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Limited Sufficiency of Antigen Presentation by Dendritic Cells in Models of Central Nervous System Autoimmunity

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Abstract

Experimental autoimmune encephalomyelitis (EAE), a model for the human disease multiple sclerosis (MS), is dependent upon the activation and effector functions of autoreactive CD4 T cells. Multiple interactions between CD4 T cells and major histocompatibility class II (MHCII)+ antigen presenting cells (APCs) must occur in both the periphery and central nervous system (CNS) to elicit autoimmunity. The identity of the MHCII+ APCs involved throughout this process remains in question. We investigated which APC in the periphery and CNS mediates disease using transgenic mice with MHCII expression restricted to dendritic cells (DCs). MHCII expression restricted to DCs results in normal susceptibility to peptide-mediated EAE. Indeed, radiation-sensitive bone marrow-derived DCs were sufficient for all APC functions during peptide-induced disease. However, DCs alone were inefficient at promoting disease after immunization with the myelin protein myelin oligodendrocyte glycoprotein (MOG), even in the presence of MHCII-deficient B cells. Consistent with a defect in disease induction following protein immunization, antigen presentation by DCs alone was incapable of mediating spontaneous optic neuritis. These results indicate that DCs are capable of perpetuating CNS-targeted autoimmunity when antigens are readily available, but other APCs are required to efficiently initiate pathogenic cognate CD4 T cell responses.

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7. DISCLOSURES

The authors have no financial conflict of interest.

Keywords

Dendritic Cells; Experimental Autoimmune Encephalomyelitis/MS; Autoimmunity; Neuroimmunology; Immunopathology; Antigen Presenting Cells

1. INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system (CNS) that models the human disease, multiple sclerosis (MS) [1]. CD4 T cells are critical mediators of disease, as they are required for EAE and pathogenic IL-17- and IFN- γ -producing CD4 T cells can transfer disease passively [2]. The activation and differentiation of effector myelin-reactive CD4⁺ T cells depends upon interactions with major histocompatibility class II (MHCII)⁺ antigen presenting cells (APCs) [3]. MHCII⁺ APCs must interact with CD4 T cells at least twice during disease: to activate naïve lymphocytes and to re-activate effector T cells following migration into the CNS [4]. Uncovering the identity and characteristics of these MHCII⁺ APCs is important for understanding the pathogenesis of CNS inflammatory diseases such as MS.

MHCII is expressed on a number of professional and non-professional APCs that have been implicated in EAE. These include DCs and B cells in the periphery [5,6] and CNS resident APCs such as astrocytes and microglia [4]. DCs are sufficient as APCs to prime naïve T cells and initiate a wide spectrum of effector functions [7]. Antigen presentation by DCs can sustain previously activated CD4 T cell responses targeting the CNS and support effector phases of EAE [5]. However, neuro-inflammation is also modulated by other APCs, such as B cells and microglia [8,9]. For example, depletion of B cells prior to the induction of EAE results in greater disease [6] and elevating the efficiency by which myelin oligodendrocyte glycoprotein (MOG) is presented by B cells to T cells results in spontaneous EAE [10]. However, pre-clinical and clinical evidence has emerged to suggest that B cells contribute to neuro-inflammation [11,12]. Additionally, neutralization of microglia prior to immunization reduces the severity of active EAE [8]. Thus, the contribution by each APC subset to antigen presentation during all phases of disease is currently not known [13,14].

Several findings have highlighted the importance of DCs in EAE [5,15-17]. In particular, DCs participate in the late effector phases of EAE. For example, DCs can mediate disease that follows passive transfer of encephalitogenic T cells that have already been primed [5]. As well, DCs contribute to epitope spreading that occurs within the CNS in a relapsing-remitting model of EAE [16]. In addition, DCs may function outside of the CNS compartment as critical regulators of autoreactive T-cell responses directed at antigens within the CNS [18]. Importantly, the capacity of antigen presentation by DCs to engender all phases of CD4 T cell auto-reactivity, from protein processing and priming of MOG-specific CD4 T cells, to CNS recruitment and regulation of effector function, remains to be tested.

We, therefore, used a genetic system that restricts MHCII expression to dendritic cells [19] to examine the sufficiency of MHCII-dependent antigen presentation by DCs in directing all phases of CD4 T cell-mediated autoimmune CNS demyelination. We found that antigen presentation by radiation-sensitive DCs alone mediates the entire spectrum of disease following peptide immunization. However, DCs do not efficiently mediate disease induced by protein or the development of spontaneous optic neuritis, prompting a re-evaluation of the role of other APCs during autoimmune CD4 T cell responses targeting the CNS.

2. MATERIALS AND METHODS

2.1 Mice

WT C57Bl/6, 2D2 and $\text{TCR}\alpha^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). CD11c-eYFP mice [20] were provided by M. Nussenzweig (Rockefeller University). $\text{MHCII}^{-/-}$ mice [21], 23rd generation backcrossed to the C57Bl/6 background, along with CD11c/ $\text{A}\beta^b$ mice [19], backcrossed at least 11 generations to the C57Bl/6 background, were bred and maintained in our animal facility. $\text{MHCII}^{+/-}$ littermates of CD11c/ $\text{A}\beta^b$ mice with WT levels of MHCII were used as control mice. CD11c/ $\text{A}\beta^b$ mice were bred to $\text{TCR}\alpha^{-/-}$ and 2D2 mice in our facility. Bone marrow chimeras were generated as described [22]. Briefly, two 500 Rad doses of γ -irradiation were delivered to mice four hours apart. Four hours after the second dose of irradiation, mice received $1-2 \times 10^6$ bone marrow cells. Chimeric mice were treated with enrofloxacin ("Baytril", Bayer, Shawnee Mission, KS) via drinking water for two weeks. Chimeric mice were utilized for experimentation 7-8 weeks after irradiation. All experiments were performed according to animal welfare guidelines set by University of Pennsylvania Institutional Animal Care and Use Committee.

