Myeloid Cell Plasticity in the Evolution of Central Nervous System Autoimmunity

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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 proinflammatory, alternatively polarized subsets can serve noninflammatory 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 inducible nitric oxide synthase (iNOS), indicative of a proinflammatory 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 proinflammatory markers to expression of noninflammatory 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 proinflammatory to a noninflammatory phenotype.

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Myeloid cells, including macrophages (MΦs) and dendritic cells (DCs), are a major component of white matter lesions in multiple sclerosis (MS) and the animal model experimental autoimmune encephalomyelitis (EAE).[1,2] Our laboratory and others have established a critical role of myeloid cells in early EAE pathogenesis.[3–6] Myeloid cells may serve as antigen-presenting cells for reactivation of myelin-specific $CD4^+$ T cells,[7,8] secrete cytokines such as IL-6, IL-1β, and TNFα,[9] and directly inflict damage through release of toxic factors such as reactive oxygen species generated by inducible nitric oxide synthase (iNOS).[10,11] iNOS-expressing myeloid cells are often described as classically activated, and considered "proinflammatory," based on their similarity to bone marrow–derived macrophages (BMDMs) that were polarized with lipopolysaccharide or interferon-γ (IFNγ) in vitro.[12] It is generally thought that classically activated myeloid cells (CAMCs) are predominant in active MS and EAE lesions, where they act as pathogenic effector cells.[13–15] Early studies of autopsied MS brains revealed iNOS-immunoreactive macrophages in active lesions.[14,16,17] 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

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spectrum of lineages with diverse, and even opposing, properties.[18–20] 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 "proregenerative" properties. Arg1/CD206-expressing myeloid cells play a critical role in wound healing.[21] They are frequently classified as alternatively activated based on their similarity to BMDMs generated in vitro by polarization with IL-4 or IL-13 via a STAT6-dependent pathway.[12,22] Alternatively activated myeloid cells (AAMCs) may regulate the inflammatory environment by secreting IL-10 and/or TGFβ1,[9] while promoting tissue regeneration by clearing debris[23,24] and secreting growth factors.[25] 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.[2,19,25,26] Primary human macrophages acquire a foamy morphology and produce immunosuppressive factors following ingestion of myelin in vitro.[19]

We questioned whether CNS myeloid cells evolve during disease progression and shift from a proinflammatory phenotype at onset to a noninflammatory or immunosuppressive state in anticipation of clinical remission/stabilization. Consistent with this hypothesis, CNS-infiltrating DCs were found to upregulate the AAMC-associated genes *Arg1, Chi3l3*, and *Ms4a8a* at the peak of EAE, shortly prior to remission.[27] Arg1 is the most significantly upregulated gene in the CNS at peak EAE.[28] Adoptive transfer of AAMC-polarized macrophages or microglia can ameliorate EAE,[29,30] and the therapeutic effects of estrogen, glatiramer acetate, and other agents in EAE were found to correlate with the expansion of AAMCs in the periphery and/or CNS.[31–34] Less is known about endogenous AAMCs that spontaneously accumulate in the CNS during the course of EAE or MS. In the current paper, we compare the spatial distribution of AAMCs 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.

Materials and Methods

Mice

C57Bl/6 and B6.Ly5.1 mice were from Charles River Laboratories (Wilmington, MA). Arg1-eYFP,[35] Rosa-LSL-eYFP, 2D2 TCR transgenic, and STAT6$^{-/-}$ mice were from Jackson Laboratory (Bar Harbor, ME). iNOS-TdTomato-Cre[36] mice were from the European Mouse Mutant Archive (INFRAFRONTIER, Neuherberg/Munich, Germany). SJL mice were from Harlan Laboratories (Horst, the Netherlands). Both male and female mice, age 6 to 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 institutional animal care and use committee–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} (Biosynthesis Lewisville, TX) in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Mice were injected intraperitoneally with 300ng pertussis toxin (List Biological Laboratories, Campbell, CA) 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 to 14 days postimmunization. Lymph node cells were cultured for 96 hours in the presence of 50 μg/ml MOG_{35-55}, 8ng/ml IL-23 (R&D Systems, Minneapolis, MN), 10ng/ml IL-1α (PeproTech, Rocky Hill, NJ), and 10 μg/ml anti-IFNγ (clone XMG1.2; Bio X Cell, West Lebanon, NH). At the end of culture, $CD4^+$ T cells were purified with CD4-positive selection magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and 3 to 5×10^6 $CD4^+$ T cells were transferred intraperitoneally into naive 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 adjuvant (Difco Laboratories) without pertussis toxin. EAE was assessed by a clinical score of disability: 1, limp tail; 2, hindlimb weakness; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; and 5, moribund state.

Mixed Bone Marrow Chimeras

B6.Ly5.1 ($CD45.1^+$) congenic hosts were lethally irradiated with 1300 Rad split into 2 doses and reconstituted by tail vein injection of 4×10^6 bone marrow cells. Cells were a 50:50 mix of B6.Ly5.1 wild-type (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 phosphate-buffered saline (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 18Ga needle and syringe in a solution containing 1mg/ml collagenase A (Roche, Basel, Switzerland) and 1mg/ml DNase 1 (Sigma-Aldrich, St Louis, MO) in Hank balanced salt solution and incubated at 37 °C for 20 minutes. Mononuclear

cells were separated from myelin over a 27% Percoll gradient (GE Healthcare, Chicago, IL).

Flow Cytometry

Mononuclear cells were labeled with fixable viability dye (eFluor506 or eFluor780; eBioscience, San Diego, CA), 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 (Franklin Lakes, NJ). 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), and CD86 (clone GL1). Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PeCy7, allophycocyanin (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) followed by an Alexa Fluor 647–conjugated donkey antisheep secondary antibody (Thermo Fisher Scientific, Waltham, MA). Data were acquired using a FACSCanto II flow cytometer or FACSAria III flow sorter (BD Biosciences) and analyzed with FlowJo software (Tree Star, San Carlos, CA). Cells were sorted with a FACSAria III flow sorter (BD Biosciences).

