

Neutrophil-related factors as biomarkers in EAE and MS

Julie M. Rumble,¹ Amanda K. Huber,¹ Gurumoorthy Krishnamoorthy,⁶ Ashok Srinivasan,² David A. Giles,¹ Xu Zhang,³ Lu Wang,³ and Benjamin M. Segal^{1,4,5}

¹Holtom-Garrett Program in Neuroimmunology, Department of Neurology,²Department of Radiology,³Department of Biostatistics, and ⁴Graduate Program in Immunology, University of Michigan, Ann Arbor, MI 48109

⁵Neurology Service, VA Ann Arbor Healthcare System, Ann Arbor, MI 48105

⁶Department of Neuroimmunology, Max Planck Institute for Neurobiology, 82152 Martinsried, Germany

A major function of T helper (Th) 17 cells is to induce the production of factors that activate and mobilize neutrophils. Although Th17 cells have been implicated in the pathogenesis of multiple sclerosis (MS) and the animal model experimental autoimmune encephalomyelitis (EAE), little attention has been focused on the role of granulocytes in those disorders. We show that neutrophils, as well as monocytes, expand in the bone marrow and accumulate in the circulation before the clinical onset of EAE, in response to systemic up-regulation of granulocyte colony-stimulating factor (G-CSF) and the ELR⁺ CXC chemokine CXCL1. Neutrophils comprised a relatively high percentage of leukocytes infiltrating the central nervous system (CNS) early in disease development. G-CSF receptor deficiency and CXCL1 blockade suppressed myeloid cell accumulation in the blood and ameliorated the clinical course of mice that were injected with myelin-reactive Th17 cells. In relapsing MS patients, plasma levels of CXCL5, another ELR⁺ CXC chemokine, were elevated during acute lesion formation. Systemic expression of CXCL1, CXCL5, and neutrophil elastase correlated with measures of MS lesion burden and clinical disability. Based on these results, we advocate that neutrophil-related molecules be further investigated as novel biomarkers and therapeutic targets in MS.

CORRESPONDENCE

Benjamin M. Segal:
bmsegal@umich.edu

Abbreviations used: BBB, blood-brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability status scale; G-CSF, granulocyte-colony stimulating factor; MS, multiple sclerosis; p.i., post immunization; PTx, *Bordetella pertussis* toxin.

It is widely believed that myelin-reactive CD4⁺ T cells initiate the formation of demyelinating lesions in the central nervous system (CNS) during multiple sclerosis (MS). That premise is supported by extensive circumstantial evidence from animal models and genome-wide association studies (Steinman and Zamvil, 2006; Sawcer et al., 2011), and by the mechanism of action of disease-modifying agents (DMAs) that suppress clinical relapses by targeting lymphocytes (Stüve, 2008; Kowarik et al., 2011). Having crossed the blood-brain barrier (BBB), myelin-reactive CD4⁺ T cells induce chemokines and vasoactive molecules, resulting in the local recruitment of a heterogeneous population of myeloid cells. Infiltrating myeloid cells secrete factors that escalate the inflammatory response and present antigen to reactivate encephalitogenic T cells within the CNS (Kwakami et al., 2004). Thus, MS disease activity is dependent on an intricate interplay between the adaptive and innate immune systems. Nevertheless, none of the FDA-approved

DMAs used to treat MS were designed to target innate immune cells.

Monocytes and macrophages can inflict damage in the CNS by phagocytosing the myelin sheath and by releasing factors that are toxic to oligodendrocytes and axons (Epstein et al., 1983; Lin et al., 1993; Toft-Hansen et al., 2004; Mantovani et al., 2011). Several studies have revealed dysregulation of peripheral monocytes and monocyte-derived dendritic cells in MS, manifested by increased expression of costimulatory molecules and polarizing cytokines (Balashov et al., 1997; Comabella et al., 1998; Karni et al., 2002, 2006; Vaknin-Dembinsky et al., 2006). Granulocytes have received less attention because they are relatively rare in mature MS and experimental autoimmune encephalomyelitis (EAE)

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lesions. However, a major function of Th17 cells, identified as critical effector cells in EAE and MS, is to induce the expression of neutrophil activating molecules such as granulocyte-colony stimulating factor (G-CSF) and ELR⁺ CXC chemokines (Kolls and Lindén, 2004; Khader et al., 2009; Onishi and Gaffen, 2010; Pelletier et al., 2010; Becher and Segal, 2011). Indeed, cerebrospinal fluid (CSF) samples obtained from newly diagnosed MS patients at clinical relapse had elevated IL-17A levels which positively correlated with CSF neutrophil counts (Kostic et al., 2014). A pathogenic role of neutrophils in human autoimmune demyelinating disease is further suggested by the occurrence of severe exacerbations in some MS and NMO patients when given recombinant G-CSF (Openshaw et al., 2000; Burt et al., 2001; Jacob et al., 2012). Transcripts encoding G-CSF are expressed in MS lesions but not normal appearing white matter (Lock et al., 2002), and the neutrophil-attracting chemokine CXCL8 has been detected in CSF of MS patients (Ishizu et al., 2005; Campbell et al., 2010). It was recently reported that circulating neutrophils are more numerous, and exhibit a primed state, in individuals with MS (Naegele et al., 2012). These observations echo prior studies that documented enhanced neutrophil protease activity and integrin receptor expression in patients with MS during relapse when compared with MS patients in remission, healthy controls, or individuals with other neurological diseases (Aoki et al., 1984; Guarnieri et al., 1985; Ziaber et al., 1998).

Despite the paucity of neutrophils in typical mature MS lesions, studies in the EAE model indicate that they comprise a higher frequency of infiltrating cells during the preclinical phase and could play a role in nascent lesion development by mediating BBB and blood-CSF barrier breakdown, and/or by stimulating the maturation of local APCs (Carlson et al., 2008; Christy et al., 2013; Steinbach et al., 2013). In the vast majority of MS tissue samples obtained by biopsy or autopsy, lesions are subacute or chronic. Hence, the impression that neutrophils do not comprise a significant leukocyte population in the CNS during MS might reflect a sampling bias. Irrespective of their presence in the CNS, neutrophils could, conceivably, promote disease activity from the periphery. Hence, activation of neutrophils within the bone marrow during autoimmune demyelinating disease would drive the mobilization of monocytes, as well as neutrophils themselves, into the circulation, thereby increasing the pool of myeloid cells available for recruitment to the CNS (Singh et al., 2012).

In the current study, we serially analyzed the cellular composition of bone marrow, blood, and CNS infiltrates after induction of EAE by active immunization or Th17 transfer. We found that neutrophils and monocytes expanded in the bone marrow and accumulated in the circulation during the preclinical phase of EAE in response to systemic up-regulation of G-CSF and the CXCR2 binding chemokine CXCL1. G-CSF receptor (G-CSFR) deficiency and CXCR2 blockade suppressed the accumulation of circulating myeloid cells and ameliorated the clinical course. In a model of spontaneous EAE, circulating neutrophils also expanded early in the clinical course. The translational relevance of these results is underscored by

