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MHC Class I-Restricted Myelin Epitopes Are Cross-Presented by Tip-DCs That Promote Determinant Spreading to CD8+ T Cells

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Abstract

Myelin presentation to T cells within the central nervous system (CNS) sustains inflammation in multiple sclerosis (MS). $CD4^+$ and $CD8^+$ T cells contribute to MS; however, only cells that present myelin to $CD4^+$ T cells have been identified. We show that MHC class I-restricted myelin basic protein (MBP) was presented by oligodendrocytes and cross-presented by Tip-dendritic cells (DCs) during experimental autoimmune encephalomyelitis (EAE), an animal model of MS initiated by $CD4^+$ T cells. Tip-DCs activated naïve and effector $CD8^+$ T cells *ex vivo*, and naïve MBP-specific $CD8^+$ T cells were activated within the CNS during $CD4^+$ T cell-induced EAE. These results demonstrate that $CD4^+$ T cell-mediated CNS autoimmunity leads to determinant spreading to myelin-specific $CD8^+$ T cells that are capable of direct recognition of oligodendrocytes.

Introduction

Multiple sclerosis (MS) is a central nervous system (CNS) autoimmune disease characterized by inflammatory, demyelinating lesions and axonal loss. MS is believed to be triggered by exposure to environmental factors that activate peripheral myelin-specific T cells, allowing them to cross the blood brain barrier that normally restricts entry of naïve T cells. The pathogenesis of MS has been studied in experimental autoimmune encephalomyelitis (EAE), an animal model of MS that is induced by immunization with myelin antigens[1]. Infiltrating $CD4^+$ T cells are re-activated within the CNS by local antigen-presenting cells (APCs) presenting MHC class II-restricted myelin epitopes[2,3]. $CD11c^+$ dendritic cells (DCs) are critical for this re-activation step[4]. The ensuing inflammatory response recruits both monocytes that differentiate into MHC class II^+ APCs after entering the CNS[5,6], and naïve T cells that recognize myelin epitopes distinct from those recognized

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The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Q.J. conducted most of the experiments and analyzed the data; L.C. assisted with some RT-PCR experiments and performed and analyzed *in vivo* experiments demonstrating activation of $CD8^+$ T cells within the CNS and critiqued the manuscript; Q.J. and J.M.G. designed the study and wrote the manuscript, J.M.G. secured the funding.

by the initial T cell population. Activation of these naive T cells leads to a phenomenon known as determinant spreading[7,8] that is important for sustaining chronic inflammation.

The mechanisms studied in EAE center on the activity of myelin-specific $CD4^+$ T cells. However, $CD8^+$ T cells are strongly implicated in the pathogenesis of MS[9,10]. $CD8^+$ T cells outnumber $CD4^+$ T cells in both acute and chronic MS lesions[11,12]. The number of $CD8^+$ T cells and macrophages also correlates with the extent of axonal damage[13,14]. $CD8^+$ T cells appear more antigen-experienced than $CD4^+$ T cells in the CNS and blood of MS patients, and $CD8^+$ but not $CD4^+$ T cell clones persist over time in both of these compartments[11,15–17]. Neuroantigen-specific $CD8^+$, but not $CD4^+$, T cells are also more abundant in the CNS in MS patients compared to healthy controls[18,19]. The contribution of $CD8^+$ T cells to CNS autoimmunity is not well understood. Some studies attribute an immunoregulatory function to $CD8^+$ T cells in $CD4^+$ T cell-induced EAE[10], although the antigen specificity of the $CD8^+$ T cells in these studies was not defined. Other studies have shown that myelin-specific $CD8^+$ T cells can not only potentiate $CD4^+$ T cell-mediated EAE[20], but also mediate CNS autoimmune disease on their own[21–23]. These diverse results suggest that $CD8^+$ T cells may play a complex role in CNS autoimmunity that can result in exacerbation or amelioration of disease.

Myelin-specific $CD8^+$ T cells must recognize their cognate antigen within the CNS to influence inflammatory responses; however, APCs presenting MHC class I-restricted myelin epitopes have not been identified. We generated unique reagents to detect a MHC class I-restricted epitope of myelin basic protein (MBP) on the surface of APCs. We analyzed CNS cells from mice with EAE induced by $CD4^+$ rather than $CD8^+$ T cells to eliminate the possibility that activated $CD8^+$ T cells may lyse APCs presenting their cognate antigen and prevent their detection. We found that MBP was cross-presented by Tip-DCs derived from inflammatory monocytes that accumulated in the CNS during $CD4^+$ T cell-mediated EAE. These DCs were the only CNS cells capable of activating naïve $CD8^+$ T cells directly *ex vivo*. Consistent with this, we show that naïve $CD8^+$ MBP-specific T cells were recruited to the CNS during EAE and activated *in situ*, demonstrating that determinant spreading to $CD8^+$ T cells can occur during $CD4^+$ T cell-induced EAE. MHC class I-restricted MBP was also directly presented by oligodendrocytes that were induced to express MHC class I during EAE, indicating that these cells as well as the Tip-DCs are potential targets of myelin-specific $CD8^+$ T cells in the CNS.

RESULTS

$CD11c^+$ DCs presented MHC class I-restricted MBP

To identify CNS cells that present MHC class I-restricted MBP, we utilized 8.6 TCR transgenic T cells generated in C3HeB/Fej mice that are specific for MBP79–87 presented by H-$2K^k$ (referred to as MBP H2-K^k)[24]. $CD8^+$ TCR transgenic 8.6 T cells become tolerized in wild-type mice; however, they can be isolated from MBP-deficient mice and used to detect cells presenting endogenous MBP epitopes directly *ex vivo*[24]. To maximize the sensitivity of detection of MBP H2-K^k epitope in this assay, we generated effector $CD8^+$ 8.6 T cells that secrete interferon-γ (IFN-γ) rapidly upon exposure to APCs presenting MBP–H2-K^k. We first assessed whether bulk CNS cells from mice with $CD4^+$ T cell-mediated

EAE or from healthy mice could stimulate $CD8^+$ 8.6 T cell responses directly *ex vivo*. CNS cells were isolated from C3HeB/Fej mice at the peak of EAE induced by immunization with recombinant myelin oligodendrocyte glycoprotein (rMOG), which activates encephalitogenic $CD4^+$ T cells in this strain[25]. Exposure of 8.6 T cells to bulk mononuclear CNS cells isolated from mice with EAE triggered IFN- γ production (Fig. 1a). CNS cells from healthy mice triggered a smaller but detectable percentage of 8.6 effector T cells to produce IFN- γ, indicating that MBP–H2-K^k is presented constitutively at low abundance in the CNS.

We also generated an antibody specific for the MBP–H2-K^k complex by immunizing C3HeB/Fej mice with recombinant H2-K^k protein that was refolded with β2 microglobulin and MBP79–87 peptide. A cell-based ELISA assay was employed to identify antibodies that bind specifically to RMA-S–H2-K^k cells pulsed with MBP79–87 peptide[26]. Over 20 MBP–H2-K^k–specific hybridomas were generated; the antibody designated 12H4 was used in the majority of our studies. Specificity of the 12H4 antibody was confirmed by showing that 12H4 binds to RMA-S–H2-K^k cells pulsed with MBP79–87 but not to RMA-S–H2-K^k cells pulsed with SV40 T antigen (TAg) peptide that also associates with H2-K^k (Fig. 1b). The 12H4 antibody also specifically inhibits IFN- γ production by 8.6 effector T cells in response to either MBP79–87-pulsed splenocytes or CNS cells presenting endogenous MBP, but did not inhibit the response of TAg-specific T cells to splenocytes pulsed with TAg peptide (Fig. 1c).

The 12H4 antibody was used to identify which CNS cell types express MBP–H2-K^k, and these populations were sorted and incubated with 8.6 effector T cells to determine whether these cell types could trigger functional T cell responses. CNS cells from EAE mice were stained with 12H4 and a panel of antibodies specific for cell-surface markers that identify DCs, macrophages and microglia. Gating strategies used for analyses and sorting of the CNS cell types are shown in Supplementary Fig. 1. $CD11c^+$ cells accounted for the highest percentage of $12H4^+$ cells and stimulated the most IFN- γ production by 8.6 effector T cells (Fig. 2a, b). The percentage of $12H4^+$ macrophages was less compared to $12H4^+$ $CD11c^+$ DCs (Fig. 2a and Supplementary Fig. 2a), which may account for the reduced IFN- γ production by 8.6 T cells incubated with macrophages (Fig. 2b). Only a small percentage of microglia were $12H4^+$ (Fig. 2a), and the IFN- γ production elicited by microglia was only slightly above background (Fig. 2b). Although there were fewer DCs than macrophages in the CNS infiltrate, the absolute number of $12H4^+$ DCs was greater than the number of $12H4^+$ macrophage (Supplementary Fig. 2b). Thus, $CD11c^+$ DCs are the predominant APCs presenting MBP–H2-K^k shortly after onset of $CD4^+$ T cell-mediated EAE and microglia contribute only marginally toward presenting MHC class I-restricted MBP at this time point.