Quantitative Real-Time Polymerase Chain Reaction

Sorted cells were resuspended in RLT buffer, and RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated by reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on an iQ Thermocycler (Bio-Rad Laboratories, Hercules, CA) 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 naive $CD4^+$ T cells, lymph nodes and spleen were collected from naive 2D2 TCR transgenic mice, and $CD4^+$ T cells were enriched by positive selection with magnetic beads (Miltenyi Biotec). To further isolate naive 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 $CD45^+CD11b^-CD3^+CD4^+MHC\text{-}II^-$ T cells. T cells were labeled with carboxy fluorescein succinimidyl ester according the manufacturer's instructions (Thermo Fisher Scientific). Myeloid cell populations were sorted with markers as indicated in the text. Myeloid cells and T cells were cocultured 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 ($MOG_{35\text{-}55}$ peptide [Biosynthesis]).

Multiplex Immunoassays

Cell culture supernatants were analyzed with the Milliplex Mouse Th17 Cytokine Panel (EMD Millipore, Billerica, MA) using the Bio-Plex 200 System (Bio-Rad Laboratories). 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.

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.[37] 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. Postmortem intervals were between 5.5 and 24 hours. Brain tissue was fixed with 10% formalin for 2 to 4 weeks and embedded in paraffin. Formalin-fixed, paraffin-embedded sections were cut, quenched with hydrogen peroxide, 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, Burlingame, CA), and counterstained with hematoxylin. The following antibodies were used in this study: MBP (rabbit polyclonal, Dako [Carpinteria, CA] A0623), CD68 (mouse monoclonal, Dako M0876), iNOS (rabbit polyclonal, Novus Biologicals [Littleton, CO] NB 120-15203), CD206 (mouse polyclonal, Abcam [Cambridge, MA] ab117644), and Iba-1 (goat polyclonal, Abcam ab5076). Adequate controls using isotype control antibodies were performed with each primary antibody. Bright-field images were acquired with a Leica (Wetzlar, Germany) DM5000 B microscope using a Leica color camera DFC310 Fx and the Leica Application Suite (v4.2.0) imaging software. For fluorescence images, sections were incubated with fluorescent-labeled secondary antibodies and subsequently treated with 0.7% Sudan Black in ethanol and $CuSO_4$ to quench autofluorescence. Sections were counterstained with 4,6-diamidino-2-phenylindole and mounted with VectaShield mounting medium (VectaShield Kit, Vector Laboratories). Images were acquired with an UltraVIEW VoX spinning disc confocal microscope (Perkin Elmer, Waltham, MA) and a Ti-E Eclipse inverted microscope (Nikon, Tokyo, Japan). Image acquisition, visualization, and quantification were performed using the Volocity 6.3 software (Improvision, Waltham, MA). Images were processed with the ImageJ software (NIH, Bethesda, MD). For 3-dimensional 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,

