Distinct Myeloid Cell Subsets with Divergent Functions in Central Nervous System Autoimmunity

by

David A. Giles

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor in Philosophy
(Immunology)
in the University of Michigan
2018

Doctoral Committee:

Professor Benjamin M. Segal, Chair
Associate Professor Irina L. Grigorova
Professor Andrew P. Lieberman
Professor Nicholas W. Lukacs
Professor Pavan R. Reddy

David A. Giles

dagiles@umich.edu

ORCID iD: 0000-0002-2745-4374

Dedication

Soli Deo gloria

To the glory of God alone

Acknowledgements

To those who have supported and encouraged in my academic career, I say thank you:

- Ben Segal, my thesis mentor - Thank you for challenging me to think more critically and more creatively. You gave me incredible freedom and were unsatisfied with anything less than excellence. Your example and mentorship has fundamentally changed the way that I approach science and ask questions of the world.
- Members of the Segal lab, past (Stephen, Julie, Kevin, Heather, Josh, Tina, Lisa) and present (Andrew, Alina, Patrick, Jesse, Ying, Niki) – Thank you for making my time in lab a joy. I came for the science but stayed for the quality peers and friendships. Jesse, Ying, and Niki, thank you for all of your technical help. These experiments wouldn't have happened without you. Patrick, thank you for all the times you helped with experiments, listened to my ideas, and provided insights. I am a better scientist after working with you.
- My committee members (Irina Grigorova, Andrew Lieberman, Nick Lukacs, and Pavan Reddy) – Thank you for your critical input and advice throughout my time in grad school.
- Richard Goodman and Stephen Magill, my first research mentors – Thank you for treating me like a researcher and introducing me to the world of biomedical research. It has been as exciting and challenging as you promised.
- My peers in the MD/PhD and Immunology programs – Thank you for being comrades in this program. Heather Schofield, thank you for all the lunches and conversations. You have been a faithful friend, and it is always a comfort to have you to sympathize with and share these experiences.

- My community at Knox Presbyterian Church – Thank you for your prayers and encouragement. Thank you for giving me perspective and encouraging me to find my hope and worth in Christ.
- My parents and family – Thank you for giving me every opportunity. I am only here because of the foundation you provided.

I am ever so grateful for you.

Table of Contents

List of Figures

Abstract

Multiple sclerosis (MS) is a disabling disorder of the central nervous system (CNS) characterized by motor, visual, and sensory deficits. It is believed to be an autoimmune process in which immune cells infiltrate the brain and spinal cord leading to demyelination and cell death. Myeloid cells, such as macrophages and dendritic cells, are a major component of the cellular infiltrates in the CNS. Initial studies described these myeloid cells as pathogenic, but recent evidence in other systems has revealed that myeloid cells may also be anti-inflammatory and even pro-regenerative. Little is known about the heterogeneity of myeloid cells in MS. We set out to investigate the diverse phenotypes and functional properties of myeloid cells in CNS autoimmunity.

Pro-inflammatory and anti-inflammatory myeloid cells have been termed classically or alternatively activated myeloid cells (CAMC, AAMC), respectively. We began by identifying markers of CAMC and AAMC in MS lesions. We observed a spectrum of activation such that CAMC were present at the active lesion rim while AAMC were predominant in the relatively quiescent lesion core. Using the animal model experimental autoimmune encephalomyelitis, we identified similar CAMC and AAMC in the CNS with the markers iNOS and Arg1, respectively. Myeloid cell phenotypes evolved such that $iNOS^+$ CAMC were present early in disease while $Arg1^+$ AAMC were present at peak and late disease. We further demonstrated that individual cells were plastic and shifted from expression of iNOS to Arg1 over the course of disease.

One function of myeloid cells is the activation of T cells. Activation is critical for the initiation of disease. We compared the functional properties of $Arg1^+$ and $Arg1^-$ cells and found

that $Arg1^+$ cells were deficient in activating $CD4^+$ T cells compared with $Arg1^-$ cells. Investigation of the mechanism of this defect led to the elucidation of additional myeloid cell subsets. We identified that the dendritic cell population was heterogenous, not only in activation state or phenotype, but also in developmental lineage. We identified cells of both the conventional (cDC) and monocyte-derived dendritic cell (moDC) lineages in the CNS during EAE and demonstrated functional specialization between these subsets. iNOS and Arg1 expression were restricted to the moDC subset, and this subset as a whole was deficient in T cell activation but proficient in phagocytosis. Conversely, cDC were highly efficient in activating T cells but weak phagocytes.

It is not clear which antigen presenting cell activates T cells to initiate disease. We observed that cDC were present in the naïve brain and meninges and that these cells from the naïve CNS could activate myelin-specific T cells. We further demonstrated that specific depletion of cDC reduces the incidence of EAE. This identifies cDC as critical antigen presenting cells for the initiation of CNS autoimmunity. Together, these studies clarify the diversity of myeloid cells and demonstrate functional differences with implications for clinical disease. The results presented herein provide a foundation for the development of myeloid cell-targeted therapies for MS.

Chapter 1 - Introduction

Multiple sclerosis (MS) is a disorder of the central nervous system (CNS) which causes debilitating motor, visual, and sensory deficits. It is a leading cause of disability in young adults and affects ~400,000 individuals in the United States[1] and over 2.3 million worldwide[2]. Patients are typically diagnosed between 20 and 40 years of age, and MS can have a devastating impact on their quality of life. Patients with MS are 3.3-fold more likely to not be employed, lose an average of 10 quality adjusted life years[3], and have life expectancy reduced by 7-14 years[4]. MS is also the second most costly chronic condition in the United States, with costs ranging from $8,528-$54,244 per patient per year[5]. MS carries significant personal and financial costs, and while current treatments offer hope, there is great need for new therapies which can prevent and even reverse the pathology of MS.

Epidemiology of MS

MS patients follow one of four different clinical courses: relapsing-remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS), or progressive-relapsing (PRMS)[6]. 85% of patients have a relapsing-remitting course at onset in which bouts of disability may persist for weeks or months but resolve[7,8]. Within 10 years, half of patients with RRMS will advance to a secondary progressive course which is characterized by increasing disability without a return to baseline. Another 10% of patients are diagnosed with primary progressive disease characterized by gradual accumulation of disability without distinct relapses and remissions. The rarest form is progressive-relapsing MS which affects ~5% of MS patients. In

this course, patients experience distinct relapses and remissions superimposed on a progressive increase in disability such that attacks leave permanent deficits without a return to baseline. It is currently unclear whether these clinical courses are distinct or whether they represent different stages or presentations of a fundamental similar inflammatory process.

The etiology of MS is uncertain but both genetic and environmental factors may contribute. Pointing to a role for genetics, the risk for first degree relatives is 20 times higher than the general population, and several susceptibility genes have been identified including genes in the MHC loci and those related to T cell function[9]. However, genes are not the only factor as the prevelance in monozyogotic twins is only 20%. Environment also plays a role. For example, incidence is higher in northern latitudes[10], for those with low levels of Vitamin D[11], or with past infection with Epstein Barr virus[12,13].

Pathogenesis of MS

A pathologic feature of MS is damage of the myelin which insulates nerve axons in the brain and spinal cord[14]. The CNS lesions were first described by Jean Cruveilhier and Robert Carswell in the 1830s followed by Jean-Martin Charcot in 1868[15]. Charcot was the first to connect these pathologic lesions with clinical findings and to identify MS as a distinct disease. Histological examination of lesions in the CNS reveal demyelination, blood-brain barrier breakdown, axonal degeneration, and reactive gliosis. Lesions are infiltrated by peripheral immune cells including T lymphocytes and macrophages and can be classified by the degree of demyelination and inflammation[16]. Active lesions are characterized by loss of myelin with diffuse infiltration of the complete lesion area with macrophages and microglia; inactive lesions are similarly demyelinated but hypocellular with few macrophages[17]. An intermediate

phenotype has also been observed with macrophages localized to the active lesion rim but absent in the quiescent lesion center. MS disease progression correlates with a shift in predominance of active to mixed to inactive lesions demonstrating the evolution of neuroinflammation over the course of disease[18].

Given the central role of the immune system in the CNS pathology, MS is believed to be autoimmune, and several disease-modifying therapies have been developed to target the immune system. To date, 15 drugs have been approved by the FDA for the treatment of MS, and though the specific mode of action for many are still under investigation, the drugs can generally be grouped by proposed mechanism[19]. Daclizumab, alemtuzumab, and ocrelizumab are monoclonal antibodies which target specific leukocyte subsets for depletion. Fingolimod and natalizumab block immune cell release for the lymph nodes or access to the CNS, respectively, and teriflunomide targets proliferating lymphocytes. IFNβ, glatiramer acetate, and dimethyl fumarate are believed to shift the quality of the immune response from pro- to anti-inflammatory.

These therapies have proved to be effective at reducing the relapse rate in RRMS, highlighting the critical role for inflammation in the pathology of MS. However, few show efficacy in the progressive stage of disease and none reverse disability or promote recovery[20]. There is a significant gap in this therapeutic arsenal in that none of the approved therapies specifically target myeloid cells. These are cells of the innate immune system which are derived from common myeloid precursors in the bone marrow and include monocytes, macrophages, dendritic cells, neutrophils, basophils, eosinophils, erythrocytes, and megakaryocytes. Monocytes, macrophages, and dendritic cells, in particular, are prominent populations in MS lesions and are key effectors in the pathologic process by breaking down and phagocytosing myelin. Several recent studies have also demonstrated that myeloid cells may be dysregulated

specifically in progressive stages of disease[21–24]. But while the pathologic role of myeloid cells is clear, there is a growing body of evidence that differentially activated myeloid cells may be anti-inflammatory or even pro-regenerative.

Myeloid cell phenotypes *in vitro*

Myeloid cells acquire different phenotypes in response to different environmental cues. In the 1980s, Nathan and colleagues first demonstrated that macrophages activated by stimulation with IFNγ have enhanced cytotoxic properties[25]. In the 1990s, Stein, Doyle, and colleagues noted that IL-4 and IL-13 also activated macrophages, but these activated macrophages differed from those stimulated by IFNγ by reduced secretion of pro-inflammatory cytokine and enhanced expression of the mannose receptor[26,27]. In 2000, Mills and colleagues further characterized these as classically or alternatively activated macrophages and assigned the names M1 and M2, respectively, given their association with cytokines produced by Th1 or Th2 cells[28]. M1 macrophages were generated by stimulation with IFNγ and identified by expression of the enzyme inducible nitric oxide synthase (iNOS); M2 macrophages were generated in response to IL-4 and identified by expression of the enzyme arginase-1 (Arg1) (Figure 1.1).

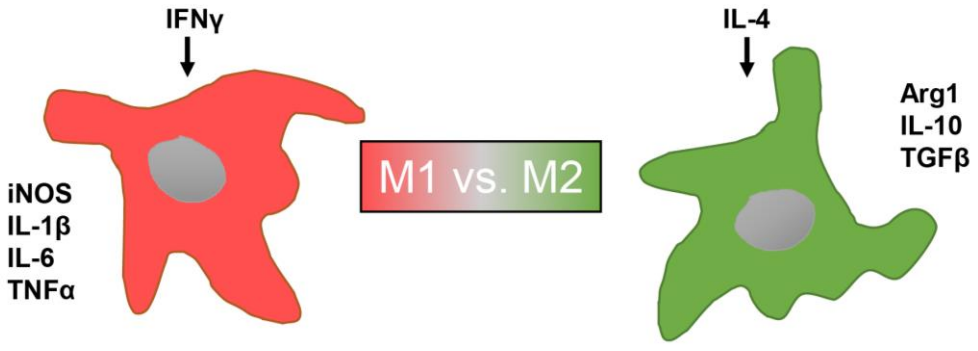


Figure 1.1 – *In vitro* macrophage polarizations.

M1 and M2 macrophages have a different impact on inflammatory response. M1 macrophages produce cytokines such IL-1β, IL-6, and TNFα to promote ongoing inflammation while M2 macrophage produce IL-10 and TGFβ1 to tamp down to the inflammatory response[29]. Even their canonical enzymes have distinct effects. Both iNOS and Arg1 metabolize the amino acid arginine but generate different products. iNOS generates toxic nitric oxide radicals while Arg1 generates trophic polyamines[30,31]. Given their influence on the inflammatory response, M1 macrophages were characterized as "pro-inflammatory" and M2 macrophages were characterized as "anti-inflammatory".

While the M1/M2 nomenclature provided a framework to understand macrophage activation, it quickly became clear that this framework was incomplete as additional cytokines and factors were shown to influence macrophage polarization. To address this, new subsets were proposed including IL-4-stimulated M2a, immune complex-stimulated M2b, and IL-10-stimulated M2c[32]. Further study also revealed that polarization of macrophages is not fixed. For example, macrophages stimulated with IFNγ to induce iNOS expression can be converted to produce Arg1 by secondary stimulation with IL-4, and vice versa[33]. It is unclear, however, whether this represents a reprogramming of individual cells or an expansion of different cells within a heterogenous population.

Myeloid cell phenotypes *in vivo*

In the complex inflammatory milieu *in vivo*, macrophages exist in a spectrum of phenotypes. The markers and functional attributes of these myeloid cells are highly dependent on the tissue and disease model. For example, macrophages present early in the response to neoplasia are similar to classically-activated, pro-inflammatory myeloid cells and can be

protective by eradicating transformed cells[34,35], but as tumors grow, an anti-inflammatory macrophage population develops. These cells are often described as tumour-associated macrophages and can be anti-inflammatory by secreting high amounts of IL-10[36]. As an alternative example, macrophages in non-obese adipose tissue appear anti-inflammatory with low expression of cytokines and high expression of Arg1. However in obesity, adipose macrophages begin to produce pro-inflammatory cytokines such as TNFα and IL-6 which fundamentally alter the inflammatory milieu[37]. A similar shift from anti- to pro-inflammatory macrophages has been observed in atherosclerosis[37]. The phenotypes of myeloid cells in disease can have critical influence on the inflammatory process, and thus extensive study is necessary to understand the properties and functions of myeloid cells in a manner specific to tissue and disease process.

Experimental autoimmune encephalomyelitis

The animal model experimental autoimmune encephalomyelitis (EAE) has been used to investigate the role of different immune population in CNS autoimmunity. EAE is a model of autoimmunity driven by myelin-specific $CD4^+$ T cells, and several related models have been developed using mice, rats, and non-human primates[38]. In C57Bl/6 mice, there are two disease induction methods. Disease may be induced by active immunization with a peptide of myelin oligodendrocyte glycoprotein (MOG_{35-55}) in combination with Complete Freund's Adjuvant and two doses of pertussis toxin. Mice generate a $CD4^+$ T cell response toward the myelin peptide which leads to activation of the T cells, immune infiltrating into the CNS, and the initiation of inflammation and damage. Histological examination reveals demyelinating lesions and dense infiltrates in the brain and spinal cord[39]. The clinical outcome is an ascending paralysis

approximately two weeks after immunization, and the level of disability is scored on a scale 0-5. It begins with a limp tail and hind-limb weakness that progresses to full hind-limb paralysis with occasional fore-limb involvement. The mice are permanently disabled and do not recover, so this is described as a chronic model of CNS autoimmunity (Figure 1.2A). Disease may also be induced by adoptive transfer of myelin-specific T cells. In this method, donor mice are immunized with myelin peptide and adjuvant. After two weeks, immune cells are collected from the lymph nodes which drain the immunization sites and cultured with myelin peptide and polarizing factors for 4 days. At the end of culture, $CD4^+$ T cells are purified and transferred intraperitoneally into a naïve recipient. Mice develop paralysis approximately 1 week after cell transfer but follow a similar chronic clinical course[40] (Figure 1.2B).

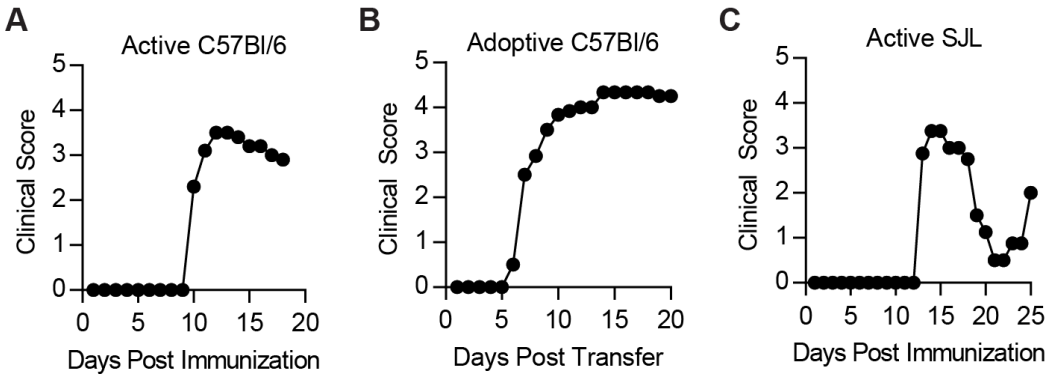


Figure 1.2 - Clinical courses of EAE models.

While these models in C57Bl/6 mice recapitulate the demyelinating lesions of MS, they lead to chronic disability and do not have the relapsing-remitting course observed in the majority of MS patients. To model the course of RRMS, a different strain can be used[41]. SJL mice immunized with a peptide of the myelin proteolipid protein ($PLP_{139-151}$) develop hind limb paralysis approximately 2 weeks after immunization but regain function by 3 weeks post immunization (remission). At four weeks post immunization, many of the mice will begin to

develop paralysis for the second time (relapse). In this way, the SJL mice display a relapsing remitting course (Figure 1.2C). The inflammatory cell infiltrate in the CNS correlates with the degree of severity, expanding during relapse and contracting during remission[42].

Myeloid cells in MS and EAE

Our laboratory and others have demonstrated that myeloid cells are critical for early EAE pathogenesis. The numbers of infiltrating monocytes in the CNS correlate with disease severity[43]. Depleting monocytes or blocking their recruitment abrogates disease[44–47] whereas enhancing Ly6C$^+$ monocytes in the blood exacerbates disease[48]. There are several effector functions of myeloid cells that may be pathogenic in EAE. Myeloid cells may re-activate myelin-specific CD4$^+$ T cells[49,50], secrete pro-inflammatory cytokines such as IL-6, IL-12, and IL-23[29,51,52], and release toxic factors such as TNFα and nitric oxide to directly inflict damage[53]. Monocyte-derived macrophages are in close proximity to demyelinating axons and contain myelin debris, suggesting an active role in demyelination[54]. Similar observations have been made in active MS lesions including oxidative damage[55] and phagocytosed myelin debris[56].

The pathogenic role for myeloid cells is clear, but there is increasing evidence that myeloid cells may also be protective in EAE. Peripheral injection of macrophages stimulated with immune complexes[57] or with IL-10[58] can suppress EAE. Similarly, intracerebroventricular transfer of microglia stimulated with IL-4 can reduce the severity of EAE[59]. Monocytes incubated with glatiramer acetate, one of the approved therapies for MS, can also reduce the severity of EAE[60]. How are the myeloid cells exerting this positive influence? These alternatively-activated myeloid cells may produce higher levels of anti-inflammatory cytokines such as IL-10 and TGFβ1 or reduced levels of pro-inflammatory factors such as IL-12[57,60]. The

cells may produce trophic factors to promote oligodendrocyte maturation[61] or axon growth[62]. The phagocytic function of macrophages may itself have protective role as blockade of TREM2, a key phagocytic receptor, exacerbates EAE[63]. These studies demonstrate the dual role myeloid cells may have in neuroinflammation, and more investigation is necessary to understand the functional and phenotypic heterogeneity of myeloid cells in CNS autoimmunity.

Phenotype vs. lineage

At this point, an important distinction must be made between cell phenotypes and developmental lineages. Phenotypes are transcriptional programs induced by stimuli in the setting of inflammation, such as classically or alternatively activated. The same cell may be polarized to distinct phenotypes by different signals, and thus there is the potential for plasticity between these phenotypes. For example, a macrophage may be secreting a pro-inflammatory cytokine at one moment and an anti-inflammatory cytokine the next but is always a macrophage. Developmental lineages are cell fates that diverge at the point of differentiation from a progenitor in a hematopoietic organ. B cells and T cells, for example, are derived from the same precursor but differentiate in the bone marrow to distinct lineages. It is easy to appreciate this difference because we have identified markers unique to these lineages. However, this is not always the case.

Myeloid cell lineages

A recent study set out to determine the origin of myeloid cell subsets in the naïve CNS. Using transgenic mice to fate map cell lineages, Goldmann et al[64] compared the source of microglia, perivascular macrophages, choroid plexus macrophages, and meningeal macrophages.

These populations were defined as myeloid cells by expression of the marker Iba-1. The nomenclature, however, is complicated as many of the cells are not derived from common myeloid precursors in the bone marrow. Microglia are a tissue resident population that comprises the majority of myeloid cells in the naïve CNS. Though microglia perform as mononuclear phagocytes like macrophages and express many of the same markers, microglia are not derived from the bone marrow. Instead, microglia are derived from the yolk sac during embryogenesis[65,66] and are locally sustained in the adult[67]. Microglia are located within the parenchyma of the tissue, but other macrophages have been identified at interfaces with the periphery such as the blood vessels, meninges, and choroid plexus. These border macrophages can be distinguished from microglia by higher expression of the cell markers CD45 and CD11b. It was thought that the border macrophages were classical myeloid cells derived from the bone marrow, but Goldmann et al[64] demonstrated their origin is mixed. Some of these cells persist from embryogenesis like microglia but others are replenished from the bone marrow. The functional implications of this heterogeneity are unknown. This study provides an example of the diversity of lineages in the naïve CNS. In the setting of neuroinflammation, many of these resident populations are indistinguishable from the infiltrate using standard markers, and analysis is further complicated. If consideration is not given to identification of myeloid cell lineages, analysis of phenotypes may be confounded by the heterogeneity of the population.

The distinction between cell lineages is especially difficult but not less important in a subset of myeloid cells called dendritic cells (DC). DCs were initially described by their large nuclei and extended dendrites[68]. This umbrella classification includes cells diverse in both phenotype and lineage. With the discovery of cell surface markers, DCs were further refined in mice to be cells with high expression of MHC-II and CD11c. This classification remains

heterogeneous, and many attempts have been made to split this group into meaningful subsets. A primary split can be made between cells of the monocyte-derived versus conventional DC lineages[69] (Figure 1.3). Monocyte-derived DC (moDC) differentiate from monocytes such that the cells leave the bone marrow as monocytes, travel to the site of inflammation as monocytes, and differentiate in tissues in response to inflammatory stimuli such as GM-CSF[70,71]. In contrast, conventional dendritic cells (cDC) are derived from a pre-DC population in the bone marrow and populate tissues and lymphoid organs during homeostasis[72]. cDCs in tissues are continuously replenished by progenitors from the bone marrow in a Flt3-dependent manner[73] and may expand in the setting of inflammation[74]. moDC and cDC share many of the same surface markers, so it has been difficult to distinguish these lineages. As a result, it is unclear whether these subsets have distinct or overlapping functional roles.

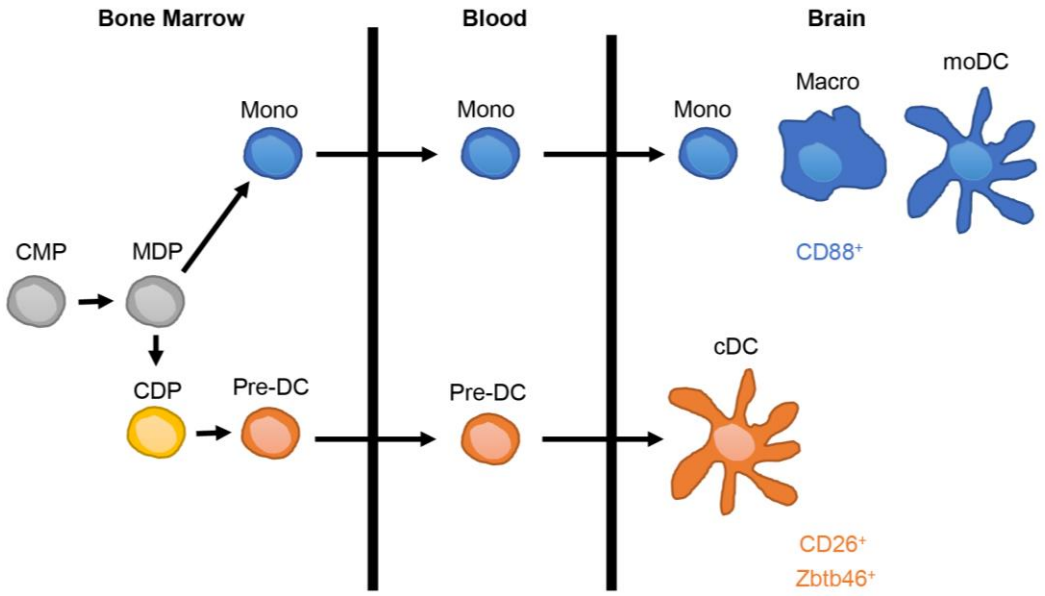


Figure 1.3 – Dendritic cell developmental lineages.
CMP, common myeloid precursor; MDP, macrophage DC progenitor; CDP, common DC progenitor; Mono, monocyte; Macro, macrophage; moDC, monocyte-derived DC; pre-DC, precursor DC, cDC, conventional DC.

Markers to identify DC lineages

CD64 and CD88 have recently been identified as markers of the monocyte lineage[75–78]. These markers are not specific to dendritic cells but do specifically identify monocyte-derived cells within the $CD11c^{+}MHCII^{+}$ population. Some reports question whether CD64 effectively discriminates moDC and cDC in all tissues[79], but the specificity of CD88 has confirmed in the lung, spleen, kidney, liver, and small intestine[78]. CD88 (C5ar1) is a complement receptor expressed on neutrophils, eosinophils, monocytes, and even some non-hematopoietic cells such as neurons and astrocytes[80]. Conversely, CD26 (dipeptidyl peptidase 4), an enzyme involved in peptide hydrolysis, has been identified as a marker with expression on cDC but not moDC. In addition to cDC, CD26 is expressed by activated T and B cells[81], plasmacytoid DCs, and many non-hematopoietic cells including stromal cell of the CNS meninges[82]. Though CD88 and CD26 are widely expressed, the combination of these markers permits the distinction of the moDC and cDC lineages within the DC compartment and is therefore a vital tool in discriminating functional roles for moDC and cDC in inflammation.

