GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

G-CSF and Neutrophils Are Nonredundant Mediators of Murine Experimental Autoimmune Uveoretinitis

Gabrielle L. Goldberg,^{*} Ann L. Cornish,^{*} Jane Murphy,^{*} Ee Shan Pang,^{*} Lyndell L. Lim,[†] Ian K. Campbell,[‡] Karen Scalzo-Inganti,[‡] Xiangting Chen,[§] Paul G. McMenamin,[§] Eugene Maraskovsky,[‡] Brent S. McKenzie,[‡] and Ian P. Wicks^{*,¶||}

From the Department of Medical Biology,^{*} The Walter and Eliza Hall Institute of Medical Research, University of Melbourne, Parkville, Victoria; the Centre for Eye Research Australia,[†] University of Melbourne, Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria; the CSL Limited,[‡] Bio21 Institute, Parkville, Victoria; the Department of Anatomy and Developmental Biology,[§] School of Biomedical Sciences, Monash University, Clayton, Victoria; the Department of Rheumatology,[¶] Royal Melbourne Hospital, Parkville, Victoria; and the Department of Medicine,^{||} University of Melbourne, Parkville, Victoria, Australia

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Address correspondence to
Ian P. Wicks, Ph.D., Walter
and Eliza Hall Institute of
Medical Research, 1G Royal
Parade, Parkville, VIC 3052,
Australia. E-mail: [wicks@
wehi.edu.au](mailto:wicks@wehi.edu.au).

Granulocyte colony-stimulating factor (G-CSF) is a regulator of neutrophil production, function, and survival. Herein, we investigated the role of G-CSF in a murine model of human uveitis—experimental autoimmune uveoretinitis. Experimental autoimmune uveoretinitis was dramatically reduced in G-CSF-deficient mice and in anti-G-CSF monoclonal antibody-treated, wild-type (WT) mice. Flow cytometric analysis of the ocular infiltrate in WT mice with experimental autoimmune uveoretinitis showed a mixed population, comprising neutrophils, macrophages, and T cells. The eyes of G-CSF-deficient and anti-G-CSF monoclonal antibody-treated WT mice had minimal neutrophil infiltrate, but no change in other myeloid-derived inflammatory cells. Antigen-specific T-cell responses were maintained, but the differentiation of pathogenic type 17 helper T cells in experimental autoimmune uveoretinitis was reduced with G-CSF deficiency. We show that G-CSF controls the ocular neutrophil infiltrate by modulating the expression of C-X-C chemokine receptors 2 and 4 on peripheral blood neutrophils, as well as actin polymerization and migration. These data reveal an integral role for G-CSF-driven neutrophil responses in ocular autoimmunity, operating within and outside of the bone marrow, and also identify G-CSF as a potential therapeutic target in the treatment of human uveoretinitis. (*Am J Pathol* 2016, 186: 172–184; <http://dx.doi.org/10.1016/j.ajpath.2015.09.008>)

Uveitis is a leading cause of blindness in developed countries. Intermediate/posterior uveoretinitis damages the retina and accounts for most visual morbidity. Infections cause approximately 50% of uveitis cases,¹ but the remainder are believed to be autoimmune.² Although current therapies control autoimmune uveitis in many patients, some fail to respond, or treatment is limited by adverse effects. There is consequently a need for alternative therapeutic approaches. Experimental autoimmune uveoretinitis (EAU), a widely used murine model of noninfectious posterior uveoretinitis, is CD4⁺ T-cell-mediated and mimics the clinical and histopathological features of human disease.³ Type 1 helper T (Th1) cells contribute to EAU, but recent studies suggest

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that Th17 cells (but not IL-17 itself) and IL-23, a potent stimulator of Th17 cell differentiation, are nonredundant in this disease model.⁴ In contrast, although innate immune cells, including neutrophils and macrophages, feature prominently in the ocular infiltrate in EAU,^{5,6} the pathogenic role of myeloid cells, or regulators of myeloid cell production and function, has been far less studied.

Granulocyte colony-stimulating factor (G-CSF) is a major regulator of steady-state neutrophil production and survival,⁷ and granulopoiesis is reduced in G-CSF- and G-CSF receptor (G-CSF-R)-deficient mice. During infection or inflammation, neutrophil production and export increases, under the influence of G-CSF, in a response known as emergency granulopoiesis.⁸ G-CSF can be produced by multiple cells throughout the body, including macrophages, bone marrow (BM) stromal cells, and endothelial cells.^{9–11} Homeostatic regulation of the neutrophil pool is complex, with proposed feedback mechanisms related to clearance of apoptotic neutrophils in BM, spleen, liver, and mucosal sites.¹² Regulation of C-X-C chemokine receptors (CXCRs) 2 and 4 and their ligands is critical to neutrophil retention and release from the BM.¹³ CXCR4-deficient mice exhibit constitutive neutrophil mobilization.¹³ Myelokathexis, a congenital cause of human neutropenia, is caused by a gain-of-function mutation in CXCR4, increasing BM neutrophil retention.¹⁴ CXCR2 is also necessary for neutrophil mobilization,¹⁵ and CXCR2-deficient neutrophils are selectively retained in the BM. G-CSF regulates CXCR2 and CXCR4 expression on BM neutrophils¹³ and increases production of chemokines CXCL1 (mediated via thrombopoietin) and CXCL2.^{16,17} Conversely, G-CSF administration reduces CXCR4 and CXCL12 expression in the BM.¹³

G-CSF also influences neutrophil function outside of the BM compartment. Neutrophil phagocytosis and oxidative burst are increased by G-CSF,¹⁸ as is neutrophil survival, the latter mediated by induction of Mcl-1 or A1.¹⁹ G-CSF enhances migration into peripheral tissues via induction of CXCR2 ligands.²⁰ G-CSF up-regulates expression of integrins, such as CD11b/CD18, promoting adhesion and transmigration,²¹ and stimulates glycosylation of myeloperoxidase (MPO), converting MPO to a cell surface ligand for E-selectin.²² Serum G-CSF is elevated during infection and in sterile inflammatory conditions,^{8,23,24} and G-CSF and G-CSF-R are elevated in the ocular tissue and blood of patients with uveitis.²⁴ In patients with active Behçet's disease, peripheral blood (PB) mononuclear cell G-CSF mRNA, CXCR2 expression on circulating neutrophils, and serum CXCL1/growth-regulated oncogene α (GRO α) increase.^{25–28} The neutrophil chemoattractant CXCL8/IL-8 also increases in uveitis patients.^{27,28} Administration of G-CSF to patients can induce or exacerbate preexisting inflammatory conditions, including uveitis.^{29,30}

