CNS resident classical DCs play a critical role in CNS autoimmune disease

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Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS), induced by the adoptive transfer of myelin-reactive CD4\(^+\) T cells into naïve syngeneic mice. It is widely used as a rodent model of multiple sclerosis (MS). EAE lesion development is initiated when transferred CD4\(^+\) T cells access the CNS and are reactivated by local antigen presenting cells (APC) bearing endogenous myelin peptide/ MHC Class II complexes. The identity of the CNS resident, lesion-initiating APC is widely debated. Here we demonstrate that classical dendritic cells (cDC) normally reside in the meninges, brain, and spinal cord in the steady state. These cells are unique among candidate CNS APC in their ability to stimulate naïve, as well as effector, myelin-specific T cells to proliferate and produce pro-inflammatory cytokines directly ex vivo. cDC expanded in the meninges and CNS parenchyma in association with disease progression. Selective depletion of cDC led to a decrease in the number of myelin-primed donor T cells in the CNS and reduced the incidence of clinical EAE by half. Based on our findings, we propose that cDC, and the factors that regulate them, be further investigated as potential therapeutic targets in MS.

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS), induced by the adoptive transfer of myelin-reactive CD4+ T cells into naïve syngeneic mice. It is widely used as a rodent model of multiple sclerosis (MS). EAE lesion development is initiated when transferred CD4+ T cells access the CNS and are reactivated by local antigen presenting cells (APC) bearing endogenous myelin peptide/MHC Class II complexes. The identity of the CNS resident, lesion-initiating APC is widely debated. Here we demonstrate that classical dendritic cells (cDC) normally reside in the meninges, brain, and spinal cord in the steady state. These cells are unique among candidate CNS APC in their ability to stimulate naïve, as well as effector, myelin-specific T cells to proliferate and produce pro-inflammatory cytokines directly ex vivo. cDC expanded in the meninges and CNS parenchyma in association with disease progression. Selective depletion of cDC led to a decrease in the number of myelin-primed donor T cells in the CNS and reduced the incidence of clinical EAE by half. Based on our findings, we propose that cDC, and the factors that regulate them, be further investigated as potential therapeutic targets in MS.
INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE), an autoimmune demyelinating disease of the central nervous system (CNS), is widely used as an animal model of multiple sclerosis (MS). EAE can be induced by the adoptive transfer of highly purified, myelin-reactive CD4+ T-helper (Th)1 or Th17 effector cells into otherwise unmanipulated, syngeneic hosts. Lesion formation in adoptively transferred EAE is triggered when myelin-specific CD4+ T cells access the naïve CNS and are reactivated by local antigen presenting cells (APCs) bearing endogenous myelin peptide/ MHC Class II (MHCII) complexes (1). The identity of the CNS-resident, lesion-initiating APC is widely debated. By definition, the cell type in question must express MHCII and co-stimulatory molecules and possess the machinery necessary to process immunogenic peptides from larger myelin proteins. In order to mediate epitope spreading during clinical relapse and/ or progression, a candidate APC would also have to be capable of activating naïve CD4+ T cells specific for secondary myelin epitopes and of polarizing them toward encephalitogenic Th1 or Th17 lineages (2).

Microglia have been posited as the critical resident APC of the CNS (3). It has become increasingly recognized that microglia are heterogeneous, and that distinct subsets may play different roles during the evolution of disease (4). However, the ability of microglia, particularly when in a resting state, to efficiently activate T cells has been questioned by several laboratories (5, 6). Although astrocytes and cerebrovascular endothelium were reported to express MHCII in response to inflammatory stimuli (7, 8), they do not do so during homeostasis, nor do they express molecules necessary for antigenic processing and loading. Furthermore, experiments with reciprocal WT/ MHCII−/− bone marrow chimeric mice indicate that induction of EAE by
adoptive transfer requires MHCII expression on radiosensitive hematopoietic host cells, while expression on radioresistant non-hematopoietic host cells is dispensable (9).

Several subsets of bone marrow-derived MHCII⁺ cells normally populate the CNS, including perivascular, meningeal, and choroid plexus macrophages (10). In addition, MHCII⁺ cells with characteristics of dendritic cells (DC), based on cell surface marker expression, morphology, and/or ultrastructural characteristics, are normal constituents of the choroid plexus, meninges, and perivascular spaces in the uninjured CNS of both humans and rodents (9, 11–17). These CNS resident DC are optimally positioned to interact with infiltrating T cells since the choroid plexus and meninges, as well as CNS parenchymal blood vessels, are important portals of leukocyte entry during EAE and MS (18–21). The lineage(s) and biological properties of putative CNS DC have yet to be delineated. Genetically engineered mice in which MHCII expression is restricted to CD11c⁺ cells are susceptible to EAE, suggesting that DC alone are sufficient to present antigen to encephalitogenic T cells in vivo and, thereby, promote their local expansion and effector functions (9). DC are generally considered potent APC, due to their ability to activate and polarize naïve T cells, which have an elevated threshold for T cell receptor (TCR) signaling compared to effector and/or memory T cells. However, the potential role of DC in EAE pathogenesis is complicated by the fact that DC are heterogeneous with a range of functional phenotypes, and can even be tolerogenic under certain circumstances (22). It is also unclear whether CNS resident CD11c⁺MHCII⁺ cells can independently activate encephalitogenic T cells in situ. Indeed, depletion of CD11c⁺ cells in transgenic mice that express diphtheria toxin receptor (DTR) under control of the CD11c promotor has been variably reported to ameliorate or exacerbate the clinical course of EAE, or to have no impact whatsoever (23, 24).
CD11c^+ MHCII^+ DC include monocyte-derived DC (moDC) and classical DC (cDC) subsets. A third DC subset, plasmacytoid DC (pDC), express low levels of CD11c and MHCII, limiting their ability to present antigen to CD4^+ T cells. moDC are not normally present in healthy parenchymal tissues but differentiate from infiltrating Ly6C^hi monocytes in the setting of inflammation. We and others have previously shown that moDC accumulate in the CNS during EAE and that their depletion or inactivation ameliorates clinical disability (25–28). However, the fact that moDC primarily emerge in the setting of active inflammation precludes their role in lesion initiation. Unlike moDC, cDC populate lymphoid, as well as some non-lymphoid, tissues in the steady state. They are derived from a common DC precursor, called the pre-DC, in the bone marrow and expand in response to the hematopoietin, FMS-like receptor tyrosine kinase 3 (FLT3) ligand. DC that have been detected in the murine CNS under physiological conditions are FLT3 dependent, differentiate from transferred pre-DC, and express a transcriptome consistent with cDC (29). FLT3 ligand antagonists suppress, while FLT3 ligand agonists exacerbate, clinical EAE, suggesting that cDC can modulate disease severity (9, 30). However, the ability of CNS-resident cDC to directly stimulate and polarize myelin-reactive T cells \textit{in vivo}, and their relative importance in EAE pathogenesis by comparison to infiltrating moDC or other APC subsets, has yet to be elucidated.

