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EAE mediated by a non-IFN-γ/non-IL-17 pathway

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Previous studies have shown that EAE can be elicited by the adoptive transfer of either IFN-γ-producing (Th1) or IL-17-producing (Th17) myelin-specific $CD4^+$ T-cell lines. Paradoxically, mice deficient in either IFN-γ or IL-17 remain susceptible to EAE following immunization with myelin antigens in CFA. These observations raise questions about the redundancy of IFN-γ and IL-17 in autoimmune demyelinating disease mediated by a diverse, polyclonal population of autoreactive T cells. In this study, we show that an atypical form of EAE, induced in C57BL/6 mice by the adoptive transfer of IFN-γ-deficient effector T cells, required IL-17 signaling for the development of brainstem infiltrates. In contrast, classical EAE, characterized by predominant spinal cord inflammation, occurred in the combined absence of IFN-γ and IL-17 signaling, but was dependent on GM-CSF and CXCR2. Our findings contribute to a growing body of data, indicating that individual cytokines vary in their importance across different models of CNS autoimmunity.

Key words: Autoimmunity · EAE · IL-17 · IFN-γ · MS

Introduction

EAE is an inflammatory demyelinating disease of the CNS that shares clinical and histopathological characteristics with MS. EAE is induced in laboratory animals by active immunization with myelin antigens or by the adoptive transfer of myelin-specific $CD4^+$ T cells. EAE was historically viewed as a prototypical Th1-driven autoimmune disorder until it was demonstrated that mice deficient in key components of the Th1 pathway, such as IFN-γ, the β2 chain of the IL-12 receptor, or the p35 subunit of IL-12, remain fully susceptible to disease [1–3]. In fact, mice with IFN-γ deficiencies exhibit a severe, atypical form of inflammatory demyelination characterized by neutrophil-rich infiltrates in the brainstem [4, 5].

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Th17 cells were subsequently implicated in EAE pathogenesis, following the demonstration that mice deficient in IL-23 (a monokine that stabilizes and expands Th17 cells) fail to succumb to the disease and that in several experimental paradigms, IL-23 stimulation directly confers encephalitogenicity to myelin-specific T cells [6, 7]. Furthermore, Th17 cells accumulate in EAE and MS lesions, and IL-17 is expressed at high levels in circulating leukocytes and cerebrospinal fluid mononuclear cells from patients with active MS [8, 9]. However, analogous to the situation with IFN-γ, neither deficiency nor neutralization of IL-17 completely prevents EAE [10, 11]. Conversely, overexpression of IL-17A in myelin-specific T cells did not enhance their encephalitogenicity [12]. Finally, T-bet-deficient mice are resistant to EAE despite mounting a robust Th17 response against myelin antigens [1, 13, 14].

A number of recent studies have revisited the relative importance of Th1 and Th17 cells in inflammatory demyelination. Th1-polarized myelin-specific T-cell lines were found to be more potent than Th17-polarized lines of the same reactivity in

the adoptive transfer of EAE [15]. These Th17 cells induced a relatively mild and delayed form of EAE, and did so only once they acquired the ability to secrete IFN-γ in host mice. Similar observations have been made in a Th17-mediated model of diabetes [16].

Collectively, the above data suggest that both Th1- and Th17-polarized T-cell lines are capable of initiating EAE. Paradoxically, however, neither IFN-γ nor IL-17 is required for disease induction following active immunization of susceptible mice with myelin antigens in CFA. This raises the question of whether one effector cytokine compensates for the absence of the other in the context of a diverse, polyclonal autoimmune response. In this article, we show that classical EAE, characterized by an ascending paralysis, can occur independent of the activities of both IFN-γ and IL-17. In this setting, GM-CSF and CXCR2 play critical roles in the pathogenic process. In contrast, atypical EAE, with prominent brainstem signs, is IL-17 dependent. Our findings challenge traditional notions regarding the association between the encephalitogenic potential of autoreactive T cells and their production of individual Th1 and/or Th17-related cytokines. They further expand the spectrum of autoimmune diseases to include those mediated by an IFN-γ/IL-17-independent, GM-CSF-dependent pathway.

Results

IL-12-polarized Th1 cells induce conventional EAE independent of IL-17A/F signaling

Myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$/CFA-primed $CD4^+$ T cells were cultured for 96 h with antigen alone or with antigen plus IL-12, IFN-γ and neutralizing antibody to IL-4. The IL-12-stimulated cells produced large quantities of IFN-γ, but no detectable IL-17 or IL-4, upon secondary *in vitro* challenge with MOG peptide (Fig. 1A). In addition, they upregulated T-bet mRNA approximately 16-fold over the level expressed in T cells harvested from the same mice that were stimulated under neutral conditions (Fig. 1B). Only IL-12-stimulated T cells induced EAE, manifested as an ascending paralysis (Fig. 1C and data not shown). Disease was not dependent on IL-17 since it was induced in 100% of IL-17 receptor deficient as well as WT mice with comparable kinetics and severity (Fig. 1C).