We have previously reported that G-CSF-deficient mice are resistant to collagen-induced arthritis.³¹ Wild-type (WT) mice treated with anti-G-CSF also had reduced collagen-

induced arthritis, even when anti-G-CSF was administered therapeutically.³¹ In this study, we investigated the role of endogenous G-CSF and neutrophils in EAU. Our data show a strong relationship between neutrophilia and elevated G-CSF in PB, and in the eye, in WT mice. In contrast, G-CSF-deficient mice did not develop EAU-associated neutrophilia and were markedly protected from disease, as were WT mice treated with a neutralizing anti-G-CSF monoclonal antibody (mAb; anti-G-CSF). Analysis of the ocular infiltrate revealed a neutrophil-rich infiltrate in WT mice with EAU, which was almost absent in G-CSF-deficient mice and in WT mice treated with anti-G-CSF. Furthermore, expression of CXCR2 on circulating neutrophils was markedly decreased in G-CSF-deficient mice and anti-G-CSF-treated mice with EAU. Lower CXCR2 expression was associated with reduced neutrophil actin polymerization and reduced *in vitro* and *in vivo* neutrophil migration. Intriguingly, decreased activation of the neutrophil compartment impaired the differentiation of pathogenic Th17 cells, linking innate and adaptive immunity. These results reveal a fundamental contribution of endogenous G-CSF and neutrophils to the pathogenesis of EAU.

Materials and Methods

Mice

Experiments were performed in accordance with National Health and Medical Research Council of Australia guidelines and approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute (WEHI; Parkville, VIC, Australia). C57BL/6 mice were obtained from WEHI. G-CSF-deficient (B6;129P2-*Csf3*^{tm1Ard}/J) mice were kindly provided by Ashley Dunn (Ludwig Institute for Cancer Research, Parkville, VIC, Australia),³² and G-CSF-R-deficient [B6.129X1(Cg)-*Csf3r*^{tm1Link}/J] mice were kindly provided by Daniel Link (Washington University Medical School, St. Louis, MO).³³ C57BL/6 RAR-related orphan receptor (ROR) γ T-enhanced green fluorescence protein (eGFP) mice were kindly provided by Gabrielle Belz, and the G-CSF-deficient/ROR γ T-eGFP mice were bred at WEHI by crossing the G-CSF-deficient mouse (B6;129P2-*Csf3*^{tm1Ard}/J) with the C57BL/6 ROR γ T-eGFP reporter mice. G-CSF-deficient and G-CSF-R-deficient mice were backcrossed onto the C57BL/6 background for >20 and >8 generations, respectively. LysM-cre-eGFP mice were kindly provided by Michael Hickey (Monash University, Clayton, VIC, Australia) and did not carry the rd8 mutation in the *Crb1* gene.³⁴ Mice were housed under standard conditions in the WEHI animal facility.

Induction of EAU

Unless otherwise stated, EAU was induced as described previously.³⁵ Each mouse received 150 μ g interphotoreceptor

retinoid-binding protein (IRBP), 250 µg *Mycobacterium tuberculosis* in complete Freund’s adjuvant, given by s.c. injection, and 0.5 µg pertussis toxin i.p. In some experiments, complete Freund’s adjuvant was replaced by incomplete Freund’s adjuvant (IFA).

G-CSF Enzyme-Linked Immunosorbent Assay

Rat anti–G-CSF mAb (clone 67604) and biotinylated rabbit anti–G-CSF polyclonal Ab (R&D Systems, Minneapolis, MN) were used in a sandwich enzyme-linked immunosorbent assay. Recombinant murine G-CSF (Peprotech, Rocky Hill, NJ) was used as a standard.

Bio-Plex Analysis of Cytokines Including IL-17

Vitreous humor samples and serum were evaluated for cytokine and chemokine levels with the Bio-Plex mouse cytokine 23-plex panel, used according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). The assay was analyzed using a Bio-Plex 200 instrument and Bio-Plex Manager software version 5.0 (Bio-Rad Laboratories). IL-17 concentrations were measured using this kit.

Ab Blockade Studies

Rat anti-murine G-CSF mAb (clone 67604, as above) was used for G-CSF blockade in EAU, on the basis of our previous G-CSF neutralization experiments.³¹ mAb (0.25 mg) (or a rat IgG1 control mAb) was injected i.p. on day 8 and then every second day up to day 20 of EAU.

G-CSF Administration

For chemokine receptor expression and migration experiments, 2 µg G-CSF per mouse (Neupogen; Amgen, Thousand Oaks, CA) was administered s.c. twice, 12 hours apart, and organs were harvested the next day. For the IFA and G-CSF experiment, 2 µg G-CSF per mouse was administered s.c. for seven doses, 12 hours apart, over 3.5 days.

MPO Assay

MPO was quantified in enucleated, homogenized whole eyes with a mouse ELISA test kit (Hycult Biotech, Uden, the Netherlands), used according to the manufacturer’s instructions.

Histological Assessment of EAU

Eyes were harvested from mice 21 days after immunization. Hematoxylin and eosin sections were scored by two separate observers (blinded to experimental group; X.C., P.G.M.) and averaged. Infiltrative and structural scores were combined to give the total histological score. The scoring system used was on the basis of the system described by Dick et al.³⁶

Table 1 Flow Cytometry Reagents

Antigen/fluorophore	Clone	Supplier
Products		
CD45.2-PercP-Cy5.5	104	BD Biosciences (San Jose, CA)
CD11b-FITC	M1/70	BD Biosciences
Gr-1–APC	RB6.8C5	BD Biosciences
F480-FITC	F4/80	WEHI monoclonal antibody facility
CXCR2-PE	242216	R&D Systems
CD62L-PE-TX-RED	MEL-14	Invitrogen
CXCR4-APC	2B11	eBioscience (San Diego, CA)
γδ-TCR–PE	B1	BD Biosciences
IL-17–APC	eBio17B7	eBioscience
GM-CSF-PE	MP1-22E9	BD Biosciences
IFN-γ–FITC	XMG1.2	eBioscience
Reagent		
AnnexinV-FITC		BD Biosciences
FluoroGold		Sigma-Aldrich (St. Louis, MO)

APC, allophycocyanin; Cy5.5, cyanine5.5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PercP, peridinin-chlorophyll proteins; TX-RED, Texas Red.