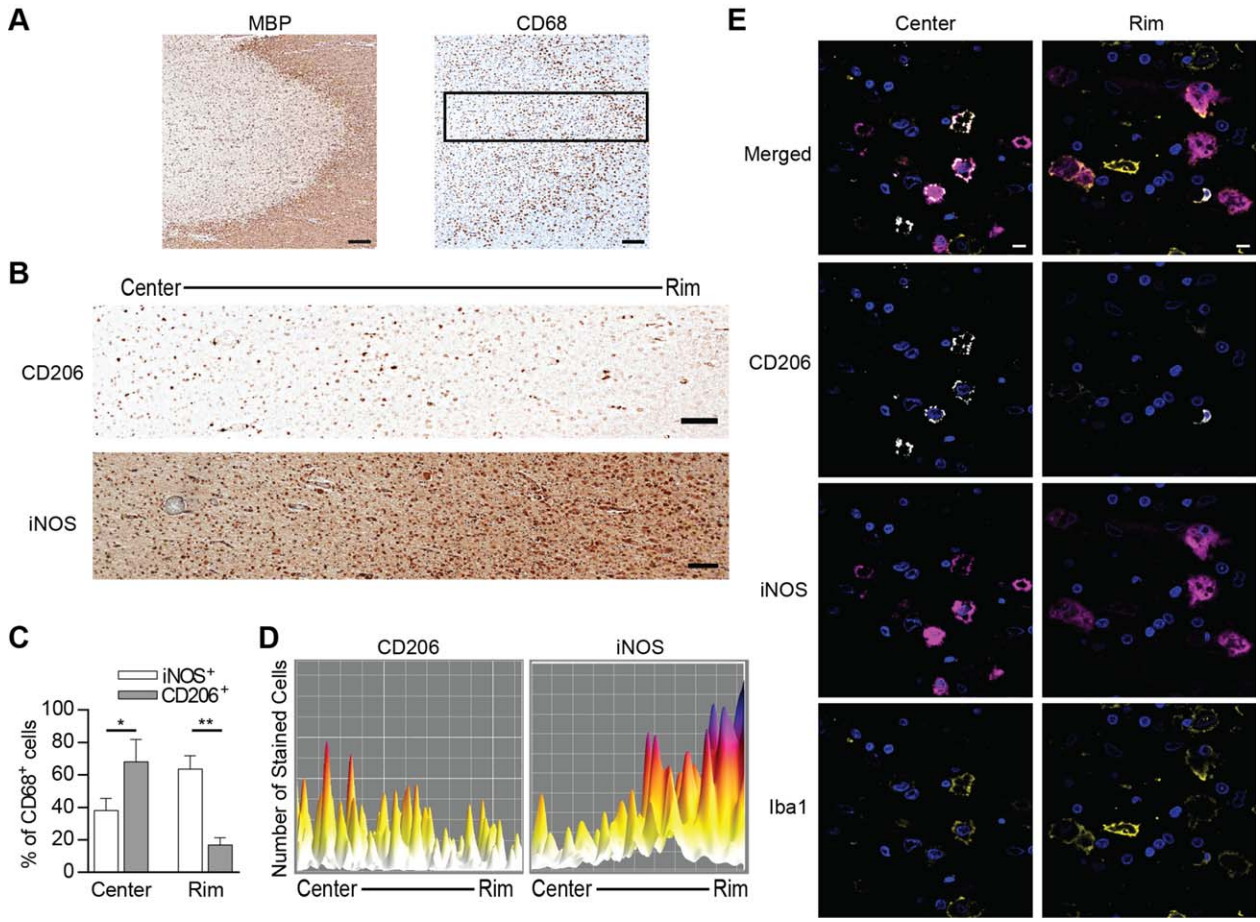

FIGURE 1: CD206$^+$ and iNOS$^+$ macrophages are distributed from the center to the rim of active multiple sclerosis (MS) lesions. (A) Chronic active MS lesion immunolabeled for myelin basic protein (MBP) and CD68. (B) Cross sections of the same lesion, extending from the lesion center to the lesion rim, immunolabeled for CD206 and iNOS. (C) Quantification of iNOS$^+$ and CD206$^+$ cells at the lesion center and lesion rim. The frequency 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 *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).

and the resulting image was processed with the Interactive Surface Plot v2.4 ImageJ plugin.

Statistics

Statistical analysis was performed using paired or unpaired, 2-tailed Student *t* test, as indicated in the figure legends.

Results

Macrophage Polarization Shifts from the Core to the Rim of MS Lesions

To characterize the polarization state of CD68$^+$ macrophage in chronic active MS lesions, we immunolabeled brain tissue sections from 4 patients (Table) with antibodies against iNOS and CD206, the standard markers of classically and alternatively activated human myeloid cells, respectively.[2,14,38,39] 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.[37] We found that a high percentage of CD68$^+$ macrophages at the lesion rim were iNOS$^+$, whereas relatively few macrophages expressed CD206 (see Fig 1A–D). Conversely, the percentage 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 (see Fig 1E). Consistent with our results, Vogel and colleagues discovered foamy macrophages and activated microglia in a panel of active MS lesions that consistently coexpressed AAMC and CAMC markers.[2]

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

TABLE. Clinical Data of MS Patients Included in This Study

Case	Age, yr	Gender	Disease Course	Disease Duration, yr	Postmortem Interval, h
1	42	M	RRMS	20	5.5
2	37	M	RRMS	15	24
3	50	F	RRMS	10	6
4	63	F	SPMS	Unknown	8

F = female; M = male; MS = multiple sclerosis; RRMS = relapsing–remitting MS; SPMS = secondary progressive MS.

myeloid cells that accumulate in the CNS do not convincingly express CD206 at onset, peak, or late time points of EAE, but a subset of splenic myeloid cells inconsistently expressed CD206 at high levels (data not shown). Because Arg1 is commonly used to identify murine AAMCs,[12] we measured expression of iNOS and Arg1 at serial time points following active immunization with myelin peptide. We detected iNOS, but not Arg1, in mononuclear cells isolated from the brain or spinal cord at clinical onset (Fig 2A). In contrast, at peak disease we detected Arg1, but not iNOS, in the CNS mononuclear 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 (see Fig 2B). The $CD45^{hi}CD11b^{+}$ myeloid subset was further divided into $Ly6G^{+}$ neutrophils, $CD11c^{-}$ monocytes/MΦs, and $CD11c^{+}$ DCs. Arg1 was expressed by a significant percentage of the infiltrating MΦs and DCs, but not by neutrophils. Similarly, among the CNS mononuclear cells isolated at clinical onset, only microglia, MΦs and DCs expressed iNOS. (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).

We sorted $Arg1^{+}$ and $Arg1^{-}$ MΦs ($CD45^{hi}$ $CD11b^{+}Ly6G^{-}CD11c^{-}$) or DCs ($CD45^{hi}CD11b^{+}$ $Ly6G^{-}CD11c^{+}$) from the CNS of Arg1-eYFP reporter mice[35] during peak stage EAE, and we measured the expression of candidate genes by qRT-PCR. Genes associated with *in vitro* generated AAMCs, including *Ym1 and Mrc1*, were enriched in $Arg1^{+}$ CNS DCs and MΦs, compared with their $Arg1^{-}$ counterparts, whereas CAMC-related genes (*iNOS*, *IL-1β*, *IL-6*) were unchanged or reduced (see Fig 2C). The genetic profiles of $Arg1^{+}$ and $Arg1^{-}$ CNS myeloid cells did not precisely mirror those of in vitro generated AAMCs and CAMCs, respectively. For example, mRNA encoding the AAMC-associated cytokines IL-10 and TGFβ1 were expressed at similar levels by $Arg1^{-}$ 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,[40] 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.[41] 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 (see Fig 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 complete Freund adjuvant and pertussis toxin, which could directly modulate innate immune cells. 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. Naive 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

