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MALT1 is an intrinsic regulator of regulatory T cells

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Regulatory T cells (Tregs) are crucial for the maintenance of immunological self-tolerance and their absence or dysfunction can lead to autoimmunity. However, the molecular pathways that govern Treg biology remain obscure. In this study, we show that the nuclear factor-κB signalling mediator mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) is an important novel regulator of both Tregs originating in the thymus ('natural' or nTregs) and Tregs induced to differentiate from naive thymocyte helper (Th) cells in the periphery ('induced' or iTregs). Our examination of mice deficient for MALT1 revealed that these mutants have a reduced number of total Tregs. In young *Malt1$^{-/-}$* mice, nTregs are totally absent and iTreg are diminished in the periphery. Interestingly, total Treg numbers increase in older *Malt1$^{-/-}$* mice as well as in *Malt1$^{-/-}$* mice subjected to experimentally induced inflammation. iTregs isolated from *WT* and *Malt1$^{-/-}$* mice were indistinguishable with respect to their ability to suppress the activities of effector T cells, but *Malt1$^{-/-}$* iTregs expressed higher levels of Toll-like receptor (TLR) 2. Treatment of *WT* and *Malt1$^{-/-}$* Th cells *in vitro* with the TLR2 ligand Pam3Cys strongly enhanced the induction and proliferation of *Malt1$^{-/-}$* iTregs. Our data suggest that MALT1 supports nTreg development in the thymus but suppresses iTreg induction in the periphery during inflammation. Our data position MALT1 as a key molecule that contributes to immune tolerance at steady-state while facilitating immune reactivity under stress conditions.

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Regulatory T cells (Tregs) are a rare T-cell population that helps to maintain immunological self-tolerance throughout life.[1] A key function of these cells is to suppress the proliferation and activities of effector T cells (Teffs) such as thymocyte helper 1 (Th1) and Th17 cells during the later stages of inflammation.[2,3] Tregs differ from other T-cell subpopulations in their transcriptional, functional and phenotypical features. For example, forkhead box P3 (FoxP3) is not expressed by most T cells but is the major transcription factor governing Treg development and function.[4] The absence or mutation of FoxP3, such as occurs in FoxP3-mutated *Scurfy* mice or humans with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome,[5,6] leads to a deficit in Tregs and severe autoimmunity. In addition to FoxP3, Tregs are distinguished from most other T cells by their constitutive surface expression of CTLA-4, GITR, cluster of differentiation (CD)-4 and CD25.[7–9]

The total pool of Treg cells can be divided into two major subpopulations. The first group arises directly in the thymus and consists of $CD4^+CD25^+FoxP3^+$ natural Treg (nTreg) cells. The second group is derived from cells that leave the thymus as $CD4^+CD25^-$ naive T cells but then acquire their suppressive capacity and FoxP3 expression in the periphery. The generation of these so-called inducible Treg (iTreg) cells is strongly dependent on the microenvironment.[9,10] This microenvironment can be mimicked *in vitro* by treating naive peripheral Th cells with anti-CD3 and anti-CD28 antibodies (Abs) plus cytokines to generate iTregs.[9,11] For example, naive Th cells exposed to tumor growth factor-β (TGFβ) start to express FoxP3, which in turn triggers the iTreg differentiation programme. This effect is CTLA4 dependent.[12] Similarly, interleukin-2 (IL-2) treatment upregulates the expression of the IL-2 receptor CD25 on cells *in vitro* and promotes the development of suppressive capacity in differentiating iTregs. The high levels of CD25 present on iTregs work as a 'cytokine sink', binding IL-2 in the immediate microenvironment such that nearby Teff become deprived of IL-2 and subsequently undergo apoptosis.[13] In addition to cytokines, the innate

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Abbreviations: Ab, antibody; APC, antigen-presenting cell; BCL10, B-cell lymphoma/leukemia 10; Carma1, CARD-containing MAGUK protein 1; CD, cluster of differentiation; CFA, complete Freund's adjuvant; CNS, central nervous system; EAE, experimental autoimmune encephalitis; FoxP3, forkhead box P3; IL-2, interleukin-2; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MCMV, mouse cytomegalovirus; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NF-κB, nuclear factor-κB; nTreg, natural Treg; Pam, *Pam3Cys*-Ser-(Lys)4; Teff, effector T cell; Th, thymocyte helper; TGFβ, tumor growth factor-β; TLR, Toll-like receptor; Treg, regulatory T cells; VCT, violet cell tracer

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pattern recognition receptor family of Toll-like receptors (TLRs) has been implicated in iTreg induction[14,15] but the effects of TLR stimulation are multifaceted and not yet completely defined. Although TLR-8 stimulation suppresses Treg function, the engagement of other TLRs such as TLR4, 5 and 2 has been reported to increase Treg function, survival and/or proliferation.[16–19] As an example TLR2 deficiency leads to decreased Treg function and as a consequence to a better clearance of *Candida albicans* infection.[20] The generation of iTregs is thus the outcome of a myriad of environmental influences.

In response to TCR engagement, the nuclear factor-κB (NF-κB) signalling mediator mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) forms a complex with CARD-containing MAGUK protein 1 (CARMA1; CARD11) and B-cell lymphoma-10 (BCL10) called the CARMA1-BCL10-MALT1 (CBM) complex.[21] Formation of the CBM complex leads to activation of downstream canonical NF-κB signalling. In addition to its scaffolding function within the CBM complex, MALT1 possesses paracaspase activity that allows it to cleave targets such as A20, BCL10, Regnase-1 and Roquin-1.[21–24] Although MALT1 has long been implicated in naive Th cell activation and proliferation, and in the differentiation of Th17 effectors,[24–27] the role of MALT1 in Treg development and function has yet to be investigated. Several other molecules in the canonical NF-κB pathway, including PKCθ, BCL10 and CARMA1, are known to be crucial for Treg generation,[28–32] suggesting that MALT1 might also be required. Here we present evidence that MALT1 has a dual role in Treg biology by promoting nTreg development but downmodulating iTreg induction. MALT1 therefore appears to define a threshold for Treg induction and acts as a molecular switch helping to ensure both immune tolerance and immune reactivity.

