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Nutrient deprivation based on the loss of essential amino acids by catabolic enzymes in the microenvironment is a critical means to control inflammatory responses and immune tolerance. Here we report the novel finding that Tph-1 (tryptophan hydroxylase-1), a synthase which catalyses the conversion of tryptophan to serotonin and exhausts tryptophan, is a potent regulator of immunity. In models of skin allograft tolerance, tumor growth, and experimental autoimmune encephalomyelitis, Tph-1 deficiency breaks allograft tolerance, induces tumor remission, and intensifies neuroinflammation, respectively. All of these effects of Tph-1 deficiency are independent of its downstream product serotonin. Because mast cells (MCs) appear to be the major source of Tph-1 and restoration of Tph-1 in the MC compartment in vivo compensates for the defect, these experiments introduce a fundamentally new mechanism of MC-mediated immune suppression that broadly impacts multiple arms of immunity.

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Abbreviations used: 5-HTP, 5-hydroxytryptophan; ANOVA, analysis of variance; BMMC, BM-derived MC; CNS, central nervous system; DST, donor-specific transfusion; EAE, experimental autoimmune encephalomyelitis; i.d., intradermal(ly); IDO, indoleamine 2,3-dioxygenase; MC, mast cell; mTOR, mammalian target of rapamycin; PCPA, parachlorophenylalanine; qRT-PCR, quantitative RT-PCR; TDO, tryptophan 2,3-dioxygenase.

One well-documented method to control immunity and tolerance is through the regulation of nutrients in their immune microenvironment. Best described is the tryptophan deficiency mediated by the catabolic enzyme indoleamine 2,3-dioxygenase (IDO), which locally depletes tryptophan and liberates the immunoregulatory metabolites known as kynurenines. T cell activation is exquisitely sensitive to local tryptophan catabolism, and thus this enzyme exerts profound protective effects in allo-fetal rejection, autoimmunity, and inflammation. IDO can also be detected in tumors and draining LNs, and DC expression of IDO limits T cell responsiveness to antigen (Munn and Mellor, 2007; Katz et al., 2008). In addition to IDO, some human cancers express tryptophan 2,3-dioxygenase (TDO), which also utilizes tryptophan as a substrate to produce kynurenines (Pilotte et al., 2012). Furthermore, cysteine and arginine deficiency in tumors can inhibit T cell activation (Rodriguez et al., 2004; Srivastava et al., 2010), suggesting that loss of any number of amino acids may serve as a common tumor escape mechanism.

In accordance with these findings, in a model of skin allograft tolerance, it was observed that multiple catabolic enzymes were up-regulated that consume a litany of essential amino acids. It was shown that these enzymatic activities could dampen T cell proliferation through nutrient deprivation (Cobbold et al., 2009). One of the many enzymes that were up-regulated was Tph-1 (tryptophan hydroxylase-1), a synthase which utilizes tryptophan as a substrate to produce serotonin and melatonin (Yao et al., 2011), and it was speculated that Tph-1 may precipitate the loss of tryptophan in the local microenvironment (Zelenika et al., 2001). In the present study, we sought to determine whether Tph-1, the isoform expressed in the periphery (Walther et al., 2003), could function as a regulator of immunity through the control of tryptophan metabolism and uncovered a novel and profound immunoregulatory function for this enzyme.

E.C. Nowak, V.C. de Vries, and A. Wasiuk contributed equally to this paper.

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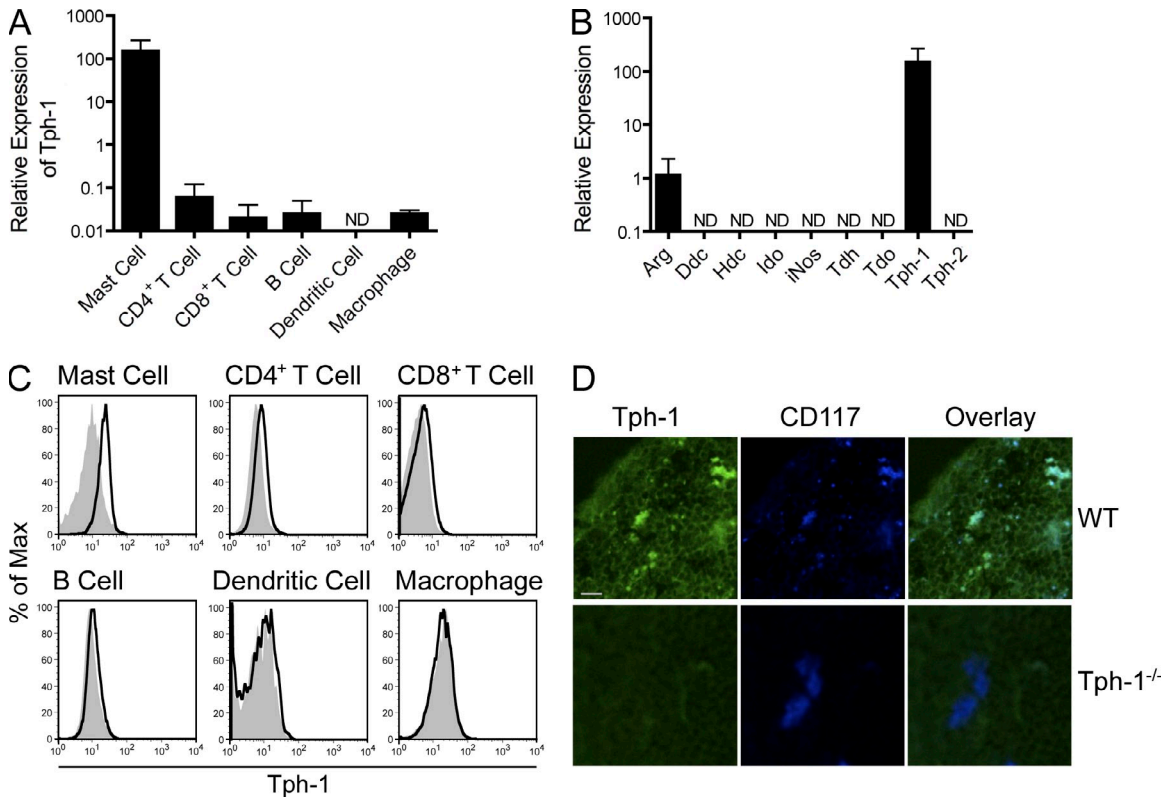