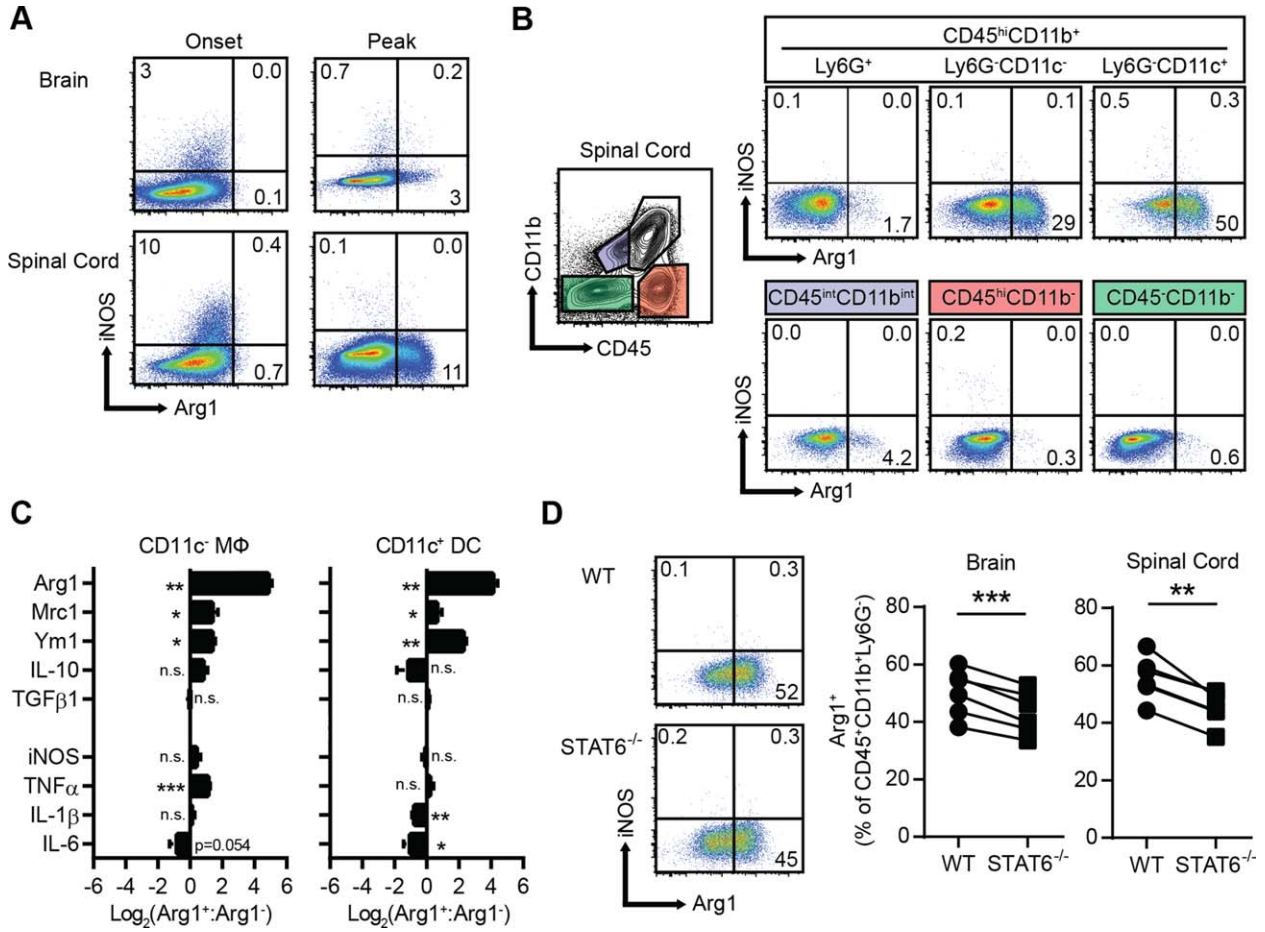

FIGURE 2: Arg1 expression defines a distinct myeloid cell population that accumulates in the central nervous system (CNS) during later stages of experimental autoimmune encephalomyelitis (EAE). (A, B) EAE was induced in mice by active immunization with myelin peptide. Inflammatory cells were isolated from the CNS at the onset or peak of clinical disability. Representative intracellular staining of Arg1 and iNOS in CNS immune cells from wild-type (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 at peak EAE. M1- and M2-related transcripts were measured by quantitative real-time polymerase chain reaction. 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 and analyzed by flow cytometry. Representative dot plots and quantification of intracellular staining of Arg1 and iNOS are shown in WT or $STAT6^{-/-}$ cells, gated on the $CD45^{hi}CD11b^+Ly6G^-$ population. Percentages are of $Arg1^+$ 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 *t* test. Data are representative of at least 2 experiments; n = 3–5 mice per group. All values are mean ± standard error of the mean. DC = dendritic cells; MΦ = macrophages.

onset, whereas $iNOS^-Arg1^+$ cells were prominent during peak and late disease (Fig 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 MΦs and DCs, as well as in $CD45^{int}CD11b^{int}$ resident microglia (see Fig 3B).

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 had accumulated by the onset of the first exacerbation, contracted during peak disease through remission, and rebounded at the onset of relapse (see Fig 3C–E). In contrast, an $iNOS^-Arg1^+$ subset expanded between clinical onset and peak of the first episode of EAE, waned during remission, and reemerged 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

Zhu and colleagues described immunosuppressive $Ly6C^{hi}$ monocytes that expand in the periphery, accumulate in

