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Regulation of Experimental Autoimmune Encephalomyelitis by TPL-2 Kinase

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) expression is required for efficient polarization of naive T cells to Th1 effector cells in vitro, as well as for Th1-mediated immune responses. In the present study, we investigated the potential role of TPL-2 in Th17 cells. TPL-2 was found to be dispensable for Th17 cell differentiation in vitro, and for the initial priming of Th17 cells in experimental autoimmune encephalomyelitis (EAE), a Th17 cell–mediated disease model for multiple sclerosis. Nevertheless, TPL-2-deficient mice were protected from EAE, which correlated with reduced immune cell infiltration, demyelination, and axonal damage in the CNS. Adoptive transfer experiments demonstrated that there was no T cell–intrinsic function for TPL-2 in EAE, and that TPL-2 signaling was not required in radiation-sensitive hematopoietic cells. Rather, TPL-2 signaling in radiation-resistant stromal cells promoted the effector phase of the disease. Importantly, using a newly generated mouse strain expressing a kinase-inactive form of TPL-2, we demonstrated that stimulation of EAE was dependent on the catalytic activity of TPL-2 and not its adaptor function to stabilize the associated ubiquitin-binding protein ABIN-2. Our data therefore raise the possibility that small molecule inhibitors of TPL-2 may be beneficial in multiple sclerosis therapy.


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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; FICZ, 6-formylindolo[3,2-b]carbazole; IKK, IκB kinase; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; Mtb, heat-inactivated Mycobacterium tuberculosis; PdBu, phorbol dibutyrate; TPL-2, tumor progression locus 2; WT, wild-type.

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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; FICZ, 6-formylindolo[3,2-b]carbazole; IKK, IκB kinase; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; Mtb, heat-inactivated Mycobacterium tuberculosis; PdBu, phorbol dibutyrate; TPL-2, tumor progression locus 2; WT, wild-type.
(12). We found that TPL-2 was dispensable for cytokine-induced differentiation of naïve 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−/− (2), Tnip2−/− (14), and Ifnar−/− mice were fully backcrossed to C57Bl/6 mice. Map3k8−/− Rag1−/− and Ifnar−/− Map3k8−/− mice were generated by intercrossing the appropriate single knockout mice. For the generation of Map3k8−/− Rag1−/− 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 ClaI-BamHI fragment containing exon 5, which encodes D270A, was isolated and subcloned into pBluescript SK− (Invitrogen) to create pSK-RA ("left arm"). From this plasmid, a 3.2-kb PsI fragment was subcloned into pBluescript SK− to create pSK-RA.PsiI. PCR was used to mutate the sequence encoding D270A 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 reintroduced into pSK-LA to create pSK-RA.LN. D270A, and from here a 5.2-kb PstI fragment containing the mutation was cloned into a unique XhoI cloning site of the pLox-AP1 vector to produce the pLox-AP1-LA (left arm). The pLox-AP1 vector contains a neomycin resistance gene flanked by two loxP sites and the HSV thymidine kinase gene, both driven by the PGK1 promoter. A 4.7-kb BamHI-SalI fragment was isolated and subcloned into pBluescript SK− to create pSK-RA.ERK-1/−2 (right arm). From this plasmid, a 4.4-kb HpaI-Bsu36I fragment was subcloned into pLox-AP1-LA to create the pLox-AP1-Tpol2D270A targeting vector (Supplemental Fig. 4D). The vector was linearized with NotI and transfected into embryonic stem cells (carried out by PolyGene, Rümlang, Switzerland). C57BL/6 (CD45.2−, wild-type [WT]), CD45.1 C57BL/6, CD45.1 Rag1−/−, Tera−/−, and μMT−/− mice were bred 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-wk-old male mice were used for all experiments.

