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Notch Signaling Regulates T Cell Accumulation and Function in the Central Nervous System during Experimental Autoimmune Encephalomyelitis

Ashley R. Sandy,* † Josh Stoolman,†‡§ Kelli Malott,* Prae Pongtornpipat,* Benjamin M. Segal,‡§ and Ivan Maillard*§‖

Systemic inhibition of Notch signaling was previously shown to attenuate experimental autoimmune encephalomyelitis (EAE), a disease model of multiple sclerosis in mice. Different studies attributed these effects to decreased T-bet and IFN-γ expression, enhanced regulatory T cell function, reduced T cell chemotaxis to the CNS, or impaired Th9 cell differentiation. Interpretation of these heterogeneous findings is difficult because past experimental strategies did not ensure complete Notch inhibition in T cells and because many cell populations could be affected by systemic Notch blockade. To resolve the role of Notch in T cells during EAE, we used the pan-Notch inhibitor dominant-negative form of Mastermind-like 1 (DNMAML), as well as several complementary loss-of-function approaches specifically in myelin-reactive T cells. Notch inhibition in T cells profoundly decreased EAE incidence and severity. Notch-deprived myelin-reactive T cells had preserved activation and effector differentiation in secondary lymphoid tissues. However, Notch-deprived T cells failed to accumulate in the CNS after immunization. Parking wild-type and DNMAML T cells together in bone marrow chimeras increased accumulation of Notch-deprived T cells in the CNS after immunization but did not prevent EAE, indicating the absence of dominant suppression by DNMAML T cells. Analysis of CNS-infiltrating DNMAML T cells revealed markedly defective IL-17A and IFN-γ production, despite preserved T-bet expression. Collectively, our findings capture the profound overall effects of Notch signaling in myelin-reactive T cells and demonstrate that Notch controls the accumulation and pathogenic functions of CD4+ T cells within their target organ but not in lymphoid tissues during EAE. The Journal of Immunology, 2013, 191: 000–000.

Notch signaling plays multiple roles in health and disease (1, 2). Notch ligands of the Delta-like (Dll) or Jagged family interact with Notch receptors, resulting in sequential proteolysis and release of intracellular Notch. In the nucleus, intracellular Notch interacts with CSL/RBP-Jk (encoded by Rbpj) and Mastermind-like (MAML) coactivators to activate target genes. In the hematopoietic system, Notch regulates development of early T cell progenitors and several other innate and adaptive immune system lineages (3–9). Additionally, mounting evidence indicates a context-dependent role for Notch in T cell differentiation and function (10, 11).

Prior studies showed that systemic Notch blockade could attenuate experimental autoimmune encephalomyelitis (EAE, a mouse model of multiple sclerosis), but with conflicting information about the intensity and mechanisms of this effect. Using γ-secretase inhibitors (GSIs) to ubiquitously inhibit Notch signaling as well as Notch1 activation and a Notch1 antisense strategy, Osborne and colleagues (12) reported that Notch directly regulates expression of Tbx21 (encoding T-bet) in peripheral T cells during EAE. GSIs were also observed to enhance remyelination and axonal survival in EAE, indicating the existence of nonimmune effects of these drugs (13, 14). Another study using GSIs and anti-Notch3 neutralizing Abs described Notch3 as a dominant receptor influencing EAE via protein kinase C0 expression in Th1/Th17 CD4+ T cells (15). Systemic blockade of the Notch ligand Dll4 was shown to bolster T regulatory cell (Treg) function during EAE, whereas others using a similar approach reported altered T cell differentiation or chemotaxis (16–18). Jagged2 activation was reported to reduce IL-17A in secondary lymphoid organs and increase Treg responses (19). Finally, Notch was linked to Th9 differentiation in EAE (19). These discrepant results might reflect the use of heterogeneous experimental systems based on systemic Notch modulation or gain-of-function, which can trigger unintended off- and on-target effects and hinder accurate conclusions about Notch function specifically in T cells. This is particularly important in EAE because Notch affects many immune and nonimmune cells that contribute to disease pathogenesis (11, 20). Additionally, experimental strategies that focus on individual Notch ligands or receptors may fail to completely block Notch signaling in myelin...
Notch Signaling in T Cells During EAE