Figure 1. MCs have specific expression of Tph-1. (A) Tph-1 expression (as determined by qRT-PCR) of the indicated cell types standardized to β-actin expression (mean ± SEM) is shown. For each cell type, two independent FACS-sorted samples were used. Each MC and macrophage sample was pooled from the peritoneal lavage of 20 mice. All other samples were pooled from the LN and spleen of two to four mice. (B) Peritoneal MC expression of the following genes was determined by qRT-PCR: arginase (Arg), dopa decarboxylase (Ddc), histidine decarboxylase (Hdc), IDO, inducible nitric oxide synthase (iNos), L-threonine dehydrogenase (Tdh), TDO, Tph-1, and Tph-2. Two independent samples were used to determine mean ± SEM shown. (A and B) ND indicates that a signal was not detected. (C) Intracellular staining for Tph-1 was performed for MCs, $CD4^+$ T cells, $CD8^+$ T cells, B cells, $CD11c^{hi}$ DCs, and $F4/80^+$ $CD11b^+$ macrophages in WT (bold lines) and Tph-$1^{-/-}$ (shaded areas) mice (n = 3–4 mice/group, and each cell type was tested in at least two independent experiments). (D) Naive LN sections were sectioned and stained for Tph-1 expression on MCs. CD117 staining is blue, Tph-1 staining is green, and overlap is white. Results are representative of three different mice/group. Bar, 10 µm.

RESULTS

Mast cells (MCs) express Tph-1

A study from our laboratory previously reported that MCs are critical in maintaining regulatory T cell–dependent skin allograft tolerance (Lu et al., 2006). Concordant with these observations, gene array analyses performed by two different groups established that tolerant skin allografts display an MC signature with heightened signal for Tph-1 and other MC gene products (Zelenika et al., 2001; Lu et al., 2006). Pilot experiments showed that purified MCs from tolerant allografts expressed heightened levels of Tph-1 compared with MCs from syngeneic grafts (not depicted). It was also observed that peritoneal MCs from naive mice express ∼1,000-fold higher expression for Tph-1 message than any other hematopoietic cell type examined by quantitative RT-PCR (qRT-PCR) and that Tph protein expression appears to be restricted to MCs (Fig. 1). Heightened Tph-1 expression in MCs appeared unique, as the expression for other catabolic enzymes was absent or minimal (Fig. 1 B).

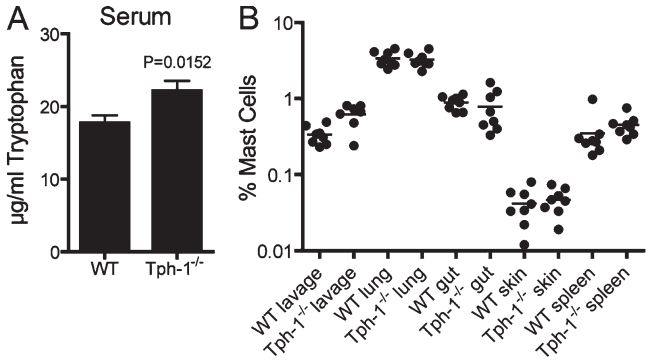

Figure 2. Tph-$1^{-/-}$ mice have elevated serum tryptophan levels and a normal MC compartment. (A) Serum tryptophan levels from in-house-bred naive WT and Tph-$1^{-/-}$ mice (n = 13–14 mice/group from three independent experiments) were determined by ELISA. Mean ± SEM is shown. The p-value was determined by Student's t test. (B) Peritoneal lavage, lungs, intestines, spleen, and skin of Tph-$1^{-/-}$ mice were analyzed by flow cytometry for the relative number of MCs and compared with WT mice. Data are pooled from two independent experiments (n = 7–8 mice/group), and mean is indicated.

Based on these findings and the recognition that Tph-1 can locally deplete tryptophan, the potential functional involvement of Tph-1 in mediating immune tolerance within the skin microenvironment was studied in Tph-1$^{-/-}$ mice. Tph-1$^{-/-}$ mice have been previously described as harboring no gross physical abnormalities except diminished cardiac function (Côté et al., 2003) and enhanced liver microcirculation (Lang et al., 2008). However, the immune compartments of Tph-1$^{-/-}$ mice have not been well characterized. The genetic absence of Tph-1 appears to impact the steady-state levels of tryptophan as serum from Tph-1$^{-/-}$ mice contains ~5 µg/ml more tryptophan in serum than that of WT controls (P = 0.0152; Fig. 2 A). Although systemic levels of tryptophan are generally attributed to the activity of TDO, it appears that IDO can modulate the relative amounts of tryptophan under inflammatory conditions (Schröcksnadel et al., 2006). Therefore, it appears that constitutive loss of Tph-1 can also alter tryptophan levels in the periphery. The analysis of immune cell phenotypes on WT and Tph-1$^{-/-}$ mice revealed that these mice were indistinguishable across all tissues examined (not depicted). Furthermore, the lack of Tph-1 does not appear to impact the frequency (Fig. 2 B) or numbers (not depicted) of MCs, which have been described as the cells most abundantly expressing Tph-1 (Stoll et al., 1990; Mathiau et al., 1994; Csaba et al., 2006).

