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Th1 Cell Diversity in Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis

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Multiple sclerosis (MS) is believed to be initiated by myelin-reactive CD4+ Th cells. IL-12–polarized Th1 cells, IL-23–polarized Th17 cells, and Th17 cells that acquire Th1 characteristics were each implicated in autoimmune pathogenesis. It is debated whether Th cells that can drive the development of demyelinating lesions are phenotypically diverse or arise from a single lineage. In the current study, we assessed the requirement of IL-12 or IL-23 stimulation, as well as Th plasticity, for the differentiation of T cells capable of inducing CNS axon damage. We found that stable murine Th1 and Th17 cells independently transfer experimental autoimmune encephalomyelitis (widely used as an animal model of MS) in the absence of IL-23 and IL-12, respectively. Plastic Th17 cells are particularly potent mediators of demyelination and axonopathy. In parallel studies, we identified MS patients who consistently mount either IFN-γ– or IL-17–skewed responses to myelin basic protein over the course of a year. Brain magnetic resonance imaging revealed that patients with mixed IFN-γ and IL-17 responses have relatively high T1 lesion burden, a measure of permanent axon damage. Our data challenge the dogma that IL-23 and Th17 plasticity are universally required for the development of experimental autoimmune encephalomyelitis. This study definitively demonstrates that autoimmune demyelinating disease can be driven by distinct Th-polarizing factors and effector subsets, underscoring the importance of a customized approach to the pharmaceutical management of MS. The Journal of Immunology, 2015, 195: 2552–2559.

Multiple sclerosis (MS) is widely believed to be an autoimmune disease mediated by CD4+ T cells reactive against myelin Ags. Significant advances have been made in the development of immunomodulatory agents that decrease MS relapse rates. However, none of those agents target encephalitogenic T cells while sparing protective immune responses. An increased understanding of the factors that drive the differentiation and function of myelin-reactive T cells would help to guide the development of more refined therapeutic modalities. IFN-γ–producing CD4+ T cells of the Th1 lineage were initially thought to be the critical effector cells in MS and the animal model, experimental autoimmune encephalomyelitis (EAE) (1, 2). The putative role of Th1 cells in EAE was buttressed by the finding that in vitro stimulation of ordinarily innocuous myelin-reactive CD4+ T cells with the Th1-polarizing factor IL-12 could confer encephalitogenicity (3). Furthermore, C57BL/6 mice deficient in the Th1-associated transcription factor T-bet have a decreased incidence of EAE following immunization with an epitope of myelin oligodendrocyte glycoprotein (MOG)35–55 (4). However, a universal role for Th1 effectors in autoimmune demyelination was challenged by the discovery that IL-17–producing Th17 cells also accumulate in EAE and MS lesions and can transfer EAE (5, 6). Actively immunized C57BL/6 mice that are deficient in the Th17-polarizing factor IL-23 are completely EAE resistant, and those deficient in the Th17-associated transcription factor RORγt are partially resistant (7, 8).

In an attempt to reconcile these data, we (9) and other investigators (10) argued that EAE and MS are heterogeneous disorders and that the importance of specific leukocyte subsets and/or proinflammatory factors in disease development is context dependent. A link between Th17- and Th1-mediated autoimmunity was revealed by the demonstration that some Th17 cells are plastic and acquire Th1-like characteristics after several rounds of activation (11). These ex-Th17 cells downregulate IL-17 and RORγt and upregulate IFN-γ and T-bet. Fate-mapping experiments demonstrated that ex-Th17 cells make up the majority of CD4+ lymphocytes that infiltrate the CNS in MOG15–55-immunized C57BL/6 mice (12). Although this observation prompted some investigators to portray myelin-specific ex-Th17 cells as the critical effectors in EAE, the relative capacities of Th1, stable Th17, and plastic Th17 cells to induce demyelination and axonopathy have not been directly tested. In this study, we interrogated the contributions of IL-12 and IL-23 signaling, as well as Th plasticity, to the acquisition of encephalitogenic properties by myelin-reactive T cells. In parallel, we conducted a longitudinal study to investigate myelin-specific cytokine profiles of patients with MS.