In order to assess the role of IFN-γ in the encephalitogenicity of the transferred T cells, the above experiments were repeated using IFN-γ-deficient donors. IL-12-stimulated, MOG-specific IFN-$\gamma^{-/-}$ $CD4^+$ T cells produced no detectable IL-4 upon secondary challenge (Fig. 1A). A small percentage of these cells

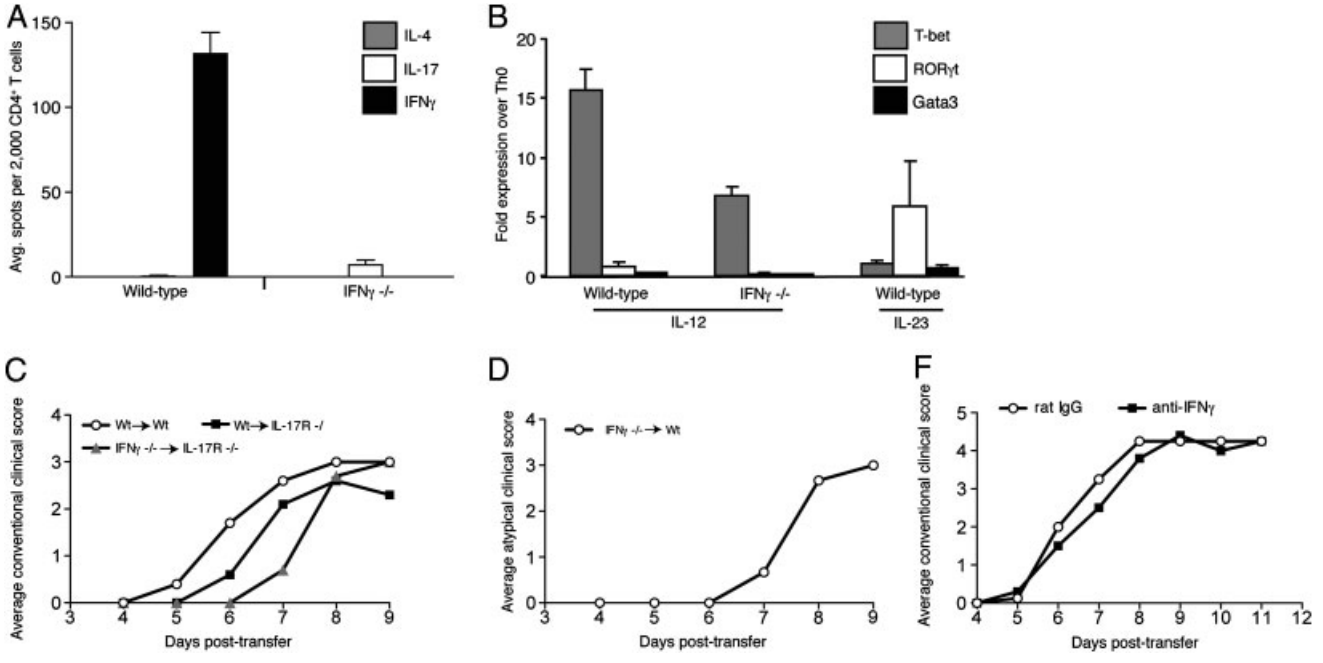

Figure 1. Induction of atypical EAE by IL-12-polarized, IFN-γ-deficient T cells is IL-17-dependent. WT or IFN-$\gamma^{-/-}$ $MOG_{35–55}$/CFA-primed T cells were cultured with antigen under conditions favorable to the generation of Th1 cells (IL-12, IFN-γ, and αIL-4) for 4 days. (A) $CD4^+$ T cells were isolated after 4-day *in vitro* polarization and restimulated in ELISPOT assays for IL-4, IL-17, and IFN-γ with naïve T-depleted splenocytes ±50 μg/mL $MOG_{35–55}$. Background counts with media alone were subtracted. (B) RNA was isolated from purified WT or IFN-$\gamma^{-/-}$ $CD4^+$ T cells after polarization with IL-12 (*left*) or from WT $CD4^+$ T cells after polarization with IL-23 (*right*). Transcription factor expression was assessed by real-time RT-PCR. Data were normalized to GAPDH and are shown as fold expression over the levels measured in $CD4^+$ T cells that were stimulated with $MOG_{35–55}$ alone. (C) MOG-specific WT or IFN-$\gamma^{-/-}$ $CD4^+$ T cells were transferred to either WT or IL-17RA$^{-/-}$ mice. The recipients were scored on a daily basis according to the conventional EAE scale (five to seven mice *per* group). (D) MOG-specific IFN-$\gamma^{-/-}$ $CD4^+$ T cells were transferred to WT mice. The recipients were scored according to the atypical EAE scale (four mice *per* group). (E) IL-17RA$^{-/-}$ mice were injected with MOG-specific IFN-$\gamma^{-/-}$ $CD4^+$ T cells and treated with a neutralizing antibody to IFN-γ or isotype-matched rat IgG. All data shown are representative of two to four independent experiments.

produced IL-17. T-bet mRNA was significantly upregulated after 96 h of stimulation, but to levels 50% lower than those induced in WT cells under the same Th1 polarizing conditions (Fig. 1B). Gata3 and RORγt were expressed at a fraction of the levels measured in unpolarized T cells. Consistent with the previous reports [4, 5], IL-12-stimulated, MOG-specific IFN-$\gamma^{-/-}$ $CD4^+$ T cells triggered an atypical form of EAE in WT hosts, characterized by gait imbalance and neutrophil-rich brainstem infiltrates (Fig. 1D and Fig. 2E, left panel).