Western Blot Analysis for Ocular IRBP

Eye homogenates were clarified by centrifugation, and sample proteins (20 µg) were run on 4% to 12% Bis-Tris NOVEX gels (Invitrogen, Carlsbad, CA), then transferred to a polyvinylidene difluoride membrane. IRBP was detected with rabbit–anti-IRBP (Santa Cruz Biotechnology, Santa Cruz, CA), followed by sheep–anti-rabbit Ig–horseradish peroxidase (Silenus, Melbourne, VIC, Australia). Immunoblots were developed with Amersham ECL (GE Healthcare, Little Chalfont, UK).

Flow Cytometry

Single-cell suspensions of PB, BM, and spleen were made by passage through a 70-µm cell strainer. Single-cell suspensions were made by crushing enucleated eyes between two frosted slides. Antibodies used to stain leukocyte subsets are listed in Table 1.

Intracellular Cytokine Staining

Single-cell suspensions of the eye and blood were stimulated for 4 hours with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO) and 500 ng/mL ionomycin, 1 mg/mL brefeldin A, and 2 mg/mL monensin. Cells were stained with surface antibodies (Table 1). Cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA), used according to the manufacturer’s instructions, and stained with anti-cytokine antibodies (Table 1), followed by resuspension in 1× BD Perm/Wash buffer (BD Biosciences). Unstimulated cells were used as a control.

Antigen-Specific Proliferation Assay for EAU

Whole splenocytes from WT and G-CSF-deficient mice, harvested 21 days after the induction of EAU, were stained with 5 $\mu\text{mol/L}$ CellTrace Violet (Invitrogen). Cells (2×10^5 per well) were cultured for 5 days in the presence or absence of 5 $\mu\text{g/mL}$ IRBP 1-20. On day 5, cells were stained for CD3, CD4, and CD8 and propidium iodide to exclude dead cells. Stained cells were analyzed on a BD Fortessa flow cytometer.

Retinal Disease Grading (*in Vivo* Imaging)

The severity of retinal disease was assessed on the basis of examination of bright-field and fluorescent fundal images obtained from *in vivo* examination (Micron III; Phoenix Laboratories, Palmdale, CA). A modified version of a previously described grading scheme was used to score fundal images³⁷ (Supplemental Table S1) by two observers (blinded to experimental group; X.C., P.G.M.), and the scores were averaged.

In Vitro Stimulation of PB Neutrophils

Splenocytes (2×10^6) were stimulated with 5 ng/mL human G-CSF (Neupogen) and murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech), or incubated in media alone for 16 hours. Live neutrophils were defined as AnnexinV[−]FluoroGold[−]Gr-1^{hi}CD11b⁺F4/80[−] (BD Biosciences).

Transwell Neutrophil Migration Assay

A total of 1×10^6 cells (1×10^7 cells/mL) were placed in the top chamber of a 3- μm pore, 24-well transwell plate (Corning, Tewksbury, MA). Media (600 μL) containing 500 ng/mL recombinant murine CXCL2/macrophage inflammatory protein (MIP)-2 (R&D Systems), or media alone, were placed in the bottom chamber, and the plate was incubated for 30 minutes at 37°C/5% CO₂. The ViaLight plus kit (Lonza, Basel, Switzerland) was used, according to the manufacturer's directions, to calculate migrating cells. The remaining cells were stained and analyzed by flow cytometry, with neutrophils defined as Gr-1^{hi}CD11b⁺F4/80[−].

Neutrophil Actin Polymerization Assay

A previously reported actin polymerization assay was adapted.³⁸ White blood cells were stained (neutrophils defined as Gr-1^{hi}CD11b⁺F4/80[−]) and resuspended at $2 \times 10^7/\text{mL}$, and 50 μL was transferred to 5-mL polystyrene tubes on ice. Samples were incubated at 37°C for 2 minutes, then murine MIP-2 (R&D Systems) was added (final concentration of 500 ng/mL). Cells were incubated for 15, 30, 60, or 120 seconds before BD Cytofix/Cytoperm was added. Samples were stained with phalloidin-fluorescein isothiocyanate and analyzed by flow cytometry.

In Vivo Neutrophil Migration Assay

B6/G-CSF-deficient and B6/G-CSF-R-deficient mice were injected s.c. with either 2 μg hG-CSF or phosphate-buffered saline. Sixteen hours later, mice were injected i.p. with 500 ng murine CXCL2/MIP-2 (Peprotech). After 4 hours, mice were bled and peritoneal lavages were performed with cold phosphate-buffered saline. Cell counts and flow cytometry were performed after lysis of red blood cells (RBCs). Cells were analyzed on an ADVIA blood analyzer (Siemens, Munich, Germany) and BD Fortessa. Neutrophils were defined as CD45⁺Gr-1^{hi}CD11b⁺F4/80[−].

Statistical Analysis

Prism software version 6 (GraphPad, La Jolla, CA) was used for statistical analysis. The *U*-test was used to compare sample groups, unless otherwise stated. Analysis of variance was used for measurements taken at multiple time points. Data shown are means $\pm$ SEM.

Results

G-CSF and Neutrophils Play Pivotal Roles during EAU

EAU was induced in age- and sex-matched C57BL/6 mice, and PB leukocytes were analyzed by automated cell counting (Figure 1A). Neutrophils increased approximately 35-fold at day (d) 7, 20-fold at d11, and 10-fold at d15 and d21. In contrast, the increase in lymphocyte numbers was modest (2.5-fold) on d7 and normalized by d15. G-CSF levels increased markedly by d7 and remained high throughout disease, correlating closely with increased circulating neutrophils (Figure 1B). In mice, MPO generation in tissues arises mainly from neutrophils, because monocytes/macrophages down-regulate MPO production outside of the BM.³⁹ MPO was, therefore, used as a surrogate marker for neutrophil infiltration in the eye. Ocular MPO⁴⁰ increased throughout EAU and correlated with increased G-CSF levels within the eye (Figure 1C).

