The NF-κB regulator MALT1 determines the encephalitogenic potential of Th17 cells

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Effectors of inflammatory IL-17-producing Th (Th17) cells have been linked to autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS). However, what determines Th17 cell encephalitogenicity is still unresolved. Here, we show that after EAE induction, mice deficient for the NF-κB regulator MALT1 (Malt1−/− mice) exhibit strong lymphocytic infiltration in the CNS, but do not develop any clinical signs of EAE. Loss of Malt1 interfered with expression of the Th17 effector cytokines IL-17 and GM-CSF both in vitro and in vivo. In line with their impaired GM-CSF secretion, Malt1−/− Th cells failed to recruit myeloid cells to the CNS to sustain neuroinflammation, whereas autoreactive WT Th cells successfully induced EAE in Malt1−/− hosts. In contrast, Malt1 deficiency did not affect Th1 cells. Despite their significantly decreased secretion of Th17 effector cytokines, Malt1−/− Th17 cells showed normal expression of lineage-specific transcription factors. Malt1−/− Th17 cells failed to cleave RelB, a suppressor of canonical NF-κB, and exhibited altered cellular localization of this protein. Our results indicate that MALT1 is a central, cell-intrinsic factor that determines the encephalitogenic potential of inflammatory Th17 cells in vivo.

Introduction

CD4+ Th cells can be categorized into 3 major subsets that make complementary contributions to immunity. Th1 cells predominantly mediate cellular immunity and are characterized by their production of the signature cytokine IFN-γ. Th2 cells mainly support humoral immunity and secrete IL-4, IL-5, and IL-13 (1). Th17 cells are key inflammatory drivers and are characterized by their production of IL-17A, IL-17F, IL-21, TNF, and GM-CSF (2–4). In particular, Th17 cells are regarded as the principal cell type responsible for the induction of EAE, an important mouse model of the human disease MS (5–7). The development of EAE is type responsible for the induction of EAE, an important mouse model of multiple sclerosis (MS). However, what determines Th17 cell encephalitogenicity is still unresolved. Here, we show that after EAE induction, mice deficient for the NF-κB regulator MALT1 (Malt1−/− mice) exhibit strong lymphocytic infiltration in the CNS, but do not develop any clinical signs of EAE. Loss of Malt1 interfered with expression of the Th17 effector cytokines IL-17 and GM-CSF both in vitro and in vivo. In line with their impaired GM-CSF secretion, Malt1−/− Th cells failed to recruit myeloid cells to the CNS to sustain neuroinflammation, whereas autoreactive WT Th cells successfully induced EAE in Malt1−/− hosts. In contrast, Malt1 deficiency did not affect Th1 cells. Despite their significantly decreased secretion of Th17 effector cytokines, Malt1−/− Th17 cells showed normal expression of lineage-specific transcription factors. Malt1−/− Th17 cells failed to cleave RelB, a suppressor of canonical NF-κB, and exhibited altered cellular localization of this protein. Our results indicate that MALT1 is a central, cell-intrinsic factor that determines the encephalitogenic potential of inflammatory Th17 cells in vivo.
As expected, WT Th17 cells produced IL-17, but no IFN-\(\gamma\) and ELISA analysis of culture supernatants (Supplemental Figure 1D). Interestingly, by intracellular staining and flow cytometry (Figure 2B), RT-PCR analyses at 30 days after MOG immunization showed dense immune cell infiltrates in the CNS tissue in both WT and Malt1–/– mice (Figure 1, B and C). There was no obvious disparity in T cell infiltrates observed in WT and Malt1–/– brains, but the distribution of these infiltrates exhibited striking differences. The white matter of WT brains contained a perivascularly centered, diffuse, widespread infiltrate consisting mainly of CD3+ T cells (Figure 1B). In contrast, in Malt1–/– brain tissue, most T cells were located in very close proximity to blood vessels. As expected, we did not detect any B cell infiltrates in WT or Malt1–/– brains (Figure 1B). Infiltrating cells were also clearly visible in the spinal cords of WT and Malt1–/– mice, although the differences between the genotypes were less pronounced (Figure 1C).

