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Neutrophils Amplify Autoimmune Central Nervous System Infiltrates by Maturing Local APCs

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Multiple sclerosis is considered to be initiated by a deregulated, myelin-specific T cell response. However, the formation of inflammatory CNS lesions and the contribution of different leukocyte subsets in setting up these lesions are still incompletely understood. In this study, we show that, in the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis, neutrophil granulocytes are important contributors in preparing CNS inflammation. Preclinical single-dose Ab-mediated depletion of neutrophils delayed the onset and continuous depletion attenuated the development of experimental autoimmune encephalomyelitis, whereas the generation of a myelin-specific T cell response remained unaffected. Neutrophil-related enzymes such as myeloperoxidase and neutrophil elastase did not contribute in mounting CNS inflammation, as analyzed by using respective knockout mice and inhibitors. CNS-infiltrating neutrophils secreted proinflammatory molecules and matured bone marrow–derived dendritic cells in vitro, which in turn enhanced their ability to restimulate myelin-specific T cells. This was mirrored in vivo, in which depletion of neutrophils specifically impaired maturation of microglia and macrophages into professional APCs, resulting in a diminished amplification of early CNS inflammation. Therefore, inside the CNS neutrophils provide local cofactors that are required for the maturation of myeloid cells into professional APCs representing an essential step for the local restimulation of myelin-specific T cells and the development of autoimmune disease. *The Journal of Immunology*, 2013, 191: 4531–4539.

M ultiple sclerosis (MS) is an inflammatory disease of the CNS, in which leukocyte infiltration results in demyelination and neurodegeneration (1). MS is thought to occur in genetically predisposed individuals under certain environmental conditions, leading to the development of CNS Ag-specific Th1 and Th17 cells (2), which can then be reactivated by their cognate Ag in the CNS and cause autoimmune disease (3). This development of CNS autoimmunity can be modeled in susceptible mouse strains by immunizing them with CNS Ags, for example, myelin peptides, resulting in myelin-specific Th1 and Th17 responses and experimental autoimmune encephalomyelitis (EAE) (4).

The development of an encephalitogenic T cell response in the CNS is a multistep process, which depends on the encounter of cognate professional APCs with CNS-infiltrating T cells (3). However, it is incompletely understood how a proinflammatory environment that facilitates reactivation of autoreactive T cells is generated in the CNS. Several leukocyte subsets mainly of the innate immune system have been shown to play essential roles in this process, among them CNS-resident cells such as microglia (5, 6) as well as infiltrating myeloid cells like dendritic cells (DCs) (7, 8) and DC-like macrophages (9, 10). Recently, a similar role for neutrophil granulocytes has been established in EAE in SJL and BALB/c mice, because neutrophil-depleting Abs and blockade of the neutrophil-specific chemokine receptor CXCR2 render mice resistant to EAE induction (11, 12). Although neutrophils are generally not detected in MS lesions, neutrophil infiltration is prominent in early active demyelinating spinal cord lesions of neuromyelitis optica patients (13). Furthermore, neutrophil-attracting chemokines like CXCL8 can be detected in cerebrospinal fluid of opticospinal and classical MS patients (14). However, it is currently unknown by which means neutrophils contribute to the development of CNS inflammation. They secrete proinflammatory enzymes, such as myeloperoxidase (MPO) or neutrophil elastase (ELANE), reactive oxygen species (ROS), and are able to produce a diverse array of proinflammatory mediators such as the cytokines TNF-α, IL-6, IL-12/23, IFN-γ, and IL-17A (15), but also the anti-inflammatory cytokine IL-10 (16), depending on the inflammatory environment and infectious challenge.