Atypical EAE is IL-17RA dependent

Next, we investigated whether IL-17 assumes a compensatory role in EAE initiated by IFN-γ-deficient donor cells. $CD4^+$ T cells were harvested from $MOG_{35–55}$ immunized IFN-γ-deficient mice, cultured with antigen and IL-12, and adoptively transferred into IL-17RA-deficient hosts. IL-17RA-deficient mice succumbed to EAE, but the disease reverted to the conventional clinical phenotype of an ascending paralysis (Fig. 1C). Treatment of IL-17RA-deficient adoptive transfer recipients with a neutralizing antibody against IFN-γ had no impact on EAE severity or incidence, demonstrating that host-derived IFN-γ was not responsible for the classical EAE phenotype (Fig. 1E). Based on these data, we concluded that brainstem, but not spinal cord, inflammation is dependent on IL-17 signaling in host cells. Our findings corroborate the previous observations that a high ratio of IL-17 to IFN-γ-producing cells in the lymph nodes of myelin immunized mice is predictive of brainstem inflammation and that IL-17 neutralization prevents atypical EAE [17].

IL-12-polarized, IFN-γ-deficient cells fail to accumulate in the brains of IL-17RA-null mice

Histopathological studies revealed a similar pattern and degree of spinal cord inflammation in WT and IL-17RA-deficient recipients of IL-12-stimulated WT T cells (Fig. 2A and B). As expected, brainstem infiltrates were prominent in WT mice that developed atypical EAE, following the transfer of IL-12-polarized IFN-γ-deficient effector cells (Fig. 2E, left panel). Despite the predominance of brainstem signs exhibited by these mice, they developed large spinal cord infiltrates that actually extended deeper into the spinal cord parenchyma than the infiltrates in the paraparetic mice that had been injected with WT effector cells (Fig. 2C). IL-12-polarized IFN-$\gamma^{-/-}$ T cells induced large parenchymal infiltrates in the spinal cords, but not the brainstems, of IL-17RA-deficient recipients (Fig. 2D and E, right panel). Collectively, these data indicate that brainstem parenchymal inflammation is predicated on two conditions: (i) IL-17 signaling pathways are intact and (ii) donor cells are unable to produce IFN-γ. In contrast, extensive spinal cord infiltration occurs in the setting of IFN-γ deficiency irrespective of IL-17 signaling.

IFN-γ-deficient effector T cells induce CNS infiltrates that are neutrophil-rich and macrophage-poor

$CD11b^+$ MHC class II^+ cells comprised a significant component of the CNS infiltrates induced by WT effector cells. The absolute number and percentage of CNS $CD11b^+$ class II^+ cells was comparable between WT and IL-17RA-deficient hosts (Fig. 3A,

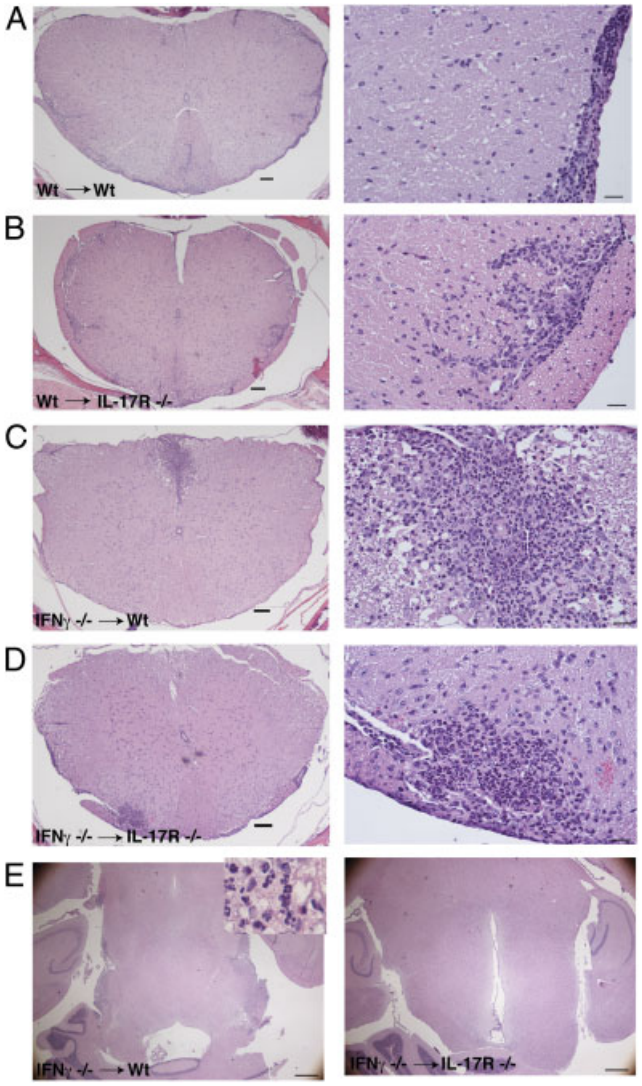

Figure 2. IL-12-polarized IFN-γ-deficient T cells fail to induce brain inflammation in the absence of IL-17A/F signaling. $MOG_{35–55}$/CFA-primed lymph node cells from WT or IFN-$\gamma^{-/-}$ mice were cultured with peptide under Th1-polarizing conditions for 4 days, and transferred to WT or IL-17$RA^{-/-}$ mice. (A–D) Spinal cord and (E) brainstem tissue was fixed at peak EAE, sectioned, and stained with hematoxylin and eosin. (A, B) Spinal cord sections from (A) WT or (B) IL-17$RA^{-/-}$ mice injected with WT Th1 cells. (C, D) Spinal cord sections from (C) WT or (D) IL-17$RA^{-/-}$ mice injected with IL-12-polarized IFN-$\gamma^{-/-}$ cells. Left panels: 4× magnification, bar = 100 μm; right panels: 40×, bar = 20 μm. (E) Brainstem sections from WT (*left*) or IL-17$RA^{-/-}$ (*right*) hosts following transfer of IL-12-polarized IFN-$\gamma^{-/-}$ effector T cells. Brainstem sections were taken at 2× magnification, bar = 500 μm. Inset image was taken at 40× magnification and is 40 μm wide. Representative sections are shown from two or more independent experiments.