Anti-Gr-1 mAb⁴¹ was administered to WT mice to examine the effect of neutrophil depletion on EAU. As previously reported,⁴² disease severity decreased ($P \leq 0.01$) (Figure 1D). EAU was, therefore, compared in WT and G-CSF-deficient mice. A significant reduction in the total histological score (comprising infiltrative and structural scores³⁶) was observed in G-CSF-deficient mice (Figure 1E). Although the infiltrative score (Figure 1F) did not differ between groups, the structural score was markedly reduced in G-CSF-deficient mice (Figure 1G). Figure 1H shows representative histological sections of WT and G-CSF-deficient mice. Consistent with these data, G-CSF-deficient mice maintained high IRBP levels (normalized to actin) compared with WT mice with EAU, indicating less retinal damage (Figure 1, I and J).

Figure 1 A critical role for granulocyte colony-stimulating factor (G-CSF) and neutrophils in experimental autoimmune uveoretinitis (EAU). **A–C:** Experimental autoimmune uveoretinitis (EAU) was induced in wild-type (WT) mice. Peripheral blood (PB) leukocytes were evaluated by ADVIA and G-CSF levels in serum and vitreous humor measured by BioPlex. **A:** PB white blood cell, lymphocyte, and neutrophil numbers throughout EAU. **B:** Increased serum G-CSF correlates with PB neutrophilia in EAU. **C:** Myeloperoxidase (MPO) activity (in total eye homogenate) and G-CSF levels in vitreous humor. **D:** EAU was induced in WT mice treated with either 0.5 mg RB6 mAb or isotype control [days (d) –1, 2, 5, and 7]. Disease severity was assessed histologically at d21. **E–K:** EAU was induced in WT or G-CSF-deficient mice. **E–G:** Eyes were examined histologically on d21 and scored for disease severity. Total histological score (**E**) assessed as the total of infiltrative histological score (**F**) and structural histological score (**G**). **H:** Representative examples of eye histology from WT or G-CSF-deficient mice with EAU. **I:** IRBP levels were determined in the eye by Western blot analysis in WT and G-CSF-deficient mice d21 after disease induction. **J:** Graphical representation of Western blot IRBP density. Eyes from WT and G-CSF-deficient mice were dissociated into single-cell suspensions and analyzed by flow cytometry d21 after induction of EAU. **K and L:** Infiltrating neutrophils (CD45⁺ F4/80[–] CD11b^{hi} Gr1^{hi}) (**K**) and neutrophil proportions and numbers (**L**). **M and N:** Resident macrophages (CD45⁺ CD11b⁺ F4/80[–] Ly6G[–] Ly6C[–]) (**M**) and inflammatory macrophages (CD45⁺ CD11b⁺ F4/80[–] Ly6G⁺ Ly6C⁺) (**N**). Data are representative of three independent experiments, $n = 8$ to 17 (**B**); $n = 4$ to 8 (**D**); data are representative of two experiments, $n = 9$ to 10 (**E–G**); $n = 3$ (**I** and **J**, fold change relative to naive WT mice); $n = 7$ to 12 (**M** and **N**). * $P < 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ by *U*-test.

Enucleated eyes were processed into single-cell suspensions and analyzed by flow cytometry. A substantial population of neutrophils was found in WT eyes on d21 of EAU, but was almost absent in G-CSF-deficient mice (Figure 1K), translating into a significant decrease in neutrophil numbers in the eyes of G-CSF-deficient mice (Figure 1L). In contrast, neither the proportion nor number of resident or inflammatory macrophages was altered in G-CSF-deficient mice (Figure 1, M and N). These data show that, in the absence of G-CSF, neutrophil infiltration into the eye is markedly (and selectively) reduced and ocular damage is much less severe, without any apparent change in the monocyte-macrophage response.

To further explore the role of G-CSF in this model, EAU was induced in WT mice with pertussis and IFA alone or pertussis and IFA combined with seven doses of 2 μ g per mouse G-CSF over 3.5 days (days 0 to 3). Mice treated with IFA alone (with or without G-CSF) developed mild

(3 of 20) or no EAU (17 of 20) (data not shown), suggesting the early administration of G-CSF is not sufficient to replace the effect of complete Freund's adjuvant in the initiation of EAU.

G-CSF Deficiency Does Not Affect T-Cell Proportion, Number, or Antigen-Specific T-Cell Proliferation But Th Differentiation Is Altered in the Absence of G-CSF

IRBP peptide-induced EAU is considered a CD4⁺ T-cell-mediated disease.⁴³ There was no difference in the proportion (Figure 2, A and B) or number (Figure 2, C and D) of total or CD4⁺ ocular infiltrating T cells when comparing WT with G-CSF-deficient mice 21 days after induction of EAU. Global antigen-specific proliferation was assessed after EAU induction. There was no difference between IRBP-immunized WT and G-CSF-deficient mice in

Figure 2 Global T-cell responses are maintained in granulocyte colony-stimulating factor (G-CSF)-deficient mice with experimental autoimmune uveoretinitis (EAU), but pathogenic Th17 cells are reduced in the eye. EAU was induced in wild-type (WT) or G-CSF-deficient mice. Proportion of CD45⁺CD3⁺ (A) and CD45⁺CD3⁺CD4⁺ (B) T cells. Number of CD45⁺CD3⁺ T cells (C) and CD45⁺CD3⁺CD4⁺ T cells (D). E: IL-17A, interferon (IFN)-γ, and GM-CSF production by CD3⁺CD4⁺ T cells in the eye after 4 hours stimulation with PMA/ionomycin. Each dot plot is data concatenated from 7 to 10 mice [FlowJo software version 9.8.2 (FlowJo LLC, Ashland, OR)]. F: Proportions of CD45⁺CD3⁺CD4⁺ IFN-γ, IL-17A, GM-CSF, IL-17A/IFN-γ, IL-17A/GM-CSF, and IL-17A/IFN-γ/GM-CSF producing cells in the eye. G: Number of CD45⁺CD3⁺CD4⁺ IFN-γ, IL-17A, GM-CSF, IL-17A/IFN-γ, IL-17A/GM-CSF, and IL-17A/IFN-γ/GM-CSF producing cells in the eye. H: Serum IL-17 levels throughout EAU. I: Vitreous humor IL-17 levels throughout EAU. J: Proportion of CD3⁺γδ-TCR⁺ splenic T cells. K: Proportion of RORγT⁺CD3⁺γδ-TCR⁺ splenic T cells. L: IL-17A and IFN-γ production by CD3⁺γδ-TCR⁺ splenic T cells after 4 hours stimulation with PMA/ionomycin. M: Proportion of IFN-γ-producing CD3⁺γδ-TCR⁺ splenic T cells after 4 hours stimulation with PMA/ionomycin. N: Proportion of IL-17-producing CD3⁺γδ-TCR⁺ splenic T cells after 4 hours stimulation with PMA/ionomycin. O: Proliferation of splenic T cells after 5 days without peptide, with IRBP peptide or with PMA. Data plotted as individual scores are representative of two experiments, $n = 5$ to 10 (I); $n = 5$ to 6 (N); data representative of three separate experiments, $n = 4$ (O). * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$, U-test. GM-CSF, granulocyte-macrophage colony-stimulating factor; PMA, phorbol myristate acetate.