Abs

Abs to TPL-2, Isl1, ERK-1, ERK-2, and actin were purchased from Santa Cruz Biotechnology, whereas p-p105 (Ser332), p-p38, and p-ERK (Thr180/Tyr182) 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, TCRβ–PECy5, TCRα–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, CD45.2–FITC, CD45–Alexa Fluor 450, biotinylated CD44, CD11c–PEC, CD11b–PE, biotinylated PE, and biotinylated MHC class II were obtained from BioLegend. 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 MOG35-55 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 d after immunization. For passive EAE experiments, Map3k8−/− or WT mice were injected i.v. with 30 × 10⁶ Th17-polarized MOG35-55-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−/− or WT mice were collected 10 d after immunization with MOG35-55 peptide plus CFA. Cells were cultured for 3 d (5 × 10⁶ cells/ml; 24-well plates) in IMDM (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated PCS (LabTech), 5 mM l-glutamine, 50 μM 2-ME, and antibiotics (all Life Technologies) in the presence of 10 μg/ml MOG35-55 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−/− or WT control mice were depleted of T cells with biotinylated TCRβ mAb (H57-597; BD Pharmingen) and were transferred by i.v. injection into lethally irradiated (400 rad) Rag1−/− or Tpl2−/− Rag1−/− hosts. For mixed bone marrow chimeras, WT or Map3k8−/− bone marrow cells were mixed with Tera−/− or μMT−/− bone marrow cells at a ratio of 1:4, prior to injection into sublethally irradiated (500 rad) Rag1−/− 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 Lulox fast blue, anti-CD3 (Serotec), anti–Mac-3, anti-B220 (both BD Pharmingen), and amylod precursor protein (APP; Chemicon).

T cell recall assay

LN cells were isolated from mice 9 d after immunization with MOG35-55. Cells were cultured for 24 h in complete medium (2 × 10³ cells/ml; 96-well flat-bottom plates) containing 10 μg/ml MOG35-55 peptide, and supernatants were collected for quantification of IL-17 and IFN-γ by ELISA. For proliferation assays, LN cells were labeled with CFSE (Molecular Probes) 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 RNA later stabilization buffer (Qiagen) 15 d after MOG35-55 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) and cultured for a total of 72 h.

Protein analyses

Purified bone marrow-derived macrophages (BMDMs), bone marrow-derived dendritic cells (BMDCs), and T cells were serum-starved for 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+ T cells were stimulated 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
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; GolgiStop; BD Pharmingen), or with MOG35-55 peptide for 12 h, adding brefeldin A for the last 4 h of culture. Cells were stained for surface Abs as indicated, fixed for 15 min in 4% (v/v) paraformaldehyde (Sigma-Aldrich), and permeabilized with 0.1% (v/v) Nonidet P-40 for 4 min. Intracellular Abs were added in PBS containing 0.01% (v/v) sodium azide and 24G2 cell supernatant to block Fc receptor binding. Four- and seven-color cytometric staining was analyzed on FACSCalibur and Cyan instruments (Becton Dickinson), respectively. Data analysis was performed with FlowJo v8.5 software (Tree Star).

Cell culture and purification

Macrophages and myeloid DCs were generated from BM stem cells as described previously (17), with purities of ≥95% for BMDM (F4/80+) and BMDC (CD11c+) populations. For biochemical analyses, CD4+ T cells were isolated to purities of ≥95% for BMDM (F4/80+) 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 naïve 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 (RM45; BD Biosciences), anti-CD25 (PC61.5; BD Biosciences), and anti-CD44 (IM7; BD Biosciences), and CD4+CD44-CD25- naïve 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 2% L929 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 × 10^5 in 24-well plates and stimulated the following day. Adherent astrocytes were trypsinized, seeded at densities of 1.0 × 10^5 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+ stained, 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’s 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 FlowJo 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 Th1-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-formylindolo[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). The orphan nuclear receptor retinoic acid–related orphan receptor (RORγt), which is selectively expressed in Th17 cells, induces the development of Th17 cells, in conjunction with RORα (23, 24). Expression of mRNAs encoding RORγt and RORα was comparable in Map3k8−/− and WT CD4+ T cells (Fig. 1D). TPL-2, therefore, was not essential for in vitro differentiation of CD4+ T cells to the Th17 cell lineage.

TPL-2 regulates the onset and severity of EAE

To investigate the physiological role of TPL-2 in Th17 cell development and function, and 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. 2A, 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). Immunohistochemistry confirmed reduced infiltration of T and B cells and macrophages in the spinal cords of Map3k8−/− mice, accompanied by significantly reduced degrees of demyelination and neuronal damage (Fig. 2D, 2E).