Table 1). The presence of MHC class II^+ cells correlated with high levels of CD74 (invariant chain) mRNA in the spinal cord at peak disease (Table 2). It is worth noting that CD74 facilitates trafficking of mature class II molecules to the cell surface [18, 19]. The $CD11b^+$ class II^+ subset was less prevalent in CNS infiltrates induced by IFN-$\gamma^{-/-}$ effectors (Fig. 3A and Table 1).

CNS-infiltrating cells from mice injected with IFN-$\gamma^{-/-}$ effector cells contained a prominent subpopulation of neutrophils, constituting 40–50% of infiltrating cells, irrespective of IL-17 receptor expression (Fig. 3B). In contrast, the percentage of neutrophils in infiltrates induced by WT effector cells ranged between 2 and 25%. The absolute number of neutrophils was significantly lower in IL-17RA$^{-/-}$ than WT recipients of either WT or IFN-$\gamma^{-/-}$ effector cells, respectively (Table 1). Reduced neutrophil numbers in the CNS of IL-17RA$^{-/-}$ hosts correlated with relatively low expression of CXCL1 and CXCL2 (Table 2 and data not shown). Hence, the capacity of effector T cells to produce IFN-γ is the dominant factor determining the cellular composition of the CNS infiltrates they trigger; IL-17 impacts the cellular composition of infiltrates induced by IFN-γ sufficient cells (whereby it facilitates neutrophil enrichment).

There was no consistent difference in the percentage or absolute number of spinal cord-infiltrating $CD4^+$ cells or $B220^+$ cells between the four experimental groups (Fig. 3C and Table 1). $CD8^+$ cells and $NK1.1^+$ cells were not detected in the CNS of any experimental group (data not shown).

GM-CSF is required in EAE induced by the transfer of IFN-$\gamma^{-/-}$ effectors into IL-17RA$^{-/-}$ mice

As shown earlier, IL-12-polarized IFN-$\gamma^{-/-}$ $CD4^+$ cells produce small quantities of IL-17 and no detectable IL-4 or IFN-γ upon reactivation with MOG_{35-55} (Fig. 1A). Hence, they are neither classical Th1 nor Th17 cells, yet they remain highly encephalitogenic (Fig. 1C–E). In order to identify the factors that IL-12-polarized IFN-$\gamma^{-/-}$ cells employ to mediate inflammatory demyelination, we performed targeted PCR arrays on RNA isolated from

