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Srividya Sriskantharajah,*1 Eva Gückel,*1 Niki Tsakiri,† Katrin Kierdorf,‡ Christine Brender,* Abduelhakem Ben-Addi,* Marc Veldhoen,§ Philip N. Tsichlis,* Brigitta Stockinger,§ Anne O’Garra,∥ Marco Prinz,‡ George Kollias,* and Steven C. Ley*

Tumor progression locus 2 (TPL-2), also known as COT and MAP3K8, is expressed in both hematopoietic and nonhematopoietic cells, and it functions as a MAP3 kine-

The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; DC, dendritic cell; dLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; IFN-γ, interferon-γ; IL-17, interleukin-17; Mtb, Mycobacterium tuberculosis; M2, M2-like macrophages; NOD, non-obese diabetic; PE, phycoerythrin; PE-Cy5.5, phycoerythrin-coupled Cy5.5; RFP, red fluorescence protein; SPL, spleen; T, transgenic mouse strain; TPL-2, tumor progression locus 2; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling; WT, wild-type.

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(12). We found that TPL-2 was dispensable for cytokine-induced differentiation of naive T cells to the Th17 cell lineage in vitro. Furthermore, TPL-2 was not required for the initial activation and expansion of MOG-specific Th17 cells in the periphery during EAE. Generation of IFN-γ-producing Th1 cells, which may also play a role in EAE (11), was also independent of TPL-2 expression. Nevertheless, TPL-2 was demonstrated to regulate both the onset and severity of EAE. Cell transfer experiments established that TPL-2 regulated the effector phase of EAE in the CNS, functioning in radiation-resistant stromal cells. Importantly, using a newly generated Map3k8 knock-in mouse strain, we also showed that the development of EAE was dependent on TPL-2 catalytic activity, while ruling out any potential function of TPL-2 as scaffolding protein in the disease process. Our data therefore suggest that small molecule inhibitors of TPL-2 might be beneficial therapeutically in MS, the most common inflammatory demyelinating disease of the CNS (13).

Materials and Methods

**Mice**

Map3k8<sup>−/−</sup> (2), Tnpi2<sup>−/−</sup> (14), and Ifnar<sup>−/−</sup> mice were fully backcrossed to C57BL/6 mice. Map3k8<sup>−/−</sup> Rag1<sup>−/−</sup> and Ifnar<sup>−/−</sup> Map3k8<sup>−/−</sup> mice were generated by intercrossing the appropriate single knockout mice. For the generation of Map3k8<sup>D270A/D270A</sup> mice, a P1-derived artificial chromosome clone (519-N17), including the Map3k8 gene, was isolated from an RPC121 P1-derived artificial chromosome library (obtained from the U.K. Human Genome Mapping Project Resource Centre, Hinxton, U.K.) by hybridization with a 1.5-kb probe encompassing full-length rat Map3k8 cDNA. A 6.6-kb Clal-BamHI fragment containing exon 5, which encodes D270, was isolated and subcloned into PBLeucscript SK<sup>−</sup> (Invitrogen) to create pSK-LA ("left arm"). From this plasmid, a 3.2-kb PstI fragment was subcloned into PBLeucscript SK<sup>−</sup> to create pSK-LA.PstI. PCR was used to mutate the sequence encoding D270 to alanine. To facilitate screening for the mutation, a BssSI site was introduced next to the 3′ of the D270A mutation without altering with the coding sequence. The D270A-containing PstI fragment was subsequently reinserted into pSK-LA to create pSK-LA.PstI was used in specific pathogen-free animal facilities of the National Institute for Medical Research (London, U.K.) in accordance with the U.K. Home Office regulations. Six- to 10-week-old male mice were used for all experiments.

**Abs**

Abs to TPL-2, IeBr, ERK-1, ERK-2, and actin were purchased from Santa Cruz Biotechnology, whereas p-p105 (Ser933), p-p38, and p-ERK (Thr185/Tyr187) Abs were obtained from Invitrogen. Tubulin mAb was provided by Keith Gull (University of Oxford).

A number of fluorescently labeled Abs for flow cytometry were used against GM-CSF-PE, Gr1-FITC, CD25-PE, TCR-β-PE, streptavidin-PerCP, and streptavidin-PE, which were purchased from BD Pharmingen. IL-17A–allophycocyanin, IFN-γ–FITC, CD4–FITC, CD4–PE, F4/80–allophycocyanin, F4/80–PE, biotinylated Gr1, CD25–allophycocyanin, CD44–AF450, CD44–FITC, CD44–PE, CD45–Alexa Fluor 480, biotinylated CD45.1, CD11c–PE, CD11b–PE, biotinylated PE, and biotinylated MHC class II were obtained from eBioscience. CD4–PerCP, and CD19-Pacific Blue were purchased from BioLegend. CD4–PE/Texas Red and CD8–PE/Texas Red were obtained from Invitrogen.