To resolve these conflicting results, we investigated Notch function specifically in mature T cells during EAE using several complementary loss-of-function approaches, including expression of the pan-Notch inhibitor dominant-negative form of MAML-1 (DNMAML) and inactivation of Notch receptor genes. Additionally, we evaluated the effects of Notch inhibition in TCR transgenic mice that are sensitized to EAE by a dominant population of myelin-reactive T cells. T cell–specific Notch inhibition resulted in near complete protection from EAE, independent of T cell activation and effector differentiation effects in secondary lymphoid organs. Notch-deprived CD4+ T cells failed to accumulate in the CNS after immunization despite preserved in vitro migration. Parking wild-type (WT) and DNMAML CD4+ T cells together in bone marrow (BM) chimeras increased accumulation of Notch-deprived CD4+ T cells in the CNS but did not suppress disease. In the CNS, Notch-deprived myelin-reactive CD4+ T cells failed to produce IL-17A and IFN-γ despite preserved expression of the master transcription factor T-bet. Our findings reveal the overall effects of Notch in T cells during EAE, as complete T cell–specific Notch inhibition led to significantly more protection than reported with other methods of Notch blockade. Moreover, we demonstrate that Notch specifically regulates the secondary response of myelin-reactive CD4+ T cells in the CNS independently of effects on T-bet and Tregs during the primary response in lymphoid organs.

Materials and Methods

Mice

C57BL/6/Jpnpn (B6-SL, CD45.1+) mice were from the National Cancer Institute (Frederick, MD). C57BL/6/JgTcra2D2.Tcrb2D2.Jux1/Jux1 (2D2) TCR transgenic mice were provided by Dr. B. Segal (University of Michigan) (21). Rbpfm mice by Dr. T. Honjo (Kyoto, Japan) (6), Notch1f/f mice by Dr. R. Kopan (St. Louis, MO) (5), and Notch2f/f mice were provided by Dr. T. Gridley (Saharborad, ME) (22). ROSA26DNMAML mice (DNMAML) contain a Cre-inducible cassette encoding the DNMAML-GFP pan-Notch inhibitor (23). DNMAML, Rbpfm, Notch1f/f, and Notch2f/f mice were crossed to 2D2 mice (2D2/DN) mice. All mice were backcrossed to the B6 background (more than eight generations). The mice were crossed to 2D2 mice (2D2/DN) mice. All mice were backcrossed to the B6 background (more than eight generations). The University of Michigan’s Committee on Use and Care of Animals approved all experiments.

EAE induction

On day 0, age-matched (6–14 wk) and sex-matched mice were immunized with CPA containing heat-killed Mycobacterium tuberculosis (Thermo Fisher Scientific, Pittsburgh, PA) and myelin oligodendrocyte glycoprotein (MOG)35-55 peptide (MRC0195PFSRVRVLYRGK; 0.25 μg/site; Bio-Synthesis, Lewisville, TX). On days 0 and 2, mice received pertussis toxin (Thermo Fisher Scientific) (300 ng i.p.). Mice were scored for disease severity according to the following scale: 1, limp tail; 2, inability to right oneself; 3, hind limb weakness; 4, hind limb paralysis; 5, moribund. For disease severity according to the following scale: 1, limp tail; 2, inability to right oneself; 3, hind limb weakness; 4, hind limb paralysis; 5, moribund.

Isolation of CNS-infiltrating cells

After anesthesia, mice were perfused with PBS. Brains and spinal cords were digested with collagenase (2.125 mg/ml; Invitrogen, Grand Island, NY) and DNase I (1 mg/ml; Roche, Indianapolis, IN) followed by purification on a 30/70% Percoll gradient (Sigma-Aldrich, St. Louis, MO).

ELISPOT

Draining lymph node (DLN) cells (axial, brachial, inguinal) from immunized mice at peak disease were restimulated in MultiScreen HTS filter plates (Millipore, Billerica, MA) with and without 50 μg/ml MOG35-55 for 18 h. Abs used for cytokine detection were from BioLegend (anti–IFN-γ and IL-17A). Streptavidin-HRP was from SouthernBiotech (Birmingham, AL). HRP substrate was from Vector Laboratories (Burlingame, CA).