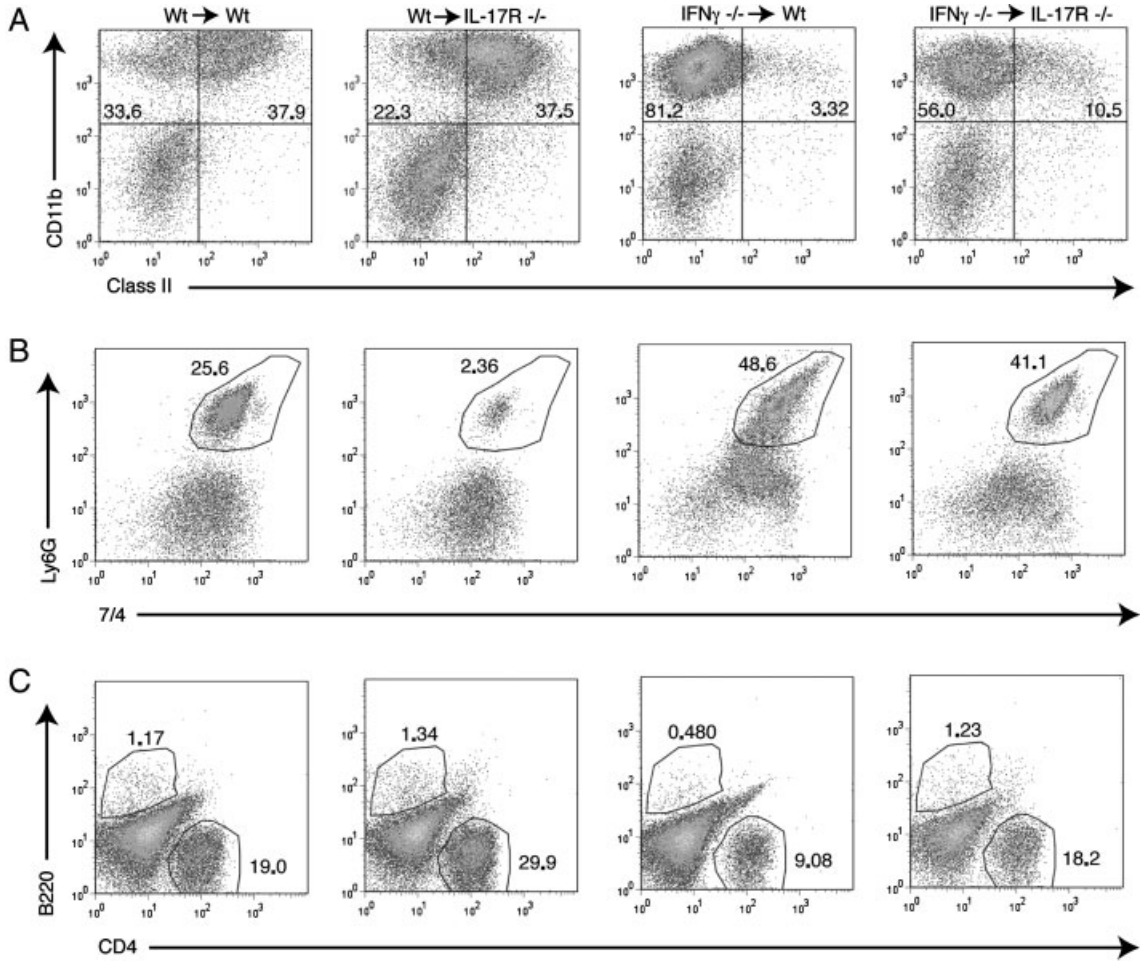

Figure 3. IFN-$\gamma^{-/-}$ effector cells induce neutrophil-rich infiltrates irrespective of IL-17 signaling. Mononuclear cells were isolated from spinal cord tissue at peak disease and analyzed by flow cytometry. IL-12-polarized WT or IFN-$\gamma^{-/-}$ effector cells were injected into WT or IL-17RA$^{-/-}$ hosts as indicated in the figure. Plots show (A) MHC class II *versus* CD11b, (B) 7/4 *versus* Ly6G (gated on $CD11b^+$ cells), or (C) CD4 *versus* B220. The data shown are representative of at least two independent experiments.

Table 1. Absolute cell numbers *per* spinal cord

	Wt→Wt	Wt→IL-17R$^{-/-}$	IFN-γ$^{-/-}$→Wt	IFN-γ$^{-/-}$→IL-17R$^{-/-}$
Total cells/cord[a)]	3.2×10^5 (8.7×10^4)	3.2×10^5 (12.9×10^4)	2.5×10^5 (2.3×10^4)	1.6×10^5 (2.9×10^4)
CD11b$^+$ class II$^-$	0.55×10^5 (3.6×10^4)	0.20×10^5 (0.17×10^4)	2.0×10^5 (2.8×10^4)	0.86×10^5 (2.4×10^4)
CD11b$^+$ class II$^+$	1.4×10^5 (5.2×10^4)	1.4×10^5 (4.7×10^4)	0.068×10^5 (0.34×10^4)	0.12×10^5 (0.048×10^4)
CD11b$^+$ LyG$^+$ 7/4$^+$ [b)]	0.35×10^5 (2.4×10^4)	0.064×10^5 (0.096×10^4)	1.4×10^5(0.77×10^4)	0.46×10^5 (0.43×10^4)
CD4$^+$	0.60×10^5 (2.1×10^4)	0.83×10^5 (5.8×10^4)	0.24×10^5 (0.096×10^4)	0.31×10^5 (0.48×10^4)
B220$^+$	0.038×10^5 (0.21×10^4)	0.044×10^5 (0.032×10^4)	0.019×10^5 (0.10×10^4)	0.019×10^5 (0.092×10^4)

[a)] Two to four experiments/group, with four to seven mice/experiment, giving a minimum total, $n = 8$ mice *per* group. Standard deviations are shown in parenthesis.
[b)] This population is also Ly6C intermediate and class II negative.

Table 2. Relative mRNA expression[a)]

	Wt→Wt	Wt→IL-17R$^{-/-}$	IFN-γ$^{-/-}$→Wt	IFN-γ$^{-/-}$→IL-17R$^{-/-}$
IL-1β	517.5	191.3	246.3	816.3
IL-1f9	11.5	2.40	21.3	66.1
GM-CSF	104.5	115.7	81.30	450.4
CCL22	3.30	3.95	6.23	20.7
CCL17	17.2	19.5	9.80	68.8
CXCL9	5700	9433	24.60	394.7
CXCR2	3.80	−1.10	17.6	28.9
CD74	507.7	512.1	10.40	83.30
CXCL1	81.0	46.8	134	47.5
CXCL2	2008	920.7	964.1	273.3

[a)] Fold induction over naïve spinal cords, normalized to GAPDH, Hprt1, and β actin.

spinal cords of IL-17RA$^{-/-}$ recipients at peak disease. CCL22 and CCL17 were among the molecules that were upregulated above levels in the naïve CNS (Table 2). CCL22 and CCL17 are downstream of the Th2 cytokine IL-13 in delayed type hypersensitivity reactions and in the formation of schistosomal granulomas [20]. However, administration of a neutralizing antiserum against IL-13 did not suppress the induction of EAE in our model (data not shown). Furthermore, IL-13, IL-4, and IL-5 transcripts were not detected either in the IL-12-polarized, IFN-γ$^{-/-}$ effector cells or in the spinal cords of host mice with EAE (data not shown). Taken together, these data indicate that EAE mediated by the non-Th1/Th17 effector cells is not Th2 driven.