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-1β, 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−/− BMDCs, similar to WT BMDCs (Supplemental Fig. 2B). TPL-2 deficiency reduced the induction of Il1b mRNA by ∼40%, whereas the induction of Il12b 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 vivo 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
TPL-2 regulates the onset and severity of EAE. (A) Mean clinical scores of WT and Map3k8−/− mice (n = 19/WT; n = 18/Map3k8−/−) at various times after immunization with MOG35–55/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−/− mice (n = 8/genotype) at the peak of disease was determined by flow cytometry using the indicated markers (mean ± SEM). (C) Intracellular staining for infiltrated IL-17A−, IFN-γ−, and GM-CSF−expressing CD4+ T cells in the spinal cords of WT and Map3k8−/− mice (n = 8/genotype) on day 12 after MOG35–55/CFA immunization. Data presented are combined from two independent experiments (n = 9–10/genotype/experiment) 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). Data in (D) and (E) are representative of two independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

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−/− mice 9 d after MOG35–55 immunization and then cultured with MOG35–55 peptide, IL-1β, and IL-23 to expand MOG35–55-specific Th17 cells (28). Intracellular cytokine staining indicated similar polarization efficiency within WT and Map3k8−/− CD4+ T cell populations (data not shown). Following i.v. injection, the onset and extent of EAE induced by transferred Map3k8−/− 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−/− mice from EAE might be due to TPL-2 signaling in B cells. 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 1. Clinical features of MOG35–55–induced EAE

<table>
<thead>
<tr>
<th>Incidence</th>
<th>Maximum Score (Mean ± SD)</th>
<th>Mean Day of Onset (Mean ± SD)</th>
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<tbody>
<tr>
<td>WT</td>
<td>19/19 (100%)</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>Map3k8−/−</td>
<td>18/18 (100%)</td>
<td>2.7 ± 0.9</td>
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TPL-2 does not function within T cells to promote EAE

Defective T cell priming did not explain the reduced susceptibility of Map3k8−/− 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−/− mice with WT or Map3k8−/− BM cells (ratio 4:1) before transferring them into Rag1−/− hosts. In the resulting chimeras, all of the hematopoietic cells were derived from the TPL-2–sufficient Tcra−/− 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.
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 cell s 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

**FIGURE 3.** TPL-2 is not required for T cell priming in EAE. WT and Map3k8−/− mice were immunized with MOG33–55/CFA, and dLNs were isolated on day 9. (A) CD4+ T cell numbers were determined by flow cytometry (mean ± SEM; n = 3). (B) Flow cytometric analysis of the proliferation of CFSE-labeled CD4+ T cells cultured for 3 d with MOG35–55 peptide (mean ± SEM; n = 3). (C) Frequencies of IL-17A– and IFN-γ–expressing CD4+ T cells were determined by flow cytometry after restimulation with PdBu/ionomycin or MOG35–55 peptide (mean ± SEM; n = 3). (D) ELISA of IFN-γ and IL-17A production by LN cells restimulated with MOG35–55 peptide (mean ± SEM; n = 3). Data are representative of at least three independent experiments.

**FIGURE 4.** TPL-2 signaling in T cells is not required for EAE development. (A) BM cells from WT and Map3k8−/− mice were mixed in a 1:4 ratio with BM cells from Tcra−/− mice and transferred into Rag1−/− hosts. After 8 wk, EAE was induced by immunization with MOG35–55/CFA, and clinical scores were determined (mean ± SEM; n = 6). (B) dLN cells from WT and Map3k8−/− mice were isolated 9 d after immunization with MOG35–55/CFA and restimulated in vitro with MOG35–55 peptide in the presence of recombinant IL-1β and IL-23 for 5 d. These Th17-polarized cells were then transferred into naive WT and Map3k8−/− mice. Graph represents the average clinical score after Th17 cell transfer (mean ± SEM; n = 5). Data in (A) and (B) are representative of two independent experiments.
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. 5A, left and middle panels). 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. 5A, middle panel). Moreover, consistent with a defect in secondary immune cell infiltration into Map3k8−/− brains, fewer CD45.2F4/80+ macrophages were detected 14 d after Th17 cell transfer (Fig. 5A, right panel). The number of CD45.1 CD4+ T cells in the spleen at these later time points was similar between the two recipient CD45.2+ genotypes, demonstrating that host TPL-2 expression was not required for CD4+ T cell survival (Fig. 5A, left panel). Furthermore, transferred CD4+ T cells in the CNS of Map3k8−/− hosts produced similar fractions of IFN-γ and IL-17 to WT hosts (Fig. 5B). Pioneer Th17 cells, therefore, could infiltrate the CNS of Map3k8−/− mice, but failed to optimally trigger a secondary infiltration of immune cells, the major mediators of myelin damage.