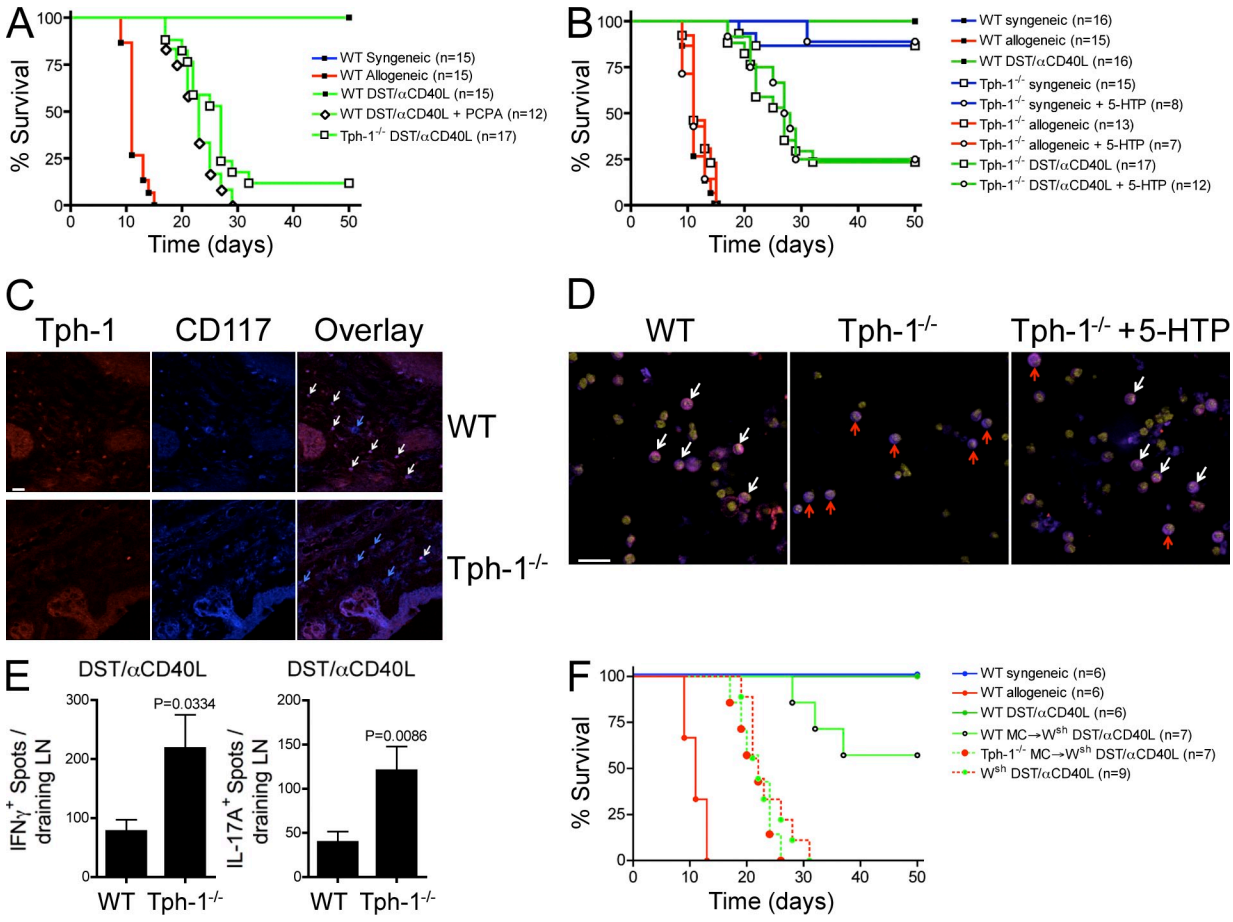

Figure 3. Tph-1 is essential for the maintenance of tolerance. (A and B) WT and Tph-1$^{-/-}$ mice were grafted with syngeneic C57BL/6 or allogeneic $CB6F_1$ skin. DST- and α-CD40L–treated mice received an allogeneic skin graft. In the indicated groups, PCPA and 5-HTP were given every other day for the duration of the experiment starting at day −8 for DST/α-CD40L–treated mice and day −1 for syngeneic and allogeneic mice. The number of mice/group is indicated, and data were pooled from two or more independent experiments. (C) Histology staining on skin allografts in DST/α-CD40L–treated mice of Tph-1 or WT mice at day 10 after grafting. Tph-1 is shown in red, and cKit (CD117) is shown in blue. In the overlay, white arrows indicated double-positive cells, and blue arrows indicate cells positive for cKit but not for Tph-1. (D) Reconstitution of serotonin in the granules of MCs was confirmed by immunofluorescent staining on purified peritoneal lavage MCs. Serotonin staining is in red, cKit staining in blue, and nuclear staining in yellow. The white arrows indicate MCs positive for serotonin, whereas the red arrows indicate MCs negative for serotonin. Bars, 20 µm. (E) Graft draining LNs from DST/α-CD40L–treated mice were depleted of antigen-presenting cells and restimulated with irradiated WT of F1 antigen-presenting cells for 18 h for IFN-γ and for 36 h for IL-17A before ELISPOT development. Data show n = 7–8 mice/group, and mean ± SEM is representative of results from two independent experiments. P-values were determined by Student's t test. (F) Mast-deficient (W^{sh}) mice were locally (skin) reconstituted with 6–8-wk-old MCs from WT or Tph-1$^{-/-}$ mice 8 wk before DST/α-CD40L treatment. After receiving a skin graft, mice were monitored for rejection for another 50 d. Data shown were combined from two independent experiments.

Donor-specific transfusion (DST)/α-CD40L–treated mice reject allogeneic skin grafts with Tph-1 deficiency

The potential role of Tph-1 deficiency in immune tolerance was studied in a model of skin allograft tolerance. Here mice were treated with a tolerance-inducing regimen of DST in combination with α-CD40L on days −7, −5, and −3 and then received an allogeneic $CB6F_1$ (C57BL/6 × BALB/c) skin graft on day 0 (Quezada et al., 2003). In the first experiments, the specific and irreversible inhibitor parachlorophenylalanine (PCPA), which has been reported to effectively block the effects of Tph with multiple treatments (Côté et al., 2003), was used. Approximately 90% of the PCPA-treated mice receiving DST/α-CD40L rejected their skin allografts within 30 d. In contrast, 100% of DST/α-CD40L–treated WT mice retained their skin allografts for >50 d (Fig. 3 A). The role of Tph-1 in allograft tolerance was then studied in Tph-$1^{-/-}$ mice (Fig. 3, A and B). It was shown that 80% of the DST/α-CD40L–treated Tph-$1^{-/-}$ mice rejected their skin grafts within 30 d as compared with DST/α-CD40L–treated WT mice ($P < 0.0001$ between these two groups by log-rank test). Tph-$1^{-/-}$ mice grafted with syngeneic grafts retained those grafts as did WT controls, establishing that Tph-1 is not involved in wound healing. Furthermore, allogeneic grafts placed on unmanipulated WT or Tph-$1^{-/-}$ mice mounted similar rejection responses with regard to the frequency and rates of graft rejection (Fig. 3 B). It was also noted that the majority of MCs in Tph-$1^{-/-}$ mice in donor skin allografts were negative for Tph-1, indicating that they were host derived (Fig. 3 C). The loss of tolerance in this model was not caused by the absence of peripheral serotonin synthesis in the Tph-$1^{-/-}$ mice as adding back 5-hydroxytryptophan (5-HTP), which bypasses the Tph-1 deficiency to restore serotonin levels, did not allow for long-lived tolerance in Tph-$1^{-/-}$ mice (Fig. 3 B). Because MCs store serotonin in their granules, reconstitution of serotonin biosynthesis by 5-HTP administration in Tph-$1^{-/-}$ mice was confirmed by histological analysis of cytospins of peritoneal lavage MCs (Fig. 3 D). It is therefore concluded that Tph-1 is required for the maintenance of peripheral tolerance independently of serotonin.