Quantitative RT-PCR

RNA from CD4+Vα3.2+Vβ11+CD44+ T cells was isolated using TRIzol (Invitrogen, Carlsbad, CA). cDNA was prepared with SuperScript II (Invitrogen). Quantitative PCR was performed with TaqMan (Applied Biosystems, Carlsbad, CA) on Mastercycler realplex (Eppendorf, Westbury, NY). Primers were from Applied Biosystems. Relative expression was calculated using the ΔΔCt method.

Transwell migration assays

The following chemokine ligands were from R&D Systems (Minneapolis, MN): CCL20/Mip-3a, CCL2/Mcp-1, CCL11/Eotaxin-2, and CXCL12/Cchemokine. CD4+ T cells from 2D2 and 2D2/DN MAML immunized mice at peak disease were purified by magnetic bead technology (Miltenyi Biotec, Auburn, CA). Purified CD4+ T cells were warmed to 37°C, plated in a ChemoTx system (Neuro Probe, Gaithersburg, MD), and allowed to migrate for 4 h before analysis of migrated CD4+Vα3.2+Vβ11+CD44+ T cells in bottom wells by flow cytometry. The number of cells migrated was normalized using a standard curve of known numbers of T cells and a fixed number of counting beads (Bangs Laboratories, Fishers, IN) by flow cytometry. Specificity of migration was determined by enumerating the number of activated CD22 CD4+Vα3.2+Vβ11+CD44+ T cells that migrated to the bottom well in the absence of chemokines.

Flow cytometry

The following Abs were from BioLegend, eBioscience (both San Diego, CA), or BD Biosciences (San Jose, CA): anti-CD4, CD8α, CD44, CD45.1, CD45.2, TCRβ, CD49d (a1), CD29 (b4), IFN-γ, IL-17A, Vβ11, Vα3.2, Foxp3, and T-bet (4B10). For T cell restimulation, we used plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) (BioLegend; 2.5 μg/ml). Intracellular flow cytometry was performed per the manufacturer’s instructions after addition of brefeldin A (>2 h) (BD Biosciences). Analysis/sorting were on FACS禀nto or FACSaria II/III (BD Biosciences). Dead cells were excluded with DAPI (Sigma-Aldrich). Files were analyzed in FlowJo (Tree Star, San Carlos, CA).

Generation of mixed BM chimeras

Lethally irradiated (900 rad) B6-CD45.1 mice received B6 CD45.2-WT and B6-CD45.1 or B6 CD45.2/DNAML mice and B6-CD45.1 BM (mixed at 1:1 or 7:3 ratio). Mice were allowed to reconstitute for 8–12 wk before EAE induction.

Statistical analysis

Comparison of two means was performed with a two-tailed unpaired Student t test or a nonparametric Mann–Whitney U test (GraphPad Prism; GraphPad Software, La Jolla, CA). For differences in disease incidence, significance was determined by the χ2 and a Fisher exact test (GraphPad Prism). A p value <0.05 was considered statistically significant.

Results

Notch inhibition in myelin-reactive CD4+ T cells prevents EAE

To overcome limitations of past studies, we used in vivo loss-of-function models to block Notch signaling specifically in T cells during EAE (Fig. 1A). We inhibited the Notch transcriptional activation complex downstream of all Notch receptors in mature T cells by expressing DNMAMAL or by inactivating Rbpj, encoding CSL/RBP-Jk (6, 23). In selected experiments, DNMAML was introduced into 2D2 TCR transgenic T cells, recognizing MOG35-55 (21). Notch blockade efficiently prevented EAE, with <8% of mice developing symptoms, as compared with ~95% of controls (Fig. 1B, 1C). When DNMAML was expressed in 2D2 T cells, disease incidence was also markedly reduced (Fig. 1D, 1E). Decreased EAE in 2D2/DNAML mice correlated with reduced demyelination (Fig. 1F) and CNS cellular infiltrates (Fig. 1G, 1H). Previous reports showed significantly less protection, perhaps because of incomplete Notch blockade or family redundancy when only Notch1, Notch3, or Dll4 was inhibited (12, 15–19). Thus, Notch inhibition...
in T cells markedly reduced EAE, even in the presence of a high frequency of myelin-reactive T cells in TCR transgenic mice.