Detailed \textit{in vivo} studies of moDC and cDC have been undermined by a dearth of distinguishing cell surface markers. It was recently reported that CD88 (complement 5a receptor 1; C5ar1) and CD26 (dipeptidyl peptidase; DPP4), an enzyme involved in peptide hydrolysis, are reciprocally expressed by moDC and cDC (31). The transcription factor ZBTB46 has also been identified as a singular marker of cDC in mice and humans (32, 33). These molecules are not exclusive to DC but are useful in delineating CD11c^+ DC lineages. In the present study, we
employ the above markers to investigate the heterogeneity of DC during EAE. We detect both moDC and cDC in the inflamed target organ but demonstrate that CNS cDC are uniquely capable of processing immunogenic peptides from larger myelin fragments and activating myelin-specific naïve, as well as effector, CD4\(^+\) T cells. We found that cDC are present in the naïve CNS and that selective depletion of that subset reduces the incidence of EAE. Hence, cDC play an important role in disease initiation. Collectively, our data suggest that cDC, and the factors that regulate them, be investigated as potential therapeutic targets in patients with MS, particularly in those individuals who are not responsive to currently available disease modifying therapies.
RESULTS

cDC, as well as moDC, are present in EAE infiltrates

In order to characterize APC subsets that accumulate in the CNS during EAE, we performed flow cytometric analysis on brain and spinal cord mononuclear cells isolated at the time of peak clinical severity. MHCII+ cells in the brain included CD45intCD11bint microglia, CD45hiCD11b+CD11c- monocytes/macrophages, CD19+ B cells, and CD45hiCD11c+ DC (Figure 1A and data not shown). Spinal cord infiltrates had a similar cellular composition (data not shown). The CNS DC population was comprised of both CD88−CD26+ cells, consistent with cDC, and CD88−CD26− cells, consistent with moDC (Figure 1A, upper right panel). Microglia and macrophage/monocytes expressed CD88 but not CD26 (Figure 1A, lower panels). We also detected CD26+ pDC; however, the majority of pDC were MHCII− and constituted <5% of the MHCII+CD26+ population in the inflamed CNS (Supplemental Figure 1). In order to confirm the lineages of the CD26+ versus CD88+ CNS DC subsets, we performed transcriptional profiling. The CD26+ DC cohort expressed high levels of genes identified by the Immunological Genome Project (ImmGen) (34) as core cDC transcripts, including Amica1, CCR7, and Kit, while the CD88+ DC cohort expressed markers associated with monocyte-derived cells, including Slc11a1 (35), CD84 (36), and Bst1 (37) (Figure 1B). CNS CD26+ DC expressed elevated levels of Flt3 and Tlr3, while CD88+ DC expressed high levels of Tlr4, which mirrors the expression of those stimulatory molecules by peripheral cDC and moDC, respectively (Figure 1B, right panel) (38). The designation of CNS CD11c−CD26+ cells as cDC was corroborated by their selective expression of the transcription factor, ZBTB46, as demonstrated via intracellular staining and flow cytometry (Figure 1C). Similarly, CD11c−CD26+, but not CD11c−CD88+, cells isolated from Zbtb46-gfp reporter mice at peak EAE were GFP+ (Figure 1D).
**CNS cDC are highly efficient antigen presenting cells**

We next compared the ability of CNS cDC and moDC to present antigen to myelin-specific CD4\(^+\) T cells *ex vivo*. MHCII\(^+\)CD11c\(^+\)CD88\(^+\) moDC and CD26\(^+\) cDC were FAC sorted from the CNS at peak EAE and co-cultured with naïve CD4\(^+\) T cells that express a transgenic T cell receptor specific for the myelin oligodendrocyte glycoprotein (MOG\(_{35-55}\)) peptide (2D2 cells) (39). 2D2 cells underwent multiple rounds of proliferation, upregulated the activation marker CD44, and expressed intracellular IFN\(\gamma\) and/ or GM-CSF upon co-culture with MOG\(_{35-55}\) peptide and CNS cDC (Figure 2A and B). In contrast, 2D2 cells neither proliferated, upregulated CD44, nor expressed effector cytokines when co-cultured with MOG\(_{35-55}\) and CNS moDC.

Similar results were obtained with cDC and moDC sorted from the spleens of the same mice (data not shown). 2D2 cells did not express FoxP3 under any of the culture conditions. In order to determine whether CNS cDC could process immunogenic epitopes from larger myelin proteins, we repeated the APC assays using a longer fragment of MOG (MOG\(_{1-125}\)) as antigen. CNS cDC were able to process MOG protein and activate 2D2 cells, whereas their moDC counterparts were incompetent (Figure 2A and B). The superior APC properties of CNS cDC over moDC are not antigen-specific since only the former were able to activate OVA-specific TCR transgenic OT-II cells upon co-culture in the presence of either OVA peptide or whole ovalbumin protein (40) (data not shown).

The majority of CD4\(^+\) T cells that infiltrate the CNS during EAE or MS are CD44\(^{hi}\) effector cells. As a transgenic T cell line, 2D2 cells do not reflect the heterogeneity of encephalitogenic T cells that infiltrate the CNS during EAE, both in terms of TCR affinity and biological properties. To more accurately simulate the local T cell-APC interactions that occur
during autoimmune demyelinating disease, we isolated CD4+ T cells from the CNS of mice at peak EAE and reconstituted them with purified DC subsets obtained from the same tissues. Notably, CNS cDC spontaneously induced the proliferation of the CNS-infiltrating effector CD4+ T cells in the absence of exogenous antigen, ostensibly due to the presence of endogenous myelin peptide/MHCII complexes on their cell surface (Figure 2C). Proliferation of the effector T cells was enhanced by pulsing the CNS cDC with MOG35-55. moDC failed to induce a significant effector T cell response, even when the co-cultures were supplemented with MOG35-55 (Figure 2C). Taken together, these data demonstrate that cDC, but not moDC, are proficient at activating both naïve and antigen experienced myelin-specific T cells.