We next assessed the functionality of the Th cells infiltrating the brains and spinal cords of MOG-immunized WT and Malt1–/– mice. At 14 days after EAE induction, we isolated CNS-infiltrating lymphocytes by density gradient centrifugation. Cells isolated from Malt1–/– brains and spinal cords showed a dramatic decrease in the percentage of infiltrating Th cells that secreted IL-17A (WT, 12.0%; Malt1–/–, 2.46%; Figure 3A). As expected, IL-17 was not detected in any GFAP+ astrocytes were detectable in brains (Figure 3A). However, compared with WT brain, there were dramatic decreases in Malt1–/– brain in the percentages of infiltrating Th cells that secreted IL-17A (WT, 12.0%; Malt1–/–, 4.10%) or GM-CSF (WT, 25.8%; Malt1–/–, 2.46%; Figure 3A).

The recruitment of bone marrow–derived myeloid cells has been associated with encephalitogenic Th17 cell responses and GM-CSF secretion (3, 5, 10, 12, 30, 31). Consistent with our observation of impaired IL-17A and GM-CSF secretion by CNS-infiltrating Malt1–/– Th17 cells, we detected a striking reduction in the number of activated macrophages/microglia in Malt1–/– brains. In contrast to the diffuse distribution of these Mac3+ cell infiltrates in WT brains, they were found only in close proximity to blood vessels in Malt1–/– brains (Figure 3B). Moreover, whereas WT brains showed large numbers of reactive GFAP+ astrocytes within the infiltrated areas (a nonspecific sign of severe CNS damage), hardly any GFAP+ astrocytes were detectable in Malt1–/– brains (Figure 3B). Thus, loss of MALT1 did not affect the capacity of Th cells to infiltrate brain tissue, but did compromise their effector functions and impair their ability to recruit other inflammatory cells. This lack of inflammatory cell infiltration could explain the resistance of Malt1–/– mice to EAE.

To consolidate these findings, we isolated brain-infiltrating cells on day 14 after EAE induction, a time point prior to the WT disease
Figure 1

*Malt1–/–* mice are resistant to EAE induction. (A) *Malt1–/–* (n = 9) and WT (n = 11) mice were immunized s.c. with MOG peptide in CFA, followed by i.p. injection of PT. Disease severity was scored daily. Data are the mean score ± SEM and are representative of 2 independent experiments. (B and C) Representative histopathological analyses of cross-sections of (B) brain and (C) spinal cord from mice in A at 30 days after MOG injection. Brain and spinal cord sections were stained with H&E (B and C) or immunostained to detect CD3+ T cells (B and C) or B220+ B cells (B). Scale bars: 100 μm. Data are from 1 mouse per group, representative of 2 independent experiments. (D) CNS-infiltrating CD4+ T cells from brains and spinal cords of *Malt1–/–* and WT mice were isolated by gradient centrifugation on day 14 after EAE induction. Expression levels of IL-17A and IFN-γ were analyzed by intracellular staining and flow cytometry. Data are gated on live CD4+ T cells. Also shown is quantitation of percent brain- and spinal cord–infiltrating CD4+ T cells producing IFN-γ and IL-17. Data are mean ± SEM (n = 4 per group).
peak (day 20). In contrast to our day 20 results, Th cell infiltrates on day 14 were reduced in Malt1−/− versus WT brains (Supplemental Figure 3A), which suggests that Malt1−/− Th cells exhibit delayed kinetics. Consistent with our histological analysis, recruitment of inflammatory macrophages (F4/80hiMHCIIhi) to the CNS was significantly impaired in Malt1−/− mice (Figure 3B and Supplemental Figure 3, A and B). However, MALT1 deficiency did not alter the recruitment of granulocytes (Supplemental Figure 3A), which are important inflammatory mediators in EAE to the CNS (32).