Results

***Malt1* is crucial for nTreg development.** To determine whether, similar to other NF-κB signalling molecules, MALT1 is required for nTreg development, we prepared single-cell suspensions of thymic tissues of 4- to 5-week-old *Malt1*$^{-/-}$ and *WT* mice and immunostained these cells to detect CD4, CD8, CD25 and FoxP3. Confirming a previous study,[25] we found no significant differences in the expression of these molecules by CD4 or CD8 single-positive thymocytes, or by double-negative or double-positive thymocytes (Figure 1a, left). However, the CD4$^+$CD25$^+$FoxP3$^+$ nTreg population was totally absent from *Malt1*$^{-/-}$ thymus (Figure 1a right and Figure 1b). These data indicate that MALT1 is indeed indispensable for nTreg development in the thymus.

iTregs can be generated in the absence of *Malt1*. To assess whether *Malt1* deficiency also affected Treg numbers in the periphery, we analysed the CD4$^+$CD25$^+$FoxP3$^+$ population in blood samples taken from 6-week-old *WT* and *Malt1*$^{-/-}$ mice. Consistent with our findings in the thymus, the blood of *Malt1*$^{-/-}$ mice showed a dramatic reduction in total Treg levels (Figure 2a). Intriguingly, the peripheral blood of aged *Malt1*$^{-/-}$ mice (1 year old) contained a CD4$^+$CD25$^+$ FoxP3$^+$ cell population that was almost as large as that in aged *WT* controls (Figure 2b), suggesting that these animals could accumulate iTregs over time. To characterize this iTreg cell population in aged mice, we measured cell surface expression of CD62L. CD62L is downregulated during Teff

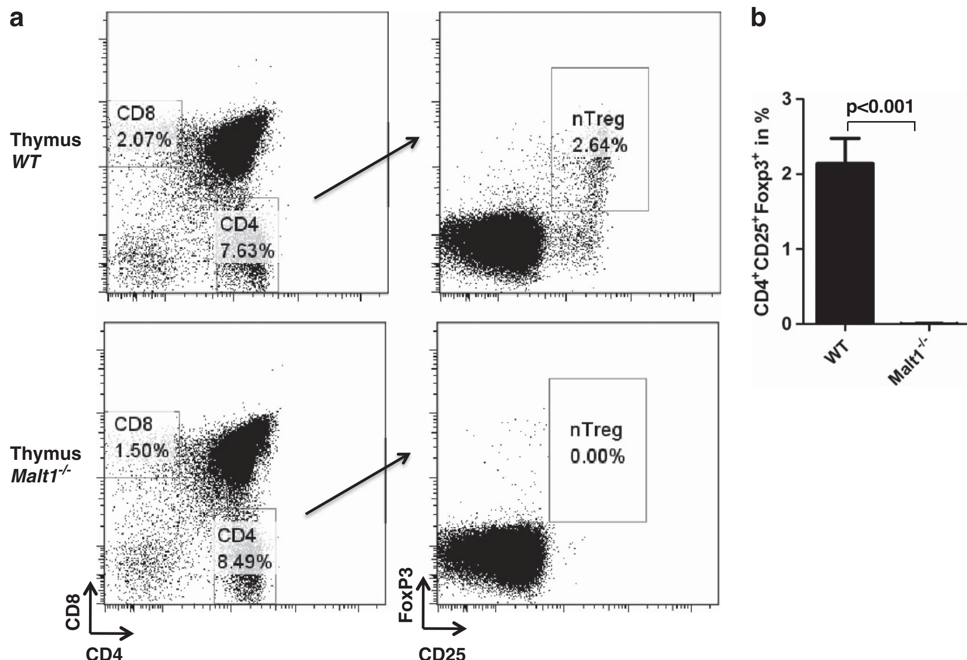

Figure 1 Natural Treg cell development is MALT1 dependent. (**a**) Left panel: flow cytometric analysis of thymocytes that were isolated from 4-week-old *Malt1*$^{-/-}$ and *WT* mice and immunostained to detect CD4 and CD8. Right panel: the cells in the left panel were gated on CD4$^+$ and immunostained to detect CD25 and FoxP3. (**b**) Quantitation of the CD4$^+$CD25$^+$FoxP3$^+$ thymocytes in the right panel of **a**. Data are the mean ± S.E.M. ($n = 4$)