To determine whether rejection in the DST/α-CD40L–treated Tph-$1^{-/-}$ mice was caused by the emergence of an allogeneic T cell response, T cells from draining LNs 14 d after the initial grafting were recalled with F1 antigen-presenting cells to assess their cytokine production (Fig. 3 E). An increase in the allogeneic-specific IFN-γ response ($P = 0.0334$) as well as IL-17A response ($P = 0.0084$) was observed in the DST/α-CD40L–treated Tph-$1^{-/-}$ mice compared with similarly prepared WT mice.

Based on the finding that the highest signal of Tph-1 message and protein expression occurs in MCs, it was envisioned that MC-derived Tph-1 is responsible for the phenotype seen in the deficient mice. W^{sh} mice harbor a MC deficiency caused by a c-Kit mutation and can be readily reconstituted with genetically deficient MCs to test the role of MC-derived genes in immune responses (Grimbaldeston et al., 2005). As such, W^{sh} mice were reconstituted with WT or Tph-$1^{-/-}$ BM-derived MCs (BMMCs). As previously reported, DST/α-CD40L–treated W^{sh} mice reconstituted with WT BMMCs show a gradual erosion of tolerance leading to rejection of ∼50% of the allografts between days 30 and 60 after transplantation (Lu et al., 2006). However, when DST/α-CD40L–treated

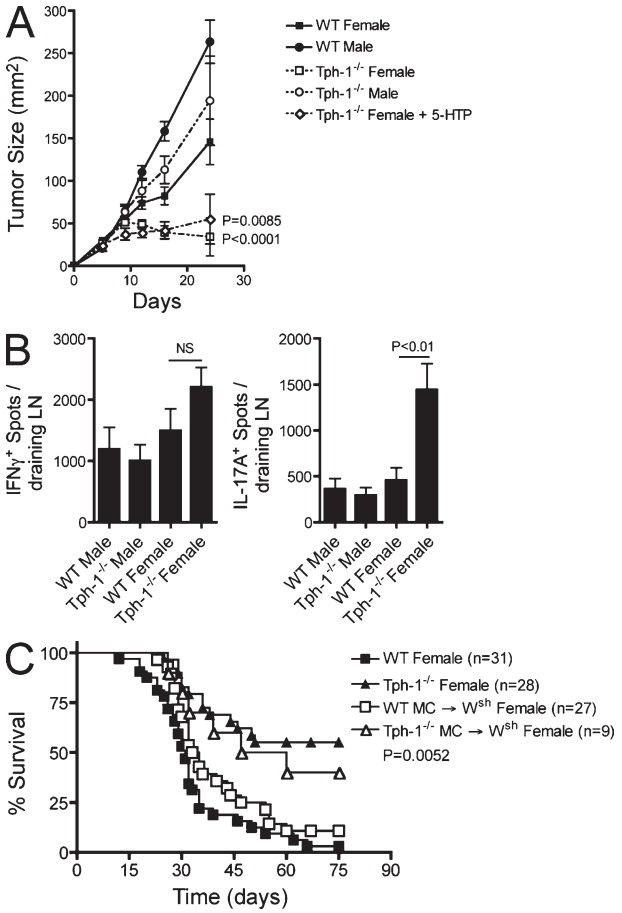

Figure 4. Tph-1 is essential for the generation of protective T cell–mediated antitumor immunity. (A) MB49 was inoculated, and tumor growth was monitored over time in all the groups. One group received 5-HTP every other day from the day before the start of experiment. Data show mean ± SEM of $n = 6$–8 mice/group and are representative of results from three independent experiments. P-values listed are for the group they are shown next to in comparison with WT female mice and were determined by two-way ANOVA. (B) On day 11 after tumor inoculation, whole tumor draining LNs were isolated, plated, and restimulated with irradiated tumor to determine IFN-γ ELISPOTS after 18 h and IL-17A ELISPOTS after 36 h, restimulated for 18 and 36 h, respectively. Data show mean ± SEM for $n = 10$–16 mice/group and were pooled from three independent experiments. The p-value shown was determined for the indicated groups by Tukey posttest after one-way ANOVA analysis. (C) MB49 was inoculated systemically through the tail vein in age-matched female WT and Tph-$1^{-/-}$ mice as well as female W^{sh} mice systemically reconstituted with BMMCs from WT and Tph-$1^{-/-}$ mice. The graph is compiled from three independent experiments with total numbers of mice indicated in the figure. The p-value was determined by log-rank comparison between the two BMMC-reconstituted groups.

W^{sh} mice were reconstituted with Tph-$1^{-/-}$ BMMCs, it was observed that all mice rejected their allograft within 30 d with P = 0.0002 in comparison with WT BMMC-reconstituted mice by log-rank test (Fig. 3 F). Therefore, it appears that MC-derived Tph-1, and not Tph-1 produced by other cells, is necessary for long-term graft tolerance.

