European Journal of
Immunology

IL-12-polarized Th1 cells produce GM-CSF and induce EAE independent of IL-23

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CD4^{+} T-helper (Th) cells reactive against myelin antigens mediate the mouse model experimental autoimmune encephalomyelitis (EAE) and have been implicated in the pathogenesis of multiple sclerosis (MS). It is currently debated whether encephalitogenic Th cells are heterogeneous or arise from a single lineage. In the current study, we challenge the dogma that stimulation with the monokine IL-23 is universally required for the acquisition of pathogenic properties by myelin-reactive T cells. We show that IL-12-modulated Th1 cells readily produce IFN-γ and GM-CSF in the CNS of mice and induce a severe form of EAE via an IL-23-independent pathway. Th1-mediated EAE is characterized by monocyte-rich CNS infiltrates, elicits a strong proinflammatory cytokine response in the CNS, and is partially CCR2 dependent. Conversely, IL-23-modulated, stable Th17 cells induce EAE with a relatively mild course via an IL-12-independent pathway. These data provide definitive evidence that autoimmune disease can be driven by distinct CD4^{+} T-helper-cell subsets and polarizing factors.

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Introduction

Multiple sclerosis (MS), an inflammatory demyelinating disease of the CNS, is believed to be autoimmune in etiology. In the animal model experimental autoimmune encephalomyelitis (EAE), active immunization with MHC Class II-restricted epitopes of myelin proteins or adoptive transfer of myelin-reactive CD4^{+} T cells results in an ascending paralysis with histopathological features that are reminiscent of MS. However, EAE studies have shown that not all myelin-reactive T cells are pathogenic [1–3]. A major goal in MS research is to elucidate the factors required for the differentiation and function of encephalitogenic T cells that might ultimately serve as biomarkers or novel therapeutic targets in the clinical setting.

It is widely believed that exposure of myelin-reactive T cells to the cytokine IL-23 is critical for their acquisition of pathogenic properties [1, 4, 5]. IL-23 promotes the expansion of IL-17-producing Th17 cells [4, 5]. Interestingly, "hallmark" Th17 cytokines (including IL-17A and IL-17F) are dispensable for the development of EAE [6]. Several recent studies suggest that the

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mechanism of action of IL-23 in EAE is to induce the production of GM-CSF, which drives the accumulation of pro-inflammatory myeloid cells in the CNS, culminating in axonopathy and demyelination [7, 8]. In contrast to the relationship between IL-23 and GM-CSF in mice, GM-CSF production is associated with the IL-12/Th1 axis in humans, including individuals with MS [9]. IL-12-polarized, IFN-γ-producing Th1 cells were once thought to be critical autoimmune effector cells in EAE as well as MS [10, 11]. However, their relevance was challenged by the observations that mice deficient in IL-12, the IL-12 receptor, or IFN-γ are highly susceptible to EAE [12–14]. The notion that EAE is IL-23-dependent and IL-12-independent is primarily based on experiments in which mice on a C57BL/6 background were actively immunized with a peptide of myelin oligodendrocyte glycoprotein (MOG$_{35-55}$) in CFA and injected with *Bordetella pertussis* toxin [5]. We questioned whether a nonredundant role of IL-23 is specific for that model.

In fact, we and others have shown that, in certain adoptive transfer paradigms, stimulation of ordinarily innocuous myelin-specific CD4$^+$ T cells with recombinant IL-12 is sufficient to confer encephalitogenicity [2, 3, 15]. IL-12-polarized donor T cells employed in those studies were derived from myelin-primed wild-type mice. This led some to speculate that disease was mediated by plastic Th17 cells that had been exposed to endogenous IL-23 in vivo and subsequently acquired Th1 characteristics during stimulation with IL-12 in vitro (so-called exTh17 cells) [16], rather than by classic Th1 cells. Fate mapping studies have shown that the majority of CD4$^+$ T cells that infiltrate the CNS of MOG-immunized C57BL/6 mice at peak EAE are exTh17 cells [17]. The goal of the current study was to determine whether classical Th1 cells, that have not been exposed to IL-23 signaling, are capable of mediating inflammatory demyelination.