Abbreviations used in this article: CAP, compound action potential; EAE, experimental autoimmune encephalomyelitis; KO, knockout; LN, lymph node; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; MS, multiple sclerosis; ON, optic neuritis; RGC, retinal ganglion cell; rm, recombinant mouse; WT, wild-type.

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Materials and Methods

Mice

Eight- to twelve-week-old C57BL/6 and CD45.1 congenic C57BL/6rsLy5.2/Cr mice were obtained from the National Cancer Institute at Frederick (Frederick, MD). C57BL/6 IL2p35cre mutant mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred in a vivarium at the University of Michigan. IL2p35cre mutant mice backcrossed on a C57BL/6 background were described previously (13). All mice were housed in microisolator cages under specific pathogen-free, barrier facility conditions.

Induction of EAE by adoptive transfer

Donor mice were anesthetized with trichloroethanol (Sigma) and injected s.c. with MOG35-55 (100 μg; Biosynthesis) emulsified in CFA containing 400 mg/ml heat-killed Mycobacterium tuberculosis (H37Ra; Difco). At 10–14 d postimmunization, a single-cell suspension was prepared from pooled draining inguinal, axillary, and brachial lymph nodes (LNs) and passed through a 70-μm cell strainer (BD Falcon). LN cells were cultured in vitro for 4 d with MOG35-55 under conditions favorable for the generation of Th1 cells (recombinant mouse [rm]IL-12, 6 ng/ml; rmIFN-γ, 2 ng/ml; anti–IL-4 [clone 11B11], 10 μg/ml) or Th17 cells (rmIL-1α, 10 ng/ml; rmIL-23, 8 ng/ml; anti–IL-4, 10 μg/ml; anti–IFN-γ [clone XMG1.2], 10 μg/ml). After 4 d of culture, LN cells were collected, washed, and injected into naive syngeneic recipients (2 × 10⁶ CD4+ T cells/mouse). Adoptive-transfer recipients were monitored daily for neurologic deficits and rated using the following criteria: 1, hypotonic and weak tail; 2, waddling gait and difficulty righting; 3, overt hindlimb weakness; 4, hindlimb paralysis; and 5, moribund.

Flow cytometry

Brains, spinal cords, and optic nerves were harvested at serial time points following the onset of neurologic deficits (1–2, 5–6, and 21–22 d after onset), homogenized in DNase (1 mg/ml) and collagenase A (2 mg/ml), and incubated for 30 min at 37°C. Mononuclear cells were isolated over a 50/50 Percoll gradient (GE Healthcare). Splenocytes were passed through a 70-μm cell strainer, lysed with ACK, and washed twice. For intracellular staining, cells were stimulated with PMA (50 ng/ml) and ionomycin (2 μg/ml) for 6 h. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin prior to incubation with fluorochrome-conjugated Abs. Flow cytometry was performed using a BD FACSCanto II, and data were analyzed using FlowJo (TreeStar). All plots were gated from live, CD45+ singlets. CD4+ T cells were further gated on CD3+ counts.

Abs and reagents

The following Abs were used for flow cytometry or immunohistochemistry: rat anti-myelin basic protein (MBP, Millipore); mouse anti-ubiquitylated neurofilament-H (SMI32; Covance); CD45 (Serotec); Brn3a (Santa Cruz); Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rat IgG (both from Life Technologies); FITC–anti-MHC class II, FITC–anti-B220, PE–anti-CD45 (Ly5), PE–anti-CD8, PerCP–anti-CD45, PE–anti-GM-CSF, PE–anti–CD11b, PE–anti–CD4, PerCP–Cy5.5–anti–Ly6C, PerCP–Cy5.5–anti–CD3e, and PerCP–Cy5.5–anti–IL-17A (all from eBioscience); and allophycocyanin–anti–CD45.2, FITC–anti–CD44, allophycocyanin–anti–Ly6G, allophycocyanin–anti–CD3e, and allophycocyanin–anti–IFN-γ (all from BD Biosciences). rIFN-γ, rIL-23, and rIL-12 were from R&D Systems.