The transcription factor Zbtb46 (BTBD4) has also been identified as a specific marker of cDC in both mice and humans[83,84]. Its expression is more specific than CD26, but there is still additional expression in definitive erythroid precursors and endothelial cells[84]. To evaluate the function of cDCs, Meredith et al generated Zbtb46-DTR mice to allow specific depletion of Zbtb46-expressing cells by administration of diphtheria toxin[83]. A single dose, however, proved lethal. To focus on the role of Zbtb46 in hematopoietic cells, Zbtb46-DTR bone marrow was used to generate chimeras. Using this system, Meredith et al demonstrated that Zbtb46-DTR specifically depletes cDC in multiple organs without depleting moDCs. Furthermore, depletion of Zbtbt46-expressing cells abrogated both $CD4^{+}$ and $CD8^{+}$ T cell responses to immunization,

pointing to a critical role for cDC in antigen presentation. This transgenic mouse provides a powerful tool to specifically target cDC and evaluate their role in homeostasis and disease.

Several groups have used these tools to investigate the roles of moDC and cDC in other disease models. Both cDC and moDC are present in the lung, and cDC displayed an enhanced capacity to activate T cells compared with moDC[78]. Both cDC and moDC are also present in the heart following myocardial infarction, but cDC are superior at activating autoreactive $CD4^+$ T cells *ex vivo*[85]. Depleting cDC with the Zbtb46-DTR mice impairs $CD4^+$ and $CD8^+$ T cell responses to immunization with functional implications on tumor responses[83]. Specific depletion of MHCII on Zbtb46-expressing cells also abrogated T cell responses to immunization and impaired antimicrobial adaptive immune responses in the gut[86]. These studies begin to demonstrate the distinct roles of moDC and cDC in inflammatory processes in diverse tissues, but many questions remain. Future study is necessary to evaluate the presence of these subsets in the CNS and to determine the functional role of these subsets in MS and EAE.

Specific Aims

Myeloid cells are a prominent component of CNS inflammatory lesions that accumulate in individuals with MS and in laboratory animals with EAE. There is increasing evidence that these myeloid cells are heterogenous in both phenotype and developmental lineage, but the implications of this heterogeneity are unclear. Myeloid cells may by pathogenic by promoting and sustaining inflammation within the CNS or have anti-inflammatory properties and play a role in the resolution of inflammation and initiation of repair. Current therapies for MS do not discriminate between myeloid cell subsets. Investigating the diversity of myeloid cell

phenotypes may reveal novel methods to specifically target pathogenic immune cells while preserving protective subsets and promoting recovery during autoimmune demyelinating disease.

In this dissertation, we undertake the investigation of myeloid cells in CNS autoimmunity in two parts:

- **Aim 1: Chapter 2:** Investigate myeloid cell phenotypes and function in MS and EAE. We use iNOS and Arg1 as markers of myeloid cell phenotypes and characterize the evolution of these phenotypes on the single cell level in disease.
- **Aim 2: Chapter 3:** Determine the roles of monocyte-derived versus conventional dendritic cells in EAE. We identify multiple DC lineages in the CNS and characterize their distinct functions and contributions to disease.

These studies provide critical insights into the immunopathology of CNS autoimmunity and can inform the development of therapies for MS.

Chapter 2 - Myeloid cell plasticity in the evolution of central nervous system autoimmunity

****Portions of this chapter are under review for publication**
Giles DA, Washnock-Schmid JM, Duncker PC, Dahlwai S, Ponath G, Pitt D, Segal BM. Myeloid cell plasticity in the evolution of central nervous system autoimmunity. 2017.

Abstract

Objective: Myeloid cells, including macrophages and dendritic cells, are a prominent component of central nervous system (CNS) infiltrates during multiple sclerosis (MS) and the animal model experimental autoimmune encephalomyelitis (EAE). Although myeloid cells are generally thought to be pro-inflammatory, alternatively-polarized subsets can serve non-inflammatory and/or reparative functions. Here we investigate the heterogeneity and biological properties of myeloid cells during central nervous system autoimmunity. **Methods:** Myeloid cell phenotypes in chronic active MS lesions were analyzed by immunohistochemistry. In addition, immune cells were isolated from the CNS during exacerbations and remissions of EAE and characterized by flow cytometric, genetic and functional assays. **Results:** Myeloid cells expressing iNOS, indicative of a pro-inflammatory phenotype, were detected in the actively demyelinating rim of chronic active MS lesions, whereas macrophages expressing mannose receptor (CD206), a marker of alternatively-polarized human myeloid cells, were enriched in the quiescent lesion core. During EAE, CNS-infiltrating myeloid cells, as well as microglia, shifted from expression of pro- to non-inflammatory markers immediately prior to clinical remissions. Murine CNS myeloid cells expressing the alternative lineage marker arginase-1 (Arg1) were partially derived from $iNOS^+$ precursors and were deficient in activating encephalitogenic T cells

compared with their Arg1$^-$ counterparts. **Interpretation:** These observations demonstrate the heterogeneity of CNS myeloid cells, their evolution during the course of autoimmune demyelinating disease, and their plasticity on the single cell level. Future therapeutic strategies for disease modification in individuals with MS may be focused on accelerating the transition of CNS myeloid cells from a pro- to a non-inflammatory phenotype.

Introduction

Myeloid cells, including macrophages (MΦ) and dendritic cells (DC), are a major component of white matter lesions in multiple sclerosis (MS) and the animal model experimental autoimmune encephalomyelitis (EAE)[87,88]. Our laboratory and others have established a critical role of myeloid cells in early EAE pathogenesis[43,45,48,89]. Myeloid cells may serve as antigen presenting cells for re-activation of myelin-specific CD4$^+$ T cells[49,50], secrete cytokines such as IL-6, IL-1β, and TNFα[29], and directly inflict damage through release of toxic factors such as reactive oxygen species generated by inducible nitric oxide synthase (iNOS)[90,91]. iNOS-expressing myeloid cells are often described as classically-activated, and considered "pro-inflammatory", based on their similarity to bone marrow derived macrophages (BMDM) polarized with LPS or IFNγ *in vitro*[28]. It is generally thought that classically-activated myeloid cells (CAMC) are predominant in active MS and EAE lesions where they act as pathogenic effector cells[92–94]. Early studies of autopsied MS brains revealed iNOS-immunoreactive macrophages in active lesions[93,95,96]. The presence of iNOS$^+$ myeloid cells generally correlated with zones of ongoing demyelination.

However, there is growing evidence that myeloid cells that accumulate in the central nervous system (CNS) are heterogeneous and likely encompass a spectrum of lineages with

diverse, and even opposing, properties[56,97,98]. In contrast to iNOS, the enzyme arginase-1 (Arg1) and the mannose receptor CD206 have been identified as markers of myeloid cells with "immunosuppressive" or "pro-regenerative" properties. Arg1/CD206-expressing myeloid cells play a critical role in wound healing[99]. They are frequently classified as alternatively-activated based on their similarity to BMDM generated *in vitro* by polarization with IL-4 or IL-13 via a STAT6-dependent pathway[26,28]. Alternatively-activated myeloid cells (AAMC) may regulate the inflammatory environment by secreting IL-10 and/or TGFβ1[29], while promoting tissue regeneration by clearing debris[100,101] and secreting growth factors[61]. Foamy (lipid-laden) macrophages, perivascular macrophages, and microglia expressing human AAMC markers, such as CD206 and CD163, have been discovered in acute and chronic active MS lesions[56,61,88,102]. Primary human macrophages acquire a foamy morphology and produce immunosuppressive factors following ingestion of myelin *in vitro*[56].

We questioned whether CNS myeloid cells evolve during disease progression and shift from a pro-inflammatory phenotype at onset to a non-inflammatory or immunosuppressive state in anticipation of clinical remission/stabilization. Consistent with this hypothesis, CNS-infiltrating DC were found to upregulate the AAMC-associated genes *Arg1, Chi3l3* and *Ms4a8a* at the peak of EAE, shortly prior to remission[103]. In fact, Arg1 is the most-significantly up-regulated gene in the CNS at peak EAE[104]. Adoptive transfer of AAMC- polarized macrophages or microglia can ameliorate EAE[58,105], and the therapeutic effects of estrogen, glatiramer acetate and other agents in EAE were found to correlate with the expansion of AAMC in the periphery and/or CNS[60,106–108]. Less is known about endogenous AAMC that spontaneously accumulate in the CNS during the course of EAE or MS. In the current paper, we compare the spatial distribution of AAMC in actively demyelinating and quiescent regions of MS lesions. In

addition, we examine the origin, kinetics and biological properties of CNS myeloid subsets from the preclinical stage of EAE through peak and remission.

Methods

Mice. C57Bl/6 and B6.Ly5.1 mice were from Charles River Laboratories. Arg1-eYFP[109], Rosa-LSL-eYFP, 2D2 TCR transgenic, and STAT6$^{-/-}$ mice were from the Jackson Laboratory. iNOS-TdTomato-Cre[110] mice were from the European Mouse Mutant Archive. SJL mice were from Harlan Laboratory. Both male and female mice, age 6-12 weeks, were used in experiments. All mice were bred and maintained under specific pathogen-free conditions at the University of Michigan, and all animal experiments were performed in accordance with an IACUC-approved protocol at the University of Michigan.

Induction and assessment of EAE. For active immunization, C57Bl/6 mice were subcutaneously immunized over the flanks with 100 µg MOG_{35-55} (Biosythesis) in complete Freund's adjuvant (Difco). Mice were injected intraperitoneally with 300 ng pertussis toxin (List Biological) on days 0 and 2. For adoptive transfer, mice were immunized as described, without pertussis toxin, and the draining lymph nodes (inguinal, brachial, and axillary) were collected at 10-14 days post-immunization. Lymph node cells were cultured for 96 hours in the presence of 50 ug/mL MOG_{35-55}, 8 ng/ml IL-23 (R&D Systems), 10 ng/ml IL-1α (Peprotech), and 10 µg/mL anti-IFNγ (Clone XMG1.2, BioXcell). At the end of culture, $CD4^+$ T cells were purified with CD4 positive selection magnetic beads (Miltenyi), and 3-5x10^6 $CD4^+$ T cells were transferred intraperitoneally into naïve recipients. For induction of relapsing-remitting EAE, SJL mice were subcutaneously immunized over the flanks with 100 µg $PLP_{139-151}$ (Biosynthesis) in complete Freund's adjuvant

(Difco) without pertussis toxin. EAE was assessed by a clinical score of disability: 1, limp tail; 2, hind-limb weakness; 3, partial hind-limb paralysis; 4, complete hind-limb paralysis; and 5, moribund state.

Mixed bone marrow chimeras. B6.Ly5.1 ($CD45.1^+$) congenic hosts were lethally irradiated with 1300 Rad split into two doses and reconstituted by tail vein injection of $4x10^6$ bone marrow cells. Cells were a 50:50 mix of B6.Ly5.1 WT ($CD45.1^+$) and $STAT6^{-/-}$ ($CD45.2^+$) bone marrow. Mice were allowed to reconstitute for 6 weeks prior to use. Expression of CD45.1 or CD45.2 was used to distinguish WT and $STAT6^{-/-}$ cells from the same animal for analysis.

Cell isolation. Mice were anesthetized with isoflurane. Mice were perfused with PBS through the left ventricle. Draining lymph nodes and spleens were isolated, and a single-cell suspension was generated by passing cells through a 70-μm mesh filter. For collection of CNS mononuclear cells, brains were dissected from the skull, and spinal cords were flushed from the spinal column with PBS. Tissues were homogenized with an 18G needle and syringe in a solution containing 1 mg/ml collagenase A (Roche) and 1 mg/ml DNase 1 (Sigma-Aldrich) in HBSS and incubated at 37°C for 20 minutes. Mononuclear cells were separated from myelin with a 27% Percoll gradient (GE Healthcare).

Flow cytometry. Mononuclear cells were labelled with fixable viability dye (eFluor506 or eFluor780, eBioscience), blocked with anti-CD16/32 (Clone 2.4G2, hybridoma), and stained with fluorescent antibodies. For intracellular staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained with fluorescent antibodies.

Antibodies for CD62L (Clone MEL-14) and Ly6G (Clone 1A8) were obtained from BD Biosciences. The following antibodies were obtained from eBioscience: CD45 (Clone 30-F11), CD11b (Clone M1/70), CD11c (Clone N418), NOS2 (Clone CXNFT), CD44 (Clone IM7), CD4 (Clones RM4-5, GK1.5), MHC-II (Clone AF6-120.1), CD40 (Clone HM40-3), CD80 (Clone 16-10A1), CD86 (Clone GL1). Antibodies were conjugated to FITC, PE, PeCy7, APC, APC-Cy7, PerCP-Cy5.5, PerCP-eFluor710, PE/Dazzle 594, eFluor450, or PE-eFluor610. Arginase was stained with FITC-conjugated antibody (IC5868F, R&D Systems) or with unconjugated antibody (AF5858, R&D Systems) and an AlexaFluor 647-conjugated donkey anti-sheep secondary (ThermoFisher). Data was acquired using a FACSCanto II flow cytometer or FACSAria III flow sorter (BD Biosciences) and analyzed with FlowJo software (Treestar). Cells were sorted with a FACSAria III flow sorter (BD Biosciences).

Quantitative PCR. Sorted cells were resuspended in RLT buffer, and RNA was isolated with the RNeasy Mini Kit (Qiagen). cDNA was generated by reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCR was performed on an iQ Thermocycler (Bio-Rad) using the iQ SYBR Green Supermix. Relative gene expression was determined with the ΔCT method with normalization to *Actb*.

Ex vivo cultures. For purification of naïve $CD4^+$ T cells, lymph nodes and spleen were collected from naïve 2D2 TCR transgenic mice, and $CD4^+$ T cells were enriched by positive selection with magnetic beads (Miltenyi). To further isolate naïve T cells, cells were sorted for live $CD4^+CD44^-CD62L^+$ T cells. For purification of memory T cells, mononuclear cells from the CNS were flow sorted for live $CD45^+CD11b^-CD3^+CD4^+$MHC-II^- T cells. T cells were labeled

with CFSE according the manufacturer's instructions (ThermoFisher). Myeloid cell populations were sorted with markers as indicated in the text. Myeloid cells and T cells were co-cultured in complete RPMI for 96 hours at a ratio of 1:20 (typically 5,000 myeloid cells with 95,000 T cells) in the presence or absence of myelin antigen (MOG_{35-55} peptide [Biosynthesis]).

Multiplex immunoassays. Cell culture supernatants were analyzed with the Milliplex Mouse Th17 Cytokine Panel (EMD Millipore) using the Bio-Plex 200 System (Bio-Rad). Values below the reported level of detection of the assay (minDC) were assigned a value of 0.5*minDC for statistical analysis and represented on graphs as not detectable (n.d.).

Immunohistochemistry on MS brain tissue. Human CNS tissue was obtained at autopsy according to an Institutional Review Board-approved protocol at Yale University. CNS tissue was obtained from 4 subjects with MS and a total of 5 chronic active white matter lesions were examined. Lesions were identified as "chronic active" according to the classification proposed by Bruce Trapp[111]. Specifically, lesions were characterized by ongoing demyelination at the lesion rim, with dense infiltration of CD68-positive myeloid cells that contained myelin degradation products. The lesion center was demyelinated but still contained lipid-positive myeloid cells. Post mortem intervals were between 5.5 and 24 hrs. Brain tissue was fixed with 10% formalin for 2 to 4 weeks and embedded in paraffin. Formalin-fixed, paraffin-embedded (FFPE) sections were cut, quenched with hydrogen peroxide and blocked with normal serum, incubated with primary antibodies overnight and finally processed with the appropriate biotinylated secondary antibody and avidin/biotin staining kit with diaminobenzidene as chromogen (Vector ABC Elite Kit and DAB Kit, Vector Laboratories), and counterstained with

haematoxylin. The following antibodies were used in this study: MBP (Rabbit polyclonal, Dako A0623), CD68 (Mouse monoclonal, Dako M0876), iNOS (Rabbit polyclonal, Novus NB 120-15203), CD206 (Mouse polyclonal, Abcam ab117644), and Iba-1 (Goat polyclonal, Abcam ab5076). Adequate controls using isotype control antibodies were performed with each primary antibody. Brightfield images were acquired with a Leica DM5000 B microscope using a Leica colour camera DFC310 Fx and the Leica Application Suite (version 4.2.0) imaging software. For fluorescence images, sections were incubated with fluorescent-labelled secondary antibodies and subsequently treated with 0.7% Sudan Black in ethanol and $CuSO_4$ to quench auto-fluorescence. Sections were counterstained with DAPI and mounted with VectaShield mounting medium (VectaShield Kit, Vector Laboratories). Images were acquired with an UltraVIEW VoX (Perkin Elmer) spinning disc confocal Nikon Ti-E Eclipse microscope. Image acquisition, visualization and quantification were performed using the Volocity 6.3 soft- ware (Improvision). Images were processed with the ImageJ software (Schneider et al., 2012). For 3D Surface plots, colors were split into red, green and blue, and only the red color images were processed further. Background was removed by setting an appropriate threshold and the resulting image was processed with the "Interactive Surface Plot v2.4" ImageJ plug-in.

Statistics. Statistical analysis was performed using paired or un-paired, 2-tailed Student's t test, as indicated in the legends.

Results

Macrophage polarization shifts from the core to the rim of MS lesions

In order to characterize the polarization state of $CD68^+$ macrophage in chronic active MS

lesions, we immunolabelled brain tissue sections from 4 patients (Table 2.1) with antibodies against iNOS and CD206, the standard markers of classically and alternatively activated human myeloid cells, respectively[88,93,112,113]. MS lesions tend to grow centrifugally over time, with inflammatory activity gradually moving outward. Chronic active lesions have a relatively quiescent and hypocellular lesion center, due to waning of the earlier inflammatory response, surrounded by a rim of macrophages with early myelin degradation products, signifying ongoing demyelination[111]. We found that a high percentage of $CD68^+$ macrophages at the lesion rim were $iNOS^+$, while relatively few macrophages expressed CD206 (Figure 2.1A-D). Conversely, the ratio of $CD206^+$ cells was higher than $iNOS^+$ cells among those macrophages in the center of the lesion. We detected iNOS/CD206 double positive macrophages in all 5 MS lesions, which were most numerous in the lesion center (Figure 2.1E). Consistent with our results, Vogel and colleagues discovered foamy macrophages and activated microglia in a panel of active MS lesions that consistently co-expressed AAMC and CAMC markers[88].

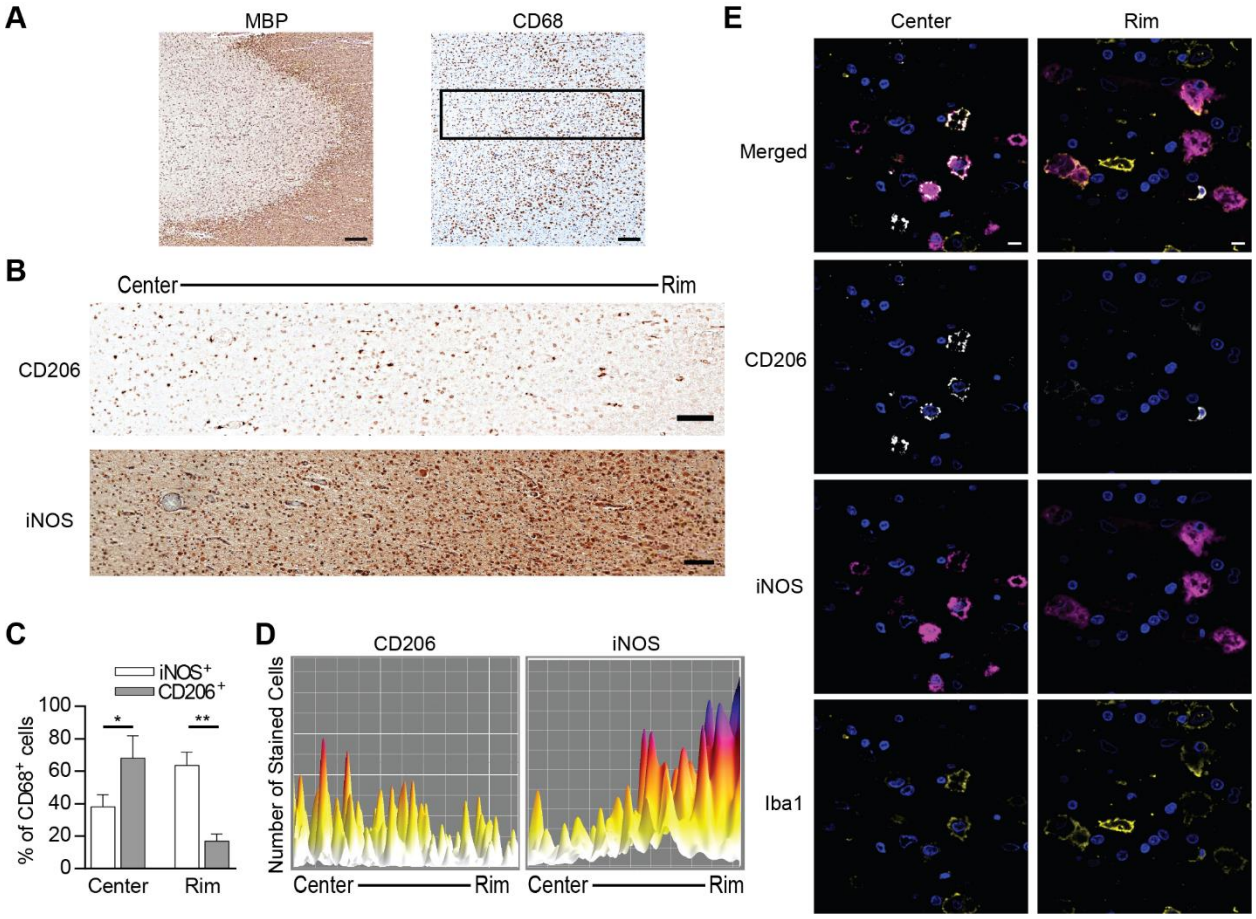


Figure 2.1 - CD206$^+$ and iNOS$^+$ macrophages are distributed from the center to the rim of active MS lesions.

(A) Chronic active MS lesion immunolabeled for myelin basic protein (MBP) and CD68. (B) Cross sections of the same lesion, extending from the lesion center to the lesion rim, immunolabeled for CD206 and iNOS. (C) Quantification of iNOS$^+$ and CD206$^+$ cells at the lesion center and lesion rim. The number of cells is expressed as a percentage of CD68$^+$ cells. N=5 lesions quantified. *P<0.05, **P<0.01 by paired, 2-tailed Student's t test. (D) Surface plots of sections in B with the number of stained cells expressed as height, processed with the "Interactive Surface Plot v2.4" ImageJ plug-in. (E) Representative immunofluorescent labeling for CD206 (white), iNOS (magenta), and Iba1 (yellow). Scale bars indicate 150 µM (A); 100 µM (B) and 10 µM (E).

Table 2.1 – Clinical data of MS patients included in this study.

Case	Age	Gender	Disease Course	Disease duration	Post-mortem Interval
1	42	Male	RRMS	20 yrs	5.5 hrs
2	37	Male	RRMS	15 yrs	24 hrs
3	50	Female	RRMS	10 yrs	6 hrs
4	63	Female	SPMS	unknown	8 hrs

Arg1 is expressed by a subset of CNS-infiltrating myeloid cells at peak EAE

Next, we investigated the phenotypes of CNS myeloid cells during the course of EAE. We found that the myeloid cells that accumulate in the CNS do not reliably express CD206, but a subset of splenic myeloid cells consistently expressed CD206 at high levels (data not shown). Since Arg1 is commonly used to identify murine AAMC[28], we measured expression of iNOS and Arg1 in inflammatory cells isolated from the spinal cord at serial time points following active immunization with myelin peptide. We detected iNOS, but not Arg1, in mononuclear cells isolated from the spinal cord at clinical onset (Figure 2.2A). In contrast, at peak disease we detected Arg1, but not iNOS, in the CNS myeloid cells. Arg1 was expressed by $CD45^{hi}CD11b^{+}$ infiltrating myeloid cells and $CD45^{int}CD11b^{int}$ resident microglia, but not by $CD45^{+}CD11b^{-}$ lymphoid cells or $CD45^{-}$ nonhematopoietic cells (Figure 2.2B). The $CD45^{hi}CD11b^{+}$ myeloid subset was further divided into $Ly6G^{+}$ neutrophils, $CD11c^{-}$ monocytes/ macrophages (MΦ), and $CD11c^{+}$ dendritic cells (DC). Arg1 was expressed by a significant percent of the infiltrating MΦ and DC, but not by neutrophils. At clinical onset, expression of iNOS followed a similar pattern both in spinal cord and brain mononuclear cells (data not shown). $Arg1^{+}$ and $iNOS^{+}$ myeloid cells were restricted to the CNS as no $Arg1^{+}$ or $iNOS^{+}$ cells were detected in the spleen, draining lymph nodes or blood (data not shown).