**Induction and assessment of EAE**

Active EAE was induced by immunizing mice s.c. with 250 μg MOG<sub>35-55</sub> peptide (Cambridge Research Biochemicals), emulsified in CFA, containing 250 μg heat-killed *Mycobacterium tuberculosis* (H37RA; Difco Laboratories). Mice received 200 ng pertussis toxin (Calbiochem) i.p. on day 0 and 2 after immunization. For passive EAE experiments, MOG<sub>35-55</sub> WT or WT mice were injected i.v. with 30 × 10<sup>6</sup> Th17-polarized MOG<sub>35-55</sub>-specific T cells and i.p. with pertussis toxin (days 0 and 2). To generate Th17 cell populations, draining lymph node (DLN) cells from Map3k8<sup>−/−</sup> WT or WT mice were collected 10 d after immunization with MOG<sub>35-55</sub> peptide plus CFA. Cells were cultured for 3 d (5 × 10<sup>5</sup> cells/ml; 24 wells) in IMDM (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated FCS (LabTech), 5 mM l-glutamine, 50 μM 2-ME, and antibiotics (all Life Technologies) in the presence of 10 μg/ml MOG<sub>35-55</sub> peptide under Th17-polarizing conditions (20 ng/ml recombinant murine IL-23 [R&D Systems] plus 20 ng/ml recombinant murine IL-1β [Invitrogen]) or Th1-polarizing conditions (20 ng/ml recombinant murine IL-12 [R&D Systems] and 1 μg/ml anti-IL-23p19 [eBioscience]). Clinical signs of disease were assigned daily: 0, no symptoms of disease; 1, loss of tail tonicity; 2, hindlimb weakness/impaired gait; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, forelimb paralysis or moribund.

**Generation of bone marrow radiation chimeras**

Bone marrow cells from Map3k8<sup>−/−</sup> or WT control mice were depleted of T cells with biotinylated TCRβ mAb (H57-597; BD Pharmingen) and streptavidin-labeled magnetic beads (Dynal/Invitrogen). Five to 10 × 10<sup>6</sup> cells were then transferred by i.v. injection into lethally irradiated (twice 400 rad) Rag1<sup>−/−</sup> or Tp2<sup>−/−</sup> Rag1<sup>−/−</sup> hosts. For mixed bone marrow chimeras, WT or Map3k8<sup>−/−</sup> bone marrow cells were mixed with Tera<sup>−/−</sup>, or μMT<sup>−/−</sup> bone marrow cells at a ratio of 1:4, prior to injection into sublethally irradiated (500 rad) Rag1<sup>−/−</sup> mice. EAE was induced 6–8 wk after cell transfer.

**Isolation of CNS cells**

Brains and spinal cords were mechanically homogenized and passed through a 70-μm cell strainer (BD Pharmingen). Cells were centrifuged at 400 × g for 5 min at 4°C, and lymphocytes were separated on a 36.5% Percoll gradient (GE Healthcare) prior to flow cytometric staining.

**Histological analysis**

Spinal cords were removed and fixed in 4% formalin. Paraffin-embedded sections were stained, as previously described (15), with Luxol fast blue, anti-CD3 (Serotec), anti–Mac-3, anti-B220 (both BD Pharmingen), and amyloid precursor protein (APP; Chemicon).

**T cell recall assay**

LN cells were isolated from mice 9 d after immunization with MOG<sub>35-55</sub> peptide. Cells were cultured for 24 h in complete medium (2 × 10<sup>6</sup> cells/ml; 96-well flat-bottom plates) containing 10 μg/ml MOG<sub>35-55</sub> peptide, and supernatants were collected for quantification of IL-17 and IFN-γ by ELISA. For proliferation assays, LN cells were labeled with CFSE (Mohawk) and re-stimulated in vitro with MOG<sub>35–55</sub> peptide plus CFA. Cells were cultured for 3 d (5 × 10<sup>6</sup> cells/ml). Spinal cord tissue was collected in RNA stabilization buffer (Qiagen) and mechanically homogenized and passed through a 70-μm strainer. Isolation of CNS cells was performed as described previously (16), and cultured for a total of 72 h.

**ELISA quantitation of cytokines**

Levels of IL-17 and IFN-γ were quantified by ELISA according to the manufacturer’s protocol (eBioscience).

**Real-time quantitative PCR**

Spinal cord tissue was collected in RNALater stabilization buffer (Qiagen) 15 d after MOG<sub>35-55</sub> peptide/CFA immunization. Total RNA was isolated from spinal cords, cultured T cells, and primary cultures of microglia and astrocytes (RNeasy kit; Qiagen). After treatment with DNAAse I (Invitrogen), cDNA was synthesized (1 μg RNA; SuperScript First Strand Synthesis System, Invitrogen), and expression of mRNA was determined using an ABI Prism 7000 sequence detection system and commercial FAM-labeled probes (Applied Biosystems). Gene expression is displayed in arbitrary units (2<sup>ΔΔCT</sup>). Levels of disease were assigned daily: 0, no symptoms of disease; 1, loss of tail tonicity; 2, hindlimb weakness/impaired gait; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, forelimb paralysis or moribund.

**Protein analyses**

Purified bone marrow–derived macrophages (BMDMs), bone marrow–derived dendritic cells (BMDCs), and T cells were cultured at 12 h (1% FCS) to reduce basal ERK activation. BMDMs and BMDCs were stimulated with 1 μg/ml heat-inactivated *M. tuberculosis* (Difco Laboratories), whereas CD4<sup>+</sup> T cells were cultured with soluble anti-CD3 (1 μg/ml; BD Pharmingen) plus anti-CD28 (1 μg/ml; BD Pharmingen). Cultured primary microglia and astrocytes were stimulated with LPS (100 ng/ml; Enzo), recombinant murine TNF (50 ng/ml; R&D Systems), IFN-γ.
(100 ng/ml; R&D Systems), IL-1β (20 ng/ml; PeproTech), and IL-17A (100 ng/ml; R&D Systems), alone or in the indicated combinations. Cells were washed once in PBS before lysis in buffer A (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM NaVO₄, 100 mM okadaic acid; Calbiochem, 2 mM Na₃P₂O₇ plus protease inhibitors) containing 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. Centrifuged lysates were mixed with an equal volume of 2× Laemmli sample buffer, resolved by SDS-PAGE, and immunoblotted. Protein concentration in lysates was determined by Bradford assay (Bio-Rad).