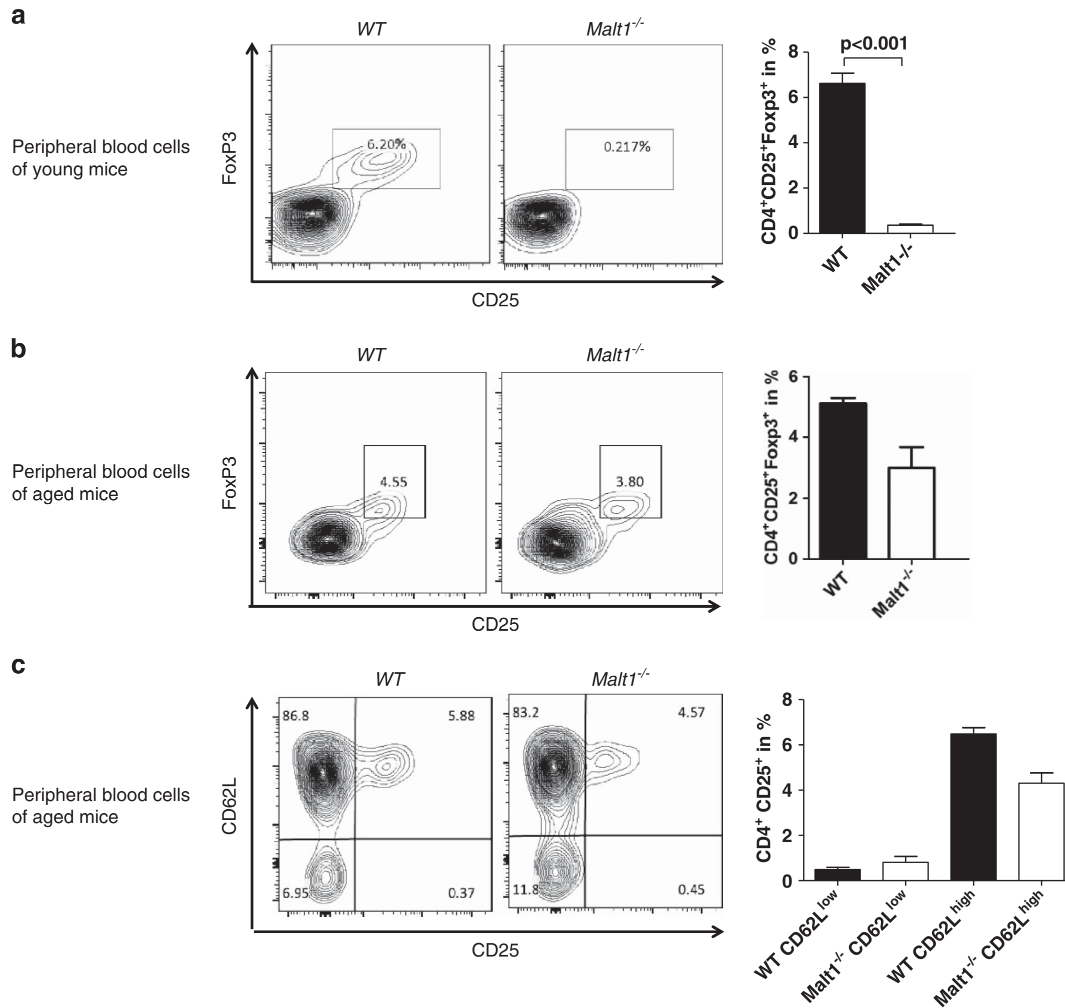

Figure 2 iTregs are present in the periphery of *Malt1*$^{-/-}$ mice. Left panels: flow cytometric analyses of total peripheral blood cells that were isolated from (**a**) 6–8-week-old or (**b** and **c**) 1-year-old *Malt1*$^{-/-}$ and *WT* mice, gated on viable CD4$^+$ cells and immunostained to detect (**a** and **b**) CD25 and FoxP3 or (**c**) CD25 and CD62L. Right panels: quantitation of the CD4$^+$CD25$^+$FoxP3$^+$ or CD4$^+$CD25$^+$ cells in the left panels. Data are gated on live CD4$^+$ cells and are the mean ± S.E.M. ($n = 5$)

activation but is upregulated on iTregs with high suppressive capacity.[33] We found that most of the CD4$^+$CD25$^+$ cells in steady-state *WT* and *Malt1*$^{-/-}$ mice expressed equivalent high levels of CD62L (Figure 2c). In addition Helios, a marker for nTregs,[34] was strongly reduced in CD4$^+$CD25$^+$Foxp3$^+$ cells (Supplementary Figure 1) Thus, as age of the mice progresses, *Malt1*$^{-/-}$ iTregs are generated successfully *in vivo* in the absence of *Malt1* and have a surface phenotype that indicates normal functionality.

MALT1 sets the threshold of iTreg differentiation but is dispensable for iTreg functionality. We have previously shown that MALT1 is indispensable for the differentiation of Th17 cells but not for that of other Teff subpopulations.[26] To determine MALT1's role in iTreg differentiation, we isolated naive peripheral CD4$^+$CD62L$^+$ T cells from *Malt1*$^{-/-}$ and *WT* mice and treated them *in vitro* with various concentrations of anti-CD3 Ab in the presence of constant concentrations of anti-CD28 Ab, IL-2 and TGFβ. Cells stimulated under the same conditions without TGFβ were used as a 'Th0' control. Strong TCR stimulation with high concentration of anti-CD3 Ab led to equal levels of FoxP3 expression by *WT* and *Malt1*$^{-/-}$ iTregs (Figure 3a, left). At lower TCR-stimulation conditions, this induction of FoxP3 was diminished by MALT1 deficiency but still clearly superior to that in non-TGFβ-treated Th0 controls (Figure 3a).

To determine whether MALT1 influences the suppressive capacity of *in vitro*-induced iTregs, we co-cultured equal numbers of *in vitro*-induced, violet cell tracer (VCT)-labelled *WT* and *Malt1*$^{-/-}$ iTregs with *WT* naive peripheral T cells. Irradiated antigen-presenting cells (APCs) and soluble anti-CD3 Ab and IL-2 were added to cultures to engage the TCR, deliver the required costimulatory signal and generate Teff. As expected, the Teff generated by these activated naive T cells proliferated vigorously in the absence of Tregs (Figure 3b, grey line) and this proliferation was suppressed in the presence of *WT* iTregs (Figure 3b, blue line). Significantly, *Malt1*$^{-/-}$ iTregs were just as proficient at inhibiting the proliferation of Teff as *WT* iTregs (Figure 3b, red line). This result indicates that once naive *Malt1*-deficient T cells differentiate into iTregs, their

deficit in MALT1 has no detrimental effect on their suppressive capacity.

To determine whether this conclusion was valid *in vivo*, we isolated iTregs from the peripheral blood of aged *WT* and *Malt1*$^{-/-}$ mice and compared the suppressive capacities of these cells using the same *in vitro* suppression assay. Again, an absence of *Malt1* did not reduce the ability of iTregs to inhibit *WT* Teff proliferation (Figure 3c). Taken together, these data indicate that MALT1 lowers the threshold for iTreg induction but has no influence on the actual function of these iTregs.

The absence of *Malt1* enhances TLR2-induced iTreg induction and proliferation. The above data suggested