Tph-1 and immune suppression to tumor

As MC expression of Tph-1 is critical for allograft tolerance and MCs have been implicated in suppressing antitumor immunity (Maltby et al., 2009), experiments were designed to determine whether MC-derived Tph-1 mediated immune tolerance to an intradermal (i.d.) skin tumor model. MB49 is a bladder cell carcinoma that expresses male minor histocompatibility antigen and readily grows and kills male mice because they are centrally tolerant to H-Y (Summerhayes and Franks, 1979; Halak et al., 1999). In contrast, female WT mice have delayed tumor growth kinetics in comparison with WT males yet nonetheless ultimately succumb to tumor. Strikingly, female Tph-$1^{-/-}$ mice have reduced tumor growth kinetics, and ~50% completely reject MB49. It was also confirmed that the effect of Tph-1 deficiency was independent of serotonin biosynthesis by the lack of effect of administration of 5-HTP (Fig. 4 A). 11 d after tumor inoculation, tumor-specific recall responses from T cells from the draining LNs were measured (Fig. 4 B). Female Tph-$1^{-/-}$ mice had significantly higher numbers of IL-17A–specific spots ($P < 0.01$) in comparison with all other groups, and IFN-γ–specific spots were elevated in female Tph-$1^{-/-}$ mice although it did not reach statistical significance. Through reconstitution of W^{sh} mice with either BMMCs from either WT or Tph-$1^{-/-}$ mice, it was confirmed that Tph-$1^{-/-}$ BMMCs conferred protection to the same extent as female Tph-$1^{-/-}$ mice with P = 0.0052 between WT and Tph-$1^{-/-}$ BMMC-reconstituted mice (Fig. 4 C). The data establish that MCs maintain a suppressive antitumor microenvironment and that Tph-1 is a major mediator within this context.

Tph-1 deficiency exacerbates experimental autoimmune encephalomyelitis (EAE)

The question arose as to whether Tph-1 only enhances immunity under immune-tolerant or -suppressive conditions, or can it function in tempering inflammation in general? To address this question, the impact of Tph-1 deficiency on a central nervous system (CNS) inflammatory disease was studied. EAE is a model of CNS inflammation in which encephalitogenic Th17 lineage T cells infiltrate the CNS, mediate damage, and cause ascending paralysis. Using suboptimal disease conditions, it was found that Tph-$1^{-/-}$ mice develop earlier and more severe disease than controls (P = 0.0064; Fig. 5 A). As seen in all other models tested, restoration of serotonin levels by administration of 5-HTP had no effect on the immunological impact of Tph-1 deficiency. In this specific case, it was shown that serotonin levels in serum were restored to the same levels of controls after just one injection with 5-HTP (Fig. 5 B). Furthermore, long-term treatment of 5-HTP restored serotonin to a level greater than WT controls ($P < 0.001$; Fig. 5 C). The finding that serotonin is not involved is also supported by the observation that serotonin transporter–deficient mice have attenuated EAE (Hofstetter et al., 2005).

Further characterization of immune responses during EAE found that Tph-$1^{-/-}$ mice have an enhanced frequency of encephalitogenic T cells. Analysis of T cell responses during EAE revealed that on day 7 after immunization (when neither group showed disease), Tph-$1^{-/-}$ mice had greater numbers of $CD4^+$ T cells expressing CCR6 (P = 0.0484), one of the adhesion molecules necessary to be on the first wave of T cells that infiltrate the CNS (Fig. 6 A; Liston et al., 2009; Reboldi et al., 2009). Tph-$1^{-/-}$ mice also had elevated recall IL-17A production by $CD4^+$ T cells (P = 0.0002). At late stages of disease, Tph-$1^{-/-}$ mice also had elevated numbers of $CD4^+$ and $CD8^+$ T cells that were infiltrating the CNS, which have high IL-17A and IFN-γ recall responses (Fig. 6 B). To determine whether this effect is just caused by differences in T cell

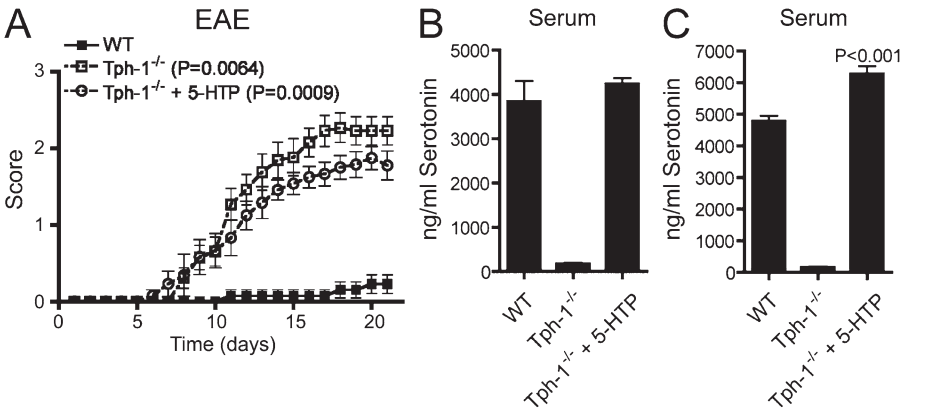

Figure 5. Tph-$1^{-/-}$ mice have exacerbated EAE independent of their serotonin level. (A) WT, Tph-$1^{-/-}$, and Tph-$1^{-/-}$ + 5-HTP–treated (starting on day −1 and continuing every other day for the duration of the experiment) mice were monitored and scored. Data are pooled from three independent experiments and show mean ± SEM for *n* = 13 mice/group. P-values indicate significance in comparison with WT mice and were calculated by Mann–Whitney *U* test. (B) Serum serotonin levels of WT, Tph-$1^{-/-}$, and Tph-$1^{-/-}$ + 5-HTP–treated (one shot 2 d prior) mice on day 1 after EAE induction were determined by ELISA (*n* = 11–12 mice/group pooled from three independent experiments). (C) Serum serotonin levels of WT, Tph-$1^{-/-}$, and Tph-$1^{-/-}$ + 5-HTP–treated (last shot given 2 d before sample collection) mice on day 21 after EAE induction were determined by ELISA (*n* = 13 mice/group pooled from three independent experiments). The p-value was determined by one-way ANOVA analysis and indicates difference from WT mice. (B and C) Mean ± SEM is shown.