Flow cytometry
Single-cell suspensions were obtained from LNs, spleen, brain, or spinal cords of mice via gentle homogenization through nylon mesh filters (70 μm; BD Pharmingen). Cell concentrations were determined using a Casy counter (Scharfe Instrument Systems). Erythrocytes in spleen samples were lysed prior to staining.

For analysis of surface markers, cells were stained with the indicated Abs in PBS (2% [w/v] BSA). For intracellular cytokine staining, cells were restimulated for 4 h with phorbol dibutyrate (PdBu; 0.5 μg/ml; Sigma-Aldrich), ionomycin (0.5 μg/ml; Sigma-Aldrich), and brefeldin A (1 μg/ml; GolgiPlug; BD Pharmingen), or with MOG 35–55 peptide for 12 h, and BMDC (CD11c+) populations. For biochemical analyses, CD4+ T cells were purified (≥95% for BMDM (E4/80)+ and BMDC (CD11c+) populations. For biochemical analyses, CD4+ T cells were purified (≥95% CD4+) from single-cell suspensions prepared from LNs by negative selection as described (16). For the isolation of naive T cells, CD4+ T cells were prepared from pooled LNs and spleens by negative selection, as described above. Cells were then stained with anti-CD4 (RM4/5; BD Biosciences), anti-CD25 (PC61.5; eBioscience), and anti-CD44 (IM7; BD Biosciences), and CD4+CD44-CD25- naive cells were isolated to purities of >98% on a MoFlo cytometer (Dako Cytomation). Naïve T cells were differentiated into Th17 cells as described (18, 19).

Mixed glial cultures were prepared from 1- to 2-d-old mice using a published protocol (20). In brief, brains were dissected and meninges were removed. Brains were mechanically homogenized and passed through a 70-μm cell strainer (BD Pharmingen). The resulting cell suspension was cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS, antibiotics, and 20% L929 cell supernatant, with medium changes every 3–4 d. After 10–14 d, the floating and loosely adherent microglial cells were harvested by vigorous shaking of culture flasks (250 rpm, 3 h, 37°C), before being replated at 2.0 × 10⁶ in 24-well plates and stimulated the following day. Adherent astrocytes were trypsinized, seeded at densities of 1.0 × 10⁶ per six-well plate, rested overnight in 1% serum, and stimulated the following day. Microglial cell populations were ≥98% CD11b+, whereas astrocyte cell populations were ≥85% glial fibrillary acidic protein+ staining, as determined by flow cytometry.

Statistical analyses
Data are presented as means ± SEM. For analysis of clinical scores, a two-way ANOVA with Bonferroni correction was applied. An unpaired Student t test with two-tailed p values was used for statistical analysis of in vitro assays. A nonparametric Mann–Whitney U test was used for statistical analyses of flow cytometric data. All statistical analyses were calculated using GraphPad Prism 5 software. A p value <0.05 was considered significant.

Results
TPL-2 is not essential for in vitro generation of Th17-polarized cells
TPL-2 is required for efficient polarization of naïve CD4+ T cells to IFN-γ+ producing Th1 cells in vitro, and in vivo after T. gondii infection (8). To initially determine whether TPL-2 was required for polarization to the Th17 cell lineage, CD25−CD44+CD4+ T cells from WT and Map3k8−/− mice were cultured under Th17-polarizing conditions (19). Intracellular staining revealed that TPL-2 deficiency did not significantly reduce the fraction of IL-17A+ cells (Fig. 1A, 1B). The amount of IL-17A in culture supernatants was also equivalent between WT and Map3k8−/− cells (Fig. 1C). Similarly, TPL-2 expression was not required for production of IL-17A+ cells or IL-17 protein in culture supernatants when either IL-1β or the aryl hydrocarbon receptor ligand 6-formylindololo[3,2-b]carbazole (FICZ) (18, 21) were added to the culture medium (Fig. 1).

Quantitative real-time PCR was used to determine whether TPL-2 regulated the induction of other Th17 signature cytokines and transcription factors (22). Steady-state levels of Il17a, Il17f, and Il21 mRNAs were equivalent in Map3k8−/− and WT CD4+ T cells in each of the conditions tested (Fig. 1D). The abundance of Il22 mRNA in IL-1β or FICZ containing cultures was also similar between Map3k8−/− and WT CD4+ cell cultures (18).

TPL-2 regulates the onset and severity of EAE
To investigate the physiological role of TPL-2 in Th17 cell development and function, we tested the susceptibility of Map3k8−/− mice to EAE, a Th17 cell-mediated animal model for MS (11). Map3k8−/− mice and control C57BL/6 WT mice were immunized with MOG35–55, and disease progression was monitored (13). Despite 100% incidence, Map3k8−/− mice showed a delay in the onset and a reduced disease severity relative to controls (Fig. 2, Table I). Accordingly, flow cytometric analyses at the peak of disease revealed significantly fewer CD45+ hematopoietic cells, including CD4+ and CD8+ T cells, CD19+ B cells, DCs, CD45+CD11b+ macrophages, and neutrophils, in the spinal cords of Map3k8−/− mice compared with WT mice (Fig. 2B).