Malt1−/− Th17 cells are not pathogenic in vivo. Our observations suggested that T cells are the main determinants of EAE resistance in Malt1−/− mice. We reinforced this contention by demonstrating that Malt1−/− bone marrow–derived DCs elicited normal T cell proliferation and cytokine secretion and showed no defects in the expression of molecules related to antigen presentation, including CD86 and MHCII (Supplemental Figure 4). To confirm our theory experimentally, we injected WT 2D2 CD4+ T cells into Malt1−/− mice and induced EAE. Consistent with our previous data, transfer of WT 2D2 T cells enabled EAE induction in the formerly resistant Malt1−/− mice (Figure 4A). Histopathological analyses of brain tissue from Malt1−/− mice injected with WT 2D2 CD4+ T cells revealed strong infiltration of immune cells (Figure 4B), with cellular infiltrate distribution similar to that in WT mice (Figure 1B). T cell infiltrates were diffuse and widespread, and Malt1−/− macrophage/microglia infiltration and/or activation was prominent (Figure 4B). Punctate GFAP staining of reactive astrocytes was also observed, indicative of extensive neuroinflammation. Thus, although other mechanisms could theoretically contribute, we believe the EAE resistance of Malt1−/− mice is caused primarily by impaired T cell responses.

Based on the above-described dissection of Th cell populations, we reasoned that the EAE resistance of Malt1−/− mice might be due to their nonfunctional Th17 cells. To test this hypothesis in vivo, WT and Malt1−/− mice were injected with MOG plus PT to induce EAE and sacrificed on day 10 after immunization. Cells were isolated from spleens and draining LNs and expanded under Th1- or Th17-polarizing conditions. These MOG-specific Th1 and Th17 cells of Malt1−/− and WT origin were injected into WT recipients, and animals were monitored for signs of EAE. Injection of MOG-specific WT Th1 or WT Th17 cells resulted in severe EAE, as expected (Figure 4C and Supplemental Figure 5A). However, in accordance with our previous results, MOG-specific Malt1−/− Th17 cells failed to cause any disease in their WT hosts (Figure 4C), which indicates that Malt1−/− Th17 cells are not pathogenic. Surprisingly, transfer of MOG-specific Malt1−/− Th1 cells also did not elicit any signs of EAE (Supplemental Figure 5A). One potential interpretation of these results is that Malt1 deficiency causes a general decrease in antigen-specific Th cell generation. To explore this issue, we took advantage of the fact that CD40L upregulation can be used to identify antigen-specific T cells in EAE (33). However, we detected no differences in the frequencies of CD40L+ MOG-specific WT and Malt1−/− Th cells at day 18 after transfer (Figure 4D and Supplemental Figure 5B), which indicates that antigen-specific Th cell generation is not altered by Malt1 deficiency.

Plasticity between the Th1 and Th17 cell subsets in terms of overlapping cytokine production profiles is crucial in several dis-
eases, including EAE (34, 35). To test whether Th1/Th17 plasticity is affected by Malt1 deficiency, we injected CD45.1 transgenic WT mice with MOG-specific CD45.2+ Th cells that were derived from WT or Malt1−/− mice and polarized in vitro toward the Th1 or Th17 subset. Mice were sacrificed at day 18 after injection and analyzed for frequencies of Th1 and Th17 cells. In mice receiving WT Th1 or Th17 cells, a small population producing both IFN-γ and IL-17A was detected (Figure 4E and Supplemental Figure 5C), indicative of Th1/Th17 cell plasticity. In contrast, in mice receiving Malt1−/− Th1 or Th17 cells, there were no Th cells with the capacity to produce both IFN-γ and IL-17A. This lack of double IFN-γ/IL-17A producers in the absence of MALT1 suggests a defect in Th1/Th17 cell plasticity that could explain the inability of MOG-specific Malt1−/− Th1 and Th17 cells to cause EAE in WT mice. In any case, these in vivo data clearly showed that Malt1−/− Th cells were not pathogenic and were the main determinants of EAE resistance in Malt1−/− mice. We therefore conclude that MALT1 is critical for the proinflammatory and encephalitogenic functions of WT Th cells in vivo.

**MALT1 deficiency does not alter expression of transcription factors crucial for Th17 cell differentiation.** TGF-β and IL-6 are essential for Th17 cell development in vitro (11, 12). As shown in Supplemental Figure 1B, TGF-β-driven Treg induction was normal in the absence of MALT1, which suggests that TGF-β signaling is intact in these cells. IL-6 signaling triggers phosphorylation and activation of the transcription factor STAT3 (36). To determine whether defective IL-6 signaling caused a defect in Malt1−/− Th17 cell differentiation, we stimulated Malt1−/− and WT naive CD4+ T cells with IL-6 and monitored STAT3 phosphorylation by intracellular staining and flow cytometry. Phospho-STAT3 levels were comparable between Malt1−/− and WT CD4+ T cells (Figure 5A), which indicates that the dysfunctional Th17 cell differentiation of Malt1−/− naive T cells is not due to altered IL-6 signaling.