Results and discussion

### Th1 cells and Th17 cells differentiate independent of IL-23 and IL-12, respectively

IL-12 and IL-23 are heterodimers composed of a common IL-12p40 chain and a unique IL-12p35 or IL-23p19 chain, respectively. We immunized C57BL/6 mice deficient in the IL-12p40 chain (and therefore unable to produce either bioactive IL-12 or IL-23) with MOG$_{35-55}$ emulsified in CFA. In order to generate pure populations of Th1 and Th17 cells, draining lymph node cells (LNC) were obtained 10 days later and challenged ex vivo with antigen plus either recombinant IL-12 and IFN-γ, or IL-23, IL-1α, and anti-IFN-γ, respectively. As expected, a significant percentage of IL-12-polarized IL-12p40$^{-/-}$ Th1 cells produced IFN-γ but not IL-17, while the converse was true of their IL-23-polarized Th17 counterparts (Fig. 1A). Th1 cells expressed high levels of *tbx21*, while Th17 cells expressed high levels of *rorc* (Fig. 1B). Relatively few Th1 cells produced GM-CSF compared with Th17 cells derived from the same IL-12p40$^{-/-}$ donor pool (Fig. 1A). However, IL-12 can suppress GM-CSF production by murine Th1 cells [8]. We questioned whether committed Th1 cells would upregulate GM-

CSF upon reactivation in the absence of IL-12. Indeed, a high percentage of Th1 cells, derived from either IL-12p40$^{-/-}$ or WT donors, expressed GM-CSF during secondary antigenic challenge in vitro under neutral conditions (Fig. 1C). The reactivated Th1 cells expressed a high IFN-γ/low IL-17 profile, irrespective of donor phenotype. IL-23-polarized T cells derived from IL-12p40$^{-/-}$, but not from WT, donors maintained a high level of IL-17 and low level of IFN-γ production during in vitro reactivation, indicating that they had not converted into exTh17 cells (Fig. 1C and D). Similar results were obtained in parallel experiments with Th1 and Th17 cells derived from donors deficient in the IL-23 receptor (IL-23R) or IL-12 receptor (IL-12Rβ2), respectively (Fig. 1D–G). IL-23-independent, IL-12-polarized CD4$^+$ T cells express negligible levels of *rorc* during polarizing culture, consistent with a classical Th1 phenotype and not an exTh17 phenotype (Supporting Information Fig. 1).

Th1 cells can induce EAE independent of IL-23

Next, we assessed the ability of highly polarized Th1 cells to induce EAE by an IL-23-independent pathway. Thakker et al. previously demonstrated that Th1 cells derived from IL-23-deficient donors are encephalitogenic in wild-type hosts [18]; however, this study does not rule out the possibility that host-derived IL-23 rescues T-cell encephalitogenicity following transfer. In order to eliminate IL-23 at all stages of disease, MOG-primed T cells derived from IL-12p40$^{-/-}$ mice were polarized with recombinant IL-12 and injected into IL-12p40$^{-/-}$ hosts. For controls, some T cells from the same donor pool were cultured in the presence of IL-23, or in the absence of any recombinant cytokines, prior to adoptive transfer into other groups of IL-12p40-/- mice. The IL-12-polarized cells reproducibly induced EAE in 100% of recipients, with an accelerated and more severe course than the disease induced by their IL-23-polarized counterparts (Fig. 2A). T cells cultured under neutral conditions failed to induce clinical EAE in any of the recipients (Supporting Information Fig. 2). Consistent with these results, IL-12-polarized, MOG-specific IL-23R$^{-/-}$ T cells transferred clinical EAE to WT recipients at high incidence (Fig. 2D). In both experimental systems, donor Th1 cells continued to produce IFN-γ, but little IL-17, after infiltrating the CNS (Fig. 2B, C, E, and F). They also produced GM-CSF, reminiscent of Th1 cells reactivated under neutral conditions in vitro (Fig. 1C). These data demonstrate that classical Th1 cells, bereft of IL-23 signaling during the priming, expansion, and effector stages, are potent encephalitogenic effectors. To our knowledge, this is the first report of EAE induction in the complete absence of IL-23.