2.2 MOG Protein

Recombinant rat MOG1-125 cDNA was a gift of Dr. N. Ruddle (Yale University, New Haven, CT). Protein expression and purification of rat MOG1-125 (rMOG1-125) was performed according to protocol [13] by the Children's Hospital of Philadelphia Protein Core. Recombinant human MOG1-125 (hMOG1-125) was produced as described [14].

2.3 Thymic Transplantation

Donor thymuses were obtained from 1-3 day old C57Bl/6 pups and placed into ice-cold, sterile PBS. A lateral incision was made at the mid-axillary line at the 4th intercostal space. Following subcutaneous dissection, the thymus was placed into the axilla. The wound was covered with topical antibiotics (Bacitracin, Clay-park Labs, Bronx, NY) and closed with nylon sutures. Prior to immunization, thymic engraftment was verified by peripheral blood CD4 T cell numbers. Mice routinely reconstituted peripheral CD4 T cells to 75% of WT levels.

2.4 Induction of EAE

Active EAE was induced according to standard protocol [23]. Briefly, female mice were injected over the four flanks with a total of 200 μg of rodent MOG35-55 (MEVGWYRSPFSRVVHLYRNGK; CSBio, CA) or 150-400 μg MOG protein emulsified in 500 μg CFA. Mice received 200 ng Pertussis toxin (List Biological Laboratories, Campbell, CA) intraperitoneally at the time of immunization and 48 hours later. Passive EAE was performed as described [23]. Mice were clinically scored on a five point scale for signs of disease (0=no weakness, 1=limp tail, 2=mild hindlimb paresis, 3=moderate to severe hindlimb paresis, 4=hindlimb paralysis, 5=moribund or dead). EAE was induced in CD11c/ $\text{A}\beta^b \times \text{TCR}\alpha^{-/-}$ and $\text{TCR}\alpha^{-/-}$ mice 18-24 hours after i.v. transfer of 2×10^7 polyclonal CD4 T cells isolated from naïve C57Bl/6 as described [24].

2.5 Histologic evaluation of spinal cords and optic nerves

Following perfusion with PBS, spinal cords were dissected and placed in 4% paraformaldehyde prior to embedding in paraffin. Luxol fast blue (AKA Solvent Blue 38, Sigma, St. Louis, MO) staining and Hematoxylin and eosin (H&E) counterstaining were performed on 8 μm sections. Alternatively, following sacrifice, isolated optic nerves were fixed in 4% paraformaldehyde in PBS, and then processed for histology. Nerves were embedded in paraffin, cut into 5 μm thick longitudinal sections, and stained with H&E.

Optic neuritis was detected by the presence of inflammatory cells infiltrating the optic nerve and optic nerve sheath using a relative 0-4 point scale similar to prior studies [25,26]: No inflammatory cells observed = 0; rare inflammatory cells = 1; moderate infiltration with foci of multiple inflammatory cells occupying up to 1/3 of the optic nerve = 2; severe infiltration occupying 1/3 to 2/3 of the nerve = 3; massive infiltration of over two thirds of the nerve = 4. Each nerve was graded by a blinded investigator (KSS), and any nerve receiving a score of 2 or higher was considered to have definitive histological confirmation of optic neuritis. Because optic neuritis can occur in either one or both eyes [25], each eye was considered independently for statistical comparisons.

2.6 Flow Cytometry

CD4, CD8, IFN- γ , CD45, CD11b, CD11c, CD103, IA^b, and IL-17 antibodies were purchased from BD Biosciences (San Jose, CA). Intracellular staining for IL-17 and IFN- γ was performed using the BD Cytfix/Cytoperm™ kit (BD Biosciences, San Jose, CA) after direct ex vivo stimulation of $5-10 \times 10^6$ lymphocytes or CNS mononuclear cells for 4 hours with MOG35-55. Stimulation with 200 ng/ml of PMA (Sigma, St. Louis, MO) with 300 μ g/ml Ionomycin (Sigma, St. Louis, MO) was performed in parallel. In order to account for variations in quantities of MHCII-bearing APCs between mice, equal numbers of CHB3 cells [27] were added as “stimulators” to ex vivo cultures for each mouse group. Samples were acquired using a FACSCalibur™ (BD) or FACSCanto™ (BD) and analyzed using FloJo (Tree Star, Inc., Ashland, OR).

2.7 MOG-specific ELISA

Blood was collected from mice via cardiac puncture and allowed to clot. Serum collected after centrifugation at 14,000 RMP for 20 minutes was diluted 1:500 and applied in triplicate to 96-well flat bottom plates coated overnight at 4°C with hMOG protein (10 mg/ml) according to protocol [14]. After overnight incubation at 4°C, plates were washed with PBS-0.5% Tween 20 (PBT) and Streptavidin-Alkaline Phosphatase conjugated goat anti-mouse IgM or IgG antibody (Jackson ImmunoResearch; West Grove, PA) was added at 1:10,000 in PBT. Following two hours at 22°C, and washing with PBT, substrate was added and plates were incubated for four hours at 22°C protected from light. An ELISA plate reader was used to read absorbance at 405 nm on with plate correction (550 nm) subtracted.

2.8 Statistics

Two-tailed Student t-tests were performed for comparisons between serum antibody levels and cumulative disease scores of different groups of mice. Fisher's exact test was used for the comparison of optic neuritis frequency.