splenic T-cell proliferation after *in vitro* stimulation with IRBP peptide (Figure 2O). Recent studies have implicated the production of interferon (IFN)-γ and/or GM-CSF by IL-17A-producing CD4⁺ T cells in the pathogenesis of experimental allergic encephalomyelitis (EAE).⁴⁴ Cytokine production by CD4⁺ T cells 21 days after EAU induction was, therefore, analyzed (Figure 2E). The proportion and number of IFN-γ alone, GM-CSF alone, and IL-17A/IFN-γ-producing ocular CD4⁺ T cells was reduced in G-CSF^{-/-} mice (Figure 2, F and G). IL-17A levels were also analyzed in the serum and vitreous humor throughout the course of EAU.

There was no difference between WT and G-CSF-deficient mice in baseline levels, but from d7, serum IL-17A increased markedly in G-CSF-deficient mice compared with WT mice, and this difference was maintained throughout the course of EAU (Figure 2H). By d21, IL-17A was detectable in vitreous humor, and the levels appeared higher in G-CSF-deficient mice (Figure 2I).

Increased serum IL-17A seemed paradoxical given the decreased EAU we observed and, thus, was further investigated. γδ T cells are the predominant source of IL-17 in collagen-induced arthritis⁴⁵ and play an important role in

Figure 3 Granulocyte colony-stimulating factor (G-CSF) antagonism reduces the severity and incidence of experimental autoimmune uveoretinitis (EAU). Anti-G-CSF or isotype control monoclonal antibody (mAb; 250 μ g) was administered to wild-type (WT) mice every second day between days 8 to 20 after EAU induction. **A–C:** Eyes were examined histologically on day (d) 21 and scored for disease severity. **A:** Total histological score. **B:** Infiltrative histological score. **C:** Structural histological score. **D:** Representative histological sections of eyes from mice treated with either isotype-matched control mAb or anti-G-CSF mAb. **E:** The fundus of anesthetized mice under bright field microscopy. **F:** Severity of EAU, as assessed by bright field microscopy of anesthetized mice on d14 and d21, scored by an observer blinded to experimental group (X.C., P.G.M.). **G–K:** Whole eyes were analyzed by flow cytometry on d21 of EAU. **G:** Dot plot of intraocular neutrophils (defined as CD45⁺ F4/80⁻ CD11b^{hi} Gr1^{hi}). **H:** Neutrophil proportions and numbers. **I** and **J:** Activation markers CD62L and CD11b were analyzed on infiltrating (**I**) and circulating (**J**) neutrophils. **K:** Macrophage and monocyte (CD45⁺ Gr-1⁻ CD11b⁺ and/or F4/80⁺) proportions and numbers. Data are representative of two experiments. $n = 9$ to 10 (**A–C**); $n = 5$ (**E** and **F**); $n = 8$ (**H–K**). * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ U-test; $^{\dagger}P \leq 0.05$ analysis of variance test.

EAU.⁴⁶ IL-17 production by $\gamma\delta$ T cells has been reported to increase serum G-CSF and circulating neutrophils in a metastatic tumor model.⁴⁷ We, therefore, evaluated whether $\gamma\delta$ T-cell proportions or IL-17A production by $\gamma\delta$ T cells was altered in G-CSF-deficient mice. Splenic $\gamma\delta$ T-cell proportions were unchanged in naïve G-CSF-deficient mice when compared with WT B6 mice (Figure 2J). However, there was an increase in splenic ROR γ T⁺ $\gamma\delta$ T cells in naïve G-CSF-deficient ROR γ T-eGFP mice compared with WT ROR γ T-eGFP mice (Figure 2K). Splenocytes from WT B6 mice and G-CSF-deficient mice were stimulated with phorbol myristate acetate and ionomycin, and representative plots of $\gamma\delta$ T-cell cytokine production are depicted in Figure 2L. There was no difference in IFN- γ production by $\alpha\beta$ or $\gamma\delta$ T cells (Figure 2M) or in IL-17 production by $\alpha\beta$ T cells (Figure 2N), but there was a marked increase in IL-17 production by splenic $\gamma\delta$ T cells from G-CSF-deficient mice (Figure 2N). These data demonstrate that conventional CD4⁺ T-cell proportions and antigen-specific proliferative responses are preserved in the absence

of G-CSF. IL-17A production is enhanced in G-CSF deficiency, but appears to arise from an expanded population of ROR γ T⁺ $\gamma\delta$ T cells. In contrast, the differentiation of pathogenic Th17 cells infiltrating the eye in EAU is reduced with G-CSF deficiency.

G-CSF Antagonism after the Initiation of EAU Reduces Disease Severity and Local Neutrophil Trafficking and Activation

In preliminary studies, we found that histological changes in the eye commence approximately 8 days after immunization with IRBP. To further explore the contribution of G-CSF, and to model a more clinically relevant disease and therapeutic scenario, WT mice were treated with anti-G-CSF from d8 to d20 after initiation of EAU. The Micron III retinal imaging system was used for *in vivo* imaging of eyes on d14 and d21, and histological evaluation and flow cytometric analysis were performed on d21. Treatment of