Preserved effector differentiation of myelin-reactive Notch-deprived CD4+ T cells in lymphoid tissues

Previous studies in EAE suggested that Notch modulates effector T cell differentiation in secondary lymphoid tissues (12, 16, 19). We assessed T cell responses at peak disease using 2D2 transgenic mice, in which myelin-reactive T cells can be tracked based on expression of a Vα3.2+Vβ11+ TCR (21). DNMAML expression preserved 2D2 CD4+ T cell activation in DLNs as measured by increased CD44 expression (Fig. 2A). Analysis of DLN IFN-γ– and IL-17A–producing cells, two important EAE drivers, revealed no significant difference between WT and Notch-inhibited CD4+ T cells by ELISPOT (Fig. 2B) (24). Intracellular IL-17A and IFN-γ expression by activated 2D2/DNMAML T cells was also largely preserved (Fig. 2C). These data suggest that Notch inhibition in myelin-reactive CD4+ T cells did not significantly impact IFN-γ and IL-17A production in DLNs. These results are similar to data showing that anti-Dll4 treatment during EAE did not alter cytokine production by proteolipid protein–specific T cells (17).

FIGURE 1. Inhibition of Notch signaling in myelin-reactive CD4+ T cells markedly attenuates EAE. (A) Experimental design. (B) Mean clinical EAE score (two or more experiments). (C) Percentage disease incidence (score of ≥2) of immunized WT, DNMA M (DN), or CSL/RBP-Jk-deficient (RBKO) mice (pooled results from two or more experiments for each strain). In all models, Cd4-Cre–mediated Notch inactivation was achieved specifically in mature T cells. (D) Mean clinical score in TCR transgenic 2D2 or 2D2/DN mice (expressing DNMA M in T cells) (three or more experiments). (E) Percentage disease incidence (score of ≥2) of immunized 2D2 and 2D2/DN mice (two or more experiments). (F) Luxol fast blue (original magnification ×100) and (G) H&E staining of spinal cord lumbar sections from 2D2 and 2D2/DN mice (original magnification ×20; representative of n = 3 mice/group; two experiments). (H) Number of white matter infiltrates per H&E section (counted blindly). **p < 0.01, ***p < 0.001.

FIGURE 2. Notch inhibition in myelin-reactive CD4+ T cells does not alter initial activation or effector T cell differentiation. (A) Percentage and absolute number of Vβ11+CD4+ T cells in DLNs at peak disease (n = 3–4 mice/group; two or more experiments). (B) Number of IFN-γ– and IL-17A–secreting cells as assessed by ELISPOT in DLNs from immunized WT, DNMA M (DN), 2D2, and 2D2/DN mice (n = 3–4 mice/group; two or more experiments). (C) Frequency of Vβ11+CD4+ T cells after restimulation with anti-CD3/CD28 and staining for intracellular cytokines (n = 3–4 mice/group; two experiments). (D) Tbx21 and Rorc mRNA in activated CD4+ T cells and inactivated CD4+ T cells (n = 3–4 mice/group; two experiments). (E) Intracellular T-bet and IFN-γ in Vβ11+CD4+ T cells as assessed by intracellular flow cytometry (n = 3–4 mice/group; two experiments). Representative flow cytometry plots are shown. MFI, mean fluorescence intensity. ***p < 0.001.
Prior studies suggested that Notch1 regulates expression of Tbx21 (encoding T-bet) in Th1 cells, whereas Dll4-mediated signaling can increase Rorc mRNA (encoding Rorγt) in Th17 cells (12, 25). However, we found no significant change in Tbx21 transcripts and a trend for increased Rorc mRNA in activated 2D2/DNMAML CD4+ T cells after immunization (Fig. 2D). We next studied T-bet expression after verifying Ab specificity in Tbx21−/− mice during EAE (Supplemental Fig. 1) (26). We observed a preserved frequency of T-bet+ cells and normal staining intensity among 2D2/DNMAML CD4+ T cells in CNS-infiltrating leukemic T cells (29). Other chemokine receptors have been linked to CD4+ T cell infiltration into the CNS (28). We found no difference in αβ1 expression between 2D2 and 2D2/DNMAML CD4+ T cells after immunization (Fig. 4D), although these data do not rule out a defect in integrin conformation or function. Prior work reported that anti-Dll4 inhibits chemotaxis due to decreased Ccr2, Ccr5, and Ccr6 expression (17). Additionally, Notch can regulate Ccr7 expression in CNS-infiltrating leukemic T cells (29). Other chemokine receptors have been linked to CD4+ T cell infiltration into the inflamed CNS, such as Cxcr3 (30). However, we found no significant change in expression of these chemokine receptors by 2D2/DNMAML T cells during EAE (data not shown). Next, we assessed responses of Notch-deprived myelin-reactive CD4+ T cell to candidate chemokines in vitro (Fig. 4E). After immunization, activated 2D2/DNMAML CD4+ T cells migrated as well as 2D2 T cells in response to chemokines that interact with Ccr2, Ccr5, Ccr6, Ccr7, and Cxcr3. These data indicate that Notch-deprived myelin-reactive CD4+ T cells can migrate toward chemotactic signals at least in vitro, although they do not rule out defective migration in vivo. Our conclusions differ from results with Dll4 blockade (17). These differences could reflect specific effects of individual Notch ligands or incomplete Notch inhibition in past studies, as well as effects on T cell migration induced by bystander cells.

