT22), CD45-FITC, CD45–PE-Cy5 (both 30F11), and Ly6C-6 (AL-21) and secondary reagents Strp-PE, Strp-PerCP, and Strp-Cy5 were from BD Bioscience. Anti–IFN-γ–FITC (AN18.17.24), anti–CD11b-Cy5 (M1/70.15.11), anti–Gr1–FITC (RB6-8C5), anti–CD4-biotin (GK1.5), and anti–CD4-Cy5 (GK1.5) were purified from respective hybridomas and conjugated to fluorochromes in the Core Antibody Facility at DRFZ. CD11c-bio Ab was purchased from eBioscience. Foxp3 staining was performed using a Foxp3 staining kit from eBioscience. Cells were acquired using flow cytometers FACSCalibur and LSRII (both from BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

Bone marrow-derived macrophages cultures and activation

Bone marrow cells were cultured in DMEM containing 20% horse serum and 30% L929-conditioned supernatant for 7 d. Cells were harvested and activated for 8 h with murine TNF (100 ng/ml), washed twice with DMEM, and stimulated with LPS (100 ng/ml) for 18 h prior to harvesting and isolation of mRNA.

Real-time PCR analysis

RNA from the indicated tissues and cells was isolated using TRI reagent, according to the manufacturer’s instructions (Sigma-Aldrich). One microgram of RNA was treated with RQ1 Dnase 1 (Promega), and cDNA was synthesized using Im-Prom II reverse transcriptase (Promega). Real-time PCR was performed using Brilliant II SYBR Green QPCR Master Mix (Agilent). PCR was performed using Stratagene Real-time PCR amplifier. All reactions were run using the following program: 95°C for 10 min and 50 cycles of 95°C for 10 s and 60°C for 30 s. The following primers were used: CCL1_Fw: 5′-CTTCCAGCCTTCAATAGCTGC, CCL1_Rv: 5′-CCTGAACTCTCCTGACTACACAC-3′; CCL2_Fw: 5′-ACC-TGTCCTACTTACATCACC-3′; CCL2_Rv: 5′-CACTGTACACAGTGTCACTCC-3′; CCL3_Fw: 5′-GCTGCGCTTACAAGAATTTG, CCL3_Rv: 5′-CAGTAGGCTCCTACACTCTC-3′; CCL5_Fw: 5′-ACTAACCTGATCTAGTGTTCG, CCL5_Rv: 5′-GCTCGCTACTGATCTACACAC-3′; CCL7_Fw: 5′-GAGGAGAAGGATGATAAGAAC, CCL7_Rv: 5′-CCTCAGCGACGGTACCTCTG-3′; CCL10_Fw: 5′-GGTCGGGAGGAGTATTTCA-3′; CCL19_Fw: 5′-GGAAGGAGCTTCCACAGGATGTT-3′; CCL19_Rv: 5′-CTTACGGGCTCTTACCTTACCC-3′; IL-12p40_Fw: 5′-AGATGGAGTGTTACAGGCTCTG-3′; IL-12p40_Rv: 5′-CAATTTCCTTCTTGTTGGAGCA-3′; IL-6_Fw: 5′-GTTTCTGGGAAAATCTGGTA-3′; IL-6_Rv: 5′-TGACTCTCAGGGATGTATG-3′; TNF_Fw: 5′-GGGAGGAGCTTCCACAGGATGTT-3′; TNF_Rv: 5′-CCTCAGCGACGGTACCTCTG-3′; β-actin_Fw: 5′-CTTCCAGGCGCAAGAATCTGCTG-3′; β-actin_Rv: 5′-TAAAACGGCACTGATGAACCTGCC-3′.

Statistical analysis

Statistical analysis was done using the two-tailed unpaired Student t test, unless otherwise stated. The p values < 0.05 were considered statistically significant.