Intracellular staining showed similar proportions of CD4+ T cells expressing IFN-γ and GM-CSF in the spinal cord between WT and Map3k8−/− mice (Fig. 2C, upper panel). However, the fraction of CD4+ cells producing IL-17A was significantly decreased by ~50% in the absence of TPL-2. Consistent with the overall decrease in CD4+ T cell number, the total numbers of CD4+ T cells expressing each of these encephalitogenic cytokines were significantly reduced in Map3k8−/− mice (Fig. 2C, lower panel). The proportions of γδ T cells expressing IL-17A and IFN-γ in the CNS were unaffected by TPL-2 deficiency, and total γδ T cell numbers were not statistically significantly different from WT (Supplemental Fig. 1A).

A number of proinflammatory cytokines and chemokines are produced in the spinal cord by infiltrating reactivated CD4+ T cells and activated CNS-resident cells (25, 26). Quantitative RT-PCR demonstrated significantly reduced amounts of mRNAs encoding most of these proteins in the spinal cords of Map3k8−/− mice at the peak of disease (Supplemental Fig. 1B). In contrast, steady-state levels of Il12a and Il12b mRNAs were increased, consistent with earlier in vitro studies (27).

Taken together, these data indicate that TPL-2 deficiency protected mice from EAE by limiting CNS inflammation, thereby reducing demyelination and axonal damage.
TPL-2 is not required for T cell priming during EAE

The disease course of EAE can be considered to occur in two stages: a priming phase, in which immunization leads to activation and expansion of peripheral myelin-specific T cells, and an effector phase, in which infiltrating inflammatory cells cause CNS damage. The priming phase involves the stimulation and expansion of myelin-responsive T cells by activated Ag-presenting DCs in LNs. The production of IL-18, IL-6, and IL-23 by DCs, and other innate immune cells, is critical for the initial induction of Th17 cells during an immune response (10), and signaling via each of these cytokines is essential for EAE induction (21, 25). Because TPL-2 has an established signaling function in DCs, we initially investigated the effect of TPL-2 deficiency on the induction of Th17-polarizing cytokines by BMDCs in response to heat-inactivated M. tuberculosis (Mtb).

Immunoblotting of cell lysates revealed that TPL-2 deficiency blocked the early activation of ERK by Mtb, whereas a second wave of ERK-1/2 phosphorylation was TPL-2–independent (Supplemental Fig. 2A). p38 activation was also reduced in Map3k8−/− BMDCs, similar to the reported effects of TPL-2 deficiency after LPS stimulation (27). Despite these decreases in MAPK activation, quantitative RT-PCR revealed that Mtb was still able to induce Il6 and Il23p19 mRNAs to similar levels in Map3k8−/− and WT BMDCs (Supplemental Fig. 2B). TPL-2 deficiency reduced the induction of Il1b mRNA by ~40%, whereas the induction of Il22b mRNA was increased. TPL-2 deficiency had similar effects on Mtb induction of ERK-1/2 activation and cytokines expression in BMDMs (Supplemental Fig. 3A, 3B). These data indicated that TPL-2 was not essential for the induction of Th17-polarizing cytokines by Mtb in BMDCs and BMDMs, although Mtb activation of ERK was largely dependent on TPL-2 expression in both cell types. Consistently, Map3k8−/− BMDCs were able to induce IL-17A production by CD4+ T cells to the same extent as WT BMDCs in an in vitro Th17 differentiation assay (Supplemental Fig. 2C, 2D) (19).

Our in vitro experiments suggested that TPL-2 expressed in Ag-presenting DCs and in responding CD4+ T cells might not be essential for initial generation of Th17 cells in EAE. To investigate this, the in vivo priming and differentiation of Th17 cells was examined in WT and Map3k8−/− mice during the early stages of EAE. Analysis of dLNs isolated from mice 9 d after MOG35–55 immunization revealed that both the total cellularity and CD4+ T cell numbers were unaffected by TPL-2 deficiency (Fig. 3A). This suggested that the activation and expansion of MOG35–55–specific Map3k8−/− CD4+ T cells occurred normally. To quantify the Ag-specific T cell response, dLN cells were labeled with CFSE and restimulated in vitro with MOG35–55. Consistent with normal priming in EAE, a similar fraction of MOG35–55–specific CD4+ T cells divided within the WT and Map3k8−/− LN cell populations (Fig. 3B).

Although T cell activation and expansion in EAE were unaffected by TPL-2 deficiency, it was possible that the ability of responding T cells to differentiate into Th17 and/or Th1 cells following MOG35–55 immunization was impaired. However, restimulation of dLN cells with either MOG35–55 peptide or PdBu and ionomycin revealed a similar fraction of IFN-γ– and IL-17A–producing CD4+ T cells, as well as a similar production of both cytokines by MOG35–55–specific CD4+ T cells from immunized WT and Map3k8−/− mice (Fig. 3C, 3D).