Histology

Eyes and optic nerves were harvested, fixed in 4% paraformaldehyde, and cryoprotected in 30% sucrose. Specimens were embedded in OCT Tissue-Tek Medium (Sakura Finetek) and cut into 12-μm (nerves) or 20-μm (eyes) sections on a Thermo Scientific Microm HM550 cryostat. Immunofluorescence staining was performed with primary Abs against CD45 (1:100, BMS1, 1:200), and SMI-32 (1:1000). Citrate buffer Ag retrieval was performed for SMI-32 staining. Goat anti-mouse IgG Alexa Fluor 594 (1:500) and goat anti-rat IgG Alexa Fluor 488 (1:500) were used as secondary Abs. Sections were incubated with DAPI (100 ng/ml) prior to washing and mounting on slides (ProLong Gold Antifade Reagent; Life Technologies).

Fluorescent images were acquired with a Nikon Eclipse Ti microscope, CoolSNAP EZ camera, and NIS-Elements Basic Research v3.10 software. Confocal images were acquired using a Nikon A1 confocal microscope. (Nikon Plan Fluor 10×/0.30 or Nikon Plan Apo VC 60×/1.40 oil) with a diode-based laser system and NIS Elements software. Three-dimensional reconstruction images were constructed from confocal Z-stack images using Biplane software (Imaris). Additional image processing was performed with Adobe Photoshop CS5.1 and applied equally to all samples and controls. For tracing studies, anesthetized (trichloroethanol) mice received intravital injections (2 μl) of 1 μg/μl Alexa Fluor 594–conjugated cholera toxin β (Invitrogen) in both eyes. Mice were euthanized 24 h postinjection, and their optic nerves were dissected, postfixed in 4% paraformaldehyde overnight, and cryoprotected in 30% sucrose.

Quantification of histological parameters

Brn3a+ cells were counted in the ganglion cell layer in four to nine cross-sections of the entire retina perpendicular to the corneoscleral divide. Contiguous longitudinal sections of optic nerves (from the nerve head to the chiasm) were stained for CD45 or SM32. Swollen SM32+ axons were manually counted in 500-μm-long segments. The mean fluorescence intensity of CD45 staining was measured in the corresponding segment using ImageJ. Total SM32+ axons were enumerated in three or more coronal sections/nerve, ~1 mm proximal to the chiasm.

Electrophysiology

Optic nerve recording of compound action potentials (CAPs) was performed as described previously (14). Nerves were harvested at the same time following onset, but before peak, of disease. Four nerves were

FIGURE 1. IL-12– and IL-23–polarized CD4+ T cells are both capable of mediating ON. (A–C) C57BL/6 mice were immunized with MOG35-55 emulsified in CFA. Ten to fourteen days later, draining LN cells were harvested and cultured for 96 h with MOG35-55 and either rIL-12 or rIL-23 to generate Th1 and Th17 cells, respectively. (A) Intracellular staining and flow cytometric analysis of cultured cells at 96 h, gating on the CD3+CD4+ population. (B and C) Following culture, CD4+ T cells were transferred into naive syngeneic recipients. Mice in each group were euthanized at day 9 posttransfer, the day after clinical EAE onset. Mononuclear cells isolated from optic nerves (OptiNev) and spinal cords (SC) were assessed for CD4+ T cell cytokine production (B), and the percentage of CD4+ T cells and CD11b+ myeloid cells among CD45+ leukocytes (C) was assessed by flow cytometry. Data are representative of three independent experiments with at least three mice/group.
measured/group in each of three or four independent experiments. Briefly, mice were euthanized with CO2, and optic nerves were dissected and incubated in artificial cerebrospinal fluid containing NaCl (125 mM), NaH2PO4 (1.25 mM), glucose (25 mM), NaHCO3 (25 mM), CaCl2 (2.5 mM), MgCl2 (1.3 mM), and KCl (2.5 mM) and saturated with 95% O2/5% CO2. Nerves were drawn into suction electrodes for stimulation and recording at 37˚C. Signals were amplified and acquired with a Digidata 1440A using Clampex software (Axon instruments). Analysis was performed offline using Clampfit. Amplitude and conduction velocity values for individual components of the CAP were derived by fitting with multiple Gaussians using OriginPro (15). Statistical analysis was performed with Microsoft Excel and GraphPad Prism.

**MS subjects**

Patients diagnosed with relapsing MS (n = 36), based on the revised McDonald Diagnostic Criteria (16), were recruited from the Multiple Sclerosis Clinics at the University of Michigan and the University of Rochester. All subjects had a moderate degree of disability (Expanded Disability Status Scale score 2.5–4.5), and none were treated with disease-modifying therapies.