Results

TNF prevents chronic EAE development and inhibits autoreactive T cell responses

To investigate the role of TNF in CNS autoimmunity, we first compared the clinical score of EAE in WT versus TNF-deficient mice (TNF KO), because the gene-targeting design used to produce these mutant animals differed from that used in previously

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tested TNF KO strains (7, 10, 19). Because our TNF KO strain was originally generated from TNF\textsuperscript{flox/flox} mice (19), we felt that it was the most appropriate control for the entire study. Upon EAE induction, WT animals developed classical monophasic EAE characterized by ascending paralysis 12–16 d after immunization and prominent leukocytic infiltration in the CNS. In accordance with previous reports (6, 10), TNF-deficient mice displayed delayed disease onset but then developed an exacerbated form of EAE compared with WT mice (Fig. 1A, Table I). TNF KO mice exhibited delayed disease induction due to impaired accumulation of inflammatory cells in the CNS, as revealed by H&E staining of spinal cord sections at day 14 after immunization (Fig. 1B). Consistently, the CNS infiltrates isolated from TNF KO mice at this time point contained lower numbers of both CD4\textsuperscript{+} T cells and CD11b\textsuperscript{+} myeloid cells, compared with their WT counterparts (data not shown). Next, we analyzed whether TNF influenced the distribution of myeloid cells within the CNS during disease progression. We found that TNF deficiency had no apparent effect on the localization of inflammatory macrophages (Mac-3\textsuperscript{+} cells) in CNS tissue during EAE (Supplemental Fig. 1). Altogether, these data indicated that TNF is required for initial recruitment of inflammatory cells to the CNS.

**FIGURE 1.** TNF prevents development of chronic EAE and limits autoactive Th development. A, Development of EAE in TNF-deficient mice. Data are representative of four independent experiments (six mice/group). B, H&E staining of spinal cords of WT and TNF KO mice at day 14 after immunization (original magnification \( \times 25 \)). Arrows denote infiltration of lymphoid cells to the CNS. Data are representative of two independent experiments (five mice/group). C, Numbers of total CNS-infiltrating lymphocytes to the CNS. Data are representative of two independent experiments (five mice/group). D, Numbers of total CNS-infiltrating lymphocytes in the CNS of WT and TNF KO mice at days 120 and 35 after immunization. Data (mean \( \pm \) SEM) are representative of three experiments (three to six mice/group). Representative FACS plots of CD11b\textsuperscript{+} cells, CD4\textsuperscript{+} cells, and IFN-\( \gamma \)- and IL-17–producing T cells among CNS-infiltrating cells in TNF KO and WT mice at days 20 (D) and 35 (E) after immunization. Data (mean \( \pm \) SEM) are representative of three experiments (three to six mice/group). MOG-specific Th1 (F) or Th17 (G) CD4\textsuperscript{+} T cell numbers in spleens of WT and TNF-deficient mice at days 20 and day 35 after immunization. Data (mean \( \pm \) SEM) are representative of four experiments (three to six mice/group). *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
To gain insight into how TNF deficiency may ultimately result in more severe disease manifestation, we analyzed CNS-infiltrating cells at later time points after EAE induction. Remarkably, total numbers of infiltrated cells were significantly higher in the CNS of TNF KO mice on day 35 after EAE induction compared with WT mice (Fig. 1C). This was associated with an increase in numbers of CNS cells in TNF KO mice from days 20 to 35, whereas this number decreased during the same time period in WT mice (Fig. 1C). Detailed analysis of immune cell infiltration revealed that, on day 20, TNF-deficient mice had a greater frequency of infiltrating CD4+ T cells, but fewer CD11b+ cells, compared with WT mice (Fig. 1D). At a later time point (day 35), TNF KO mice exhibited markedly increased frequencies of both CD4+ T cells and CD11b+ myeloid cells in the CNS (Fig. 1E). TNF KO mice also showed more Th1 cells in the CNS than WT mice at this time point (Fig. 1E). Of note, TNF-deficient mice also had greater absolute numbers of Th17 cells (Figs. 1E, 2E). Collectively, these findings indicated that TNF ultimately limits the progression of established disease by inhibiting late accumulation of pathogenic immune cells in CNS.