IL-23-polarized T cells derived from IL-12p40$^{-/-}$ or IL-12Rβ2$^{-/-}$ donors expressed a high ratio of IL-17 to IFN-γ in the CNS of IL-12p40$^{-/-}$ and WT recipients, respectively, demonstrating that the majority of transferred cells had retained a Th17 phenotype yet were still capable of mediating clinical EAE (Fig. 2B, C, E, and F). Interestingly, most IL-12Rβ2$^{-/-}$ donor T cells did not transition to an exTh17 phenotype in WT hosts, despite their accessibility to IL-23 signaling. This
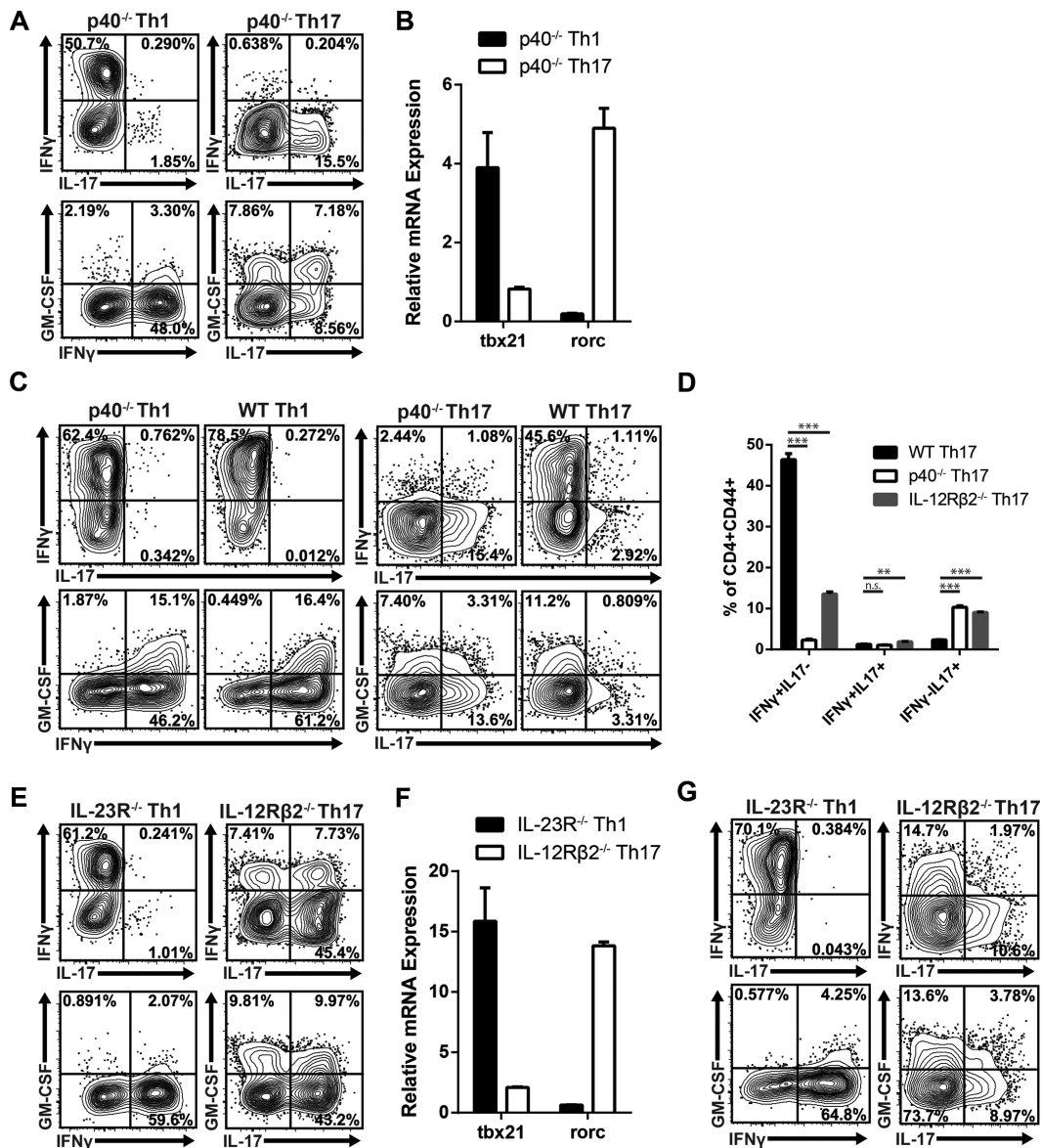
Figure 1. IL-12 and IL-23 promote the differentiation of stable Th1 and Th17 cells, respectively, in the absence of the reciprocal polarizing factor. (A–D) LNC were harvested from IL-12p40$^{-/-}$ mice 10 days following immunization with MOG$_{35-55}$ in CFA. Cells were cultured with antigen plus IL-12 and IFN-γ (Th1) or IL-23, IL-1α, and anti-IFN-γ (Th17). Cells were harvested 4 days later for flow cytometric analysis and RNA extraction. (A) Cytokine expression in CD4$^+$CD44$^+$ T cells following 4 h of stimulation with PMA/Ionomycin ex vivo was determined by flow cytometry. (B) Transcription factor expression in purified CD4$^+$ T cells, measured by quantitative