In this study, we investigated the role of neutrophils in the initiation of a chronic CNS inflammation and were able to demonstrate an important contribution of neutrophils in setting up early, preclinical CNS inflammation in myelin oligodendrocyte gp35–55 (MOG_{35-55})-immunized C57BL/6 mice. CNS-infiltrating neutrophils produce several proinflammatory molecules and are able to mature bone marrow–derived DCs (BM-DCs) in vitro, thereby enhancing their capacity to restimulate myelin-specific T cells. In the absence of CNS-infiltrating neutrophils in vivo, the maturation of microglia and infiltrating monocytes is significantly decreased,

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Abbreviations used in this article: BM-DC, bone marrow–derived dendritic cell; BM-N, bone marrow–derived neutrophil; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; CNS-N, CNS-derived neutrophil; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; ELANE, neutrophil elastase; MOG, myelin oligodendrocyte glycoprotein; MPO, myeloperoxidase; MS, multiple sclerosis; ROS, reactive oxygen species.

resulting in a strong impairment of leukocyte recruitment to the CNS and amelioration of clinical disease.

Materials and Methods
Mice

We obtained wild-type C57BL/6J mice from The Jackson Laboratory and bred them in the animal facility of the University Medical Center Hamburg-Eppendorf. *Mpo*-deficient mice were previously described (17), as well as 2D2-transgenic mice (18). We performed all animal experiments in accordance with the guidelines of the local authorities (Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz Hamburg).

Experimental autoimmune encephalomyelitis

We injected 6- to 10-wk-old mice s.c. at two sites in the flanks with 100 μl 200 μg MOG$_{35-55}$ emulsified in CFA supplemented with 2 mg ml^{-1} *Mycobacterium tuberculosis H37Ra*. We administered immunized animals 300 ng pertussis toxin i.v. the same day and i.p. 2 d later. EAE developed after approximately 10 d and was scored daily based on a 5-point EAE scale (0, no disease symptoms; 1, limp tail; 2, hind limb paresis; 3, partial hind limb paralysis; 3.5, complete hind limb paralysis; 4, hind limb paralysis and fore limb paresis; 5, moribund). We assured food and water access for severely disabled animals. We euthanized mice with complete hind limb paralysis continuing over 3 d or that suffered from tetraparesis. For neutrophil depletion, we treated animals i.v. at indicated time points with 100 μg anti–Ly-6G (clone 1A8) or isotype control (rat IgG2a, clone 2A3; both from BioXCell) in PBS. For blockade of neutrophil elastase, we treated immunized mice i.p. with sivelestat (0.5 mg daily) or GW311616A (0.1 mg every other day) or vehicle control.

Immunohistochemistry

We prepared cervical spinal cord samples from PBS-perfused animals at indicated time points after immunization and fixed them in 4% paraformaldehyde overnight at 4°C and then transferred them into 30% sucrose for 24 h. We incubated cryosections (14 μm) in blocking solution for 1 h at room temperature, washed them once in PBS, and incubated them overnight at 4°C with anti-Gr1 (RB6-8C5) and anti-CD3 (145-2C11) diluted in PBS. We washed sections three times in PBS for 5 min and incubated them for 1 h at room temperature with fluorescently labeled secondary Abs (all from The Jackson Laboratory) diluted in PBS and stained nuclei with H 33258. We washed stained sections three times in PBS for 5 min, mounted them in Fluoromount G, and documented them on an AxioVert40 equipped with an ApoTome (Zeiss). For immunohistochemical analysis of MHC class II expression on CNS-infiltrating neutrophils, paraformaldehyde-fixed cryosections were blocked in PBS supplemented with 5% normal donkey serum and 0.05% Triton X-100 for 30 min at room temperature. Sections were incubated with anti–Ly-6G (1A8; BioXcell) for 16 h at 4°C, followed by donkey anti–rat-Cy3 secondary Ab (The Jackson Laboratory) for 2 h at room temperature. Stained sections were blocked with rat IgG2a (2A3; BioXcell) and then incubated with FITC anti-MHC class II (M5/115.15.2; BioLegend) for 2 h at room temperature. Stained sections were mounted in Fluoromount G and documented on an AxioVert40 equipped with an ApoTome (Zeiss).