IL-1 was dramatically upregulated in the CNS of mice with IL-17/IFN-γ-independent EAE (Table 2). IL-1R antagonism was previously shown to ameliorate EAE induced by active immunization of C57BL/6 WT mice with $MOG_{35–55}$ [21]. IL-1β is an effective inducer of CXCL1/2 in the CNS [22], suggesting that it might be involved in the recruitment of neutrophils to the CNS in our model. Nonetheless, treatment of IL-17RA-deficient mice with the IL-1R antagonist Anakinra (Kineret^{TM}) failed to suppress EAE transferred by IL-12-polarized IFN-γ$^{-/-}$ MOG-specific T cells (Fig. 4A). In contrast, we reproduced the earlier studies demonstrating that Anakinra prevents MOG-specific T-cell priming and the development of neurological deficits when given prophylactically to actively immunized mice (our unpublished observations). This suggests that the importance of IL-1 in EAE is model dependent.

Finally, GM-CSF transcripts were expressed at high levels in the spinal cords of IL-17RA$^{-/-}$ hosts (Table 2). IL-12-polarized IFN-γ-deficient lymph node cells secreted large quantities of GM-CSF protein in response to antigenic challenge *in vitro* (Fig. 4B). Intracellular staining revealed that CD4$^+$ T cells were the major source of GM-CSF protein in CNS infiltrates (Fig. 4C).

In order to determine whether GM-CSF is critical for the manifestation of conventional EAE following adoptive transfer of IL-12-polarized IFN-γ-deficient cells, we treated IL-17RA-deficient adoptive transfer recipients with neutralizing antibody to GM-CSF or rat IgG isotype control. The mice treated with monoclonal antibody to GM-CSF were completely protected from EAE and remained healthy after the cessation of therapy (Fig. 4D). Therefore, production of GM-CSF by infiltrating CD4$^+$ T cells promotes induction of EAE in the absence of IFN-γ and IL-17A/F signals.

EAE is CXCR2 dependent in the absence of CD4-derived IFN-γ and IL-17A/F signaling

We found that neutrophils constitute 40–50% of the spinal cord-infiltrating cells in the non-Th1/Th17 model (Fig. 3B). In order to assess the requirement of neutrophil trafficking to the CNS for the manifestation of clinical EAE, we treated IL-17RA-deficient mice with a neutrophil-depleting CXCR2 anti-serum, following adoptive transfer of IL-12-modulated MOG-specific IFN-γ$^{-/-}$ cells. Flow

cytometric analyses demonstrated that the anti-CXCR2 treatments depleted neutrophils from the blood and spleen, but spared other leukocyte subsets (data not shown). EAE was completely abrogated in anti-CXCR2-treated hosts (Fig. 4E), demonstrating the importance of neutrophils in the non-Th1/Th17 model of EAE.

Discussion

Drugs that neutralize T-cell-derived cytokines have been proposed as putative therapies for autoimmune disease. Our findings suggest that the emergence of redundant immunopathogenic pathways could undermine such a strategy in MS. In this article, we demonstrate that autoimmune demyelination can occur in the combined absence of both IFN-γ and IL-17. By extension, autoreactive T cells other than those that fall within the classic Th1 and Th17 lineages are capable of mediating CNS autoimmunity. Th9 cells were recently heralded as a novel encephalitogenic subset [23]. However, the IL-12-stimulated IFN-$\gamma^{-/-}$ T cells used in our studies did not secrete IL-9, nor was IL-9 detectable in the CNS of the host mice into which they were injected (unpublished data). We found that disease induction in our model is dependent on GM-CSF, and that $CD4^+$ T cells were the primary source of this mediator in the inflamed CNS. This suggests that the MOG-specific donor T cells produced GM-CSF themselves and/or induced its production in bystander host T cells. Hence, myelin-specific