**Myelin-reactive Notch-deprived CD4+ T cells do not suppress EAE induced by WT CD4+ T cells**

Past studies relying on systemic Notch ligand fusion proteins and Abs suggested that Notch modulation increased Treg frequency (16). To assess whether this contributed to protection from EAE in our T cell–specific genetic model of Notch inhibition, we assessed Foxp3 expression, focusing on nontransgenic T cells that have the most Treg activity in TCR transgenic mice (31). No change in Treg frequency was observed in 2D2/DNMAML mice after immunization (Fig. 5A). We observed a slight but significant increase in Treg frequency in DNMAML compared with WT CD4+ T cells after immunization (Fig. 5B). To assess the overall suppressive capacity of DNMAML T cells after immunization, we generated mixed BM chimeras to park WT and DNMAML T cells in the same recipients (Fig. 5C). WT/DNMAML chimeras succumbed to EAE at the same frequency as did mice containing only WT T cells, suggesting the absence of dominant suppressor function in DNMAML T cells (Fig. 5D, SE).

The very low abundance of DNMAML T cells in the CNS prevented accurate assessment of their effector function. It was previously shown that Ccr6-deficient T cells failed to traffic to the...
CNS during EAE (32). However, bystander T cells induced Ccr6-independent T cell migration into the CNS. To determine whether WT T cells could overcome the inability of Notch-deprived CD4+ T cells to accumulate in the CNS, we measured T cell numbers in the CNS of WT/DNMAML BM chimeras at peak disease. DNMAML CD4+ T cells partially regained their ability to accumulate in the CNS in the presence of WT CD4+ T cells (Fig. 5F). These data suggest that bystander WT T cells can induce Notch-deprived T cells to accumulate in the CNS.

Notch inhibition in CNS-infiltrating CD4+ T cells blocks IL-17A and IFN-γ expression independently of T-bet

Decreased T cell reactivation in the CNS can result in reduced EAE severity (33, 34). Because DNMAML CD4+ T cells accumulate in the CNS in the presence of WT T cells (Fig. 5F), we could study the impact of Notch inhibition on T cell effector differentiation in the CNS. In immunized mixed BM chimeras, WT CD45.1+ competitor T cells functioned as an internal positive control. Whereas DNMAML CD4+ T cells produced IFN-γ and IL-17A in DLNs (Figs. 2B, 2C, 2F, 6B), they had markedly reduced production of IFN-γ and IL-17A in the CNS (Fig. 6A). The blunted cytokine response occurred without defect in T-bet expression, as evidenced by the presence of many T-bet+IFN-γ− cells (Fig. 6B). Our results suggest that Notch-mediated regulation of IFN-γ production is an important feature of its effects in EAE, but through mechanisms that are T-bet-independent and CNS-restricted. Moreover, decreased T cell migration into the CNS could account for some of the effects of Notch inhibition. However, it cannot fully explain protection from EAE, as Notch-inhibited CD4+ T cells that enter the CNS in the presence of bystander WT T cells have markedly reduced effector function.