**ELISPOT assays**

PBMCs were obtained from study participants on a monthly basis over the course of 1 y. They were isolated using CPT Vacutainer tubes (Fisher Scientific), suspended in FBS with 20% DMSO, and stored in liquid nitrogen until thawed for analysis. ELISPOT assays were performed to enumerate the frequencies of MBP-specific IFN-γ and IL-17 producers using a protocol that we described previously (17). Subjects were classified as IFN-γ skewed if the frequency of MBP-specific IFN-γ producers in their PBMCs exceeded that of IL-17 producers by ≥2-fold in at least two thirds of samples. Conversely, IL-17–skewed subjects had ≥2-fold the frequency of MBP-specific IL-17 producers than IFN-γ producers in at least two thirds of PBMC samples. Subjects who did not meet either of the above criteria were classified as mixed.

**Magnetic resonance imaging protocol and imaging analysis**

All patients were evaluated by cranial magnetic resonance imaging (MRI) examination on a 1.5-T magnet using axial T2-weighted and axial and sagittal T1-weighted sequences. Brain parenchymal and T1 and T2 lesion volumes were measured using commercial software developed by VirtualScopics (Rochester, NY), as previously described (17).

**Statistics**

Analyses were performed using GraphPad Prism software. Leukocyte cell numbers and percentages were compared using the unpaired Student t test. Axon counts were analyzed by one-way ANOVA, and electrophysiological recordings were analyzed by the Mann–Whitney U test. MRI lesion volumes were analyzed with one-way ANOVA (Kruskal–Wallis) and the Dunn multiple-comparisons test. The p values < 0.05 were considered significant.

**Study approval**

All EAE experiments were performed in compliance with local and national animal care guidelines and approved by the University of Michigan Committee on the Use and Care of Animals.
Boards of the University of Michigan and the University of Rochester approved our human study protocol. Informed consent was obtained from each subject.

**Results**

**Characteristics of optic nerve inflammation in Th1 and Th17 adoptive-transfer recipients**

We showed previously that the clinical course of EAE is comparable between C57BL/6 mice injected with IL-12– or IL-23-polarized, MOG35–55-reactive Th1 and Th17 cells (9, 10, 18). The same T cells activated under neutral conditions do not induce CNS infiltrates or clinical EAE. In preliminary experiments, we performed flow cytometric analyses of optic nerves and spinal cords harvested from Th1 and Th17 adoptive-transfer recipients at serial time points. The majority of host mice (>90%) had bilateral optic neuritis (ON) on the day of onset of clinical EAE, irrespective of donor Th phenotype (K.S. Carbajal and B.M. Segal, unpublished observations). A significant percentage of IL-12–polarized donor T cells produced IFN-γ immediately prior to transfer, as well as in host spinal cords and optic nerves (Fig. 1A, 1B). In contrast, intracellular IL-17 staining rarely exceeded background levels. As expected, a high percentage of IL-23–polarized T cells expressed IL-17 prior to and following adoptive transfer. However, there were significant subpopulations of IL-17/IFN-γ double producers and IFN-γ single producers among IL-23–polarized donor cells harvested after 96 h of culture, consistent with transition to ex-Th17 cells (Fig. 1A). The percentage of IFN-γ single producers within the CNS of mice injected with IL-23–polarized T cells equaled or exceeded the percentage of IL-17 single producers (Fig. 1B).

Consistent with earlier studies, high percentages of infiltrating CD4+ T cells produced GM-CSF, irrespective of Th lineage (Fig. 1B) (19–21). In both Th1 and Th17 adoptive-transfer recipients, the cellular composition of CNS mononuclear cells and cytokine profile of infiltrating T cells were comparable in the optic nerve and spinal cord (Fig. 1B, 1C). Hence, in subsequent experiments we focused on the pathology of optic nerves because of their accessibility for axonal tracing and electrophysiological studies.