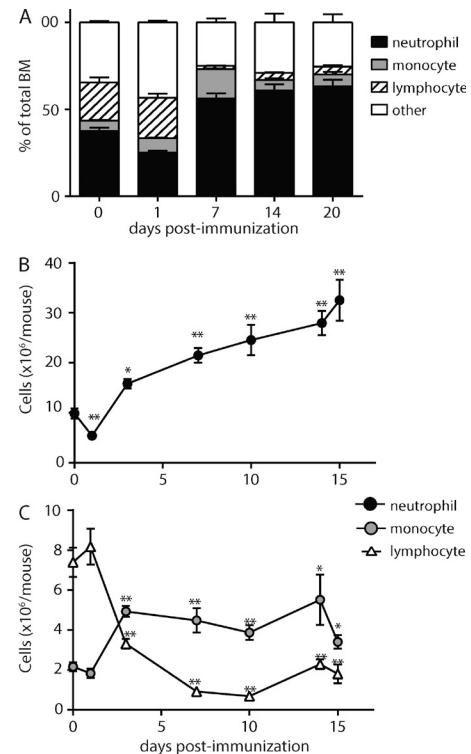


Figure 1. Neutrophils and monocytes expand in the bone marrow after EAE induction. (A–C) WT mice were immunized with MOG_{35–55} in CFA and injected with PTx on days 0 and 2 p.i. Bone marrow cells were flushed from femurs and tibiae of representative mice at serial time points and analyzed by flow cytometry to enumerate neutrophils (CD31[−]Ly6C^{int}Ly6G⁺, black bars/closed circles), monocytes (CD31⁺Ly6C^{hi}Ly6G[−], gray bars and circles), and lymphocytes (CD31⁺Ly6C[−], diagonal stripes/open triangles). (A) Percentage of leukocyte subsets within bone marrow cells. Data are representative of six experiments ($n \geq 3$ per time point). (B and C) Absolute numbers of neutrophils (B) and monocytes and lymphocytes (C) recovered per mouse. Data are representative of nine experiments ($n \geq 3$ per time point). All graphs indicate means; error bars denote SEM. *, $P < 0.05$; **, $P < 0.01$ compared with unimmunized mice.

our additional finding that plasma levels of CXCL5, another CXCR2 ligand, are elevated in relapsing MS patients coincident with acute lesion development. Furthermore, expression of CXCL1, CXCL5, and neutrophil elastase correlated with measures of MS lesion burden and clinical disability. The results of our study endorse further evaluation of myeloid-related molecules as novel biomarkers and therapeutic targets in MS and other inflammatory demyelinating disorders.

RESULTS

Intramedullary neutrophils and monocytes expand after active immunization with myelin antigens

Myeloid cells are short-lived after tissue infiltration, raising the question of how they are replenished in the setting of relapsing or chronic autoimmune disease. We have previously shown that CD11b⁺CD115⁺Ly-6C^{hi} blood monocytes are a precursor of CNS DCs and macrophages in EAE lesions (King et al., 2009). These cells are dynamically regulated during the

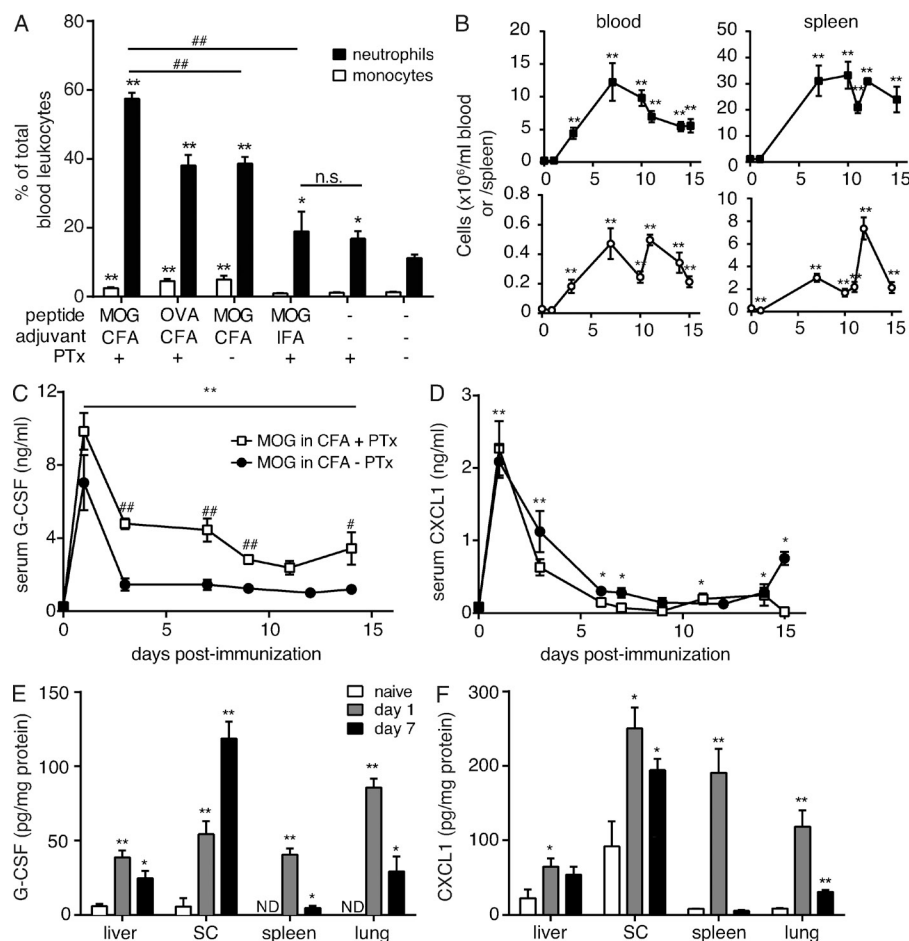


Figure 2. Serum G-CSF and CXCL1 are up-regulated and myeloid cells are mobilized into the circulation during EAE.

(A–D) Peripheral blood cells and sera were collected from mice that had been primed with MOG_{35–55} or OVA_{323–339} in CFA or MOG_{35–55} in IFA, with or without administration of PTx. (A) Percentage of circulating neutrophils (CD11b⁺Ly6C^{int}Ly6G⁺, black bars) and monocytes (CD11b⁺Ly6C^{hi}Ly6G[–], white bars) on day 7 p.i. Shown is a representative of three experiments ($n \geq 6$ mice per group). (B) Numbers of neutrophils (top panels, closed squares) and monocytes (bottom panels, open circles) per ml of blood or per spleen at serial time points after active immunization with MOG_{35–55} in CFA. Data are pooled from 10 experiments (blood, $n \geq 6$ per time point) or 5 experiments (spleen, $n \geq 3$ per time point). (C and D) Serum levels of G-CSF (C) and CXCL1 (D) were measured by ELISA. Data were pooled from 10 experiments ($n \geq 3$ mice per time point). (E and F) G-CSF (E) and CXCL1 (F) were measured in tissue homogenates and normalized to total protein. Shown is a representative of two experiments ($n = 4$ mice per time point). All graphs indicate means; error bars denote SEM. *, $P < 0.05$; **, $P < 0.01$ compared with unimmunized mice. #, $P < 0.05$; ##, $P < 0.01$ between groups. ND = not detectable.

course of EAE, accumulating in the blood and CNS immediately before clinical episodes. However the source of the inflammatory monocytes (i.e., from the bone marrow or extramedullary sites), and the factors that modulate their frequency and migration patterns, have yet to be investigated in detail. Similarly, relatively little is known about the activation and distribution of neutrophils, or their interactions with other myeloid populations, in myelin-immunized mice.

We speculated that intramedullary myeloid cells expand in the bone marrow and then migrate into the circulation to support new CNS lesion formation. To assess our hypothesis, C57BL/6 mice were actively immunized with myelin oligodendrocyte glycoprotein peptide (MOG_{35–55}) in CFA and bone marrow cells were analyzed at serial time points thereafter. We found that the frequencies and absolute numbers of intramedullary neutrophils and monocytes rose during the preclinical phase (Fig. 1, A–C). The numbers of both myeloid subsets remained elevated above baseline at clinical onset (days 10–14 post immunization [p.i.]). In contrast, the number and percentage of intramedullary lymphocytes fell over the same time frame (Fig. 1, A and C).