Cell isolation

We prepared single-cell suspensions of splenocytes or lymph nodes by passing tissue through a 40-μm cell strainer (BD Falcon). We isolated and quantified CNS-infiltrating leukocyte subsets, as previously described (19). Briefly, we minced CNS from PBS-perfused animals, digested them with collagenase and DNase I (Roche Applied Science), and then triturated them. We isolated CNS-infiltrating leukocytes by Percoll gradient centrifugation (30 and 78%) and isolated CNS-infiltrating neutrophils or bone marrow neutrophils from CNS-infiltrating leukocytes or bone marrow cells using the anti–Ly-6G microbead kit (Miltenyi Biotec), according to manufacturer's instructions.

Ex vivo flow cytometry analyses

We quantified CNS-infiltrating leukocytes using TruCount tubes (BD Biosciences), as previously described (19). We stained for surface Ags in the presence of anti-CD16 and anti-CD32 (Fc-Block). After surface staining, we incubated EDTA blood samples from mice with FACS lysing solution (BD Biosciences) for 10 min at room temperature. For detection of intracellular cytokines in T cells, we stimulated leukocytes with PMA and ionomycin (both Sigma-Aldrich) for 5 h in the presence of brefeldin A (eBioscience). We fixed and permeabilized cells for intracellular staining of

cytokines or FoxP3 by using intracellular fixation and permeabilization kit or FoxP3 staining buffer set (both from eBioscience), according to manufacturer's instructions. For quantification of cytokine production by CNS-infiltrating neutrophils, cells were cultured for 5 h in the presence of brefeldin A without further stimulation. We used the following Abs for flow cytometric analyses: anti-CD3e Pacific Blue (500A2), anti-CD3e PerCPCy5.5 (145-2C11), anti-CD4 FITC/Pacific Blue (GK1.5), anti-CD8a Pacific Blue/PECy7 (53-6.7), anti-CD11b FITC/PerCPCy5.5 (M1/70), anti-CD11c allophycocyanin/PECy7 (N418), anti-CD25 allophycocyanin (PC61.5), anti-CD45 allophycocyanin-eFluor750 (30-F11), anti-CD45R(B220) PECy5.5 (RA3-6B2), anti-CD62L PECy7 (MEL-14), anti-CD69 FITC (H1.2F3), anti-CD80 PE (16-10A1), anti-CD86 allophycocyanin (GL1), anti-FoxP3 PE (FJK-16s), anti-Gr1 FITC/Pacific Blue/V405 (RB6-8C5), anti–IFN-γ PE (XMG1.2), anti–IL-6 PE (MP5-20F3), anti–IL-10 allophycocyanin (JES5-16E3), anti–IL-12p40 PE (C15.6), anti–IL-17A Alexa647 (eBio17B7), anti–Ly-6C Alexa647 (ER-MP20), anti–Ly-6G PE/Pacific Blue/V405 (1A8), anti-MHCII FITC (M5/115.15.2), anti-NK1.1 PECy7 (PK138), and anti–TNF-α PE (MP6-XT22). We determined IFN-γ secretion by bone marrow–derived neutrophils (BM-N) after 3 h of in vitro stimulation with 1 μg ml^{-1} LPS (Sigma-Aldrich) or 20 μg ml^{-1} heat-inactivated *M. tuberculosis* (Difco) using the cytokine secretion assay (Miltenyi Biotec), according to manufacturer's instructions. We excluded cells with impaired membrane integrity from analysis by 7-aminoactinomycin D staining (Beckman Coulter) and analyzed samples on a LSRII flow cytometer (BD Biosciences) using appropriate compensation controls and doublet discrimination.