**Morphological and ultrastructural abnormalities in inflamed optic nerves of mice with Th1- and Th17-mediated disease**

Although MS was characterized previously as a primary demyelinating disorder, axonal transections and swellings are pervasive features of the disease, and axonal loss correlates with disability (22–24). The relative contribution of myelin-reactive Th1 and Th17 cells to axonopathy has yet to be assessed. We detected axonal swellings in recipients of both IL-12– and IL-23–polarized T cells shortly following the onset of clinical EAE (Fig. 2A, 2B). Confocal microscopy demonstrated that some of the swellings were located at the tips of transected axons (Fig. 2C). These swellings were also apparent in sections stained for unphosphorylated neurofilament-H (SMI-32) (Fig. 2E–G). Demyelination was evident in areas of inflammation early in the clinical course; however, we frequently observed swollen or degenerating axons surrounded by myelin, suggesting that axonopathy can occur without overt myelin damage (Fig. 2H–J, data not shown). Similar to other regions of the CNS, inflammation of the optic nerve is multifocal and discontinuous in mice with EAE (Fig. 2A, 2B). The frequency of axonal swellings correlated with staining for the pan-

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**FIGURE 3.** Axon loss and faulty nerve conduction occur in both Th1- and Th17-mediated ON. (A–D) Optic nerves harvested immediately before the peak of EAE were stained with Abs against SMI32 (green) and CD45 (red). Representative cross-sections of nerves from mice with Th17-mediated ON (A) or Th1-mediated ON (B), as well as from a naive mouse (C), are shown. The number of axons per section was quantified (D). Scale bars, 100 μm (inset, 25 μm). (E) Brn3a+ RGCs were counted in retinas harvested from naive mice and from ON mice at serial time points posttransfer. **p < 0.01, t test. (F) Representative traces of CAPs of optic nerves acutely isolated from naive and ON mice. CAP velocities (G and I) and amplitudes (H and J) were averaged over 7–12 nerves/group. Data are mean ± SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001, Mann–Whitney test.
leukocyte marker CD45, a measure of inflammation (Fig. 2D).

The relationship between axonal swellings and inflammation was comparable in IL-12– and IL-23–mediated ON. Optic nerve axon loss was already apparent in both groups of transfer recipients at clinical presentation (Fig. 3A–D). The number of retinal ganglion cells (RGCs), the neurons that give rise to optic nerve axons, was reduced late in disease (21–22 days after onset), but not at earlier time points (Fig. 3E). There were no significant differences in the extent of axon or RGC loss between groups of mice with ON (Fig. 3D, 3E).

Conduction deficits in optic nerves of mice with Th1- and Th17-mediated EAE

EAE lesions are heterogeneous with regard to location, volume, and severity of damage. Quantification of myelin breakdown across multifocal lesions via histological or immunohistochemical techniques is challenging and prone to sampling error. Furthermore, the relative contributions of demyelination and axonopathy to disability cannot be measured by those approaches. To overcome these limitations, we performed functional studies by subjecting acutely isolated optic nerves from Th1 and Th17 adoptive-transfer recipients to electrophysiological recordings. We measured velocities and amplitudes of CAPs at disease onset. A reduction in CAP amplitude is indicative of axon loss and/or conduction block, whereas a reduction in velocity is indicative of demyelination. CAP amplitudes and velocities of fast-, intermediate-, and slow-conducting fibers (AMP1/CV1, AMP2/CV2, and AMP3/CV3, respectively) were reduced in both groups of transfer recipients compared with naive mice (Fig. 3F–J).

Stable Th17 cells are capable of inducing ON

ex-Th17 cells have been portrayed as the critical effector cells in autoimmune demyelinating disease. Conversion of Th17 cells into ex-Th17 cells is promoted by IL-12 (11). To determine whether