Discussion

Our findings highlight an essential role for Notch signaling in CD4+ T cells mediating EAE. We used genetic pan-Notch inhibition as well as inactivation of individual Notch receptor genes specifically in T cells to fully determine the impact of Notch signaling in CD4+ T cells during EAE. We found that complete Notch inhibition in transgenic MOG-reactive T cells or polyclonal T cells nearly completely protected mice from EAE. This protection was not due to an effect on myelin-reactive T cell activation or differentiation in secondary lymphoid tissues, nor to a dominant...
suppressor function of DNMAML T cells. In contrast, Notch-deprived T cells failed to accumulate in the CNS, despite preserved in vitro chemotaxis. Parking WT and DNMAML T cells together in BM chimeras resulted in enhanced accumulation of DNMAML T cells in the CNS. Functional analysis of DNMAML T cells in the CNS of BM chimeras revealed a significant defect in IL-17A and IFN-γ production, despite preserved T-bet expression. Collectively, Notch signaling regulates the accumulation and effector function of myelin-reactive T cells in their target organ during EAE. These findings are reminiscent of past observations with Dll4 blockade (17). However, decreased T cell accumulation cannot by itself explain all the consequences of Notch blockade in EAE, as additional effects on cytokine production by T cells were detected specifically in the CNS.

Past work suggested that Notch regulates the differentiation of myelin-reactive T cells in secondary lymphoid organs through effects on T-bet expression, IFN-γ and IL-17A production, or Treg function (12, 16, 19, 35). In contrast, our work reveals a function for Notch signaling that predominate in myelin-reactive T cells infiltrating the CNS, and not in secondary lymphoid tissues. The reasons for these differences are unclear, but they could reflect the use of heterogeneous methods to modulate Notch signaling in previous studies, with no capacity to assess the role of all Notch receptors specifically in T cells. For instance, several past reports have relied on systemic pharmacological modulation of Notch signaling with GSIs, blocking Abs, or agonistic fusion proteins. Many cell populations in the immune system require Notch signaling with Dll4 blockade (17). However, that report described defects in in vitro T cell chemotaxis that we did not replicate using stringent genetic loss-of-function approaches in T cells.

Notch signaling has previously been suggested to regulate T-bet expression (12). Despite drastically decreased IFN-γ production in the CNS by Notch-inhibited T cells, myelin-reactive Notch-deprived T cells had preserved expression of T-bet. This is similar to previous work published by our laboratory using models of graft-versus-host disease in which allogeneic Notch-deprived T cells had preserved expression of T-bet, but failed to produce IFN-γ (36–38). Other signaling cascades such as IL-12 and IL-27 have been shown to elicit T-bet expression and could account for preserved T-bet expression in Notch-deprived T cells (39, 40). Overall, our findings suggest that Notch can control the production of inflammatory cytokines without directly controlling T helper lineage determination, but rather by influencing the responsiveness of Ag-specific T cells in vivo (38, 41).

Chemotaxis to the CNS is regulated by the cooperative effects of many signaling cascades (42). Using systemic inhibition of the Notch ligand Dll4, Notch has been suggested to regulate Ccr1, Ccr2, Ccr5, and Ccr6 expression during EAE, whereas another study described the ability of Notch to regulate Ccr7 expression in CNS-homing leukemia cells (17, 29). In contrast, we found that genetic pan-Notch inhibition in myelin-reactive T cells did not affect expression of these chemokine receptors or migration in response to their chemokine ligands, at least in vitro. However, our work does not rule out defective migration in vivo in response to chemotactic signals. These discrepant results may reflect differences in the effects of systemic Dll4 blockade as opposed to specific genetic Notch inhibition in T cells. For example, inhibition of Dll4 in other cell populations in secondary lymphoid organs could elicit chemotaxis changes in T cells that do not result from direct cell-autonomous effects in T cells.