3. RESULTS

3.1 Experimental model systems

To determine the capacity of DCs to function as the sole APC during active EAE, we employed a genetic approach using mice with MHCII expression restricted to DCs, termed CD11c/A β^b mice [19]. Only CD11b+ and CD8 α + DCs express MHCII in the periphery of these mice; macrophages and B cells are MHCII negative [19]. CD11c/A β^b mice do not express MHCII in the thymus and, therefore, have no CD4 T cells (FIGURE 1). Two different approaches to restore CD4 T cells in CD11c/A β^b mice were taken. To reconstitute CD4 T cell development in CD11c/A β^b mice, syngeneic neonatal C57Bl/6 thymi were implanted subcutaneously into the axilla of these mice [28]. A substantial CD4 T cell compartment was established in CD11c/A β^b mice following grafting (FIGURE 1); absolute numbers of CD4 T cells reached wild-type (WT) levels within the cutaneous lymph nodes

between 6 and 9 weeks post-transplant (data not shown). Unless otherwise noted, CD11c/ $A\beta^b$ mice will be recipients of thymus transplantation. In addition, CD11c/ $A\beta^b$ mice without thymic transplants were crossed with TCR $\alpha^{-/-}$ mice, which lack the entire T cell compartment [29] (referred to as CD11c/ $A\beta^b$ ×TCR $\alpha^{-/-}$ mice). Hence, we were able to reconstitute the CD4 T cell compartment with the transfer of 2×10^7 CD4 T cells.

3.2 CD11c+ DCs are sufficient for active EAE induction after MOG peptide immunization

To test the sufficiency of DCs to mediate peptide-induced EAE, MHCII $^{+/-}$, MHCII $^{-/-}$, and CD11c/ $A\beta^b$ mice were immunized with MOG35-55 peptide [23]. As expected, MHCII $^{-/-}$ mice with thymic transplants did not display clinical signs of EAE. As well, in the absence of CD4 T cells, CD11c/ $A\beta^b$ mice are also resistant to disease (FIGURE 2A). Following immunization, MHCII $^{+/-}$ mice developed progressive hind-limb weakness. CD11c/ $A\beta^b$ mice were equally susceptible to clinical disease as their littermates with normal expression of MHCII (FIGURE 2A). The onset of symptoms (day 14) and the peak of disease (clinical score = 2) were similar in both groups of mice. In addition, following intravenous transfer of 2×10^7 CD4 T cells (isolated from naïve B6 mice), CD11c/ $A\beta^b$ ×TCR $\alpha^{-/-}$ mice were immunized with MOG peptide. Onset of disease and maximal disease severity was identical between TCR $\alpha^{-/-}$ mice with WT levels of MHCII and CD11c/ $A\beta^b$ ×TCR $\alpha^{-/-}$ mice when immunized with MOG peptide (FIGURE 2B). Thus, MHCII expression by other APCs is not required for typical clinicopathologic disease in EAE. As well, cognate interactions between DCs and CD4 T cells are sufficient for mediating disease during active EAE induced by MOG peptide.

3.3 Peptide-induced active EAE is similar in CD11c/ $A\beta^b$ and WT mice

Ascending paralysis is a common phenotypic endpoint in EAE [1,30,31]. However, multiple immunologic pathways involving different lineages of CD4 T cells can result in EAE that is clinically indistinguishable [30]. We asked if any pathologic differences exist during EAE between CD11c/ $A\beta^b$ and WT mice. Histological examination of spinal cords from CD11c/ $A\beta^b$ mice revealed the typical pathology of EAE, including demyelination associated with inflammatory infiltrates (data not shown). CD4 T cell production of the cytokines IFN- γ and IL-17 are implicated in the pathogenesis of EAE. Fifteen to 21 days after immunization, lymphocytes from draining lymph nodes were re-stimulated *ex vivo* with WT APCs and MOG 35-55 and production of IFN- γ and IL-17 was assessed by flow cytometry. The percentage of IFN- γ - and IL-17-producing CD4 T cells in CD11c/ $A\beta^b$ and MHCII $^{+/-}$ mice was similar at disease onset (day 15) (FIGURE 3A) and later timepoints (> day 30; data not shown). We also determined the cytokine profile of effector T cells infiltrating the CNS of diseased mice. Mononuclear cells from the CNS of mice with EAE on day 21 were re-stimulated with MOG35-55. In both CD11c/ $A\beta^b$ and MHCII $^{+/-}$ mice, ~20% of CD4 T cells within the CNS produced IFN- γ . Further, CNS infiltration of IL-17+ CD4 T cells was observed in CD11c/ $A\beta^b$ mice in similar proportion (~7%) compared to MHCII $^{+/-}$ mice (FIGURE 3B). Thus, expression of MHCII by DCs alone not only promotes the peripheral generation of pathogenic CD4 T cells, but also is sufficient for the induction of characteristic cytokines by CD4 T cells within the CNS during peptide-induced EAE.

3.4 Peptide-induced EAE does not require radiation-resistant APCs

MHCII antigen processing and presentation in the CNS compartment is necessary for effector T cell function during EAE [32,33]. A diverse set of APCs express MHCII in WT mice during EAE [17], yet our results demonstrate that the expression of MHCII mediated by the CD11c promoter in CD11c/ $A\beta^b$ mice is sufficient for typical disease during all phases of EAE after peptide immunization. Microglia are resident antigen presenting cells of the CNS that can express CD11c and contribute to inflammatory destruction in EAE [8]. We

sought to define the cellular expression of MHCII by APCs within the CNS of CD11c/A β^b and MHCII $^{+/-}$ mice with EAE.