Although transfer of encephalitogenic Th17 cells demonstrated that the TPL-2–deficient host environment was protective in passive EAE (Fig. 4B), it did not reveal whether TPL-2 promoted EAE by signaling in hematopoietic cells other than T or B cells. This was investigated by generating BM chimeras via transferring WT or Map3k8−/− BM cells into lethally irradiated Rag1−/− or Rag1−/−/Map3k8−/− recipients. In these chimeras, all lymphocytes, as well as >99% of MHC class II+ cells, were of donor origin (data not shown). Upon EAE induction, deletion of TPL-2 in radiation-sensitive cells (Map3k8−/− > Rag1−/− > Rag1−/−/Map3k8−/−) was not able to protect against disease (Fig. 6A). However, transferring WT BM cells into Rag1−/− hosts that additionally lacked TPL-2 resulted in delayed EAE onset. Furthermore, lack of TPL-2 expression in the radiation-resistant compartment resulted in a reduction in immune infiltrates in the spinal cord (Fig. 6B) but did not affect polarization of CD4+ T cells to produce IL-17A and IFN-γ (Fig. 6C).

Therefore, TPL-2 promoted the effector phase of EAE by signaling in radiation-resistant nonhematopoietic cells, which induced the secondary infiltration of immune cells into the CNS parenchyma.

**TPL-2 signals in both microglia and astrocytes**

CNS-resident microglia and astrocytes have been reported previously to play pivotal roles in EAE disease manifestation (20, 37, 38). These radiation-resistant cell types produce a number of proinflammatory cytokines and chemokines during EAE, which promote the secondary influx of hematopoietic cells into the CNS (34, 36, 39). Defective activation of one or both of these cell types, therefore, could account for the protection of Map3k8−/− mice from EAE development. We explored this possibility by determining whether TPL-2 deficiency affected cytokine and chemokine induction in primary in vitro–derived microglia and astrocytes.

Map3k8−/− microglia expressed significantly less Il6, Il1b, Cxcl1, Ccl2, and Ccl20 mRNAs in response to LPS stimulation than did their WT counterparts (Fig. 7A). Additionally, induction of Il1b and Ccl2 mRNAs was reduced by TPL-2 deficiency following stimulation with IFN-γ or with TNF plus IFN-γ (Fig. 7A, 7B). LPS upregulation of mRNAs encoding IL-6, IL-1β, CCL2, CXCL1, VCAM1, and MMP9 was also significantly reduced in Map3k8−/− astrocytes compared with WT control cells (Fig. 7C). Furthermore, IL-1β induction of Il6, Ccl2, and Vcam1 mRNAs, as well as TNF induction of Ccl2 and Mmp9 mRNAs, was reduced by TPL-2 deficiency in astrocytes (Fig. 7C). Defective TNF induction of Ccl2, Vcam1, and Mmp9 mRNAs in Map3k8−/− astrocytes was not rescued by costimulation with IL-17A (Fig. 7D). In line with earlier studies, stimulation of astrocytes with IL-17A alone had minimal effect on the expression of mRNAs encoding proinflammatory cytokines or chemokines in astrocytes (20, 40).

We next analyzed the activation of MAPK signaling pathways in microglia and astrocytes to gain insight into how TPL-2 deficiency might impair proinflammatory gene expression in these cell types. In line with earlier studies using primary macrophages and DCs (2, 3, 27), ERK-1/2 and p38 phosphorylation following LPS or TNF stimulation of microglia was reduced by TPL-2 deficiency (Fig. 8A). Analysis of NF-κB p105 phosphorylation (data not shown) indicated that this decrease did not result from impaired activation of the IkB kinase (IKK) complex (41). Phosphorylation of ERK-1/2 and p38 was not induced by stimulation with either IFN-γ or IL-17A (data not shown).
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 p38a 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 p38a phosphorylation in astrocytes (data not shown).