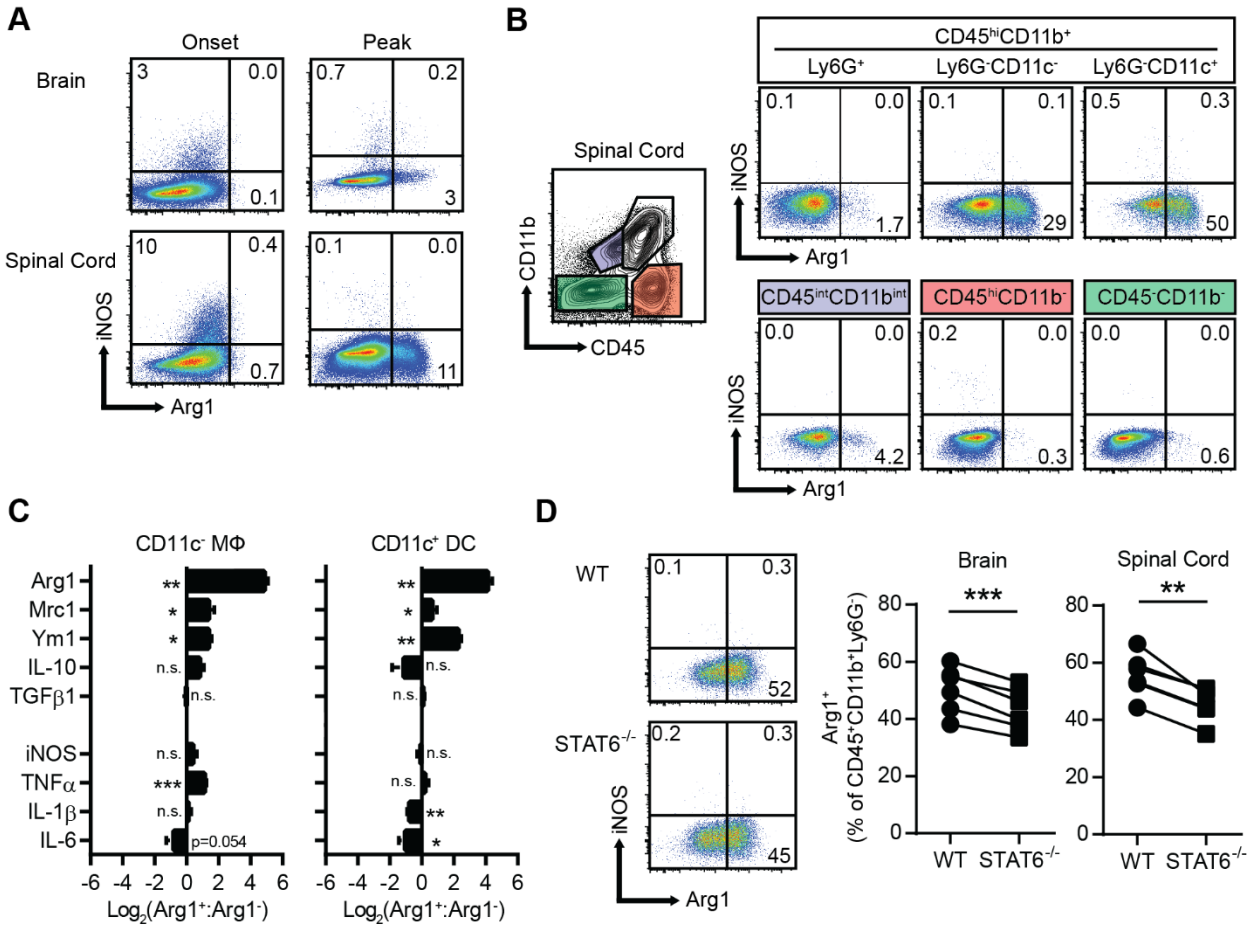


Figure 2.2 - Arg1 expression defines a distinct myeloid cell population that accumulates in the CNS during later stages of EAE.

(A, B) EAE was induced in mice by active immunization with myelin peptide. Inflammatory cells were isolated from the CNS at the onset or peak of clinical disability. Representative intracellular staining of Arg1 and iNOS in CNS immune cells from WT mice gating on (A) all viable cells at onset or peak or (B) cell subsets at peak disease. (C) $Arg1^+$ and $Arg1^-$ myeloid cells were flow sorted from the CNS of Arg1-eYFP reporter mice. M1 and M2 related transcripts were measured by qRT-PCR. Data are represented as the ratio of gene expression in $Arg1^+$ cells over $Arg1^-$ cells. (D) Inflammatory cells were isolated from the CNS of $STAT6^{-/-}$:WT→WT mixed bone marrow chimeric mice at the peak of EAE. Representative flow and quantification of intracellular staining of Arg1 and iNOS in WT or $STAT6^{-/-}$ cells, gated on the $CD45^{hi}CD11b^+Ly6G^-$ population. Percentages are $Arg1^+$ cells among WT or $STAT6^{-/-}$ CNS myeloid cells isolated from individual chimeric mice. Connected dots indicate cells derived from the same mouse. *P<0.05, **P<0.01, ***P<0.001 and n.s. = P>0.05, by paired, 2-tailed Student's t test. Data are representative of at least 2 experiments. N=3-5 mice per group. All values are mean ± SEM.

We sorted $Arg1^+$ and $Arg1^-$ MΦ ($CD45^{hi}CD11b^+Ly6G^-CD11c^-$) or DC ($CD45^{hi}CD11b^+Ly6G^-CD11c^+$) from the CNS of Arg1-eYFP reporter mice[109] during peak stage EAE, and we measured the expression of candidate genes by quantitative RT-PCR. Genes

associated with *in vitro* generated AAMC, including Ym1 and Mrc1, were enriched in $Arg1^+$ CNS DC and MΦ, compared with their $Arg1^-$ counterparts, while CAMC-related genes (iNOS, IL-1β, IL-6) were unchanged or reduced (Figure 2.2C). The genetic profiles of $Arg1^+$ and $Arg1^-$ CNS myeloid cells did not precisely mirror those of *in vitro* generated AAMC and CAMC, respectively. For example, mRNA encoding the AAMC-associated cytokines IL-10 and TGFβ1 were expressed at similar levels $Arg1^-$ and $Arg1^+$ cells, and the CAMC-associated cytokine TNFα was expressed at relatively high levels in the $CD11c^-Arg1^+$ cohort. Reminiscent of these results, an $Arg1^+$ CNS myeloid subset with a mixed gene profile was recently described in traumatic brain injury[114], which may signify a distinct myeloid cell phenotype induced within the CNS microenvironment.

$Arg1^+$ myeloid cells accumulate in the CNS independent of STAT6

STAT6 has been identified as a critical regulator of Arg1 expression in BMDMs in response to IL-4 and IL-13 *in vitro*[115]. To determine whether STAT6 is also critical for the accumulation of $Arg1^+$ CNS myeloid cells during EAE, we constructed mixed bone marrow chimeric mice by reconstituting lethally irradiated WT mice with a combination of $STAT6^{-/-}$ and WT bone marrow cells. Brain and spinal cord mononuclear cells were isolated from individual chimeric mice at peak EAE for flow cytometric analysis. The frequency of $Arg1^+$ cells was modestly reduced among $STAT6^{-/-}$, compared with WT, myeloid cells isolated from the same CNS tissue (Figure 2.2D). Nonetheless, the accumulation of $Arg1^+$ CNS myeloid cells was largely preserved in the absence of STAT6.

Myeloid cells shift from iNOS to Arg1 expression during the evolution of adoptively transferred and relapsing remitting EAE

Active immunization involves the administration of CFA and pertussis toxin which could directly modulate innate immune cells. In order to determine how iNOS and Arg1 expression evolves in CNS myeloid cells during EAE in the absence of adjuvant, we utilized an adoptive transfer model. Naïve C57BL/6 mice were injected with myelin-primed syngeneic Th17 cells, and CNS mononuclear cells were isolated at serial time points for analysis by flow cytometry. Similar to our observations in the active immunization model, $iNOS^{+}Arg1^{-}$ cells were prominent during the preclinical stage and at clinical onset, while $iNOS^{-}Arg1^{+}$ cells were prominent during peak and late disease (Figure 2.3A). A population of $iNOS^{+}Arg1^{+}$ CNS myeloid cells also emerged at clinical onset but had contracted by peak EAE. This dynamic shift in the expression of iNOS and Arg1 occurred in CNS-infiltrating MΦ and DC, as well as in $CD45^{int}CD11b^{int}$ resident microglia (Figure 2.3B).

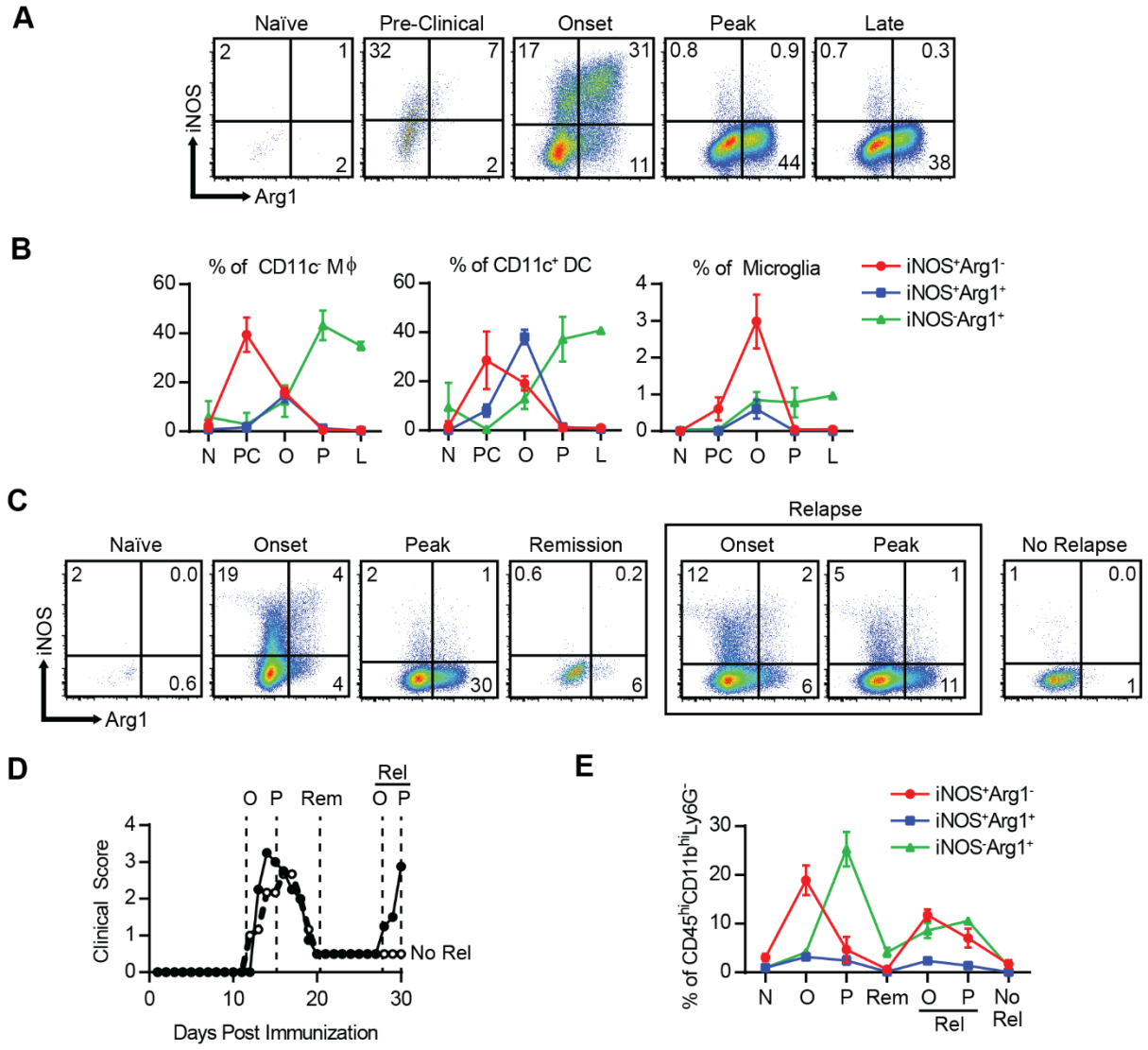


Figure 2.3 - Myeloid cells shift from iNOS to Arg1 expression in adoptive transfer and relapsing models of EAE.

(A, B) EAE was induced by adoptive transfer of WT myelin-primed Th17 cells into naïve syngeneic hosts. CNS inflammatory cells were isolated at serial time points and analyzed by flow cytometry. (A) Representative intracellular staining of Arg1 and iNOS, gating on the $CD45^{hi}CD11b^{+}Ly6G^{-}$ population. (B) Percentages of $CD45^{hi}CD11b^{+}CD11c^{-}$ MΦ, $CD45^{hi}CD11b^{+}CD11c^{+}$ DC and $CD45^{mid}CD11b^{+}CD11c^{-}$ microglia expressing iNOS and/or Arg1 in naïve mice (N) and in mice with EAE during the pre-clinical (PC), onset (O), peak (P), and late (L) stages of disease. (C-E) EAE was induced in SJL mice by active immunization with $PLP_{139-151}$ peptide, and CNS inflammatory cells were collected at serial time points, including onset (O), peak of first episode (P), remission (Rem), and relapse (Rel). Cells were also collected from mice that experienced an initial exacerbation followed by remission but did not subsequently relapse (No Rel). (C) Representative dot plots of intracellular iNOS and Arg1 expression gating on the $CD45^{hi}CD11b^{+}Ly6G^{-}$ population. (D) Representative relapsing-remitting clinical course of SJL mice. (E) Percentages of $CD45^{hi}CD11b^{+}Ly6G^{-}$ CNS myeloid cells expressing iNOS and/or Arg1 at serial time points. Data are representative of at least 2 experiments. N=3-5 mice per time point. All values are mean ± SEM.

Next, we used a relapsing remitting model of EAE in SJL mice to determine whether myeloid cells undergo cyclical shifts in iNOS and Arg1 expression through multiple relapses. We observed that an $iNOS^{+}Arg1^{-}$ subset accumulated immediately prior to the first exacerbation, contracted during peak disease, and rebounded at relapse (Figure 2.3C-E). In contrast, an $iNOS^{-}Arg1^{+}$ subset expanded between the preclinical stage and peak EAE, waned during remission, and re-emerged at the peak of relapse. Collectively, these results demonstrate that a shift in myeloid cell phenotype parallels clinical exacerbations and remissions/ plateaus and is consistent across multiple models of EAE.

Individual myeloid cells convert from iNOS to Arg1 expression

Khoury and colleagues described immunosuppressive $Ly6C^{hi}$ monocytes that expand in the periphery, accumulate in the CNS during EAE, and have the potential to differentiate into either $iNOS^{+}$ or $Arg1^{+}$ cells upon *ex vivo* culture with different polarizing factors[98]. As shown in Figures 2.1 and 2.3, we detected myeloid cells in MS and EAE lesions that co-express AAMC and CAMC markers. It is unclear if these cells represent a distinct, stable lineage or an intermediate stage during the transition between polarized subsets. The experiments in Figures 3 demonstrate the evolving characteristics of CNS myeloid cells on a population level; however, they do not address plasticity at the level of individual cells. To this end, we permanently labelled $iNOS^{+}$ cells and their descendants by crossing NOS2-TdTomato-Cre reporter mice[110] with Rosa26-LSL-eYFP reporter mice to generate iNOS fate-mapping mice ($iNOS^{FM}$). Disease was induced in these mice by adoptive transfer of myelin-primed Th17 cells. A subset of myeloid cells isolated from the CNS of the fate mapping mice at peak EAE expressed eYFP, indicative of previous expression of iNOS ($iNOS^{FM+}$) (Figure 2.4A-B). At this point, iNOS

protein is no longer detectable. Approximately 45% of the iNOS^{FM+} subset co-expressed Arg1 (Figure 2.4C), demonstrating that individual CNS-infiltrating myeloid cells are capable of transitioning from expression of iNOS to expression of Arg1 during the course of disease. However, Arg1 expression is not absolutely dependent on prior expression of iNOS since ~15% of the iNOS^{FM-} cells were also Arg1^{+}. Notably, a higher percentage of Arg1^{+} than Arg1^{-} cells were derived from iNOS^{FM+} precursors (33% versus 9%). These data demonstrate that Arg1-expressing cells may be derived from iNOS-expressing cells or from iNOS-naïve cells.

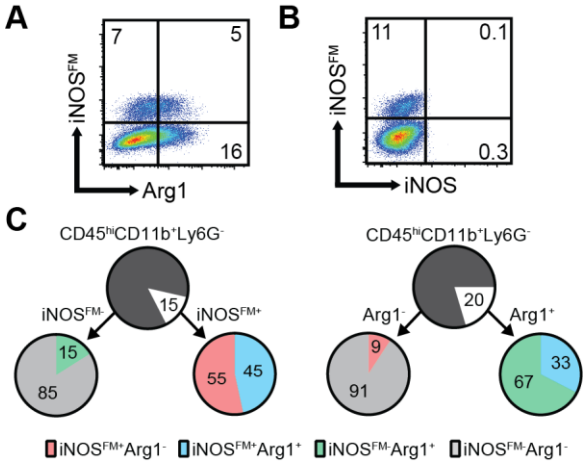


Figure 2.4 - Arg1-expressing CNS myeloid cells are derived, in part, from iNOS-expressing precursors.
EAE was induced in mice by adoptive transfer of WT myelin-primed Th17 cells. CNS inflammatory cells were analyzed by flow cytometry. (A-C) Inflammatory cells were isolated from the spinal cords of iNOS^{FM} mice at peak disease. (A-B) Representative dot plots showing iNOS^{FM} and (A) Arg1 or (B) iNOS expression, gated on CD45^{hi}CD11b^{+}Ly6G^{-} myeloid cells. (C) Percentages of CNS myeloid cell subpopulations defined according to iNOS^{FM} and Arg1 expression. Subpopulations were initially segregated based on expression of iNOS^{FM} (left) or Arg1 (right). Data are representative of at least 2 experiments. N=3-5 mice per group. Mean values are shown.

Arg1-expressing CNS myeloid cells are inefficient antigen presenting cells

DCs have been identified as potent antigen presenting cells (APC) in the CNS during EAE[49,116]. We sought to compare the APC function of Arg1^{+} versus Arg1^{-} CNS DC by measuring the ability of each subset to promote myelin-specific T cell proliferation *ex vivo*.

Sorted CNS DC subsets were co-cultured with naïve 2D2 $CD4^+$ T cells, which bear a transgenic T cell receptor specific for myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}), in the presence of myelin peptide. Compared with their $Arg1^-$ counterparts, $Arg1^+$ CNS DC were inefficient in promoting the proliferation of, or cytokine production by, myelin-specific T cells (Figure 2.5A-B). Similar results were obtained when MOG_{1-125} protein was used as the recall antigen (data not shown). The majority of $CD4^+$ T cells within the CNS during EAE are $CD44^+CD62L^{lo}$ effector cells. In order to simulate antigen presentation within the CNS more closely, we next isolated $CD4^+$ effector T cells from the spinal cords of mice at peak EAE and co-cultured them with $Arg1^+$ or $Arg1^-$ CNS DC. Similar to naïve 2D2 T cells, the $CD4^+$ effector T cells proliferated and secreted cytokines at reduced levels in response to antigen presentation by $Arg1^+$ versus $Arg1^-$ CNS DC (Figure 2.5C-D). The poor APC capacity of $Arg1^+$ CNS DC did not appear to be due to low expression of MHC-II or co-stimulatory molecules (Figure 2.5E-F). Essentially all of the $Arg1^+$ and $Arg1^-$ CNS DC expressed MHC-II, though $Arg1^+$ cells expressed a slightly lower level than their $Arg1^-$ counterparts. $Arg1^+$ CNS DC expressed similar levels of the co-stimulatory marker CD40 and slightly higher levels of CD80 and CD86 compared with $Arg1^-$ CNS DC. Arginase enzyme activity has been shown to suppress T cell proliferation in other experimental systems[117,118]; however, inhibition of arginase during co-cultures of T cells with $Arg1^+$ CNS myeloid cells did not rescue T cell activation (data not shown).

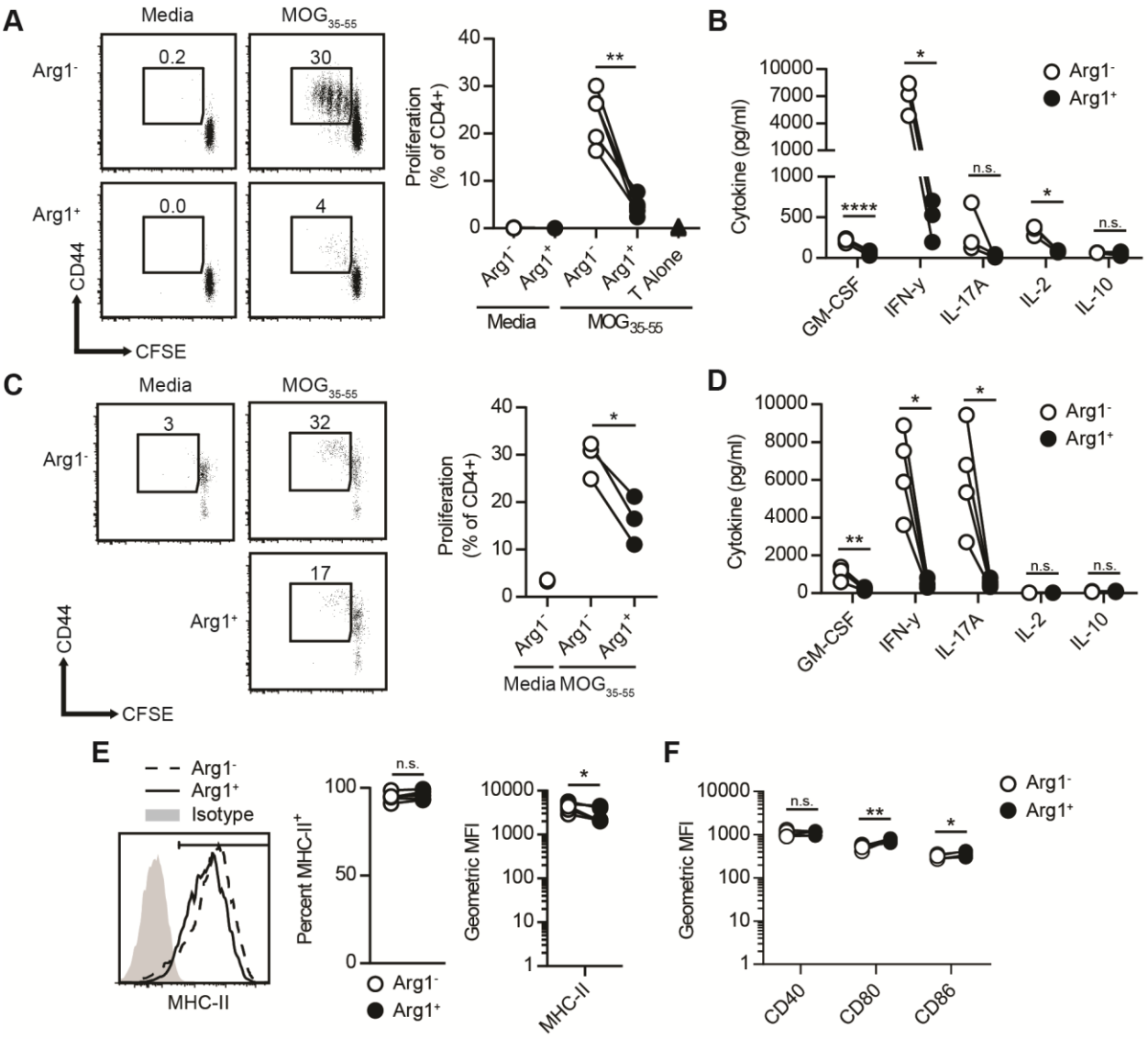


Figure 2.5 - Arg1$^+$ cells are deficient at antigen presentation.
EAE was induced in Arg1-eYFP reporter mice by active immunization with myelin peptide, and immune cells were isolated from the CNS at peak disease. Arg1$^+$ and Arg1$^-$ CD45^{hi}CD11b$^+$Ly6G$^-$CD11c$^+$ myeloid cells were flow sorted and co-cultured with MOG-reactive T cells in the presence of myelin peptide (MOG_{35-55}). (A-B) CNS myeloid cell subsets were co-cultured with CD44$^-$CD62L$^+$ CD4$^+$ T cells from naïve 2D2 TCR transgenic mice. (C, D) CNS myeloid subsets were co-cultured with CD4$^+$ T cells isolated from the CNS of actively immunized WT mice at the peak of EAE. Graphs show (A, C) proliferation measured by CFSE dilution and (B, D) cytokine levels in culture supernatants, measured by multiplex bead assay. (E, F) MHC-II and co-stimulatory marker expression were measured by flow cytometry on Arg1$^+$ and Arg1$^-$ CD45^{hi}CD11b$^+$Ly6G$^-$CD11c$^+$ myeloid cells from the spinal cord at the peak of disease. (E) MHC-II expression quantified as both a percentage and by geometric MFI. (F) Co-stimulatory marker expression quantified by geometric MFI. *P<0.05, **P<0.01, and n.s. = P>0.05, by paired, 2-tailed Student's t test. Data are representative of at least 2 experiments. N=3-5 mice per group.

We next investigated whether Arg1$^+$ CNS myeloid cells are immunosuppressive or simply incompetent APCs. Arg1$^+$ CNS myeloid cells did not induce 2D2 T cells to upregulate

FoxP3 (data not shown) or to produce IL-10 (Figures 2.5B, D). Furthermore, the addition of $Arg1^+$ CNS myeloid cells to co-cultures of $Arg1^-$ myeloid cells and 2D2 T cells did not impede antigen presentation by the $Arg1^-$ cells (data not shown). Hence, we concluded that $Arg1^+$ cells are intrinsically poor APC but do not actively block T cell activation by competent APC.