The expansion of monocytes and neutrophils in the bone marrow was mirrored in the blood and spleen, from preclinical time points through EAE onset (Fig. 2, A and B). Interestingly,

coadministration of *Bordetella pertussis* toxin (PTx), which is necessary for the clinical manifestation of disease in MOG/CFA immunized C57BL/6 mice, augmented the frequency of peripheral blood neutrophils (Fig. 2 A). Mice immunized with MOG peptide in IFA and injected with PTx on days 0 and 2 p.i. had comparable percentages of circulating myeloid cells to mice treated with PTx alone (Fig. 2 A). This suggests that myeloid cells accumulate in the blood largely in response to mycobacterial components in CFA. Immunization with a peptide of ovalbumin in CFA induced a significant expansion of circulating neutrophils and monocytes, demonstrating that this phenomenon is not antigen-specific (Fig. 2 A).

G-CSF and CXCL1 levels rise in the serum during the preclinical stage of EAE

We next investigated the factors responsible for stimulating myeloid cell expansion in the bone marrow and their mobilization into the circulation in our model. G-CSF levels rose dramatically in the serum of actively immunized mice, peaking at day 1 p.i. and remaining elevated through day 14 (Fig. 2 C). Reminiscent of its effects on the frequency of circulating neutrophils (Fig. 2 A), PTx enhanced the expression of serum G-CSF. The neutrophil-attracting chemokine CXCL1, but not CXCL2, spiked in the serum on day 1 p.i. and fell to near baseline levels

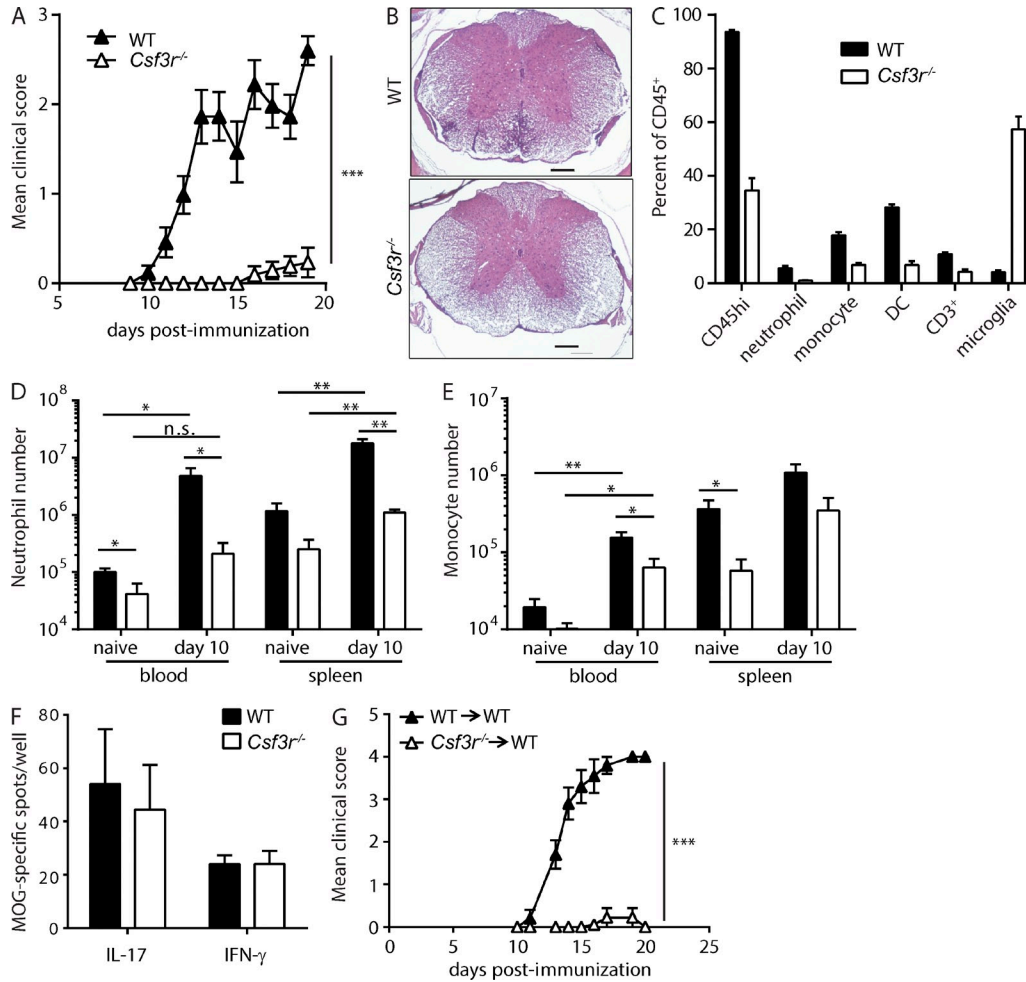


Figure 3. EAE is dependent on G-CSF signaling in hematopoietic cells. (A–E) WT (closed triangles, black bars) and *Csf3r*^{-/-} (open triangles, white bars) mice were actively immunized with MOG_{35–55} in CFA. (A) Mean clinical scores (*n* = 25 WT, 21 *Csf3r*^{-/-} pooled from five independent experiments). (B) Representative paraffin sections of spinal cords stained with H&E. (C) Cell subsets recovered from spinal cords at peak of disease, shown as a percentage of total CD45⁺ cells. Cell types were defined as follows: neutrophil (CD45^{hi}, CD11b⁺, Ly6G⁺), monocyte (CD45^{hi}, CD11b⁺, CD11c⁻, Ly6G⁻), DC (CD45^{hi}, CD11b⁺, CD11c⁺), CD3⁺ (CD45^{hi}, CD3⁺), and microglia (CD45^{mid} CD11b⁺). Data are representative of two experiments (*n* ≥ 4 mice/group). (D and E) Circulating and splenic neutrophils (D) and monocytes (E) were enumerated by flow cytometry. Data were pooled from two independent experiments (*n* ≥ 5 per group). (F) MOG-specific cytokine production by draining lymph node cells measured by ELISpot. Data are representative of three experiments (*n* = 3–5 mice per group). In the experiment shown there were 2.4 × 10⁵ total cells/well. (G) Mean clinical scores of WT to WT (closed triangles, *n* = 10) or *Csf3r*^{-/-} to WT (open triangles, *n* = 9) bone marrow chimeric mice after active immunization with MOG_{35–55} in CFA. Data are representative of three experiments. All graphs indicate means; error bars denote SEM. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. Bars, 100 μm.

by day 7 (Fig. 2 D). Co-administration of PTx did not alter CXCL1 levels. To determine the source of G-CSF and CXCL1, we harvested an array of tissues on days 1 and 7 p.i. and performed ELISAs on homogenate supernatants. G-CSF and CXCL1 production were up-regulated in the spleen, lungs, liver, and spinal cord at both time points (Fig. 2, E and F).