Three populations of CNS mononuclear cells can be distinguished by expression levels of CD45 and CD11b: resting microglia (CD45 low , CD11b low ; population I in FIGURE 4A), activated microglia/infiltrating radiosensitive APCs, (including macrophages, monocytes and most DCs; CD45 high , CD11b high ; population II in FIGURE 4A) and lymphocytes (CD45 high , CD11b low) [34]. During disease, CD11c $^{+}$ cells are predominantly located within populations I and II (FIGURE 4A). The pattern and number of CD11c $^{+}$ cells labeled with CD45 and CD11b were similar in CD11c/A β^b and MHCII $^{+/-}$ mice (FIGURE 4A). We next asked whether the expression of MHCII by APCs differed between CD11c/A β^b and MHCII $^{+/-}$ mice. Overall, there was less MHCII expression on CD11c $^{+}$ mononuclear cells from the CNS of CD11c/A β^b mice with EAE (FIGURE 4B) compared to WT. MHCII expression was similar in CD11c $^{+}$ resting microglia (Population I, FIGURE 4B). However, MHCII expression in Population II (infiltrating APCs and activated microglia) was reduced in CD11c/A β^b mice with EAE compared with MHCII $^{+/-}$ mice (FIGURE 4B). In particular, the MHCII hi expression by CD11c $^{+}$ CNS cells was reduced (FIGURE 4B). Thus, expression of MHCII by CD11c $^{+}$ cells within the CNS is less in CD11c/A β^b mice compared to MHCII $^{+/-}$ mice but still sufficient to promote neuro-inflammation during EAE.

We sought to determine whether activation of microglia accounts for the difference in MHCII expression by CD11c $^{+}$ cells between CD11c/A β^b and MHCII $^{+/-}$ mice during EAE. Microglia are resistant to lethal irradiation. In contrast, DCs, like B cells, are sensitive to gamma-irradiation. We utilized this relative radio-resistance to further categorize the relevant APCs during EAE. To do this, we generated bone marrow chimeras in which host radio-resistant microglia could be distinguished from donor radio-sensitive DCs. Bone marrow from CD11c-eYFP mice [20], in which DCs express eYFP, was transferred into lethally-irradiated WT mice. In these mice, radiation-sensitive DCs will be eYFP $^{+}$, whereas radiation-resistant microglia expressing CD11c will not express eYFP. CNS APCs were analyzed 15 days after EAE was induced in the chimeras. Approximately half of the CD11c $^{+}$ cells within the CNS of mice with EAE were eYFP $^{+}$ (FIGURE 4C). Bone-marrow-derived CD11c $^{+}$ cells had displayed the highest expression of MHCII (FIGURE 4C). Thus, the majority of MHCII hi -expressing CD11c $^{+}$ cells are bone marrow derived. Given the reduction of MHCII hi DCs in the CNS of CD11c/A β^b mice during EAE (FIGURE 4B), the functional relevance of bone marrow-derived DCs is called into question.

Our results indicate that a sizable fraction of MHCII $^{+}$ APCs in CD11c/A β^b mice is composed of microglial cells (FIGURE 4). To test whether microglia are required for antigen presentation during EAE in CD11c/A β^b mice, lethally irradiated MHCII $^{-/-}$ mice were reconstituted with T-depleted CD11c/A β^b bone marrow. This generated mice with MHCII expression limited to DCs within the radiation-sensitive compartment. Control WT $\rightarrow$ MHCII $^{-/-}$ or CD11c/A β^b $\rightarrow$ CD11c/A β^b bone marrow chimeras were also generated. Eight weeks after thymic transplantation, these chimeras were immunized with MOG 35-55. CD11c/A β^b $\rightarrow$ MHCII $^{-/-}$ and WT $\rightarrow$ MHCII $^{-/-}$ mice were equally susceptible to active EAE (FIGURE 5). In a separate experiment, EAE was similar in CD11c/A β^b $\rightarrow$ MHCII $^{-/-}$ and CD11c/A β^b $\rightarrow$ CD11c/A β^b mice (data not shown). Thus, radiation-resistant DCs are not required for DC-mediated active EAE, consistent with earlier work in passive EAE [5]. Instead, radiation-sensitive DCs are sufficient for all phases of EAE.

3.5 Antigen presentation by DCs results in sub-optimal protein-induced EAE

Antigen presentation by DCs alone is sufficient for disease induction after immunization with peptide. However, the contributions made by other APCs to antigen processing, particularly in an antigen-specific fashion via the B cell receptor, are notable [35,36]. We

hypothesized that by immunizing with MOG peptide containing the immunodominant CD4 T cell epitope, protein uptake and processing is circumvented, allowing for efficient disease induction by DCs. Therefore, we asked if MHCII antigen presentation by DCs alone could mediate active EAE following whole protein immunization using recombinant MOG protein (amino acids 1-125; MOG1-125). Human recombinant MOG protein (hMOG1-125) differs from rodent recombinant MOG protein by one amino acid within the CD4 T cell immunodominant domain (a proline instead of a serine at position 42). This amino acid difference results in the inability to induce EAE in B cell-deficient mice (H-2^b) after immunization with hMOG [14]. However, immunization with rat recombinant MOG protein (rMOG1-125), as opposed to hMOG, results in strong priming toward endogenous MOG rodent epitopes [13] and is not dependent up on B cells.

To test the hypothesis that DCs are a sufficient APC for protein-induced EAE, CD11c/A β ^b mice, along with MHCII^{+/-} littermates, were immunized with rMOG1-125 in CFA. Littermate controls with WT expression of MHCII on average succumbed to hindlimb paralysis on day 13. Disease severity ranged from a score of 2 to 5 with an average overall cumulative disease score of 37.3 at day 25 post-immunization (FIGURE 6A and TABLE 1). rMOG1-125-immunized CD11c/A β ^b mice were also susceptible to protein-induced active EAE with equal frequency (FIGURE 6A and TABLE 1). However, CD11c/A β ^b mice had a delayed onset (average onset = day 18) and reduced severity of disease (average cumulative disease score = 7.4 at day 25), which was statistically much more mild when compared to MHCII^{+/-} mice (TABLE 1). To equalize the number of CD4 T cells present prior to immunization with MOG protein in CD11c/A β ^b and WT mice, we again employed the CD11c/A β ^b×TCR α ^{-/-} system. TCR α ^{-/-} mice (with WT expression of MHCII) receiving 2×10⁷ CD4 T cells prior to immunization with rMOG1-125 developed hindlimb weakness several days before CD11c/A β ^b×TCR α ^{-/-} mice receiving the same number of CD4 T cells (TABLE 1, p<0.05). Again, there was also reduced maximal clinical disease in CD11c/A β ^b×TCR α ^{-/-} mice (average cumulative score at day 25 = 10.3) compared to TCR α ^{-/-} mice receiving identical donor CD4 T cells (average cumulative score at day 25 = 29.3, p < 0.05 compared to CD11c/A β ^b×TCR α ^{-/-}mice) (TABLE 1 and FIGURE 6B). Thus, CD11c/A β ^b mice, with MHCII restricted to DCs, are much less susceptible to protein-induced active EAE compared to mice with expression of MHCII on all APCs.