We next measured a panel of selected cytokines in supernatants from the APC assays. Co-culturing 2D2 cells with CNS cDC, in the presence of either MOG35-55 or MOG1-125, resulted in the production of IL-2, IL-17A, IFNγ, and GM-CSF (Figure 2D). Similar results were obtained when CD4+ effector T cells, isolated from the inflamed CNS, were co-cultured with MOG35-55 and CNS cDC (Figure 2E). In contrast, we did not detect any cytokines in supernatants from co-cultures of 2D2 cells and moDC with MOG peptides (Figure 2D). moDC did elicit production of GM-CSF (but none of the other cytokines in the panel), when co-cultured with CNS-infiltrating effector T cells and MOG35-55 (Figure 2E). The amount of GM-CSF produced was significantly lower than the amount elicited by CNS cDC.

We performed APC assays with resident microglia, and splenic and CNS-infiltrating B cells, as a foil to the CNS DC subsets. B cells, isolated from either the CNS or spleen at peak EAE, induced 2D2 cell proliferation in response to exogenous MOG35-55 peptide (Supplemental Figure 2A). Conversely, they were inefficient at processing and presenting the larger MOG1-125 protein to the naïve myelin-reactive T cells. CNS-infiltrating B cells induced the spontaneous
proliferation of myelin primed effector T cells, but this was not enhanced by the addition of exogenous antigen (Supplemental Figure 2B). MHCII⁺CD45intCD11bint microglia did not stimulate the proliferation of either naïve or effector T cells, even when cultured with MOG₃₅₋₅₅ (Supplemental Figure 2C and D).

cDC express high levels of H2M molecules

We next investigated the mechanism underlying the disparate APC capacities of CNS cDC versus moDC. First, we measured the cell surface density of MHCII molecules on each subset via mean fluorescence intensity (MFI). This analysis revealed the presence of two populations within the CNS CD26⁺ cDC subset, that were distinguished by expression of either high (CD11cintMHCII++) or comparable (CD11хиMHCII⁺) levels of MHCII in comparison to their CD88⁺ counterparts (Figure 3A). The CD26⁺MHCII⁺+ subpopulation also expressed elevated levels of the co-stimulatory markers, CD40, CD80, and CD86 (Figure 3B). In order to determine whether these disparities in MHCII and co-stimulatory molecule expression translated into functional differences, we performed APC assays with the CNS DC subsets side by side. There was no significant difference in the proliferation of 2D2 cells co-cultured with the CD26⁺MHCII⁺ versus CD26⁺MHCII+++ cDC subpopulations. Both of the CD26⁺ cDC subpopulations promoted more 2D2 cell activation than CD88⁺ moDC sorted from the same CNS mononuclear suspension (Supplemental Figure 3). We also measured expression of the inhibitory ligand, PDL1, and found that it was expressed on all three subsets. Blockade of PDL1 did not rescue myelin-specific T cell activation by CNS moDC (data not shown). Based on these results, we concluded that heightened MHCII and/ or co-stimulatory molecule expression was not responsible for the superior antigen presenting capacity of CNS cDC.
H2M (HLA-DM in humans) is a non-classical MHC molecule that facilitates antigen loading into the binding groove of MHCII. It functions by catalyzing the exchange of class II-associated invariant chain peptide (CLIP, a space holding peptide that is inserted into the binding groove during the assembly of MHCII to stabilize its structure), and endosomal peptides (41). H2M-deficient mice are defective in the processing of native MOG for presentation to encephalitogenic T cells and are resistant to EAE, induced either by active immunization or adoptive transfer (42). Therefore, we questioned whether CNS moDC are incompetent APC due to reduced expression of H2M. We found that CNS cDC expressed high levels of H2M protein (Figure 3C, left panel). Conversely, we did not detect H2M protein in CNS moDC. In addition, CNS cDC expressed much higher levels of the transcripts encoding H2M subunits than CNS moDC (Figure 3C, right panel). Hence, insufficient processing and binding of immunogenic myelin peptides to MHCII may underlie the relative inability of CNS moDC to activate encephalitogenic T cells.

cDC and moDC have distinct cytokine profiles

In order to further characterize the immunological properties of the CNS DC subsets, we measured intracellular expression of candidate polarizing factors. We observed distinctive cytokine profiles among the DC subsets, in that CD26+ cDC expressed IL-12p40 following short term incubation with Brefeldin A, while CD88+ moDC expressed IL-23p19 and IL-10 (Figure 3D). IL-12p40 is a subunit of both IL-12 and IL-23. Production of IL-12p40 by CNS CD26+ cDC is consistent with their induction of IFNγ in myelin-reactive T cells (Figure 2B, D, and E). Measurement of IL-12p35, the second subunit of bioactive IL-12 heterodimer, was limited by available methods. IL-23 is a heterodimer of IL-12p40 and IL-23p19 (43), and polarizes T cells
toward IL-17 production and the Th17 phenotype (44). Although CNS CD88+ moDC express IL-23p19, they would be unable to synthesize bioactive IL-23 in the absence of IL-12p40. This might explain the failure of CNS moDC to induce IL-17 production upon co-culture with myelin-specific T cells (Figure 2D and E). Instead, CD88+ moDC production of IL-10 may exert a regulatory influence on the inflammatory process. Stimulation of the CNS DC subsets with LPS altered the level, but not the pattern, of cytokine production (Figure 3D).

moDC efficiently phagocytose myelin

Having established CNS-derived cDC as superior APC, we questioned the role of moDC in neuroinflammatory disease. We recently reported that CD11b+CD11c+ DC evolve during the course of EAE, and shift from a proinflammatory phenotype (denoted by expression of the enzyme, inducible nitric oxide synthase (iNOS)) at clinical onset, to a non-inflammatory or immunosuppressive state (denoted by expression of the alternative enzyme, arginase-1 (Arg1)), in anticipation of clinical remission/ stabilization (4). During this transition, some of the CNS DC acquire an iNOS+Arg1+ intermediary phenotype. Our published study did not address the lineage of the CNS DC populations. Upon revisiting this issue, we found that that iNOS and/ or Arg1 expression is restricted to moDC throughout the disease course (Figure 4A).

Since the monocyte/ macrophage lineage is specialized in phagocytosis, we also compared the capacity of CNS moDC and cDC to internalize extracellular myelin. We isolated total CNS mononuclear cells from mice with EAE and cultured them overnight with purified myelin that had been obtained from a naïve mouse and labeled with the pH-sensitive dye, pHrodo. In a representative experiment, we detected myelin in the cytoplasm of approximately 55% of CD88+ moDC compared with ~25% of cDC (Figure 4B). Similar results were obtained
when FAC sorted moDC and cDC were cultured independently (data not shown). Myelin phagocytosis by both DC subsets was inhibited by the addition of cytochalasin D, demonstrating dependence on actin polymerization (45) (data not shown). These data indicate that the inability of CNS moDC to present antigen to myelin-specific T cells is not secondary to a defect in myelin phagocytosis. Moreover, our results suggest potential roles for CD88+ moDC in modulation of the inflammatory milieu and clearance of myelin debris.