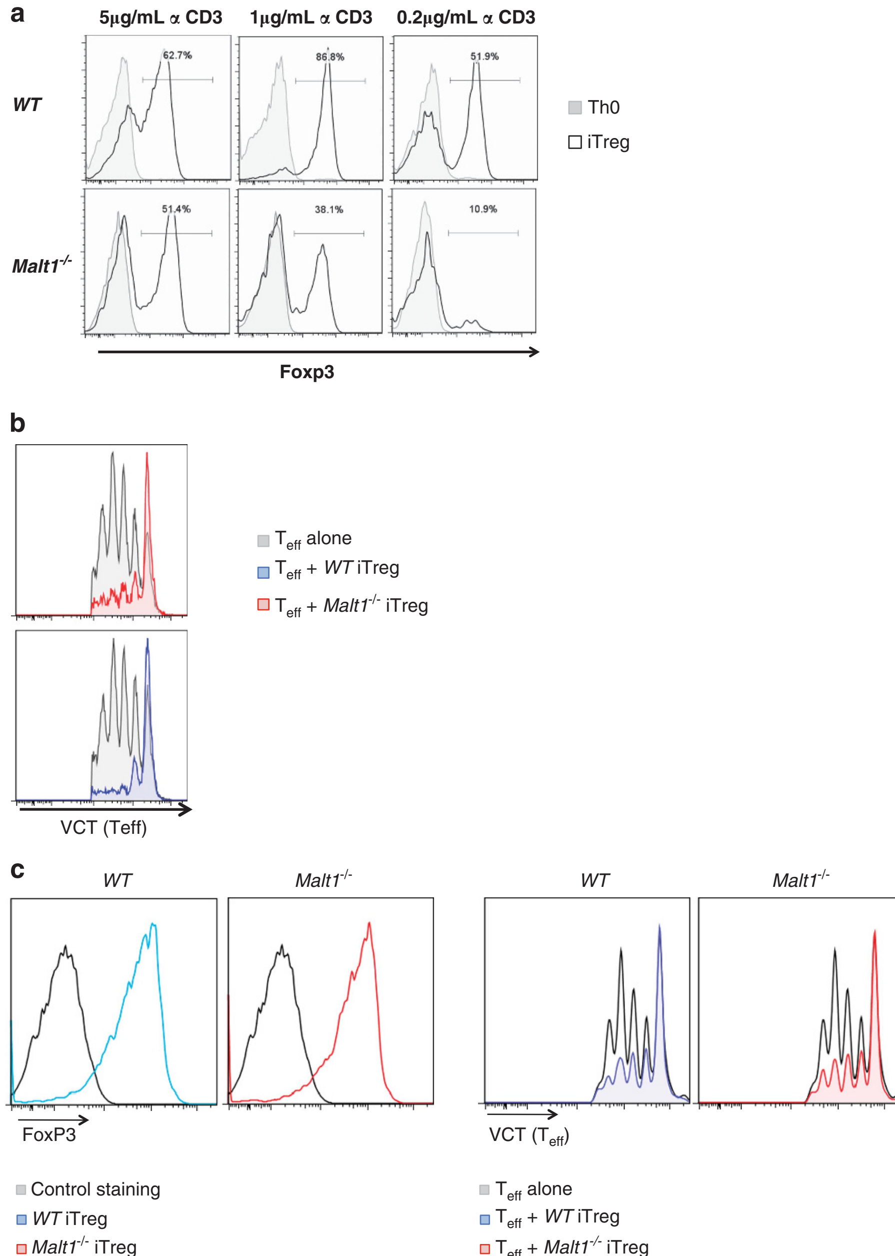

Figure 3 MALT1 is not required for iTreg function. (**a**) Flow cytometric analysis of induction of iTregs from naive *Malt1*$^{-/-}$ and *WT* T cells that were differentiated for 72 h with the indicated doses of anti-CD3 Ab plus 1 μg/ml anti-CD28 Ab, 3 ng/ml TGF-β and 50 U IL-2. FoxP3 was detected by intracellular staining. Data are gated on live CD4^{+} cells and are representative of three independent experiments. (**b**) Flow cytometric analysis of iTreg function. VCT-labelled naive *WT* T cells were co-cultured with *Malt1*$^{-/-}$ or *WT* iTreg cells generated as in **a**, in the presence of irradiated APCs, and 3 μg/ml anti-CD3 Ab and 50 U/ml IL-2. Proliferation of *WT* Teff was measured by flow cytometry. Grey curve, *WT* Teff proliferation without iTregs; blue, *WT* Teff co-cultured with *WT* iTregs; red, *WT* Teff co-cultured with *Malt1*$^{-/-}$ iTregs. Data are gated on live VCT^{+} *WT* Teff and are representative of three independent experiments. (**c**) Left panel: CD4^{+}CD25^{+} cells were isolated from 1-year-old *Malt1*$^{-/-}$ and *WT* mice by FACS and immunostained to detect FoxP3^{+} Tregs. Right panel: these *in vivo*-generated iTregs were co-cultured *in vitro* with VCT-labelled live *WT* naive CD4^{+} T cells and APCs as described in **b**. Proliferation of *WT* Teff was measured by flow cytometry. Grey curve, *WT* Teff proliferation without Tregs; blue, co-cultured with *WT* iTregs; red, co-cultured with *Malt1*$^{-/-}$ iTregs. Data are gated on live VCT^{+} Teff and are representative of three independent experiments

that the threshold of TCR-dependent iTreg induction is regulated by MALT1. We therefore expected that the induction of *Malt1*$^{-/-}$ iTregs would require a strong TCR stimulus. However, we had also observed an age-dependent increase in iTreg numbers in the blood of *Malt1*$^{-/-}$ mice that were at steady-state, suggesting that this increase was unlikely to be due to a vigorous TCR signal. During age progression, mice are exposed to many potential immune-relevant stimuli, even under SPF conditions. Besides TCR engagement, various other stimuli have been reported to induce iTreg proliferation and survival, in particular TLR activation.[35] We therefore analysed the expression of various TLRs on *Malt1*$^{-/-}$ and *WT* total Tregs. To this end, we immunostained single-cell suspensions of splenocytes of 3- to 4-month-old *Malt1*$^{-/-}$ and *WT* mice, to detect the CD4^{+} CD25^{high} total Treg cell population. We then used flow cytometry to determine the surface levels of TLR2 and TLR4 proteins on these cells. Interestingly, although TLR4 was not expressed by CD4^{+}CD25^{high} total Tregs, TLR2 was upregulated on *Malt1*$^{-/-}$ (but not on *WT*) total Tregs (Figure 4a). These data pointed towards a potential role for TLR2 in controlling iTreg differentiation and/or function.

To investigate the role of TLR2 in iTregs *in vitro*, we cultured *WT* and *Malt1*$^{-/-}$ naive peripheral T cells in the presence of IL-2, TGFβ and increasing doses of Pam3Cys -Ser-(Lys)4, a well-described TRL2 ligand of bacterial origin,[36] or LPS, a ligand for TLR4. We stimluated these cells with a low dose of anti-CD3 Ab plus anti-CD28 Ab, to promote suboptimal FoxP3 induction. In accordance with our hypothesis, TLR2 ligation by Pam3Cys, but not TLR4 ligation by LPS, markedly increased the differentiation of *Malt1*-deficient iTregs (Figure 4b). Pam3Cys treatment also increased iTreg numbers in *WT* cultures but the effect was much less dramatic. Thus, MALT1 regulates TLR2 signalling that can support iTreg generation.