RT-PCR and normalized to *gapdh*. (C, D) CD4$^+$ T cells were purified from Th-polarized IL-12p40$^{-/-}$ or WT primary cultures, washed, rested for 2 days, and re-challenged with antigen and T-depleted IL-12p40-/- or WT splenocytes, respectively, under neutral conditions. CD4$^+$CD44$^+$ T cells were analyzed for cytokine expression at 24 h by flow cytometry. The bar graph represents biological replicates; n = 3 per group. (E–G) LNC were harvested from MOG/CFA-immunized IL-23R$^{-/-}$ or IL-12Rβ2$^{-/-}$ mice and cultured with antigen under Th1 or Th17 polarizing conditions, respectively, for 4 days. (E) Cytokine expression in PMA/Ionomycin-stimulated CD4$^+$CD44$^+$ T cells was analyzed by flow cytometry. (F) Transcription factor levels in purified CD4$^+$ T cells were measured by quantitative RT-PCR as described above. (G) MOG-specific cytokine production in CD4$^+$CD44$^+$ T cells following rest and re-challenge with WT T-depleted splenocytes, as analyzed by flow cytometry. Data are representative of at least 10 (A,E), or 2–4 (B,C,D,F,G) independent experiments with n≥3 mice per group. (B, D, F) Data are shown as mean ± SEM. *p≤0.05, **p≤0.01, ***p≤0.001 using the two-tailed, unpaired *t*-test.