**cDC are present in the naïve CNS and expand during autoimmune demyelinating disease**

We hypothesized that resident cDC are the primary APC encountered by encephalitogenic T cells as they enter the uninflamed CNS, and that cDC drive T cell activation at the inception of neuroinflammation, and possibly during epitope spreading. In support of this theory, FLT3-dependent, radiosensitive DC were recently discovered in the meninges under steady state conditions (16, 29, 46). Similarly, we detected MHCII⁺CD11c⁺CD26⁺ DC in the naïve meninges and brain and, to a lesser extent, in the naïve spinal cord, by flow cytometric analysis (Figure 5A). The CNS DC population in naïve C57BL/6 mice was predominantly CD88⁻CD26⁺ (Figure 5A). CD26⁺, but not CD88⁺, CD11c⁺ DC isolated from the CNS of unmanipulated *Zbtb46-gfp* reporter mice expressed GFP (Figure 5B). MHCII⁺CD11c⁺CD26⁺ cDC isolated from uninflamed CNS tissues readily activated naïve, myelin-specific CD4⁺ T cells directly *ex vivo* and enhanced T cell survival during short term culture (Figure 5C).

Time course studies revealed that cDC and moDC progressively expand from baseline through the onset and peak of EAE in every CNS compartment that we examined (Figure 5D). Although cDC consistently accumulated in the brain and spinal cord in association with increasing neurological disability, their expansion was overshadowed by a dramatic rise in the
frequency of moDC. Consequently, moDC were the predominant DC subset in the brain and spinal cord by peak disease. In contrast, the frequency of meningeal cDC exceeded that of moDC at both the pre-clinical and peak stages of EAE. We and others have found the choroid plexus and meninges to be the initial portal of entry of CNS-infiltrating T cells during EAE (unpublished data and 17, 19, 20). Meningeal inflammation is widespread in the early stage, as well as progressive forms, of MS, and is spatially associated with cortical pathology (14, 47). In a survey of postmortem brain and spinal cord tissues from 11 patients with MS, cells expressing mature DC markers were consistently detected in meningeal infiltrates and were often in close proximity to, or in contact with, proliferating lymphocytes (14). Therefore, the presence of cDC in the meninges might facilitate the development of nascent demyelinating lesions in the subpial grey matter in addition to the white matter.

cDC are critical for initiation of experimental autoimmune encephalomyelitis

To definitively investigate the role of cDC in EAE, we employed transgenic mice with diphtheria toxin receptor (DTR) expressed under control of the ZBTB46 promoter (Zbtb46-dtr mice) (32). ZBTB46 is expressed by endothelial cells as well as DC (33). Consequently, we generated Zbtb46-dtr→WT bone marrow chimeric mice to restrict diphtheria toxin (DT) to the cDC population. In parallel, we generated CD11c-dtr→WT bone marrow chimeric mice, which target both cDC and moDC (48), as a positive control, and WT→WT bone marrow chimeric mice as a negative control. We optimized the DT dosing strategy to deplete DC in the CNS prior to disease induction, and to maintain depletion through the clinical course. Following three doses of DT, CD26+ cDC counts were reduced by over 50% in the brain, spinal cord, and meninges of
both sets of DTR bone marrow chimeras (Figure 6A). As expected, CD88\(^+\) moDC were also diminished in the brain and spinal cords of DT treated CD11c-\(dtr\), but not Zbtb46-\(dtr\), chimeras.

All of the chimeric mice were treated with 3 daily doses of DT before injection with highly purified myelin-primed Th17 cells. Daily DT injections were continued through the experimental time course. Global depletion of DC completely prevented clinical EAE in CD11c-\(dtr\)\(\rightarrow\)WT bone marrow chimeras (Figure 6B). Strikingly, selective depletion of cDC in Zbtb46-\(dtr\)\(\rightarrow\)WT chimeras reduced the incidence of clinical EAE by half in comparison to the WT\(\rightarrow\)WT chimeras (40% versus 80%). Histological findings reflected the clinical scores in that there was no evidence of CNS parenchymal inflammation or tissue damage in spinal cord sections from Zbtb46-\(dtr\)\(\rightarrow\)WT mice that remained free of neurological deficits (Supplemental Figure 4). In fact, we did not detect any MHCII\(^+\) APC in those sections. The susceptibility of some of the DT treated Zbtb46-\(dtr\)\(\rightarrow\)WT mice to EAE may reflect incomplete cDC depletion, as a small number of CD11c\(^+\)CD26\(^+\) cells persisted in the CNS of DT treated mice (Figure 6A). DT treatment resulted in decreased numbers of donor CD4\(^+\) T cells in the brain, spinal cord, meninges, and the CNS draining cervical lymph nodes of Zbtb46-\(dtr\)\(\rightarrow\)WT, as well as CD11c-\(dtr\)\(\rightarrow\)WT, adoptive transfer recipients (Figure 6C). Collectively, our data indicate that cDC promote the accumulation/ expansion of myelin reactive T cells in the CNS during the effector stage of EAE, thereby increasing susceptibility to clinical disability. The Zbtb46-\(dtr\)\(\rightarrow\)WT mice that did develop disease had a similar day of onset, maximum score, and degree of weight loss compared with symptomatic WT\(\rightarrow\)WT adoptive transfer recipients (Supplemental Figure 5 and data not shown). This suggests that cDC play a pivotal role in the inception of the neuroinflammatory response, but that other APC subsets, such as infiltrating B cells, might be able to perpetuate disease activity thereafter.
DISCUSSION

The current study adds to a growing body of literature that challenges the traditional portrayal of the uninjured CNS as an immune privileged site. Numerous laboratories have documented the presence of a network of MHCII+ innate immune cells, many of which have DC characteristics, in human, as well as rodent, brain and spinal cord under steady state conditions (11, 15–17). These cells are concentrated in the meninges, choroid plexus, and perivascular space, regions that interface with the periphery, where they are optimally positioned to serve as sentinels and first responders to foreign threats. The possibility that the same leukocytes could be subverted to support autoimmune neuroinflammation is supported by the fact that DC are enriched in perivascular infiltrates within MS white matter lesions as well as in the meninges overlying cortical lesions (13–15).