FIGURE 4. Stable Th17 cells induce ON. (A) Intracellular cytokine production by CD4+ T cells derived from MOG-immunized IL-12–KO mice after 96 h of culture with MOG35–55 and rIL-23. (B) Intracellular cytokine production by CD4+ T cells derived from MOG-immunized IL-12–KO donors after infiltrating the spinal cords and optic nerves of IL-12–KO hosts. (C) Clinical courses of IL-12–KO mice injected with myelin-specific CD4+ Th17 cells derived from IL-12–KO donors versus WT mice injected with WT effector Th17 cells. The data shown are pooled from three experiments with n = 10 WT and n = 17 IL-12–KO host mice/group. (D) Percentages of CD4+ T cells and CD11b+ myeloid cells among CD45+ leukocytes infiltrating the optic nerve and spinal cord of IL-12–KO hosts shortly after the onset of clinical EAE. (E) Contiguous sections of an optic nerve obtained from an IL-12–KO host at onset of clinical EAE and stained with CD45 (red) or SMI-32 (green). Scale bars, 50 μm. (F) Representative section stained with toluidine blue, Scale bar, 25 μm. Areas with normal appearing axons are outlined, asterisks indicate swollen axons, and arrowheads point to empty myelin sheaths. (G–I) CAPs were measured in acutely isolated optic nerves from IL-12–KO recipients of stable Th17 cells at onset of clinical EAE. (G) Representative waveforms of optic nerve CAPs from a naive IL-12–KO mouse and an IL-12–KO adoptive transfer recipient with acute ON. The data were averaged over seven to nine nerves/group. Data are mean ± SEM. *p < 0.05, **p < 0.01, Mann–Whitney test.
plasticity contributes to the acquisition of encephalitogenic properties by Th17 cells, we polarized CD4\(^+\) T cells from MOG-primed IL-12-deficient (IL-12–knockout [KO]) donors with Ag and rIL-23. After 96 h of culture, a relatively high percentage of those cells expressed IL-17 (Fig. 4A). Unlike primed wild-type (WT) cells cultured under Th17-polarizing conditions (Fig. 1A), MOG-reactive CD4\(^+\) T cells derived from IL-12–KO donors had low frequencies of IFN-\(\gamma\) producers in vitro (Fig. 4A). Furthermore, these cells maintained a Th17 phenotype after infiltrating the spinal cords and optic nerves of syngeneic IL-12–KO hosts (Fig. 4B). Hence, we refer to IL-23–polarized cells from WT and IL-12–KO donors as plastic and stable Th17 cells, respectively. Stable Th17 cells induced EAE with a clinical course that was indistinguishable from that induced by WT Th17 cells (Fig. 4C). Stable Th17 cells also resembled their plastic counterparts by producing GM-CSF, as well as IL-17, and inducing infiltrates of similar cellular composition in the optic nerves and spinal cord (Figs. 1B, 1C, 4B, 4D). At clinical onset, we isolated comparable numbers of CD45\(^+\) cells from the optic nerves of hosts injected with stable or plastic Th17 effectors (data not shown). Reminiscent of our findings with plastic Th17 cells, stable Th17 cells induced axonal swellings and demyelination in the optic nerves of adoptive-transfer recipients at early time points (Fig. 4E, 4F). This was corroborated by electrophysiological studies, which showed significant reductions in CAP amplitudes and velocities compared with naive IL-12–KO controls (Fig. 4G–I). The reduction in CAP velocities induced by stable Th17 cells was modest compared with the degree of slowing induced by plastic Th17 cells (Figs. 3G, 4H). Plastic Th17 cells also were more effective at reducing the CAP amplitudes of fast conducting fibers (Figs. 3H, 4I). These observations could reflect an enhanced pathogenicity of ex-Th17 cells compared with stable Th17 cells. Alternatively, Th1 cells

**FIGURE 5.** Bona fide Th1 cells are capable of inducing ON. (A) Intracellular cytokine production by CD4\(^+\) T cells derived from MOG-immunized IL-23–KO mice after 96 h of culture with MOG35–55 and rIL-12. (B) Intracellular cytokine production by CD4\(^+\) T cells derived from MOG-immunized IL-23–KO donors after infiltrating the spinal cords and optic nerves of IL-23–KO hosts. (C) Clinical courses of IL-23–KO mice injected with myelin-specific CD4\(^+\) Th1 cells derived from IL-23–KO donors versus WT mice injected with WT effector Th17 cells. The data were pooled from three experiments with \(n = 9\) WT and \(n = 22\) IL-23–KO hosts. * \(p < 0.05\), ** \(p < 0.01\), Holm–Sidak multiple t test. (D) Percentages of CD4\(^+\) T cells and CD11b\(^+\) myeloid cells among CD45\(^+\) leukocytes infiltrating the optic nerve and spinal cord. (E) Contiguous sections of a representative optic nerve obtained from an IL-23–KO host at onset of clinical EAE and stained with CD45 (red) or SMI-32 (green). Scale bars, 50 \(\mu m\) (inset, 25 \(\mu m\)). (F) Representative section stained with toluidine blue. Examples of areas with normal-appearing axons are outlined, asterisks mark examples of swollen axons, and arrowheads point to myelin sheaths left behind by degenerated axons. Scale bar, 25 \(\mu m\). (G–I) CAPs were measured in optic nerves from the IL-23–KO recipients of Th1 cells at onset of clinical EAE. The data were averaged over seven to nine nerves/group. (G) Representative waveforms. CAP velocities (H) and amplitudes (I) were measured in 8–12 nerves/group. Data are mean ± SEM. * \(p < 0.05\), ** \(p < 0.01\), Mann–Whitney test.
might contaminate the pool of IL-23-stimulated WT donor cells and act synergistically with Th17 cells to induce inflammatory infiltration and CNS tissue damage (25).