In conclusion, our biochemical analyses demonstrated that TPL-2 contributed to p38a 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<sup>−/−</sup> mice revealed that TPL-2 is required to maintain steady-state expression of ABIN-2, and both splenocytes and BMDMs from Map3k8<sup>−/−</sup> mice have substantially reduced amounts of ABIN-2 compared with WT (Fig. 9A). This suggested that the resistance of Map3k8<sup>−/−</sup> mice to EAE might result from reduced ABIN-2 expression. However, EAE disease severity was similar between Tnip2<sup>−/−</sup> (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<sup>−/−</sup> mice were sufficient to promote maximal disease.

Taken together, our analyses of Map3k8<sup>−/−</sup> and Tnip2<sup>−/−</sup> 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-2<sup>D270A</sup> (Supplemental Fig. 4D), which is catalytically inactive (43). In contrast to Map3k8<sup>−/−</sup> cells, Map3k8<sup>D270A/D270A</sup> 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 Map3k8<sup>D270A/D270A</sup> mice (Fig. 9C), similar to Map3k8<sup>−/−</sup> 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<sup>−/−</sup> Iftan<sup>−/−</sup> 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<sup>−/−</sup> Iftan<sup>−/−</sup> and Map3k8<sup>−/−</sup> Iftan<sup>−/−</sup> 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 \textit{Mtb}\textsuperscript{H1} (28). \textit{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

\begin{figure}[h]
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\caption{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 \textit{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 \leq 0.05, **p \leq 0.01.}
\end{figure}
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 Mtb HI was largely dependent on TPL-2 expression in BMDCs. However, TPL-2 deficiency only partially reduced Mtb HI 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 Mtb HI induction of Th17-polarizing cytokines in BMDCs and BMDMs. Furthermore, mixed BM chimaera 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 LNs, 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 p38α 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

FIGURE 8. TPL-2 is required for optimal MAPK activation in microglia and astrocytes. Lysates of microglia (A) and astrocytes (B), generated from WT and Map3k8−/− mice and stimulated for the indicated time points with LPS or TNF, were immunoblotted. Phosphorylated protein bands were quantified by laser densitometry using the Quantity One software package and are presented as relative density normalized to the respective total protein. Data are representative of three to four independent experiments with similar results.

FIGURE 9. TPL-2 kinase activity is required to induce EAE. (A) Lysates were prepared from splenocytes (left panel) and BMDMs (right panel) generated from WT, Map3k8−/−, Map3k8D270A/D270A and TNIP2−/− mice and immunoblotted for the indicated Ags. (B) Mean clinical scores of WT and Tnip2−/− mice (mean ± SEM; n = 7–8). Data are compiled from two different experiments (n = 3–4/genotype/experiment). (C) Mean clinical scores of WT, Map3k8−/−, Map3k8D270A/D270A, and Map3k8−/− mice at various times after immunization with MOG35-55/CFA (mean ± SEM; n = 8–9). Data are compiled from two different experiments (n = 3–4/genotype/experiment). *p ≤ 0.05, ***p ≤ 0.001. (D) Mean clinical scores of WT, Map3k8−/−, Ifnar−/−, and Map3k8−/− Ifnar−/− mice after immunization with MOG35-55/CFA (mean ± SEM; n = 9–10). Data are compiled from two different experiments (n = 4–5/genotype/experiment).
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 Taki, 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 inhibitor NF-κB to p105 (50, 51) and directly phosphorylating a key regulatory serine in the TPL-2 C terminus (52). The protective effects of IKK2/NEMO deficiency in astrocytes or TAK1 deficiency in astrocytes and microglia, therefore, may be mediated in part by blockade of TPL-2 signaling. The generation of conditional knockout mouse strains lacking TPL-2 expression in astrocytes or microglia will be essential to determine whether TPL-2 signaling in either or both these cell types is important for development of EAE.

Earlier studies of Map3k8−/− mice identified TPL-2 as a potential drug target in septic shock and inflammatory bowel disease (2, 5). Interestingly, Mapk1 was recently identified as a disease susceptibility gene locus in MS (53). Mapk1 encodes ERK2, a major 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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