finding is consistent with previous reports of EAE mediated by IL-23-polarized, T-bet-deficient CD4$^+$ T cells, which also retain a polarized Th17 phenotype post-transfer, and suggests that IL-12 might play a distinctive role driving Th17 plasticity in vivo [19–21].

IL-23-independent Th1 cells induce cytokine production and monocyte-rich infiltrates in the CNS

We next interrogated the characteristics of CNS infiltrates and the cytokine/chemokine profile induced by Th1 cells in our
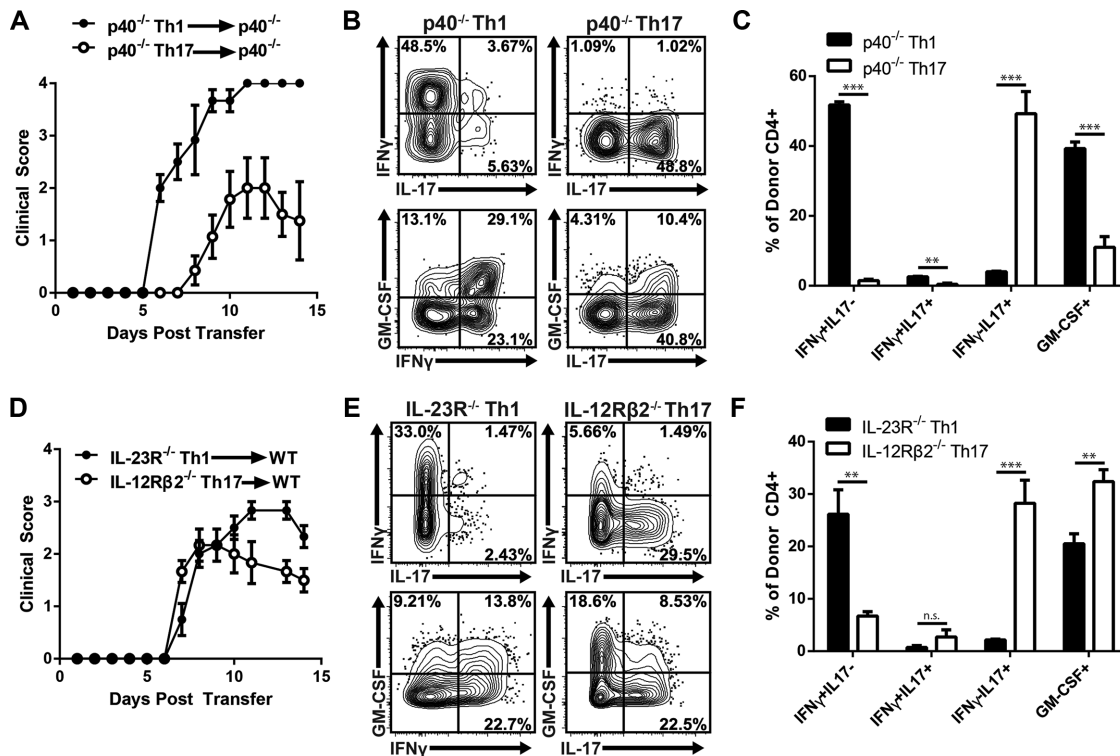
Figure 2. IL-12-polarized T cells induce EAE independent of IL-23 signaling. (A–C) LNC from MOG/CFA-immunized CD45.2+ IL-12p40−/− mice were cultured for 4 days with antigen under Th1 or Th17 polarizing conditions. Purified CD4+ T cells were adoptively transferred into naïve CD45.1+ IL-12p40−/− hosts. (A) Mean clinical scores; $n = 6$–7 mice per group, from one of four experiments with similar results. (B, C) At peak disease, leukocytes were recovered from the spinal cord, and stimulated with PMA/Ionomycin ex vivo. The figures show cytokine expression in CD45.2+ CD4+ donor T cells as determined by flow cytometry. (D–F) MOG$_{35-55}$-reactive CD4+ IL-23R−/− Th1 or IL-12Rβ2−/− Th17 cells were purified and adoptively transferred into naive CD45.1+ WT hosts. (D) Mean clinical scores; $n = 6$ mice per group, from one of three independent experiments with consistent results. (E,F) At peak disease, leukocytes were recovered from the spinal cord, and stimulated with PMA/Ionomycin ex vivo. The figures show cytokine expression in CD45.2+ CD4+ donor T cells as determined by flow cytometry. (B, C, E, F) Data are representative of two independent experiments with $n \geq 3$ mice per group. Bar graphs represent biological replicates. Data in A, C, D, F are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using the two-tailed, unpaired t-test.

IL-23-independent EAE models. CNS tissues obtained from mice injected with IL-12-independent Th17 cells were analyzed as a foil. As in conventional models of EAE, inflammation was concentrated in the thoracolumbar spinal cord of both Th1 and Th17 recipients (Fig. 3A). The percent of MHC Class II+ monocytes and myeloid dendritic cells tended to be higher in infiltrates induced by IL-23-independent Th1 cells compared with stable Th17 cells (Fig. 3B). Th1 cells induced elevated levels of CCL2 in CNS homogenates, as well as other myeloid-related factors including TNF-α, GM-CSF, IL-6, and IL-1α (Fig. 3C). Such an inflammatory milieu is likely to favor the recruitment of monocytes. CCR2−/− mice were relatively resistant to EAE following the transfer of either IL-23R−/− Th1 or IL-12Rβ2−/− Th17 donor cells, signifying a damaging role of inflammatory monocytes in both forms of EAE (Fig. 3D).