The clinical manifestation of EAE is dependent on intact G-CSF signaling in hematopoietic cells

As mentioned earlier, administration of G-CSF has been associated with severe exacerbation of MS (Openshaw et al., 2000). To directly assess the role of endogenous G-CSF in the development of EAE, we compared the clinical courses of

MOG-immunized C57BL/6 WT and G-CSFR-deficient (*Csf3r*^{-/-}) mice (Liu et al., 1996). *Csf3r*^{-/-} mice were highly resistant to EAE (Fig. 3 A). Whereas 80% of WT mice manifested neurological deficits beginning on day 10, only 14% of *Csf3r*^{-/-} mice succumbed to clinical EAE, with the earliest signs presenting on day 15. Histological analyses revealed multifocal inflammatory disease in the spinal cords of WT mice at peak clinical EAE but no pathological changes in *Csf3r*^{-/-} mice euthanized on the same day p.i. (Fig. 3 B). Consistent with these findings, cells isolated from the CNS of WT mice were composed primarily of infiltrating hematopoietic cells, whereas microglia were the most prevalent cell type isolated from the CNS of *Csf3r*^{-/-} mice (Fig. 3 C). As previously

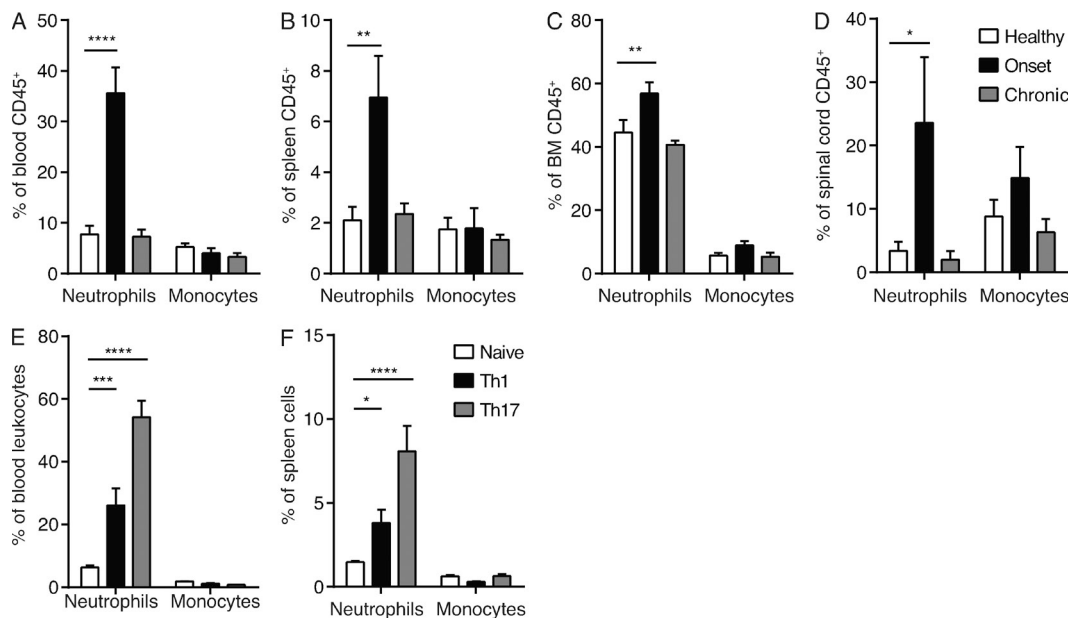


Figure 4. Neutrophils accumulate at onset of disease in adjuvant-free models of EAE. (A–D) OSE mice were sacrificed when healthy ($n = 5$; white bars), within 2 d of the onset of clinical EAE ($n = 5$; black bars) or during the chronic stages of EAE ($n = 4$; gray bars). Peripheral blood cells (A), splenocytes (B), BM cells (C) and spinal cord-infiltrating cells (D) were collected. Neutrophils and monocytes were enumerated by flow cytometry. (E and F) WT mice were injected with IL-12-polarized (Th1; black bars) or IL-23-polarized (Th17; gray bars) MOG-specific T cells. At day 7 after transfer, blood (E) and spleens (F) were collected and neutrophils and monocytes were enumerated by flow cytometry. Data are pooled from four (Th17 transfers) or two (Th1 transfers) experiments ($n \geq 10$ mice per group). All graphs indicate means; error bars denote SEM. *, $P < 0.05$; **, $P < 0.005$; ***, $P = 0.001$; ****, $P < 0.0001$, by two-way ANOVA, correcting for multiple comparisons.

described in infectious disease models (Basu et al., 2000), “emergency” mobilization of myeloid cells occurred in $Csf3r^{-/-}$ mice after immunization with MOG in CFA in that peripheral pools of neutrophils and monocytes expanded over baseline. However, at the time of clinical onset, the absolute numbers of circulating and splenic neutrophils, and of circulating monocytes, were significantly lower in $Csf3r^{-/-}$ mice than in WT mice (Fig. 3, D and E). The low incidence and mild course of disease in $Csf3r^{-/-}$ mice was not due to insufficient $CD4^{+}$ Th priming because MOG-immunized $Csf3r^{-/-}$ and WT mice mounted comparable IL-17 and IFN- γ responses upon antigenic challenge ex vivo (Fig. 3 F).

Although primarily expressed on neutrophils, G-CSFR has been detected on nonhematopoietic cells, including glia and subsets of neurons (Kadota et al., 2012). Therefore, we constructed bone marrow chimeric mice to establish whether G-CSFR deficiency in immune cells alone is sufficient to confer resistance to EAE. Lethally irradiated WT hosts were reconstituted with either WT or $Csf3r^{-/-}$ bone marrow cells. After active immunization with MOG in CFA, WT \rightarrow WT bone marrow chimeras developed severe EAE at 100% incidence. In contrast, $Csf3r^{-/-}$ \rightarrow WT bone marrow chimeras were highly resistant to disease induction (11% incidence), simulating the phenotype of germline $Csf3r^{-/-}$ mice (Fig. 3 G).

Peripheral neutrophils expand at clinical onset in adjuvant-free models of EAE

The data in Fig. 2 A suggest that TLR ligands in CFA drive the systemic up-regulation of G-CSF and consequent shifts in

myeloid cell populations that we observed in MOG-immunized mice. This prompted us to question whether peripheral myeloid cells expand and mobilize in models of EAE that do not require the administration of exogenous antigen or adjuvant. Approximately 50% of C57BL/6 mice that coexpress MOG-specific T cell receptor and B cell receptor transgenes (OSE mice) spontaneously develop inflammatory demyelinating lesions and neurological deficits by 12 wk of age when maintained under specific pathogen-free conditions (Krishnamoorthy et al., 2006). We found that PBMC, splenocytes, bone marrow cells, and CNS mononuclear cells harvested from OSE mice with acute EAE (1–3 d after onset) had elevated percentages of neutrophils, but not monocytes, when compared with analogous cell preparations from healthy OSE mice (Fig. 4, A–D). The frequency of neutrophils fell to baseline during chronic EAE (>14 d after onset).

As an alternative to active immunization, EAE can be induced via the injection of myelin-primed, IL-23 modulated $CD4^{+}$ Th17 cells or IL-12 modulated Th1 cells into naive WT C57BL/6 hosts (Kroenke et al., 2008; Kroenke and Segal, 2011). Similar to our findings in OSE mice with spontaneous EAE, the frequencies of circulating and splenic neutrophils, but not monocytes, rose above baseline in adoptive transfer recipients of either Th1- or Th17-polarized T cells at clinical onset (Fig. 4, E and F). Th17 effector cells mediated the most profound neutrophil expansion, consistent with the established role of IL-17A as an inducer of granulocyte mobilizing factors (Kolls and Lindén, 2004; Onishi and Gaffen, 2010; Pelletier et al., 2010). The frequency of bone marrow neutrophils did