Cross-presenting DCs are derived from inflammatory monocytes

The majority of $CD11c^+$ cells in the CNS of rMOG-immunized C3HeB/Fej mice expressed CD11b (Supplementary Fig. 3), consistent with the predominance of myeloid DCs (mDCs) in the CNS reported for other $CD4^+$ T cell-mediated EAE models[4,27–29]. $12H4^+$ DCs were also $CD11b^+$; the few $CD11c^+CD8^+$ and $CD11c^+B220^+$ DCs detected in the CNS did not present MBP–H2-K^k (Supplementary Fig. 3). Previous studies demonstrated that both

mDCs and macrophages that accumulate in the CNS during $CD4^+$ T cell-mediated EAE arise primarily from $CD11b^+Ly6C^+CD62L^+F4/80^+$ inflammatory monocytes that infiltrate the CNS via a CCR2-dependent mechanism[5,6,30]. The $12H4^+$ DCs also exhibited a $CD11c^+CD11b^+Ly6C^+F4/80^+$MHC class $II^+CX3CR1^-$ phenotype consistent with cells differentiated from inflammatory monocytes (Fig. 3), although the $12H4^+$ DCs were $CD62L^-$. CCR2 expression on $12H4^+$ DCs also differed from monocytes in that it ranged from intermediate to negative. Down-regulation of CCR2 by monocytes entering an environment with elevated expression of interleukin 1 (IL-1) and tumor necrosis factor (TNF) has been previously described[31], and this may be the case for CD62L as well.

Because not all of the mDCs in the CNS were $12H4^+$, we investigated whether this subset exhibited any similarities to other DC subsets known to be proficient in cross-presentation. The spleen contains a DC subset that is proficient in cross-presentation and exhibits a unique $CD8\alpha\alpha^+CD11b^-$ $CD205^+$ phenotype[32] with variable expression of CD103 (ref. [33]). This DC subset is generated via a distinct differentiation program dependent on the transcription factors Batf3 and IRF8 as well as the inhibitor of DNA protein Id2 (ref. [34]). Non-lymphoid tissues also contain Batf3–IRF8–Id2 lineage DCs that are proficient in cross-presentation and are phenotypically similar ($CD11b^-$ $CD103^+CD205^+$) to splenic $CD8^+$ DCs except that they do not express CD8 (ref. [34]). The MBP–H2-K^k APCs in the CNS of EAE mice differ from these DC subsets in that they were $CD11b^+$ (Fig. 3) and $CD205^-$ (data not shown). We analyzed expression of CD103 on $12H4^+$ cells to determine if expression of this integrin was shared by cells proficient in cross-presentation. A broad range of CD103 expression was observed on $CD11c^+$ cells in the CNS, with cells that expressed an intermediate amount of CD11b expressing the most CD103 (Fig. 4a, top). However, these $CD103^{Hi}CD11b^{Int}$ cells did not present MBP–H2-K^k. Instead, MBP–H2-K^k was expressed only on cells expressing intermediate amounts of CD103 (Fig. 4a, bottom), which corresponded to cells expressing the most CD11b. Together, the expression patterns of CD11b and CD103 define a unique $CD11b^{Hi}CD103^{Int}$ phenotype for MBP–H2-K^{k+} cells that differs from that seen on other DCs specialized in cross-presentation.

To further confirm that MBP–H2-K^k DCs are distinct from peripheral DCs specialized in cross-presentation, we compared the expression of Batf3, IRF8 and Id2 in $12H4^+CD103^{Int}$ and $12H4^-$ $CD103^-$ DCs sorted from the CNS of EAE mice to that seen in $CD8^+$ and $CD8^-$ DCs sorted from the spleen. We also analyzed expression of TLR3 and the chemokine receptor XCR1 that is expressed on $CD8\alpha\alpha^+$ DCs in the mouse and the human homolog of these DCs in the human, both of which are frequently observed on cross-presenting DCs in the periphery. As expected, splenic $CD8^+$ DCs expressed significantly more IRF8, Id2, TLR3, Batf3 and XCR1 transcripts compared to $CD8^-$ DCs, (Fig. 4b). In contrast, no significant differences in expression of any of these transcripts were observed between $12H4^-$ $CD103^-$ and $12H4^+CD103^{Int}$ CNS DCs (Fig. 4b). Taken together, the phenotype and gene expression pattern are not consistent with MBP–H2-K^{k+} DCs arising from the same Batf3–IRF8–Id2 lineage as peripheral DCs that are specialized in cross-presentation, supporting the notion that MBP–H2-K^k DCs originate from inflammatory monocytes. We then analyzed the expression in MBP–H2-K^k APCs of ZBTB46, a transcription factor whose expression distinguishes classical DCs from other immune cells[35, 36]. Both $CD8^+$ and $CD8^-$ splenic DCs expressed ZBTB46 and low expression was detected in 12H4 $CD103^-$

DCs as well. In contrast, ZBTB46 was not detected in 12H4^{+}CD103^{Int} DCs (Fig. 4b), indicating that they are not derived from the classical DC lineage.

MBP is cross-presented by Tip-DCs

We investigated whether MBP–H2-K^{k+} DCs were Tip-DCs characterized by TNF and inducible nitric oxide synthase (iNOS) expression[37] that are derived from activated monocytes rather than the classical DC lineage[35,36]. Approximately 50% of MBP–H2-K^{k+} DCs isolated from EAE mice expressed TNF directly *ex vivo*, and iNOS was also preferentially expressed in 12H4^{+}CD103^{Int} DCs compared to 12H4^{-} CD103^{-} DCs (Fig. 5a). Only low amounts of IL-12 were detected in 12H4^{+} and 12H4^{-} CNS DCs in EAE mice (data not shown). Interestingly, MBP H2-K^{k+} DCs lacked CCR7 expression (Fig. 5a), while 12H4^{-} CD103^{-} DCs express high levels of transcripts encoding this chemokine receptor. Together, these data indicate that MBP–H2-K^{k+} APCs are Tip-DCs derived from inflammatory monocytes that infiltrate the inflamed CNS and do not traffic to draining lymph nodes.

To confirm that the Tip-DCs are cross-presenting MBP rather than synthesizing MBP and directly presenting it, we analyzed 12H4^{+}CD103^{Int} and 12H4^{-} CD103^{-} cells isolated from EAE mice for expression of classic and golli-MBP transcripts. Golli-MBPs are a family of proteins that are not incorporated into myelin but that are encoded in part by exons shared with classic MBPs, including the exon that encodes MBP79–87. Golli-MBPs are expressed in the nervous system and by multiple types of immune cells, including DCs[38]. Oligonucleotide primers specific for a classic-MBP-specific transcript amplified a strong signal from CNS tissue but did not amplify transcripts from either 12H4^{+}CD103^{Int} or 12H4^{-} CD103^{-} DCs (data not shown). As expected, golli-MBP transcripts were detected in DCs, however; the signal was actually stronger in the 12H4^{-} CD103^{-} DCs, which do not express MBP H2-K^{k} on the surface (Fig. 5b). Similarly, we have not detected 12H4^{+} thymocytes or 12H4^{+} T cells in peripheral lymphoid tissues, even though these cells synthesize golli-MBP (data not shown). This result indicates that the expression of MBP–H2-K^{k} on the surface of DC occurs via cross-presentation of exogenous myelin.