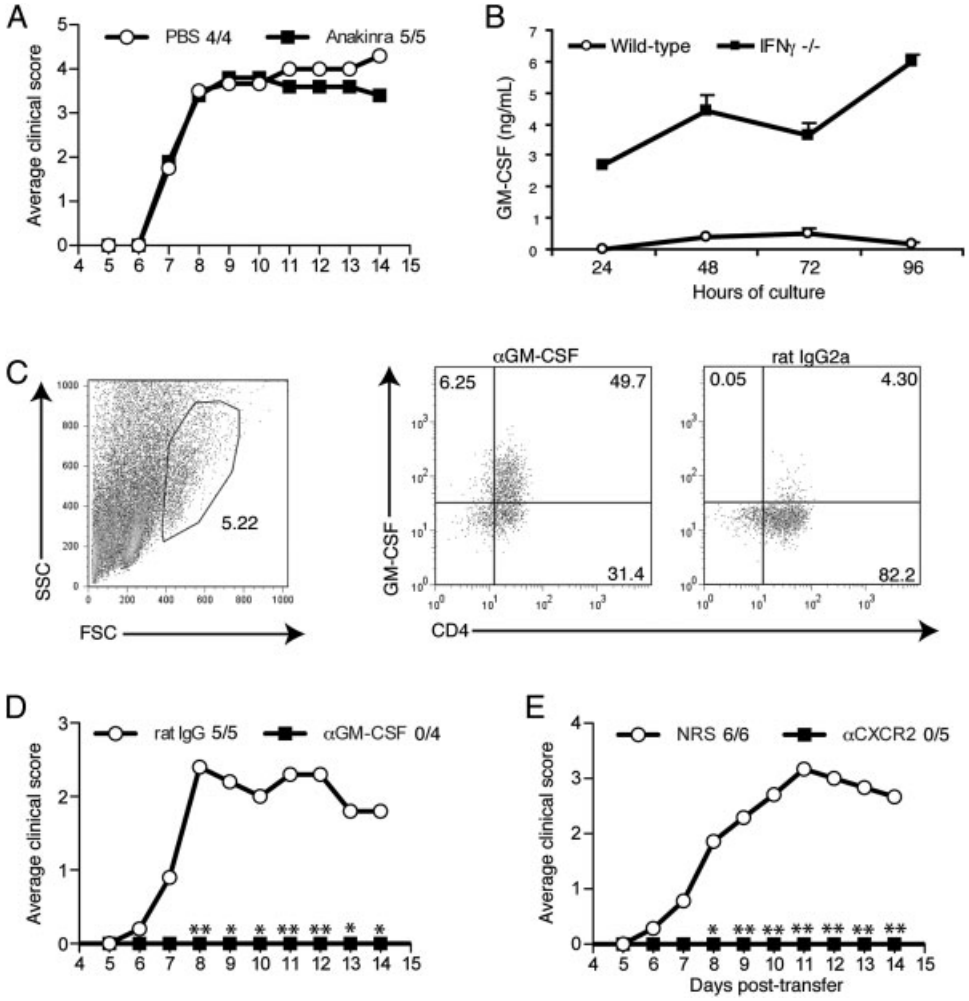

Figure 4. EAE induced *via* an IFN-γ/IL-17-independent pathway is GM-CSF dependent. $MOG_{35–55}$/CFA-primed lymph node cells were harvested from IFN-$\gamma^{-/-}$ mice and cultured with antigen and IL-12 prior to adoptive transfer into IL-17RA$^{-/-}$ hosts. (A) Clinical scores of recipient mice that were treated with the IL-1R antagonist, Anakinra, or vehicle daily from day 0 to day 10 post-transfer (2.5 mg/dose). (B) Supernatants were collected from the lymph node cell cultures every 24 h prior to adoptive transfer. GM-CSF levels were measured by ELISA. (C) Mononuclear cells were isolated from the spinal cords of IL-17R$^{-/-}$ mice at peak EAE following the adoptive transfer of MOG-specific, IFN-$\gamma^{-/-}$ $CD4^+$ T cells. GM-CSF expression was assessed by intracellular staining and flow cytometry. Dot plots (GM-CSF stain, *middle*; rIgG2a isotype, *right*) are gated on blasting lymphocytes (*left*). About 83% of GM-CSF-producing cells were $CD4^+CD3^+$, irrespective of the gate used (data not shown) (D, E) Mice were treated with (D) GM-CSF-neutralizing monoclonal antibodies or (E) anti-serum to CXCR2 from day 0 to day 10 post-transfer. Anti-GM-CSF (0.5 mg/dose) and CXCR2 anti-serum (0.5 mL/dose) were administered every other day. For all *in vivo* experiments, each treatment group contained eight to ten mice. Disease incidence is shown at the right-hand side of the relevant symbol. The data shown are representative of two or more experiments with similar results. $^{**}p<0.01$, $^{*}p<0.05$, compared with the control group.

T cells with a diverse array of cytokine profiles have encephalitogenic potential. In some forms of EAE, one cell type might predominate. In others, cells of multiple phenotypes might contribute to CNS injury in an additive or synergistic manner. A common downstream mediator that is critical for demyelination has yet to be identified.

It was previously reported that C57BL/6 mice deficient in GM-CSF or GM-CSF receptor are resistant to EAE induced by active immunization with MOG peptide [24–26]. GM-CSF could, theoretically, foster inflammation in the CNS by stimulating local and/or infiltrating APC to upregulate MHC class II and costimulatory molecules. There is also evidence that GM-CSF can trigger IL-6 production by dendritic cells, and hence enhance survival of autoreactive T cells [27]. In addition, we have found that GM-CSF facilitates the mobilization of classII$^{-/lo}$Ly6C^{hi} bone marrow cells in MOG-immunized mice [24]. These immature myeloid cells ultimately accumulate in the CNS and differentiate into dendritic cells. However, as with IL-17, a requirement for GM-CSF is not universal across all EAE models. For example, EAE triggered by the adoptive transfer of PLP-specific Th1 cells in SJL mice is GM-CSF independent [28]. In that model, IFN-γ production by donor T cells results in upregulation of ELR$^-$ CXC chemokines and the recruitment of classII^{hi} CD11b$^+$ monocytes to the CNS. This could obviate the requirement for GM-CSF for the establishment of a local population of activated APC.

The fact that depletion of CXCR2$^+$ cells suppressed EAE substantiates a nonredundant role for neutrophils in our model. It is likely that this intervention will be most beneficial in EAE mediated by IFN-γ$^{-/-}$ effectors, since neutrophils are sparse in CNS infiltrates induced by WT T cells [29]. Our data also suggest that neutrophils may contribute significantly to atypical EAE, since the percentage and absolute number of neutrophils in the CNS of WT hosts injected with IFN-γ$^{-/-}$ effector cells was remarkably elevated (Fig. 2E and 3B). Neutrophils mediate blood–brain barrier breakdown in animal models of viral encephalitis and following intracerebral injection of IL-1β [30, 31]. We speculate that depletion of neutrophils prevents the increased blood–brain barrier permeability that ordinarily heralds the onset of clinical EAE. Other plausible mechanisms of action of neutrophils include altering the cytokine/chemokine milieu in the CNS microenvironment and facilitating the mobilization of myeloid cell precursors from the bone marrow.