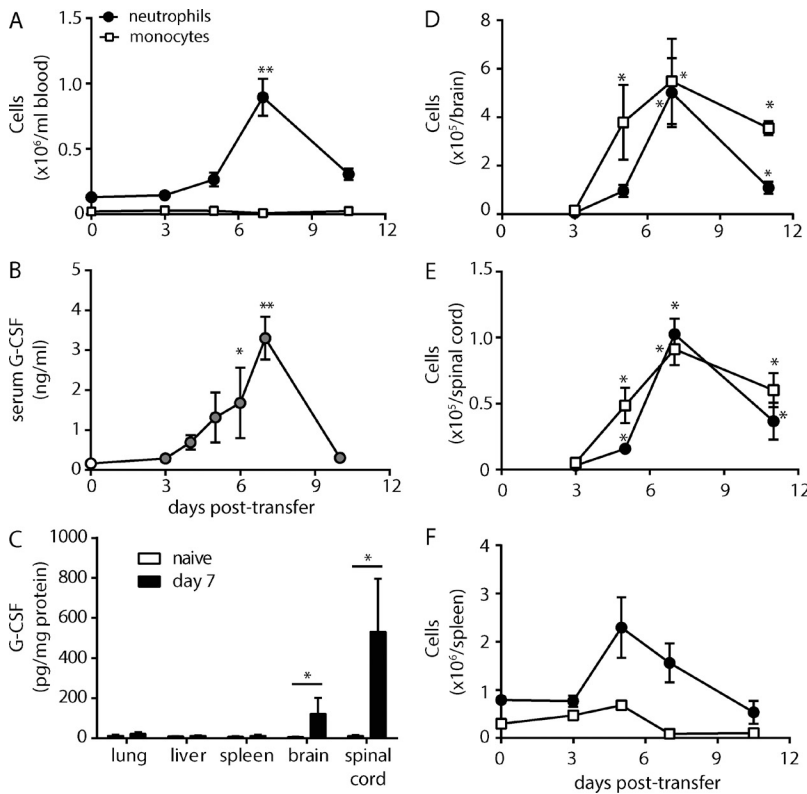


Figure 5. Adoptive transfer of encephalitogenic Th17 cells induces the systemic up-regulation of G-CSF and neutrophil mobilization. (A–F) WT mice were injected with IL-23 polarized, MOG-specific CD4⁺ Th17 cells. (A) Circulating neutrophils (closed circles) and monocytes (white squares) were enumerated by flow cytometry at serial time points. Data were pooled from three experiments ($n \geq 7$ mice per group). (B) Serum G-CSF levels were measured by ELISA. Data were pooled from three experiments ($n \geq 10$ mice per group). (C) G-CSF levels in tissue homogenates obtained from naive mice or from host mice on day 7 after transfer, measured by ELISA and normalized to total protein ($n = 5$ mice per group). (D–F) Number of monocytes and neutrophils in brain (D), spinal cord (E), and spleen (F), determined by flow cytometry. Data were pooled from two experiments ($n \geq 6$ per group). All graphs indicate means; error bars denote SEM. *, $P < 0.05$; **, $P < 0.01$ compared with naive or day 3 after transfer.

not change significantly after the transfer of either Th1 or Th17 myelin-reactive T cells (unpublished data). Collectively, the above studies demonstrate for the first time that peripheral neutrophils are modulated during the development of clinical EAE in the absence of adjuvant.

Plasma G-CSF levels rise and neutrophils expand in the circulation after the adoptive transfer of encephalitogenic Th17 cells

We next interrogated the kinetics of neutrophil expansion after the adoptive transfer of encephalitogenic Th17 cells. We found that the number of circulating neutrophils consistently increased ~1 wk after transfer around the time of clinical onset (Fig. 5 A). Concomitantly, G-CSF levels rose in the blood and the CNS (Fig. 5, B and C) and the numbers of infiltrating myeloid cells peaked in the brain and spinal cord (Fig. 5, D and E). The number of splenic neutrophils also rose but not to a statistically significant extent (Fig. 5 F). In contrast to actively immunized mice, adoptive transfer recipients did not up-regulate G-CSF in non-CNS tissues (Fig. 5 C).

G-CSFR deficiency confers resistance to Th17-mediated EAE, which is partially rescued by overexpression of CXCL1

We compared the clinical manifestation of EAE in *Csf3r*^{-/-} and WT adoptive transfer recipients of MOG-reactive Th17 cells. *Csf3r*^{-/-} hosts experienced a milder disease course (Fig. 6 A), which correlated with a relative diminution in the number of neutrophils infiltrating the spinal cord at the time of peak EAE and accumulating in the circulation and spleen on day 7 after

transfer (Fig. 6, B and C). At clinical onset, circulating neutrophils had expanded approximately threefold over baseline in *Csf3r*^{-/-} hosts and approximately sevenfold over baseline in WT hosts (Fig. 6 D). The cytokine profile of CNS-infiltrating donor cells was comparable between the groups (Fig. 6 E).

Serum CXCL1 levels were elevated in *Csf3r*^{-/-} versus WT mice both at baseline and on day 7 after transfer (Fig. 6 F). Similarly, CXCL1 expression was significantly higher in the CNS of *Csf3r*^{-/-} compared with WT hosts at clinical onset (Fig. 6 G). This led us to question whether CXCL1 drives the residual neutrophil mobilization and CNS recruitment that occur in *Csf3r*^{-/-} hosts. In fact, treatment of *Csf3r*^{-/-} hosts with CXCR2 blocking antisera beginning on the day of transfer prevented the expansion of neutrophils in the circulation and development of neurological deficits (Fig. 6 H and not depicted). Mice succumbed to clinical disease 3 d after the final treatment, concurrent with rebound recovery of circulating neutrophils (data not shown). These data suggest that heightened expression of CXCL1 partially compensates for deficient G-CSF signaling in *Csf3r*^{-/-} hosts.

Neutrophil-related markers correlate with new lesion formation and measures of CNS injury in patients with relapsing MS

As shown in Figs. 2 and 6, CXCL1 levels are elevated in the blood at the onset of clinical EAE, whether induced by active immunization or Th17 transfer. To assess the association between the expression of neutrophil-related factors and lesion formation during relapsing MS, we measured the plasma levels of a panel of chemokines in patients with active or inactive