**Bona fide Th1 cells can induce EAE in the absence of IL-23**

We next questioned whether Th1 cells are capable of inducing EAE independent of stable Th17 cells and/or ex-Th17 cells. Because the IL-12–polarized T cells used in our earlier experiments were derived from WT donors, it is possible that they were contaminated with ex-Th17 cells that had been exposed to IL-23 during priming in vivo. To generate a pure population of bona fide Th1 cells, we primed IL-23–KO donors with MOG35–55 in CFA and cultured draining LN cells with Ag and rIL-12. CD4+ T cells were then transferred into naive syngeneic IL-23–KO hosts. These Th1-polarized cells, never exposed to IL-23, produced IFN-γ and GM-CSF, but no significant IL-17, before and after transfer (Fig. 5A, 5B, data not shown). They induced EAE at 90–100% incidence in repeated experiments, although peak severity was slightly lower than we observed with WT to WT transfers (Fig. 5C). Optic nerve and spinal cord infiltrates induced by IL-23–KO Th1 cells had a similar composition to WT, with IFN-γ+ and TNF-α+ infiltrates, but no significant IL-17, before and after transfer (Fig. 5A, 5B, data not shown). They induced EAE at 90–100% incidence in repeated experiments, although peak severity was slightly lower than we observed with WT to WT transfers (Fig. 5C). Optic nerve and spinal cord infiltrates induced by IL-23–KO Th1 cells had a similar cellular composition (Fig. 5D). At clinical onset, we isolated comparable numbers of CD45+ cells from the optic nerves of WT and IL-12–KO hosts injected with WT or IL-12–KO Th1 effectors, respectively (data not shown). Bona fide Th1 cells were still capable of inducing axonal swelling and demyelination (Fig. 5E, 5F) and caused reductions in CAP amplitudes (Fig. 5G, 5I); however, they were relatively ineffective at inducing CAP slowing (Fig. 5G, 5H).

**Anti-myelin cytokine responses in MS patients**

MS is a heterogeneous disease with regard to the clinical course, extent, and pattern of CNS injury, as well as therapeutic responsiveness to disease-modifying therapy. Our EAE studies raise the question of whether autoreactive Th responses can be used to define subsets of MS patients that are pathophysiologically and/or clinically meaningful. As a first step in addressing that issue, we performed a longitudinal exploratory study to measure MBP-specific IFN-γ and IL-17 responses in a cohort of relapsing MS patients with moderate disability and a history of ON and myelitis. PBMCs were collected on a monthly basis over the course of 1 y. The frequency of MBP-specific cytokine-producing cells was quantified by ELISPOT. We found that 23% of patients consistently mounted IFN-γ–skewed responses, 17% had an IL-17–dominant pattern, and the remainder had comparable or oscillating frequencies of IFN-γ and IL-17 producers (Fig. 6A). Cerebral MRI scans were obtained from each subject and analyzed as previously described (17). Average MRI T2 lesion load was similar across the three groups (Fig. 6B). T1 lesion load, which is associated with severe CNS injury and axonal loss, was relatively high in patients with the mixed IL-17/IFN-γ pattern (Fig. 6C).