We recently reported that altered MHCII expression in skin-resident DCs in CD11c/A β ^b mice leads to inefficient T cell activation in response to subcutaneous immunization with protein in CFA [22]. We therefore considered whether CD11c/A β ^b mice immunized with rMOG protein might have limited disease secondary to poor T cell priming. We, therefore, examined the activation and proliferation of MOG-specific 2D2 TCR transgenic CD4 T cells in CD11c/A β ^b mice immunized subcutaneously with rMOG protein. 1×10⁶ CFSE-labeled 2D2 CD4 T cells (with specificity for MOG35-55) were transferred into MHCII^{+/-} or CD11c/A β ^b mice one day prior to subcutaneous immunization with 200 μ g MOG peptide or 150 μ g rMOG1-125 (both standard doses for disease induction). Similar to our published data [22], up-regulation of CD69 and CD25 at 24 and 48 hours post-immunization was similar in WT and CD11c/A β ^b mice (data not shown). However, ~5% of 2D2 CD4 T cells in MHCII^{+/-} controls remained undivided within draining lymph nodes four days after immunization with rMOG1-125, while as many as 30% of antigen-specific cells in CD11c/A β ^b mice (FIGURE 6C) did not divide. The percentage of cells without CFSE dilution showed greater similarity in peptide-immunized CD11c/A β ^b (6.6%) and MHCII^{+/-} (1.4%) mice (FIGURE 6C). Thus, subcutaneous immunization of CD11c/A β ^b mice with standard doses of MOG protein leads to sub-optimal priming of CD4 T cells. Not surprisingly, inadequate peripheral priming is associated with sub-maximal clinical disease in EAE. We hypothesized that an increase in rMOG protein immunogen should overcome this priming deficit. However, at greater than two-fold higher doses of rMOG (400 μ g), the severity of

disease was still reduced (TABLE 1). Overall, these results demonstrate that while CD11c/A β^b mice can develop EAE following protein immunization, there is a limited functional capacity of DCs to act as APCs when immunized with standard doses of protein.

To explore the contribution of B cell antigen presentation during MOG protein-induced EAE, we used hMOG1-125 protein as immunogen. EAE resulting from hMOG1-125 immunization is B cell dependent, though it remains unclear whether the lack of disease in B cell-deficient mice immunized with hMOG1-125 [14] is a consequence of antigen presentation by B cells. To address this, MHCII $^{+/-}$ and CD11c/A β^b mice were immunized with hMOG1-125. MHCII $^{+/-}$ mice succumbed to typical hindlimb weakness 11 to 13 days after immunization with 150 μ g of hMOG protein (FIGURE 6D, Table 1). In contrast, following immunization with hMOG protein, CD11c/A β^b mice did not display signs of clinical disease. These results suggest that APCs other than DCs must express MHCII for disease to occur in protein-induced EAE and that the presence of B cells lacking MHCII is not sufficient for hMOG-induced EAE.

B cell expression of MHCII has several functions including antigen presentation to T cells and CD40-dependent T cell help for germinal center reactions and immunoglobulin class switching [19,37]. Transfer of immune sera can supplant the requirement for B cells in hMOG-induced EAE [14]. Limited MOG-specific IgG would be expected following hMOG immunization. As shown in FIGURE 6E, equivalent amounts of MOG-specific IgM levels were produced in CD11c/A β^b mice, MHCII $^{+/-}$ littermates, and WT C57Bl/6 mice immunized with hMOG protein. However, MOG-specific IgG produced by CD11c/A β^b mice was less than half of MHCII $^{+/-}$ and WT mice (FIGURE 6E). Thus, MHCII expression by DCs alone, with reduced MOG-specific IgG, cannot support MOG protein-induced EAE. Overall, our data indicate a requirement for B cell antigen presentation during protein-induced CNS autoimmunity.

3.6 Antigen presentation by DCs alone does not mediate spontaneous optic neuritis

Given the attenuated EAE observed in CD11c/A β^b mice following protein immunization, we hypothesized that antigen presentation by DCs alone may be incapable of generating encephalitogenic CD4 T cell responses in vivo from endogenous myelin antigens. Optic neuritis spontaneously develops in 2D2 mice with a transgenic TCR specific for MOG35-55 [38]. To determine whether antigen presentation by DCs alone could support the development of spontaneous optic neuritis, we crossed CD11c/A β^b mice to 2D2 mice (CD11c/A β^b ×2D2 mice). Optic nerves were harvested from CD11c/A β^b mice and CD11c/A β^b ×2D2 mice five to six months after thymic transplantation and from age-matched 2D2 mice. Inflammatory changes were evaluated histologically as reported [25,26]. In 2D2 mice, foci of inflammatory cell infiltration were detected in 20% of optic nerves. In contrast, none of the CD11c/A β^b or CD11c/A β^b ×2D2 mice demonstrated evidence of optic neuritis (TABLE 2). Thus, spontaneous inflammatory demyelination resulting from endogenous antigen uptake and processing is limited when antigen is presented by DCs alone.