Two recent studies suggested that a population of DC normally present in the healthy mouse meninges and choroid plexus is of the cDC lineage (16, 29). Parabiont experiments indicate that CNS resident DC originate from a pre-DC bone marrow precursor and have a half-life of 5-7 days (29). However, the role of those cells in autoimmune demyelination was not addressed. The current study corroborates these earlier findings by demonstrating that the dominant DC population in the uninflamed CNS expresses markers and transcripts typical of cDC (Figures 1 and 5). Our data indicate that CD26+ZBTB46+ DC are unique among CNS APC subsets in their ability to process immunogenic peptides from larger myelin fragments and activate myelin-specific naïve, as well as effector, CD4+ T cells to proliferate and produce pro-inflammatory cytokines (Figure 2). Most importantly, selective depletion of cDC led to a reduction in the frequency of transferred myelin-primed CD4+ T cells in the meninges, brain,
spinal cord, and cervical lymph nodes, and significantly lowered the incidence of clinical EAE (Figure 6).

DT treatment of Zbtb46-dtr→WT adoptive transfer recipients targets cDC in the periphery as well as in the CNS. However, we believe that cDC depletion in the CNS is most likely responsible for the results shown in Figure 6B. This is supported by prior evidence that MOG-specific donor T cells, analyzed via flow cytometry at serial time points following adoptive transfer, first upregulate activation markers and proliferate within the CNS, as opposed to peripheral lymphoid tissues, 1-2 days prior to expected clinical onset (unpublished data). Our current data demonstrate that cDC support the expansion/survival of encephalitogenic T cells within the CNS and play an important role in disease initiation (Figure 6 B and C). An analogous role of CNS DC as APC in the pathogenesis of human autoimmune demyelinating disease is suggested by the presence of myelin-laden cells expressing mature DC markers in close proximity to, or in contact with, proliferating lymphocytes within active MS lesions, as well as in the overlying meninges (15). The choroid plexus and meninges have been increasingly recognized as portals of entry for the infiltration of encephalitogenic T cells into the CNS (18–21). Furthermore, physical interactions between acutely activated myelin-reactive T cells and perivascular phagocytes have been directly visualized within the meningeal space at EAE onset using 2 photon microscopy (21, 49). We found that cDC accumulate rapidly in the meninges during EAE (Figure 5D). We are currently investigating whether activated encephalitogenic T cells drive the proliferation and maturation of CNS cDC, possibly via production of FLT3 ligand and/or GM-CSF (50).

Deficiency in H2M impedes endocytic processing and the loading of MHCII molecules with native peptides (51). Hence, low H2M expression may be, in part, responsible for the
inability of MOG1-125-pulsed CNS moDC to activate MOG-reactive T cells (Figure 2). In support of that hypothesis, it was previously shown that APC isolated from H2M-deficient mice are unable to process whole recombinant MOG protein into immunogenic epitopes (42, 52). We found that MOG35-55-pulsed CNS moDC are also poor APC. This observation is consistent with published studies that show APC from H2M-deficient C57BL/6 mice to be impaired in the presentation of short peptides (including MOG35-55), as well as whole proteins, to CD4+ T cells (42, 51, 53). The inefficient presentation of exogenous peptides by H2M-deficient APC may reflect the need for those peptides to displace high affinity CLIP peptides, which are bound to cell surface MHCII at an elevated density in the absence of H2M (51). H2M-independent pathways undoubtedly also contribute to APC dysfunction of CNS moDC. Based on the data in Figure 4, moDC production of immunosuppressive cytokines might represent one such pathway.

The success of anti-CD20 B cell depleting monoclonal antibodies in suppressing MS lesion development and clinical exacerbations underscores the importance of B cells in MS pathogenesis (54). These reagents spare plasma cells, and therapeutic responsiveness does not correlate with a reduction in circulating or cerebrospinal fluid antibody levels, indicating an antibody independent mechanism of action (55). A leading hypothesis is that B cell depletion ameliorates relapsing MS by limiting antigen presentation to encephalitogenic T cells. Meningeal B cell follicles have been discovered adjacent to large subcortical lesions in some patients with secondary progressive MS (56). CD3+ T cells are a regular component of the meningeal follicles, raising the possibility that B cells also serve as APC in that context (57). Our data is consistent with a potential role of B cells as APC in EAE. B cells isolated from the CNS during EAE were able to present exogenous MOG35-55 peptide to autoreactive T cells and stimulate their proliferation ex vivo (Supplemental Figure 2). However, in contrast to CNS cDC, they were
inefficient at processing larger MOG proteins for presentation of immunogenic epitopes. This may be explained by the different pathways employed by B cells to acquire peptide versus protein antigen. Protein antigen uptake by B cells is primarily mediated through the B cell receptor (BCR) (58), such that larger myelin fragments might only be efficiently internalized by myelin-specific B cells. However, the frequency of myelin-specific B cells is highly variable in MS, and appears to be low at early time points in EAE (59). In fact, we found that B cells, isolated from the CNS at EAE onset or peak, failed to phagocytose pHrodo-labeled myelin ex vivo (data not shown). Based on these collective data, we speculate that infiltrating B cells can promulgate neuroinflammation in the setting of established autoimmune demyelinating disease, once myelin peptides are released into the CNS microenvironment via proteolysis. Conversely, the ability of CNS cDC to process large myelin peptides/proteins for presentation to naïve T cells may make them uniquely qualified to serve as APC when antigen load is low. In support of that theory, encephalitogenic donor T cells are incapable of initiating neuroinflammation in naïve adoptive transfer recipients when polyclonal B cells are the sole APC, unless the precursory frequency of MOG-specific B cells is artificially heightened (60). We have previously shown that CNS-infiltrating myeloid cells, including CD11c+ DC, shift from a pro-inflammatory phenotype during early EAE to an alternatively activated phenotype immediately prior to clinical remissions, which correlates with changes in APC function (4). In future studies we plan to investigate how APC evolve, on the cellular subset as well as the population level, across successive stages of EAE.