Discussion

Collectively, the data presented in our study demonstrate the heterogeneity and plasticity of myeloid cells within the CNS in MS and EAE. We identified an $Arg1^+$ CNS myeloid population during the peak and later stages of EAE which is deficient in the capacity to activate myelin-specific T cells. The appearance of $Arg1^+$ myeloid cells in the CNS immediately prior to disease remission was consistent across multiple disease induction methods (active and adoptive) as well as across mouse strains (C57Bl/6 and SJL). CNS $Arg1^+$ DC were not immunosuppressive in the sense that they did not actively suppress T cell activation by their immunocompetent $Arg1^-$ counterparts. Nevertheless, the poor antigen presenting ability of these myeloid cells may passively contribute to the resolution of inflammation, and hence to clinical remission, following peak disease. The mechanism underlying ineffective antigen presentation by CNS $Arg1^+$ myeloid cells remains to be elucidated. We are currently interrogating the efficiency of myelin antigen loading on MHC-II molecules in CNS DC subsets. It is also possible that the $Arg1^+$ cells play an immunoregulatory or pro-regenerative role independent of T cell activation. Although the $Arg1^+$ cells do not produce IL-10 and express comparable levels of TGFβ1 to $Arg1^-$ cells, we are currently investigating whether they secrete other regulatory molecules. CNS AAMC may play a role in ameliorating the clinical course by clearing debris[100,101] and/or releasing trophic factors[61].

Arg1$^+$ CNS myeloid cells differ from *in vitro* polarized AAMC in that they differentiate in the absence of STAT6, express low levels of CD206 and high levels of MHC-II, and have a distinct genetic profile. The factors that drive their polarization *in vivo* are a subject of ongoing research. Candidates include HIF1α and PPARγ, which have been shown to promote AAMC differentiation in other experimental systems[119,120]. Alternatively, studies with primary human macrophages suggest that ingestion of myelin could trigger their development[56]. Unexpectedly, we found that the Arg1$^+$ CNS myeloid cells that accumulate in the CNS during EAE are derived, in part, from iNOS$^+$ precursors. Although plasticity of CAMC and AAMC has previously been demonstrated in other experimental systems on the population level, the current study is the first to our knowledge to directly demonstrate the transition of CNS myeloid cells from iNOS to Arg1 expression *in vivo* on the single cell level. Furthermore, during the transition from iNOS to Arg1 expression, CNS myeloid cells entered an intermediary stage characterized by expression of both markers. The demonstration by us and others[88] that myeloid cells in MS lesions also co-express CAMC and AAMC markers raises the possibility that they undergo a dynamic transformation from a pro- to a non-inflammatory state. Such a transformation is further suggested by the increasing gradient of iNOS versus CD206 expression in macrophages extending from the active rim to the quiescent center of chronic active MS lesions.

Second generation disease modifying therapies (DMT) in MS suppress clinical exacerbations by depleting peripheral lymphocytes or blocking their trafficking to the CNS. While these reagents are highly effective in many individuals with relapsing-remitting MS, they are not cures, with up to 30% non-response rates. Furthermore, DMT currently used in the clinic are generally ineffective in progressive forms of MS. Myeloid cells and related factors have been under-utilized as candidate therapeutic targets in MS clinical trials. This is particularly

relevant to secondary progressive MS, since numerous studies point to dysregulation of the innate arm of the immune system and, in particular, cells of the myeloid lineage as a distinctive feature of that stage of disease[21–24]. The current findings suggest an opportunity for slowing, or even halting, progressive disability in patients with MS via the development of novel therapeutics that suppress pathogenic myeloid subsets in a targeted manner and/or promote their conversion to a non-inflammatory phenotype.

Chapter 3 – Conventional dendritic cells are critical for the initiation of experimental autoimmune encephalomyelitis

****Portions of this chapter are being prepared for publication**
Giles DA, Duncker PC, Wilkinson N, Washnock-Schmid JM, Segal BM. Conventional dendritic cells are critical for the initiation of experimental autoimmune encephalomyelitis. 2017.

Abstract

Antigen presenting cells (APC) are required for the initiation of experimental autoimmune encephalomyelitis (EAE); however, the identity of the initiating APC is unknown. In this study, we investigate the role of dendritic cells (DC) as APC in EAE. DC may arise from distinct lineages. Monocyte-derived DC (moDC) differentiate from monocytes while conventional DC (cDC) differentiate from pre-DC precursors. While the presence of moDC in EAE is known, it is not clear whether cDC are present in EAE or how cDC contribute to neuroinflammation. Here, we identified cDC in the central nervous system (CNS) during EAE, and we demonstrated that CNS-derived cDC, but not moDC, were proficient at activating myelin-specific T cells. We further characterized antigen-presenting cDC in the naïve CNS and showed that targeted depletion of these cDC was sufficient to reduce the incidence of adoptive EAE. Our results indicate that cDC are present and critical for the initiation of adoptive EAE.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is widely used as an animal model of multiple sclerosis (MS)[38]. In this model, adoptive transfer of myelin-primed T cells is sufficient to promote neuroinflammation and an ascending paralysis. T cells are activated in an antigen-specific manner by interaction with an antigen presenting cell (APC)[121,122]. It remains unclear, however, what APC is required to initiate the neuroinflammatory process.

APC are identified by expression of MHCII which allows presentation of antigen and recognition by $CD4^+$ T cells. There are several candidate APC which may be involved in MS and EAE. Dendritic cells (DC), macrophages, and B cells are considered professional antigen presenting cells as these cells express both MHCII and co-stimulatory molecules requisite for T cell activation[123]. Non-hematopoietic cells such as astrocytes have also been shown to express MHCII[124,125], though it is unclear whether these cells possess the rest of the machinery necessary to be a competent APC[126]. As a functional demonstration of this, Greter et al[49] showed that MHCII expression on hematopoietic cells was required for adoptive transfer EAE while expression on non-hematopoietic cells was dispensable. Furthermore, restricting MHCII expression to $CD11c^+$ DCs was sufficient to permit disease induction.

The $CD11c^+$ DC population is heterogeneous and includes both monocyte-derived (moDC) and conventional (cDC) DC subsets[127]. moDC are rare in homeostasis but differentiate from monocytes in the setting of inflammation. In fact, monocytes, macrophages, and moDC may represent a continuum of activation as the cells differentiate and up-regulate MHCII and CD11c[128,129]. In contrast, cDC are resident in both lymphoid and non-lymphoid tissues during homeostasis and expand in a Flt3-dependant manner[130]. cDC are derived from a pre-DC precursor in the bone marrow and constitutively express MHC-II and CD11c[131]. Plasmacytoid

DC (pDC) are a third population of DC derived from a similar pre-DC precursor; however, pDC express low levels of CD11c and MHCII, limiting their role as APC[132].

It has been difficult to distinguish moDC and cDC *in vivo*, and thus it is unclear how these distinct subsets contribute to an inflammatory response. Recent work, however, has identified several markers which allow such discrimination. CD88 (C5ar1), a complement receptor, and CD26 (DPP4), an enzyme involved in peptide hydrolysis, are expressed by moDC and cDC, respectively[78]. These markers are not specific to DC but are effective in segregating the $CD11c^+$ DC population. The transcription factor Zbtb46 has also been identified as a marker of cDC in both mice and humans[83,84].

We and others have previously shown that $CD11c^+$ cells accumulate in CNS during EAE[89] and are derived from $Ly6C^{hi}$ monocytes[48,133], suggesting the presence of moDC in disease. It is not known whether cDC are also present. A $CD11c^+MHCII^+$ DC population has been described in the naïve meninges in rodents[134–136] and humans[137]. Furthermore, recent studies have identified DC populations in the naïve meninges which expand in response to Flt3L, suggesting their identity as cDC[138,139]. It is not clear whether or how these cells may contribute to neuroinflammation.

Several studies have evaluated the role of DCs in EAE using CD11c-DTR mice, which allow depletion of $CD11c^+$ cells by administration of diphtheria toxin (DT); however, the results have varied. Depleting $CD11c^+$ DC may ameliorate disease[140,141], exacerbate disease[142], or have no impact on the disease course[44,143]. These studies differ in the DT dosing regimens and may thus differ in the efficiency of DC depletion in the CNS and periphery. Furthermore, given the distinct types of DC, it is advantageous to separately target moDC and cDC, but CD11c-DTR does not distinguish between moDC and cDC. Attempts have been made to target moDC with

CCR2-DTR mice[44], but this is confounded by the simultaneous depletion of CCR2-expressing monocytes and macrophages. To specifically target cDC, Zbtb46-DTR mice have been generated which allow focused study of the role of cDC in inflammation[83].

In the present study, we investigate the DC subsets present in the CNS during EAE. We identify the presence of both moDC and cDC and demonstrate the CNS-derived cDC, but not moDC, are capable of activating myelin-specific $CD4^+$ T cells. We show that cDC are present in the naïve CNS and that targeted depletion of cDC reduces the incidence of disease, identifying cDC as critical for the initiation of EAE.

Methods

Mice. C57Bl/6 and B6.Ly5.1 mice were from Charles River Laboratories. Zbtb46-GFP, Zbtb46-DTR, CD11c-DTR, and 2D2 TCR transgenic mice were from the Jackson Laboratory. Both male and female mice, age 6-12 weeks, were used in experiments. All mice were bred and maintained under specific pathogen-free conditions at the University of Michigan, and all animal experiments were performed in accordance with an IACUC-approved protocol at the University of Michigan.

Induction and assessment of EAE. For adoptive transfer, C57Bl/6 mice were subcutaneously immunized over the flanks with 100 µg MOG_{35-55} (Biosythesis) in complete Freund's adjuvant (Difco). At 10-14 days post-immunization, the draining lymph nodes (inguinal, brachial, and axillary) were collected and cultured for 96 hours in the presence of 50 ug/mL MOG_{35-55}, 8 ng/ml IL-23 (R&D Systems), 10 ng/ml IL-1α (Peprotech), and 10 µg/mL anti-IFNγ (Clone XMG1.2, BioXcell). At the end of culture, $CD4^+$ T cells were purified with CD4 positive

selection magnetic beads (Miltenyi), and 3-5x10^6 $CD4^+$ T cells were transferred intraperitoneally into naïve recipients. For active EAE, mice were immunized as before and injected with 300 ng of pertussis toxin (List Biological) on days 0 and 2. EAE was assessed by a clinical score of disability: 1, limp tail; 2, hind-limb weakness; 3, partial hind-limb paralysis; 4, complete hind-limb paralysis; and 5, moribund state. Daily weight measurements were also taken.

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

Cytokine production by DC subsets. Mononuclear cells were isolated as above and cultured in complete RPMI with media, BFA (10 µg/mL), or BFA + LPS (6.7 µg/ml) for 4 hours. At the end of culture, cells were collected and stained for cytokines by intracellular flow cytometry.

Ex vivo cultures. Mononuclear cells were isolated as above, and DC subsets, microglia, and B cells were flow sorted from the CNS and spleen according to the indicated surface markers. For purification of naïve $CD4^+$ T cells, lymph nodes and spleen were collected from naïve 2D2 TCR transgenic mice. $CD4^+$ T cells were enriched by positive selection with magnetic beads (Miltenyi), and naïve T cells were further purified by flow sorting for live $CD4^+CD44^-CD62L^+$ T cells. For purification of effector T cells, mononuclear cells from the CNS were flow sorted for live $CD45^+CD11b^-CD3^+CD4^+$MHC-II^- T cells. T cells were labeled with CFSE according the manufacturer's instructions (ThermoFisher). APC and T cells were co-cultured for 96 hours in complete RPMI at a ratio of 1:20 (typically 5,000 myeloid cells with 95,000 T cells) with media, myelin peptide (MOG_{35-55} peptide [Biosynthesis]), or myelin protein (MOG_{1-125} [Anaspec]). At the end of culture, cells were cultured with PMA (50 ng/ml), ionomycin (2 µg/ml), and BFA (10 µg/ml) for 4 hours to stimulate cytokine production. Cells were collected and stained for activation by surface markers and cytokine production by intracellular staining.

Flow cytometry. Cells were labelled with fixable viability dye (eFluor506, eBioscience), blocked with anti-CD16/32 (Clone 2.4G2, hybridoma), and stained with fluorescent antibodies. Antibodies for CD62L (Clone MEL-14), FoxP3 (Clone MF23), and Zbtb46 (Clone U4-1374) were obtained from BD Biosciences. Antibodies for CD26 (Clone H194-112), CD88 (Clone 20/70), and PDL1 (Clone 10F.9G2) were obtained from Biolegend. The following antibodies were obtained from eBioscience: CD3 (Clone 145-2C11), CD4 (Clones RM4-5, GK1.5), CD11b (Clone M1/70), CD11c (Clone N418), CD19 (Clone MB19-1), CD40 (Clone HM40-3), CD44 (Clone IM7), CD45 (Clone 30-F11), CD45.1 (Clone A20), CD45.2 (Clone 104), CD80 (Clone 16-10A1), CD86 (Clone GL1), GM-CSF (Clone MP1-22E9), IFNγ (Clone XMG1.2), IL-10

(Clone JES5-16E3), IL-12p35 (Clone 4D10p35), IL-12p40 (Clone C17.8), IL-17 (Clone eBio17B7), IL-23p19 (Clone fc23cpg), MHC-II (Clone M5/114.15.2), NOS2 (Clone CXNFT). Isotype control antibodies were matched for each. Antibodies and isotype controls were conjugated to FITC, PE, PeCy7, APC, APC-Cy7, PerCP-Cy5.5, PerCP-eFluor710, PE/Dazzle 594, eFluor450, eFluor660, AlexaFluor 647, or AlexaFluor 700. Arginase was stained with FITC-conjugated antibody (IC5868F, R&D Systems) or with unconjugated antibody (AF5858, R&D Systems) and an AlexaFluor 647-conjugated donkey anti-sheep secondary (ThermoFisher). For intracellular staining of cytokines and enzymes, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained with fluorescent antibodies. For intracellular staining of Zbtb46, cells were fixed and permeabilized with the Transcription Factor Buffer Set (BD Pharmingen). Data was acquired using a FACSCanto II flow cytometer or FACSAria III flow sorter (BD Biosciences) and analyzed with FlowJo software (Treestar). Cells were sorted with a FACSAria III flow sorter (BD Biosciences).

Nanostring gene expression analysis. Sorted cells were resuspended in RLT buffer, and cell lysates were directly analyzed for expression of 750 immune-related genes with the nCounter PanCancer Immune Panel (Nanostring Technologies). Data was processed using the nSolver Analysis Software by normalization to the geometric mean of positive controls and housekeeping genes. R was used to perform paired Student's t-tests and calculate Benjamini & Hochberg's false discovery rate (FDR), comparing the gene expression of the $CD26^+$ and $CD88^+$ populations.

Myelin phagocytosis. Myelin was purified from naïve mice as previous[144] and labelled with pHrodo Red succinimidyl ester (ThermoFisher) according the manufacturer's instructions. Mononuclear cells from the CNS were resuspended in complete RPMI, plated in a flat bottom 96-well plate, and cultured overnight with 1-5 µg of myelin per well. Unlabelled myelin was used as a control. Cells were collected by scraping, and pHrodo uptake was analyzed by fluorescence on a FACS Aria III cytometer.

Bone marrow chimeras. B6.Ly5.1 ($CD45.1^+$) congenic hosts were lethally irradiated with 1300 Rad split into two doses and reconstituted by tail vein injection of $4x10^6$ $CD45.2^+$ bone marrow cells from WT, CD11c-DTR, or Zbtb6-DTR donors. Mice were allowed to reconstitute for 6 weeks prior to use.

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

Statistics. Statistical analysis was performed using paired or un-paired, 2-tailed Student's t test, as indicated in the legends.

Results

cDC are present in the CNS during EAE

We began by investigating what myeloid cell subsets are present in the CNS during EAE. Disease was induced by adoptive transfer of myelin-primed Th17 cells, and mononuclear cells were isolated from the brain and spinal cord at peak disease. Among $MHCII^+$ antigen presenting cells, we identified multiple myeloid populations including $CD45^{int}CD11b^{int}$ microglia, $CD45^+CD11b^+CD11c^-$ macrophages, and $CD45^+CD11c^+$ dendritic cells (DC) in the brain (Figure 3.1A). Similar results were observed in the spinal cord. The DC population included both $CD26^+CD88^-$ conventional DC (cDC) and $CD88^+CD26^-$ monocyte-derived DC (moDC). The macrophage and microglial populations expressed CD88 but not CD26. Plasmacytoid DCs(pDC) also express CD26; however, the majority of pDC were $MHCII^-$ and contributed <5% of the $MHCII^+CD26^+$ population in the CNS during EAE (Supplemental Fig 3.1). To confirm the identities of the DC populations, we performed transcriptional profiling of the CNS-derived $CD26^+$ and $CD88^+$ DC subsets (Figure 3.1B). The $CD26^+$ population expressed several core cDC transcripts recently identified by the ImmGen Project[145], including Amica1, CCR7, and Kit, while the $CD88^+$ population expressed several markers found on monocyte-derived cells including Slc11a1[146], CD84[147], and Bst1[148].

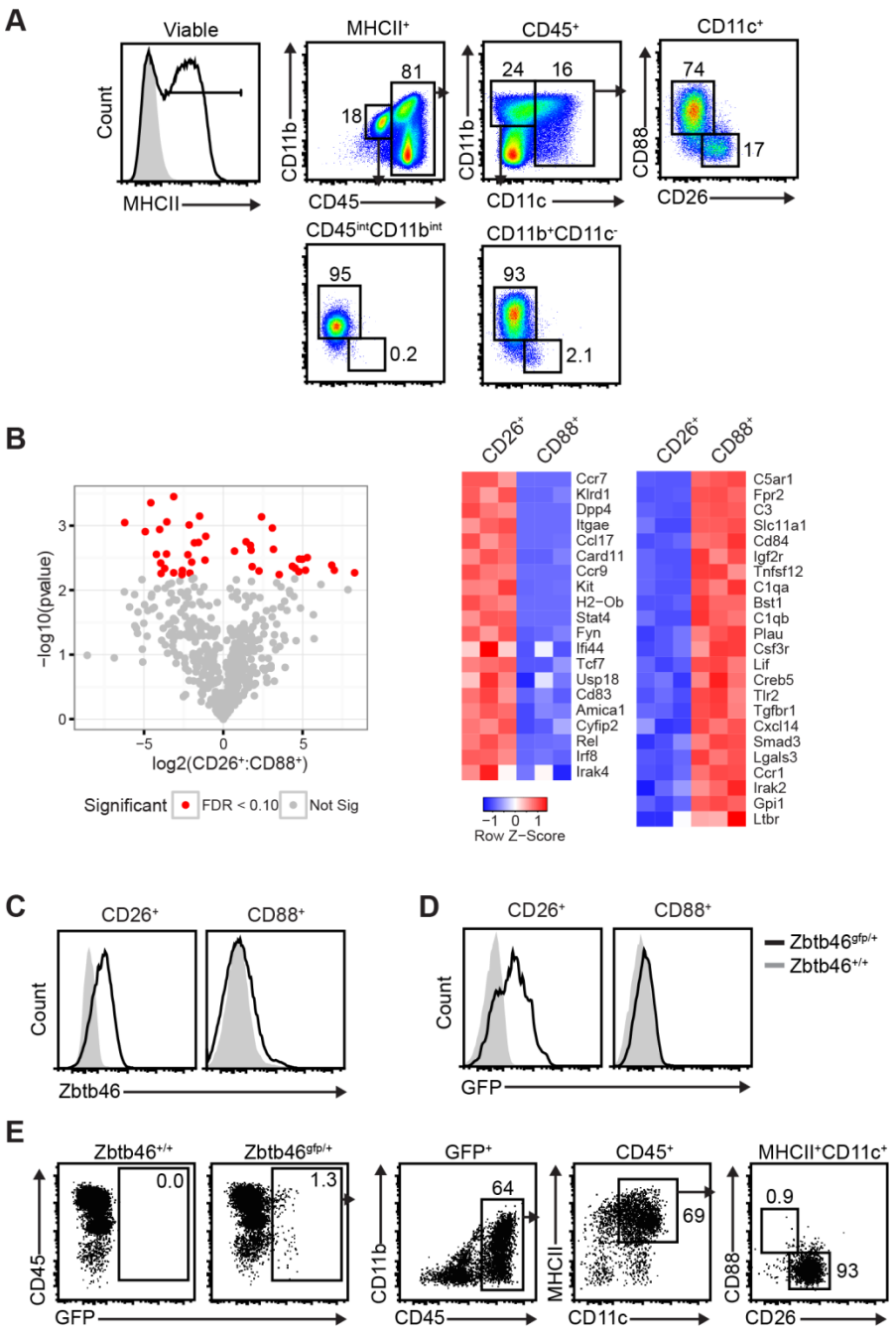


Figure 3.1 – Expression of CD26 and Zbtb46 identifies cDC in the CNS in EAE.
EAE was induced by adoptive transfer of WT myelin-primed Th17 cells. (A) Gating of MHCII$^+$ myeloid populations in the brain at the peak of adoptive EAE. Numbers show percentage of parent population, as indicated above each plot. (B) MHCII$^+$CD11c$^+$ CD26$^+$ or CD88$^+$ were purified by flow sorting, and gene expression was measured Nanostring nCounter analysis. P value determined by paired, 2-tailed Student's t test. Genes with a false discovery rate (FDR) < 0.10 are identified in the heatmaps. (C, D) Expression of Zbtb46 by (C) flow cytometry or (D) with the Zbtb46$^{gfp/+}$ reporter in MHCII$^+$CD11c$^+$ CD26$^+$ and CD88$^+$ cells from the brain at the peak of adoptive EAE. The shaded grey histogram is the (C) isotype or (D) non-reporter control. (E) Identification of Zbtb46-expressing cells in the brain during adoptive EAE using the Zbtb46$^{gfp/+}$ reporter. Numbers show percentage of parent population, as indicated above each plot.

Zbtb46 was recently identified as a specific marker of cDC, so we assayed expression of Zbtb46 in the DC subsets by flow cytometry. We identified Zbtb46 expression by $CD26^+$ cDC but not $CD88^+$ moDC in the brain during EAE (Figure 3.1C). A Zbtb46-GFP reporter mouse has also been generated, and we confirmed reporter expression in $CD26^+$ cDC but not in $CD88^+$ moDC (Figure 3.1D). A portion of the CD26 population was GFP^{lo}; this may reflect the small pDC population or weak expression of the reporter. We next analyzed all GFP^+ cells in the CNS during EAE. Both $CD45^+$ hematopoietic and $CD45^-$ non-hematopoietic cells were labelled. The $CD45^-$ population may include endothelial cells[84], but within the $CD45^+$ population, reporter expression was enriched in $CD26^+$ cDC. These data confirm the identity of the $CD26^+$ cells as cDCs which are present in the CNS during EAE.

cDC but not moDC are competent antigen presenting cells

We next questioned what roles cDC and moDC may play in disease. Dendritic cells are professional antigen presenting cells, so we first investigated the antigen presenting capacity of the DC subsets. We sorted $MHCII^+CD11c^+$ $CD88^+$ moDCs and $CD26^+$ cDC from the CNS at the peak of disease and co-cultured the cells *ex vivo* with naïve $CD4^+$ 2D2 T cells, which have a T cell receptor specific for the MOG_{35-55} peptide[149]. cDC cultured with T cells and MOG_{35-55} peptide promoted high levels of proliferation while moDC did not (Figure 3.2A). T cells co-cultured with cDC also expressed higher levels of the activation marker CD44 (Figure 3.2A) and the cytokines IFNγ and GM-CSF (Figure 3.2B). No IL-17 or FoxP3 were detected (data not shown). Similar results were obtained with cDC and moDC sorted from the spleen (data not shown). Peptide and protein may be processed and loaded on MHCII through different pathways[150], so we also assessed presentation of the MOG_{1-125} protein. cDC were again superior

to moDC in presentation of this antigen (Figure 3.2A-B). This deficit was not antigen-specific as cDC also displayed a higher capacity to activate OVA-specific OT-II cells[151] when cultured with both OVA peptide and ovalbumin protein (data not shown).

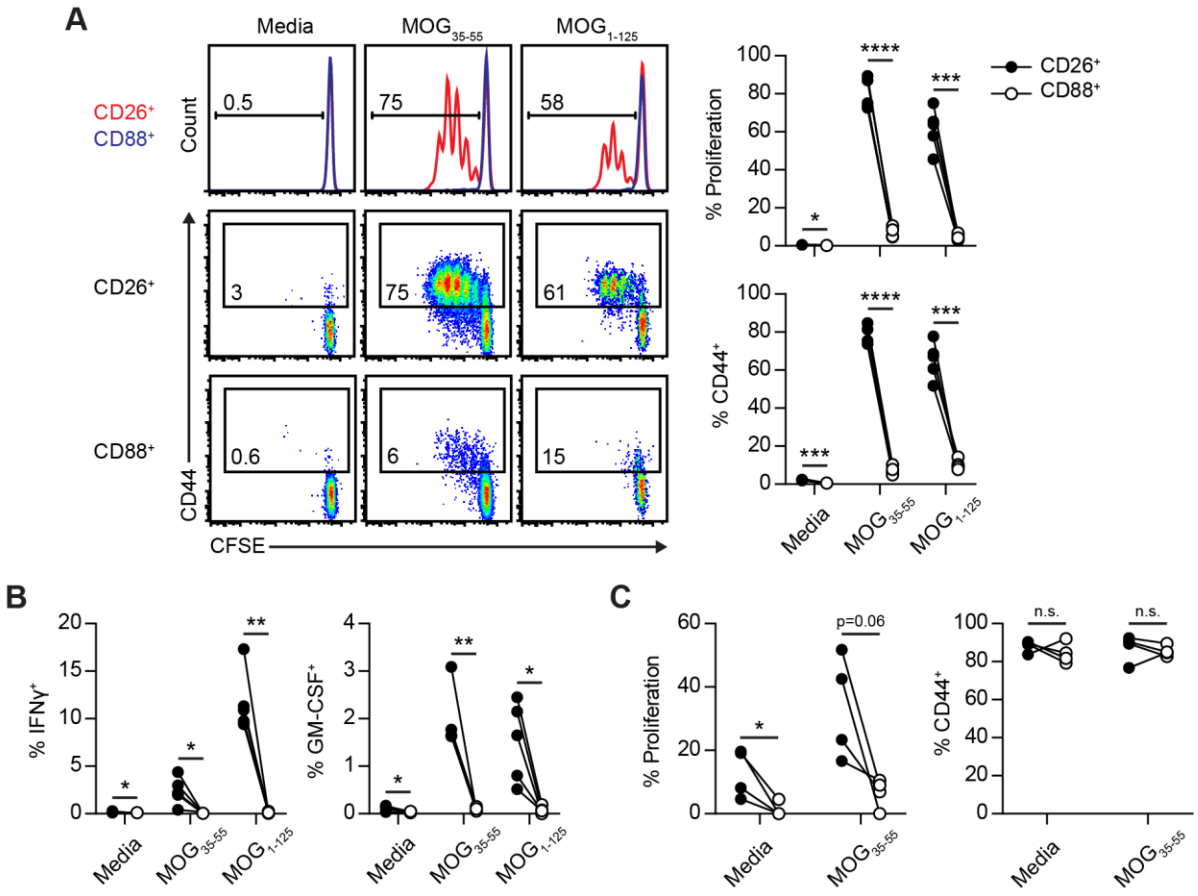


Figure 3.2 – Myelin-specific T cells are activated in co-culture with cDC but not moDC.
EAE was induced by active immunization with myelin peptide, and immune cells were isolated from the CNS at peak disease. CD26$^+$ or CD88$^+$ DC (CD45$^+$MHCII$^+$CD11c$^+$) were purified by flow sorting and co-cultured with MOG-reactive T cells in the presence of myelin peptide (MOG_{35-55}) or myelin protein (MOG_{1-125}). (A,B) DC subsets were co-cultured with CD44$^-$CD62L$^+$CD4$^+$ T cells from naïve 2D2 TCR transgenic mice. (A) T cell proliferation was measured by CFSE dilution, and T cell activation was measured by CD44 expression, represented as a % of total CD4$^+$ T cells. (B) T cell cytokine production was measured by intracellular flow cytometry and represented as a % of total CD4$^+$ T cells. (C) DC subsets were co-cultured with CD4$^+$ T cells isolated from the CNS of actively immunized WT mice at the peak of EAE. T cell proliferation and activation are shown before. Connected dots indicate paired samples from the same mouse. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and n.s. = P>0.05, by paired, 2-tailed Student's t test. N=3-5 mice per group.