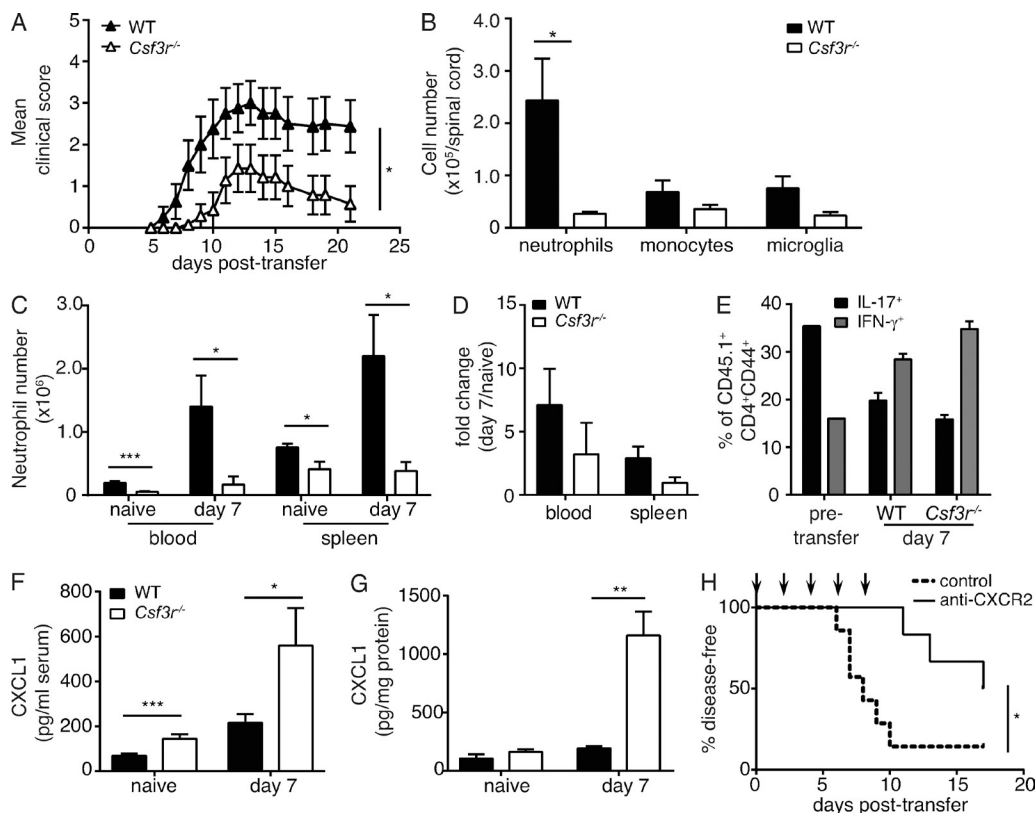


Figure 6. ELR⁺ CXC chemokines partially compensate for loss of G-CSF signaling in *Csf3r*^{-/-} adoptive transfer recipients. (A–G) WT (closed triangles, black bars) and *Csf3r*^{-/-} (open triangles, white bars) mice were injected with MOG-specific Th17 cells. (A) Mean clinical scores, representative of seven independent experiments ($n \geq 7$ mice per group). (B) Absolute number of neutrophils, monocytes, and microglia recovered from the spinal cord on day 7 after transfer, assessed by flow cytometry ($n \geq 7$ per group, pooled from two experiments). (C) Number of neutrophils per milliliter of blood or per spleen at baseline and on day 7 d after transfer, assessed by flow cytometry ($n \geq 7$ per group, pooled from two experiments). (D) Fold change in the number of circulating and splenic neutrophils over baseline on day 7 after transfer. (E) Proportion of donor cells expressing IL-17 and IFN- γ immediately before adoptive transfer, and after isolation from the spinal cords of WT or *Csf3r*^{-/-} hosts on day 7 after transfer ($n = 5$ per group). (F) Levels of CXCL1 in sera from naive mice and adoptive transfer recipients, measured by ELISA ($n \geq 9$, pooled from three experiments). (G) CXCL1 levels in spinal cord homogenates were measured by ELISA and normalized to total protein ($n \geq 4$). (H) *Csf3r*^{-/-} recipients of WT Th17 cells were treated with control serum ($n = 7$) or anti-CXCR2 ($n = 6$) every other day from days 0–8 (arrows). Data are representative of two independent experiments. All graphs indicate means; error bars denote SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

disease, as determined by cerebral magnetic resonance imaging (MRI) and clinical course. We found that expression of CXCL5, a CXCR2 binding chemokine which activates and attracts neutrophils (Liu et al., 2011; Mei et al., 2012), was elevated in active patients concomitant with the presence of acute inflammatory lesions on MRI scan when compared with inactive patients with no inflammatory lesions (Fig. 7 A). In contrast, there was no significant association between new lesion formation and expression of CXCL10 or CCL2, chemokines that primarily target lymphocytes and monocytes, respectively (Fig. 7, B and C). The expanded disability status scale (EDSS) score, a measure of MS clinical disability (Kurtzke, 1983), correlated directly with plasma levels of CXCL1, CXCL5, and neutrophil elastase, as well as CCL2 (Table 1). CXCL1, CXCL5, and neutrophil elastase, but not CXCL10 or CCL2, correlated directly with cumulative MRI lesion volume on T1 weighted sequences, indicative of extensive tissue damage and axonal loss. CXCL5 and neutrophil elastase expression

also correlated with overall brain lesion volume, as visualized on T2 weighted sequences. In contrast, brain parenchymal tissue volume correlated inversely with CXCL5, CXCL1, and neutrophil elastase but was not significantly related to CXCL10 or CCL2.

DISCUSSION

The formation of inflammatory demyelinating lesions is initiated by the trafficking of encephalitogenic T cells across the BBB and their reactivation within the CNS (Kawakami et al., 2004). These events are critical in the pathogenesis of relapsing-remitting MS, as suggested by the therapeutic efficacy of anti- $\alpha 4$ integrin monoclonal antibodies that suppress exacerbations, ostensibly by preventing effector CD4⁺ T cells from infiltrating the CNS (Stüve et al., 2006). However, adoptive transfer experiments with labeled myelin-reactive effector cells have shown that donor T cells comprise a small percentage of infiltrating leukocytes in established EAE lesions and tend to

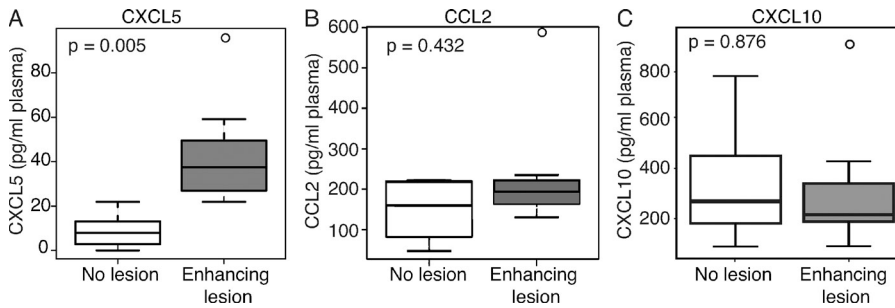


Figure 7. Plasma CXCL5, but not CCL2 or CXCL10, levels increase in association with new MS lesion formation. Patients with relapsing MS were classified as having “active” or “inactive” disease based on clinical course, neurological examination, and MRI scanning. Plasma levels of CXCL5, CCL2, and CXCL10 were measured by multiplex assay. Patients with active disease had enhancing MRI lesions and patients with inactive disease had no enhancing lesions on the day of phlebotomy. Box plots show median, interquartile range, sample minimum, and maximum. Circles show outliers.

cluster in the perivascular or subpial space. Host phagocytes migrate to the CNS in a secondary wave, penetrate deep into the CNS parenchyma, and directly inflict damage to myelin, glial cells, and axons. The recruitment of innate immune cells from the periphery correlates with BBB breakdown and the clinical onset of autoimmune demyelinating disease (Ajami et al., 2011). In the current study, we show that neutrophils and monocytes expand in the bone marrow and accumulate in the circulation immediately before clinical exacerbations after either active immunization of C57BL/6 mice or injection of Th1- or Th17-polarized encephalitogenic T cells. This substantiates our earlier observation that the frequency of circulating myeloid progenitor cells (measured as GM-CFU) increases in concert with the onset and relapse of EAE (King et al., 2009). These shifts in peripheral myeloid populations are driven by systemic up-regulation of G-CSF and ELR⁺ CXC chemokines. Reminiscent of their role in infectious diseases (Wengner et al., 2008), G-CSF and CXCL1 act synergistically to promote neutrophil mobilization during EAE. We found that *Csf3r*^{-/-} mice are relatively resistant to EAE induced by adoptive transfer, consistent with a role of neutrophils during the effector phase (McColl et al., 1998). Administration of pharmacological doses of recombinant G-CSF during EAE has yielded conflicting results (Lock et al., 2002; Zavala et al., 2002; Verda et al., 2006), reflecting the pleiotropic effects of the molecule. Compensatory pathways may be engaged in G-CSF-treated mice depending on the dosing regimen and timing of administration.

In the active immunization model, mycobacterial components (most likely pathogen-associated molecular patterns) in CFA stimulate expression of G-CSF and CXCL1 in numerous tissues, including the spleen, liver, and lungs, as well

as the spinal cord. The risk of MS relapse is higher in the setting of infection (Sibley et al., 1985; Rapp et al., 1995). Our results suggest that this association could be secondary, at least in part, to the release of Toll-like receptor ligands that modulate myeloid-related factors. Campbell et al. (2010) detected increased hepatic expression of CXCL1, accompanied by neutrophil recruitment to the liver, in Biozzi mice that were immunized with spinal cord homogenate in CFA to induce EAE. Those authors also detected neutrophil infiltration in postmortem liver tissue from MS patients. Conversely, we found the CNS to be the primary site of G-CSF expression in the Th17 adoptive transfer model. Interestingly, Lock et al. (2002) found that G-CSF transcripts are present in MS lesions but not normal-appearing white matter. Because encephalitogenic Th17 cells secrete cytokines, such as IL-17A and TNF, that directly induce G-CSF and CXCL1 (Witowski et al., 2000; Kolls and Lindén, 2004; Iwakura et al., 2011), they could drive local production of neutrophil activating/chemoattractant factors upon being reactivated in the CNS. The cellular source of G-CSF and CXCL1 in the CNS may be meningeal epithelial cells (as previously reported in MOG-immunized mice; Soulika et al., 2009), astrocytes, neurons, or cerebrovascular endothelial cells (Lenhoff and Olofsson, 1996; Jacob et al., 2012). We are currently performing experiments to distinguish between those possibilities.