Despite the significant advances that have been made in MS therapeutics over the past 15 years, none of the medications approved for the management of MS, including anti-CD20 monoclonal antibodies, are cures, and none are effective in all patients. Up to the present,
pharmaceutical development has focused on lymphocytes, and myeloid cells have largely been ignored. We and others have shown that the pathological mechanisms that drive CNS injury in MS are diverse, and that the relative contribution of specific cytokine pathways and immune effector cell subsets can vary from one patient to another (61, 62). Such differences may translate into different patterns of therapeutic responsiveness to individual disease modifying therapies. For example, B cell targeting approaches may be particularly effective in MS patients who harbor a high frequency of anti-myelin antibody expressors in their B cell repertoire, which would favor the uptake and presentation of myelin antigens by B cells (63). Conversely, cDC modulating agents might be effective in individuals who are early in the disease course, when there is a lower lesion burden and less myelin breakdown, thereby limiting the accessibility of immunogenic peptides to B cells and lending a competitive advantage to CNS resident cDC as APC. We would argue that inactivation of cDC in some individuals with MS might abort the escalation of neuroinflammation and have long lasting benefits. Furthermore, the discovery of myelin-laden DC in MS lesions in chronic progressive, as well as recently diagnosed, patients raises the possibility that cDC targeting might be therapeutically beneficial over a broad range of MS clinical subsets and disease stages (15, 47, 56).
METHODS

Mice. C57Bl/6 and B6.Ly5.1 mice were from Charles River Laboratories. Zbtb46-gfp, Zbtb46-dtr, CD11c-dtr, and 2D2 TCR transgenic mice were from the Jackson Laboratory. Both male and female mice, age 6-12 weeks, were used in experiments. All mice were bred and maintained under specific pathogen-free conditions at the University of Michigan.

Induction and assessment of EAE. For adoptive transfer, C57Bl/6 mice were subcutaneously immunized over the flanks with 100 µg MOG35-55 (Biosythesis) in complete Freund’s adjuvant (Difco). At 10-14 days post-immunization (p.i.), the draining lymph nodes (inguinal, brachial, and axillary) were collected and cultured for 96 hours in the presence of 50 µg/mL MOG35-55, 8 ng/ml IL-23 (R&D Systems), 10 ng/ml IL-1α (Peprotech), and 10 µg/mL anti-IFNγ (Clone XMG1.2, BioXcell). At the end of culture, CD4+ T cells were purified with CD4 positive selection magnetic beads (Miltenyi), and 3-5x10^6 CD4+ T cells were transferred intraperitoneally into naïve recipients. For active EAE, mice were immunized as above and injected with 300 ng of pertussis toxin (List Biological) on days 0 and 2 p.i.. EAE was assessed by a clinical score of disability: 1, limp tail; 2, hind-limb weakness; 3, partial hind-limb paralysis; 4, complete hind-limb paralysis; and 5, moribund state.

Cell Isolation. Mice were anesthetized with isoflurane and perfused with PBS. Meninges were isolated by removing the calvarium, placing the calvarium in a dish with PBS, and stripping the meninges from the inner surface. The meninges tissue and loosely adherent cells released in the PBS were collected, pelleted, and incubated in a solution of HBSS with 1 mg/ml collagenase A (Roche) and 1 mg/ml DNase 1 (Sigma-Aldrich) for 20 minutes at 37°C. The meninges were then
passed through a 70-µm mesh filter to remove debris and generate a single cell suspension. The brain was removed from the skull, and the spinal cord was flushed from the spinal column with PBS. The brain and spinal cord were homogenized with an 18G needle in the collagenase solution and incubated at 37°C for 20 minutes. Mononuclear cells were separated from myelin with a 27% Percoll solution (GE Healthcare). Spleens were isolated and passed through a 70-µm mesh filter to generate a single cell suspension. Red blood cells from the spleen were lysed by a brief incubation in ACK lysis buffer (Quality Biological) followed by a wash in PBS.

**Cytokine production by DC subsets.** Mononuclear cells were isolated as above and cultured with Brefeldin A (BFA) (10 µg/mL) or BFA + LPS (1 µg/ml) for 4 hours. At the end of culture, cells were collected and stained for cytokines by intracellular flow cytometry.

**Ex vivo cultures.** Mononuclear cells were isolated as above, and DC subsets, microglia, and B cells were FAC sorted from the CNS and spleen according to the indicated surface markers. For purification of naïve CD4+ T cells, lymph nodes and spleen were collected from naïve 2D2 TCR transgenic mice. CD4+ T cells were enriched by positive selection with magnetic beads (Miltenyi), and naïve T cells were further purified by flow sorting for live CD4+CD44+CD62L+ T cells. For purification of effector T cells, mononuclear cells from the CNS were flow sorted for live CD45+CD11b−CD3+CD4+MHCII− T cells. T cells were labeled with CFSE according the manufacturer’s instructions (ThermoFisher). APC and T cells were co-cultured for 96 hours at a ratio of 1:20 (typically 5,000 myeloid cells with 95,000 T cells) with media, myelin peptide (MOG35-55 peptide [Biosynthesis]), or myelin protein (MOG1-125 [Anaspec]). At the end of culture, cells were cultured with PMA (50 ng/ml), ionomycin (2 µg/ml), and BFA (10 µg/ml) for
4 hours to stimulate cytokine production. Cells were collected and stained for activation by surface markers and cytokine production by intracellular staining.

*Multiplex cytokine analysis.* Cytokine levels were measured using Luminex multiplex bead-based analysis (Millipore) used the Bio-Plex 200 system (BD Biosciences) according to the manufacturer’s protocols. Total protein was measured via Bradford assay (ThermoFisher) and used to normalize analyte concentrations to total protein.

*Phagocytosis.* Myelin was purified from the naïve mouse brain by ultracentrifugation as previously described (64). Purified myelin was conjugated to the pH-sensitive dye pHrodo Red, succinimidyl ester (ThermoFisher) per the manufacturer protocol. Mononuclear cells were isolated from the CNS at the peak of adoptive EAE and cultured overnight with the unlabeled or pHrodo-labeled myelin (1 µg/200 µl). Cells were collected, washed, and stained for flow cytometry. Phagocytosis was determined by pHrodo Red fluorescence.