Figure 4 Granulocyte colony-stimulating factor (G-CSF) signaling regulates C-X-C chemokine receptors (CXCRs) 2 and 4 expression on bone marrow and peripheral blood neutrophils in mice with experimental autoimmune uveoretinitis (EAU) and in human neutrophils. **A–F**: EAU was induced in WT and G-CSF-deficient mice or isotype mAb-treated and anti-G-CSF mAb-treated WT mice (250 μ g every second day, days 8 to 20). Cells in bone marrow (BM; **A–C**) and peripheral blood (PB; **D–F**) were analyzed by flow cytometry on day 21. Neutrophils were defined as CD11b⁺F4/80[−]Gr-1⁺. **A**: Representative dot plots of CXCR4 and CXCR2 expression on BM neutrophils. **B**: CXCR4 expression on BM neutrophils. **C**: CXCR2 expression on BM neutrophils. **D**: Representative histograms of CXCR4 and CXCR2 expression on PB neutrophils. **E**: CXCR4 expression on PB neutrophils. **F**: CXCR2 expression on PB neutrophils. **G**: CXCL1/KC concentration in serum and eye fluid of WT and G-CSF-deficient mice throughout EAU. **H**: Spleen cells stimulated with G-CSF, GM-CSF, or media alone and CXCR4 expression was analyzed on CD11b⁺F4/80[−]Gr-1⁺ neutrophils at baseline and 20 hours. **I**: Spleen cells stimulated with G-CSF, GM-CSF, or media alone and CXCR2 expression was analyzed on CD11b⁺F4/80[−]Gr-1⁺ neutrophils at baseline and 20 hours. **J**: Human WBCs stimulated with G-CSF, GM-CSF, or media alone. CXCR2 expression on CD16⁺CD66b⁺FluoroGold[−]AnnexinV[−] neutrophils at baseline and 20 hours. Data plotted as individual scores with means $\pm$ SEM and representative of two experiments (**A–J**). $n = 6$ to 7 (**A–F**); $n = 4$ to 7 (**G**); $n = 8$ (**H** and **I**); $n = 4$ (**J**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ compared to baseline; ‡ $P < 0.05$, ‡‡ $P < 0.01$ compared to unstimulated; §§§ $P < 0.001$ compared to G-CSF, *U*-test.

WT mice with anti-G-CSF significantly reduced the severity of EAU (Figure 3A). The infiltrative histological score was significantly reduced (Figure 3B), and the structural score was reduced (but did not reach significance) (Figure 3C). Figure 3D shows representative histological sections of WT and anti-G-CSF-treated mice. *In vivo* imaging on d14 of LysM-Cre-eGFP mice (used later to assess neutrophil infiltrate), treated with either anti-G-CSF or an isotype control, showed patchy perivascularitis, some focal infiltrates, and mild vitreous haze in both WT and anti-G-CSF-treated mice (Figure 3, A–C and H–K). By d21, abnormalities had progressed in control mice to widespread retinal edema, extensive retinal folds, focal granulomas, and perivascularitis, but were much less evident in anti-G-CSF-treated mice (Figure 3E). Although both isotype-treated and anti-G-CSF-treated WT mice had equivalent disease features at d14, progression to severe EAU by d21 was reduced with anti-G-CSF treatment (Figure 3F).

Remarkably, neutrophil infiltration of the eye was almost completely abrogated by treatment of WT mice with anti-G-CSF from d8 to d20 of EAU (Figure 3, G and H). This effect could not be explained by the decrease in proportion of neutrophils in the PB (WT versus anti-G-CSF EAU, 46.6% $\pm$ 5.1% versus 12.1% $\pm$ 2.0%). Infiltrating neutrophils in the isotype-mAb-treated mice with EAU were activated (CD62L^{lo}CD11b^{hi}) (Figure 3I). In contrast, infiltrating neutrophils from the anti-G-CSF-treated mice

had a less activated phenotype (CD62L^{hi}CD11b^{lo/int}), suggesting G-CSF antagonism regulates infiltrating neutrophil activation. However, these differences were not observed in blood neutrophils (Figure 3J), implying local regulation. Again, the proportions of monocytes/macrophages in the eyes of anti-G-CSF-treated mice were similar to those of WT mice with EAU (Figure 3K), although the absolute number of these cells was decreased because of a decrease in the total number of CD45⁺ cells infiltrating the eye.

Neutrophil CXCR4 and CXCR2 Expression Is Altered by G-CSF Signaling

CXCR2 and CXCR4 chemokine receptors mediate neutrophil release from the BM and entry into inflamed peripheral tissues.¹³ Given the specific reduction we observed in neutrophil trafficking into the eye with G-CSF deficiency or blockade, we analyzed neutrophil expression of CXCR2 and CXCR4 in the PB of mice with EAU and compared this with BM (Figure 4, A–F). A population of Gr-1⁺CD11b⁺ neutrophils from G-CSF-deficient and anti-G-CSF-treated WT mice expressed CXCR4 in the BM, as did those from WT mice. Neutrophils from G-CSF-deficient and anti-G-CSF-treated BM failed to up-regulate CXCR2 (Figure 4A). CXCR4 expression was increased (Figure 4B) and CXCR2 decreased (Figure 4C) on BM neutrophils in both G-CSF-deficient and anti-G-CSF-treated mice. Similar

Figure 5 Granulocyte colony-stimulating factor (G-CSF) mediated changes in C-X-C chemokine receptor 2 (CXCR2) expression have functional consequences for neutrophil migration. **A** and **B**: Transwell migration assays performed on neutrophils from isotype- or anti-G-CSF-treated WT mice d21 of experimental autoimmune uveoretinitis. CXCL2/macrophage inflammatory protein 2 (MIP-2) was used as a chemotactic stimulus. Neutrophils (Gr-1^{hi}CD11b^{hi}F4/80^{neg}) from spleen (**A**) and peripheral blood (PB; **B**). **C**: Actin polymerization in response to CXCL2/MIP-2 assessed by flow cytometry in mice treated with anti-G-CSF or control. **D** and **E**: G-CSF-deficient and G-CSF-R-deficient mice were injected with G-CSF or phosphate-buffered saline (PBS). Sixteen hours later, mice were injected i.p. with CXCL2/MIP-2 to induce neutrophil migration into the peritoneum. Expression of CXCR2 on PB neutrophils (**D**) and migrating neutrophils (**E**), expressed as a migration index. **F**: Intraocular eGFP expressing cells at day (d)14 and d21 following treatment with anti-G-CSF or control. Quantification of LysM-cre-eGFP (neutrophils are eGFP^{hi}) in eyes d14 and d21 after EAU induction. **G** and **H**: Total retinal fluorescence (**G**) and fluorescence in the optic nerve region (**H**). **I**: Total fluorescence minus optic nerve region. * $P \leq 0.05$, ** $P \leq 0.01$. $n = 3$, each replicate contains three to four pooled organs/PB (**A** and **B**); $n = 5$ (**F**).

trends were observed in PB neutrophils (Figure 4, D–F), in steady-state G-CSF-deficient mice, and in WT B6 mice treated with anti-G-CSF mAb (Supplemental Figure S1). Serum levels of CXCL1/KC/GRO α , a ligand for CXCR2, were increased in G-CSF-deficient mice as early as 7 days and remained elevated throughout EAU (Figure 4G). In the vitreous humor, CXCL1, a ligand for CXCR2 that is produced by ciliary epithelial cells,⁴⁸ was also increased on d7 and d21 in G-CSF-deficient mice (Figure 4G).