While most T cells in lymphoid organs are naive, $CD4^+$ T cells in the CNS during EAE are $CD44^{hi}$ effector cells. To better reconstitute the immune synapse in the CNS during EAE, we next used effector T cells sorted from the CNS during EAE. cDC induced proliferation of effector T cells while moDC did not (Figure 3.2C). The effector T cells retained high expression of CD44 in both cultures. Notably, this proliferation of effector T cells did not absolutely require the addition of exogenous antigen but could be enhanced by the addition of MOG peptide, suggesting the cDC acquired sufficient antigen *in vivo* to activate the effector T cells. This is in contrast to activation of naïve T cells, which required the addition of exogenous peptide or protein. Together, these data demonstrate that cDC but not moDC are proficient at activating myelin-specific T cells.

Microglia and B cells may also express MHCII and act as APCs within the CNS, so we performed similar assays on these subsets. We compared B cells derived from the CNS and the spleen, and we found that both were capable of activating naïve myelin-specific T cells when cultured with MOG peptide but not with MOG protein (Supplemental Fig 3.2A). CNS-derived B cells were also capable of inducing effector T cell proliferation with and without exogenous antigen (Supplemental Fig 3.2B). In contrast, $MHCII^+CD45^{int}CD11b^{int}$ microglia were deficient in promoting proliferation of either naïve or effector T cells (Supplemental Fig 3.2C-D). This identifies B cells but not microglia as additional candidate APC in EAE. However for the given study, we chose to focus on discriminating the DC subsets.

cDC express higher levels of co-stimulatory markers

We next investigated the reason for differential T cell activation between cDC and moDC. Naïve T cells activation is commonly broken into three 'signals' derived from the

APC[152]. Signal 1 is the presentation of antigen on MHC-II that is recognized by the T cell receptor. Signal 2 is co-stimulation through ligands and receptors on the APC and T cell. Signal 3 is cytokine release by the APC which influences the differentiation of the T cell. We investigated each signal in sequence.

First, we explored antigen presentation. DC were $MHCII^{+}CD11c^{+}$ by definition, so we analyzed the level of expression of MHCII by mean fluorescence intensity (MFI). Two $MHCII^{+}$ populations were noted within the $CD26^{+}$ cDC subset: $CD11c^{hi}MHCII^{+}$ and $CD11c^{int}MHCII^{++}$ (Figure 3.3A). The $CD26^{+}MHCII^{+}$ population expressed similar levels of MHCII as the $CD88^{+}$, but MHCII expression was elevated among $CD26^{+}MHCII^{++}$ population.

Second, we investigated expression of co-stimulatory markers – CD40, CD80, CD86, and PDL1. CD40, CD80, and CD86 provide positive co-stimulation[153]. Similar to MHCII, expression of these markers was commensurate between $CD88^{+}$ moDC and $CD26^{+}MHCII^{+}$ cDC but elevated in $CD26^{+}MHCII^{++}$ cDC (Figure 3.3B). CD86, in particular, was not detected on $CD88^{+}$ moDC or $CD26^{+}MHCII^{+}$ cDC but was expressed by $CD26^{+}MHCII^{++}$ cDC. Reduced MHCII and co-stimulatory receptor expression could explain the difference in antigen presenting capacity, so we compared the function of the $CD26^{+}MHCII^{++}$ population with the $CD26^{+}MHCII^{+}$ and $CD88^{+}$ population. There was no difference in T cell activation in co-culture with the $CD26^{+}MHCII^{+}$ and $MHCII^{++}$ populations, and both $CD26^{+}$ populations promoted more T cell activation than the $CD88^{+}$ (data not shown). Therefore, the difference in expression of these surface receptors does not explain the difference in antigen presenting capacity.

The co-stimulatory receptor PDL1 provides a negative signal which can block T cell proliferation[154]. All the DC subsets expressed PDL1, but in a pattern distinct from the positive co-stimulatory receptors, the $CD88^{+}$ and $CD26^{+}MHCII^{++}$ subsets expressed higher levels

compared to the CD26^{+}MHCII^{+} subset (Figure 3.3B). As both CD88^{+} and CD26^{+} subsets express PDL1, it is also unlikely to explain the functional differences between these subsets.

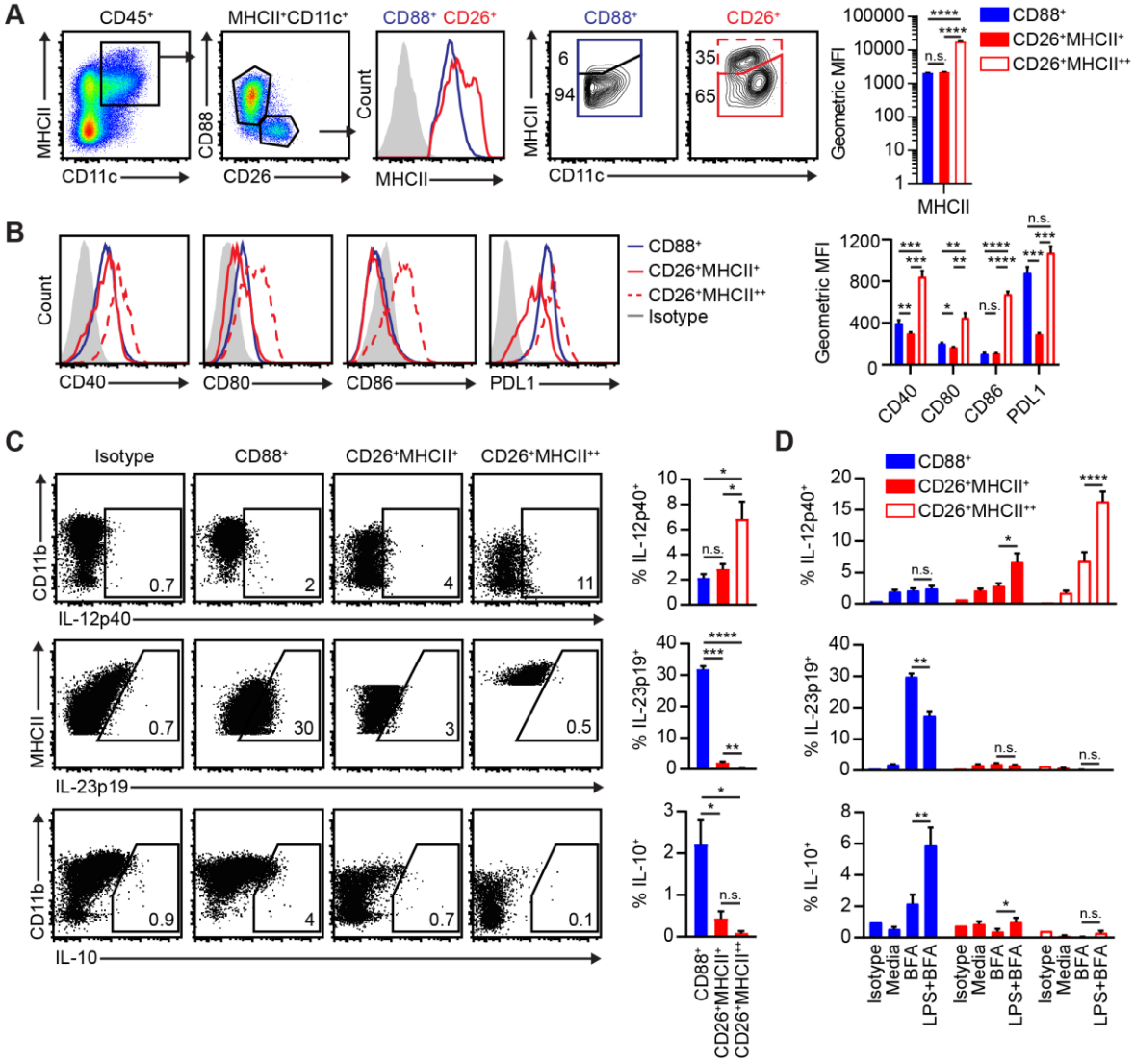


Figure 3.3 – cDC and moDC differ in expression of co-stimulatory molecules and cytokines. EAE was induced by adoptive transfer of WT myelin-primed Th17 cells, and mononuclear cells were isolated from the CNS at the peak of EAE. (A) Gating of DC populations based on MHCII expression in the brain. MHCII expression quantified by geometric mean fluorescence intensity (MFI). (B) Representative expression of co-stimulatory molecules on indicated DC subsets in the brain. Quantified as geometric MFI. (C) Cells from the CNS were cultured for 4hr with brefeldin A (BFA), and cytokine production was assessed by intracellular flow cytometry. Dots plots of representative flow for each cytokine with the isotype control or staining of the indicated DC subsets. Quantified as a % of the indicated DC population. (D) Comparison of cells cultured *ex vivo* in media, BFA, or BFA+LPS. Isotype also shown as reference for non-specific staining. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and n.s. = P>0.05, by paired, 2-tailed Student's t test. N=3-5 mice per group or condition. All values are mean ± SEM.

cDC and moDC have distinct patterns of cytokine expression

Third, we measured cytokine production by the DC subsets. Mononuclear cells from the CNS were cultured *ex vivo* in media alone, with Brefeldin A (BFA) to trap cytokines prior to secretion, or with BFA plus LPS to stimulate cytokine production. The condition with BFA alone reflects continued cytokine production from the CNS microenvironment. We observed distinct patterns of cytokine production among the DC subsets such that $CD26^+$ cDC expressed IL-12p40 while $CD88^+$ moDC expressed IL-23p19 and IL-10 (Figure 3.3C). IL-12p40 is a subunit of both IL-12 and IL-23. IL-12 is a heterodimer of IL-12p40 and IL-12p35[155] and polarizes T cells toward IFNγ production and the Th1 phenotype[156]. Production of IL-12p40 by the $CD26^+$ cDC corresponds with the IFNγ production by the co-cultured T cells (Figure 3.2B). We attempted to measure expression of IL-12p35, the second subunit of IL-12, but were limited by available methods. IL-23 is a heterodimer of IL-12p40 and IL-23p19[157] and polarizes T cells toward IL-17 production and the Th17 phenotype[158]. $CD88^+$ moDC express IL-23p19 but, in the absence of IL-12p40, are unlikely to express the heterodimer IL-23, consistent with the absence of IL-17 production by the co-cultured T cells. Instead, $CD88^+$ moDC production of IL-10 may exert an anti-inflammatory influence on the inflammatory process.

Next, we questioned whether the DC subsets may be stimulated to produce additional cytokines. Stimulation increased expression of IL-12p40 by $CD26^+$ cells and IL-10 by $CD88^+$ cells but decreased expression of IL-23p19 by $CD88^+$ cells. Importantly, it did not alter the pattern of expression (Figure 3.3D). $CD26^+$ cDC expressed IL-12p40 but not IL-23p19 or IL-10. $CD88^+$ moDC expressed IL-23p19 and IL-10 but not IL-12p40.

In summary, differences in these three signals alone do not appear sufficient to explain the differential capacity of $CD26^+$ cDC to promote T cell activation compared to $CD88^+$ moDC. Further study is necessary to elucidate this mechanism.

moDC are superior phagocytes

Having established CNS-derived cDC as superior antigen presenting cells, we questioned the role of moDC in disease. Much has been made of myeloid cell polarizations – classical or alternative, M1 or M2, iNOS- or Arg1-expressing cells – and the influence of these polarizations on the quality of the immune response[159]. We found expression of the markers iNOS and Arg1 exclusively in $CD88^+$ moDC in the CNS during EAE (Figure 3.4A).

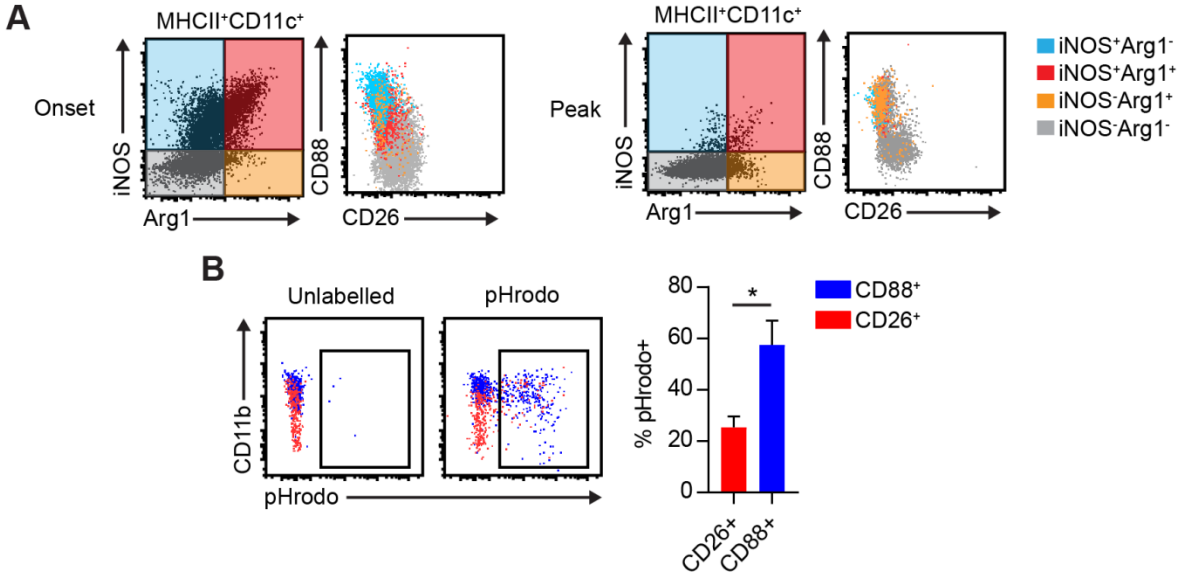


Figure 3.4 – moDC express iNOS and Arg1 and phagocytose myelin.
(A) EAE was induced by active immunization with myelin peptide, and immune cells were isolated from the CNS at onset or peak disease. Expression of iNOS and Arg1 was determined by intracellular flow cytometry. $MHCII^+CD11c^+$ DC were colored based on iNOS or Arg1 expression (left panel) and shown in reference to CD88 and CD26 expression (right panel). (B) EAE was induced by adoptive transfer of WT myelin-primed Th17 cells, and mononuclear cells were isolated from the CNS at the peak of EAE. Cells were culture overnight with unlabelled or pHrodo-labelled purified myelin. Phagocytosis measured as % $pHrodo^+$ of the gated $CD26^+$ or $CD88^+$ DC populations. *$P<0.05$ by paired, 2-tailed Student's t test. N=3-5 mice per group or condition. All values are mean ± SEM.

The monocyte/macrophage lineage is also specialized in phagocytosis. Myelin phagocytosis may be pathogenic if removed from axons[54] or protective if clearing debris[160]. We evaluated myelin phagocytosis *ex vivo* by isolating mononuclear cells from the CNS and culturing the cells overnight with pHrodo-labelled purified myelin, obtained from a naïve mouse. ~55% of $CD88^+$ moDC took up myelin compared to ~25% of cDC (Figure 3.4B). Similar results were obtained when moDC and cDC populations were sorted and cultured independently (data not shown). Phagocytosis was inhibited by the addition of cytochalasin D, demonstrating myelin uptake was an active process requiring actin polymerization[161] (data not shown). Though $CD88^+$ moDC are deficient in antigen presentation, these results suggest roles for $CD88^+$ moDC in modulation of the inflammatory milieu and in myelin phagocytosis.

cDC are present in the naïve and expand in disease

At the peak of EAE, the number of moDC dominate the number of cDC (Figure 3.1A); however, we questioned how this balance may change over the course of disease. A Flt3-dependent, radiosensitive DC population has been described in the meninges[138,139], so we investigated the DC subsets in the naïve brain, skull meninges, and spinal cord. There was a population of $MHCII^+CD45^+$ cells which included $CD11b^-CD11c^-$ lymphoid cells, $CD11b^+CD11c^-$ macrophages, and $CD11c^+$ DCs (Figure 3.5A). The DC population was predominantly $CD26^+CD88^-$, and this population was abundant in both the brain and skull meninges but low in the spinal cord. To validate the identities of these subsets, we evaluated expression of the Zbtb46-GFP reporter. Expression was restricted to the $CD26^+$ subsets (Figure 3.5B), confirming their classification as cDC. Furthermore, we evaluated antigen presentation as

before and found that MHCII$^+$CD11c$^+$CD26$^+$ cDC from the naïve CNS are capable of activating naïve, myelin-specific CD4$^+$ T cells (Figure 3.5C).

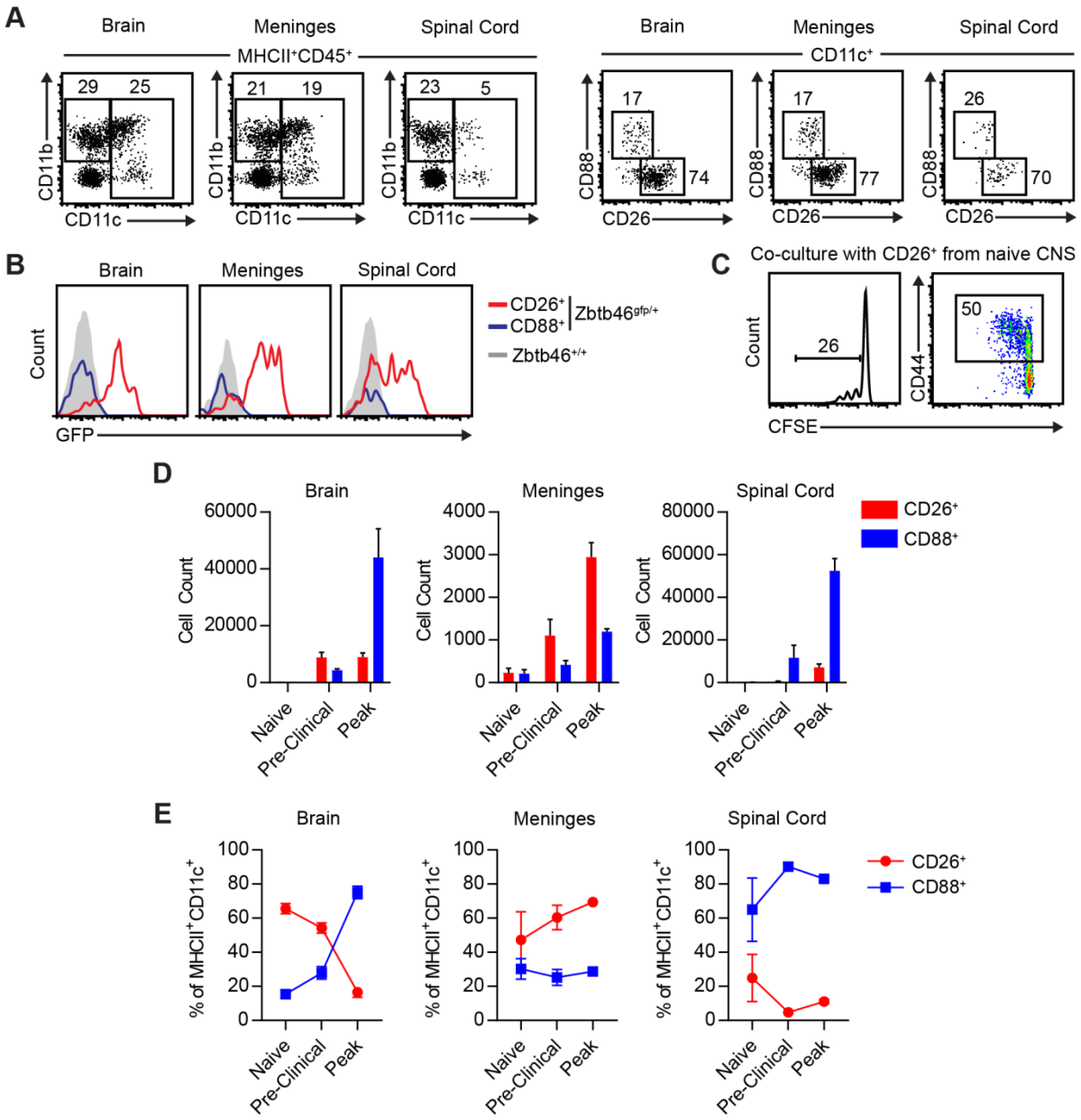


Figure 3.5 – cDC are present in the naïve CNS and increase during EAE.
(A) Mononuclear cells were isolated from the naïve brain, meninges, and spinal cord and gated on all MHCII$^+$CD45$^+$ cells (left) or further gated on CD11c+ cells (right). (B) Zbtb46$^{gfp/+}$ reporter expression in MHCII$^+$CD11c$^+$ DC subsets in the naïve CNS. (C). MHCII$^+$CD11c$^+$CD26$^+$ cDC were isolated from the naïve CNS and co-cultured with naïve 2D2 T cells. Representative proliferation and activation are shown as a % of total CD4$^+$ cells. (D, E) Cells were isolated from the naive CNS or from the CNS at pre-clinical or peak adoptive EAE. (D) Cell counts are shown for the DC subsets. (E) DC populations are represented as a percent of total MHCII$^+$CD11c$^+$ cells. N=3-5 mice per group or condition. All values are mean ± SEM.

We next compared these populations at preclinical and peak time points in the course of adoptive EAE. Comparing the brain, meninges, and spinal cord, we observed different patterns. In the brain and spinal cord, $CD26^+$ cDC expanded from naïve to preclinical to peak time points, but this expansion was dwarfed by the dramatic increase in the $CD88^+$ moDC (Figure 3.5D). As a result, $CD88^+$ moDC were dominant in the brain and spinal cord at peak disease (Figure 3.5E). In contrast in the meninges, $CD26^+$ cDC showed a greater increase in number compared to $CD88^+$, so $CD26^+$ cDC remained the dominant DC population at peak disease.

cDC are critical for initiation of experimental autoimmune encephalomyelitis

Given the antigen presenting capacity of cDC and their presence in the naïve CNS, we hypothesized that cDC are critical for initiating EAE. To investigate the role of DCs in EAE, we used transgenic mice with DC-specific expression of the diphtheria toxin receptor – CD11c-DTR[162] and Zbtb46-DTR[83]. CD11c-DTR depletes both cDC and moDC while Zbtb46-DTR specifically targets only cDC[83]. Both mice have expression on non-hematopoietic cells and experience significant toxicity with repeated dosing; therefore, we generated bone marrow chimeras with WT recipients and DTR-expressing bone marrow. In these mice, a single dose of DT is sufficient to deplete all the DC in the spleen; however, repeat dosing is necessary to deplete the DC populations within the CNS. We optimized the DT dosing strategy to deplete DCs in the CNS prior to disease transfer and maintain depletion through the disease course. Following three doses of DT in the DTR chimeras, $CD26^+$ cDC in the brain and meninges were reduced by over 50% in both chimeras (Figure 3.6A). $CD88^+$ moDC were also reduced in the CD11c-DTR chimeras; however, there was a trend toward increased $CD88^+$ moDC in the

Zbtb46-DTR meninges. The rarity of CD11c^{+} DC in the spinal cord limited analysis of this tissue.