^{3}H-thymidine incorporation assay

We cultured lymph node cells from immunized animals in 96-well plates at 2×10^5 cells/well in RPMI 1640 supplemented with 10% FCS and 50 μM 2-ME and stimulated them with different concentrations of MOG$_{35-55}$ peptide or 0.2 μg ml^{-1} anti-CD3 (145-2C11). After 2 d, we pulsed cells with 1 μCi [methyl-^{3}H]-thymidine (Amersham) per well for 16 h, harvested cells, and spotted them on filtermats using Harvester 96 MACH III M (Tomtec), according to manufacturer's instructions. We dried spotted filtermats and sealed them in bags containing betaplate scintillation liquid (Perkin-Elmer). We assessed incorporated activity per 96 wells in a beta counter (1450 Microbeta; Perkin-Elmer) in cpm and calculated stimulation indices of applied peptides or Abs by dividing the mean incorporated activity of stimulated wells by the mean of unstimulated control wells.

APC maturation assay

We differentiated DCs from neutrophil-depleted bone marrow cells in RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME, glutamine, penicillin, streptomycin, and 20 ng ml^{-1} murine rGM-CSF (Immunotools). After 4 d, we harvested adherent cells and plated them without GM-CSF. After day 6, we cocultured DCs at a 1:1 ratio with neutrophils isolated from the inflamed CNS (day 10 after immunization) or from bone marrow (unprimed control). In transwell experiments, we separated DCs and neutrophils by a 0.4-μm polycarbonate membrane (Corning). We harvested cells after 16 h of coculture and analyzed surface expression of maturation markers by flow cytometry. Alternatively, we stimulated BM-DCs at day 5 with supernatants of overnight cultures derived from bone marrow neutrophils or CNS-infiltrating neutrophils. For cytokine-blocking experiments, Abs against IFN-γ (XMG1.2; eBioscience), IL-6 (MP5-20F3; BioLegend), TNF-α (MP6-XT22; BioLegend), and/or IL-12/23p40 (C17.8; BioLegend), or corresponding isotype control rat IgG1 (RTK2071; BioLegend) or rat IgG2a (2A3; BioXCell) were added at a concentration of 10 μg ml^{-1} each to cocultures or supernatant stimulations. Neutralizing activity of Abs was verified by stimulating BM-DCs with a pool of cytokines (IL-6, IL-12, TNF-α [all 10 ng ml^{-1}], and IFN-γ [100 ng ml^{-1}]) and coincubation with respective Abs.

For heat denaturation, cell culture supernatants of bone marrow neutrophils or CNS-infiltrating neutrophils were incubated at 95°C for 15 min, followed by centrifugation at 14,000 rpm for 5 min. For neutralization of ROS, cultures derived from bone marrow neutrophils or CNS-infiltrating neutrophils were treated with 0.5 mM *N*-acetylcysteine (Sigma-Aldrich) for indicated time periods. After overnight incubation of BM-DCs with indicated supernatants, cells were harvested and subjected to flow cytometric analyses.

Quantification of ROS production by neutrophils

Neutrophils were isolated from inflamed CNS or bone marrow, according to the aforementioned protocol. Cells were loaded with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Life Technologies), according to manufacturer's instructions. Briefly, cells were incubated in PBS containing 10 μM CM-H$_2$DCFDA for 15 min at 37°C. Remaining reagent was removed by centrifugation and, subse-

quently, cells were supplemented with growth medium and subjected to incubation at 4°C or 37°C for 30 min. Oxidation of CM-H$_2$DCFDA by intracellular ROS leads to an increase in fluorescence intensity, which was quantified by flow cytometry.