IFN-γ has been reported to have both protective and pathogenic effects in inflammatory demyelination [13, 22, 23]. The role of IFN-γ in IL-23-independent EAE has yet to be determined. We found that neutralization of IFN-γ did not suppress EAE induction by IL-23-independent Th1 cells (data not shown). These data contribute to a growing body of data demonstrating the dispensability of individual "hallmark" cytokines in autoimmune disease

[6, 13]. Similarly, iNOS was highly upregulated in Th1 recipients, yet iNOS−/− mice remained highly susceptible to Th1-mediated, IL-23-independent EAE (data not shown).

Concluding remarks

The current study challenges the dogma that IL-23 is universally critical for the development of EAE. Collectively, our data substantiate and expand previous studies indicating that clinically similar forms of EAE may be mediated by distinct autoreactive T-cell subsets via parallel inflammatory pathways, in a more definitive manner than previously shown [23, 24]. If these findings are translatable to human autoimmune disease, they suggest that the therapeutic targeting of proximal factors that promote the differentiation and/or stabilization of encephalitogenic T cells should be customized based on the predominant Th subset in an individual's autoreactive repertoire. Conversely, we found that CNS GM-CSF production and monocyte recruitment are common features of EAE initiated by disparate effector T cells. Hence, targeting innate
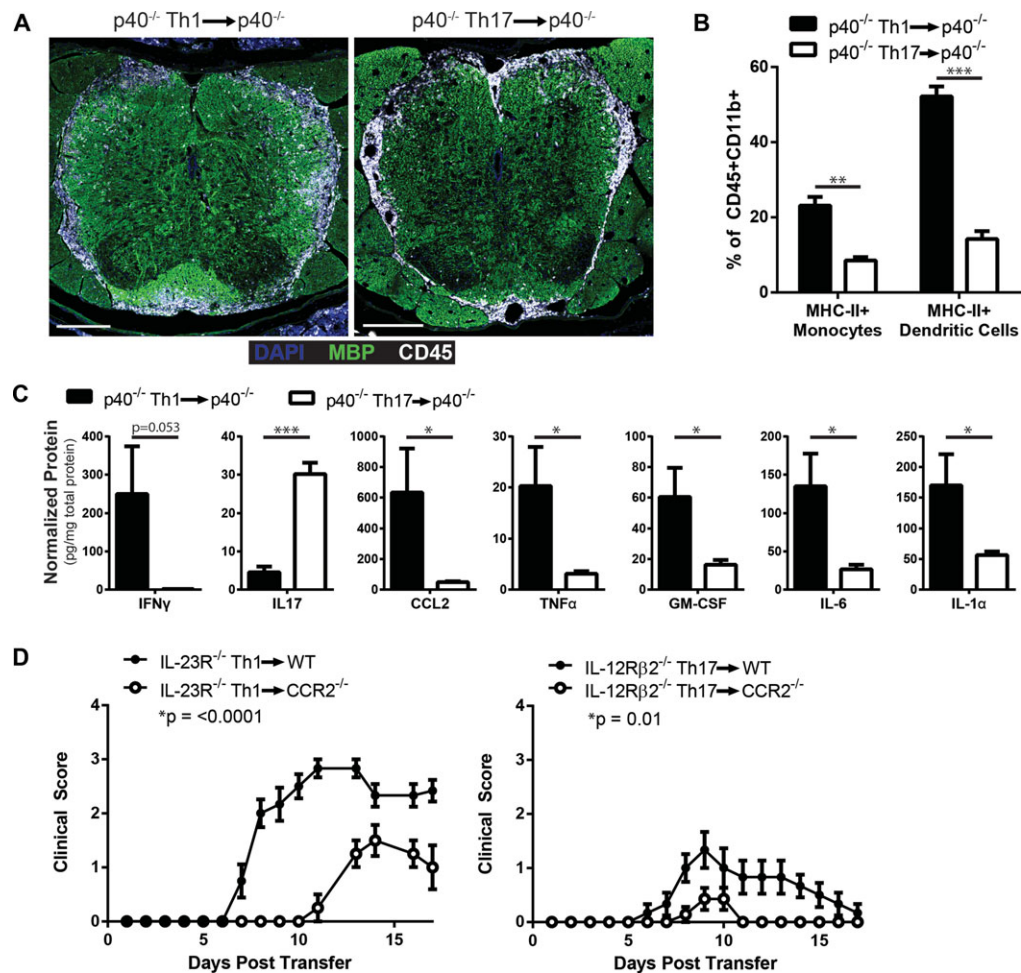