Previous studies of determinant spreading showed that naive, myelin-specific CD4^{+} T recruited to the CNS during EAE are activated *in situ* rather than in peripheral lymph nodes[28]. To determine if the MBP–H2-K^{k+} DCs could promote determinant spreading to naive CD8^{+} T cells, we investigated whether they could activate naive CD8^{+} T cells. CD11c^{+} DCs, macrophages and microglia were sorted from the CNS of EAE mice and cultured with naïve 8.6 T cells. The 8.6 T cells proliferated only in response to DCs presenting endogenous MBP (Fig. 5c). To determine if naive CD8^{+} T cells infiltrate the CNS during EAE and become activated by MBP–H2-K^{k+}APCs, we utilized a different TCR transgenic line in which the T cells are specific for the same MBP–H2-K^{k} epitope but do not undergo T cell tolerance, allowing the periphery to be populated with non-activated MBP-specific CD8^{+} T cells (8.8 mice)[24]. EAE was induced by adoptive transfer of genetically marked CD4^{+} rMOG-specific T cells into 8.8 mice, and cells isolated from the CNS and spleen at the peak of disease were analyzed by flow cytometry. Host 8.8 T cells represented an average of 11% of the total T cell population in the CNS (data not shown, $n = 9$),

demonstrating that $CD8^+$ 8.8 T cells that had not been activated in the periphery enter the CNS during $CD4^+$ T cell-induced EAE. While the 8.8 T cells in the spleen exhibited a naive phenotype, the 8.8 T cells in the CNS exhibited an activated phenotype ($CD44^{Hi}CD62L^{Lo}CD69^{Hi}$) in the CNS (Fig. 5d). It is possible that the 8.8 $CD8^+$ T cells are activated in the cervical lymph nodes rather than within the CNS; however, $12H4^+$ DCs were barely detectable in cervical lymph nodes and the percentage of $12H4^+$ DCs in CNS cells was typically much higher than that seen in lymph nodes (Supplementary Fig. 4). Together these results support the notion that MBP–H2-K^{k+} DCs generated in the CNS during $CD4^+$ T cell-induced EAE are capable of activating $CD8^+$ T cells specific for a different myelin epitope that infiltrate the inflamed tissue.

Oligodendrocytes are induced to express MBP–H2-K^k in EAE

Under healthy conditions, non-hematopoietic CNS cells do not express MHC molecules. We investigated whether the inflammatory milieu generated during $CD4^+$ T cell-mediated EAE induced MHC class I expression on these cells, allowing them to present MBP–H2-K^k. Oligodendrocytes are of particular interest as they synthesize MBP. Astrocytes also present antigen to $CD8^+$ and $CD4^+$ T cells under some circumstances[39]. Cerebral endothelial cells have also been reported to present peptide that was non-invasively injected into the CNS to $CD8^+$ T cells[40], suggesting that these cells might present MBP peptides derived from degraded myelin during EAE. The 12H4 antibody was used to detect presentation of MBP–H2-K^k by these cells, and the individual cell types were sorted from the CNS of EAE mice and cultured with effector 8.6 T cells to detect functional antigen presentation. No MBP H2-K^k complexes were detected on astrocytes or endothelial cells and neither cell type stimulated IFN- γ production by effector 8.6 T cells (Supplementary Fig. 5). In contrast, MBP–H2-K^k was detected on oligodendrocytes in EAE mice (Fig. 6a), and these cells triggered IFN- γ production by 8.6 effector T cells (Fig. 6b), indicating that oligodendrocytes could be direct targets of MBP-specific $CD8^+$ T cells under inflammatory conditions.

The observation that some oligodendrocytes express MBP–H2-K^k shortly after the onset of clinical disease suggested the possibility that CD8 T cell-mediated lysis of oligodendrocytes may be required to provide a source of MBP that is then presented by DCs. To investigate this possibility, we analyzed MBP–H2-K^k expression on oligodendrocytes and $CD45^+$ APCs during the preclinical stage of EAE. In mice induced for $CD4^+$ T cell-mediated EAE, MBP–H2-K^k expression was detected on $CD45^+$ CNS cells harvested from the mice prior to onset of clinical signs (Supplementary Fig. 6). In contrast, a low amount of MHC class I was detected on oligodendrocytes but the cells did not yet express MBP–H2-K^k. These data indicate that MBP–H2-K^k expression on oligodendrocytes is not required prior to expression on DCs and macrophages.

Distinct DCs present MBP–H2-K^k in the healthy CNS

The healthy CNS must contain MBP–H2-K^{k+} APCs because adoptive transfer of MBP-specific $CD8^+$ T cells triggers autoimmunity in the absence of $CD4^+$ T cells[41]. Staining with 12H4 detected MBP–H2-K^k on DCs and macrophages in naive mice, while microglia expressed very little MHC class I and no detectable MBP H2-K^k (Fig. 7a). Different cell

types were sorted from the CNS of healthy mice and cultured with effector 8.6 T cells; DCs and macrophages were sorted together due to their low numbers in the healthy CNS. Only the pooled DC-macrophage fraction triggered IFN- γ production by effector $CD8^+$ T cells (Fig. 7b), indicating that resident DCs and/or macrophages in the healthy CNS constitutively present MBP–H2-K^k.

Consistent with a lack of inflammation, CNS DCs in healthy mice expressed less CD80 and CD86 compared to CNS DCs from sick mice (Fig. 7c). The MBP–H2-K^{k+} CNS DCs in healthy mice were $Ly6C^-$ $CD11b^+F4/80^+CD103^-$ and expressed CX3CR1 and CCR2 (Fig. 7d). Thus, MBP H2-K^{k+} DCs in the healthy CNS exhibit a non-inflammatory phenotype distinct from both cross-presenting DCs in peripheral tissues and from CNS DCs observed under the inflammatory conditions of EAE.

DISCUSSION

APCs that present myelin antigen in the CNS activate the T cells that propagate the inflammatory response and therefore could be therapeutic targets in MS. Although there is compelling evidence that both $CD8^+$ and $CD4^+$ T cells contribute to the pathogenesis of MS, only APCs that present myelin antigen to $CD4^+$ T cells have been characterized. In animal models of MS, both pathogenic and immunoregulatory activities have been attributed to $CD8^+$ T cells. While the mechanisms underlying either of these potential activities are not known, they may reflect interactions with different types of APCs.

We used $CD8^+$ TCR transgenic T cells specific for a MHC class I-restricted epitope of MBP and a newly generated antibody specific for the same epitope to identify cells that present MHC class I-restricted MBP to $CD8^+$ T cells. Although it is not known whether $CD4^+$ or $CD8^+$ T cells initiate MS, we chose to use a $CD4^+$ T cell-mediated EAE model for these studies for two reasons. First, mice expressing myelin-specific $CD4^+$ transgenic TCRs develop spontaneous EAE with many similarities to MS, supporting the idea that models in which $CD4^+$ T cells initiate disease are physiologically relevant to MS[1]. Second, the disease would initially involve predominantly activated $CD4^+$ T cells, minimizing the possibility that activated myelin-specific $CD8^+$ T cells infiltrating the CNS would lyse APCs presenting their cognate antigen and prevent their detection. We show in $CD4^+$ T cell-mediated EAE that MHC class I-restricted MBP is predominantly presented by DCs whose phenotype and function indicate that they are Tip-DCs, and that these DCs can activate naive MBP-specific $CD8^+$ T cells. Macrophages are much less efficient in presenting MBP–H2-K^k, and microglia, astrocytes and endothelial cells do not present MBP to $CD8^+$ T cells. Oligodendrocytes are induced to express MHC class I and present MBP–H2-K^k under the inflammatory conditions generated during $CD4^+$ T cell mediated EAE.

Presentation of MBP–H2-K^k largely by DCs is consistent with the critical role that DCs play in promoting inflammation during CNS autoimmune disease. In MS patients, DCs accumulate in the meninges and parenchymal lesions[4,42,43], and mature DCs are observed lining the blood vessels in non-inflamed CNS tissue where they are well-positioned to re-activate infiltrating T cells[4]. In $CD4^+$ T cell-mediated EAE, DCs are required to initiate disease[4], and $CD11b^+$ mDCs were the only cells that could activate naive $CD4^+$ T cells,

even though macrophages phagocytosed comparable amounts of myelin and could activate effector $CD4^+$ T cells[28]. Interestingly, the pro-inflammatory DCs present early in $CD4^+$ T cell-mediated EAE appear to be replaced later in the disease course with more immature DCs that are less efficient in stimulating $CD4^+$ T cells[29]. The mechanism responsible for the decline in pro-inflammatory DCs has not been described.