In the current manuscript we demonstrate, for the first time that spikes in plasma levels of CXCL5, an ELR⁺ CXC chemokine, correspond with the development of new inflammatory lesions in relapsing MS patients. Furthermore, expression of CXCL1, CXCL5, and neutrophil elastase correlated with clinical and radiological measures of CNS injury in MS. These findings are consistent with recent reports of elevated

Table 1. Relationship between chemokine levels and clinical/radiological parameters

Parameter	CXCL1	CXCL5	Neutrophil elastase	CXCL10	CCL2
	Correlation (R)/Significance (p-value)				
EDSS	0.58/<0.00001	0.43/<0.0001	0.26/0.029	NS	0.38/0.0006
T2 lesion volume	NS	0.47/0.0009	0.46/0.0043	NS	NS
T1 lesion volume	0.47/0.0039	0.46/0.0005	0.42/0.0097	NS	NS
BPV	-0.54/<0.0001	-0.38/0.0019	-0.45/<0.0001	NS	NS

NS, not significant; BPV, brain parenchymal tissue volume.

numbers and enhanced priming of circulating neutrophils in patients with active MS (Naegele et al., 2012), as well as with the association between MS relapse and G-CSF administration (Openshaw et al., 2000). Expression of CCL2, as well as CXCL1, CXCL5, and neutrophil elastase, all correlated with EDSS, possibly reflecting complementary roles of neutrophils and monocytes in MS pathogenesis.

Previous studies on the role of innate immunity in MS and EAE have focused on the monocyte/macrophage lineage because that subset is prominent in established white matter lesions. Although CNS-infiltrating neutrophils are prevalent in alternative forms of autoimmune demyelinating disease, such as neuromyelitis optica and acute disseminated encephalomyelitis, they are scarce in typical MS infiltrates. This might be interpreted as inconsistent with a role of neutrophil-related factors in MS (Godiska et al., 1995). However, EAE studies from our laboratory and others indicate that neutrophils participate in autoimmune demyelination during early lesion development, preceding the development of overt neurological deficits (Carlson et al., 2008; Soulika et al., 2009; Christy et al., 2013). 1–2 d before expected clinical onset, neutrophils comprise a significant percentage of CNS-infiltrating cells. By the time mice exhibit their first neurological signs, neutrophils are greatly outnumbered by monocytes/macrophages. Hence, the paradox that neutrophils appear to be functionally important despite their scarcity in MS lesions may be a consequence of sampling bias, in that the vast majority of autopsied CNS tissues are from patients with long-standing progressive disease, and biopsies are generally performed after lesions have matured over days, if not weeks. An alternative, but not mutually exclusive, explanation is that neutrophils primarily promote neuroinflammation from the periphery. Activation of neutrophils in the bone marrow indirectly triggers the mobilization of monocytes and hematopoietic progenitor/stem cells (HPSCs) into the blood, thereby making them accessible for recruitment to the CNS (Singh et al., 2012). Some monocytic cells and HPSCs express G-CSFR themselves and, thus, can be directly stimulated by G-CSF to expand and migrate from the bone marrow (Demetri and Griffin, 1991; Christopher et al., 2011). It is also possible that neutrophils mediate increased permeability at the cerebrovascular interface without actually crossing the BBB. Neutrophils crawl on the luminal endothelial surface of CNS blood vessels before, and at the time of, clinical EAE onset (Richard et al., 2011). The adhesion of activated neutrophils to cerebrovascular endothelial cells alone may impair interendothelial cell-to-cell contacts via secretion of proteases and free oxygen radicals or by conferring conformational changes to adherens junctional proteins (Smedly et al., 1986; Tinsley et al., 1999; Scholz et al., 2007). Neutrophils have been shown to mediate BBB breakdown in animal models of brain trauma, cerebral hemorrhage, and viral encephalitis, and in response to intracerebral injection of recombinant IL-1 β or CXCL2 (Bell et al., 1996; Anthony et al., 1997, 1998; Zhou et al., 2003; Scholz et al., 2007; Moxon-Emre and Schlichter, 2011). A comparable role of neutrophils in autoimmune demyelinating disease is suggested by the fact that

BBB breakdown is prevented in myelin-immunized mice by treatment with neutrophil-depleting or blocking antibodies (Carlson et al., 2008).

Recent advances in MS therapeutics have revolved around strategies that target lymphocytes. Agents that block lymphocyte trafficking to the CNS (such as natalizumab and fingolimod) or deplete lymphocytes in the circulation (such as alemtuzumab and ocrelizumab) are currently used in the clinic to decrease the risk of MS relapse, or have yielded promising results in clinical trials. However, none of those agents fully suppress disease activity or are effective in all patients, underscoring the need for innovative medicinal approaches. The current study supports a growing body of evidence suggesting that myeloid cells and the factors critical for their survival, expansion, activation, and mobilization should be investigated as an alternative source of novel biomarkers and therapeutic targets in MS.

MATERIALS AND METHODS

Mice. C57BL/6 and B6-Ly5.2/Cr mice were obtained from the National Cancer Institute. *Csf3r*^{-/-} mice were initially provided by D.C. Link (Washington University School of Medicine, St. Louis, MO; Liu et al., 1996; Christopher et al., 2011) and bred in our vivarium. Double transgenic OSE mice (Krishnamoorthy et al., 2006) were bred in animal facilities at the Max Planck Institute of Neurobiology. All animals were housed under specific pathogen-free conditions.

Antibodies and reagents. The following monoclonal antibodies were used for flow cytometry: anti-Ly6C, anti-CD31, and anti-Ly6G (BD); and anti-CD4, anti-CD11b, anti-CD45.1, and anti-CD45.2 (eBioscience). The following monoclonal antibodies were used for ELISpot assays: anti-IL-17 (TC11-18H10), biotinylated anti-IL-17 (TC11-8H4), anti-IFN- γ (AN18), and biotinylated anti-IFN- γ (R4-6A2; e-Bioscience). Recombinant mouse IFN- γ and IL-12 were from R&D Systems.

Induction and assessment of EAE. Mice were immunized subcutaneously over the flanks with 100 μ g MOG₃₅₋₅₅ (MEVGWYRSP-FSRVHLYRNGK; Biosynthesis) in CFA containing 250 μ g *Mycobacterium tuberculosis* H37RA (BD). For induction of EAE by active immunization, animals were injected i.p. with heat inactivated PTx (List Biological Laboratories) on days 0 and 2.

For induction of EAE by adoptive transfer, B6-Ly5.2/Cr mice were immunized as described, but without administration of PTx. 10–14 d later, draining lymph nodes (inguinal, brachial, and axillary) were harvested and dissociated into single cell suspensions. Cells were cultured in standard medium with 50 μ g/ml MOG₃₅₋₅₅ and either Th17-polarizing factors (rmIL-23 at 8 ng/ml, rmIL-1 α at 10 ng/ml, anti-mIFN- γ [clone XMG1.2] at 10 μ g/ml, and anti-IL-4 [clone 11B11] at 10 μ g/ml) or Th1-polarizing factors (rmIL-12 at 6 ng/ml, rmIFN- γ at 2 ng/ml, and anti-IL-4 [clone 11B11] at 10 μ g/ml). After 4 d in culture, 2.5–5 \times 10⁶ CD4⁺ T cells were injected i.p. into naive congenic recipients. Clinical assessment of EAE was performed according to the following scale: 0, no disease; 1, limp tail; 2, hind-limb weakness; 3, partial hind-limb paralysis; 4, complete paralysis of hind-limbs; and 5, moribund state.