4. DISCUSSION

We have demonstrated that expression of MHCII by DCs is sufficient to mediate all phases of EAE induced by a short peptide. More precisely, we have defined a minimally sufficient APC population – radiation sensitive DCs – capable of mediating neuro-inflammation after peptide immunization. In contrast, we find that antigen presentation by MHCII $^{+}$ DCs alone cannot support full disease following immunization with either rat or human MOG protein and cannot mediate spontaneous optic neuritis. These findings strengthen our understanding of the role of DCs in EAE and bring into question the contribution of other APCs during neuro-inflammation.

Decreased proliferation of MOG-specific T cells in protein-immunized CD11c/A β^b mice correlates with poor induction of disease. Thus, disease attenuation could result from inadequate subcutaneous immunization in CD11c/A β^b mice that do not reconstitute MHCII expression on migratory DCs in the skin [22]. Consequently, too few encephalitogenic CD4 T cells are generated and a threshold for disease induction is not reached. Thereby, reduced protein-induced EAE in CD11c/A β^b mice may be a direct consequence of limitations in CD4 T cell number. In a similar vein, the diversity of the CD4 T cell repertoire targeting CNS antigens in EAE may also be critical for optimal disease induction. Antigen presentation by DCs limits the repertoire of antigen-specific T cells when compared to B cells in vitro [36]. Thus, in CD11c/A β^b mice, distinct CD4 T cell populations with specificities directed at MOG may be absent. This includes type B T cells that recognize only exogenous peptide, which are known to target myelin epitopes in EAE [39,40]. Overall, these limitations, resulting from initial CD4 T cell priming only by DCs, result in sub-optimal disease induction.

However, it is unlikely that the lack of MHCII⁺ DCs from the skin is entirely responsible for the reduction in neuro-inflammation. Immunization with higher doses of MOG protein, circumventing the need for migratory skin DCs, does not completely restore disease. Furthermore, the absence of optic neuritis in CD11c/A β^b × 2D2 mice indicates that spontaneous autoimmunity is also limited when cognate interactions are limited to DCs and CD4 T cells. Since the development of spontaneous optic neuritis in 2D2 mice is most likely independent of skin-resident APCs, additional factors, including antigen presentation by other professional APCs or antibody, are relevant to maximal priming of MOG-specific CD4 T cells. In any case, this deficiency probably develops from early APC interactions with antigen during the initiation of disease, since CD11c/A β^b mice are equally susceptible to passive EAE [5] and peptide immunization results in robust disease.

It follows that other APCs may be required for efficient antigen processing during disease initiation. B cells are critical regulators of EAE onset particularly after rMOG immunization [12]. Specifically, depletion of B cells, including activated pro-inflammatory populations, in protein-induced EAE reduced the frequency of encephalitogenic T cells and clinical disease [12]. This is in agreement with previous speculation that B cells are required during the pathogenesis of EAE [13] and recent human clinical trials in which depletion of B cells significantly improves outcomes in MS [11]. However, depletion of B cells after the induction of EAE results in greater disease [6], and may modulate disease by performing critical antigen presentation functions [9]. An important consideration is the degree to which B cell involvement in EAE is dependent upon antigen presentation, particularly after protein immunization, which remains unclear [13,14].

B cells may function to complement antigen presentation by DCs during protein-induced EAE via several mechanisms. During disease induction, B cells could be necessary for lymphopoiesis, contribute to antigen presentation, or act as a source of antigen-specific IgG. Immunoglobulin class switching does not occur in CD11c/A β^b mice due to the lack of MHCII-dependent CD4 T cell-B cell interactions [19] and MOG-specific IgG, which has been suggested to potentiate EAE [41], is quite reduced in CD11c/A β^b mice after protein immunization. In preliminary experiments, transfer of immune sera from hMOG-immunized WT or MHCII-sufficient mice into CD11c/A β^b mice immunized with hMOG worsened clinical disease but did not normalize it (data not shown). Thus, MHCII-dependent immunoglobulin class-switching by B cells may provide a parallel contribution to the development of EAE after protein immunization. Induction of EAE with hMOG is associated with unique histo-pathologic features, including greater recruitment of neutrophils. Our results demonstrate that rather than the presence of B cells per se, this

pathology depends on MHCII expression by B cells and is not rescued by DC antigen presentation.

Another key consideration is the timing of B cell involvement during neuro-inflammation. The contribution by B cells during EAE has been shown to differ when comparing the initiation and effector phases. During the induction of peptide-induced EAE, B cells are suppressive of neuro-inflammation [6,12]. This is mediated by IL-10 [42], which in large part is derived from B10 cells (CD1d^{hi}, CD5+ B cells capable of producing IL-10)[43]. In latter phases of disease, B cells promote encephalitogenic T cell responses, and depletion of B cells results in the reduction of clinical disease in both murine [6] and marmoset [44] models of EAE. Furthermore, the capacity of B cells to influence the generation of T regulatory cells during EAE [12], in combination with the emergence of inhibitory effects by T regulatory cells late in disease [43], demonstrates that B cell modulation during EAE is persistent and extensive. Thus, the complex regulation of inflammatory events during EAE is critically dependent upon the timing of involvement by various subsets of B cells.