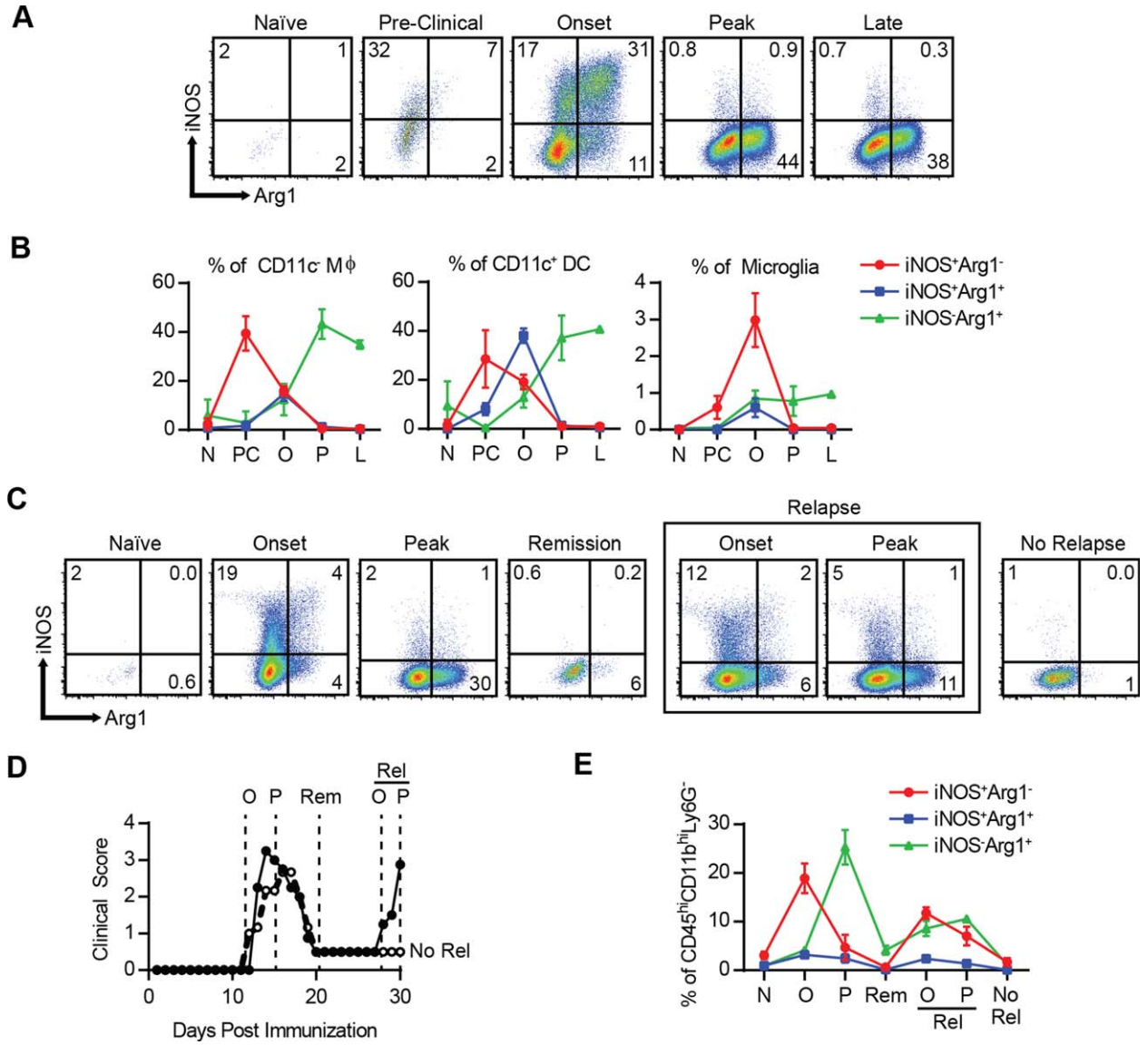

FIGURE 3: Myeloid cells shift from iNOS to Arg1 expression in adoptive transfer and relapsing models of experimental autoimmune encephalomyelitis (EAE). (A, B) EAE was induced by adoptive transfer of wild-type myelin-primed Th17 cells into naive syngeneic hosts. Central nervous system (CNS) inflammatory 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 $CD45^{hi}CD11b^{+}CD11c^{-}$ macrophages (MΦ), $CD45^{hi}CD11b^{+}CD11c^{+}$ dendritic cells (DC), and $CD45^{mid}CD11b^{+}CD11c^{-}$ microglia expressing iNOS and/or Arg1 in naive mice (N) and in mice with EAE during the preclinical (PC), onset (O), peak (P), and late (L) stages of disease. (C–E) EAE was induced in SJL mice by active immunization with $PLP_{139\text{-}151}$ 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 ± standard error of the mean.

the CNS during EAE, and have the potential to differentiate into either $iNOS^{+}$ or $Arg1^{+}$ cells upon ex vivo culture with different polarizing factors.[20] As shown in Figures 1 and 3, we detected myeloid cells in MS and EAE lesions that coexpress AAMC and CAMC markers. It is unclear whether these cells represent a distinct, stable lineage or an intermediate stage during the transition between polarized subsets. The experiments in Figure 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 labeled $iNOS^{+}$ cells and their descendants by crossing NOS2-TdTomato-Cre reporter mice[36] with Rosa26-LSL-eYFP reporter mice to generate iNOS fate-

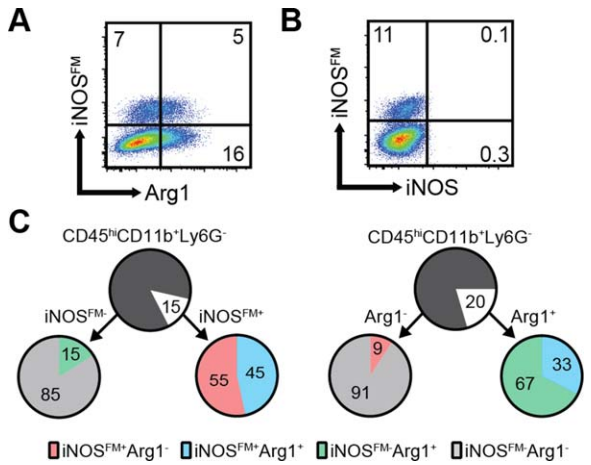

FIGURE 4: Arg1-expressing central nervous system (CNS) myeloid cells are derived, in part, from iNOS-expressing precursors. Experimental autoimmune encephalomyelitis was induced in mice by adoptive transfer of wild-type myelin-primed Th17 cells. CNS inflammatory cells were analyzed by flow cytometry. Inflammatory cells were isolated from the spinal cords of iNOS fate-mapping ($iNOS^{FM}$) 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.