Notch-inhibited myelin-reactive T cells failed to suppress disease induced by WT T cells in mixed BM chimeras. This is in contrast to prior work suggesting that Dll4 blockade expanded Tregs, which resulted in slightly reduced EAE severity (16). These discrepancies could reflect differences in experimental strategy. In our observations, Notch-inhibited T cells were mixed with WT T cells in BM chimeras. In this context, a mild increase in the suppressive capacity of Notch-deprived T cells may have been overcome by the large population of WT T cells in the same animal. However, our findings remain inconsistent with a dominant suppressive effect as the sole explanation for the protective effects of Notch inhibition in EAE.
If Notch signaling does not directly affect chemokine receptor expression or signaling in myelin-reactive T cells, then what accounts for the markedly decreased accumulation of Notch-deficient T cells in the CNS during EAE? One possibility is that Notch signaling modulates integrin expression and/or function. For example, the integrin αβ4 is required for T cell chemotaxis to the CNS during EAE and MS (28, 43). Although Notch-deprived T cells expressed similar levels of surface αβ4, impaired integrin activation or defective downstream signaling could account for their decreased accumulation in the CNS. Another possibility is that Notch-deprived T cells fail to produce inflammatory cytokines such as IFN-γ when infiltrating the CNS, and thus fail to induce expression or activation of integrins (e.g., the αβ4 ligand VCAM-1) in endothelial cells of the CNS (44, 45). This scenario would be consistent with the rescue of DNAMAML T cell accumulation in the CNS that we observed in the presence of bystander WT T cells. Alternatively, Notch-deprived T cells may have preserved migration into the CNS but fail to survive or proliferate in their target organ during local reactivation, either immediately after crossing the blood-brain barrier or during subsequent exposure to tissue Ags. Of note, failure of myelin-reactive T cells to be locally reactivated can ultimately result in decreased T cell accumulation in the CNS, even after successful initial migration (33, 34). This scenario could account for our observation that Notch-deprived CNS-infiltrating T cells in mixed BM chimeras had markedly decreased production of inflammatory cytokines, which cannot be explained solely by a migration defect. Instead, defective T cell accumulation and cytokine production in the CNS could be linked to a role of Notch in enhancing T cell reactivation that becomes apparent predominantly in the target organ and not in secondary lymphoid tissues. We speculate that myelin-reactive T cells get exposed to a unique source or density of Notch ligands in the CNS to which they do not have access in lymph nodes. Alternatively, cytokines or other signaling pathways could compensate for the effects of Notch deprivation in lymph nodes but be missing in the CNS during EAE, resulting in a functional defect that becomes apparent only in the brain and spinal cord.

In conclusion, our work provides definitive experimental evidence to understand the overall effects of Notch signaling in CD4+ T cells during EAE. By using multiple loss-of-function approaches restricted to T cells, we demonstrate a profound requirement for Notch in CD4+ T cells to elicit EAE. The degree of protection observed in our study is markedly higher than reported in all past studies in the field, most likely because we were able to achieve complete inhibition of signaling downstream of all Notch receptors in myelin-reactive T cells. The constellation of mechanisms largely differed from previous reports and was independent of the master transcription factors of T helper lineages. Of note, these data in EAE are reminiscent of our findings in graft-versus-host disease, as markedly defective IFN-γ production by Notch-deprived allo-reactive T cells was observed despite preserved T-bet expression (36–38). In EAE, our results suggest a function for Notch in T cells that is CNS-restricted, possibly due to local exposure to Notch ligands during T cell restimulation in the CNS. Because effects on both EAE and graft-versus-host disease outcome were observed upon interfering with the transcriptional activation complex, future studies will work to elucidate direct transcriptional Notch targets in mature T cells that regulate T cell–mediated immune disorders.

Disclosures

The authors have no financial conflicts of interest.

References

**Supplemental Figure 1. Specificity of T-bet detection.** WT and T-bet knockout mice were immunized. Draining lymph node cells were stained with isotype or anti-T-bet antibody at peak disease. Representative flow cytometry plot is shown.