Th17 cells are characterized not only by their distinct cytokine secretion profile, but also by their expression of lineage-specific transcription factors. In particular, RORyt, RORα, and IRF4 have been implicated in Th17 cell differentiation (18). We used RT-PCR and intracellular flow cytometry to determine whether decreased expression of these transcription factors correlates with the inability of Malt1−/− CD4+ T cells to differentiate into Th17 cells. We primed Malt1−/− and WT naive CD4+CD62L+ Th cells
in the presence of IL-6 plus TGF-β to induce Th17 cell differentiation. As controls, parallel cultures of Malt1−/− and WT CD4+ Th cells were treated with IL-12 and IL-2 to generate Th1 cells. As expected, Th1 cells of either Malt1−/− or WT origin expressed the Th1 lineage–specific transcription factor T-bet (encoded by Tbx21), but not RORγt or RORα (Figure 5, B and C). However, to our surprise, there were no significant alterations to the expression of Rorc, Rora, or Irf4 in WT or Malt1−/− CD4+ Th cells treated with Th17-polarizing cytokines (Figure 5, B and C). These results imply that Malt1 deficiency does not abrogate the initiation of Th17 cell differentiation per se, but might interfere with the development of Th17 cell effector functions.

MALT1 is highly expressed in Th17 cells and cleaves the NF-κB subunit RelB. To gain more mechanistic insight into MALT1’s role in Th17 cell differentiation, we first measured MALT1 protein levels in various WT Th cell subsets. Surprisingly, MALT1 expression was substantially higher in Th17 cells than in Th1 cells or Tregs, as determined by immunoblotting (Figure 6A). Because we found that MALT1 influenced the differentiation of Th17 cells, but not Th1 cells, we speculated that canonical NF-κB signaling might not be of equal importance in these subsets. Although it is believed that MALT1’s structural components are generally important for IκBα degradation (21, 37), Th subset–specific differences in this involvement have yet to be investigated. We found that IκBα degradation in Malt1−/− Th17 cells was delayed but still clearly obvious at 30 minutes after restimulation with PMA plus ionomycin (PMA/Iono), whereas restimulated Malt1−/− Th1 cells showed almost no IκBα degradation (Figure 6B). In addition, restimulated WT Th17 cells showed almost complete IκBα degradation and faster resynthesis of the bona fide NF-κB target IκBα compared with restimulated WT Th1 cells. These results suggest that IKK activation is normally stronger in Th17 cells than in Th1 cells. Next, we induced the differentiation of naive WT T cells into Th1 or Th17 cells in the presence or absence of the pharmacological inhibitor
Nemo-binding domain (NBD), which prevents IKK assembly and therefore blocks canonical NF-κB activation (38). Th17-polarized WT Th cells differentiating in the presence of NBD showed a dramatic reduction in IL-17A secretion, whereas secretion of IFN-γ by Th1-polarized cells differentiating in the presence of NBD was not affected (Figure 6C). These data support our hypothesis that IKK signaling and canonical NF-κB activation is more important for Th17 cells than for Th1 cells.

The paracaspase activity of MALT1 is important for NF-κB activation in antigen receptor–stimulated T cells (24, 25) and for the survival of an aggressive subset of diffuse large B cell lymphomas (39, 40). In contrast to MALT1’s scaffolding function, its protease activity is not crucial for IκBα degradation and acts downstream of IKK (41). We found that inhibition of MALT1’s paracaspase activity by the peptide caspase inhibitor Z-VRPR-FMK reduced IL-17A production by Th17-polarized WT Th cells, but had no effect on Th1 cell differentiation (Figure 6C). Thus, IKK-driven canonical NF-κB activation (Figure 6, B and C) and IKK-independent MALT1 protease activity (Figure 6D) were crucial for the differentiation of Th17 cells, but not Th1 cells. These data further suggest that Th17 cell differentiation is regulated by 2 independent pathways that both involve MALT1. A logical scenario might be that MALT1 paracaspase activity is important for cleaving negative regulators of Th17 cell differentiation.