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 ($iNOS^{FM+}$; Fig 4A, B). At this point, iNOS protein is no longer detectable (see Fig 4B). Approximately 45% of the $iNOS^{FM+}$ subset coexpressed Arg1 (see Fig 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, because ~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% vs 9%). These data demonstrate that Arg1-expressing cells may be derived either from iNOS-expressing cells or from iNOS-naive cells.

Arg1-Expressing CNS Myeloid Cells Are Inefficient Antigen-Presenting Cells

DCs have been identified as potent antigen-presenting cells in the CNS during EAE.[7,42] We sought to compare the antigen-presenting cell function of $Arg1^{+}$ versus $Arg1^{-}$ CNS DCs by measuring the ability of each subset to promote myelin-specific T-cell proliferation ex vivo. Sorted CNS DC subsets were cocultured with naive 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 DCs were inefficient in promoting the proliferation of, or cytokine production by, myelin-specific T cells (Fig 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. 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 cocultured them with $Arg1^{+}$ or $Arg1^{-}$ CNS DCs. Similar to naive 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 DCs (see Fig 5C, D). The poor antigen-presenting cell capacity of $Arg1^{+}$ CNS DCs did not appear to be due to low expression of MHC-II or costimulatory molecules (see Fig 5E, F). Essentially all of the $Arg1^{+}$ and $Arg1^{-}$ CNS DCs expressed MHC-II, though $Arg1^{+}$ cells expressed a slightly lower level than their $Arg1^{-}$ counterparts. $Arg1^{+}$ CNS DCs expressed similar levels of the costimulatory marker CD40 and slightly higher levels of CD80 and CD86 compared with $Arg1^{-}$ CNS DCs. Arginase enzyme activity has been shown to suppress T-cell proliferation in other experimental systems[43,44]; however, inhibition of arginase during cocultures of T cells with $Arg1^{+}$ CNS myeloid cells did not rescue T-cell activation (data not shown).

We next investigated whether $Arg1^{+}$ CNS myeloid cells are immunosuppressive or simply incompetent antigen-presenting cells. $Arg1^{+}$ CNS myeloid cells did not induce 2D2 T cells to upregulate FoxP3 (data not shown) or to produce IL-10 (see Fig 5B, D). Furthermore, the addition of $Arg1^{+}$ CNS myeloid cells to cocultures 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 antigen-presenting cells but do not actively block T-cell activation by competent antigen-presenting cells.

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 that is deficient in the capacity to activate myelin-specific T cells. The appearance of $Arg1^{+}$ myeloid cells