In vivo experiments therefore indicated that CD4+ T cell activation, expansion, and differentiation into Th1 and Th17 effector cells occurred independently of TPL-2. These results were consistent with the ability of Map3k8−/− CD4+ T cells to activate ERK-1/2 (Supplemental Fig. 3C) and proliferate normally in response to CD3/CD28 crosslinking (16), and for Map3k8−/− BMDCs to induce...
FIGURE 2. TPL-2 regulates the onset and severity of EAE. (A) Mean clinical scores of WT and Map3k8<sup>−/−</sup> mice (n = 19/WT; n = 18/Map3k8<sup>−/−</sup>) at various times after immunization with MOG<sub>35-55</sub>/CFA. Data presented are combined from two independent experiments (n = 9–10 genotype/experiment). (B) Immune cell infiltration in the spinal cords of WT and Map3k8<sup>−/−</sup> mice (n = 8/genotype) at the peak of disease (mean ± SEM). (C) Intracellular staining for infiltrated IL-17A–, IFN-γ–, and GM-CSF–expressing CD4<sup>+</sup> T cells in the spinal cords of WT and Map3k8<sup>−/−</sup> mice (n = 8/genotype) on day 12 after MOG<sub>35-55</sub>/CFA immunization. In (D) and (E), EAE was induced in WT and Map3k8<sup>−/−</sup> mice, and spinal cords were removed at the peak of disease. (D) Spinal cord sections were stained with Luxol fast blue (LFB) to monitor demyelination. Scale bars, 200 μm in the first panels and 50 μm in the lower panels. Immune cell infiltration was revealed by Ab staining: CD3 for T cells, Mac-3 for macrophages, and B220 for B cells. Axonal damage was visualized by staining for amyloid precursor protein (APP). (E) Quantitation of demyelination, immune infiltration, and axonal damage (mean ± SEM). Data in (B) and (C) are compiled from two independent experiments (n = 4/genotype/experiment). Data in (D) and (E) are representative of two independent experiments. *p = 0.05, **p = 0.01, ***p = 0.001.

TPL-2 does not function within T cells to promote EAE
Defective T cell priming did not explain the reduced susceptibility of Map3k8<sup>−/−</sup> mice to EAE. Nevertheless, it remained possible that TPL-2 had a T cell–intrinsic function at a later stage in EAE pathogenesis. This was investigated by mixing BM cells from Tcra<sup>−/−</sup> mice with WT or Map3k8<sup>−/−</sup> BM cells (ratio 4:1) before transferring them into Rag1<sup>−/−</sup> hosts. In the resulting chimeras, all of the T cells developed from either WT or TPL-2–deficient donor BM, whereas most (80%) of the other hematopoietic cells were derived from the TPL-2–sufficient Tcra<sup>−/−</sup> BM. Upon induction, the onset and severity of EAE were essentially identical in both sets of mixed BM chimeras (Fig. 4A), indicating that there was no T cell–intrinsic function for TPL-2 in EAE pathogenesis.

Table I. Clinical features of MOG<sub>35-55</sub>–induced EAE

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<tr>
<th>Incidence</th>
<th>Maximum Score (Mean ± SD)</th>
<th>Mean Day of Onset (Mean ± SD)</th>
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<tr>
<td>WT</td>
<td>19/19 (100%)</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>Map3k8&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>18/18 (100%)</td>
<td>2.7 ± 0.9</td>
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The potential function of TPL-2 in T cells was also investigated in a passive EAE model, in which encephalitogenic myelin-responsive T cells were transferred into naive recipients (13). DLN cells were isolated from Map3k8<sup>−/−</sup> mice 9 d after MOG<sub>35-55</sub> immunization and then cultured with MOG<sub>35-55</sub> peptide, IL-1β, and IL-23 to expand MOG<sub>35-55</sub>-specific Th17 cells (28). Intracellular cytokine staining indicated similar polarization efficiency within WT and Map3k8<sup>−/−</sup> CD4<sup>+</sup> T cell populations (data not shown). Following i.v. injection, the onset and extent of EAE induced by transferred Map3k8<sup>−/−</sup> and WT Th17-polarized cells were very similar (Fig. 4B), consistent with the results of active EAE in mixed BM chimeras.

CD20 Ab depletion has demonstrated that B cells promote EAE disease progression via production of IL-6 (29, 30), in addition to their late-acting regulatory role (31, 32). TPL-2 is known to regulate ERK-1/2 activation in B cells following CD40 and TLR stimulation (3, 33), raising the possibility that the protection of Map3k8<sup>−/−</sup> mice from EAE might be due to TPL-2 signaling in B cells. To investigate this, chimeric mice were generated in which BM from μMT<sup>−/−</sup> mice was mixed with Map3k8<sup>−/−</sup> BM cells (ratio 4:1) and injected into lethally irradiated Rag1<sup>−/−</sup> recipients. The onset and severity of EAE were similar between mice lacking TPL-2 expression in B cells and WT controls (Supplemental Fig. 4A). Therefore, the protection from EAE observed in Map3k8<sup>−/−</sup> mice was not attributable to the absence of TPL-2 in T or B cells.
TPL-2 signaling in radiation-resistant cells promotes the effector phase of EAE

Because the priming phase of EAE was normal in Map3k8−/− mice, this suggested that TPL-2 functioned during the effector phase of the disease. To investigate this, the ability of WT MOG35–55 peptide-primed Th17 cells to induce passive EAE in WT and Map3k8−/− recipient mice was determined. A delay in onset of clinical symptoms and a reduction in disease severity were observed upon transfer of encephalitogenic Th17-polarized cells into Map3k8−/− mice (Fig. 4B). TPL-2–deficient mice were also protected from EAE induced by WT MOG35–55 peptide–primed Th1 cells, suggesting the protection from disease did not involve a Th cell–specific factor (Supplemental Fig. 4B, 4C).