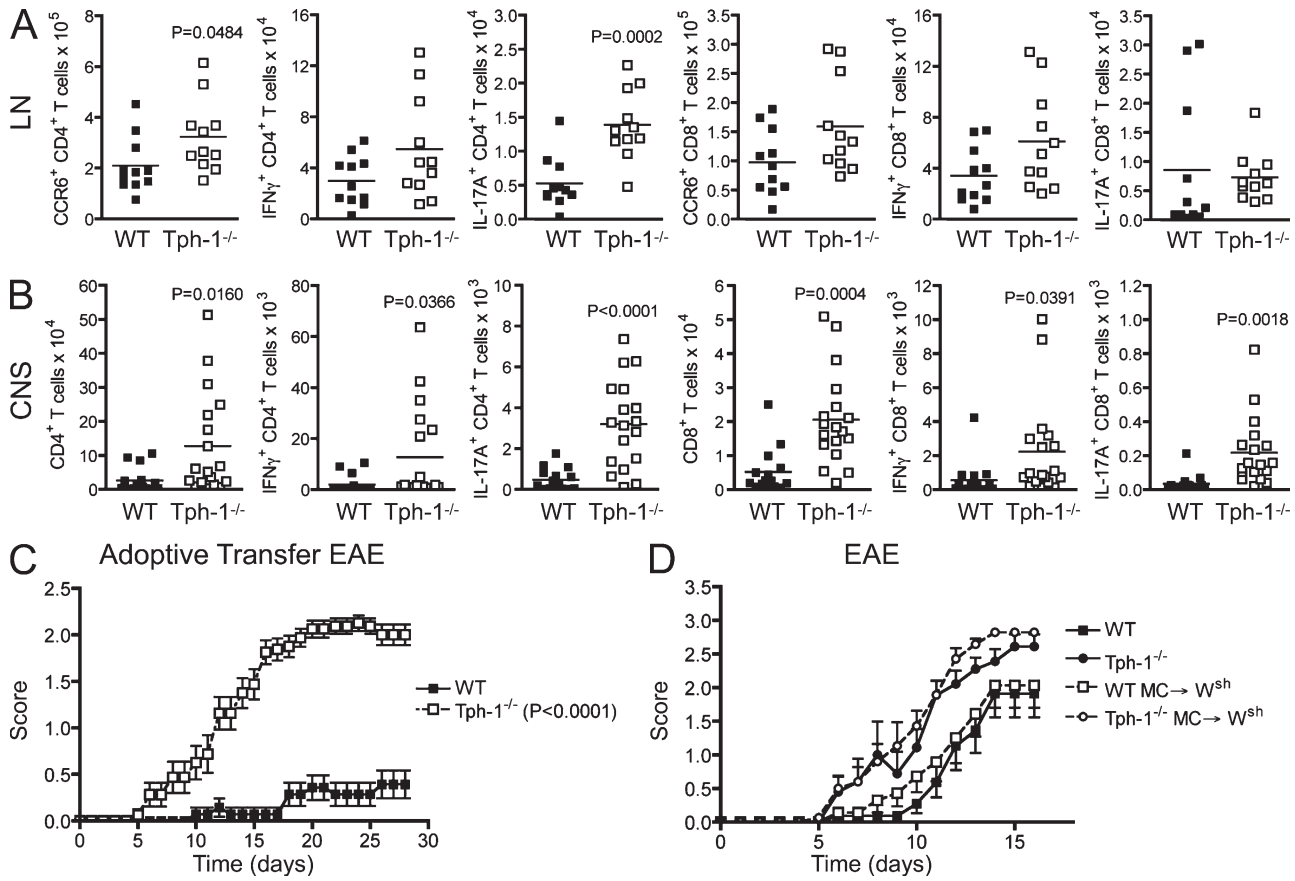

Figure 6. Tph-1$^{-/-}$ mice have increased encephalitogenic T cell responses. (A) Day 7 after inoculation for EAE, mice were taken down and draining LNs were stained for the indicated parameters (after restimulation for cytokine stains). Data are pooled from three independent experiments (n = 11 mice/group), and mean is indicated. (B) Day 21 after inoculation, lymphocytes from the CNS were isolated and stained for the indicated parameters (after restimulation for cytokine stains). Data are pooled from three independent experiments (n = 15–18 mice/group), and mean is indicated. (A and B) P-values were determined by Student's t test. (C) Mice were injected with activated MOG_{35-55}-specific T cells, and disease severity was monitored over time. Data are pooled from two independent experiments and show mean ± SEM for n = 14–16 mice/group. The p-value was determined by Mann–Whitney U test. (D) W^{sh} mice were given WT or Tph-1$^{-/-}$ BMMCs systemically and given 8 wk to reconstitute. These mice and age-matched controls were immunized for active EAE and monitored for disease severity over time. Data show mean ± SEM for n = 10–15 mice/group pooled from two independent experiments.

priming, myelin-specific Th17 cells were adoptively transferred into WT and Tph-1$^{-/-}$ mice. Data show that these T cells could effectively initiate disease in Tph-1$^{-/-}$ mice but not controls ($P < 0.0001$), showing that Tph-1$^{-/-}$ mice are better able to sustain and facilitate encephalitogenic T cell–mediated pathogenesis (Fig. 6 C). Although hampered by differences in EAE severity caused by the increased age of the mice, the phenotype of W^{sh} mice reconstituted with Tph-1$^{-/-}$ BMMCs looked consistent with that of Tph-1$^{-/-}$ mice, suggesting MC-derived Tph-1 is responsible for this effect (Fig. 6 D).

Tph-1$^{-/-}$ mice have enhanced signaling of mammalian target of rapamycin (mTOR)

The mTOR pathway can promote CD4 T cell differentiation to Th1 and Th17 cells (Delgoffe et al., 2009), and its activity is inhibited in vitro with loss of essential amino acids (Cobbold et al., 2009). Therefore, it presented itself as a potential target for mediating the differences observed in WT and Tph-1$^{-/-}$ mice. The phosphorylation of S6 riboprotein, one of the targets of mTOR, in CD4$^+$ T cells was measured in the draining LNs 4 d after EAE immunization, and we found that it was elevated in Tph-1$^{-/-}$ mice ($P = 0.04$; Fig. 7). This suggests that comparatively there is a reduction of mTOR activity in WT mice that is consistent with there potentially being a tryptophan-deficient microenvironment imposed by Tph-1.