There is a requirement for de novo antigen processing within the CNS during the effector phase of EAE, as H-2DM-deficient mice incapable of presenting MHCII-restricted antigens are resistant to active or passive EAE, yet can serve as a source of pathogenic CD4 T cells after MOG35-55 immunization [32,33]. Radiation-resistant APCs, and microglial cells in particular, are not required for antigen presentation during EAE (FIGURE 5 and [5]). Rather, radiation-sensitive DCs are sufficient for both the initiation and propagation of CNS inflammation induced by peptide. Thus, sufficient accumulation of DCs within the CNS may be a critical requirement during the development of EAE. The accrual of DCs within the CNS during autoimmune inflammation could result from an influx of fully differentiated DCs. Alternatively, DC precursors mobilized from the periphery could be a major source of DCs during disease [45,46]. Whether interactions between primed myelin-reactive CD4 T cells and DCs at the blood-brain barrier are required for efficient entry into the CNS remains to be determined. However, the partially-radiation-sensitive, CD11c+ perivascular macrophage could serve as the optimal intermediate APC for promoting entry and re-activation of encephalitogenic CD4 T cells. Overall, radiation-sensitive DC accumulation in the CNS is a critical event during EAE that may serve as a potential target for therapeutic intervention during CNS autoimmune disease.

The role of plasmacytoid DCs (pDCs) is an important consideration for the pathogenesis of EAE and MS. pDCs produce large amounts of type I interferon – currently used as one therapy for MS – and localize to the CNS during EAE [47]. In one report, depletion of pDCs results in elevates the severity of peptide-induced EAE [48]. Further, mice in which pDCs lack MHCII exhibit heightened susceptibility to EAE, with greater priming of encephalitogenic CD4 T cells [49]. CD11c/A β ^b mice express MHCII on pDCs at comparable levels to MHCII^{-/-} mice [19]. However, in our experiments, absence of MHCII expression by pDCs did not result in heightened disease in CD11c/A β ^b mice at any point, even when clinical scores were carried out beyond 30 days post-immunization (data not shown). In contrast, depletion of pDCs prior to immunization with MOG protein has been demonstrated to promote Th17 responses and exacerbate EAE [50]. The possibility that pDCs modulate CD4 T cell responses differentially following protein and peptide immunization is open for exploration based on our findings.

5. CONCLUSIONS

In conclusion, there are three main findings in this study. First, DCs alone are fully sufficient to mediate standard EAE after immunization with a 21 amino acid peptide of MOG. In the setting of robust priming, the clinico-pathologic phenotype engendered only by

DCs during EAE is remarkably similar to disease in mice with MHCII expression on all APCs. Second, bone marrow-derived, radiation-sensitive DCs are a minimally sufficient APC for EAE. Thus, a minimally sufficient APC population is defined for all phases of EAE. Finally, DCs cannot efficiently elicit pathogenic CD4 T cell responses following protein immunization or during spontaneous optic neuritis. Overall, these findings point to a modular organization of APCs relevant during the initiation of inflammatory neurologic disease states.

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Abbreviations used in this paper

APC	antigen presenting cell
CNS	central nervous system
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
MHCII	major histocompatibility class II
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
TCR	T cell receptor
WT	wild-type

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Figure 1. Thymic transplantation reconstitutes the CD4 T cell compartment of CD11c/A β^b mice
 CD11c/A β^b mice were recipients of day one post-natal C57Bl/6 thymic transplants. Eight weeks later, peripheral blood was analyzed by FACS for CD4 and MHCII expression. A representative animal pre- (left panel) and post- (right panel) transplant is shown. FACS plots are representative of more than four separate experiments.

A.

B.

Figure 2. CD11c/A β^b and MHCII+/- mice are equally susceptible to MOG peptide- induced EAE
 Mice were immunized with MOG 35-55 according to standard protocol [23]. **A.** Disease onset and course of EAE in MHCII+/- (open diamonds, n=5) or CD11c/A β^b mice receiving thymic transplant (black squares, n=4). MHCII-/- mice (black triangles, n=2) and CD11c/A β^b mice without thymic transplants (open circles, n=3) were asymptomatic. Disease course is representative of three separate experiments. **B.** CD11c/A β^b mice crossed to TCR α -/- mice (CD11c/A β^b ×TCR α -/- mice), or control TCR α -/- mice, received 2×10^7 CD4 T cells 24 hours prior to immunization with MOG peptide. Disease course shown is representative of at least three separate experiments.

Figure 3. Pathogenic cytokine production by CD4 T cells in CD11c/A β^b and MHCII $^{+/-}$ mice with peptide-induced EAE is equivalent

A. CD4 production of IL-17 was measured by intracellular staining without stimulation (left panels) or following ex-vivo stimulation with MOG35-55 peptide (middle panels) or PMA/Ionomycin (right panels). Fractions of IL-17-producing CD4 T cells from lymph nodes at day 15 post-immunization were similar between WT (top row) and CD11c/A β^b (bottom row) mice. FACS plots are representative of three separate experiments. **B.** Following mononuclear cell isolation from the CNS of perfused mice, cells were stimulated ex vivo in the presence of MOG35-55. Surface staining for CD4 along with intracellular cytokine staining was performed (plots shown are from CD4 $^{+}$ gated cells). Similar fractions of CD4 T cells produced IL-17 and IFN- γ in both MHCII $^{+/-}$ (left panel) and CD11c/A β^b (right panel) mice. FACS plots are representative of two separate experiments.

A.

B.

C.

Figure 4. CNS DC expression of MHCII during EAE is reduced in CD11c/A β^b compared to MHCII $^{+/-}$ mice

A. Mononuclear cells were isolated from the CNS of CD11c/A β^b and MHCII $^{+/-}$ mice with peptide-induced EAE. After gating on CD11c $^{+}$ cells, DCs were further analyzed for expression of CD45 and CD11b (left panel = MHCII $^{+/-}$, right panel = CD11c/A β^b).