It was recently shown in a tumor cell line setting that MALT1 cleaves the noncanonical NF-κB subunit RelB (23), but the physiological role of this cleavage is not known. To analyze the RelB status during Th cell differentiation, we treated differentiating Th1 cells, Th17 cells, and Tregs with the protease inhibitor MG132 for 3 hours and analyzed RelB protein levels by immunoblotting. Interestingly, RelB levels were dramatically reduced in WT Th1 cells, Th17 cells, and Tregs (Figure 6E). In addition, the small amount of RelB present in these cells was already cleaved, and therefore primed for degradation. In contrast, RelB was highly expressed in Malt1–/– Th1 cells, Th17 cells, and Tregs and was not cleaved at all (Figure 6E), which indicates that RelB remains functional in these cells. Importantly, expression levels of the NF-κB subunits p65, p50, and cRel were not altered by Malt1 deficiency (Figure 6E).

Several studies have demonstrated that RelB inhibits the canonical NF-κB pathway by directly interfering with p65 (23, 42, 43). In cellular fractionation experiments, we found that p65 and RelB were reciprocally regulated in Malt1–/– Th17 cells (Figure 6F).
In the absence of MALT1, only a minor fraction of p65 translocated to the nucleus upon stimulation. In sharp contrast, RelB was constitutively detected in cytosolic and nuclear fractions of Malt1–/– Th17 cells, whereas in WT Th17 cells, RelB expression was far lower and exclusively found in the cytosol (Figure 6F), which indicates that MALT1 also regulates RelB translocation. Taken together, these results show that IKK-induced canonical NF-κB activation and the paracaspase activity of MALT1 are important for Th17 cell differentiation. We conclude that RelB is inactivated during Th cell differentiation in WT cells, but not in Malt1–/– cells, and serves as a transcriptional repressor of canonical p65, which is of particular importance for the generation of Th17 cells.

**Discussion**

How Th17 cells are generated in vivo is the subject of much ongoing debate, particularly as to the identity of the cytokines involved.
In vitro, IL-6, IL-21, and IL-23 — with or without TGF-β — have been reported to induce Th17 cell differentiation or stabilization (5, 11, 12, 44, 45). However, the roles of TCR stimulation and costimulatory signaling in the differentiation of specific Th subsets have yet to be clearly delineated (46–49). Engagement of both the TCR and CD28 results in activation of the I KK complex, which relieves NF-κB from its suppression by the I kB proteins. MALT1 is known to be an important modifier in the signaling pathway linking the TCR to NF-κB activation (22). In the present study, we revealed what we believe to be previously unknown functions for MALT1 and canonical NF-κB in the development of Th17 cells and their effector functions.

Our in vitro and in vivo studies showed that Malt1–/– mice exhibited normal differentiation of Th1 cells capable of normal IFN-γ production, but a profound reduction in the frequency of IL-17–producing Th cells. Malt1–/– mice were also completely resistant to the development of EAE, a disease linked to the effector functions of Th17 cells. However, previous research has established that IL-17 production alone is not sufficient for EAE induction (50). Several reports have shown that Th17 cell functionality is needed to recruit myeloid cells into the CNS (5, 10, 12, 30, 31) and that the GM-CSF produced by Th17 cells is the key driver of this recruitment and subsequent EAE onset (3, 4, 51). Indeed, in addition to their IL-17 deficit, we found that Malt1–/– Th17 cells produced much less GM-CSF than did WT Th17 cells, consistent with the substantially fewer inflammatory macrophages observed in histological sections of brains of Malt1–/– mice subjected to EAE induction. All these data point to a defective inflammatory Th17 cell response in the absence of MALT1, with the observed defects in IL-17 and GM-CSF production accounting for the EAE resistance of Malt1–/– mice.

The proliferation of peripheral T cells has been previously reported to depend on MALT1 (20, 21). In line with these studies, we found that Malt1–/– cells cultured under Th17-polarizing conditions proliferated only slightly less vigorously than did WT cells. In vivo, Malt1–/– Th cells infiltrated the CNS with delayed kinetics. At day 14 after EAE induction, T cell infiltration in Malt1–/– CNS was significantly reduced compared with WT animals, whereas at the disease peak (day 20), Malt1–/– CNS showed only a modest reduction in T cell infiltration. However, since these Malt1–/– mice never developed clinical signs of EAE, we can conclude that these Malt1–/– Th cells are no longer pathogenic, despite their near-normal expansion and CNS infiltration.