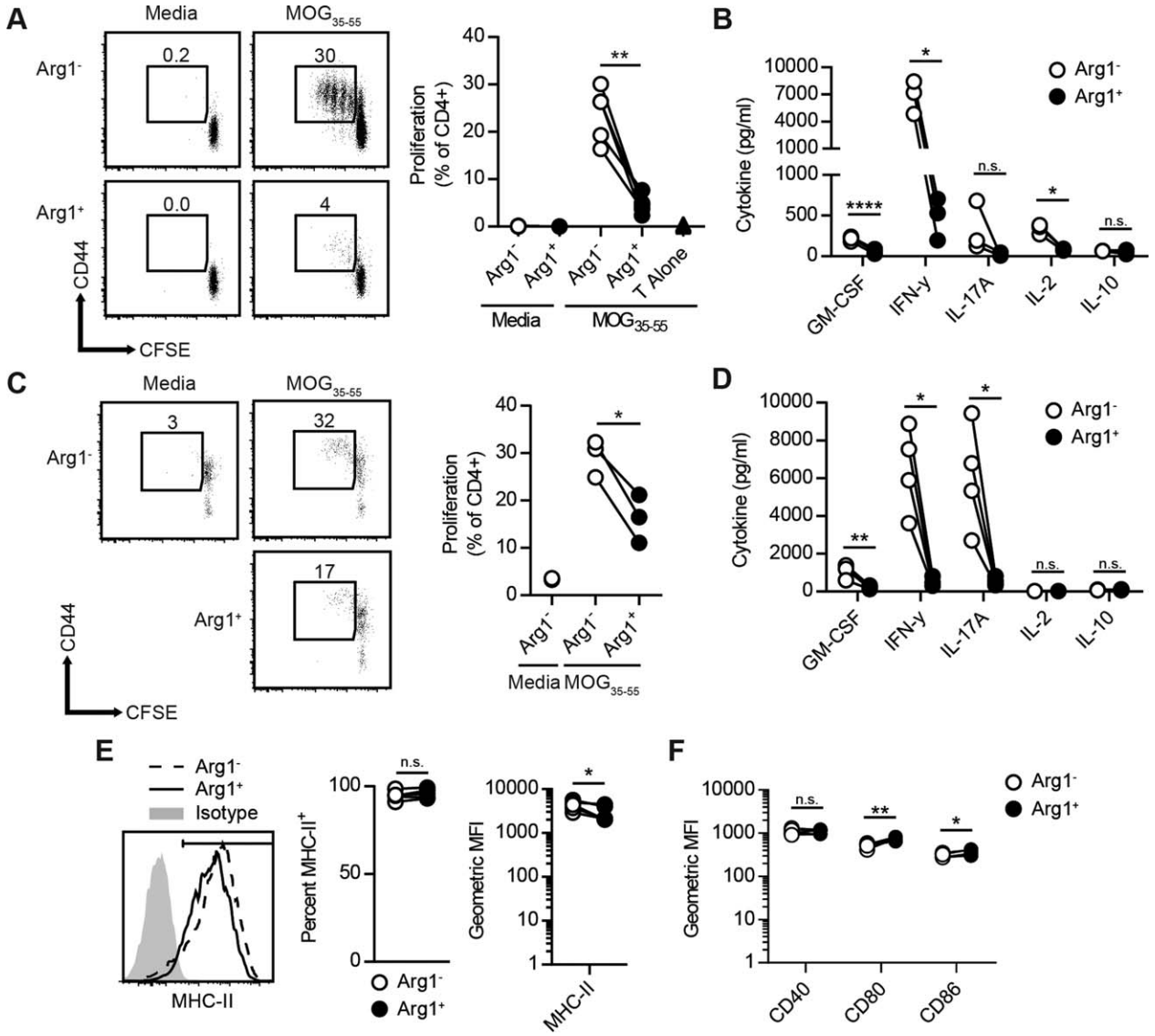

FIGURE 5: Arg1$^+$ cells are deficient at antigen presentation. Experimental autoimmune encephalomyelitis (EAE) was induced in Arg1-eYFP reporter mice by active immunization with myelin peptide, and immune cells were isolated from the central nervous system (CNS) at peak disease. Arg1$^+$ and Arg1$^-$ CD45^{hi}CD11b$^+$Ly6G$^-$CD11c$^+$ myeloid cells were flow sorted and cocultured with myelin oligodendrocyte glycoprotein (MOG)-reactive T cells in the presence of myelin peptide (MOG_{35-55}). (A, B) CNS myeloid cell subsets were cocultured with CD44$^-$CD62L$^+$ CD4$^+$ T cells from naive 2D2 TCR transgenic mice. (C, D) CNS myeloid subsets were cocultured with CD4$^+$ T cells isolated from the CNS of actively immunized wild-type mice at the peak of EAE. Graphs show (A, C) proliferation measured by carboxy fluorescein succinimidyl ester dilution and (B, D) cytokine levels in culture supernatants, measured by multiplex bead assay. (E, F) MHC-II and costimulatory marker expression were measured by flow cytometry on Arg1$^+$ and Arg1$^-$ CD45^{hi}CD11b$^+$Ly6G$^-$CD11c$^+$ myeloid cells, isolated from the spinal cord at the peak of disease. (E) MHC-II expression quantified as both a percentage of DCs that were positive, and by geometric mean fluorescence intensity (MFI). (F) Costimulatory marker expression quantified by geometric MFI. *$p<0.05$, **$p<0.01$, ****$p<0.0001$, and n.s. = $p>0.05$, by paired, 2-tailed Student *t* test. Data are representative of at least 2 experiments; n = 3–5 mice per group. CFSE = carboxy fluorescein succinimidyl ester; GM-CSF = granulocyte-macrophage colony–stimulating factor.

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$^+$ DCs were not immunosuppressive in the sense that they did not actively inhibit 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 proregenerative 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 AAMCs may play a role in ameliorating the clinical course by clearing debris[23,24] and/or releasing trophic factors.[25]

Arg1$^+$ CNS myeloid cells differ from in vitro polarized AAMCs 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 hypoxia-inducible factor 1α and peroxisome proliferator-activated receptor γ, which have been shown to promote AAMC differentiation in other experimental systems.[45,46] Alternatively, studies with primary human macrophages suggest that ingestion of myelin could trigger their development.[19] 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 CAMCs and AAMCs 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[2] that myeloid cells in MS lesions also coexpress CAMC and AAMC markers raises the possibility that they undergo a dynamic transformation from a proinflammatory to a noninflammatory state. Such a transformation is further suggested by the increasing gradient of iNOS versus CD206 expression in macrophages extending from the quiescent center to the active rim of chronic active MS lesions.

Second generation disease-modifying therapies (DMTs) in MS suppress clinical exacerbations by depleting peripheral lymphocytes or blocking their trafficking to the CNS. Although these reagents are highly effective in many individuals with relapsing–remitting MS, they are not cures, with up to 30% nonresponse rates. Furthermore, DMTs currently used in the clinic are generally ineffective in progressive forms of MS. Myeloid cells and related factors have been underutilized as candidate therapeutic targets in MS clinical trials. This is particularly relevant to secondary progressive MS, because 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.[47–50] 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 noninflammatory phenotype.

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Author Contributions

D.A.G., P.C.D., and B.M.S. contributed to study concept and design. D.A.G., J.M.W.-S., S.D., G.P., and D.P. contributed to data acquisition and analysis. D.A.G. and B.M.S. contributed to drafting the manuscript and figures.

Potential Conflicts of Interest

Nothing to report.

References

1. Sobel RA, Blanchette BW, Bhan AK, Colvin RB. The immunopathology of experimental allergic encephalomyelitis. I. Quantitative analysis of inflammatory cells in situ. J Immunol 1984;132:2393–2401.
2. Vogel DY, Vereyken EJ, Glim JE, et al. Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. J Neuroinflammation 2013;10:35.
3. Deshpande P, King IL, Segal BM. 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 2007;178:6695–6699.
4. King IL, Dickendesher TL, Segal BM. Circulating Ly-6C+myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. Blood 2009;113:3190–3197.
5. Ajami B, Bennett JL, Krieger C, et al. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci 2011;14:1142–1149.
6. Fife BT, Huffnagle GB, Kuziel WA, Karpus WJ. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. J Exp Med 2000;192:899–906.
7. Greter M, Heppner FL, Lemos MP, et al. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nat Med 2005;11:328–334.
8. Katz-Levy Y, Neville KL, Girvin AM, et al. Endogenous presentation of self myelin epitopes by CNS-resident APCs in Theiler's virus–infected mice. J Clin Invest 1999;104:599–610.
9. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol 2014;5:491.
10. Cross AH, Keeling RM, Goorha S, et al. Inducible nitric oxide synthase gene expression and enzyme activity correlate with disease activity in murine experimental autoimmune encephalomyelitis. J Neuroimmunol 1996;71:145–153.
11. Li S, Vana AC, Ribeiro R, Zhang Y. Distinct role of nitric oxide and peroxynitrite in mediating oligodendrocyte toxicity in culture and

in experimental autoimmune encephalomyelitis. Neuroscience 2011;184:107–119.