*Flow cytometry.* Cells were labeled with fixable viability dye (eFluor506, eBioscience), blocked with anti-CD16/32 (Clone 2.4G2, hybridoma), and stained with fluorescent antibodies. For intracellular staining of cytokines and enzymes, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained with fluorescent antibodies. For intracellular staining of ZBTB46, cells were fixed and permeabilized with the Transcription Factor Buffer Set (BD Pharmingen). Data was acquired using a FACSCanto II flow cytometer or FACSaria III flow sorter (BD Biosciences) and analyzed with FlowJo software (Treestar). Cells were sorted with a FACSaria III flow sorter (BD Biosciences).
**Antibodies.** The following antibodies were obtained from BD Biosciences: αH2M [2E5A], APC-Cy7-(αIFNγ [XMG1.2], αLy6G [1A8]), Biotin-(αRat IgG1 [RG11/39.4]), FITC-(αCD40 [HM40-3], αCD62L [MEL-14], αRat IgG1 [RG11/39.4]), PE-(αCD4 [GK1.5 and RM4-5], αZBTB46 [U4-1374]). The following antibodies were obtained from Biolegend: APC-(αCD26 [H194-112], αCD88 [20/70]), FITC-(αCD26 [H194-112]), Biotin-(αCD88 [20/70]), PE-(αCD88 [20/70], αPD-L1 [10F.9G2]), PE-DAZZLE-(αCD11c [N418]). The following antibodies and reagents were obtained from ThermoFisher: APC-(αCD19 [MB19-1], αCD44 [IM7], αIL-23p19 [fc23cpg], Streptavidin), APC-Cy7/ APC-eF780-(αCD11b [M1/70], αCD45.2 [104], αMHCII [M5/114.15.2], Biotin-(α-I-Ab [AF6-120.1]), BV510-(αCD45 [30F11], αCD45.1 [A20]), eF450-(αCD4 [RM4-5]), eF700-(Streptavidin), FITC-(αCD45.2 [104], αCD317 [eBio927], αMHCII [M5/114.15.2], Streptavidin), PE-(αCD86 [GL1], αIL-10 [JES5-16E3], αGM-CSF [MP1-22E9]), PE-Cy7-(αCD11b [N418], Streptavidin), PE-eF610-(αiNOS [CXNFT]), PerCP-Cy5.5-(αCD11c [N418], αIL-12p40 [C17.8], αMHCII [M5/114.15.2]), PerCP-eF710-(α-I-Ab [AF6-120.1]), V506 Fixable Viability Dye). The following antibodies were obtained from R&D Systems: αArg1 [sheep IgG], FITC-(αArg1 [sheep IgG]). Alexa-488-Donkey-αSheep was obtained from Life Technologies.

**Nanostring gene expression analysis and qPCR.** Sorted cells were resuspended in RLT buffer, and cell lysates were directly analyzed for expression of 750 immune-related genes with the nCounter PanCancer Immune Panel (Nanostring Technologies). Data was processed using the nSolver Analysis Software by normalization to the geometric mean of positive controls and housekeeping genes. R was used to perform paired Student’s t-tests and calculate Benjamini &
Hochberg’s false discovery rate (FDR), comparing the gene expression of the CD26+ and CD88+ populations. Cells used to confirm the Nanostring results via qPCR were resuspended in RLT buffer before Qiagen RNeasy RNA purification. Relative mRNA levels were quantified by SYBR Green qPCR performed on an iQ Thermocycler (Bio-Rad).

**Bone marrow chimeras.** B6.Ly5.1 (CD45.1+) congenic hosts were lethally irradiated with 1300 Rad split into two doses and reconstituted by tail vein injection of 4x10^6 CD45.2+ bone marrow cells from WT, CD11c-dtr, or Zbtb46-dtr donors. Mice were allowed to reconstitute for 6 weeks prior to use.

**DT Ablation.** Diphtheria toxin (Sigma) was administered in two stages. Three daily doses of 1 µg/20 g mouse (50 µg/kg) in 200 µl of PBS were given i.p. prior to the assessment of DC depletion or to the induction of EAE. Daily doses of 100 ng/20 g mouse (500 ng/kg) in 200 µl of PBS were given i.p. starting on the day of adoptive transfer and continued until the end of the experiment.

**Histology.** Spinal columns were harvested from mice perfused intracardially with 1X PBS and 4% PFA, post fixed with 4% PFA, decalcified with 0.5 M EDTA, cryopreserved with sucrose, and embedded in OCT for cryosectioning. 12 µm sections were stained with the following primary antibodies; rat αMBP_{82-87} (Millipore) and biotinylated rat αMHCII (ThermoFisher). Avidin/biotin block (ThermoFisher) was used to prevent streptavidin binding to endogenous biotin. Normal goat serum (Sigma) was used to block non-specific binding of secondary goat α-rat IgG Alexa Fluor 488 (ThermoFisher). Steptavidin-APC (ThermoFisher) was used to visualize
bound biotinylated αMHCII. Confocal images were acquired using an Olympus IX83 with Fluoview 31 software.

Statistics. Statistical analysis was performed in GraphPad Prism (v7) using paired or un-paired 2-tailed Student’s t test, or 1-way or 2-way ANOVA with correction for multiple comparisons with Tukey’s post-hoc test, as indicated in the legends. Disease curves were compared by 2-way ANOVA. Outliers were identified by ROUT analysis and removed when indicated. A p value < 0.05 (*) was considered significant. p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****).

Study Approval. All animal experiments were performed in accordance with an IACUC-approved protocol at the University of Michigan.
AUTHOR CONTRIBUTIONS

D.A.G., P.C.D., and B.M.S. contributed to study concept, design, and data interpretation.

P.C.D., and B.M.S. contributed to drafting the manuscript and figures.
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Figure 1 – CD26\textsuperscript{+}ZBTB46\textsuperscript{+} cDC accumulate in the CNS during adoptively transferred EAE.

EAE was induced by adoptive transfer of WT myelin-primed CD4\textsuperscript{+} Th17 cells into naive syngeneic hosts. (A) Brain mononuclear cells were harvested at peak EAE and analyzed by flow cytometry. Dot plots are gated on the population indicated directly above each plot. The numbers indicate percent of the gated population. The data are representative of 3 experiments. (B) MHCII\textsuperscript{+}CD11c\textsuperscript{+} CD88\textsuperscript{+} or CD26\textsuperscript{+} cells were purified from the CNS (N=3 per group) by flow sorting, and gene expression was measured by Nanostring nCounter analysis. Genes with a false discovery rate (FDR) < 0.10 are identified in the heatmaps. The right panel shows Flt3, Tlr3, and Tlr4 mRNA levels in paired DC subsets from individual mice. P values were determined by paired, 2-tailed Student’s t test. **p<0.01. (C, D) Expression of ZBTB46 was measured in MHCII\textsuperscript{+}CD11c\textsuperscript{+} CD26\textsuperscript{+} or CD88\textsuperscript{+} brain mononuclear cells, harvested at peak EAE, by flow cytometry. The open histograms reflect intracellular staining with anti-ZBTB46 antibodies (C) or GFP expression in cells from Zbtb46\textsuperscript{gfp\textsuperscript{+/+}} reporter mice (D). The shaded grey histograms reflect the (C) isotype or (D) non-reporter control.
Figure 2 – CNS cDC stimulate naïve and effector myelin-specific T cells to proliferate and produce pro-inflammatory cytokines, while CNS moDC are incompetent APC.