To further understand how TNF may limit the immune processes driving pathogenesis of EAE, we analyzed the development of MOG-reactive T cells in spleen of WT and TNF-deficient animals at various time points. TNF KO mice showed markedly more Th1 MOG-reactive T cells in spleen on day 20 after immunization, and this level was sustained at day 35 (Fig. 1F, 1G). MOG-reactive Th17 cells were also present in greater numbers in spleens of TNF KO mice compared with WT mice on day 20 (Fig. 1F). However, this difference was no longer significant at day 35 after

<table>
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<tr>
<th>Mouse Strain</th>
<th>Incidence (%)</th>
<th>Day of Onset (Mean ± SD)</th>
<th>Disease Severitya (Mean ± SD)</th>
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<tr>
<td>WT</td>
<td>100 (13/13)</td>
<td>14.7 ± 1.4</td>
<td>3.2 ± 0.7</td>
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<tr>
<td>T-TNF KO</td>
<td>93 (14/15)</td>
<td>14.3 ± 1.4</td>
<td>2.5 ± 0.7**</td>
</tr>
<tr>
<td>M-TNF KO</td>
<td>100 (14/14)</td>
<td>16.8 ± 2.1**</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>MT-TNF KO</td>
<td>93 (13/14)</td>
<td>17.8 ± 2.3***</td>
<td>2.3 ± 1.0*</td>
</tr>
<tr>
<td>TNF KO</td>
<td>88 (14/16)</td>
<td>17.1 ± 1.5***</td>
<td>2.5 ± 1.0*</td>
</tr>
</tbody>
</table>

Data are representative of at least two experiments.

aClinical score for EAE at day 20 after immunization (peak of disease for WT mice).

*p < 0.05, **p < 0.01, ***p < 0.001, versus WT; Student t test.
immunization (Fig. 1G). Therefore, TNF might protect from EAE by limiting the expansion of pathogenic T cell responses.

Collectively, these results showed that TNF mediates several functions during EAE. It promotes disease by accelerating the initial recruitment of inflammatory cells into the CNS. However, TNF also plays protective roles, downregulating the late accumulation of cells in the CNS, as well as inhibiting the development of autoreactive Th1 and Th17 CD4+ T cells in secondary lymphoid tissues.

**T cells and myeloid cells are critical TNF sources during EAE**

T cells and myeloid cells are major players in the pathogenesis of EAE and MS (20). Therefore, we asked whether TNF production by T cells and myeloid cells is critical for EAE development. Induction of EAE in MT-TNF KO mice resulted in delayed disease onset compared with WT mice (Fig. 2A, Table I). In addition to that, we observed a trend toward greater disease severity in MT-TNF KO mice compared with control mice during the later stages of EAE. In contrast, TNF ablation in B cells had no significant influence on disease course or autoreactive T cell responses at day 35 after immunization (Supplemental Fig. 2). Analysis of CNS-infiltrating cells at day 20 postimmunization (i.e., at peak of disease for WT animals) revealed elevated numbers of CD4+ T cells and decreased infiltration of CD11b+ cells in MT-TNF KO mice (Fig. 2B), as previously observed in completely TNF-deficient animals. Similarly to TNF KO mice, MT-TNF KO mice also harbored increased numbers of total CNS-infiltrating cells, including CD11b+ inflammatory cells (Fig. 2C, 2D), as well as MOG-reactive IFN-γ- and IL-17-producing CD4+ T cells at day 35 after immunization (Fig. 2E). Consistent with this, we also found greater numbers of Th1 CD4+ T cells in spleens of TNF KO and MT-TNF KO animals at day 35 after immunization (Fig. 2F). Taken together, these findings demonstrated that MT-TNF–deficient mice mostly recapitulate the disease course and immunological abnormalities observed in the CNS and spleen of completely TNF-deficient animals. From these results, we concluded that myeloid cells and T cells are the critical sources of TNF during EAE. In contrast, provision of TNF by B cells did not appear to play a major role in this disease model.