DISCUSSION

The findings presented herein describe Tph-1 as an important and novel MC-derived regulator of immunological tolerance. This single molecule exerts striking alterations in immunological outcomes in models of transplantation tolerance, tumor growth, and autoimmunity. Tph-1 metabolizes

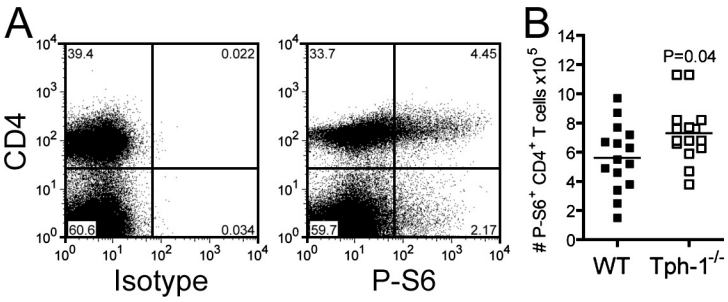

Figure 7. Tph-1$^{-/-}$ mice have enhanced mTOR activity. (A) Example staining of P-S6 and its isotype control antibody is shown. (B) Day 4 after inoculation for EAE, mice were taken down, draining LNs were harvested, and CD4+ T cells were stained for P-S6 expression (and isotype control). Data are pooled from three independent experiments with $n = 15$ mice/group. Mean is indicated, and the p-value was determined by Student's *t* test.

tryptophan for the purpose of producing serotonin. However, the experiments presented here clearly show that the major immunological impact of this pathway is not through the regulation of serotonin levels and therefore must be caused by its ability to exhaust tryptophan as suggested by the mTOR experiments.

There are several catabolic enzymes that have been suggested to contribute to the establishment of immune tolerance. For example, during skin allograft tolerance, DCs activated by regulatory T cells can express enzymes to consume 9 of the 10 essential amino acids and cause a reduction in mTOR signaling (Cobbold et al., 2009). Extensive work on IDO, one of the tryptophan-catabolizing enzymes, also shows that it can limit immune responses through the induction of the GCN2 stress response, which promotes anergy, as well as the production of tryptophan metabolites that suppress inflammation (Munn and Mellor, 2007). There are also some indications that different catabolic enzymes may counter-regulate the activity of each other. For example, the production of nitric oxide by inducible nitric oxide synthase can prevent IDO activity (Katz et al., 2008). However, in the case of Tph-1 expression by MCs, this does not appear to be occurring because MCs have little to no messenger RNA expression for other catabolic enzymes, so competition for substrate is not likely contributing to the immunosuppressive activity of Tph-1.

In the case of Tph-1, the question arises as to which cell type is the target of the tryptophan deficiency in the tolerant microenvironment. Although we observe enhancement of mTOR activity in CD4 T cells with Tph-1 deficiency during EAE, it is not yet clear whether this is the case in our transplant and tumor models. In addition, there could be additional targets of Tph-1 activity. For example, we have recently reported that within the tolerant allograft, graft-derived DCs mediate regional allo-specific unresponsiveness. Upon analysis of gene expression of these DCs, it appears that they have experienced a nutrient-stressed environment (de Vries et al., 2011). We would contend that this is caused by Tph-1 activities on DCs from the increased MCs that infiltrate the tolerant allograft. It would also be of interest to determine the factors that up-regulate Tph-1 expression in MCs as well as additional cell types to potentially mediate immune tolerance.

The experiments presented provide a new prospective on this immunologically important enzyme. Its end product serotonin can impact inflammation, particularly in the gut (O'Connell et al., 2006; Nakamura et al., 2008). It has also been observed in Tph-1$^{-/-}$ mice that there is an enhanced ability to clear lymphocytic choriomeningitis virus (Lang et al., 2008) and reject the MC-38 colon cancer line (Nocito et al., 2008). However, in these cases, this is dependent on the loss of serotonin in these mice. Therefore, Tph-1 likely regulates immunity by regulating serotonin levels or by exhausting tryptophan depending on the nature of the immune response invoked. Certainly, the fact that Tph-1 can so profoundly impact tolerance and inflammation provides compelling incentive to consider Tph-1 as a novel target in immune intervention.

MATERIALS AND METHODS

Mice. Tph-1$^{-/-}$ mice fully backcrossed to a C57BL/6 background were provided by N. Horseman (University of Cincinnati, Cincinnati, OH) and maintained at the Dartmouth College animal facility. Male and female 6–8-wk-old C57BL/6 mice were bred in-house for measurement of tryptophan in serum, all EAE experiments, and all ELISPOT experiments. In all other experiments, C57BL/6 mice were purchased from the National Cancer Institute. 2D2 TCR transgenic mice were provided by V. Kuchroo (Harvard University, Cambridge, MA) and maintained in-house. C57BL/6 Kit$^{W\text{-}sh}$ (W^{sh}) mice were purchased from the Jackson Laboratory. CB6F$_1$ (C57BL/6 × BALB/c hybrid) were purchased from the National Cancer Institute. Experiments were performed under protocols approval by the Institutional Animal Care and Use Committee of Dartmouth College, and mice were maintained in a specific pathogen–free facility at Dartmouth Medical School.

MC reconstitution. BMMCs for MC reconstitution were generated by culturing BM cells with 20 ng/ml IL-3 (PeproTech) and 50 ng/ml stem cell factor (SCF; PeproTech) for 5–8 wk as shown previously (Lu et al., 2006). Purity was assessed by anti-CD117 and anti-FCεRI staining on BMMC cultures (Lu et al., 2006). A total of 4–5 × 10^6 BMMCs were then injected i.d., i.v., and i.p. into W^{sh} recipients. Mice were allowed to rest for 8–12 wk before use in experiments. At the end of the experiment, reconstitution was confirmed by flow cytometry or histology.

Skin grafting. Skin grafting was performed as previously described (Markees et al., 1998). In brief, 1-cm^2 full-thickness tail skins were collected from CB6F$_1$ allogeneic donor or C57BL/6 syngenic donor mice. Skins were then stored on PBS-soaked gauze and were on the following day applied to the dorsal surface of age-matched WT or Tph-1$^{-/-}$ host mice. Indicated groups were treated 7 d before grafting, via the i.v. injection of 3 × 10^7 DST in conjunction with an injection of 250 μg anti-CD40L (clone MR1), followed by further injections on days −5 and −3 before graft. This regimen results in long-term tolerization of the mice to alloantigen.

Cell culture, tumor challenge, and vaccination. Murine bladder carcinoma cell line MB49 was maintained in complete medium (RPMI 1640 containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol). Mice were injected with 2.5 × 10^5 MB49 tumor cells i.d. on the right flank, and tumor diameters were measured with a caliper thrice weekly. Alternatively, mice were challenged with 2.5 × 10^5 MB49 tumor cells i.v. in the tail vein and were monitored for survival.