EAE was induced by active immunization with MOG35-55 peptide in CFA. CNS mononuclear cells were harvested at peak disease. CD26+ or CD88+ DC subsets (CD45+MHCII+CD11c+) were purified by FAC sorting and co-cultured with MOG-reactive T cells in the presence or absence of myelin peptide (MOG35-55) or myelin protein (MOG1-125). (A, B, D) The CNS DC subsets were co-cultured with CD44-CD62L+ CD4+ T cells that had been isolated from the spleens and lymph nodes of naïve 2D2 TCR transgenic mice. (A, B) T cell proliferation was measured by CFSE dilution. The percent of CD4+ T cells that underwent 1 or more division, or that expressed the activation marker CD44, is shown for each group. (B) Cytokine production was measured by
intracellular flow cytometry. The percent of cytokine producers among total CD4+ T cells is shown. (D) Cytokine levels were measured in culture supernatants via a multiplex Luminex bead-based assay. (C, E) CNS DC subsets were co-cultured with CD4+ T cells isolated from the CNS at the peak of EAE. (C) T cell proliferation was measured as in (A). (E) Cytokine levels were measured in culture supernatants via Luminex. (B-E) Each circle represents a data point generated with CNS DC subsets isolated from a single mouse. Connected circles indicate paired samples from the same mouse. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by paired, 2-tailed Student’s t test. Data in A, B and D, and in C and E, are from individual experiments, that are representative of 2-4 independent experiments with similar results. N=3-5 mice per group per experiment.
Figure 3 – CNS moDC are deficient in expression of H2M and have a distinct cytokine profile in comparison to CNS cDC.

EAE was induced by adoptive transfer of WT myelin-primed Th17 cells into naïve syngeneic hosts. (A, B) CNS mononuclear cells were isolated at peak clinical severity and subjected to flow cytometric analysis. The geometric mean fluorescence intensity (MFI) of MHCII and costimulatory molecules (B) was measured on gated DC subsets. (C) H2M expression was assessed in CNS DC subsets by flow cytometry (left). The levels of transcripts encoding MHCII and H2M subunits were quantified in FAC sorted CD88\(^+\) and CD26\(^+\) CNS DC via qPCR (right). (D) CNS mononuclear cells, isolated from individual mice with EAE, were cultured for 4hr with Brefeldin A (BFA), with or without LPS. Cytokine production was assessed by intracellular staining and flow cytometry. The data are shown as the percentage of cytokine positive cells within the indicated DC population. Each symbol represents a data point generated from a single mouse. Connected symbols indicate paired samples from the same mouse. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical significance was determined using 1-way (D) or 2-way (A, B) ANOVA with Tukey’s post-hoc test or (C) paired 2-tailed Student’s t test. N=3-5 mice per group or condition. All data are representative of at least 2 experiments. All error bars indicate mean ± SEM.
Figure 4 – CNS moDC express iNOS and Arg1 and efficiently phagocytose myelin.

(A) EAE was induced by active immunization with myelin peptide, and CNS mononuclear cells were isolated at clinical onset (left panels) or peak disease (right panels). Expression of iNOS and ARG1 in CD88+ or CD26+ CNS DC was assessed by intracellular flow cytometry. All of the dot plots are gated on MHCII+CD11c+ cells. Cells in the CD88 versus CD26 dot plots are color coded based on patterns of iNOS and ARG1 expression. (B) EAE was induced by adoptive transfer of WT myelin-primed Th17 cells. Mononuclear cells were isolated from the CNS at the peak of EAE and cultured overnight with unlabeled or pHrodo-labeled purified myelin. Phagocytosis was measured as the percentage of pHrodo+ cells within gated CD26+ or CD88+ DC populations. Each symbol represents a data point generated from a single mouse. Connected symbols indicate paired samples from the same mouse. Data are representative of 2 experiments. *p<0.05 by paired, 2-tailed Student’s t test. N=3-5 mice per group or condition.
Figure 5 – cDC reside in the naïve CNS and expand during EAE.

(A) Mononuclear cells were isolated from the naïve brain, meninges, and spinal cord, and analyzed by flow cytometry. The dot plots are gated on MHCII⁺CD45hiCD11c⁺ cells. (B) CNS mononuclear cells harvested from naïve Zbtb46gfp/+ reporter mice or Zbtb46⁺/+ controls were analyzed for GFP expression, gating on MHCII⁺CD11c⁺ DC subsets. (C) MHCII⁺CD11c⁺CD26⁺ cDC were isolated from the naïve CNS and co-cultured with naïve 2D2 transgenic T cells in the presence or absence of MOG peptide. 2D2 cells were also cultured in the absence of APC as a negative control. 2D2 proliferation was measured by CFSE dilution, and activation by upregulation of CD44 (left). The numbers in the histograms and dot plots represent the percent of 2D2 T cells that divided or expressed CD44, respectively. Live 2D2 cells were counted at the beginning and completion of culture (right). Connected symbols indicate paired samples from the same mouse. (D) Cells were isolated from the naïve CNS or from the CNS during the pre-clinical or peak stages of adoptively transferred EAE. CNS DC subsets were quantified by flow cytometry. Each symbol represents a data point generated from a single mouse. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical significance was determined using (C) paired, 2-tailed Student’s t test or (D) 1-way ANOVA with Tukey’s post-hoc test. N=3-5 mice per group or condition. All data are representative of at least 2 experiments. Error bars indicate mean ± SEM.
Figure 6 – Depletion of cDC in adoptive transfer recipients results in a decreased number of myelin primed donor T cells in the CNS and reduces the incidence of clinical EAE.

Bone marrow chimeric mice were generated by reconstituting lethally irradiated CD45.1+ hosts with CD45.2+ WT, CD11c-dtr, or Zbtb46-dtr bone marrow cells. (A) Naïve chimeric mice in each group were treated with DT or PBS for 3 consecutive days. CNS cDC and moDC subsets were quantified by flow cytometric analysis. (B, C) Chimeric mice were treated with DT as in panel A, and EAE was induced by the adoptive transfer of WT myelin-primed Th17 cells. Daily DT injections were continued throughout the clinical course. (B) Mice were monitored on a daily basis and rated for degree of neurological disability by an examiner blinded to the identity of the experimental groups. Clinical scores and incidence are shown for each group. (C) Total number of donor (CD45.1+) CD4+ T cells were enumerated in DT treated adoptive transfer recipients 1-2 days prior to expected clinical onset. Each symbol in A and C represents a data point generated from a single mouse. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical significance was determined by (A) unpaired 2-tailed Student’s t test, (B) 2-way ANOVA, or (C) 1-way ANOVA with Tukey’s post-hoc test. Data are combined from (A), or representative of (B, C), at least 2 experiments with N=3-15 mice per group or condition. All error bars indicate mean ± SEM.