The differentiation of self-reactive T cells is a prerequisite for establishing autoimmunity. Our data suggest that the EAE resistance of Malt1–/– mice is mainly a Th cell–intrinsic effect. Transfer of transgenic WT 2D2 CD4+ T cells into Malt1–/– mice allowed for EAE induction in the formerly resistant animals. Histological analysis indicated that WT 2D2 Th cells were able to recruit inflammatory Malt1–/– macrophages to the CNS, resulting in severe neuroinflammation. This result suggests that Malt1–/– mice do not have other defects that prevent EAE development and that MALT1 regulates Th17 cell functionality in a cell-autonomous fashion. We confirmed this hypothesis by transferring Malt1–/– Th17-polarized cells into WT recipients: the mutant Th cells could not induce EAE in their WT hosts. Moreover, Malt1–/– mice showed a normal frequency of antigen-specific T cells, ruling out a deficit in this capacity as the cause of the observed EAE resistance. Thus, we conclude that MALT1 is a central regulator of Th17 cell differentiation and determines the pathogenic potential of these cells. However, EAE resistance was also observed in mice receiving MOG-specific Malt1–/– Th1 cells. Our analyses established that a substantial proportion of WT Th17 cells and a small fraction of WT Th1 cells acquired the capacity to produce both IFN-γ and IL-17. However, this plasticity was completely lost in the absence of MALT1. Th1/Th17 plasticity is reportedly important in EAE (34, 35), which might explain the inability of Malt1–/– Th1 cells to induce the disease. This theory is further supported by the work of several others showing that mouse strains with impairments of Th1 or Th17 cells are resistant to EAE independent of the functionality of the other Th subset (9, 13, 16, 52), indicative of a tight connection within the different Th subsets.

The differentiation of naïve Th cells into Th17 cells is governed by several lineage-specific transcription factors, including RORα, RORγt, and IRF4 (18). To our surprise, the expression of these factors was not influenced by Malt1 deficiency. Recent studies have suggested that c-Rel, which may act downstream of MALT1, controls Th17 cell differentiation by inducing RORγt and RORγt expression (53, 54). However, in our experimental system, MALT1 appeared to act independently of c-Rel, as we did not detect any changes in c-Rel protein levels or in RORγt expression in Malt1–/– Th cells. Importantly, we demonstrated that MALT1-driven NF-κB activity was essential for Th17 cell effector function. Although it is theoretically possible that NF-κB is a direct transcriptional activator of the Il17a gene, a previous computer-based analysis to detect putative NF-κB elements in the mouse Il17a locus identified only 1 nonfunctional sequence (53), making it unlikely that NF-κB is a direct activator of this gene.

Although IKK activity is crucial for IκB degradation, the para-caspase activity of MALT1 is important for NF-κB activation, but not for IκB degradation (22, 41). However, in our study, application of either an IKK inhibitor or a specific caspase inhibitor led to the same result: a block in Th17 cell differentiation. We propose that MALT1 independently regulates 2 pathways that influence NF-κB activation and are of equal importance for Th17 cell differentiation. Previous work in T cells has shown that the scaffolding function of MALT1 is important for IKK activation and IκB degradation (21, 37). In contrast, in a tumor cell line–based model, the catalytic activity of MALT1 targeted the noncanonical NF-κB subunit RelB (23), which was identified as a transcriptional repressor of the canonical NF-κB proteins p65 and c-Rel (42, 43). We showed under physiological conditions that MALT1 negatively regulated RelB protein stability during Th cell differentiation. Malt1–/– Th cells showed dramatically higher RelB levels than did WT Th cells. More importantly, RelB was constitutively localized in the nucleus of Malt1–/– Th17 cells, where it could potentially block p65 or c-Rel. Thus, we propose that MALT1 regulates RelB’s localization in addition to its protein stability. It should be noted that both inhibitory and stimulatory functions have been described for RelB, depending on the cell type and context (55). In that respect, it is interesting that RelB is essential for IL-17 production by γδ T cells (56).