G-CSF Regulates Expression of CXCR4 and CXCR2 on Peripheral Neutrophils, Independent of Cell Survival

G-CSF administration increased CXCR2 and decreased CXCR4 expression on PB neutrophils (Supplemental Figure S1). To determine whether G-CSF can directly mediate these responses, WT spleen cells were stimulated with G-CSF or GM-CSF for 16 hours. GM-CSF was included to control for an effect on neutrophil survival (Figure 4, H and I). To eliminate dead, dying, and apoptotic cells, cells were stained with AnnexinV and FluoroGold, and only AnnexinV⁺FluoroGold⁺GR-1^{hi}CD11b⁺F480⁺ neutrophils were analyzed. As expected, baseline CXCR4 expression was low (Figure 4H). After overnight incubation in the absence of G-CSF or GM-CSF, CXCR4 was up-regulated (Figure 4H). Addition of G-CSF, but not GM-CSF, prevented this up-regulation (Figure 4H). In contrast, mature peripheral neutrophils express high levels of CXCR2, which is typically down-regulated *in vitro*

(Figure 4I). Although GM-CSF maintained CXCR2 expression, G-CSF significantly increased its expression (Figure 4I). The effect of G-CSF on CXCR2 expression was also confirmed in human white blood cells (Figure 4J). G-CSF maintained high expression of CXCR2, whereas CXCR2 was down-regulated on human neutrophils cultured alone, or with GM-CSF (Figure 4J). These data demonstrate that G-CSF can regulate CXCR2 and CXCR4 expression on peripheral neutrophils that have exited the BM compartment.

G-CSF-Mediated CXCR2 Expression Is Required for Neutrophil Migration, *in Vitro* and *in Vivo*

To examine the functional significance of G-CSF-induced changes in chemokine receptor expression, transwell assays were performed with splenocytes or PB neutrophils from mice with EAU that had been treated with anti-G-CSF or isotype mAbs. Cells were placed in the top chamber, and CXCL2/MIP-2 in the bottom. Cells migrating to the bottom chamber were analyzed by flow cytometry and ViaLight assay, and the number of migrating neutrophils was calculated. Significantly fewer splenic (Figure 5A) and PB (Figure 5B) neutrophils from anti-G-CSF-treated mice migrated in response to CXCL2, compared with isotype control mAb treatment. Actin polymerization is essential for neutrophil migration.⁴⁹ PB cells were stimulated with CXCL2/MIP-2 and stained with phalloidin-fluorescein isothiocyanate, which binds filamentous or F-actin. GR-1⁺CD11b⁺ neutrophils from WT mice with EAU that had

been treated with isotype control mAb underwent normal actin polymerization and depolymerization (Figure 5C). In contrast, neutrophils from WT mice with EAU that had been treated with anti-G-CSF mAb failed to undergo actin polymerization in response to CXCL2/MIP-2 stimulation.

We assessed whether these *in vitro* chemotactic differences translated into a change in neutrophil migration *in vivo*. PB neutrophils from G-CSF[−] and G-CSF-R[−] deficient mice express lower levels of CXCR2. We, therefore, injected G-CSF[−] and G-CSF-R[−] deficient mice with G-CSF (or phosphate-buffered saline) s.c., 12 hours before injecting the chemoattractant CXCL2/MIP-2 into a different anatomical compartment, namely the peritoneal cavity. Four hours after injection of CXCL2, peritoneal lavages and PB were analyzed. PB neutrophil CXCR2 expression was increased after G-CSF administration to G-CSF[−] deficient, but not G-CSF-R[−] deficient, mice (Figure 5D). The number of infiltrating neutrophils in the peritoneal cavity was calculated as a percentage of PB neutrophil number to generate a migration index. Consistent with our data from the transwell assays, *in vivo* neutrophil migration in response to CXCL2 was increased after G-CSF administration in G-CSF[−] deficient mice, but not in G-CSF-R[−] deficient mice (Figure 5E).

To examine neutrophil trafficking *in vivo* during disease, ocular eGFP expression was analyzed in LysM-Cre-eGFP reporter mice with EAU. At d14, there was early perivascularitis and abnormal fluorescence of the optic nerve, which was less evident in anti-G-CSF-treated mice compared with the isotype-matched control. In contrast, on d21 of EAU, there were markedly more eGFP^{hi} neutrophils in the eyes of the isotype-treated EAU mice (Figure 5, F and G). At d14, total fluorescence (a measure of neutrophil infiltration) was decreased in mice treated with anti-G-CSF. Separating fluorescence in the region of the optic nerve from the rest of the eye revealed a decrease in eGFP fluorescence around the optic nerve at d14 and in the rest of the retina at d21 in anti-G-CSF-treated mice (Figure 5, H and I). These data demonstrate that G-CSF antagonism decreases neutrophil migration in response to the CXCR2 ligand, CXCL2, both *in vitro* and *in vivo*, and reduces retinal damage in EAU.

Discussion

Neutrophils are traditionally considered as ancillary cells during sterile inflammation, contributing to non-specific host tissue damage. In this study, we have demonstrated that neutrophils and G-CSF play critical effector roles in EAU, a classic experimental model of organ-restricted, T-cell-mediated autoimmunity. Serum G-CSF increased early in EAU, and tracked closely with elevation of the PB neutrophil count. Subsequently, ocular G-CSF levels increased in the inflamed eye, associated with increased ocular MPO. These kinetics suggest a bimodal effect and are consistent with G-CSF acting early to increase BM

granulopoiesis, and later acting locally at the inflammatory site. Histological analysis and *in vivo* imaging demonstrated that the severity of EAU was markedly decreased in G-CSF[−] deficient mice and after G-CSF antagonism, whereas flow cytometric analysis showed that in the absence of G-CSF, the ocular neutrophil population in EAU was profoundly reduced. Although the G-CSF receptor is expressed most abundantly on neutrophils, it is also found on monocytes and G-CSF can induce cytokine production in human and mouse monocytes.^{50,51} Monocytes/macrophages are known to play an important role in EAU,⁵² and it is therefore possible that the absence or blockade of G-CSF in the setting of EAU may also reduce monocyte activation.¹¹ However, we found no change in infiltrating monocyte subsets in the eye associated with G-CSF blockade or deficiency.