Our data demonstrate that the DCs generated in the CNS during $CD4^+$ T cell-induced EAE that present MHC class I-restricted MBP are $CD11b^+$ Tip-DCs that are likely derived from infiltrating inflammatory monocytes. This conclusion is based on the phenotypic similarity to inflammatory monocytes, the predominant production of TNF and iNOS without IL-12, and the lack of expression of the ZBTB46 transcription that is selectively expressed by classical DCs and their progenitors[35, 36]. The MBP–H2-K^{k+} DCs are also distinguished by their intermediate expression of CD103, an integrin whose expression is correlated with, but not required for, cross-presentation by DCs[33]. Neither $CD103^-$ nor $CD103^{Hi}$ DCs present MBP–H2-K^k, and virtually all $CD103^{Int}$ DCs are $12H4^+$, indicating that the MBP–H2-K^{k+} DCs are a distinct DC subset that can be phenotypically defined by these markers. Importantly, our data demonstrate that presentation of MBP–H2-K^k by Tip-DCs occurs via cross-presentation. Expression of classic MBP was not detected in the MBP–H2-K^{k+} DCs, and the low amount of golli-MBP expression was not sufficient to allow direct presentation of MBP H2-K^k because $12H4^-$ DCs expressed as much or more golli-MBP transcripts as $12H4^+$ DCs.

There are several mechanisms by which the Tip-DCs could acquire and present MBP–H2-K^k. Tip-DCs could phagocytose myelin debris or dead oligodendrocytes and transfer the antigen to the cytosol where it is processed and loaded onto MHC class I molecules for cross-presentation. Oligodendrocytes have also been shown to produce exosomes containing myelin epitopes under steady state conditions that are preferentially taken up by microglia[44]. If these exosomes are taken up under inflammatory conditions by macrophage and DCs, they could be a potential source of MBP peptides for these APCs during EAE, similar to bone marrow-derived DCs that obtain antigen from exosomes and load it onto their own MHC class I molecules[45]. An alternative possibility is that the Tip-DCs acquire pre-formed MBP peptide–MHC class I complexes from the membranes of neighboring cells such as oligodendrocytes via trogocytosis[46,47]. This cell-contact dependent process has recently been shown to result in “cross-dressed” DCs that are capable of activating both naïve and memory $CD8^+$ T cells *in vitro*[45]. However, cross-dressed DCs are not able to activate naïve $CD8^+$ T cells *in vivo*, at least in the context of a viral infection. As our data show that naïve $CD8^+$ cells enter the CNS and are activated *in situ*, cross-dressing seems an unlikely mechanism for presentation of MBP–H2-K^k by Tip-DCs.

Our findings indicate that determinant spreading can occur within the CNS between $CD4^+$ and $CD8^+$ myelin-specific T cells. The Tip-DCs are likely the only cell type in the CNS capable of activating the naive $CD8^+$ T cells as these are the only cells that activated naive 8.8 T cells directly *ex vivo*. $CD8^+$ T cell activation within the CNS is consistent with the lack of CCR7 on MBP–H2-K^{k+} DCs, as this chemokine receptor facilitates trafficking of classical DCs from peripheral tissues to lymph nodes. CCR7 is expressed on MBP–H2-K^k DCs in the inflamed CNS, and DCs that cross-present tumor antigens acquired in the CNS

under less inflammatory conditions migrate to peripheral lymph nodes[48]. Thus, lack of CCR7 may be a characteristic of Tip DCs in inflamed tissues.

DCs are also the predominant APCs that present MBP–H2-K^k in the healthy CNS. The MBP–H2-K^{k+} CNS DCs in healthy mice do not exhibit the inflammatory phenotype of the DCs in EAE mice, but their $CD11b^+CD103^-$ $F4/80^+$ phenotype also does not resemble other tissue resident DCs specialized in cross-presentation. The co-expression of CCR2 and CX3CR1 on these DCs is unusual, although it is not known whether either chemokine receptor is required for their localization in the healthy CNS. APCs that reactivate infiltrating myelin-specific T cells are found in the subarachnoid space[37,49]; and the role of particular chemokine receptors in facilitating entry of DCs into this compartment is not known. MBP H2-K^{k+} APCs in the healthy CNS are capable of stimulating previously activated $CD8^+$ T cells *in vitro*, suggesting that they have the potential to re-activate $CD8^+$ T cells that had been primed in the periphery.

Our data demonstrate that oligodendrocytes may be lytic targets of activated, MBP-specific $CD8^+$ T cells during EAE, consistent with reports that human MBP-specific $CD8^+$ T cells can lyse oligodendrocytes in a MHC-restricted fashion without addition of exogenous antigen[50], and that $CD8^+$ T cells containing polarized cytolytic granules are seen in close proximity to oligodendrocytes and demyelinated axons in the CNS of MS patients[14]. Because RNA transport mechanisms allow MBP synthesis to take place within myelin membranes distal from the oligodendrocyte cell body, MBP–H2-K^k complexes could be expressed on the myelin sheath where they can engage $CD8^+$ T cells. Endothelial cells and astrocytes did not present MBP–H2-K^k, suggesting that astrocytes contribute to $CD8^+$ T cell activation primarily via cytokine production[9], and that endothelial cells do not play a role in enhancing $CD8^+$ T cell recruitment to the CNS via MBP peptide presentation[40].

Our findings that MHC class-I-restricted MBP is presented by both Tip-DCs and by oligodendrocytes suggest the intriguing hypothesis that $CD8^+$ T cells could exert both pathogenic and immunoregulatory activities in the CNS during EAE. $CD8^+$ T cells activated by Tip-DCs could exacerbate disease via production of IFN-γ, and the $CD8^+$ T cells could also contribute to tissue damage by lysing oligodendrocytes. However, the Tip-DCs themselves could also become lytic targets of the newly activated $CD8^+$ T cells. Loss of these DCs may reduce inflammation by decreasing the number of APCs available to stimulate both $CD4^+$ and $CD8^+$ T cells in the CNS. Ongoing axonal destruction in inactive MS lesions that appear to have few inflammatory cells (referred to as “low-burning” axonal damage) has been described[51] and it is possible that myelin-specific $CD8^+$ T cells may play an important role in this process. It would also be of interest to determine if there is an increase in myelin-specific $CD8^+$ T cell activity during the transition from relapsing/remitting to secondary progressive MS, as this phase of disease is characterized by a decrease in inflammation with ongoing axonal degeneration.

Methods

Mice

C3HeB/Fej mice from The Jackson Laboratory were bred in house. TCR transgenic 8.6 and 8.8 mice have been described[24]. Mice expressing transgenic GFP promoted by the PLP promoter (PLP-GFP) were provided by W. Macklin (University of Colorado, Denver, CO), and mice expressing transgenic GFP promoted by the astrocyte-specific human glial fibrillary acidic protein promoter (GFAP-GFP) were from The Jackson Laboratory. PLP-GFP and GFAP-GFP mice were backcrossed 6 generations onto the C3HeB/FeJ background. All mice were bred and maintained in a specific pathogen-free facility at the University of Washington. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Peptides

MBP79–87 (DENPVVHFF) and SV40 TAg (SEFLLEKRI) were from Genemed Synthesis, Inc.

EAE induction and CNS cell isolation

EAE was induced in C3HeB/Fej mice by rMOG immunization as described[25]. EAE was induced in 8.8 mice by adoptive transfer of $CD4^+$ T cells from rMOG-immunized C3HeB/Fej mice that were re-stimulated *in vitro* with MOG97–114 as described[25]. CNS tissues were harvested from perfused naïve or EAE mice (1–3 days post onset, clinical score >3). DCs, macrophages, microglia and endothelial cells were isolated from tissues digested with 2 mg/ml collagenase D (Roche Applied Science) and 20 ng/ml of DNase in PBS for 30 min at 37 °C. Cells were isolated from mechanically dissociated tissues for CD62L staining. Oligodendrocytes and astrocytes were isolated from tissues digested with 1 mg/ml papain (Worthington Biochemical Corp.) and 20 ng/ml DNase in HBSS. Tissues were gently triturated and PBS containing 5% fetal bovine serum was added to stop digestion. Digested tissue was filtered through a stainless steel mesh and centrifuged. CNS cells were isolated from the interface of a 30/70% percoll gradient centrifuged without brake for 20 min at 250 *g*.