Figure 3. EAE mediated by IL-23-independent Th1 cells is characterized by monocyte-rich infiltrates and is partially CCR2-dependent. (A–C) EAE was induced by adoptive transfer of MOG$_{35-55}$-reactive IL-12p40$^{-/-}$ Th1 or Th17 cells. (A) Upon reaching a score of 2, mice were perfused and spinal cords isolated for immunohistochemistry. Sections of the lumbar spinal cord were stained for DAPI (blue), myelin basic protein (MBP, green), and CD45 (white) and analyzed by confocal microscopy. Scale bar = 200 μm. (B) At disease onset, leukocytes were isolated from the spinal cord, and the frequencies of MHC-II$^+$ monocytes (CD11b$^+$CD11c$^-$Ly6G$^-$) and dendritic cells (CD11b$^+$CD11c$^+$) were determined by flow cytometry. n = 3 mice per group, from one of two independent experiments. Mice from each group were matched based on clinical score. The gating strategy is shown in Supporting Information Fig. 3. (C) Spinal cords were isolated at disease onset, homogenized, and centrifuged to pellet the tissue. Supernatants were subjected to a multiplex bead assay. Data were pooled from two independent experiments with six to seven mice per group. (D) MOG$_{35-55}$-reactive IL-23R$^{-/-}$ Th1 cells or IL-12Rβ2$^{-/-}$ Th17 cells were transferred into naïve syngeneic CCR2$^{-/-}$ or WT hosts. n = 4–7 mice per group, from one of three independent experiments with consistent results. Data in B–D are shown as mean ± SEM. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ using the two-tailed, unpaired t-test (B, C). Clinic courses were compared using 2-way ANOVA, $^*p$ as indicated (C).