EAE immunization and clinical evaluation. Age-matched WT and Tph-1$^{-/-}$ mice were immunized subcutaneously with 125 µg $MOG_{35–55}$ peptide (Peptides International) emulsified in IFA (Sigma-Aldrich) supplemented with 0.5 mg/mouse *Mycobacterium tuberculosis H37* (Difco Laboratories) on day 0 and an i.p. injection of 200 ng pertussis toxin (List Biologicals) on days 0 and 2. For adoptive transfer EAE, donor 2D2 transgenic T cells received a standard immunization as previously described (Nowak et al., 2009), and lymphocytes were isolated out of mice 10 d later. Cells were stimulated with 20 µg/ml $MOG_{35–55}$, 10 µg/ml anti–IFN-γ (BioXCell), 20 ng/ml IL-23 (BD), 10 ng/ml IL-6 (PeproTech), and 10 ng/ml IL-1β (PeproTech) for 4 d before reisolation of live T cells (∼80% positive for IL-17A). Recipient mice were injected with 1.0×10^6 T cells i.v. Mice were scored as previously described (Becher et al., 2002).

Antibodies and reagents. Mouse monoclonal antibodies to CD8 (53-6.7) and CD4 (GK1.5) were purchased from eBioscience. Mouse monoclonal antibodies to CD4 (RMA4.5), CD45 (30-F11), IL-17A (TC11-18H10.1), IFN-γ (XMG1.2), CCR6 (29-2L17), CD117 (ACK2), FCεR1 (MAR-1), CD11b (M1/70), CD11c (N418), F4/80 (BM8), and CD19 (6D5) were purchased from BioLegend. Tph-1 antibody was purchased from Santa Cruz Biotechnology, Inc. Secondary F(ab′)2 anti–rabbit IgG antibody was purchased from eBioscience. P-S6 antibody and its isotype control antibody were purchased from Cell Signaling Technology.

ELISPOT antibodies for IFN-γ were purchased from Mabtech and those for IL-17A were purchased from BioLegend. Both were developed using the AEC substrate kit from BD. ELISA kits for serotonin (Enzo Life Sciences) and tryptophan (Rocky Mountain Diagnostics) were used according to the manufacturers' directions. 5-HTP (Sigma-Aldrich) and PCPA (Sigma-Aldrich) were given i.p. at 5.5 mg/mouse every other day in all indicated experiments starting the day before any other treatment.

Histology. Sections were cut and stained as previously described (Lu et al., 2006). In brief, tissues were fixed in OCT, sectioned onto slides, fixed with methanol and acetone, stained with antibody in 10% serum, washed extensively, and mounted with Prolong Gold (Molecular Probes) according to the manufacturer's directions. Unstained and single-stain sections were performed for each tissue. In addition, the same staining cocktails were used on tissue from Tph-1$^{-/-}$ mice to serve as a negative control for Tph-1 and serotonin stains. Images were taken on an LSM 510 confocal microscope (Carl Zeiss) and analyzed using LSM 5 Image Browser (Carl Zeiss).

Flow cytometry. Single cell suspensions were incubated with antibodies conjugated with FITC, PE, PerCP, APC, Alexa Fluor 647, and/or Alexa Fluor 700. Intracellular staining and restimulation for cytokine staining was performed as previously described (Nowak et al., 2009). To stain for Tph-1, cells were surface stained, washed, fixed with Fixation/Permeabilization Buffer (BD), washed, permeabilized with Perm/Wash (BD), stained with Tph-1 antibody, washed, stained with secondary antibody, washed, and resuspended for analysis. For all staining steps, 10% normal rat serum was included. P-S6 staining was performed as follows: cells were blocked in 10% serum, surface stained, washed, fixed with 4% paraformaldehyde, washed, fixed and permeabilized with methanol, washed, blocked with 10% serum, intracellularly stained, washed, and resuspended for analysis. Five-color analyses were performed on a modified FACScan (BD) running CellQuest software (BD) and Rainbow software (Cytek).

RNA preparation and qRT-PCR. All cell populations were sorted on a FACS Aria (BD). For MC samples, the cell number acquired was insufficient for postsort analysis, so the samples were assessed for expression of several MC markers and products to confirm specificity of cells. RNA was prepared according to the manufacturer's directions using an RNeasy Mini kit (QIAGEN). MC samples were further amplified and converted to cDNA using QuantiTect Whole Transcriptome kit (QIAGEN). All other samples were transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories). qRT-PCR was performed as previously described (Becher et al., 2002). All samples were standardized to expression of β-actin. Primers used are as follows: β-actin (5′-CCACACCCGCCAGTTCG-3′ and 5′-TCTGGGCCTCGTCACCCACAT-3′), Tph-1 (5′-GAAGACAACATCCCGCAACT-3′ and 5′-GTTCAGCCAAGAGAGGAACG-3′), arginase (5′-CAGAAGAATGGAAGAGTCAG-3′ and 5′-CAGATATGCAGGGAGTCACC-3′), dopa decarboxylase (5′-AGGGCAGAGAAAGAATGAAAGCA-3′ and 5′-GGAGTGGTAGTTATTTTTCTCTTTCCA-3′), histidine decarboxylase (5′-GATCAGATTTCTACCTGTGG-3′ and 5′-GTGTACCATCATCCACTTGG-3′), IDO (5′-TGGCAAACTGGAAGAAAAAG-3′ and 5′-ATTGCTTTCAGGTCTTGACG-3′), inducible nitric oxide synthase (5′-ACCCCTGTGTTCCACCAGGAGATGTT-3′ and 5′-TGAAGCCATGACCTTTCGCATTAGCA-3′), L-threonine dehydrogenase (5′-AAGCACGCGCCTGACTTC-3′ and 5′-CCGAGCATTGCTGTCATCTAGA-3′), TDO (5′-TGGGAACTAGATTCTGTTCG-3′ and 5′-TCGCTGCTGAAGTAAGAGCT-3′), and Tph-2 (5′-CAGGAGAGGGTTGTCCTTGG-3′ and 5′-TTTGCCGCTTTTCTTGTCCT-3′).

Statistical analysis. Data graphs were made using Prism software (GraphPad Software) and expressed as the mean ± SEM. Differences for graphs with one grouping variables were analyzed by Student's *t* test (two groups) or one-way analysis of variance (ANOVA) and Tukey analysis (three or more groups). Log-rank tests were used to compare skin graft survival and survival of mice inoculated with MB49. For the study of MB49 growth kinetics, two-way ANOVA was used to assess significance. In EAE time course experiments, statistical relevance was determined using Mann–Whitney *U* Test.

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