Flow cytometry

CNS cells were incubated with Fc block (clone 2.4G2; eBioscience) in 5% normal mouse serum for 15 min at 25°C, washed and stained with antibodies for 30 min at 4 °C. Biotinylated 12H4 or control mouse IgG2a or anti-K^b antibody was detected with PE-conjugated streptavidin (BD). Antibodies from BD were: APC-anti-CD8 (clone 2.43), FITC-anti-CD11c (clone HL3), APC-anti-CD31 (clone MEC 13.3), PE-Cy5-anti-B220 (clone RA3–6B2), PerCP-Cy5.5-anti-CD62L (clone MEL-14), PE-anti-CD80 (clone 16-10A1), PE-anti-CD86 (clone GL1), FITC-anti-H-$2K^k$ (clone 36-7-5), FITC-anti-Thy1.2 (clone 53-2.1); from eBioscience: FITC-anti-CD8 (clone 53.6.7), PE-Cy7-anti-CD11b (clone M1/70), FITC-anti-CD103 (clone 2E7), PerCP-Cy5.5-anti-Ly6C (clone HK1.4), APC-anti-F4/80 (clone BM8), PerCP-eFluor 710-anti-I-A^k (clone 10-3.6), purified mouse IgG2a; from Biolegend: APC-anti-CD11c (clone N418), APC-Cy7-anti-CD45 (clone 37-F11); and from

R&D: APC-anti-CCR2 (clone 475301), NL637-anti-O4 (clone O4), CX3CR1 (polyclonal). CX3CR1 staining was carried out prior to incubation with other antibodies and detected with APC-conjugated donkey anti-goat IgG (R&D). Intracellular IFN- γ staining was performed according to manufacturer's instructions (BD Biosciences) on 8.6 or TAg-specific T cells cultured with CNS cells (T cell:APC ratio 1:1; 1:2 for fixed CNS cells). Cells were analyzed with a FACSCanto flow cytometer (BD Biosciences). Intracellular staining for TNF-α was performed on CNS cells cultured in complete media for 3 h and analyzed using a LSRII flow cytometer (BD Biosciences). CNS cells were sorted using a FACS ARIA (BD Biosciences). PLP-GFP mice and GFAP-GFP mice were used to sort GFP^+ oligodendrocytes and astrocytes, respectively. Endothelial cells were sorted based on CD31 expression.

Generation of MBP–H2-K^k-specific antibodies

MBP–H2-K^k complexes were generated in house with a construct encoding H-2K^k provided by the National Institutes of Allergy and Infectious Diseases Tetramer Core Facility. C3HeB/Fej mice were subcutaneously immunized with 20 μg MBP–H2-K^k emulsified in complete Freund's adjuvant (CFA, Sigma-Adrich) and boosted twice at three week intervals with subcutaneous injections of 10 μg MBP–H2-K^k in incomplete Freund's adjuvant. Two weeks after the last boost, mice were injected intravenously with 10 μg soluble MBP–H2-K^k. Cells were harvested from spleen and draining lymph nodes four days later, fused to X63.Ag8.653 mouse myeloma cells and selected in HAT complete medium. Hybridoma supernatants were screened using a cell-based ELISA assay. RMA-S–H2-K^k cells were incubated with 5 μg/ml recombinant β-2-microglobulin and either 20 μM MBP peptide or no peptide overnight at 25° C. Cells were washed, fixed with 0.5% paraformaldehyde overnight at 4° C and plated (1×10^5 per well) in 96-well U bottom plates (Becton Dickinson) that had been blocked with complete medium. Hybridoma supernatant was added and the cells were incubated for 90 minutes at 25°C, washed with PBS/Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch) for 90 min at 25°C before development. Hybridomas demonstrating high absorbance specifically with peptide-pulsed RMA-S–H2-K^k cells were characterized by flow cytometry for binding to RMA-S–H2-K^k cells pulsed with MBP or TAg peptide.

Generation of effector $CD8^+$ T cells

Naïve 8.6 splenocytes were cultured with 0.5 μM MBP peptide in RPMI 1640 complete medium, split on day 3 with complete medium containing 20 IU/ml IL-2 and used 7–20 days after stimulation. Effector TAg-specific T cells were generated by immunizing C3HeB/FeJ mice subcutaneously with 200 μg TAg peptide in CFA and culturing cells from draining lymph nodes and spleens with 1 μM TAg peptide and 20 IU/ml IL-2. Cells were restimulated with 0.5 μM peptide on day 14 and used for experiments 7 days later.

T cell ELISPOT and proliferation assays

ELISPOT for IFN- γ was performed as previously described [25] using sorted CNS cells (1–10×10^4) and 1×10^5 effector 8.6 T cells without adding exogenous antigen. T cell proliferation was assessed by incubating sorted CNS cells with naive 8.6 $CD8^+$ T cells

(1×10^4) purified by negative selection using an autoMACS separator (Miltenyi Biotec) in a 96 well U-bottom plate for 72 h. 1 μCi of ^{3}H-thymidine was added for the last 16 h.

Quantitative RT-PCR

$CD11c^+CD103^{Int}12H4^+$ and $CD11c^+CD103^-12H4^-$ cells were sorted from EAE mice, RNA extracted using a RNeasymicro kit (Qiagen), and first-strand cDNA synthesized using SuperScript II (Invitrogen). Quantitative PCR was performed (see Supplementary Table 1 for oligonucleotide primers) on an ABI 7300 RealTime PCR System (Applied Biosystems). Gene expression was normalized to values for HPRT.

Statistics

Unpaired two-tailed Student *t* test was used for statistical analyses. $P < 0.05$ was considered significantly difference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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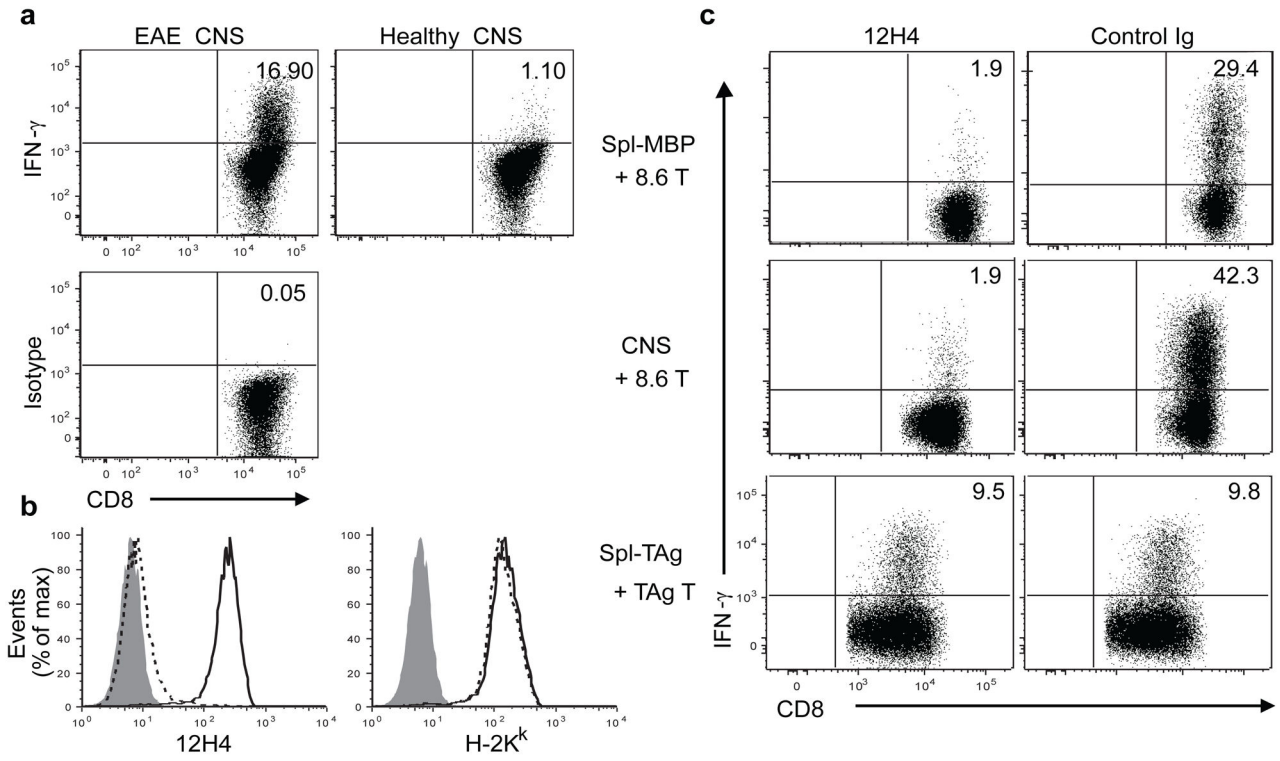

Figure 1.