Population I represents microglial cells and population II represents activated microglial cells along with infiltrating monocytes, macrophages and dendritic cells [34]. **B.** The level of MHCII expression was assessed on all CD45 $^{+}$ mononuclear cells from the CNS of mice with EAE (left plot; shaded area = MHCII $^{+/-}$, black line = CD11c/A β^b). MHCII levels were also evaluated on populations I and II gated from panel A (middle plot = population I, right plot = population II; shaded area = MHCII $^{+/-}$, black line = CD11c/A β^b). Data shown in A and B represent two separate experiments using three or more mice for each group. **C.**

CD11c-eYFP $\rightarrow$ WT radiation bone marrow chimeras were immunized with MOG35-55. On day 15 post-immunization, CNS mononuclear cells were isolated and stained for CD11c and MHCII. FACS plots are representative of two separate experiments.

Figure 5. Radiation-resistant DCs are not required for Active EAE

Clinical disease was assed in lethally irradiated MHCII^{-/-} mice receiving WT bone marrow (open triangles, n=3) or CD11c bone marrow (filled circles, n=4) after immunization with MOG35-55. Disease course is representative of two experiments.

C.

Figure 6. Mice with antigen presentation limited to DCs mice show reduced susceptibility to active EAE with protein

A. Thymic transplant-recipient CD11c/A β^b mice were immunized with rMOG1-125 according to standard protocol [13]. Clinical disease for MHCII^{+/+} (open diamonds, n=4) and CD11c/A β^b (black squares, n=5) mice was measured as described in the Materials and Methods. Disease course is representative of two separate experiments. **B.** CD11c/A β^b ×TCR $\alpha^{-/-}$ (black squares; n=3) and TCR- $\alpha^{-/-}$ (open diamonds; n=2) mice received 2×10^7 CD4 T cells 24 hours prior to immunization with rMOG1-125. Disease course is representative of two separate experiments. **C.** 1×10^6 CFSE-labeled MOG-specific 2D2 cells were transferred i.v. into CD11c/A β^b or MHCII^{+/+} mice 24 hours prior to immunizing with 200 μ g MOG35-55 (middle column) or 150 μ g rMOG protein (right column). CFSE dilution of 2D2 cells was assessed four days later following isolation from draining lymph nodes. MHCII^{+/+} mice immunized with CFA alone served as negative controls (far left panel). Data shown is representative of two separate experiments. **D.** Clinical disease was measured in MHCII^{+/+} (open diamonds; n = 4) and CD11c/A β^b mice receiving thymic transplants (black squares; n = 4) immunized with 150 μ g hMOG protein. Graph is representative of two separate experiments. **E.** MOG-specific IgM and IgG antibodies were measured from serum of CD11c/A β^b (n = 6), MHCII^{+/+} (n = 5) and WT C57Bl/6 mice (n = 4) immunized with hMOG protein. Values are combined from two separate experiments (* indicates $p < 0.0001$ when comparing CD11c/A β^b and MHCII^{+/+} mice using Student's t test).

Table 1

MOG protein-induced EAE in CD11c/Aβ^b and MHCII^{+/+} mice

Mouse group	Immunogen	Incidence	Onset (mean ± SEM) ^a	Maximal Score	Cumulative Score/mouse (mean ± SEM) ^b
CD11c/Aβ ^b	rMOG ^c (150 µg)	10/11	18.5 ± 1.1	4	7.4 ± 3.4
MHCII ^{+/+}	rMOG (150 µg)	11/11	13.2 ± 0.5	5	37.3 ± 3.4 ^d
CD11c/Aβ ^b ×TCRα ^{-/-} ^e	rMOG (150 µg)	6/6	19.7 ± 2.3	4	10.3 ± 4.7
TCRα ^{-/-}	rMOG (150 µg)	4/4	14.3 ± 1.0	5	29.3 ± 4.4 ^f
CD11c/Aβ ^b	rMOG (400 µg)	6/12	17.3 ± 1.2	4	10.7 ± 3.8
MHCII ^{+/+}	rMOG (400 µg)	2/2	12.5 ± 0.5	5	51.0 ± 5.0 ^g
CD11c/Aβ ^b	hMOG (150 µg)	1/7	17	2	2.4 ± 2.4
WT ^h	hMOG (150 µg)	8/9	12.9 ± 0.8	4	30.4 ± 5.8 ⁱ

^aInclusive of mice with clinical disease only

^bFor statistical purposes, comparisons were made at day 25 post-immunization; a majority of experiments were carried out beyond day 30 post-immunization

^crMOG (rodent MOG) differs from hMOG (human MOG) protein by one amino acid substitution at position 42. While both can serve as encephalitogenic immunogens, hMOG induces a B cell-dependent disease in B6 mice, whereas immunization with rMOG is capable of eliciting EAE in B cell deficient mice [13].

^dp < 0.0001 compared to cumulative score from CD11c/Aβ^b group

^eCD11c/Aβ^b×TCRα^{-/-} and TCRα^{-/-} mice were recipients of 2×10⁶ CD4 T cells prior to immunization

^fp < 0.05 compared to cumulative score from CD11c/Aβ^b×TCRα^{-/-} group

^gp < 0.01 compared to cumulative score from CD11c/Aβ^b group

^gWT includes MHCII^{+/+} and C57Bl/6 mice

^hp < 0.01 compared to cumulative score from CD11c/Aβ^b group

Table 2Spontaneous optic neuritis in 2D2, CD11c/A β^b and CD11c/A β^b ×2D2 mice

Mouse group	Number of mice	Number of optic nerves with optic neuritis ^a	Optic nerves affected (%)
2D2	10	4	20 ^b
CD11c/A β^b	18	0	0
CD11c/A β^b ×2D2	14	0	0

^aOptic nerves were harvested between five to six months after thymic transplant from CD11c/A β^b and CD11c/A β^b ×2D2 mice along with age-matched 2D2 mice and examined for infiltration of inflammatory cells. Histologically, optic nerves with inflammation greater than or equal to grade 2 were classified as positive for optic neuritis.

^bp = 0.02 for comparison between 2D2 and CD11c/A β^b ×2D2 groups