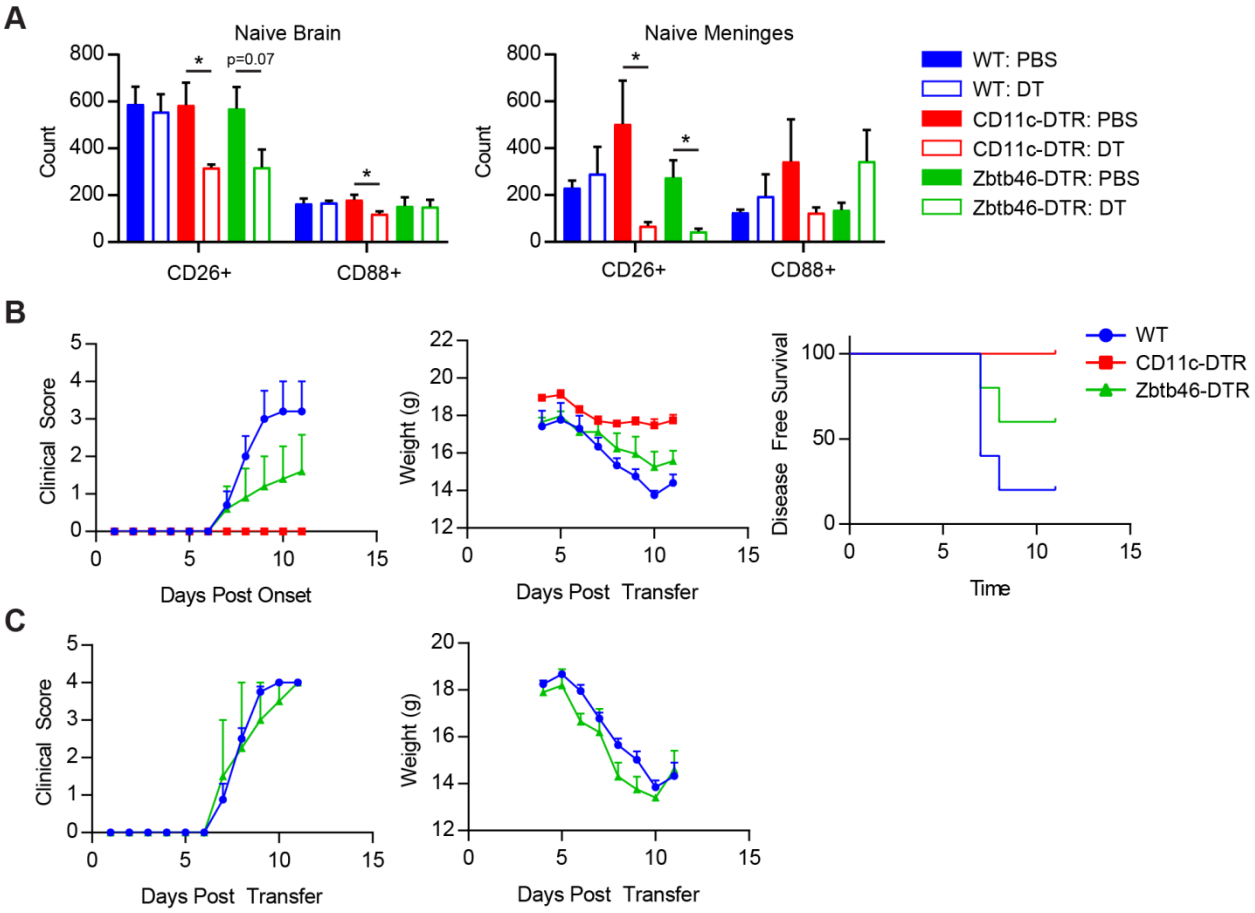


Figure 3.6 – Depleting cDC reduces the incidence of EAE.
Bone marrow chimeras were generated with CD45.1^{+} recipients and CD45.2^{+} WT, CD11c-DTR, or Zbtb46-DTR donors. (A) Counts of DC subsets from donor (CD45.2^{+}) bone marrow in the brain and meninges following DT ablation in naïve chimeras. (B) DC were ablated with DT as in A, and EAE was induced by adoptive transfer of WT myelin-primed Th17 cells. Clinical scores, weights, and incidence are shown for the three groups. (C) Clinical scores and weights for only the WT and Zbtb46-DTR which showed signs of disease. *P<0.05 by 2-tailed Student's t test. N=3-5 mice per group or condition. All values are mean ± SEM.

To evaluate the role of the DC subsets in EAE, we depleted DC with three doses of DT prior to adoptive transfer of myelin-primed Th17 cells. Daily DT doses at a reduced level were continued for the duration of the disease course. We observed that depletion of both DC subsets in the CD11c-DTR mice completely blocked disease (Figure 3.6B). Depleted mice showed no signs of paralysis and minimal weight loss. Furthermore, depletion of the cDC subset alone with

the Zbtb46-DTR reduced the incidence of disease by half (40% vs 80%). This heterogeneity in response may reflect inefficient DC depletion in some mice. The mice that did develop disease had a similar day of onset, maximum score, and degree of weight loss compared with the WT mice (Figure 3.6C). This data suggests a role for DC in EAE and highlight cDC as the critical cell for initiating adoptive transfer EAE.

Discussion

In this study, we identified cDC in the CNS during adoptive EAE. Previous studies in EAE have attempted to discriminate moDC and cDC by expression of Ly6C[141]; however, Ly6C is down-regulated by moDC differentiation in tissues[163]. We also found that some $CD26^{+}Zbtb46^{+}$ cDC in the CNS express intermediate levels of Ly6C (unpublished data), so $Ly6C^{lo}$ and $Ly6C^{int}$ cells are thus a mixture of cDC and moDC. These caveats limit the utility of Ly6C as a marker. The application of CD88 and CD26 as markers of moDC and cDC, respectively, allows more rigorous discrimination of these subsets. We further validated these markers for DC from the CNS by analysis of the cDC-specific transcription factor Zbtb46 and cDC-specific gene expression. Several recent studies have sought to identify DC subset-specific transcriptomes and to identify novel DC subsets by gene expression in mouse and humans[145,164–166]. This includes further division of cDC into cDC1 and cDC2 subsets, specialized for activation of $CD8^{+}$ or $CD4^{+}$ T cells, respectively. Several markers have been proposed to distinguish these subsets including CD8 and CD103 for cDC1 and CD4 and CD11b for cDC2[167], but expression of these markers may vary by tissue and disease model. Further study is necessary to elucidate the contribution of these subsequent DC divisions in EAE, and there is much still to learn about the functional implications of these subsets in disease.

Myelin-specific T cells must encounter an MHCII$^+$ hematopoietic cell to initiate EAE, but the identity of this initiating APC is still unknown. Dendritic cells are a leading candidate as transfer of DC can induce EAE[168] and promote myelin-specific T cell proliferation in the CNS[169]. We observed that CNS-derived CD26$^+$ cDC but CD88$^+$ moDC were competent antigen presenting cells. A similar distinction in function was observed for CD26$^+$ cDC and CD88$^+$ moDC from the lung[78]. Still others have shown, comparing Ly6C$^-$ cDC and Ly6C$^+$ moDC derived from the lymph nodes and spleen, that Ly6C$^-$ myeloid cells were more proficient at inducing T cell proliferation[44,170]. In contrast, Ly6C$^+$ promoted more production of cytokines including IL-17 and IFNγ. Our data and these studies suggest a role for cDC in T cell activation. In support of this, depleting Zbtb46-expressing cells with Zbtb46-DTR mice[83] or knocking out MHCII on Zbtb46-expressing cells[86] limits T cell responses to immunization. These data demonstrate cDC are critical for antigen-specific T cells responses and thus are a promising candidate for the initiating APC.

B cells are another APC candidate, in particular given the success of B cell targeted therapies in MS[171]. In animal studies, the role of B cells differs by strain and antigen. Some B cell-deficient strains, such as B10.PL, SJLxB10.PL, and C57Bl/6, are susceptible to disease induced by immunization with myelin peptide[172–177], suggesting B cells are dispensable. However, other B cells appear critical in other models such as C3H/Fej and C3H.SW strains[178] and in C57Bl/6 mice immunized with human MOG protein[174]. Still other studies which depleted B cells using anti-CD20 therapy have demonstrated an exacerbated disease course if depleted early or an ameliorated disease course if depleted late[179,180], suggesting stage specific pathogenic or regulatory roles for B cells.

Our studies demonstrate that CNS-derived B cells are capable of activating myelin-specific T cells. However in contrast to cDC, B cells activated naïve T cells with the addition of MOG_{35-55} peptide but not MOG_{1-125} protein. This may be due to differences in the ability to acquire peptide versus protein antigen. Protein antigen uptake by B cells is primarily mediated through the B cell receptor (BCR)[181], so acquisition of myelin antigen would require myelin-specific B cells. The number of myelin-specific B cells may be limited early in EAE; therefore, few B cells would be capable of myelin protein acquisition and presentation. In support of this, we found that CNS B cells failed to phagocytose pHrodo-labelled myelin (data not shown). Another study with TCR transgenic mice found that the incidence of spontaneous disease increased when mated to BCR transgenic mice[174]. This suggests that BCR specificity could be a limiting factor in initiating EAE. As for whether B cells are critical, additional research is necessary to clarify the role of B cells in MS and EAE.

pDC have also been proposed as APC in EAE. We indeed found $PDCA1^+$ pDC in the CNS during EAE but observed that the majority of the cells did not express MHCII. Current evidence suggests the role of pDC in EAE differs at different stages of disease. Depleting pDC in early EAE with anti-PDCA1 antibodies ameliorates EAE[182–184], but treatment with anti-PDCA1 during ongoing disease exacerbates disease severity[183]. Furthermore, pDC from myelin-primed lymph nodes can activate myelin-specific T cells but reducing MHCII expression on pDC and B cells exacerbated disease[185]. These data suggest a potential dual role for pDC: pathogenic during priming of active disease but protective in ongoing disease. Notably, pDC appear dispensable for the initiation of disease as depletion of pDC did not reduce the incidence of disease[183].

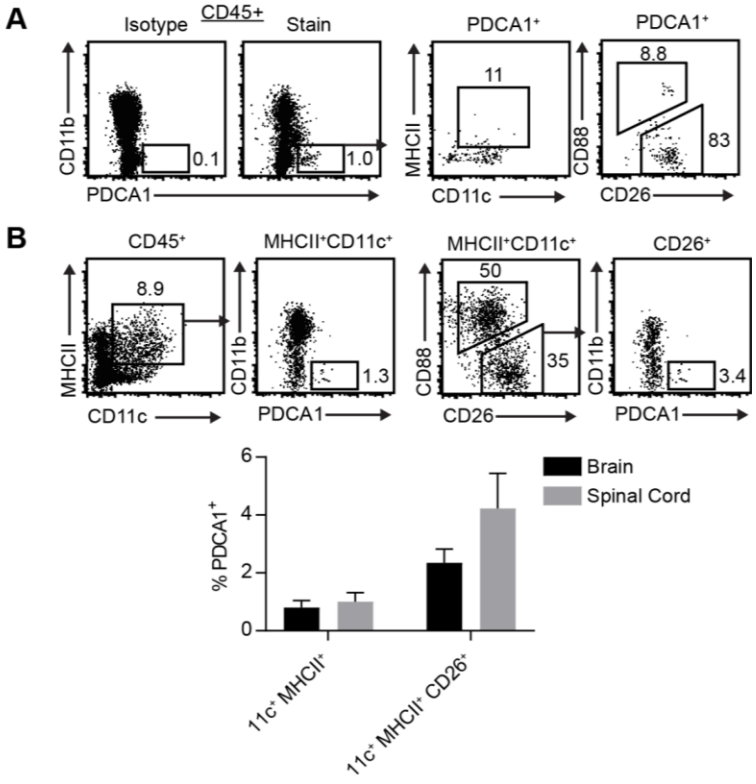
The initiating APC is likely to be present in the naïve CNS to present myelin antigens to T cells and trigger the neuroinflammatory response. One key site for this interaction is the meninges. MHCII$^+$ cells have been identified in the meninges[134–136], but to date the lineage of the cells has been unclear. Using specific markers, we demonstrate that the CD11c$^+$ DC in the naïve CNS are cDC and that these cells are capable of activating myelin-specific T cells. A recent study by Anandasabapathy et al[139] also highlighted the presence of Flt3-dependent DC in the meninges and their capacity for antigen presentation. Other have shown that CD11c$^+$ DC in the meninges are closely associated with the large dural vessels[136,138,186–188] and even present in CNS lymphatics[189]. CD11c$^+$ and MHCII$^+$ DC in these areas can interact with T cells the within vessels and promote their extravasation[141,190]. In a seminal two-photon imaging study, Bartholomaus et al[191] recorded T cells interacting with phagocytes in the meninges in the spinal cord during EAE, and a follow-up study went further to demonstrate that T cells are activated in an antigen-specific manner by interaction with leptomeningeal APC[192].

The sum of our data and the available literature supports a role for cDC in the initiation of EAE, and yet the necessity for DC in EAE is controversial due to inconsistencies in depletion studies. We found that CD11c-DTR mice are completely resistant to adoptive EAE. Published studies with high dose or high frequency, and which demonstrated DC depletion within the CNS, also found an ameliorative effect[140,141]. Those with low dose or low frequency, and which only evaluated DC depletion in the periphery, observed no difference[44,143]. This suggests the variability may be due to incomplete DC depletion.

An additional study by Yogev et al suggests that DC depletion exacerbates severity after peak EAE, suggesting a regulatory role for DC in late EAE[142]. This finding is supported by Clarkson et al[140] who also observed that depleting DC starting at the peak of EAE exacerbated

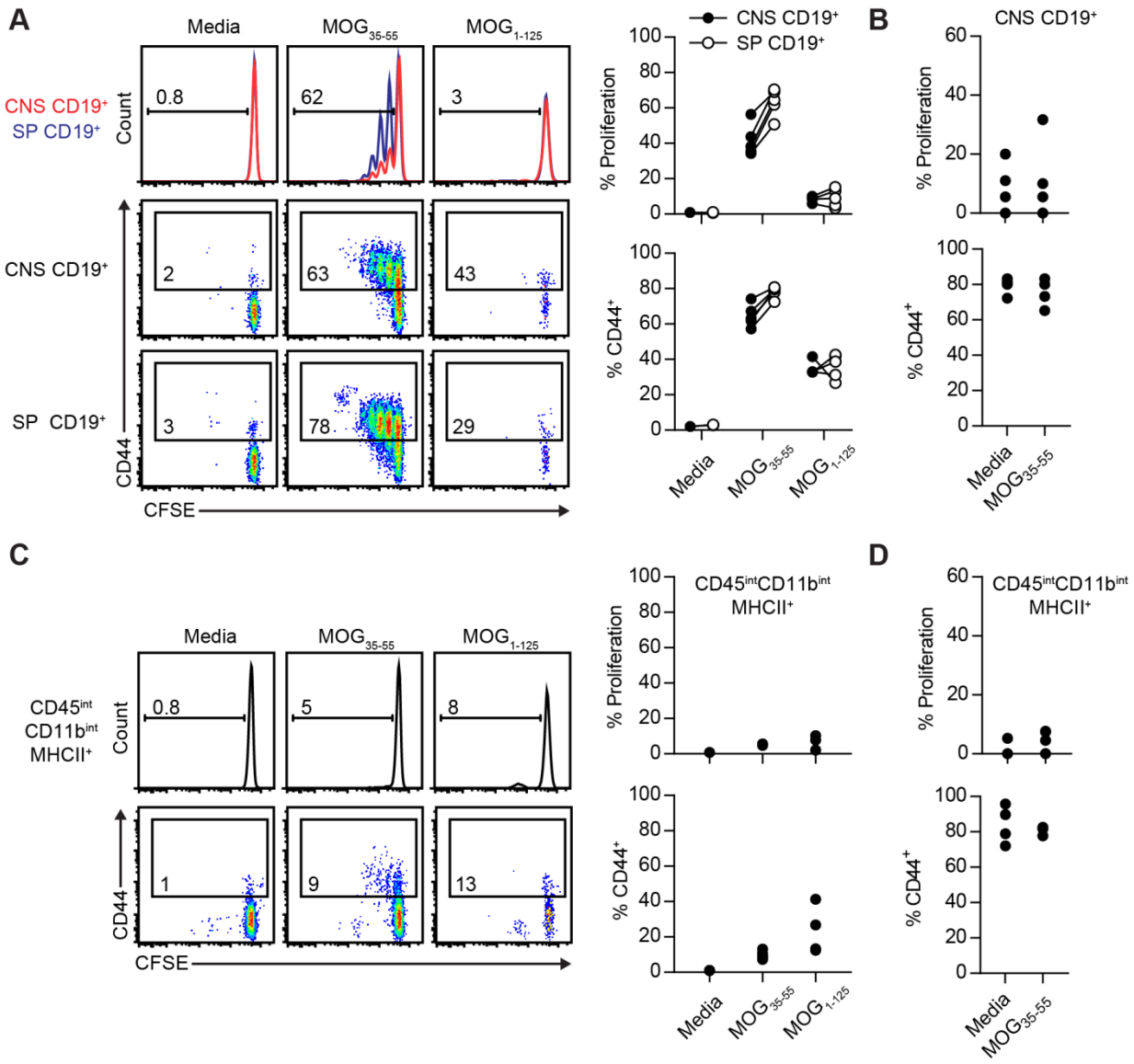
disease. It is possible that DC may play different roles during the pre-clinical and peak stages. DC may be required as APC to initiate EAE but may play a regulatory role later in disease. Consistent with this, we observed PD-L1 expression on both the moDC and cDC from the CNS at peak disease which may inhibit T cell activation or regulate Treg induction[142,154].

Having shown the $CD11c^+$ DC are required for the initiation of EAE, we further interrogated the role of cDC. By specifically targeting cDC with Zbtb46-DTR mice, we showed that depletion of cDC alone is sufficient to reduce the incidence of adoptive EAE. Together, our data demonstrate that cDC are potent APC, both in the naïve and diseased CNS, and are the critical APC for initiating adoptive EAE. These results suggest that cDC may be an attractive target for the treatment of MS.



Supplemental Figure 3.1 – pDC are present in the CNS during EAE but express low levels of MHCII.

EAE was induced by adoptive transfer of WT myelin-primed Th17 cells, and mononuclear cells were isolated from the brain and spinal cord at peak EAE. (A) pDC were identified in the brain by expression of PDCA1 and expressed CD26 but low levels of MHCII. (B) Gating and quantification of pDC as a percentage of $11c^{+}MHCII^{+}$ total DC and $11c^{+}MHCII^{+}CD26^{+}$ cDC. Parent gating is indicated above each plot. Data represented as mean ± SEM.



Supplemental Figure 3.2 – B cells but not microglia are competent antigen presenting cells.
EAE was induced by active immunization with myelin peptide, and immune cells were isolated from the CNS and spleen (SP) at peak disease. B cells ($MHCII^+CD45^+CD11b^-CD11c^-CD19^+$) and microglia ($MHCII^+CD45^{int}CD11b^{int}$) were flow sorted and co-cultured with MOG-reactive T cells in the presence of myelin peptide (MOG_{35-55}) or myelin protein (MOG_{1-125}). (A) B cells or (C) microglia were co-cultured with $CD44^-$ $CD62L^+$ $CD4^+$ T cells from naïve 2D2 TCR transgenic mice. (B) B cells or (D) microglia were co-cultured with $CD4^+$ T cells isolated from the CNS of actively immunized WT mice at the peak of EAE. T cell proliferation was measured by CFSE dilution, and T activation was measured by CD44 expression, represented as a % of total $CD4^+$ T cells. Data are representative of at least 2 experiments. Connected dots indicate paired samples from the same mouse. N=3-5 mice per group.

Chapter 4 – Discussion

Despite significant advances in MS treatment over the past decade, several challenges remain. First, the fundamental etiology of MS remains unknown, so there are not therapies which can block the disease. Second, there are no therapies which can reverse the disability caused by MS. Myeloid cells can perform both pathogenic and pro-regenerative functions; therefore, targeting myeloid cells is a promising strategy to address both therapeutic gaps in MS. Myeloid cells may initiate or perpetuate an inflammatory response, so inhibiting myeloid cells may prevent episodes of neuroinflammation. Conversely, myeloid cells may regulate the inflammatory response and stimulate neuronal or glial regeneration, so enhancing myeloid cells may facilitate repair and recovery. Development of myeloid cell-targeted therapies is limited by a fundamental lack of understanding of these dual roles in neuroinflammation. Through this thesis work, we have sought to better understand the role of myeloid cells in CNS autoimmunity.

Targeting Myeloid Cells – Prevent Neuroinflammation

Neuroinflammation is dependent on the interaction of myelin-specific $CD4^+$ T cells with APC[121,122]. We demonstrated that cDC are present in the naïve and capable of activating myelin-specific T cells. Therefore, depleting the cDC in the CNS or inhibiting their antigen presenting functions are possible strategies to block the development of neuroinflammation. In support of this, we showed that depletion of cDC reduced the incidence of adoptive EAE. Several important questions remain.

We showed that moDC were poor APC, but this did not seem to be due to low expression of MHCII, costimulatory molecules, or cytokines. It also did not appear to be due to antigen acquisition as moDC were more proficient at myelin phagocytosis. The mechanism for the difference in APC capacity between moDC and cDC is still unresolved. One possible explanation is that moDC are deficient at loading the antigen on MHCII. cDC express the protein H2-DMβ2, a non-classical MHC molecule that interacts with MHCII to load antigenic peptides[193], but moDC do not (Figure 4.1A). In fact, every subset with demonstrated APC capacity (cDC, B cells) expressed H2-DMβ2, and those deficient in presentation did not (moDC, macrophages, microglia). Further, expression of the H2-DMβ2 and other genes of the MHC loci is regulated by the transcription factor class II transactivator[194]. We observed enhanced expression of CIITA in cDC compared to $MHCII^+$ moDC, macrophages, or microglia (Figure 4.1B). Several isoforms of CIITA exist which display cell-type specific expression[195,196]. We noted that the pIII isoform was specific to the cDC population while the pI isoform was expressed by the moDC and cDC and the pIV isoform was expressed by microglia. B cells also express the pIII isoform of CIITA[197], so this provides a possible mechanism regulating H2-DMβ2 expression and APC capacity in the subsets which are competent APC. Further functional studies are necessary to investigate this mechanism.

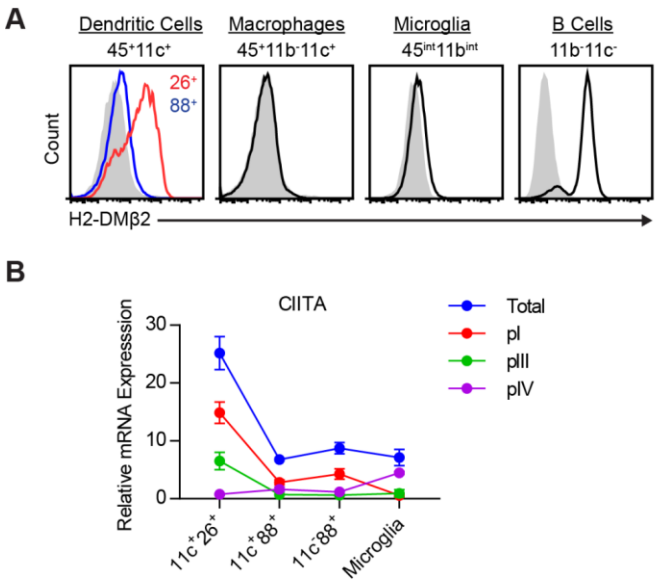


Figure 4.1 – H2-DMβ2 and CIITA are expressed by cDC and B cells.
(A) Expression of H2-DMβ2 by MHCII$^+$ cell subsets isolated from the brain at the peak of EAE, gated as indicated. (B) Expression of total CIITA or specific CIITA isoforms driven by the promoters pI, pIII, or pIV. Mononuclear cells were isolated from the CNS at the peak of EAE, and MHCII$^+$ cells subsets were flow sorted as indicated. Expression was determined by RT-qPCR. Data represented as mean ± SEM

We also observed that the numbers of both cDC and moDC increase during the course of EAE. Another important question is from where do these cells come. Since cDC are present in the naïve CNS and can be expanded by cytokines[138,139], we hypothesize that cDC are predominantly derived from *in situ* proliferation. In contrast, moDC, which are derived from peripheral monocytes, predominantly traffic from the periphery. This nuance is significant because therapies that block trafficking to the CNS may then affect the moDC but not the cDC. In addition, we identified two populations of cDC: MHCII$^+$ and MHCII^{++}. In lymphoid tissues, these represent resident and migratory cDC populations, respectively[198]. Migratory MHCII^{++} cDC traffic from an inflammatory tissue to lymph nodes in a CCR7-dependent manner[199]. We found that CNS-derived cDC, but not moDC, expressed CCR7 transcript, supporting the migratory potential for these cells. CD11c$^+$ have been identified in lymphatics within the

CNS[189], and we would hypothesize that these are MHCII^{++} cDC. Therapies which limit trafficking of the cDC in the lymphatics may be efficacious in inhibiting neuroinflammation.

In summary, our data suggest multiple ways to block the function of cDC as APC, but further research is necessary to investigate these pathways.

Targeting Myeloid Cells – Promote Repair

A complementary therapeutic strategy would be to promote repair of the CNS tissue. cDC perform a critical pathogenic role as APC, but the role of moDC and other monocyte-derived myeloid cells is unclear. Since inhibiting monocyte recruitment to the CNS blocks disease[43,45], cells of the monocyte lineage must exert a pathologic function early in disease. However, the function of myeloid cells may change as disease evolves. DC depletion early in EAE ameliorated disease, but some of the DC depletion studies found that DC depletion at peak exacerbates late disease[140,142]. The proposed mechanism was that DC express PD-L1 and promote Tregs which suppress the ongoing inflammatory process. In the absence of DC, this regulation was absent and inflammation was exacerbated. moDC express high levels of PD-L1 and may thus be involved in the resolution of neuroinflammation.

The phenotype of myeloid cells changes during course of disease such that the cells express iNOS at onset but shift to express Arg1 at peak disease. We found that expression of these markers was specific to monocyte-derived cells. Arg1 is used as a marker of an anti-inflammatory or pro-regenerative myeloid cell phenotype. Arg1-expressing cells have been shown to promote oligodendrocyte differentiation through production of Activin A[61], and we confirmed expression of Activin A in CNS myeloid cells (Figure 4.2A-B). Activin A expression was higher in moDC compared with cDC and higher in Arg1^{+} myeloid cells compared with

Arg1$^-$, highlighting the regenerative potential of these alternatively activated myeloid cells. Arg1-expressing cells may also promote axon growth by secretion of polyamines, the product of arginine metabolism by Arg1[30]. Degenerated myelin and myelin-associated glycoproteins inhibit neurite outgrowth in the CNS[200]. Polyamines from Arg1-expressing cells overcome this inhibition[31]. Further, myeloid cells may also clear this myelin debris by phagocytosis[160,201], and we've shown that moDC are proficient myelin phagocytes. These data demonstrate that myeloid cells can promote repair and remyelination of the damaged CNS.