**TNF produced by T cells exacerbates EAE and limits Th1 and Th17 cell development**

We next analyzed the individual contribution of TNF produced either by T cells or by myeloid cells in EAE. First, EAE was induced in mice lacking TNF only in T cells (T-TNF KO). Disease symptoms were significantly milder in T-TNF KO mice than in WT littermate controls, indicating that T cell-derived TNF plays a pathogenic role during EAE (Fig. 3A, Table I). Decreased demyelination visualized by Luxol fast blue staining of spinal cord sections also correlated with milder disease severity in T-TNF KO mice compared with WT animals (Fig. 3B). Diminished EAE in T-TNF KO animals could be due to reduced homing of inflammatory cells into the CNS. To address this, we analyzed CNS-infiltrating cells at day 20 after EAE induction. Intriguingly, T-TNF KO mice exhibited increased numbers of total CNS-infiltrating cells at day 20 after immunization (Fig. 3C). FACs analysis of CNS-infiltrating cells at this time point revealed significantly increased CD4+ T cell numbers and frequencies, including for IFN-γ- and IL-17–producing CD4+ T cells (Fig. 3D, 3E) but not regulatory T cells (CD4+Foxp3+) (Fig. 3F). In contrast to this augmented T cell response, the numbers of CNS-infiltrating monocytes (CD11b+ GR1lowLy6C+Ly6Chigh) and neutrophils (CD11b+GR1+Ly6C+Ly6Chigh) were strongly reduced (Fig. 3G, 3H), suggesting a unique role for T cell-derived TNF in the control of phagocyte accumulation in the CNS during EAE.

Because ablation of TNF in T cells resulted in greater numbers of MOG-reactive Th1 cells in the CNS, we asked whether T cell-derived TNF affected Th1 and Th17 development in secondary lymphoid organs. Indeed, T-TNF KO mice displayed increased development of MOG-specific Th1 and Th17 cells in spleen at day 20 after immunization (Fig. 3J). The observed effect was not due to delayed T cell activation, because T-TNF KO mice exhibited increased Th1 and similar Th17 numbers in spleen already by day 9 postimmunization (Supplemental Fig. 3).

In conclusion, TNF produced by T cells exerts a dual role during EAE: on the one hand, it promotes pathogenesis by prompting macrophage accumulation in the CNS; on the other hand, it limits disease by restricting Th1 and Th17 cell development in secondary lymphoid organs.

**TNF expressed by myeloid cells accelerates EAE onset and limits Th1 and Th17 cell development**

The onset of EAE occurred earlier in TNF KO and MT-TNF KO mice, but not in T-TNF KO mice, compared with WT animals. Of interest, CD11b+ myeloid cells were the major TNF-producing cell subset in spinal cord of WT mice at the beginning of EAE (Supplemental Fig. 1). Therefore, we hypothesized that TNF derived from myeloid cells may accelerate EAE onset. Indeed, upon EAE induction, M-TNF KO mice displayed a delayed onset of disease symptoms compared with WT animals (Fig. 4A, Table I). Delayed disease onset in M-TNF KO mice was associated with reduced numbers of both CD4+ and CD11b+ CNS-infiltrating cells at day 14 after immunization compared with littermate controls, a time point when WT animals showed the first signs of paralysis (Fig. 4B). This effect was specific to myeloid cell-derived TNF, because T-TNF KO animals exhibited similar numbers of CD4+ T cells and reduced numbers of CD11b+ cells in the CNS at day 14 after immunization (Supplemental Fig. 4). Altogether, this suggested that M-TNF regulates disease induction via recruitment of inflammatory T and myeloid cells to the CNS.

Upregulation of multiple chemokines, such as CCL1, CCL2, CCL7, and CXCL10, in the CNS precedes the appearance of the first clinical symptoms of paralysis and represents one of the critical steps for disease initiation (21). Because TNF can induce expression of chemokines and could, thereby, facilitate recruitment of inflammatory cells to the site of inflammation (6), we assessed chemokine expression in the CNS of M-TNF KO and WT mice at day 14 after immunization. We found a strong reduction in mRNA coding for chemokines, such as CCL1, CCL6, CCL7, and CCL19, in spinal cord of both M-TNF KO and TNF KO mice relative to WT animals (Fig. 4C), indicating that TNF production by myeloid cells may accelerate EAE onset via induction of chemokine expression in the CNS.