We speculated that the heightened induction of *Malt1*$^{-/-}$ iTregs promoted by Pam3Cys might be explained either by increased proliferation of the few already existing iTregs or by the *de novo* induction of additional iTregs. To distinguish between these possibilities, we stained naive peripheral *Malt1*$^{-/-}$ T cells with VCT and monitored their proliferation after culture with IL-2 plus TGFβ, with or without the addition of Pam3Cys. Flow cytometric analysis revealed that FoxP3 was increased in both proliferating (VCT-low) cells as well as in non-proliferating (VCT-high) cells (Figure 4c). Pam3Cys therefore induced FoxP3 expression in both resting and dividing *Malt1*$^{-/-}$ T cells. This result argues for a cooperative effect that includes both *de novo* iTreg generation and the expansion of existing iTregs.

To analyse the effect of Pam3Cys specifically on the proliferation of iTregs, we treated VCT-labelled *Malt1*$^{-/-}$ and *WT* naive T cells with either IL-2 alone, to generate Th0 cells (controls), or with IL-2 plus TGFβ, to initiate iTreg differentiation. We then added Pam3Cys (or vehicle) to these cultures and determined iTreg proliferation by VCT dilution and flow cytometry. We normalized the result for each Pam3Cys-stimulated culture to its counterpart lacking exogenous TLR2 stimulation and calculated a proliferation index to specifically reflect Pam3Cys-driven expansion (see Materials and Methods). Although Th0 cells of both genotypes showed little proliferation in response to Pam3Cys, *Malt1*$^{-/-}$ iTregs expanded massively in the presence of this TLR2 ligand (Figure 4d). Significantly, the proliferation of *WT* iTregs was much less markedly stimulated by TLR2 ligation. Taken together, these data suggest that an absence of *Malt1* increases TLR2 levels on iTregs, setting the stage for TLR2-dependent iTreg cell expansion if a TLR2 ligand appears in the immediate microenvironment. We speculate that a MALT1-driven NF-κB signalling cascade may normally limit TLR2 expression by iTregs and thus their expansion at sites of inflammation, which frequently exhibit elevated levels of TLR2 ligands due to pathogen invasion. This modulation of iTreg activity would ensure the full functioning of Teff and so may contribute to a strong immune response against a pathogen.

Enhanced iTreg induction during experimentally induced inflammation in *Malt1*$^{-/-}$ mice. As noted above, Tregs are crucial for the maintenance of immunological self-tolerance *in vivo*. In particular, deregulated Treg function has been described in patients with relapsing-remitting multiple sclerosis (MS).[37] On the other hand, aggressive infections have been shown to induce iTreg responses,[38] presumably to introduce an element of control over responding Teff. We speculated that MALT1 might modulate iTreg responses/numbers during an immune response by downregulating TLR2 expression. To test this hypothesis *in vivo*, we treated *WT* and *Malt1*$^{-/-}$ mice with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant (CFA) to induce experimental autoimmune encephalitis (EAE), a mouse model of MS.[26,39] The immune stimulatory function of CFA depends largely on the strong stimulation of TLRs. We then investigated iTreg induction during disease progression. We have previously shown that *Malt1*-deficient mice are resistant to EAE induction despite exhibiting Teff infiltration into the brain.[26] We therefore analysed the CD4^{+} T cells infiltrating the central nervous system (CNS) of our *WT* and *Malt1*$^{-/-}$ mice subjected to EAE induction ('EAE mice') for FoxP3 expression and determined Treg proportions. Interestingly, and in line with our *in vitro* data, *Malt1*$^{-/-}$ EAE mice displayed an increased percentage of CD4^{+}CD25^{+}FoxP3^{+} cells in the CNS, even higher than that induced in *WT* EAE controls (Figure 5a). Relative iTreg levels were also elevated in the spleens of *Malt1*$^{-/-}$ EAE mice (Figure 5b). Histological and immunostaining analyses to detect FoxP3^{+} T cells in brain sections of *Malt1*$^{-/-}$ and *WT* EAE mice confirmed these findings (Figure 5c). These data suggest that MALT1 normally represses iTreg expansion *in vivo*. Moreover, although total Tregs are present at only very low numbers in both *Malt1*$^{-/-}$ and *WT* animals at steady state, they increase proportionally more in *Malt1*$^{-/-}$ EAE mice during inflammation than they do in *WT* EAE mice. These *in vivo* findings bolster our *in vitro* findings and indicate that MALT1 has an indispensable role in balancing central and peripheral tolerance *versus* immune reactivity. Our results also suggest that MALT1 has a double-edged function in Treg biology, being a promoter of nTreg development but a suppressor of iTreg induction.