Detection of MBP–H2-K^k by the 12H4 antibody and MBP-specific CD8$^+$ T cells. (**a**) Effector CD8$^+$ 8.6 T cells were incubated with CNS cells isolated from either naïve or EAE mice and IFN- γ production was analyzed on gated CD8$^+$ cells (representative of two independent experiments). (**b**) RMA-S-K^k cells pulsed with either MBP79–87 (solid line) or TAg560–568 peptide (dotted line) were stained with 12H4 or isotype control antibody (shaded) followed by FITC-conjugated anti-mouse IgG (left panel). Peptide-pulsed cells were separately stained for K^k (right panel). Data are representative of three independent experiments. (**c**) Effector 8.6 T cells were incubated with MBP peptide-pulsed and paraformaldehyde-fixed splenocytes (top panel), or paraformaldehyde-fixed CNS cells from an EAE mouse (middle panel), and TAg-specific T cells were incubated with TAg-pulsed and fixed splenocytes (bottom panel) in the presence of either 12H4 or control IgG2a antibody. IFN- γ production was analyzed as in **a**. Data are representative of three independent experiments.

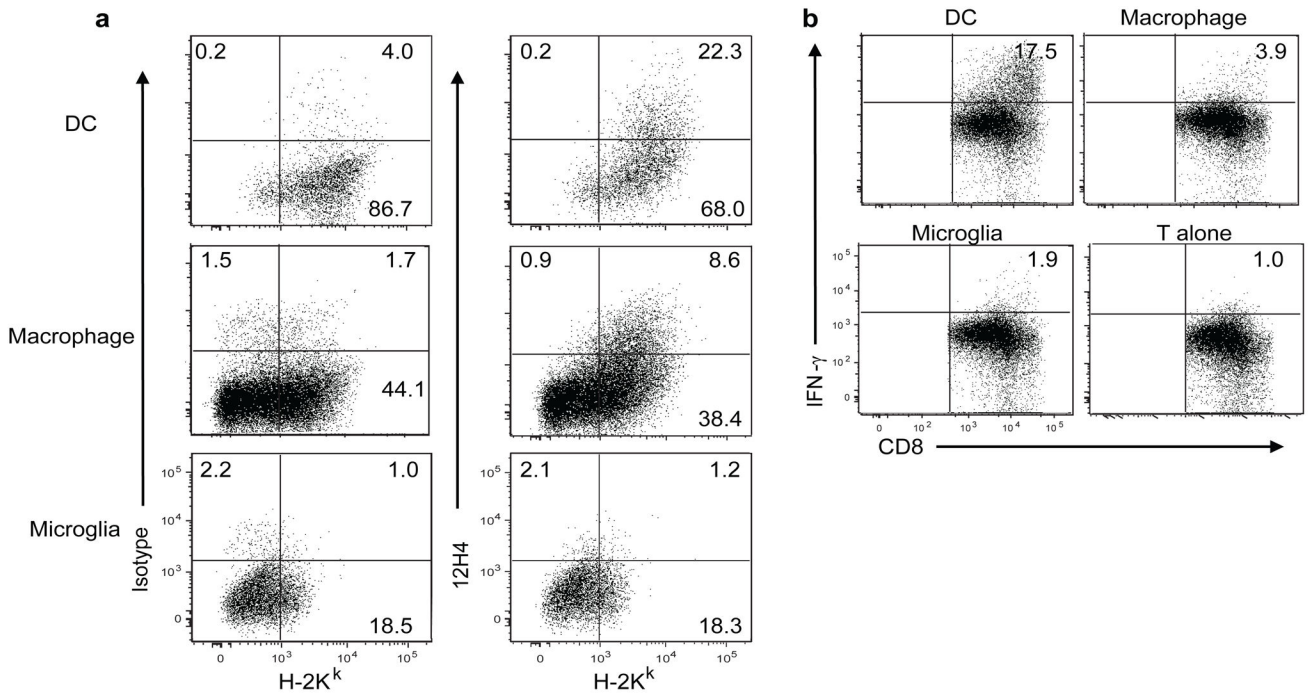

Figure 2.

MBP–H2-K^k is presented predominantly by DCs in the CNS during CD4$^+$ T cell-mediated EAE. (**a**) CNS cells from a mouse with EAE were stained with 12H4 (or isotype control), anti-K^k and antibodies used to distinguish DCs, macrophages and microglia and analyzed by flow cytometry (gating strategy is shown in Supplementary Figure 1). Data are representative of at least ten mice analyzed in five independent experiments. (**b**) 8.6 effector T cells were incubated either alone or with DCs, macrophages or microglia sorted from CNS cells from EAE mice and analyzed in an intracellular IFN- γ staining assay. Data are gated on CD8$^+$ T cells and representative of two independent experiments.

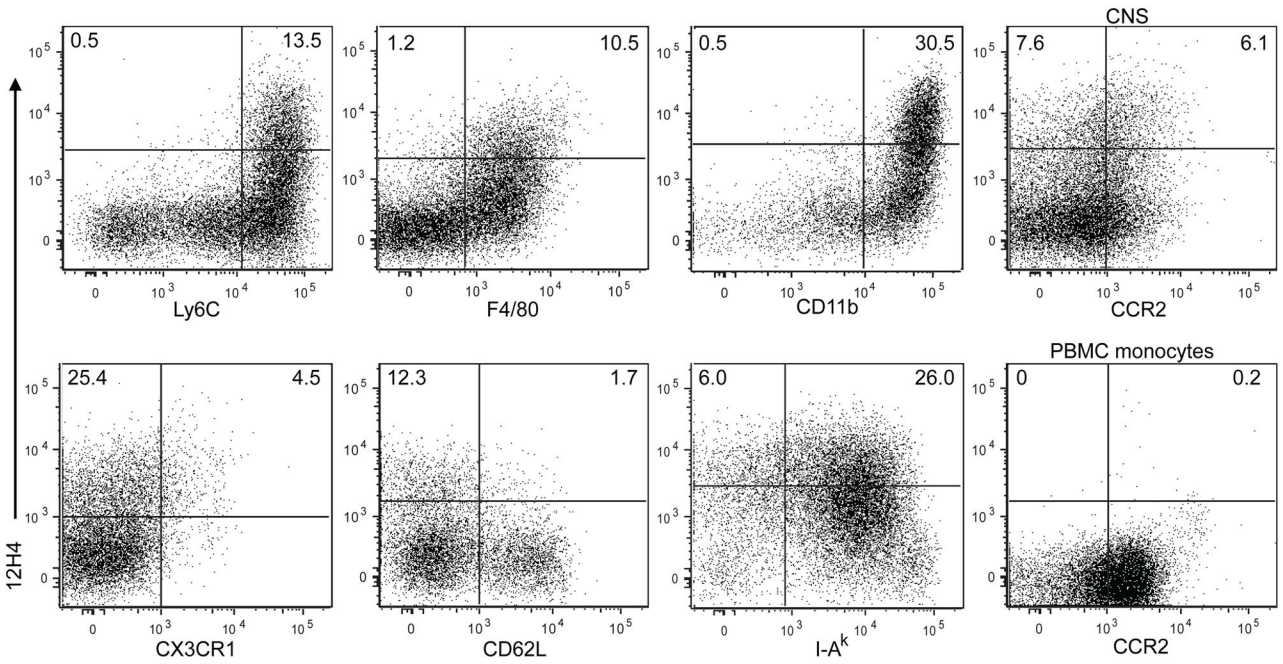

Figure 3.

MBP–H2-K^{k+} DCs in EAE mice are phenotypically similar to tissue-infiltrating inflammatory monocytes. CNS cells were isolated from EAE mice and stained with 12H4 and antibodies specific for CD11c, CD45 and the indicated cell-surface antigens. Analyses are gated on CD45^{Hi}CD11c^{+} cells. Peripheral blood monocytes were also isolated from rMOG-immunized mice and stained for CD11b, Ly6C and CCR2; CCR2 expression is shown on CD11b^{+}Ly6C^{+} gated cells in the lower right panel. Data are representative of 2–3 independent experiments for each cell marker.