Analysis of CNS-infiltrating cells at later time points after disease was developed (day 20) revealed no significant differences in the total numbers of CD4+ T cells and CD11b+ myeloid cells between WT and M-TNF KO mice (Fig. 4D, 4E). M-TNF KO mice also presented with similar numbers of IL-17–producing T cells as WT mice, yet they exhibited lower frequencies of IFN-γ–producing T cells (Fig. 4F). Thus, M-TNF specifically regulates accumulation of Th1 cells in the inflamed CNS.

Taking into account that TNF KO, MT-TNF KO, and T-TNF KO mice developed increased Ag-specific Th1 and Th17 responses during EAE, we next asked whether myeloid cell-derived TNF also controls the magnitude of Th responses during EAE. To this end, we analyzed MOG-reactive Th1 and Th17 CD4+ T cells at day 20 after EAE induction. At that time point, M-TNF KO mice...
exhibited greater numbers of MOG-specific Th1 and Th17 cells in spleen (Fig. 4G). Analysis of Th cell responses before the appearance of clinical symptoms revealed increased Th1, but normal Th17, cells in comparison with WT mice (Supplemental Fig. 3).

Taken together, TNF produced by myeloid cells promotes EAE onset via induction of proinflammatory chemokines in CNS, but it limits the peripheral induction of autoreactive Th1 and Th17 responses.

**TNF ablation in T cells or in myeloid cells is insufficient for prolonged persistence of inflammatory cells during EAE**

Given that concomitant ablation of TNF in T cells and in myeloid cells resulted in heightened persistence of inflammatory cells in CNS compared with WT animals (Fig. 2), we next tested whether TNF ablation in T cells or myeloid cells only was responsible for this effect. To address this, we analyzed mice at day 35 after EAE induction. Neither TNF deletion in T cells nor in myeloid cells...
alone resulted in persistence of inflammatory cells in the CNS (Fig. 5A, 5B).

T-TNF KO and WT animals contained similar frequencies of CD4+ and CD11b+ cells, as well as similar numbers of Th1 and Th17 T cells in their CNS at the later stage of EAE (day 35 after immunization) (Fig. 5A, 5B), which correlated with their similar clinical scores at that phase of the disease. Of note, T-TNF KO animals exhibited increased IFN-γ–producing CD4+ T cells, but similar numbers of IL-17–producing CD4+ T cells in spleen compared with WT counterparts (Fig. 5C). However, the contraction of MOG-reactive, cytokine-producing T cells in the CNS remained unaffected (Fig. 5A, 5B). From these results, we concluded that myeloid cells or T cells can independently regulate the late accumulation of immune cells in the CNS through TNF production.

TNF expressed by T cells and myeloid cells regulates IL-12p40 and IL-6 production by APCs during EAE

Next, we aimed to address the mechanism responsible for increased Th1 and Th17 CD4+ T cell development in T-TNF KO and M-TNF KO mice upon EAE induction. Increased Th cell development in vivo could be due to several factors: TNF could directly act on Th cells during Th polarization or it could affect numbers, activation status, or cytokine production of APCs (22–24). First, we tested whether TNF directly affects Th cell polarization. For this purpose, Th1 and Th17 T cells were generated in vitro from naive T cells isolated from OT-II mice on WT or TNF-deficient background. We found that absence of TNF did not have a significant impact on Th1 and Th17 differentiation (Fig. 6A), pointing to an
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FIGURE 5. TNF ablation in T cells or in myeloid cells is insufficient for prolonged persistence of immunological cells during EAE. A, Representative FACS dot plots of CNS-infiltrating CD4+ T cells and CD11b+ mononuclear phagocytes in WT, T-TNF KO, M-TNF KO, and TNF KO mice at day 35 after immunization. B, Numbers of IFN-γ- and IL-17-producing T cells in the CNS of WT, T-TNF KO, M-TNF KO, and TNF KO mice at day 35 after immunization. C, Numbers of IFN-γ- and IL-17-producing T cells in spleens of WT, T-TNF KO, M-TNF KO, and TNF KO mice at day 35 after immunization. Data (mean ± SEM) are representative of two independent experiments. *p < 0.05, **p < 0.01.