The effector phase of EAE involves two waves of immune cell infiltration into the CNS (34–36). First, “pioneer” MOG35–55-specific CD4+ T cells enter the brain via the choroid plexus. Inflammatory
cytokines produced by reactivated CD4^+ T cells then induce recruitment of a second wave of infiltrating immune cells. We investigated the recruitment of immune cells to the CNS in passive EAE. CD45.1^+ MOG33-55 peptide–primed Th17 cells generated from WT mice were transferred to CD45.2^+ WT or Map3k8^−/− mice. Similar numbers of CD45.1^+ CD4^+ T cells were detected in the spleens and brains of WT and Map3k8^−/− mice early after adoptive transfer (Fig. 6B). However, at the peak of the disease, significantly more CD45.1^+ CD4^+ T cells accumulated in the brains of WT relative to Map3k8^−/− mice (Fig. 6A). Therefore, TPL-2 promoted the effector phase of EAE by signaling in radiation-resistant nonhematopoietic cells, which in

**Figure 5.** TPL-2 regulates the effector phase of EAE. (A) MOG33-55–specific Th17 cells were generated from CD45.1^+ WT mice and then transferred into CD45.2^+ WT and Map3k8^−/− mice as in Fig. 6C. Numbers of CD45.1^+CD4^+ T cells in the spleen and brain were determined by flow cytometry at the indicated time points after Th17 cell transfer (mean ± SEM, n = 3). Macrophage numbers in the brain after transfer were determined by F4/80 staining. (B) Intracellular staining for IL-17A and IFN-γ expression in transferred Th17-polarized CD45.1^+CD4^+ T cells in the spleen and brain on day 14 after transfer (mean ± SEM, n = 3). Data are representative of at least two independent experiments. *p ≤ 0.05.

**Figure 6.** TPL-2 deficiency inhibited macrophage recruitment and cytokine expression in the CNS. (A) WT and Map3k8^−/− BM cells were transferred into WT Rag1^−/− mice, and EAE was induced. Numbers of CD45.1^+CD4^+ T cells in the spleen and brain were determined by flow cytometry at the indicated time points after Th17 cell transfer (mean ± SEM, n = 3). Macrophage numbers in the brain after transfer were determined by F4/80 staining. (B) Intracellular staining for IL-17A and IFN-γ expression in transferred Th17-polarized CD45.1^+CD4^+ T cells in the spleen and brain on day 14 after transfer (mean ± SEM, n = 3). Data are representative of at least two independent experiments. *p ≤ 0.05.

**Figure 7.** TPL-2 promoted the effector phase of EAE by signaling in radiation-resistant nonhematopoietic cells, which in

TPL-2 regulates chemokine expression in macrophages. (A) WT and Map3k8^−/− BM cells were transferred into WT Rag1^−/− mice, and EAE was induced. Numbers of CD45.1^+CD4^+ T cells in the spleen and brain were determined by flow cytometry at the indicated time points after Th17 cell transfer (mean ± SEM, n = 3). Macrophage numbers in the brain after transfer were determined by F4/80 staining. (B) Intracellular staining for IL-17A and IFN-γ expression in transferred Th17-polarized CD45.1^+CD4^+ T cells in the spleen and brain on day 14 after transfer (mean ± SEM, n = 3). Data are representative of at least two independent experiments. *p ≤ 0.05.

**Figure 8.** TPL-2 deficiency inhibited macrophage recruitment and cytokine expression in the CNS. (A) WT and Map3k8^−/− BM cells were transferred into WT Rag1^−/− mice, and EAE was induced. Numbers of CD45.1^+CD4^+ T cells in the spleen and brain were determined by flow cytometry at the indicated time points after Th17 cell transfer (mean ± SEM, n = 3). Macrophage numbers in the brain after transfer were determined by F4/80 staining. (B) Intracellular staining for IL-17A and IFN-γ expression in transferred Th17-polarized CD45.1^+CD4^+ T cells in the spleen and brain on day 14 after transfer (mean ± SEM, n = 3). Data are representative of at least two independent experiments. *p ≤ 0.05.

**Figure 9.** TPL-2 deficiency inhibited macrophage recruitment and cytokine expression in the CNS. (A) WT and Map3k8^−/− BM cells were transferred into WT Rag1^−/− mice, and EAE was induced. Numbers of CD45.1^+CD4^+ T cells in the spleen and brain were determined by flow cytometry at the indicated time points after Th17 cell transfer (mean ± SEM, n = 3). Macrophage numbers in the brain after transfer were determined by F4/80 staining. (B) Intracellular staining for IL-17A and IFN-γ expression in transferred Th17-polarized CD45.1^+CD4^+ T cells in the spleen and brain on day 14 after transfer (mean ± SEM, n = 3). Data are representative of at least two independent experiments. *p ≤ 0.05.