This study provides further evidence that IL-12 is a powerful inducer of encephalitogenicity in CD4$^+$ T cells, and that this function is independent of IFN-γ as well as IL-17A/F. It remains unclear why IL-12 does not compensate for IL-23 deficiency in MOG$_{35–55}$-immunized IL-23p19$^{-/-}$ C57BL/6 mice. One explanation is that the conditions used for active immunization (*i.e.* subcutaneous administration of antigen in CFA and systemic administration of *Bordetella pertussis* toxin) are more effective in stimulating IL-23, than IL-12, production by the APC that prime myelin-reactive T cells *in vivo* [32]. On the other hand, IL-23 has been shown to restrain regulatory T-cell populations in a colitis model [33], raising the possibility that p19$^{-/-}$ mice have a more robust regulatory T-cell compartment and are generally more resistant to autoimmunity on that basis. Similarly, a contributing factor to the susceptibility of IL-12 p35-deficient mice to EAE may be the absence of the regulatory cytokine IL-35, which shares the p35 subunit with IL-12 [34]. Our system avoids these complicating factors by analyzing the direct effects of IL-12 on the encephalitogenic properties myelin-specific CD4$^+$ T cells *ex vivo*. The mechanism by which IL-12 promotes the encephalitogenicity of IFN-γ$^{-/-}$ effector cells remains to be elucidated.

We have previously shown that myelin-reactive Th1 and Th17 cells evoke distinct cytokine and chemokine pathways to induce inflammatory demyelinating disease with a similar clinical presentation [28]. Here, we expand this concept further by showing that conventional EAE can also be mediated by a non-IFN-γ/IL-17 effector pathway. Similarly, MS likely represents a heterogeneous group of diseases, mediated by distinct cytokine pathways, with a common clinical endpoint. In support of this contention, analysis of brain specimens has demonstrated that MS can be classified into at least four subtypes based on distinctive histopathological features [35]. Furthermore, MS patients exhibit diverse responses to specific immunomodulatory interventions. In order to achieve optimal control over disease, in the future it might be necessary to stratify patients based on their immunological profiles and customize treatment accordingly.

Materials and methods

Mice

Briefly, 8- to 12-wk-old WT and IFN-γ-deficient C57BL/6 mice were obtained from NCI Frederick (Frederick, MD, USA) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively, and housed in microisolator cages. All animal protocols were approved by the University Committee on Use and Care of Animals.

Immunization and T-cell culture

Mice were injected subcutaneously with 100 μg MOG$_{35–55}$ MEVGWYRSP-FSRVVHLYRNGK (Biosynthesis, Lewisville, TX, USA) in complete Freund's adjuvant (Difco, Detroit, MI, USA). Draining lymph nodes were harvested 12–14 days post-immunization, pooled, and passed through a 70-μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Lymph node cells were cultured *in vitro* for 4 days with MOG$_{35–55}$ under conditions favorable to the generation of Th1 cells (rmIL-12, 5 ng/mL; rmIFN-γ, 2 ng/mL; anti-IL-4 (clone 11B11), 10 μg/mL).

Induction and treatment of EAE

After 4 day *in vitro* culture, lymph node cells were collected and CD4$^+$ T cells were purified using negative selection columns (Cedarlane) to a purity of 88–95%, confirmed by flow cytometry. In

total, 5–6 $\times 10^6$ $CD4^+$ T cells were injected intraperitoneally in sterile PBS, and mice were observed daily for signs of EAE. Those with conventional EAE were scored as described previously [29]. For atypical EAE, the following scale was used: 0, asymptomatic; 1, slight listing/difficulty righting; 2, obvious imbalance but able to ambulate; 3, severely impaired balance/ambulation; and 4, incapacitated due to inability to maintain upright posture/spinning. Mean clinical scores were compared using the Student's *t*-test.

Antibodies used for *in vivo* neutralization were anti-GM-CSF (clone 22E9.11) and anti-IFN-γ (clone XMG1.2). Polyclonal rabbit anti-serum against IL-13 and CXCR2 were provided by Dr. Nicholas Lukacs and Dr. Thomas Lane, respectively. Rat IgG was used as a control for monoclonal antibody treatments, and normal rabbit serum (Sigma-Aldrich, St. Louis, MO, USA) was used as a control for the IL-13 and CXCR2 anti-sera. The IL-1R antagonist Anakinra was purchased from commercial sources. All treatments were given intraperitoneally.

Quantitative RT-PCR

RT-PCR was done using 384-well mouse "inflammatory response and autoimmunity" PCR arrays (SA Biosciences, Frederick, MD, USA). Arrays were performed on individual mice (three mice/group) and genes of interest were confirmed independently by quantitative RT-PCR (five to eight mice/group). Whole spinal cords were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNA was isolated by phenol/chloroform extraction. Genomic DNA was removed by Turbo DNase (Ambion, Foster City, CA, USA) and purity of RNA was confirmed by a ratio of A260:A280. cDNA was synthesized using a RT^2 First Strand kit (SA Biosciences). Data were normalized to GAPDH, HPRT1, and β actin, and are shown as fold increase over naïve spinal cords.

Flow cytometry

Spinal cords were harvested at peak disease and incubated with DNase (1 mg/mL) and collagenase (2 mg/mL) for 1 h at 37°C. Mononuclear cells were isolated over a 30/70% Percoll gradient (GE Healthcare, Piscataway, NJ, USA). Flow cytometry was performed using a BD Facs Canto.

Histological staining

After perfusion, spinal cords were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin using standard protocols [29].

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Abbreviation: **MOG:** myelin oligodendrocyte glycoprotein

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