immune cells or factors that regulate them might have broader therapeutic efficacy.

Materials and methods

Mice

C57BL/6 WT and CD45.1 congenic mice were obtained from Charles River Laboratories (Wilmington, MA, USA). C57BL/6 IL-12Rβ2$^{-/-}$, iNOS$^{-/-}$, CCR2$^{-/-}$, and IFN-γR$^{-/-}$ mice were obtained from Jackson Labs (Bar Harbor, ME, USA). IL-23R$^{-/-}$ were the generous gift of Dr. Nico Ghilardi (Genentech, South San Francisco, CA, USA) [4]. IL-12p40$^{-/-}$ mice were originally

obtained from J. Magram (Hoffman LaRoche, Nutley, NJ, USA) [25]. All mice were bred and maintained under specific pathogen-free conditions in the University of Michigan animal housing facilities. All protocols were approved by the University of Michigan Committee on Use and Care of Animals.

Immunization and cell culture

Mice were subcutaneously immunized with an emulsion of complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA) and 100 μg of myelin oligodendrocyte glycoprotein peptide 35–55 (Biosynthesis, Lewisville, TX, USA). Draining lymph nodes were harvested and homogenized into a single cell suspension 10–14 days later.

Cells were cultured for 96 h in the presence of MOG_{35-55} at 50 µg/ml and either Th1- or Th17-polarizing factors, as previously described [2, 20]. For rest and rechallenge experiments, in vitro polarized $CD4^+$ T cells were purified by magnetic activated cell-sorting (MACS) (Miltenyi Biotech, San Diego, CA, USA), and rested for 48 h without exogenous cytokines. On the day of rechallenge, $CD4^+$ T cells were harvested and co-cultured with T-depleted splenocytes (prepared using anti-CD4 MACS microbeads) in the presence or absence of MOG_{35-55} at 50 µg/ml.

Quantitative PCR

RNA was isolated from purified $CD4^+$ T cells using TRIzol Reagent (Life Technologies, Grand Island, NY, USA) and converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCR was performed on an iQ Thermocycler (Bio-Rad, Hercules, CA, USA) with predesigned Taqman assays (Life Technologies).

Induction of EAE

Naïve C57BL/6 WT or IL-12p40$^{-/-}$ mice were injected with 3–5 $\times 10^6$ $CD4^+$ T cells isolated from MOG-reactive Th cultures. Mice were rated on a daily basis by an examiner blinded to experimental groups according to the following scale: (i), limp tail; (ii), hind-limb weakness; (iii), partial hind-limb paralysis; (iv), complete paralysis of hind-limbs; and (v), moribund state.

Isolation of CNS mononuclear cells

Mice were perfused with PBS via the intracardiac route. CNS tissues were homogenized in the presence of protease inhibitors using an 18G needle and syringe and centrifuged to pellet the tissue. Supernatants were collected and stored. The pellet was digested in a solution of collagenase A (1 mg/mL, Roche, Indianapolis, IN, USA) and DNase1 (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). Mononuclear cells were enriched on a 30%/70% Percoll gradient (GE Healthcare, Pittsburgh, PA, USA).

Flow cytometry

CNS mononuclear cells and cultured Th cells were stimulated with PMA (50 ng/mL) and ionomycin (2 µg/mL) in the presence of brefeldin A (10 µg/mL) for 4 h. For rest and rechallenge experiments, brefeldin A (10 ug/ml) was added for the final 4 h of culture. For intracellular staining, cells were fixed with 4% paraformadehyde and permeabilized with 0.5% saponin. Fluorescent antibodies were purchased from eBioscience (San Diego, CA, USA) and BD Biosciences (San Jose, CA, USA). Data were acquired using an FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR, USA).

Multiplex immunoassays

Supernatants of homogenized CNS tissues were analyzed with a customized Milliplex Mouse Cytokine/Chemokine Kit (MPXMCYTO-70K, Millipore, Billerica, MA, USA) using the Bio-Plex 200 System (Bio-Rad). Cytokine and chemokine levels were normalized to total protein as quantified by Bradford assay.

Immunofluorescence

Mice were perfused with Tyrode's solution followed by 4% paraformaldehyde in PBS. The spinal column was removed, post-fixed, decalcified, cryoprotected, embedded, and frozen in OCT (Cellpath, Newton, UK). Twelve micrometer sections were blocked with Avidin D and biotin (Vector Labs, Burlingame, CA, USA), and stained with the following primary and secondary antibodies: goat anti-MBP (1:300, Millipore), Alexa Fluor 488-conjugated rabbit anti-goat IgG (1:300, Life Technologies), biotinylated rat anti-CD45 (1:100, eBioscience), and Alexa Fluor 647-conjugated streptavidin (1:300, Life Technologies). Nuclei were labeled with DAPI (Life Technologies). Images were acquired on a Nikon A1 confocal microscope (Nikon, Tokyo, Japan).

Statistics

Immune parameters were compared using the unpaired Student's t-test with Prism 6 software (GraphPad, La Jolla, CA, USA). Clinical scores were compared by two-way ANOVA. p values: $* < 0.05$, $** < 0.01$, $*** < 0.001$.

Conflict of interest: The authors declare no financial or commercial conflict of interests.

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Abbreviations: **MOG or MOG$_{35-55}$**: myelin oligodendrocyte glycoprotein fragment 35–55 · **LNC**: lymph node cell

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