**Figure 10.** TPL-2 deficiency inhibited macrophage recruitment and cytokine expression in the CNS. (A) WT and Map3k8^−/− BM cells were transferred into WT Rag1^−/− mice, and EAE was induced. Numbers of CD45.1^+CD4^+ T cells in the spleen and brain were determined by flow cytometry at the indicated time points after Th17 cell transfer (mean ± SEM, n = 3). Macrophage numbers in the brain after transfer were determined by F4/80 staining. (B) Intracellular staining for IL-17A and IFN-γ expression in transferred Th17-polarized CD45.1^+CD4^+ T cells in the spleen and brain on day 14 after transfer (mean ± SEM, n = 3). Data are representative of at least two independent experiments. *p ≤ 0.05.
ERK-1 and ERK-2 were basally phosphorylated at high levels in astrocytes, and this was not detectably altered by stimulation with LPS or TNF (Fig. 8B). However, LPS and TNF clearly induced p38α phosphorylation, and this was partially decreased by TPL-2 deficiency following stimulation with either agonist. Addition of IFN-γ or IL-17A did not alter p38α phosphorylation in astrocytes (data not shown).

In conclusion, our biochemical analyses demonstrated that TPL-2 contributed to p38α activation in both microglia and astrocytes after stimulation with LPS and TNF, and it was also required for optimal activation of ERK-1/2 in microglia by these agonists. Together with the qRT-PCR analyses of cytokine and chemokine mRNA expression (Fig. 7), these data were consistent with the hypothesis that TPL-2 signaling in both microglia and astrocytes could contribute to EAE disease development.

TPL-2 kinase activity is required for development of EAE
TPL-2 is associated with the ubiquitin-binding protein ABIN-2, which is required to maintain TPL-2 stability (42). ABIN-2-deficient mice have reduced steady-state levels of TPL-2 in multiple cell types compared with WT mice (14). In a reciprocal fashion, analysis of cells from Map3k8−/− mice revealed that TPL-2 is required to maintain steady-state expression of ABIN-2, and both splenocytes and BMDMs from Map3k8−/− mice have substantially reduced amounts of ABIN-2 compared with WT (Fig. 9A). This suggested that the resistance of Map3k8−/− mice to EAE might result from reduced ABIN-2 expression. However, EAE disease severity was similar between Tnip2−/− (which completely lack ABIN-2 expression) and WT mice (Fig. 9B). These data indicated ABIN-2 was not required for EAE development and suggested that the low levels of TPL-2 expressed in Tnip2−/− mice were sufficient to promote maximal disease.

Taken together, our analyses of Map3k8−/− and Tnip2−/− mice demonstrated that TPL-2 expression was required for efficient EAE development. However, these experiments did not establish whether this reflected a role for TPL-2 catalytic activity, and it remained possible that TPL-2 promoted disease by functioning as an adaptor protein. To distinguish these possibilities, we generated a novel knock-in mouse strain expressing mutant TPL-2D270A (Supplemental Fig. 4D), which is catalytically inactive (43). In contrast to Map3k8−/− cells, Map3k8D270A/D270A cells expressed similar amounts of ABIN-2 as WT cells (Fig. 7A). Compared to WT controls, EAE development was delayed and reduced in severity in Map3k8D270A/D270A mice (Fig. 9C), similar to Map3k8−/− mice. TPL-2 catalytic activity was therefore required to promote EAE, and this was independent of effects on the steady-state expression of ABIN-2 protein, or any potential adaptor function of TPL-2.

TPL-2 promotes EAE independently of type I IFN signaling
Development of EAE is exacerbated in mice deficient in type I IFN receptor (IFNAR), with markedly higher inflammation and demyelination in the CNS compared with WT controls (44). TPL-2/ERK-1/2 signaling negatively regulates TLR induction of IFN-β (27), raising the possibility that TPL-2 deficiency might reduce EAE disease severity by augmenting IFN-β production. We investigated this possibility genetically by generating Map3k8−/−Ifnar−/− mice lacking expression of both TPL-2 and the receptor for type I IFNs, IFNAR.

As reported previously (44), IFNAR deficiency did not affect EAE disease onset, but increased the severity and duration of the effector phase (Fig. 9D). However, the onset of EAE and maximal clinical scores were similar in Map3k8−/− and Map3k8−/−Ifnar−/− mice. The inhibitory effects of TPL-2 deficiency on EAE development, therefore, were independent of type I IFN signaling.

Discussion
We investigated the potential role of the MAP3 kinase TPL-2 in Th17 cell generation and function. Our results show that TPL-2 was dispensable for the generation of Th17 cells in vitro and during the priming phase of EAE. Nevertheless, TPL-2 regulated both the onset and severity of EAE, functioning in the effector phase of this Th17 cell-mediated autoimmune disease model. Importantly, the effects of TPL-2 in EAE required its catalytic activity, suggesting that small molecule inhibitors of TPL-2 might be therapeutically beneficial in MS. IFN-β is widely used for treatment of relapsing-remitting MS (45). TPL-2 promoted EAE independently of type I IFN signaling, raising the possibility that administration of a TPL-2...
inhibitor in combination with the established drug IFN-β might be more effective for MS therapy.