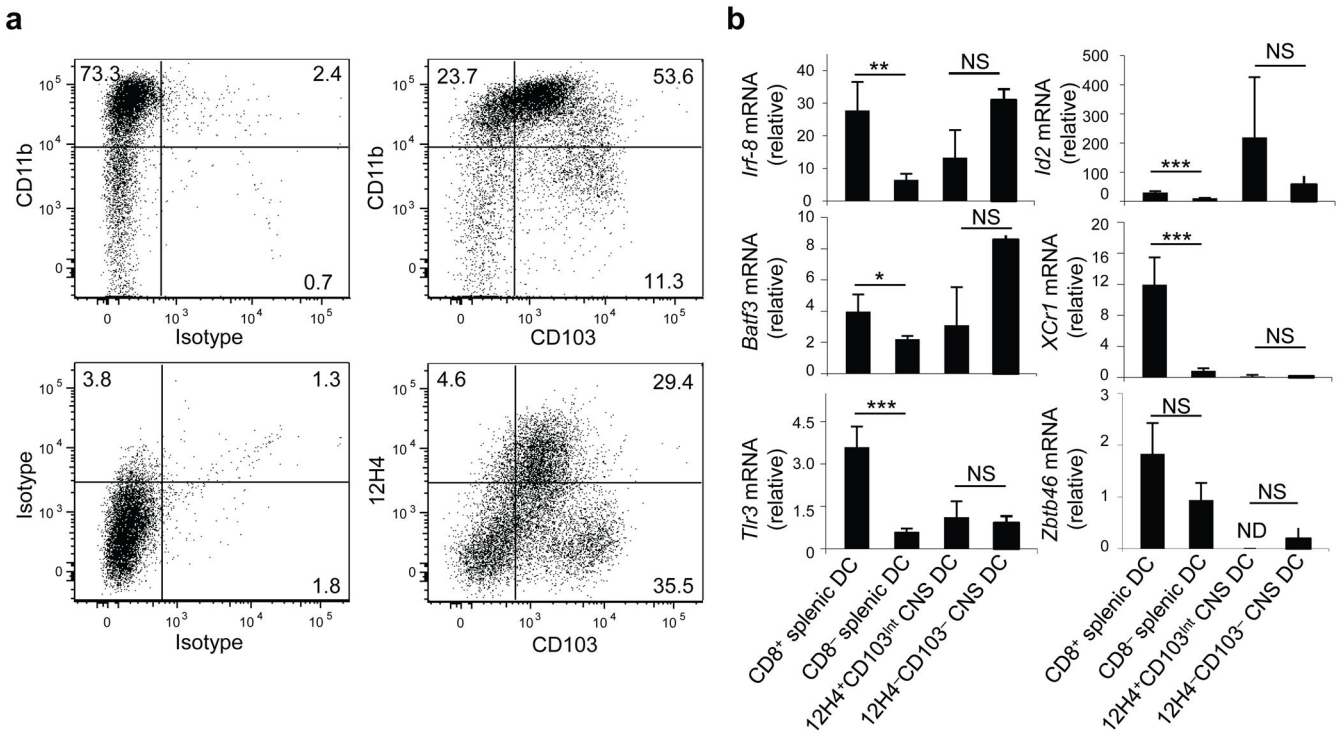

Figure 4.

MBP–H2-K^{k+} DCs are CD11b^{Hi}CD103^{Int} non-classical DCs. (**a**) CNS cells from EAE mice stained with 12H4 and antibodies specific for CD11b, CD11c, CD45 and CD103; flow cytometry analyses are gated on CD45^{Hi}CD11c^{+} cells. Data are representative of four independent experiments. (**b**) Quantitative PCR analysis of cDNA from CD45^{+}CD11c^{+} CNS cells from EAE mice sorted into 12H4^{+}CD103^{Int} and 12H4$^{−}$ CD103$^{−}$ fractions, and from splenocytes from naive mice sorted into CD11c^{+}CD8^{+} and CD11c^{+}CD8$^{−}$ fractions. Primers are shown in Supplementary Table 1; values are expressed relative to HPRT. Reactions were performed in duplicate; data are representative of two independent experiments. ND, not detected. *P < 0.02, **P < 0.005, ***P < 0.001, NS, not significant. Error bars, s.d.

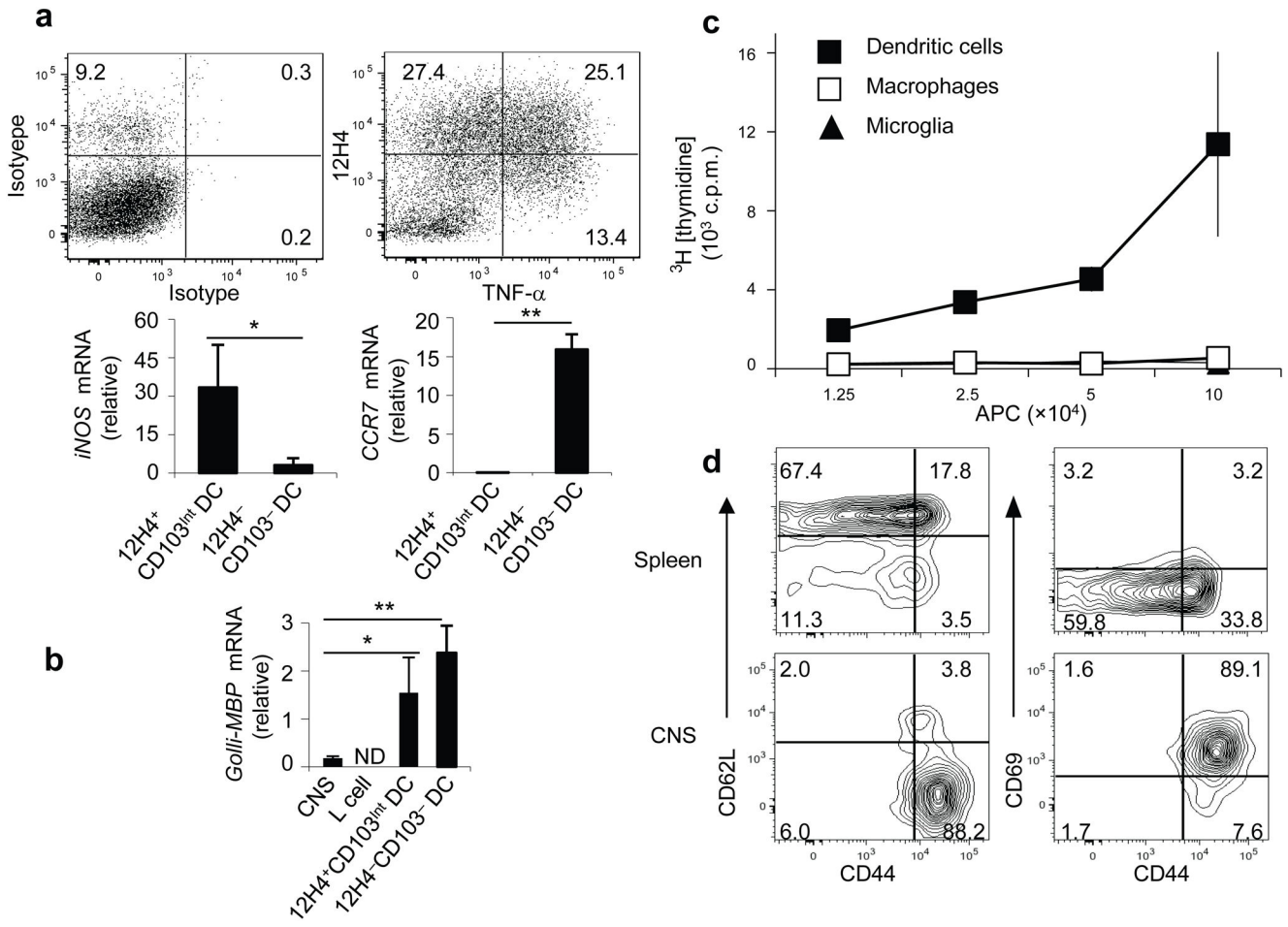

Figure 5.

MBP–H2-K^{k+} CNS DCs in mice with CD4$^+$ T cell-mediated EAE are Tip-DCs that facilitate determinant spreading to CD8$^+$ T cells. (a) CNS cells from EAE mice were stained for TNF-α (top panel). Data shown are gated on CD45^{Hi}CD11c$^+$ cells and representative of two independent experiments analyzing more than four mice. Quantitative PCR analysis of iNOS and CCR7 cDNA was performed on 12H4$^+$CD103^{Int} and 12H4$^-$ CD103$^-$ DCs sorted from the CNS of EAE mice (bottom panel). Values are expressed relative to HPRT; reactions were performed in duplicate; data are representative of two independent experiments. *P < 0.02 and **P < 0.001. Error bars, s.d. (**b**) Quantitative PCR analysis of golli-MBP cDNA from the CNS of a naive mouse, L cells, and 12H4$^+$CD103^{Int} and 12H4$^-$ CD103$^-$ DCs sorted from a mouse with EAE. ND, not detected. *P < 0.02 and **P < 0.001. (**c**) DCs, macrophages, and microglia were sorted from the CNS of EAE mice and cultured with naive CD8$^+$ 8.6 T cells. Proliferation was measured by ^{3}H-thymidine incorporation. Results are expressed as counts per minute (c.p.m) ± standard deviation (s.d.). Data are representative of two independent experiments. (**d**). EAE was induced by adoptive transfer of genetically-marked MOG-specific CD4$^+$ T into 8.8 mice. Splenocytes and CNS cells were harvested at peak of disease and stained for CD62L, CD44, and CD69. Analyses are

gated on host $CD8^+$ T cells; data are representative of two mice analyzed in two independent experiments.