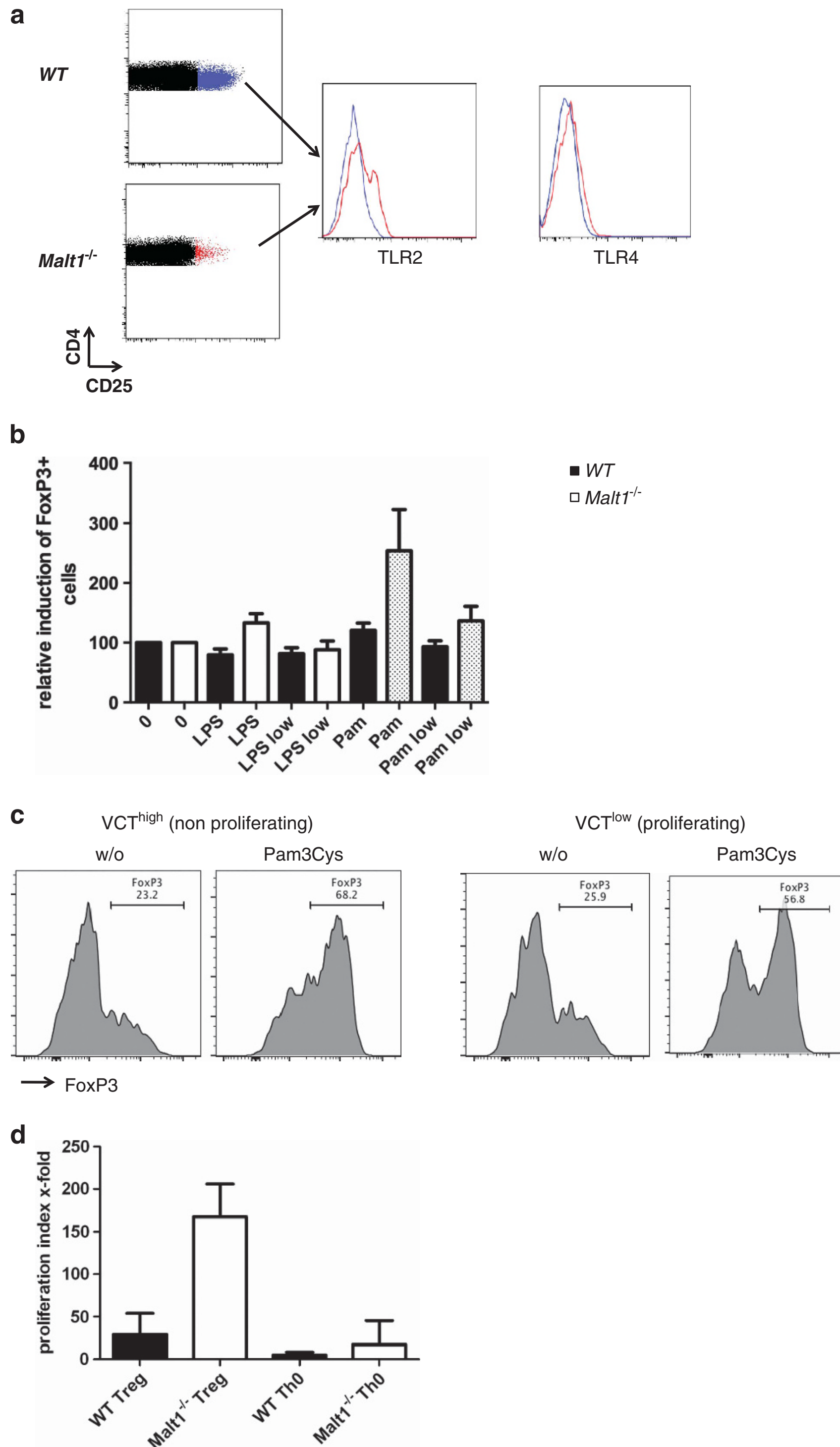

Figure 4 TLR2 stimulation promotes *Malt1*$^{-/-}$ iTreg proliferation. (**a**) Flow cytometric analysis of splenocytes that were isolated from 3- to 4-month-old *Malt1*$^{-/-}$ and *WT* mice and immunostained to detect CD4 and CD25. These CD4^{+}CD25^{+} cells were then immunostained to detect TLR2 and TLR4 expression. Data are representative of three independent experiments. (**b**) Quantitation of relative number of CD4^{+}CD25^{+}FoxP3^{+} iTregs among *Malt1*$^{-/-}$ and *WT* total splenocytes that received no exogenous TLR ligand (0) or were treated with 10 μg/ml or 10 ng/ml (low) of LPS or Pam3Cys (Pam). Data are the mean of fold induction of FoxP3^{+} $\pm$ S.E.M. ($n=4$) normalized to values for cultures without exogenous TLR stimulation. (**c**) Flow cytometric analysis of FoxP3 expression by *Malt1*$^{-/-}$ iTreg cells stained with VCT and left untreated (w/o) or stimulated with 10 μg/ml Pam3Cys. Data are gated on live non-proliferated VCT^{high} cells (left) and proliferated VCT^{low} cells (right), and are representative of three independent experiments. (**d**) Proliferation index (see Materials and Methods) of Pam3Cys-treated *WT* and *Malt1*$^{-/-}$ Th0 and Treg cells normalized to the corresponding cell populations without TLR stimulation. Data are the mean fold increase in proliferation $\pm$ S.E.M. ($n=3$)