Earlier pharmacological studies with the MEK-1/2 inhibitor U0126 have suggested that ERK-1/2 activation is required for induction of the Th17-polarizing cytokines IL-1β and IL-23 in DCs following stimulation with Mtb\textsuperscript{H1} (28). Mtb\textsuperscript{H1} can potentially activate multiple pattern recognition receptors on DCs, including TLR2, TLR4, TLR9, NOD-2, and C-type lectins (46, 47). TPL-2 is required for activation of ERK-1/2 in DCs following TLR2, TLR4, or TLR9 stimulation (4, 27), and it also contributes to the

![Figure 7. TPL-2 regulates proinflammatory gene expression in primary microglia and astrocytes. Quantitative RT-PCR of mRNA expression in primary microglia (A, B) and astrocytes (C, D) from WT and Map3k8\textsuperscript{-/-} mice, normalized to Hprt mRNA (mean ± SEM). Cells were either left untreated (nt) or stimulated with the indicated agonists for 6 h. Data are compiled from three (A, C) or two (B, D) independent experiments. *p ≤ 0.05, **p ≤ 0.01.](http://www.jimmunol.org/)
activation of ERK in macrophages following NOD-1 and NOD-2 stimulation, but it is dispensable for activation of ERK-1/2 by the C-type lectin Dectin-1 (4). Consistent with this, stimulation of ERK phosphorylation by MtbHI was largely dependent on TPL-2 expression in BMDCs. However, TPL-2 deficiency only partially reduced MtbHI induction of IL-1β, and it did not impair induction of mRNAs encoding IL-6, IL-12p35, or IL-23p19 in these cells, which were able to induce normal Th17 cell differentiation in vitro. In line with these data, the generation of Th17 cells in the dLNs during the priming phase of EAE was unaffected by TPL-2 deficiency. It is likely that low levels of TPL-2-independent ERK-1/2 activation explain the ability of Map3k8−/− BMDCs to induce mRNAs encoding IL-1β and IL-23p19.

Experiments with lethally irradiated BM chimeras demonstrated that TPL-2 functioned in a radiation-resistant cell population to promote EAE, implying that TPL-2 signaling was not required in T cells APCs (DCs, macrophages). These data are consistent with in vitro experiments showing that TPL-2 expression was dispensable for Th17 cell differentiation induced with recombinant cytokines, or for MtbHI induction of Th17-polarizing cytokines in BMDCs and BMDMs. Furthermore, mixed BM chimera experiments demonstrated that TPL-2 expression in either T or B cells was not required for EAE development. Instead, passive EAE experiments using MOG35-55-specific Th17 cells revealed that TPL-2 signaling in host cells regulated the effector phase of disease in the CNS. A similar requirement for host TPL-2 was found after adoptive transfer of WT MOG35-55-specific Th1 cells. Therefore, TPL-2 did not have a Th17-specific function in the effector phase of EAE.

After priming in LN, Ag-specific Th17 cells traffic through the choroid plexus into the subarachnoid space, where they are reactivated (35). As a consequence of productive T cell/APC interactions, Th17 cell cytokines and chemokines are then produced, which activate parenchymal vasculature. This promotes a secondary wave of leukocyte infiltration, leading to inflammatory CNS damage and EAE onset (34). Passive EAE experiments indicated that the initial trafficking of Th17 cells into the brain was not affected by TPL-2 deficiency. Instead, TPL-2 was required for the second wave of leukocyte recruitment into the CNS. The expression of several chemokines known to be involved in regulating the migration of leukocytes into the CNS was significantly reduced in intact Map3k8−/− mice during EAE, including CCL2, CCL3, CCL5, and CXCL10, which have each been implicated to have positive roles in EAE induction (34). The protective effect of TPL-2 deficiency in EAE, therefore, may result from impaired upregulation of these chemokines in CNS-resident cells.

An important outstanding question is the identity of the radiation-resistant nonhematopoietic cell type in which TPL-2 signals to promote EAE. In vitro experiments indicated that TPL-2 was required for optimal ERK-1/2 and p38 activation in microglia, resident myeloid-lineage cells in the brain and spinal cord (48). Microglia can contribute to EAE disease initiation by presenting Ags to naive T cells, and they are also a potent source of inflammatory cytokines and chemokines (37, 39). Additionally, our in vitro experiments revealed that TPL-2 signaling contributes to the activation of p38α in astrocytes, the most abundant cells of the brain that also produce cytokines and chemokines, and they have an important role in regulating the recruitment and function of T cells in the CNS (39). TPL-2 expression was found to be required for optimal expression of proinflammatory cytokines and
chemokines in both microglia and astrocytes, possibly owing to the contribution of TPL-2 to MAPK activation in these cell types. Earlier studies with knockout mice have shown the importance of the affected cytokines and chemokines for EAE disease development (34, 39). Our data raise the possibility that the reduced secondary infiltration of inflammatory cells into the CNS in Map3k8−/− mice compared with WT controls during EAE results from the decreased expression of cytokines and chemokines by microglia and astrocytes.

Genetic deletion of IKK2 or NEMO in neuroectodermal-derived cells ameliorates EAE in mice, which correlates with decreased expression of proinflammatory cytokines and chemokines by CNS-resident cells (38). Recently, it has also been shown that conditional deletion of Tak1, which functions upstream of the IKK complex, in either astrocytes or microglia also ameliorates EAE development (49). The IKK complex is a critical positive regulator of the TPL-2 signaling pathway, inducing proteolysis of its downstream target of TPL-2 signaling. These data, together with the present study, suggest that blockade of TPL-2 catalytic activity might also be therapeutically beneficial in MS.

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Disclosures

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References


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