indirect effect of TNF on Th cell development. T-TNF KO, M-TNF KO, and WT animals harbored similar numbers of CD11c+ dendritic cells, polymorphonuclear cells (CD11b+Gr1high), and inflammatory monocytes (CD11b+Gr1low) in spleen at day 20 after immunization, arguing against the possibility that T cell- or myeloid cell-derived TNF controls APC homeostasis during disease (Fig. 6B, data not shown). Moreover, we did not observe any difference in the expression of surface activation markers by CD11c+ dendritic cells from T-TNF KO and M-TNF KO animals compared with WT animals (data not shown).

To test whether TNF produced by distinct cell types influenced cytokine production during EAE, we cultured splenocytes from different groups of mice at various time points after EAE induction and quantified cytokine levels 72 h later. Strikingly, we observed significantly increased IL-12p40 and IL-6 levels in supernatants from T-TNF KO and M-TNF KO mice compared with WT mice, which correlated with decreased levels of total TNF in the organ (Fig. 6C–E). Consistent with this finding, pretreatment of bone marrow-derived macrophages (BMDMs) with TNF dampened IL-12p40 and IL-6 mRNA expression induced by LPS stimulation (Fig. 6F). Taken together, these data suggested that TNF produced by both T cells and myeloid cells regulates expression of IL-12p40 and IL-6 and, thereby, regulates development of autoreactive T cells during EAE.

Discussion

Clinical trials using TNF blockade in MS patients were terminated because of disease exacerbation (11), and several reports emphasized the protective activities of TNF during EAE (8–10). Yet, other studies demonstrated the pathogenic contribution of this cytokine to the disease course (7, 10, 25, 26). The role of TNF in CNS autoimmunity remained controversial, requiring a more precise analysis of the functions of this cytokine in such a type of pathology. In this study, using a panel of mice with cell type-specific TNF ablation, we demonstrated that TNF may exhibit pathogenic activities in the CNS, with a distinct contribution of TNF produced by myeloid cells and T cells. TNF could mediate its pathogenic functions by promoting recruitment of inflammatory cells, which are known to be critical for CNS damage (27). Importantly, we identified three distinct stages in the TNF-mediated control of lymphocyte accumulation in the CNS. In the early phase, myeloid cell-derived TNF regulates disease onset by promoting infiltration of immune cells in the CNS. In the second phase, TNF from effector T cells promotes disease exacerbation by stimulating the accumulation of myeloid cells in the CNS. In the late phase, myeloid and T cells both reduce the accumulation of immune cells in the CNS through TNF production. Considering that ablation of TNF production by myeloid cells did not affect disease severity, it is likely that the TNF-expressing macrophages observed in the CNS lesions of MS patients (15, 28) mediate direct tissue damage via TNF-independent mechanisms.

Multiple studies reported increased levels of chemokines, such as CCL1, CXCL10, CCL2, CCL19, and CCL21, in the CNS prior to the development of EAE, indicating that regulation of chemokine expression is an important step for disease induction (29). Genetic or pharmacological blockade of the aforementioned chemokines or their corresponding receptors resulted in decreased numbers of CNS-infiltrating cells and amelioration of the disease course (21, 30–33). Chemokine production during EAE has been a subject of extensive research over the last years. Various cells in CNS can express chemokines during neuroinflammation. For instance, astrocytes are major producers of CCL7 and CXCL10 during CNS inflammation (34–36), whereas microglial cells are a predominant source of CCL1 (6). Finally, inflamed venules and infiltrating lymphocytes express CCL19 (30, 37). TNF is one of the critical cytokines inducing chemokine expression during inflammation (6), and our data point to a role of TNF produced by myeloid cells in early chemokine induction in the CNS. It is very likely that TNF-expressing myeloid cells activate CNS-resident cells to produce chemokines, which attract the autoreactive T cells that then orchestrate locally the induction of disease manifestation. Additional experiments addressing the dynamics of TNF-producing myeloid cells and T cells in vivo may help to elucidate this.