12. Mills CD, Kincaid K, Alt JM, et al. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol 2000;164:6166–6173.
13. Wingerchuk DM, Lucchinetti CF, Noseworthy JH. Multiple sclerosis: current pathophysiological concepts. Lab Invest 2001;81:263–281.
14. De Groot CJ, Ruuls SR, Theeuwes JW, et al. Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. J Neuropathol Exp Neurol 1997;56:10–20.
15. Nikić I, Merkler D, Sorbara C, et al. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. Nat Med 2011;17:495–499.
16. Oleszak EL, Zaczynska E, Bhattacharjee M, 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 1998;5:438–445.
17. Hill KE, Zollinger LV, Watt HE, et al. Inducible nitric oxide synthase in chronic active multiple sclerosis plaques: distribution, cellular expression and association with myelin damage. J Neuroimmunol 2004;151:171–179.
18. Fan X, Zhang H, Cheng Y, et al. Double roles of macrophages in human neuroimmune diseases and their animal models. Mediators Inflamm 2016;2016:e8489251.
19. Boven LA, Meurs MV, Zwam MV, et al. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain 2006;129:517–526.
20. Zhu B, Bando Y, Xiao S, et al. CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. J Immunol 2007;179:5228–5237.
21. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol 2009;158:638–651.
22. 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 1992;176:287–292.
23. Kotter MR, Zhao C, van Rooijen N, Franklin RJM. Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. Neurobiol Dis 2005;18:166–175.
24. Copelman CA, Diemel LT, Gveric D, et al. Myelin phagocytosis and remyelination of macrophage-enriched central nervous system aggregate cultures. J Neurosci Res 2001;66:1173–1178.
25. Miron VE, Boyd A, Zhao J-W, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci 2013;16:1211–1218.
26. Zhang Z, Zhang Z-Y, Schittenhelm J, et al. Parenchymal accumulation of CD163 + macrophages/microglia in multiple sclerosis brains. J Neuroimmunol 2011;237:73–79.
27. Wasser B, Pramanik G, Hess M, et al. Increase of alternatively activated antigen presenting cells in active experimental autoimmune encephalomyelitis. J Neuroimmune Pharmacol 2016;11:721–732.
28. Xu L, Hilliard B, Carmody RJ, et al. Arginase and autoimmune inflammation in the central nervous system. Immunology 2003; 110:141–148.
29. Zhang X-M, Lund H, Mia S, et al. Adoptive transfer of cytokine-induced immunomodulatory adult microglia attenuates experimental autoimmune encephalomyelitis in DBA/1 mice. Glia 2014; 62:804–817.
30. Mikita J, Dubourdieu-Cassagno N, Deloire MS, 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 2011; 17:2–15.
31. Benedek G, Zhang J, Bodhankar S, et al. Estrogen induces multiple regulatory B cell subtypes and promotes M2 microglia and neuroprotection during experimental autoimmune encephalomyelitis. J Neuroimmunol 2016;293:45–53.
32. Liu C, Li Y, Yu J, et al. Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. PLoS One 2013;8:e54841.
33. Weber MS, Prod'homme T, Youssef S, et al. Type II monocytes modulate T cell–mediated central nervous system autoimmune disease. Nat Med 2007;13:935–943.
34. Kong W, Hooper KM, 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 2016;53:59–71.
35. Reese TA, Liang H-E, Tager AM, et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature 2007;447:92–96.
36. Béchade C, Colasse S, Diana MA, et al. NOS2 expression is restricted to neurons in the healthy brain but is triggered in microglia upon inflammation. Glia 2014;62:956–963.
37. Bö L, Mörk S, Kong PA, 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 1994;51:135–146.
38. Mehta V, Pei W, Yang G, et al. Iron is a sensitive biomarker for inflammation in multiple sclerosis lesions. PLoS One 2013;8: e57573.
39. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat Rev Immunol 2011;11:750–761.
40. Hsieh CL, Kim CC, Ryba BE, et al. Traumatic brain injury induces macrophage subsets in the brain. Eur J Immunol 2013;43:2010–2022.
41. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM Jr. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPβ. Gene 2005;353:98–106.
42. Bailey SL, Schreiner B, McMahon EJ, Miller SD. CNS myeloid DCs presenting endogenous myelin peptides "preferentially" polarize CD4 + TH-17 cells in relapsing EAE. Nat Immunol 2007;8:172–180.
43. Rodriguez PC, Zea AH, Culotta KS, et al. Regulation of T cell receptor CD3ζ chain expression byl-arginine. J Biol Chem 2002; 277:21123–21129.
44. Rodriguez PC, Zea AH, DeSalvo J, et al. l-Arginine consumption by macrophages modulates the expression of CD3ζ chain in T lymphocytes. J Immunol 2003;171:1232–1239.
45. Colegio OR, Chu N-Q, Szabo AL, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. Nature 2014;513:559–563.
46. Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, et al. Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. Nature 2007;447:1116–1120.
47. Karni A, Abraham M, Monsonego 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 2006;177:4196–4202.
48. Segal BM. Stage-specific immune dysregulation in multiple sclerosis. J Interferon Cytokine Res 2014;34:633–640.
49. Huber AK, Wang L, Han P, et al. Dysregulation of the IL-23/IL-17 axis and myeloid factors in secondary progressive MS. Neurology 2014;83:1500–1507.
50. Huber AK, Giles DA, Segal BM, Irani DN. An emerging role for eotaxins in neurodegenerative disease. Clin Immunol (in press).