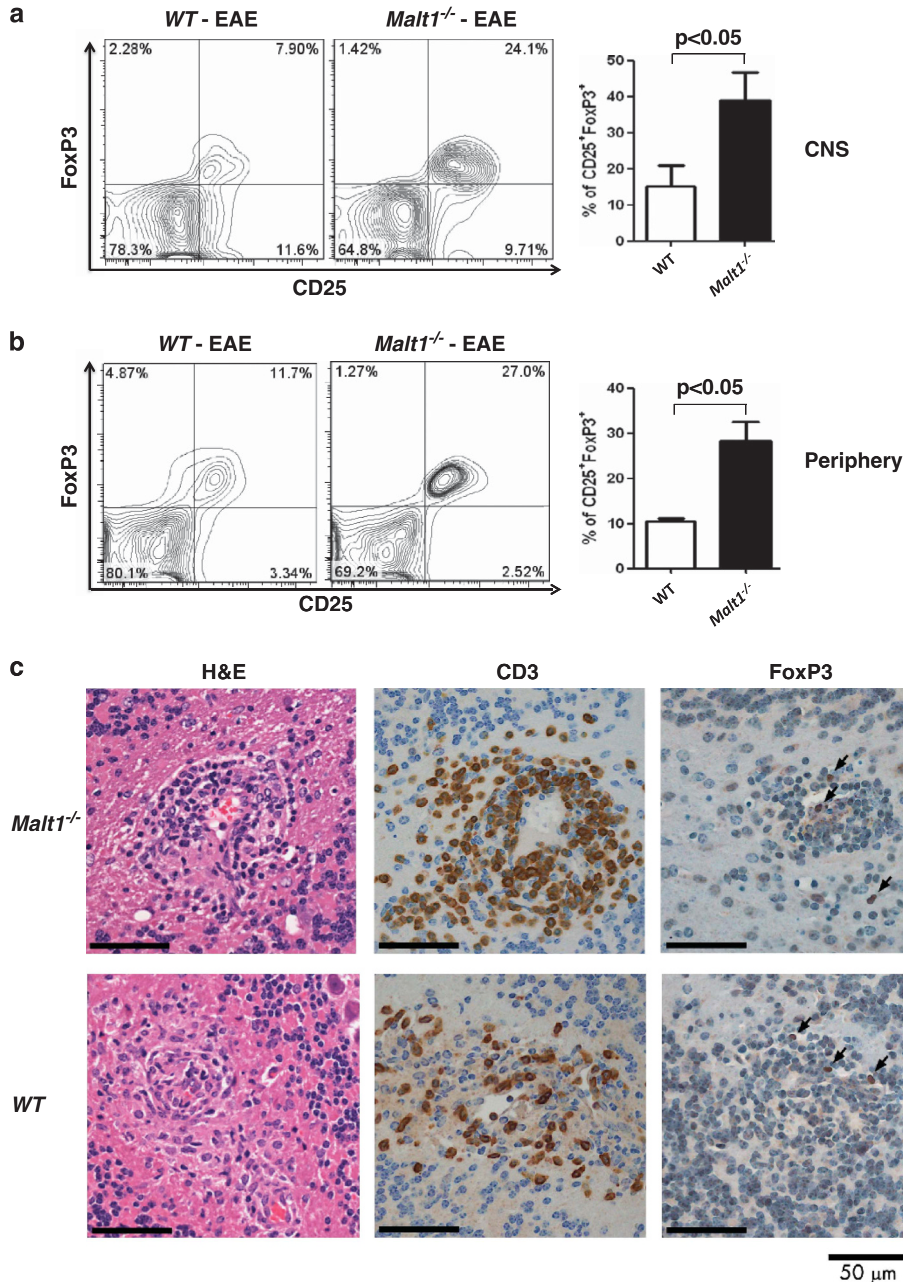

Figure 5 Inflammation associated with EAE triggers iTreg induction. Intracellular staining and flow cytometric analysis of (**a**) CNS-infiltrating CD4$^+$ T cells (isolated by gradient centrifugation) and (**b**) CD4$^+$ splenocytes, which were isolated from *Malt1*$^{-/-}$ and *WT* mice on day 14 post-EAE induction. Left panels: expression levels of CD25 and FoxP3. Data are gated on live CD4$^+$ T cells. Right panel: quantitation of percentages of the CNS-infiltrating and splenic CD4$^+$ T cells expressing CD25 and FoxP3 in the left panels. Data are the mean ± S.E.M. ($n=4$ per group). (**c**) Representative histopathological analyses of cross-sections of brains from *Malt1*$^{-/-}$ and *WT* mice at 30 days post-MOG injection. Brain sections were stained with haematoxylin and eosin (left panel), or immunostained to detect CD3$^+$ T cells (middle panels) or FoxP3$^+$ Treg cells (right panels). Scale bars, 50 μm. Black arrows indicate FoxP3-positive cells. Data are from one mouse per group ($n=5$) and are representative of two independent experiments

Discussion

Our study has revealed an important role for MALT1 as an intrinsic modulator of Treg cell responses. *Malt1*$^{-/-}$ mice are deficient in nTreg development in the thymus, indicating a crucial contribution of MALT1 to the establishment of central tolerance. However, Tregs were present in the peripheral blood of *Malt1*$^{-/-}$ mice at a steady state, albeit at significantly lower numbers than in their *WT* counterparts. Comparison of these *Malt1*$^{-/-}$ and *WT* peripheral Tregs revealed that they expressed the same FoxP3 levels per cell. In addition, CD62L, an extracellular marker correlated with high suppressive capacity of Treg,[33] was expressed equally on *WT* and *Malt1*$^{-/-}$ total Tregs. These findings support our hypothesis that the Tregs in the periphery of *Malt1*-deficient mice are iTregs induced only after naive T cells leave the thymus.

In the course of the last year, multiple independent groups published data on knock-in mutations that express a catalytic inactive form of MALT1.[40–42] All these groups describe spontaneous general autoimmunity and observe reduced Treg numbers in the periphery and in the thymus. These data indicate that the defect in Treg development is partially dependent on MALT1's paracaspase activity. Interestingly, the defective Treg development is more pronounced in *Malt1*$^{-/-}$ mice indicating that also MALT1's structural components contribute.

In addition, CARMA1 is crucial for the development of nTregs and *Carma1*-deficient mice show a defect at an early checkpoint in thymic nTreg development.[31,32] However, these studies did not provide any insights into Tregs that are induced in the periphery. Our data confirm that elements of the CBM complex are critical not only for nTreg development but also for iTreg induction both *in vitro* and *in vivo*. Furthermore, our previous work demonstrated that MALT1 regulates the strength of TCR signalling[25] and our current study shows that MALT1 defines a clear threshold for iTreg induction *in vitro*. Thus, MALT1 can be considered a gatekeeper for iTreg induction. However, once a MALT1-deficient cell has passed through this gate, its loss of MALT1 is irrelevant, because MALT1 does not affect the suppressive capacity of iTregs. This stricture applies to iTregs generated *in vitro* as well as to iTregs sorted directly from the spleen and lymph nodes of *Malt1*$^{-/-}$ mice.

Another interesting finding of our study is the striking increase in peripheral iTreg numbers that occurred in aged *Malt1*$^{-/-}$ mice. This result is in line with a study of elderly humans ($>$70 years old), each of whom displayed increased Treg numbers in peripheral blood even though the thymus showed age-related total dysfunction.[43]

Interestingly, the recent studies describing the physiological impact of MALT1's catalytic activity do not describe Treg induction after challenging these mice with inflammatory stimuli or in aged mice, which of course might be a limitation of the overall phenotype and the reduced life span of these animals.[40–42] However, in closely related *Carma1*$^{-/-}$ mice it was shown that a viral infection was followed by an expansion of Tregs in the periphery,[32] which supports our hypothesis that MALT1 (and possibly the CBM complex) suppresses iTreg induction on a non-sterile inflammation.

In our study, the relative increase in iTreg numbers in *Malt1*$^{-/-}$ mice was higher than in *WT* mice, even at steady state. This finding emphasizes that iTreg induction does not absolutely depend on an acute or highly inflammatory environment but can also arise due to minor subclinical exposures acquired throughout life. Such subclinical exposures are likely sensed by TLRs, which function as a very sensitive molecular system designed to detect low levels of pathogens or low-grade inflammation.[14] We found that TLR2 was specifically upregulated on steady-state *Malt1*$^{-/-}$ iTreg cells, and that engagement of TLR2 by Pam3Cys stimulated the proliferation of these cells as well as the differentiation of additional iTregs. A potential link between MALT1 and TLR2 could be IRF4, which has recently been linked to MALT1.[44] Blocking the caspase activity of MALT1 led to degradation of IRF4. IRF4 has been shown to actively suppress TLR4 signalling[45] and it has been shown that activation via TLR4 can upregulate TLR2.[46] This might be a possible link how MALT1 and TLR2-dependent pathways may be interconnected.