Anti-CXCR2 treatment. Adoptive transfer recipients were injected with rabbit anti-CXCR2 antisera (Biosynthesis) or control rabbit sera (Sigma-Aldrich) on alternate days beginning on the day of cell transfer. Antisera were generated against a mCXCR2 peptide (GCMGEFKVDKFNIEDFFSG; Mehrad et al., 1999; Hosking et al., 2009), and each bleed was tested for its efficacy in blocking neutrophil recruitment to the peritoneal cavity in response to thioglycollate administration. Only bleeds that showed >90% reduction in neutrophil recruitment were used in studies.

Cell isolation. Blood was collected in EDTA-coated or heparinized tubes after cardiac puncture or retro-orbital bleed. Bone marrow cells were flushed from femurs and tibiae with cold media. Splenocytes were passed through a 70- μ m nylon mesh filter to obtain a single-cell suspension. All cell preparations were ACK lysed and washed before analysis. For collection of CNS mononuclear cells, spinal cords were harvested from PBS-perfused mice, mechanically disrupted with an 18-gauge needle, and incubated with 2 μ g/ml collagenase A and 1 μ g/ml DNase in HBSS for 30 min at 37°C. Mononuclear cells were then isolated on a 30–70% discontinuous Percoll gradient.

Flow cytometry. Cells were suspended in PBS with 2% FCS and Fc Block (50 ng/ml; hybridoma 2.4G2; American Type Culture Collection) before incubation with fluorochrome-conjugated antibodies (anti-mouse Ly-6C [AL21; BD], anti-mouse Ly-6G [1A8; BD], anti-mouse CD11b [M1/70; eBioscience], anti-mouse CD4 [RM-45, eBioscience], anti-mouse CD45 [30-F11; eBioscience], and anti-mouse CD31 [MEC13.3; BD]). For intracellular cytokine staining, cells were stimulated with 50 ng/ml PMA and 2 μ g/ml ionomycin in the presence of 10 μ g/ml brefeldin A for 6 h, followed by fixation with 4% paraformaldehyde, permeabilization with 0.5% saponin, and staining with fluorochrome-conjugated anti-IFN- γ (XMG1.2; BD) and anti-IL-17 (17B7; eBioscience). Data were acquired on a FACSVerser or FACS Canto II flow cytometer (BD) and analyzed with FlowJo software (Tree Star).

ELISA. Sera were collected by cardiac puncture. Livers, spleens, spinal cords, and lungs were collected from PBS-perfused mice and homogenized in PBS with protease inhibitors (Roche). CXCL1 and G-CSF levels were measured by sandwich ELISA based on noncompeting pairs of antibodies using the manufacturer's protocol (R&D Systems). Levels in homogenates were normalized to total protein as determined by Bradford assay.

ELISpot assay. Draining lymph nodes (inguinal, axillary, and brachial) were harvested from mice on day 11 p.i., pooled, and cultured in 96-well filter plates (EMD Millipore) at $2\text{--}5 \times 10^5$ cells/well, with or without 50 μ g/ml MOG₃₅₋₅₅, for 18 h. Streptavidin-alkaline phosphatase was from SouthernBiotech and the alkaline phosphatase substrate kit was from Vector Laboratories. Spots were counted using the CTL ImmunoSpot Analyzer (Cellular Technology) with ImmunoSpot software.

Histology. After perfusion, spinal cords were fixed in 4% paraformaldehyde, decalcified, and processed for paraffin embedding. 8- μ m sections were cut with a microtome (Leica) and stained with Harris' hematoxylin and eosin by standard procedure.

Bone marrow chimeras. Femurs and tibiae from C57BL/6 WT and *Csf3r*^{-/-} females were flushed with PBS using a 26-gauge needle and ACK lysed. Cells were suspended in cold PBS for intravenous administration to 6-wk-old B6-Ly5.2/Cr mice, which had been subjected to 13 Gy of irradiation (orthovoltage x-ray source), split into two doses, 3 h apart. Recipients were given 6×10^6 total cells and allowed to reconstitute for 6 wk before further use. Chimerism was verified by flow cytometric analysis of PBMC, which was >90% in all animals tested.

MS subjects and clinical assessments. Patients diagnosed with relapsing MS ($n = 22$), based on the revised McDonald Diagnostic Criteria, were recruited from the Multiple Sclerosis Clinic at the University of Michigan. All subjects underwent bimonthly gadolinium enhanced MRI scans and phlebotomy on the same day over a 1-yr observation period. Subjects who had at least one enhancing lesion on one or more MRI scan were classified as having active disease. Subjects who had no enhancing lesions detected throughout the course of study and who had no clinical relapses were classified as having inactive disease. Every MS patient underwent complete neurological examinations, including EDSS scoring by a board-certified neurologist who was blinded to the results of MRI scanning and immune assays. On the day of phlebotomy, all subjects were afebrile and had no signs and symptoms of infection based on history, physical examination, and responses to a survey.

Multiplex assays. Plasma levels of cytokines and chemokines were measured with customized multiplex magnetic bead based arrays (EMD Millipore) according to the manufacturer's protocol. Data were collected using the Bio-Plex 200 system (Bio-Rad Laboratories). Standards were run in parallel to allow quantification of individual factors. The data shown indicates levels that fell within the linear portion of the corresponding standard curve.

MRI protocol and image analysis. All patients were evaluated with cranial MRI examinations on a 1.5 tesla strength magnet using axial T2-weighted, axial and sagittal T1-weighted sequences, and post-Gadolinium axial and coronal T1-weighted. Brain parenchymal tissue volume (defined as total brain volume subtracting CSF volume), and T1 and T2 lesion volume, were measured using commercially available software developed by VirtualScopics. This involved coregistering each MRI to a presegmented anatomical atlas with manual refinement of automated brain boundaries by an expert analyst where necessary, as previously described (Ashton et al., 2003). Lesion boundaries were identified in three dimensions using geometrically constrained region growth (GEORG; Ashton et al., 1997, 2003). T1 and T2 lesion volumes were normalized to total brain parenchymal tissue volume.

Statistical analysis. Clinical courses of WT and *Csf3r*^{-/-} mice were compared by two-way ANOVA using Prism (GraphPad Software). Disease-free survival curves of anti-CXCR2 and control antisera treated mice were generated with Prism software and analyzed by Log-rank (Mantel-Cox) test. Immune parameters were compared between groups of mice by unpaired Student's *t* tests.

Plasma chemokine levels were measured in patients with active disease on the day of their first MRI scan with a gadolinium enhancing lesion (indicative of acute lesion formation) and in inactive patients on the day of the initial blood draw, during which they had an MRI scan showing no enhancing lesions. Levels between the active and inactive groups were compared using Box plots and Wilcoxon nonparametric tests.

Comparisons were made between immune parameters and EDSS, T1 lesion volume, T2 lesion volume, or brain parenchymal tissue volume using all available measurements. The analysis was done using within-cluster resampling methodology, considering the concern of the intraclass correlation. Specifically, we have randomly chosen one observation per patient and calculated Spearman's correlations. This process was repeated 200 \times , and we merged all 200 estimated correlations to obtain the final results using established methods (Hoffman et al., 2001; Rieger and Weinberg, 2002).

Study approval. All animal experiments described herein were performed under protocols approved by the University of Michigan Committee on the Use and Care of Animals. The Institutional Review Boards of the University of Michigan and the University of Rochester approved our human study protocol. All subjects gave their written informed voluntary consent after the nature and possible consequences of the study were explained.

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