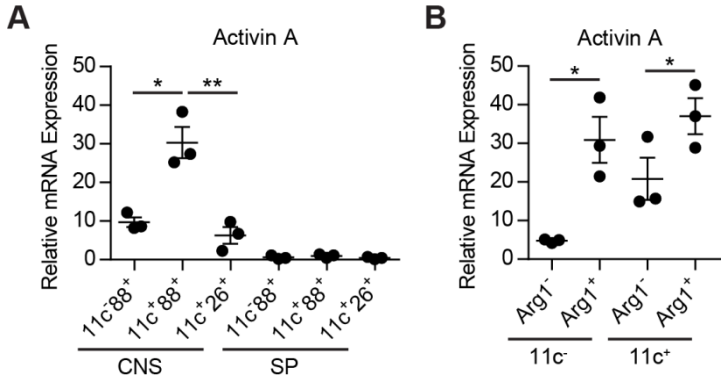


Figure 4.2 – Activin A is expressed by Arg1$^+$ monocyte-derived cells in the CNS.
(A) CD11b$^+$ macrophage (11c$^-$88$^+$) and DC (11c$^+$88$^+$ moDC or 11c$^+$26$^+$ cDC) populations were sorted from CNS and spleen (SP) at the peak of EAE, and Activin A expression was determined by RT-qPCR. (B) Arg1$^-$ and Arg1$^+$ macrophages (11c$^-$) or DC (11c$^+$) were sorted from the CNS of Arg1-eYFP reporter mice in late EAE and expression was measured by RT-qPCR. Data represented as mean ± SEM.*P<0.05, **P<0.01 by paired, 2-tailed Student's t test.

iNOS and Arg1 are also functional enzymes which have an influence on the inflammatory response. iNOS has been extensively studied in EAE, but the results are contradictory. Inhibiting iNOS with a small molecule inhibitor ameliorates disease [202] while genetic deficiency in iNOS exacerbates disease [203]. We found that adoptive EAE was exacerbated in iNOS mice (Figure 4.3A), suggesting that iNOS may play a protective role in disease. The reason for this discrepancy is unclear and requires further investigation[204]. A

similar inconsistency has been observed for Arg1. One report found that inhibiting Arg1 with the competitive inhibitor ABH delayed disease and reduced disease severity[205]; however, it is unclear whether this effect was mediated through inhibition of Arg1 in immune cells. Using hematopoietic-specific knockout of Arg1 (Vav1-Cre x $Arg1^{fl/fl}$), we showed that Arg1 is dispensable for the development of disease but that Arg1-deficient mice trend towards an enhanced severity of disease (Figure 4.3B). This suggests a protective role for Arg1 as well.

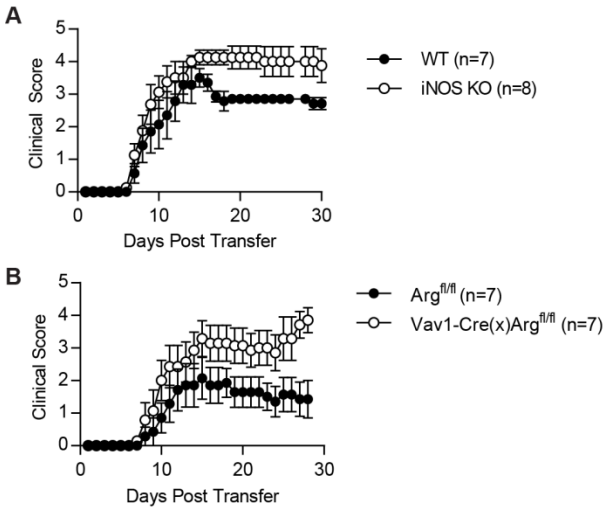


Figure 4.3 – Deficiency in either iNOS or Arg1 exacerbates EAE.
(A) Clinical course of EAE induced by adoptive transfer of myelin-primed WT Th17 cells to WT or iNOS-deficient recipients. (B) Clinical course of EAE induced by adoptive transfer of myelin-primed WT Th17 cells to mice with hematopoietic-specific Arg1 deficiency (Vav1-Cre x $Arg1^{fl/fl}$) or littermate controls $Arg1^{fl/fl}$). All values are mean ± SEM. Representative of at least two experiments.

Promoting Arg1 expression or the $Arg1^{+}$ cell phenotype is a possible therapeutic strategy, but the factors which control this myeloid cell polarization in the CNS are not known. We showed that Arg1 expression by CNS myeloid cells was independent of STAT6, distinguishing these cells from alternatively activated cells from other models[206,207]. As an alternative, hypoxia inducible factor 1α (HIF1α) has also been described to promote Arg1 in myeloid cells[119]. Furthermore, CNS tissues become hypoxic during EAE[208], providing a stimulus to promote

HIF1α stabilization[209], and HIF1α protein is enriched in macrophages during EAE[210]. We hypothesized that HIF1α may regulate Arg1 expression in the CNS. To investigate the role of HIF1α in Arg1 expression in EAE, we generated mixed bone marrow chimeras using a knockout in which HIF1α is conditionally deleted in all hematopoietic cells (Vav1-Cre x $HIF1\alpha^{fl/fl}$ = $HIF1\alpha^{-/-}$). WT and HIF1α-deficient bone marrow reconstituted similar compositions of macrophages and dendritic cells (unpublished data); however at peak disease, the frequency of $Arg1^{+}$ cells was reduced by >60% among HIF1α-deficient myeloid cells, compared with WT cells isolated from the same animal (Figure 4.4A). At onset, iNOS is also significantly reduced (Figure 4.4B). These data identify HIF1α as a critical regulator of Arg1 and iNOS in CNS-infiltrating myeloid cells. However, since HIF1α regulates both Arg1 and iNOS, the factor which promotes the conversion from iNOS to Arg1 expression in CNS myeloid cells remains to be elucidated.

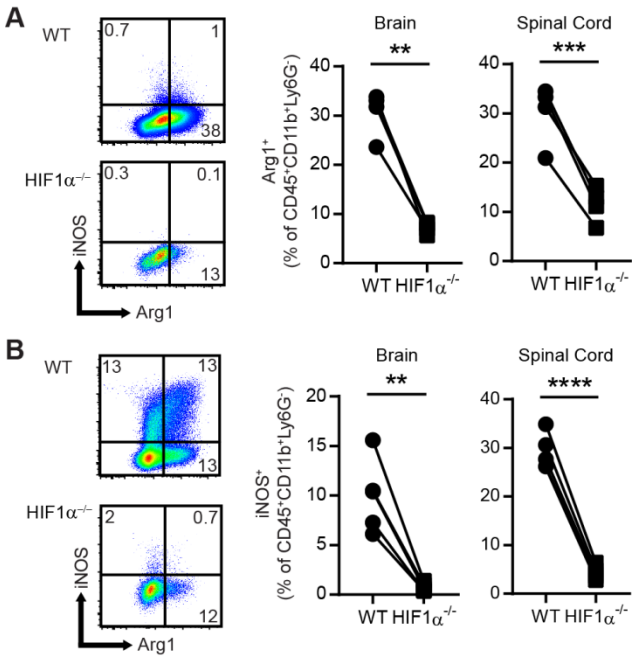


Figure 4.4 – Arg1 and iNOS expression in CNS myeloid cells is HIF1α-dependent.
Mononuclear cells were isolated from the CNS of HIF1α$^{-/-}$:WT→WT mixed bone marrow chimeric mice at the peak of EAE (A) or the onset of EAE (B). Representative flow and quantification of intracellular staining of Arg1 and iNOS in WT or KO cells, gated on the CD45^{hi}CD11b^{+}Ly6G^{-} population. Percentages are Arg1^{+} or iNOS^{+} cells among WT or KO CNS myeloid cells isolated from individual chimeric mice. Connected dots indicate cells derived from the same mouse. *P<0.05, **P<0.01, ***P<0.001 and n.s. = P>0.05, by paired, 2-tailed Student's t test. Data are representative of at least 2 experiments. N=3-5 mice per group.

In summary, monocyte-derived myeloid cells can inhibit inflammation, promote oligodendrocyte differentiation, and overcome axon growth inhibition. However, the factors which control these functional programs are unclear and must be investigated further.

Challenges in translating from EAE to MS

Animal models are essential for studying human disease. We have used EAE as a model of MS as it replicates key pathologic features of MS such as CNS-infiltration of immune cells and demyelinating lesions[38]. However, there are limitations. One of the significant differences between MS and EAE is the clinical course. Individuals with MS most often follow at relapsing-remitting course at onset and then convert to a progressive course later in disease[7]. Most

available mouse models do not replicate this progression[211]. C57Bl/6 mice follow a chronic course in which the mice maintain disability. There is not remission but there is also no progression. SJL mice follow a relapsing-remitting course but maintain their state of either remission or relapse long term. There is no progression. The closest model to the progressive course is the NOD mouse strain. NOD mice are multigenic model of autoimmune diabetes[212] which follow a relapsing-remitting and subsequently progressive course following immunization with MOG_{35-55} peptide[213]. Unfortunately, wide use of this strain is limited due to the availability of genetic models on this background.

These limitations are important as one interprets results from these models. For example, we identified cDC are critical for initiating neuroinflammation in adoptive EAE in C57Bl/6 mice. Further study is necessary to determine if cDC are subsequently also required for the initiation of relapses. This is therapeutically important as most MS patients begin treatment after having experience multiple episodes of demyelinating lesions[214]. As another example, we showed that myeloid cells shift from expression of iNOS to Arg1 during course of adoptive EAE in C57Bl/6 mice. Using the SJL model, we were also able to show that this shift occurs not only in the initial episode but also in subsequent relapses. This strengthens our observation that myeloid cell polarizations shift with the evolution of neuroinflammation.

Another significant challenge with using animal models is the differences in markers and gene expression between mice and humans. Relevant to this work are differences in the expression of Arg1. We found that Arg1 is widely expressed by CNS-infiltrating myeloid cells in EAE but expression is limited to perivascular mononuclear cells in humans (David Pitt, unpublished data). This motivated our use of CD206 as an alternative activation marker.

The surface markers used to discriminate DC subsets in mouse and human are also different[198]. CD11c expression is specific to DC in mice, but CD11c is also expressed by monocytes in humans, making it difficult to distinguish moDC with this marker[215]. Furthermore, CD88 and CD26 have not been validated as markers of moDC or cDC in humans, so further study is necessary to determine if this distinction translates. Zbtb46 has been shown in mouse and human DC on the transcript level in peripheral myeloid cells[83,84], but no studies have used Zbtb46 to identify cDC in humans *in situ*. Potential alternative markers which may be of use to discriminate cDC are specific to cDC subsets. Mouse cDC continue to be subdivided into further subsets based on tissue location, inflammatory setting, and a host surface markers including CD8, CD103, CD11b, CD24, XCR1, CD172a, and more[198]. However, human DC do not carry the same markers, so it is difficult to know how or if these subsets translate. One functional division which appears consistent between mice and humans is between cDC which present antigen to $CD8^+$ versus $CD4^+$ T cells, identified as cDC1 and cDC2 respectively. cDC1 express CD8 and/or CD103 in mice and express CD141 in humans; cDC2 express CD11b and/or CD4 in mice and express CD1c in humans. Important to our work, $CD11b^+$ cDC2 in mice are proficient at presenting antigen and activating $CD4^+$ T cells[163,216], and functional studies with $CD1c^+$ cDC2 from human peripheral blood demonstrate a similar capacity[217].

When possible, it is important corroborate observations from mouse models with human disease. Many have undertaken studies of human immune cells from peripheral blood as a surrogate for CNS myeloid cells. However, the value of this approach is limited as peripheral and CNS myeloid cells are fundamentally different. cDC are exceedingly rare in the blood[72], and it is not clear how the functions of the circulating cDC differ from tissue-resident cDC. moDC are not present in the blood but are specific to sites of inflammation. As a proof of this

specificity, we observed that expression of Arg1 and iNOS was restricted to the CNS and not observed in the blood or other peripheral organs. Some have attempted to overcome this by generating inflammatory moDC *in vitro* by culturing bone marrow or blood monocytes with GM-CSF[218]. These cells can be stimulated to produce iNOS and Arg1[33], but unlike CNS-derived moDC, we found that these *in vitro*-generated moDC are competent antigen presenting cells (unpublished data), highlighting the fundamental problem of inferring properties of CNS myeloid cells from peripheral cells.

Since functional studies on human CNS myeloid cells are not possible, immunohistochemistry provides a means to evaluate the presence of myeloid cell subsets in disease. Having demonstrated that myeloid cells are heterogenous in EAE, we further validated this observation in MS lesions. Having demonstrated that cDC are present in the CNS in naïve mice and during EAE, we are pursuing a collaboration to evaluate the presence of cDC in the human brain and meninges. These studies are critical as we seek to translate our observations from EAE to MS.

Conclusions

The results presented in this dissertation advance our understanding of myeloid cell phenotypes and functions during MS and EAE. We hope these studies will provide a foundation for the development of novel therapies that inhibit CNS autoimmunity and promote repair, moving toward the ultimate goal of promoting the health and welfare of patients with MS.

Bibliography

1. Dilokthornsakul, P. *et al.* Multiple sclerosis prevalence in the United States commercially insured population. *Neurology* **86,** 1014–1021 (2016).

2. Browne, P. *et al.* Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. *Neurology* **83,** 1022–1024 (2014).

3. Campbell, J. D. *et al.* Burden of multiple sclerosis on direct, indirect costs and quality of life: National US estimates. *Mult. Scler. Relat. Disord.* **3,** 227–236 (2014).

4. Scalfari, A. *et al.* Mortality in patients with multiple sclerosis. *Neurology* **81,** 184–192 (2013).

5. Adelman, G., Rane, S. G. & Villa, K. F. The cost burden of multiple sclerosis in the United States: a systematic review of the literature. *J. Med. Econ.* **16,** 639–647 (2013).

6. Lublin, F. D. *et al.* Defining the clinical course of multiple sclerosis. *Neurology* **83,** 278–286 (2014).

7. Weinshenker, B. G. Natural history of multiple sclerosis. *Ann. Neurol.* **36 Suppl,** S6–11 (1994).

8. Dutta, R. & Trapp, B. D. Relapsing and progressive forms of multiple sclerosis – insights from pathology. *Curr. Opin. Neurol.* **27,** 271–278 (2014).

9. Dyment, D. A., Ebers, G. C. & Dessa Sadovnick, A. Genetics of multiple sclerosis. *Lancet Neurol.* **3,** 104–110 (2004).

10. Koch-Henriksen, N. & Sørensen, P. S. The changing demographic pattern of multiple sclerosis epidemiology. *Lancet Neurol.* **9,** 520–532 (2010).

11. Munger, K. L. *et al.* Vitamin D intake and incidence of multiple sclerosis. *Neurology* **62,** 60–65 (2004).

12. Handel, A. E. *et al.* An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PloS One* **5,** (2010).

13. O'Gorman, C., Lucas, R. & Taylor, B. Environmental Risk Factors for Multiple Sclerosis: A Review with a Focus on Molecular Mechanisms. *Int. J. Mol. Sci.* **13,** 11718–11752 (2012).

14. Dutta, R. & Trapp, B. D. Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis. *Prog. Neurobiol.* **93,** 1–12 (2011).

15. Compston, A. The 150th anniversary of the first depiction of the lesions of multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* **51,** 1249–1252 (1988).

16. Van Der Valk, P. & De Groot, C. J. A. Staging of multiple sclerosis (MS) lesions: pathology of the time frame of MS. *Neuropathol. Appl. Neurobiol.* **26,** 2–10 (2000).

17. Kuhlmann, T. *et al.* An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol. (Berl.)* **133,** 13–24 (2017).

18. Frischer, J. M. *et al.* Clinical and Pathological Insights into the Dynamic Nature of the White Matter Multiple Sclerosis Plaque. *Ann. Neurol.* **78,** 710–721 (2015).

19. Vargas, D. L. & Tyor, W. R. Update on disease-modifying therapies for multiple sclerosis. *J. Investig. Med. Off. Publ. Am. Fed. Clin. Res.* **65,** 883–891 (2017).

20. Comi, G. Disease-modifying treatments for progressive multiple sclerosis. *Mult. Scler. J.* **19,** 1428–1436 (2013).

21. Karni, A. *et al.* Innate Immunity in Multiple Sclerosis: Myeloid Dendritic Cells in Secondary Progressive Multiple Sclerosis Are Activated and Drive a Proinflammatory Immune Response. *J. Immunol.* **177,** 4196–4202 (2006).

22. Segal, B. M. Stage-Specific Immune Dysregulation in Multiple Sclerosis. *J. Interferon Cytokine Res.* **34,** 633–640 (2014).

23. Huber, A. K. *et al.* Dysregulation of the IL-23/IL-17 axis and myeloid factors in secondary progressive MS. *Neurology* **83,** 1500–1507 (2014).

24. Huber, A. K., Giles, D. A., Segal, B. M. & Irani, D. N. An emerging role for eotaxins in neurodegenerative disease. *Clin. Immunol.* (2016). doi:10.1016/j.clim.2016.09.010

25. Nathan, C. F., Murray, H. W., Wiebe, M. E. & Rubin, B. Y. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* **158,** 670–689 (1983).

26. Stein, M., Keshav, S., Harris, N. & Gordon, S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* **176,** 287–292 (1992).

27. Doyle, A. G. *et al.* Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *Eur. J. Immunol.* **24,** 1441–1445 (1994).

28. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. & Hill, A. M. M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *J. Immunol.* **164,** 6166–6173 (2000).

29. Arango Duque, G. & Descoteaux, A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front. Immunol.* **5,** 491 (2014).

30. Rath, M., Müller, I., Kropf, P., Closs, E. I. & Munder, M. Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages. *Front. Immunol.* **5,** (2014).

31. Cai, D. *et al.* Arginase I and Polyamines Act Downstream from Cyclic AMP in Overcoming Inhibition of Axonal Growth MAG and Myelin In Vitro. *Neuron* **35,** 711–719 (2002).

32. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25,** 677–686 (2004).

33. Davis, M. J. *et al.* Macrophage M1/M2 Polarization Dynamically Adapts to Changes in Cytokine Microenvironments in Cryptococcus neoformans Infection. Available at: http://mbio.asm.org. (Accessed: 18th November 2017)

34. Klimp, A. H., de Vries, E. G. E., Scherphof, G. L. & Daemen, T. A potential role of macrophage activation in the treatment of cancer. *Crit. Rev. Oncol. Hematol.* **44,** 143–161 (2002).

35. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8,** 958–969 (2008).

36. Biswas, S. K. *et al.* A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). *Blood* **107,** 2112–2122 (2006).

37. Ley, K., Miller, Y. I. & Hedrick, C. C. Monocyte and Macrophage Dynamics during Atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **31,** 1506–1516 (2011).

38. Lassmann, H. & Bradl, M. Multiple sclerosis: experimental models and reality. *Acta Neuropathol. (Berl.)* **133,** 223–244 (2017).

39. Mendel, I., de Rosbo, N. K. & Ben-Nun, A. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: Fine specificity and T cell receptor Vβ expression of encephalitogenic T cells. *Eur. J. Immunol.* **25,** 1951–1959 (1995).

40. Mannara, F. *et al.* Passive Experimental Autoimmune Encephalomyelitis in C57BL/6 with MOG: Evidence of Involvement of B Cells. *PLoS ONE* **7,** e52361 (2012).

41. Miller, S. D., Karpus, W. J. & Davidson, T. S. Experimental Autoimmune Encephalomyelitis in the Mouse. *Curr. Protoc. Immunol. Ed. John E Coligan Al* **CHAPTER,** Unit–15.1 (2007).

42. Takács, K., Chandler, P. & Altmann, D. M. Relapsing and remitting experimental allergic encephalomyelitis: A focused response to the encephalitogenic peptide rather than epitope spread. *Eur. J. Immunol.* **27,** 2927–2934 (1997).

43. Ajami, B., Bennett, J. L., Krieger, C., McNagny, K. M. & Rossi, F. M. V. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat. Neurosci.* **14,** 1142–1149 (2011).

44. Ko, H.-J. *et al.* GM-CSF–Responsive Monocyte-Derived Dendritic Cells Are Pivotal in Th17 Pathogenesis. *J. Immunol.* **192,** 2202–2209 (2014).

45. Fife, B. T., Huffnagle, G. B., Kuziel, W. A. & Karpus, W. J. Cc Chemokine Receptor 2 Is Critical for Induction of Experimental Autoimmune Encephalomyelitis. *J. Exp. Med.* **192,** 899–906 (2000).

46. Huang, D., Wang, J., Kivisakk, P., Rollins, B. J. & Ransohoff, R. M. Absence of Monocyte Chemoattractant Protein 1 in Mice Leads to Decreased Local Macrophage Recruitment and Antigen-Specific T Helper Cell Type 1 Immune Response in Experimental Autoimmune Encephalomyelitis. *J. Exp. Med.* **193,** 713–726 (2001).

47. Grifka-Walk, H. M., Giles, D. A. & Segal, B. M. IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23. *Eur. J. Immunol.* **45,** 2780–2786 (2015).

48. King, I. L., Dickendesher, T. L. & Segal, B. M. Circulating Ly-6C+ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. *Blood* **113,** 3190–3197 (2009).

49. Greter, M. *et al.* Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat. Med.* **11,** 328–334 (2005).

50. Katz-Levy, Y. *et al.* Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus–infected mice. *J. Clin. Invest.* **104,** 599–610 (1999).

51. Issazadeh, S., Ljungdahl, A., Höjeberg, B., Mustafa, M. & Olsson, T. Cytokine production in the central nervous system of Lewis rats with experimental autoimmune encephalomyelitis: dynamics of mRNA expression for interleukin-10, interleukin-12, cytolysin, tumor necrosis factor α and tumor necrosis factor β. *J. Neuroimmunol.* **61,** 205–212 (1995).

52. Segal, B. M. CNS chemokines, cytokines, and dendritic cells in autoimmune demyelination. *J. Neurol. Sci.* **228,** 210–214 (2005).

53. Raivich, G. & Banati, R. Brain microglia and blood-derived macrophages: molecular profiles and functional roles in multiple sclerosis and animal models of autoimmune demyelinating disease. *Brain Res. Rev.* **46,** 261–281 (2004).

54. Yamasaki, R. *et al.* Differential roles of microglia and monocytes in the inflamed central nervous system. *J. Exp. Med.* jem.20132477 (2014). doi:10.1084/jem.20132477

55. Fischer, M. T. *et al.* Disease-specific molecular events in cortical multiple sclerosis lesions. *Brain* (2013). doi:10.1093/brain/awt110

56. Boven, L. A. *et al.* Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain* **129,** 517–526 (2006).

57. Tierney, J. B., Kharkrang, M. & La Flamme, A. C. Type II-activated macrophages suppress the development of experimental autoimmune encephalomyelitis. *Immunol. Cell Biol.* **87,** 235–240 (2008).

58. Mikita, J. *et al.* Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. *Mult. Scler. J.* **17,** 2–15 (2011).

59. Butovsky, O. *et al.* Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *J. Clin. Invest.* **116,** 905–915 (2006).

60. Weber, M. S. *et al.* Type II monocytes modulate T cell–mediated central nervous system autoimmune disease. *Nat. Med.* **13,** 935–943 (2007).

61. Miron, V. E. *et al.* M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat. Neurosci.* **16,** 1211–1218 (2013).

62. Kigerl, K. A. *et al.* Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord. *J. Neurosci.* **29,** 13435–13444 (2009).

63. Piccio, L. *et al.* Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation. *Brain* **131,** 3081–3091 (2008).

64. Goldmann, T. *et al.* Origin, fate and dynamics of macrophages at CNS interfaces. *Nat. Immunol.* **17,** 797–805 (2016).

65. Perdiguero, E. G. *et al.* Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* **518,** 547 (2015).

66. Ginhoux, F. *et al.* Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science* **330,** 841–845 (2010).

67. Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W. & Rossi, F. M. V. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat. Neurosci.* **10,** 1538 (2007).

68. Steinman, R. M. & Cohn, Z. A. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* **137,** 1142–1162 (1973).

69. Satpathy, A. T., Wu, X., Albring, J. C. & Murphy, K. M. Re(de)fining the dendritic cell lineage. *Nat. Immunol.* **13,** 1145 (2012).

70. Randolph, G. J., Inaba, K., Robbiani, D. F., Steinman, R. M. & Muller, W. A. Differentiation of Phagocytic Monocytes into Lymph Node Dendritic Cells In Vivo. *Immunity* **11,** 753–761 (1999).

71. Chapuis, F. *et al.* Differentiation of human dendritic cells from monocytes in vitro. *Eur. J. Immunol.* **27,** 431–441 (1997).

72. Liu, K. *et al.* In Vivo Analysis of Dendritic Cell Development and Homeostasis. *Science* **324,** 392–397 (2009).

73. McKenna, H. J. *et al.* Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* **95,** 3489–3497 (2000).

74. Middel, P., Raddatz, D., Gunawan, B., Haller, F. & Radzun, H.-J. Increased number of mature dendritic cells in Crohn's disease: evidence for a chemokine mediated retention mechanism. *Gut* **55,** 220–227 (2006).

75. Langlet, C. *et al.* CD64 Expression Distinguishes Monocyte-Derived and Conventional Dendritic Cells and Reveals Their Distinct Role during Intramuscular Immunization. *J. Immunol.* **188,** 1751–1760 (2012).

76. Cho, K. W. *et al.* Adipose Tissue Dendritic Cells Are Independent Contributors to Obesity-Induced Inflammation and Insulin Resistance. *J. Immunol.* **197,** 3650–3661 (2016).

77. Tamoutounour, S. *et al.* CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur. J. Immunol.* **42,** 3150–3166 (2012).

78. Nakano, H. *et al.* Complement Receptor C5aR1/CD88 and Dipeptidyl Peptidase-4/CD26 Define Distinct Hematopoietic Lineages of Dendritic Cells. *J. Immunol.* **194,** 3808–3819 (2015).

79. Schlitzer, A. *et al.* IRF4 Transcription Factor-Dependent CD11b+ Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses. *Immunity* **38,** 970–983 (2013).