In conclusion, our present findings demonstrated an essential role for MALT1 in the differentiation of functional Th17 cells and the development of EAE. Importantly, we showed that MALT1 determines the encephalitogenicity of the Th17 cells driving EAE, which may position MALT1 as an attractive drug target for the treatment of human MS.

Methods

Mice. Malt1–/– mice were originally generated in our laboratory (20, 37) and backcrossed for more than 10 generations into the C57BL/6 back-
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Malt1–/– mutations of Th17 cells. EAE was initiated in Malt1–/– and WT mice by MOG injection as described above. At 10 days after injection, mice were sacrificed, and Malt1–/– and WT CD4+ T cells from spleens and LNS were cultured for 5 days in the presence of 25 μg/ml MOG35–55 peptide (Washington Biotech) emulsified in CFA (Difco) supplemented with 400 μg/ml Mycobacterium tuberculosis (Difco). On days 0 and 2 after immunization, mice were i.p. injected with 300 ng PT (List Biological). Clinical signs of EAE were monitored daily, according to the following criteria: 0, no disease; 1, decreased tail tone; 2, hind limb weakness or partial paralysis; 3, complete hind limb paralysis; 4, front and hind limb paralysis; 5, moribund state. Mice were sacrificed when they reached an EAE disease score of 4; these animals were assigned a score of 5 for the rest of the observation period for the purpose of calculating mean EAE disease score. At the end of the 30-day observation period, any surviving mice were sacrificed, and their brains and spinal cords were subjected to histological analyses.

Histology and immunohistochemistry. Brains and spinal cords were obtained from mice at 30 days after MOG injection and fixed in 10% buffered formalin for several days. Fixed tissues were cut into approximately 1.5-mm-thick coronal sections and paraffin embedded according to standard laboratory procedures. For histological and immunohistochemical assessments, 5-μm-thick paraffin sections were cut and stained with H&E or analyzed by immunohistochemistry according to standard laboratory procedures. For immunohistochemical staining, the following Abs and dilutions were used: anti-CD3 (1:200; Dako), anti-B220 (1:1,000; BD Biosciences—Pharmingen), anti-Mac3 (1:100; BD Biosciences—Pharmingen), and anti-Gr-1 (1:2,000; Dako).

Transfer of transgenic or Th1/Th17-polarized T cells. EAE was initiated in Malt1–/– and WT mice by MOG injection as described above. At 10 days after injection, mice were sacrificed, and Malt1–/– and WT CD4+ T cells from spleens and LNS were cultured for 5 days in the presence of 25 μg/ml MOG peptide and 10 ng/ml IL-23 plus 5 μg/ml anti–IFN-γ to generate Th17 cells, or with MOG peptide and 10 ng/ml IL-12 plus 5 μg/ml anti–IL-23 to generate Th1 cells. WT CD4+ 2D2 T cells were isolated from spleens and LNS and sorted using a magnetic bead cell purification kit.
according to the manufacturer’s instructions (Milenyi). WT CD4+ 2D2 T cells (1 × 10^6 cells/mouse) or polarized Th cells (2 × 10^6 cells/mouse) were transferred into recipient mice that had been sublethally irradiated 1 day before transfer. Transplanted cells were injected with PT on the day of T cell transfer and again 2 days later. EAE progression was monitored as described above.

**DC differentiation and antigen presentation assay.** DCs were induced to differentiate from bone marrow stem cells by culture for 10 days in RPMI containing 40 ng/ml GM-CSF (Peprotech), as previously described (59). On day 10, cells were harvested, washed twice in PBS, and stimulated with 10 ng/ml LPS (Sigma-Aldrich) for 16 hours. DCs were then pulsed for 3 hours with 1 μM MOG peptide, washed twice with PBS, and cocultured with WT CD4+ 2D2 T cells labeled with 2.5 μM CellTrace Violet (Invitrogen) according to the manufacturer’s instructions. Proliferation was measured 72 hours later using a Canto II plate-reader (BD Biosciences) and analyzed using Flowjo 7.5 software.

**Statistics.** Where appropriate, all differences were evaluated using 2-tailed Student’s t test, as calculated using Prism 5.0 software (GraphPad). Data are presented as mean ± SEM. A P value of 0.05 or less was considered significant.

**Study approval.** All mouse procedures were approved by the University Health Network Institutional Animal Care and Use Committee.

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