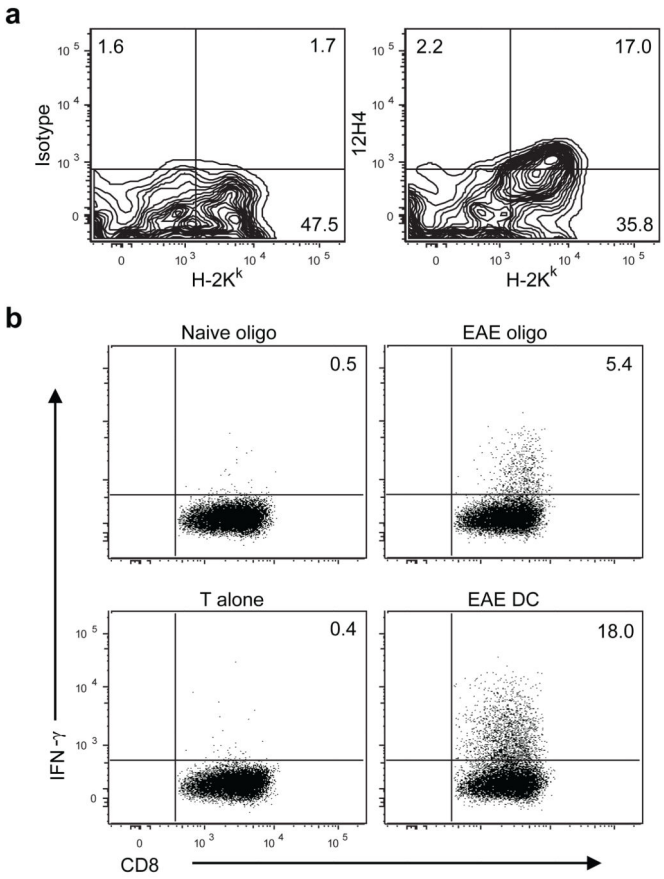

Figure 6.

Oligodendrocytes present MBP–H2-K^k during $CD4^+$ T cell-mediated EAE. (**a**) CNS cells were isolated from PLP-GFP transgenic mice (oligodendrocytes specifically express GFP) with EAE, cultured for two hours and stained with antibodies specific for CD45, K^k and either 12H4 or isotype control antibody. Data shown are gated on $CD45^-$ GFP^+ cells and representative of two independent experiments using more than four mice. (**b**) Effector 8.6 T cells were cultured with oligodendrocytes sorted from PLP-GFP transgenic naïve or EAE mice, or with DCs from EAE mice and stained for IFN- γ. Data are gated on $CD8^+$ T cells and representative of two independent experiments.

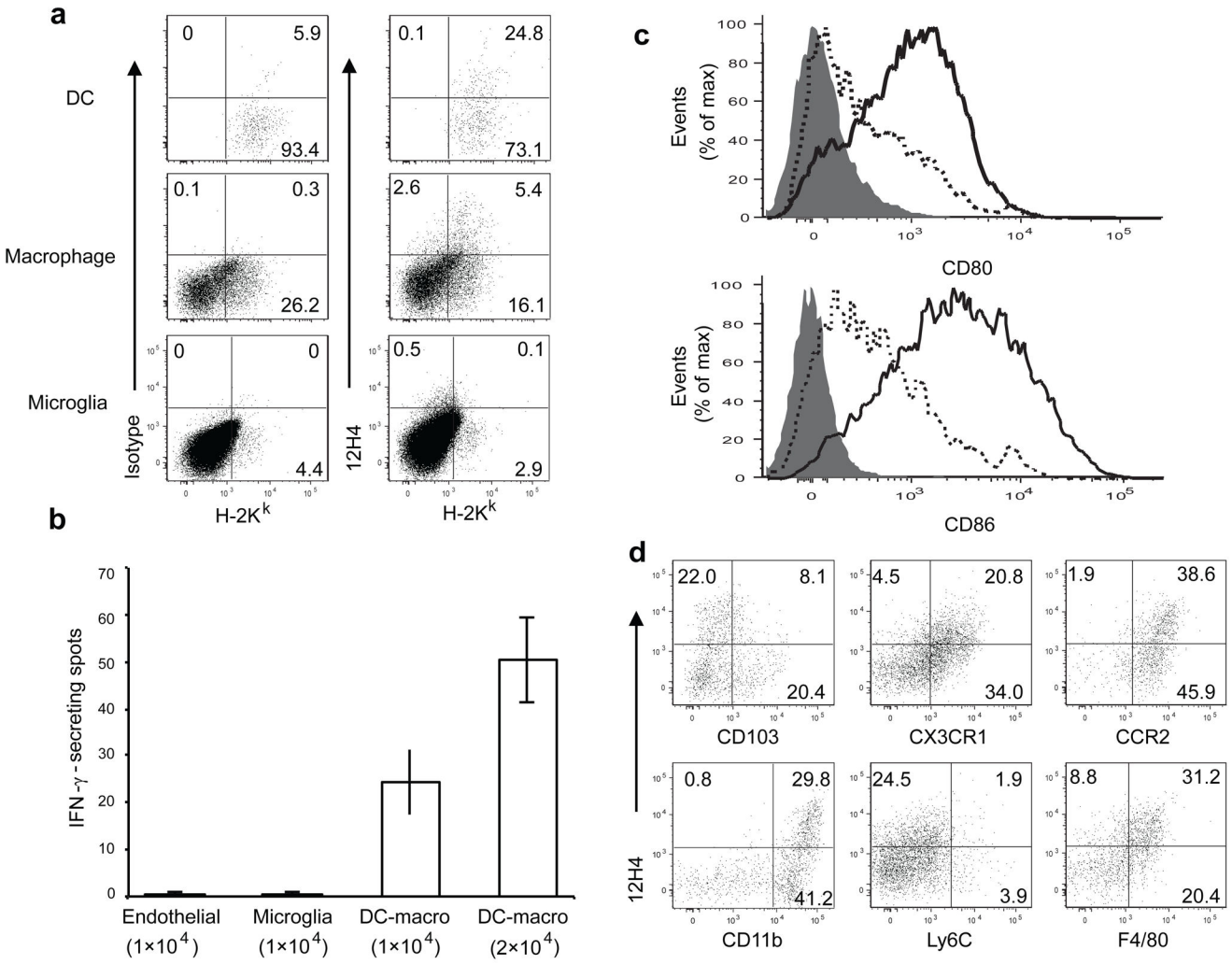

Figure 7.

DCs from the CNS of naive mice present MBP–H2-K^k. (**a**) CNS cells isolated from naïve mice were stained with antibodies specific for CD45, CD11c, CD11b, K^k and either 12H4 or isotype control antibody. CNS cell subsets were gated as in Supplementary Figure 1; data are representative of more than five independent experiments. (**b**) DCs/macrophages (CD45^{Hi}CD11b^{Hi}), microglia (CD45^{Int}CD11b$^+$), and endothelial cells (CD45$^-$ CD31$^+$) were sorted from naive CNS cells and incubated with effector 8.6 T cells in an ELISPOT assay to detect IFN-γ-secreting cells. Data are pooled from three independent experiments and expressed as the mean values ± standard deviation (s.d.). (**c**) CNS cells from naïve or EAE mice were stained for CD11c, CD45, CD80, and CD86 and analyzed by gating on CD45^{Hi}CD11c$^+$ cells from naive mice (dashed line) and EAE mice (solid line). Grey shaded area shows isotype control staining. (**d**) CNS cells from naïve mice were stained with 12H4 and antibodies specific for CD11c, CD45 and the indicated markers and analyzed by gating on CD45^{Hi}CD11c$^+$ cells. Data are representative of two independent experiments.