We found that CXCR2 neutrophil expression was markedly decreased in G-CSF[−] deficient, G-CSF-R[−] deficient, and anti-G-CSF-treated mice, whereas expression was increased in mice administered G-CSF. Conversely, CXCR4 expression was up-regulated in G-CSF[−] deficient, G-CSF-R[−] deficient, and anti-G-CSF-treated mice and was decreased in mice administered G-CSF. Reduced CXCR2 expression on peripheral neutrophils translated into functional defects in actin polymerization and reduced *in vitro* and *in vivo* neutrophil migration in response to a CXCR2 ligand. These effects were not simply because of differential neutrophil survival, because although both GM-CSF and G-CSF increased the survival of neutrophils *in vitro*, only G-CSF down-regulated CXCR4 and up-regulated CXCR2 expression on mature neutrophils. In a model of endotoxin-induced uveoretinitis, ocular leukocytic infiltrate was decreased in CXCR2-deficient mice.⁵³ Increased G-CSF-R mRNA, CXCR2, and CXCL1 have been found in the eye in EAU.⁵⁴ G-CSF is produced by endothelial cells in the eye, and these cells also express the G-CSF-R.⁵⁵ We suggest that tissue-derived G-CSF, most likely derived from ocular endothelial cells, may play several key roles during inflammatory eye disease. These include production of chemokines by endothelial cells,^{13,56} increasing the expression of adhesion receptors on these cells, fine-tuning the expression of CXCR2 and CXCR4 chemokine receptors on neutrophils to promote neutrophil trafficking, and also extending the survival of neutrophils that have entered inflamed tissues.^{19,57}

Previous studies identified important roles for Th1 cells and IL-12 in EAU and EAE.^{58–60} However, it now appears that Th17 cells are the dominant pathogenic T-cell subset in both diseases, with IL-23, rather than IL-12, playing a nonredundant role.⁶⁰ Only those T cells exposed to IL-23 were able to cause EAE,⁶¹ and IL-23-induced production of GM-CSF by Th17 cells was essential for the encephalitogenicity of T cells.^{62–64} A more recent study in EAE demonstrated that the IL-17⁺ IFN- γ ⁺ subset of CD4⁺ T cells contained the highest proportion of antigen-specific cells (as measured by tetramer staining),⁶⁴ and these cells were also the highest producers of GM-CSF.⁶⁴ Herein, we show that, although antigen-specific T-cell proliferative responses are intact in IRBP peptide-immunized

G-CSF-deficient mice, T-cell differentiation is perturbed. We found a marked increase in IL-17A levels in G-CSF-deficient mice with EAU. Further analysis showed this most likely arises from a population of ROR γ T⁺ $\gamma\delta$ T cells that appears to produce increased IL-17 with G-CSF deficiency. This interesting finding requires more investigation, but would not explain reduced EAU and, in fact, argues that a systemic increase in IL-17 is by itself insufficient to provoke neutrophil-driven inflammatory disease. In contrast, there was a decrease in proportion and number of CD4⁺ cells in the eye producing the critical combination of IL-17A and IFN- γ or GM-CSF alone, corresponding to the T cells implicated in driving EAE.⁶⁴ Our findings are in keeping with a recent report in EAE, showing G-CSF-R-deficient mice have less marked disease after transfer of myelin-reactive Th17 cells,⁶⁵ and suggest a two-way interaction between neutrophils and Th17 cells.

Potential interactions between Th17 cells and neutrophils are intriguing. Activated neutrophils can produce factors, such as IL-1, that influence Th17 polarization via effects on DC and the production of IL-23.⁶⁶ Neutrophils can produce IL-17, as well as cytokines and chemokines that attract Th17 cells⁶⁷ and, in turn, (human) Th17 cells can produce the neutrophil chemoattractant, IL-8.⁶⁸ In EAU, transgenic IRBP-specific T cells are able to penetrate the blood-ocular barrier in two waves, with the second occurring approximately 4 days after transfer.⁶⁹ At this time point, if ocular antigen-specific T cells encounter cognate antigen in the context of major histocompatibility complex class II on resident antigen presenting cells,^{70–72} an inflammatory response is initiated. The ensuing cytokine and chemokine production activates the vascular endothelium, promoting the influx of inflammatory cells. In EAE, neutrophils are required for breakdown of the blood-brain barrier.⁷³ We found that ocular infiltration of neutrophils associates with structural damage to the retina, potentially releasing IRBP for uptake and presentation by local antigen presenting cells. Interestingly, ovalbumin-presenting neutrophils can directly stimulate OVA-specific, MHC class II-restricted T cell receptor (OT-II) T cells, and ovalbumin-pulsed neutrophils stimulated both IFN- γ and IL-17 production by OT-II T cells.⁶⁰ In a mouse model of allergic contact dermatitis, T-cell priming and IFN- γ production were markedly reduced in neutrophil-depleted mice.⁷⁴ In a recent article, Warnatsch et al⁵¹ demonstrated neutrophil extracellular trap-mediated macrophage priming and the subsequent polarization/activation of Th17 cells. Furthermore, IL-17A, CXCL1, CXCL2, and chemokine ligand 2 were decreased in the atherosclerotic aortas of neutrophil extracellular trap-deficient mice. Collectively, these data demonstrate that neutrophils can play a key role in the development of the Th17 response in sterile inflammation.

We propose that endogenous G-CSF acts at multiple levels to influence the course of EAU: increasing neutrophil differentiation and production from BM progenitors, mobilizing neutrophils from the BM into PB, regulating expression of chemokine receptors on circulating neutrophils, activating

endothelial cells to become proadhesive and generate local chemokine gradients, prolonging tissue neutrophil survival, and promoting polarization of the Th17 response. These effects are likely to be enhanced when G-CSF and neutrophil levels increase during inflammation-induced granulopoiesis. As previously highlighted,⁶⁵ neutrophil number and activation status, serum levels of G-CSF, and neutrophil-related chemokines and chemokine receptors may, therefore, provide useful biomarkers in the management of patients with central nervous system autoimmunity. In contrast to decades of elegant work on T-cell-mediated mechanisms, the contribution of neutrophils to the clinical expression of autoimmune diseases, such as uveitis, has received relatively little attention. Careful manipulation of this powerful component of inflammation and autoimmunity may have therapeutic benefits in these conditions.

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Supplemental Data

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