**Discussion**

The current study provides further insight into the pathophysiology of autoimmune demyelinating disease mediated by Th1 and Th17 cells. We (9) and other investigators (10) previously demonstrated that the adoptive transfer of either IL-12– or IL-23–polarized WT Th effector cells induced EAE. These two forms of disease differ in CNS expression of downstream chemokines and proinflammatory factors, as well as therapeutic responsiveness to immunomodulatory agents. In this study, we extend those findings by showing that both Th effector cell types are capable of mediating axonopathy and demyelination. We chose to focus this study on the pathology of the inflamed optic nerve because of its accessibility for anterograde tracing experiments and electrophysiological analysis. In addition to providing a functional read-out measure, electrophysiology is particularly important to assess collective tissue damage in light of the inherent challenges of quantifying multifocal axonopathy and demyelination via histological or immunohistochemical approaches. Inflammation, demyelination, and axonopathy appeared qualitatively similar in optic nerves compared with the spinal cord, irrespective of Th-polarizing conditions or the cytokine profile of the myelin-reactive donor T cells. Furthermore, there were no significant differences in the cellular composition of optic nerve and spinal cord infiltrates isolated from the same group of mice, based on the panel of cell surface markers that we measured by flow cytometry. Nonetheless, the optic nerve has a unique anatomical structure, and only future studies will determine whether the interactions between infiltrating inflammatory cells and resident glial cells differ between CNS compartments.

Experiments that compare the properties of IL-12– and IL-23–polarized CD4+ T cells derived from primed WT donors are complicated by the possibility of contamination by T cells of the alternative lineage, as well as by the plasticity of WT Th cells. The ability of myelin-reactive Th1 cells to transfer EAE was challenged by the assertion that IL-23 signaling is universally required for the acquisition of pathogenic properties (7, 26). It could be argued that IL-12–polarized CD4+ cells, generated from MOG-primed WT donors, may be contaminated with ex-Th17 cells that are actually responsible for disease induction upon adoptive
transfer. However, contrary to the current dogma, our data demonstrate that bona fide Th1 cells, which were never exposed to IL-23 during the priming or effector stage, can induce damage to axons and myelin. A related issue is whether Th17 cells become pathogenic only after transitioning to ex-Th17 cells (12). Our results indicate that, although plasticity may enhance the potency of myelin-reactive Th17 cells, it is not absolutely required for the acquisition of disease-causing properties.

Although stable Th17 cells, derived from IL-12–KO hosts, and bona fide Th1 cells, derived from IL-23–KO hosts, were both capable of inducing ON, they were not as effective as their WT counterparts. This was particularly evident with regard to CAP slowing. One hypothetical explanation for our results is that autoreactive ex-Th17 cells are potentially potent inducers of myelin damage and, as mentioned above, may have contributed to the IL-12–polarized, as well as the IL-23–polarized, WT transfers. Alternatively, EAE studies suggest that Th1 and Th17 cells act synergistically in triggering neuroinflammation and downstream CNS pathology (25). Future studies with fate-mapping mice will be necessary to distinguish between those possibilities.

MS is a heterogeneous disease with regard to the clinical course, extent, and pattern of CNS injury, as well as the therapeutic responsiveness to disease-modifying therapies. A clearer understanding of the mechanistic basis of this diversity will be critical for the future discovery of biomarkers and the design of customized medications. An important question broached by our study is whether differences in anti-myelin Th cell cytokine responses can be used to define subsets of MS patients that are pathophysiologically and/or clinically meaningful. As a first step in answering that question, we found that the MS patients in our cohort exhibited a range of patterns of IFN-γ and IL-17 production by MBP-reactive PBMCs. Furthermore, these patterns were stable over the course of 1 y in some patients. The human studies described in this article are exploratory, and future studies with larger independent cohorts are necessary to determine whether immune profiles correlate with clinical, radiologic, and/or histopathological manifestations of disease or with responsiveness to individual disease-modifying agents.

This study demonstrates that the autoreactive Th repertoire in CNS autoimmune disease may be skewed toward a Th1 or Th17 lineage. Our EAE experiments serve as a proof of principle that highly polarized and stable Th1 or Th17 cells are capable of inducing classical features of MS, independent of the reciprocal differentiation factor. The fact that full-blown EAE can occur in the absence of IL-23, a cytokine that has been thought to play an essential role in pathogenesis, provides further evidence against a universal therapeutic target in MS. Our data underscore the importance of discovering biomarkers that define pathologically distinct subsets of individuals with MS to guide the design of more effective clinical trials and to facilitate the development of customized medicine in MS.

Disclosures

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