80. Brennan, F. H. *et al.* The Complement Receptor C5aR Controls Acute Inflammation and Astrogliosis following Spinal Cord Injury. *J. Neurosci.* **35,** 6517–6531 (2015).

81. Shipp, M. A. & Look, A. T. Hematopoietic differentiation antigens that are membrane-associated enzymes: cutting is the key! *Blood* **82,** 1052–1070 (1993).

82. Haninec, P. & Grim, M. Localization of dipeptidylpeptidase IV and alkaline phosphatase in developing spinal cord meninges and peripheral nerve coverings of the rat. *Int. J. Dev. Neurosci.* **8,** 175–185 (1990).

83. Meredith, M. M. *et al.* Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J. Exp. Med.* **209,** 1153–1165 (2012).

84. Satpathy, A. T. *et al.* Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *J. Exp. Med.* **209,** 1135–1152 (2012).

85. Borght, K. V. der *et al.* Myocardial Infarction Primes Autoreactive T Cells through Activation of Dendritic Cells. *Cell Rep.* **18,** 3005–3017 (2017).

86. Loschko, J. *et al.* Absence of MHC class II on cDCs results in microbial-dependent intestinal inflammation. *J. Exp. Med.* **213,** 517–534 (2016).

87. Sobel, R. A., Blanchette, B. W., Bhan, A. K. & Colvin, R. B. The immunopathology of experimental allergic encephalomyelitis. I. Quantitative analysis of inflammatory cells in situ. *J. Immunol.* **132,** 2393–2401 (1984).

88. Vogel, D. Y. *et al.* Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *J. Neuroinflammation* **10,** 35 (2013).

89. Deshpande, P., King, I. L. & Segal, B. M. Cutting Edge: CNS CD11c+ Cells from Mice with Encephalomyelitis Polarize Th17 cells and Support CD25+CD4+ T cell-Mediated Immunosuppression, Suggesting Dual Roles in the Disease Process. *J. Immunol.* **178,** 6695–6699 (2007).

90. Cross, A. H. *et al.* Inducible nitric oxide synthase gene expression and enzyme activity correlate with disease activity in murine experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **71,** 145–153 (1996).

91. Li, S., Vana, A. C., Ribeiro, R. & Zhang, Y. Distinct role of nitric oxide and peroxynitrite in mediating oligodendrocyte toxicity in culture and in experimental autoimmune encephalomyelitis. *Neuroscience* **184,** 107–119 (2011).

92. Wingerchuk, D. M., Lucchinetti, C. F. & Noseworthy, J. H. Multiple sclerosis: current pathophysiological concepts. *Lab. Investig. J. Tech. Methods Pathol.* **81,** 263–281 (2001).

93. De Groot, C. J., Ruuls, S. R., Theeuwes, J. W., Dijkstra, C. D. & Van der Valk, P. Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* **56,** 10–20 (1997).

94. Nikić, I. *et al.* A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat. Med.* **17,** 495–499 (2011).

95. Oleszak, E. L. *et al.* Inducible Nitric Oxide Synthase and Nitrotyrosine Are Found in Monocytes/Macrophages and/or Astrocytes in Acute, but Not in Chronic, Multiple Sclerosis. *Clin. Diagn. Lab. Immunol.* **5,** 438–445 (1998).

96. Hill, K. E., Zollinger, L. V., Watt, H. E., Carlson, N. G. & Rose, J. W. Inducible nitric oxide synthase in chronic active multiple sclerosis plaques: distribution, cellular expression and association with myelin damage. *J. Neuroimmunol.* **151,** 171–179 (2004).

97. Fan, X. *et al.* Double Roles of Macrophages in Human Neuroimmune Diseases and Their Animal Models. *Mediators Inflamm.* **2016,** e8489251 (2016).

98. Zhu, B. *et al.* CD11b+Ly-6Chi Suppressive Monocytes in Experimental Autoimmune Encephalomyelitis. *J. Immunol.* **179,** 5228–5237 (2007).

99. Munder, M. Arginase: an emerging key player in the mammalian immune system. *Br. J. Pharmacol.* **158,** 638–651 (2009).

100. Kotter, M. R., Zhao, C., van Rooijen, N. & Franklin, R. J. M. Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. *Neurobiol. Dis.* **18,** 166–175 (2005).

101. Copelman, C. A., Diemel, L. T., Gveric, D., Gregson, N. A. & Cuzner, M. L. Myelin phagocytosis and remyelination of macrophage-enriched central nervous system aggregate cultures. *J. Neurosci. Res.* **66,** 1173–1178 (2001).

102. Zhang, Z. *et al.* Parenchymal accumulation of CD163+ macrophages/microglia in multiple sclerosis brains. *J. Neuroimmunol.* **237,** 73–79 (2011).

103. Wasser, B. *et al.* Increase of Alternatively Activated Antigen Presenting Cells in Active Experimental Autoimmune Encephalomyelitis. *J. Neuroimmune Pharmacol.* 1–12 (2016). doi:10.1007/s11481-016-9696-3

104. Xu, L. *et al.* Arginase and autoimmune inflammation in the central nervous system. *Immunology* **110,** 141–148 (2003).

105. Zhang, X.-M., Lund, H., Mia, S., Parsa, R. & Harris, R. A. Adoptive transfer of cytokine-induced immunomodulatory adult microglia attenuates experimental autoimmune encephalomyelitis in DBA/1 mice. *Glia* **62,** 804–817 (2014).

106. Benedek, G. *et al.* Estrogen induces multiple regulatory B cell subtypes and promotes M2 microglia and neuroprotection during experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **293,** 45–53 (2016).

107. Liu, C. *et al.* Targeting the Shift from M1 to M2 Macrophages in Experimental Autoimmune Encephalomyelitis Mice Treated with Fasudil. *PLoS ONE* **8,** e54841 (2013).

108. Kong, W., Hooper, K. M. & Ganea, D. The natural dual cyclooxygenase and 5-lipoxygenase inhibitor flavocoxid is protective in EAE through effects on Th1/Th17 differentiation and macrophage/microglia activation. *Brain. Behav. Immun.* **53,** 59–71 (2016).

109. Reese, T. A. *et al.* Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* **447,** 92–96 (2007).

110. Béchade, C., Colasse, S., Diana, M. A., Rouault, M. & Bessis, A. NOS2 expression is restricted to neurons in the healthy brain but is triggered in microglia upon inflammation. *Glia* **62,** 956–963 (2014).

111. Bö, L. *et al.* Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions. *J. Neuroimmunol.* **51,** 135–146 (1994).

112. Mehta, V. *et al.* Iron Is a Sensitive Biomarker for Inflammation in Multiple Sclerosis Lesions. *PLoS ONE* **8,** e57573 (2013).

113. Lawrence, T. & Natoli, G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat. Rev. Immunol.* **11,** 750–761 (2011).

114. Hsieh, C. L. *et al.* Traumatic brain injury induces macrophage subsets in the brain. *Eur. J. Immunol.* **43,** 2010–2022 (2013).

115. Gray, M. J., Poljakovic, M., Kepka-Lenhart, D. & Morris Jr., S. M. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPβ. *Gene* **353,** 98–106 (2005).

116. Bailey, S. L., Schreiner, B., McMahon, E. J. & Miller, S. D. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ TH-17 cells in relapsing EAE. *Nat. Immunol.* **8,** 172–180 (2007).

117. Rodriguez, P. C. *et al.* Regulation of T Cell Receptor CD3ζ Chain Expression byl-Arginine. *J. Biol. Chem.* **277,** 21123–21129 (2002).

118. Rodriguez, P. C. *et al.* l-Arginine Consumption by Macrophages Modulates the Expression of CD3ζ Chain in T Lymphocytes. *J. Immunol.* **171,** 1232–1239 (2003).

119. Colegio, O. R. *et al.* Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* **513,** 559–563 (2014).

120. Odegaard, J. I. *et al.* Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. *Nature* **447,** 1116–1120 (2007).

121. Kawakami, N. *et al.* The Activation Status of Neuroantigen-specific T Cells in the Target Organ Determines the Clinical Outcome of Autoimmune Encephalomyelitis. *J. Exp. Med.* **199,** 185–197 (2004).

122. Archambault, A. S., Sim, J., Gimenez, M. A. T. & Russell, J. H. Defining antigen-dependent stages of T cell migration from the blood to the central nervous system parenchyma. *Eur. J. Immunol.* **35,** 1076–1085 (2005).

123. Roche, P. A. & Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat. Rev. Immunol.* **15,** 203 (2015).

124. Ransohoff, R. M. & Estes, M. L. Astrocyte Expression of Major Histocompatibility Complex Gene Products in Multiple Sclerosis Brain Tissue Obtained by Stereotactic Biopsy. *Arch. Neurol.* **48,** 1244–1246 (1991).

125. Zeinstra, E., Wilczak, N., Streefland, C. & Keyser, J. D. Astrocytes in chronic active multiple sclerosis plaques express Mhc class Ii molecules. *Neuroreport* **11,** 89–91 (2000).

126. Kambayashi, T. & Laufer, T. M. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nat. Rev. Immunol.* **14,** 719 (2014).

127. Mildner, A. & Jung, S. Development and Function of Dendritic Cell Subsets. *Immunity* **40,** 642–656 (2014).

128. Ginhoux, F. & Jung, S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat. Rev. Immunol.* **14,** 392 (2014).

129. Serbina, N. V., Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A. & Pamer, E. G. TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection. *Immunity* **19,** 59–70 (2003).

130. Waskow, C. *et al.* FMS-like tyrosine kinase 3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat. Immunol.* **9,** 676–683 (2008).

131. Diao, J. *et al.* In Situ Replication of Immediate Dendritic Cell (DC) Precursors Contributes to Conventional DC Homeostasis in Lymphoid Tissue. *J. Immunol.* **176,** 7196–7206 (2006).

132. Reizis, B., Bunin, A., Ghosh, H. S., Lewis, K. L. & Sisirak, V. Plasmacytoid Dendritic Cells: Recent Progress and Open Questions. *Annu. Rev. Immunol.* **29,** 163–183 (2011).

133. Mildner, A. *et al.* CCR2+Ly-6Chi monocytes are crucial for the effector phase of autoimmunity in the central nervous system. *Brain* **132,** 2487–2500 (2009).

134. McMenamin, P. G. Distribution and phenotype of dendritic cells and resident tissue macrophages in the dura mater, leptomeninges, and choroid plexus of the rat brain as demonstrated in wholemount preparations. *J. Comp. Neurol.* **405,** 553–562 (1999).

135. Matyszak, M. K. & Perry, V. H. The potential role of dendritic cells in immune-mediated inflammatory diseases in the central nervous system. *Neuroscience* **74,** 599–608 (1996).

136. Bulloch, K. *et al.* CD11c/EYFP transgene illuminates a discrete network of dendritic cells within the embryonic, neonatal, adult, and injured mouse brain. *J. Comp. Neurol.* **508,** 687–710 (2008).

137. Serot, J.-M., Foliguet, B., Béné, M.-C. & Faure, G.-C. Ultrastructural and immunohistological evidence for dendritic-like cells within human choroid plexus epithelium. *NeuroReport* **8,** 1995 (1997).

138. Quintana, E. *et al.* DNGR-1+ dendritic cells are located in meningeal membrane and choroid plexus of the noninjured brain. *Glia* **63,** 2231–2248 (2015).

139. Anandasabapathy, N. *et al.* Flt3L controls the development of radiosensitive dendritic cells in the meninges and choroid plexus of the steady-state mouse brain. *J. Exp. Med.* **208,** 1695–1705 (2011).

140. Clarkson, B. D. *et al.* CCR2-dependent dendritic cell accumulation in CNS during early effector EAE is essential for effector T cell restimulation in situ and disease progression. *J. Immunol. Baltim. Md 1950* **194,** 531–541 (2015).

141. Paterka, M. *et al.* Gatekeeper role of brain antigen-presenting CD11c+ cells in neuroinflammation. *EMBO J.* **35,** 89–101 (2016).

142. Yogev, N. *et al.* Dendritic Cells Ameliorate Autoimmunity in the CNS by Controlling the Homeostasis of PD-1 Receptor+ Regulatory T Cells. *Immunity* **37,** 264–275 (2012).

143. Isaksson, M., Lundgren, B. A., Ahlgren, K. M., Kämpe, O. & Lobell, A. Conditional DC depletion does not affect priming of encephalitogenic Th cells in EAE. *Eur. J. Immunol.* **42,** 2555–2563 (2012).

144. Norton, W. T. & Poduslo, S. E. Myelination in Rat Brain: Method of Myelin Isolation1. *J. Neurochem.* **21,** 749–757 (1973).

145. Miller, J. C. *et al.* Deciphering the transcriptional network of the dendritic cell lineage. *Nat. Immunol.* **13,** 888 (2012).

146. Stober, C. B., Brode, S., White, J. K., Popoff, J.-F. & Blackwell, J. M. Slc11a1, Formerly Nramp1, Is Expressed in Dendritic Cells and Influences Major Histocompatibility Complex Class II Expression and Antigen-Presenting Cell Function. *Infect. Immun.* **75,** 5059–5067 (2007).

147. Krause, S. W., Rehli, M., Heinz, S., Ebner, R. & Andreesen, R. Characterization of MAX.3 antigen, a glycoprotein expressed on mature macrophages, dendritic cells and blood platelets: identity with CD84. *Biochem. J.* **346,** 729–736 (2000).

148. Hussain, A. M. M., Lee, H. C. & Chang, C. F. Modulation of CD157 expression in multi-lineage myeloid differentiation of promyelocytic cell lines. *Eur. J. Cell Biol.* **79,** 697–706 (2000).

149. Bettelli, E. *et al.* Myelin Oligodendrocyte Glycoprotein–specific T Cell Receptor Transgenic Mice Develop Spontaneous Autoimmune Optic Neuritis. *J. Exp. Med.* **197,** 1073–1081 (2003).

150. Robinson, J. H. & Delvig, A. A. Diversity in MHC class II antigen presentation. *Immunology* **105,** 252–262 (2002).

151. Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* **76,** 34 (1998).

152. Kapsenberg, M. L. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat. Rev. Immunol.* **3,** 984 (2003).

153. Van Gool, S. W., Vandenberghe, P., Boer, M. de & Ceuppens, J. L. CD80, CD86 and CD40 Provide Accessory Signals in a Multiple-Step T-Cell Activation Model. *Immunol. Rev.* **153,** 47–83 (1996).

154. Freeman, G. J. *et al.* Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* **192,** 1027–1034 (2000).

155. Wolf, S. F. *et al.* Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.* **146,** 3074–3081 (1991).

156. Seder, R. A., Gazzinelli, R., Sher, A. & Paul, W. E. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci.* **90,** 10188–10192 (1993).

157. Oppmann, B. *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13,** 715–725 (2000).

158. Langrish, C. L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201,** 233–240 (2005).

159. Sica, A. & Mantovani, A. Macrophage plasticity and polarization: in vivo veritas. *J. Clin. Invest.* **122,** 787–795 (2012).

160. Lampron, A. *et al.* Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *J. Exp. Med.* **212,** 481–495 (2015).

161. Malawista, S. E., Gee, J. B. & Bensch, K. G. Cytochalasin B reversibly inhibits phagocytosis: functional, metabolic, and ultrastructural effects in human blood leukocytes and rabbit alveolar macrophages. *Yale J. Biol. Med.* **44,** 286–300 (1971).

162. Jung, S. *et al.* In Vivo Depletion of CD11c+ Dendritic Cells Abrogates Priming of CD8+ T Cells by Exogenous Cell-Associated Antigens. *Immunity* **17,** 211–220 (2002).

163. Plantinga, M. *et al.* Conventional and Monocyte-Derived CD11b+ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. *Immunity* **38,** 322–335 (2013).

164. Guilliams, M. *et al.* Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity* **45,** 669–684 (2016).

165. Villani, A.-C. *et al.* Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science* **356,** eaah4573 (2017).

166. See, P. *et al.* Mapping the human DC lineage through the integration of high-dimensional techniques. *Science* **356,** eaag3009 (2017).

167. Guilliams, M. *et al.* Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat. Rev. Immunol.* **14,** 571 (2014).

168. Knight, S. C., Mertin, J., Stackpoole, A. & Clark, J. Induction of immune responses in vivo with small numbers of veiled (dendritic) cells. *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6032–6035 (1983).

169. Zozulya, A. L. *et al.* Intracerebral Dendritic Cells Critically Modulate Encephalitogenic versus Regulatory Immune Responses in the CNS. *J. Neurosci.* **29,** 140–152 (2009).

170. Chow, K. V., Lew, A. M., Sutherland, R. M. & Zhan, Y. Monocyte-Derived Dendritic Cells Promote Th Polarization, whereas Conventional Dendritic Cells Promote Th Proliferation. *J. Immunol.* **196,** 624–636 (2016).

171. Ochi, H. B-cell-targeted therapy in multiple sclerosis. *Clin. Exp. Neuroimmunol.* **7,** 260–271 (2016).

172. Wolf, S. D., Dittel, B. N., Hardardottir, F. & Janeway, C. A. Experimental Autoimmune Encephalomyelitis Induction in Genetically B Cell–deficient Mice. *J. Exp. Med.* **184,** 2271–2278 (1996).

173. Dittel, B. N., Urbania, T. H. & Janeway Jr., C. A. Relapsing and Remitting Experimental Autoimmune Encephalomyelitis in B Cell Deficient Mice. *J. Autoimmun.* **14,** 311–318 (2000).

174. Molnarfi, N. *et al.* MHC class II–dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J. Exp. Med.* **210,** 2921–2937 (2013).

175. Marta, C. B., Oliver, A. R., Sweet, R. A., Pfeiffer, S. E. & Ruddle, N. H. Pathogenic myelin oligodendrocyte glycoprotein antibodies recognize glycosylated epitopes and perturb oligodendrocyte physiology. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 13992–13997 (2005).

176. Lyons, J.-A., San, M., Happ, M. P. & Cross, A. H. B cells are critical to induction of experimental allergic encephalomyelitis by protein but not by a short encephalitogenic peptide. *Eur. J. Immunol.* **29,** 3432–3439 (1999).

177. Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D. & Anderton, S. M. B cells regulate autoimmunity by provision of IL-10. *Nat. Immunol.* **3,** 944 (2002).

178. Pierson, E. R., Stromnes, I. M. & Goverman, J. M. B Cells Promote Induction of Experimental Autoimmune Encephalomyelitis by Facilitating Reactivation of T Cells in the Central Nervous System. *J. Immunol.* **192,** 929–939 (2014).

179. Matsushita, T., Yanaba, K., Bouaziz, J.-D., Fujimoto, M. & Tedder, T. F. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J. Clin. Invest.* **118,** 3420–3430 (2008).

180. Barr, T. A. *et al.* B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6–producing B cells. *J. Exp. Med.* **209,** 1001–1010 (2012).

181. Avalos, A. M. & Ploegh, H. L. Early BCR Events and Antigen Capture, Processing, and Loading on MHC Class II on B Cells. *Front. Immunol.* **5,** (2014).

182. Bailey-Bucktrout, S. L. *et al.* CNS Plasmacytoid Dendritic Cells Regulate the Severity of Relapsing Experimental Autoimmune Encephalomyelitis. *J. Immunol. Baltim. Md 1950* **180,** 6457–6461 (2008).

183. Isaksson, M. *et al.* Plasmacytoid DC promote priming of autoimmune Th17 cells and EAE. *Eur. J. Immunol.* **39,** 2925–2935 (2009).

184. Ioannou, M., Alissafi, T., Boon, L., Boumpas, D. & Verginis, P. In Vivo Ablation of Plasmacytoid Dendritic Cells Inhibits Autoimmunity through Expansion of Myeloid-Derived Suppressor Cells. *J. Immunol. Author Choice* **190,** 2631–2640 (2013).

185. Irla, M. *et al.* MHC class II–restricted antigen presentation by plasmacytoid dendritic cells inhibits T cell–mediated autoimmunity. *J. Exp. Med.* **207,** 1891–1905 (2010).

186. Chinnery, H. R., Ruitenberg, M. J. & McMenamin, P. G. Novel Characterization of Monocyte-Derived Cell Populations in the Meninges and Choroid Plexus and Their Rates of Replenishment in Bone Marrow Chimeric Mice. *J. Neuropathol. Exp. Neurol.* **69,** 896–909 (2010).

187. Nayak, D., Zinselmeyer, B. H., Corps, K. N. & McGavern, D. B. In vivo dynamics of innate immune sentinels in the CNS. *Intravital Print* **1,** 95–106 (2012).

188. Prodinger, C. *et al.* CD11c-expressing cells reside in the juxtavascular parenchyma and extend processes into the glia limitans of the mouse nervous system. *Acta Neuropathol. (Berl.)* **121,** 445–458 (2011).

189. Louveau, A. *et al.* Structural and functional features of central nervous system lymphatic vessels. *Nature* **523,** 337 (2015).

190. Kivisäkk, P. *et al.* Localizing CNS immune surveillance: Meningeal APCs activate T cells during EAE. *Ann. Neurol.* **65,** 457–469 (2009).

191. Bartholomäus, I. *et al.* Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* **462,** nature08478 (2009).

192. Pesic, M. *et al.* 2-photon imaging of phagocyte-mediated T cell activation in the CNS. *J. Clin. Invest.* **123,** 1192–1201 (2013).

193. Martin, W. D. *et al.* H2-M Mutant Mice Are Defective in the Peptide Loading of Class II Molecules, Antigen Presentation, and T Cell Repertoire Selection. *Cell* **84,** 543–550 (1996).

194. Anderson, D. A. *et al.* Revisiting the specificity of the MHC class II transactivator CIITA in classical murine dendritic cells in vivo. *Eur. J. Immunol.* **47,** 1317–1323 (2017).

195. LeibundGut-Landmann, S., Waldburger, J.-M., e Sousa, C. R., Acha-Orbea, H. & Reith, W. MHC class II expression is differentially regulated in plasmacytoid and conventional dendritic cells. *Nat. Immunol.* **5,** 899–908 (2004).

196. Muhlethaler-Mottet, A., Otten, L. A., Steimle, V. & Mach, B. Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *EMBO J.* **16,** 2851–2860 (1997).

197. Lennon, A. M. *et al.* Isolation of a B-cell-specific promoter for the human class II transactivator. *Immunogenetics* **45,** 266–273 (1997).

198. Merad, M., Sathe, P., Helft, J., Miller, J. & Mortha, A. The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. *Annu. Rev. Immunol.* **31,** (2013).

199. Jang, M. H. *et al.* CCR7 Is Critically Important for Migration of Dendritic Cells in Intestinal Lamina Propria to Mesenteric Lymph Nodes. *J. Immunol.* **176,** 803–810 (2006).

200. McKerracher, L. & Rosen, K. M. MAG, myelin and overcoming growth inhibition in the CNS. *Front. Mol. Neurosci.* **8,** (2015).

201. Takahashi, K., Prinz, M., Stagi, M., Chechneva, O. & Neumann, H. TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med.* **4,** e124 (2007).

202. Cross, A. H. *et al.* Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J. Clin. Invest.* **93,** 2684–2690 (1994).

203. Niedbala, W. *et al.* Regulation of type 17 helper T-cell function by nitric oxide during inflammation. *Proc. Natl. Acad. Sci.* **108,** 9220–9225 (2011).

204. Willenborg, D. O., Staykova, M. A. & Cowden, W. B. Our shifting understanding of the role of nitric oxide in autoimmune encephalomyelitis: a review. *J. Neuroimmunol.* **100,** 21–35 (1999).

205. Xu, L. *et al.* Arginase and autoimmune inflammation in the central nervous system. *Immunology* **110,** 141–148 (2003).

206. Kaplan, M. H., Schindler, U., Smiley, S. T. & Grusby, M. J. Stat6 Is Required for Mediating Responses to IL-4 and for the Development of Th2 Cells. *Immunity* **4,** 313–319 (1996).

207. Ohmori, Y. & Hamilton, T. A. STAT6 Is Required for the Anti-inflammatory Activity of Interleukin-4 in Mouse Peritoneal Macrophages. *J. Biol. Chem.* **273,** 29202–29209 (1998).

208. Davies, A. L. *et al.* Neurological deficits caused by tissue hypoxia in neuroinflammatory disease. *Ann. Neurol.* **74,** 815–825 (2013).

209. Greer, S. N., Metcalf, J. L., Wang, Y. & Ohh, M. The updated biology of hypoxia-inducible factor: The updated biology of HIF. *EMBO J.* **31,** 2448–2460 (2012).

210. Le Moan, N. *et al.* Hypoxia Inducible Factor-1α in Astrocytes and/or Myeloid Cells Is Not Required for the Development of Autoimmune Demyelinating Disease,,. *eNeuro* **2,** (2015).

211. Constantinescu, C. S., Farooqi, N., O'Brien, K. & Gran, B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br. J. Pharmacol.* **164,** 1079–1106 (2011).

212. Anderson, M. S. & Bluestone, J. A. The NOD mouse: a model of immune dysregulation. *Annu. Rev. Immunol.* **23,** 447–485 (2005).

213. Basso, A. S. *et al.* Reversal of axonal loss and disability in a mouse model of progressive multiple sclerosis. *J. Clin. Invest.* **118,** 1532–1543 (2008).

214. Gajofatto, A. & Benedetti, M. D. Treatment strategies for multiple sclerosis: When to start, when to change, when to stop? *World J. Clin. Cases WJCC* **3,** 545–555 (2015).

215. Collin, M., McGovern, N. & Haniffa, M. Human dendritic cell subsets. *Immunology* **140,** 22–30 (2013).

216. Tamoutounour, S. *et al.* Origins and Functional Specialization of Macrophages and of Conventional and Monocyte-Derived Dendritic Cells in Mouse Skin. *Immunity* **39,** 925–938 (2013).

217. Schrøder, M. *et al.* CD1c-Expression by Monocytes – Implications for the Use of Commercial CD1c+ Dendritic Cell Isolation Kits. *PLoS ONE* **11,** (2016).

218. Helft, J. *et al.* GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c+MHCII+ Macrophages and Dendritic Cells. *Immunity* **42,** 1197–1211 (2015).