EAE is widely viewed as a Th1- and Th17-driven disease. However, the specific functions of individual inflammatory cytokines produced by these cells during EAE remain incompletely understood. Despite the proposed contribution of IL-17- and IFN-
γ-producing Th cells in EAE development, overexpression of IL-17 in T cells had no impact on EAE severity, whereas IFN-γ deficiency exacerbated EAE (38, 39). Furthermore, GM-CSF production by both Th1 and Th17 cells was reported to be critical for EAE progression via control of myeloid cell accumulation in the CNS (40). TNF is expressed by both Th1 and Th17 cells in vitro and in vivo (41, 42). However, the significance of TNF production in the pathogenic potential of Th cells during EAE remains unknown. In this article, we demonstrated that ablation of T cell-derived TNF alone significantly reduced disability, even in the presence of increased numbers of Th1 (IFN-γ+) and Th17 (IL-17+) cells in the CNS. Our findings indicated that TNF from T cells might facilitate tissue damage by promoting accumulation of mononuclear phagocytes into the CNS. Taking into account that T cell-derived GM-CSF also regulates myeloid cell numbers during EAE (40), it would be important to understand whether GM-CSF and TNF are produced by the same pathogenic T cells and whether these cytokines have nonredundant or overlapping functions during EAE progression. It is also possible that T cell-derived TNF perpetuates disease by potentiating local inflammatory effects of IL-17. Indeed, Th17 cells were recently implicated in direct neuronal damage, and in vitro studies revealed that the proinflammatory actions of IL-17 on cells from the CNS are greatly enhanced in the presence of TNF (43, 44). Consistent with this, EAE induced by either Th1- or Th17-polarized CD4+ T cells can be inhibited by anti-TNF therapy (26). Therefore, it is becoming more evident that autoreactive Th1 and Th17 cells modulate the course of EAE via production of various proinflammatory cyto-
kines; further studies delineating the individual contributions of each cytokine for disease manifestation are required.

In addition to its profound proinflammatory effects, TNF was shown to limit IL-12p40 and IL-6 production in splenic APCs, suggesting a potential immunosuppressant function (22–24). We confirmed these findings and further showed that TNF expressed by both T cells and myeloid cells, rather than TNF from a single cellular source, limited in vivo production of IL-12p40 and IL-6, which regulated the development of Th1 and Th17 cells. Concomitant ablation of TNF in myeloid cells and T cells was sufficient to recapitulate chronic disease in mice with complete TNF ablation, characterized by persistence of inflammatory cells in the CNS. Our data shed new light on possible mechanisms of action of TNF blockers in MS patients.

Recent findings suggested the impermeability of the blood–brain barrier to large molecules, such as therapeutic Abs (45). Because TNF contributes to permeability of the brain barrier (46, 47), available anti-TNF drugs may, in fact, diminish permeability of the blood–brain barrier. This may imply that TNF inhibitors could mediate their action in patients mainly outside of the CNS, in particular, by neutralizing protective functions of TNF in secondary lymphoid organs. This could explain why administration of TNF blockers resulted in exacerbation of MS in treated patients or the appearance of neurologic symptoms in rheumatoid arthritis patients treated with such drugs. Further studies on how anti-TNF therapy affects blood–brain barrier integrity and subsequent penetration of Abs to the CNS are required to specifically address this question. In contrast, novel strategies that are able to neutralize TNF functions selectively in the target organ might be better suited to limit the formation of inflammatory lesions in the CNS.

Acknowledgments
We thank K. Horn, Y. Shebzukhov, A. Kuchmiy, and M. Drutskaia for help during this project. We are grateful to A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY), S. Grivennikov (University of California, San Diego, California, IL), and T. Geske for preparation of Abs.

Disclosures
The authors have no financial conflicts of interest.

References