The influence of TLR signalling on Treg cells is quite controversial but it is generally agreed that TLR2, 4, or 5 engagement can enhance Treg function, survival and/or proliferation.[16–19] It has been previously demonstrated that TLR2, but not TLR4, engagement stimulates iTreg proliferation, and that *Tlr2*$^{-/-}$ mice have lower iTreg numbers.[20] We have shown here that MALT1 regulates this susceptibility of iTregs to TLR2 stimulation. During an acute infection, the normal function of Tregs is to restrain the protective activities of Teff. The suppression of iTreg induction by MALT1 might be an important means of allowing immune responses to combat invading pathogens at full force. Consistent with this theory, *Tlr2*$^{-/-}$ mice, which have abnormally low Treg numbers, are more resistant to *Candida albicans* infection than *WT* mice, owing to a more efficient Teff response.[15,20]

The above observations imply that MALT1 regulates iTreg induction under inflammatory conditions *in vivo.* Indeed, we found that *Malt1*$^{-/-}$ mice, with their marginal peripheral iTreg numbers, were resistant to EAE, an inflammatory disease that clearly affects Treg numbers and functionality. Interestingly, during EAE induction, iTregs in *Malt1*$^{-/-}$ mice swelled to a greater proportion of total Tregs than occurred in *WT* EAE control mice. Our results are consistent with a study of Carma1-deficient mice, which showed that the low Treg numbers in the periphery of these mutants expanded in response to infection with mouse cytomegalovirus (MCMV).[32] However, the iTreg levels achieved by this type of experimental inflammation were far lower than those we observed in our *Malt1*$^{-/-}$ EAE mice. It is well known that even though MALT1 forms a complex with CARMA1 and BCL10, these components have different individual functions.[47] For example, *Carma1*$^{-/-}$ mice show a more complete block in TCR signalling than *Malt1*$^{-/-}$ animals.[25,48,49] One explanation for the lower iTreg numbers in *Carma1*$^{-/-}$ mice during inflammation might be the nature of inflammation itself. Infection with MCMV may lead to weaker TLR2 stimulation than treatment with MOG peptide plus CFA, which contains large amounts of bacterial extracts. Furthermore, owing to the dual function of MALT1 as an adaptor molecule and a paracaspase, MALT1 likely has to integrate more regulatory events than does CARMA1. A study using the MALT1 protease inhibitor mepazine showed no effect on Treg development. This data indicates a caspase-independent regulation of Tregs.[50] However, very recently a knock-in mouse was published, in which MALT1 was substituted by a catalytical inactive MALT1 C472A mutant. Owing to this, the scaffolding function of MALT1 and its caspase activity can be distinguished *in vivo*.[42,51] In line with our data, inactivation of MALT1's catalytic activity led to a defect in nTreg development as well as to limited peripheral Treg numbers. These findings indicate a crucial role of the caspase activity in the generation of Tregs, while the scaffolding function was connected to overall T-cell activation.

In conclusion, our data identify an unexpected role for MALT1 in modulating the balance between tolerance and immune reactivity. MALT1 is crucial for nTreg development and thus important for central tolerance. In the periphery, MALT1 determines the threshold for the differentiation of naive T cells into functional iTregs. Although MALT1 has no influence on the

suppressive function of iTregs, it does limit further induction of iTregs at sites of inflammation by downregulating TLR2 expression. This reining-in of iTregs may ensure efficient Teff function and adds another layer of complexity to MALT1 function.

Materials and Methods

Mice. *Malt1*$^{-/-}$ mice were originally generated in our laboratory[25] and have been backcrossed for more than 10 generations into the C57Bl6 background. C57Bl6 control mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Age- and gender-matched controls were used for all experiments.

CD4^{+} Th cell purification and *in vitro* differentiation. Naive CD4^{+} CD62L^{+} Th cells were isolated from mouse spleens and lymph nodes using standard procedures and sorted using a magnetic bead cell purification kit according to the manufacturer's instructions (Miltenyi, San Diego, CA, USA). Unless otherwise indicated, naive CD4^{+}CD62L^{+} Th cells were stimulated for 72 h with 1 μg/ml plate-bound anti-CD3 Ab plus 1 μg/ml soluble anti-CD28 Ab (both from BD Biosciences, Mississauga, ON, Canada) in the presence of 50 U recombinant (r) human (h) IL-2 (Peprotech, Rocky Hill, NJ, USA) to differentiate into Th0 or into iTregs by addition of 50 U rhIL-2 plus 3 ng/ml rhTGF-β (R&D, Minneapolis, MN, USA).

Intracellular staining and proliferation. Intracellular FoxP3 and Helios protein was detected using a specific immunostaining kit (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. Surface staining for CD4, CD62L, CD25, TLR2 and TLR4 was performed as accordingly. Cell division was measured by culturing up to 1×10^7 naive Th cells/ml with 2.5 μM CellTrace Violet (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Flow cytometry was performed on a Canto II instrument (BD Biosciences) and data were analysed using FlowJo 7.5 software (Tree Star, Ashland, OR, USA).

Relative proliferation after Pam3Cys induction was calculated as followed: percentage of VCT low (proliferating) cells treated with Pam3Cys (x) minus percentage of VCT low cells without Pam3Cys stimulation of the corresponding sub-population (y) devided by y times 100.

Co-culture suppression assay. Splenocytes depleted of CD4^{+} T cells using magnetic beads as described above and then irradiated (3000 rad) were defined as APCs. APCs were co-cultured with *WT* naive T cells labelled with 2.5 μM CellTrace Violet (Invitrogen) as described above, in the presence of 3 μg/ml soluble anti-CD3 Ab and 50 U/ml rhIL-2. iTregs generated as described above were added to the Teff+APC cultures at a ratio of 1 : 2 : 1 APC:Teff:iTreg. Proliferation of Teff was measured 72 h later by flow cytometry as described above.

EAE induction and histology/immunohistochemistry. EAE was induced in mice using 115 μg MOG peptide in CFA per mouse and histological sections of brains were obtained as previously described.[26,44] Haematoxylin and eosin staining and immunohistochemical analyses were performed using standard laboratory procedures.

Isolation of brain lymphocytes. On day 14 after EAE induction, mice were killed and their cardiovascular systems were flushed with 20 ml PBS applied to the left ventricle. Brains were isolated and digested for 1 h at 37 °C in RPMI containing 50 μg/ml collagenase D (Roche, Laval, QC, Canada) and 10 μg/ml DNAse (Roche). Lymphocytes were enriched by centrifugation through a two-layer Percoll gradient (40% and 70%) and washed twice in PBS before further analysis.

Statistical methods. Where appropriate, differences between groups were evaluated using the two-tailed Student's *t*-test as calculated using Prism 5.0 (GraphPad, La Jolla, CA, USA) software. Data are presented as the mean ± S.E.M. *P*-values $\leq$ 0.05 were considered significant.

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

Conflict of Interest

The authors declare no conflict of interest.

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