T cell restimulation assay

We sacrificed 6- to 10-wk-old 2D2 transgenic mice and cultivated splenocytes for 2 d in presence of 20 μg ml^{-1} MOG$_{35-55}$, 5 ng ml^{-1} IL-2, and 5 ng ml^{-1} IL-7. Stimulated cells were washed and rested in the presence of 5 ng ml^{-1} IL-2 and 5 ng ml^{-1} IL-7 for 4 d. We isolated CNS-derived neutrophils and bone marrow neutrophils, as described above, cultivated them for 16 h individually, and harvested supernatants. We differentiated BM-DCs, as mentioned above, for 4 d in the presence of GM-CSF and added, after 24 h of GM-CSF–free culture, supernatants from CNS-derived neutrophils and bone marrow neutrophil cultures for 24 h. We added 1 μg ml^{-1} MOG$_{35-55}$ peptide for 1 h, followed by addition of resting transgenic 2D2 T cells, which remained in culture for 6 h. We supplemented brefeldin A after 3 h to stop cytokine secretion and stained cells afterward for intracellular cytokines, according to the aforementioned protocol.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM software. For analysis of Ab-mediated depletion experiments, anti–Ly-6G–treated animals were compared with isotype-treated animals using two-way ANOVA, including repeated measures where applicable. Data were considered significantly different if either treatment or interaction of treatment and time was significant. In other experiments, one-way ANOVA, including repeated measures where applicable, and Student's t test were used. The specific statistical analysis is described in the respective figure legends.

Results

Neutrophils contribute to preclinical CNS inflammation during EAE

To investigate the contribution of different leukocyte subsets to the CNS-inflammatory response during EAE, we characterized the temporal dynamics of CNS infiltration by flow cytometry. We isolated leukocytes from the CNS of MOG$_{35-55}$-immunized C57BL/6 mice at different time points over the course of EAE (Fig. 1A) and quantified CD45$^+$ cells by combined flow cytometric analysis using TruCount beads. This allowed us to calculate the total number of immune cells isolated from the CNS of single animals (Fig. 1B). Among CD45$^+$ leukocytes, microglia, neutrophils, NK, and NKT cells, macrophages and DC subsets as well as T and B cells were identified using a sequential gating strategy (Fig. 1C). Over the disease course, absolute numbers of CNS-infiltrating leukocytes as well as T cell and macrophage infiltration correlated with the clinical course (Fig. 1D, 1E). Compared with healthy controls, the number of CNS-infiltrating CD45^{high} cells was already elevated 2-fold during preclinical EAE. Notably, this preclinical inflammatory infiltrate was dominated by innate immune cells, which primarily consisted of neutrophils showing almost 10-fold expansion in this phase. This observation was paralleled by an extensive amplification of neutrophil numbers in the peripheral blood and by shedding of CD62L from the neutrophil surface, which is suggestive of an activated phenotype (Supplemental Fig. 1A–C). Although the absolute number of neutrophils increased further after the onset of disease, the proportion of neutrophils among CNS-infiltrating cells did not expand simultaneously and even declined during acute EAE. By contrast, numbers and frequencies of T cells, macrophages, and professional APCs increased after onset of clinical symptoms. Of note, recovery from severe EAE and the transition to chronic disease were mainly associated with a decrease in the numbers of innate immune cells like macrophages and myeloid DCs, whereas T cells constituted one of the main cell types among CNS-infiltrating cells during recovery and in chronic disease (Fig. 1D, 1E).

As neutrophils are one of the earliest cells infiltrating the CNS, we investigated localization in the inflammatory lesions in correlation with their frequency in CNS-infiltrating leukocytes. In agreement with the elevation of neutrophil frequency in CD45^{high} CNS-infiltrating leukocytes during preclinical EAE (Fig. 1F), neutrophil infiltration was detected in meninges and perivascular spaces in the CNS (Fig. 1G). Concomitantly, early-infiltrating T cells were detected at the same anatomical site. After disease onset, with neutrophil frequencies among CNS-infiltrating leukocytes decreasing, T cells started to invade into the CNS parenchyma, whereas neutrophils stayed mostly limited to the meninges and perivascular spaces (Fig. 1G).

Ab-mediated depletion of neutrophils impedes the development of EAE

Next, we investigated the functional role of this early infiltration of neutrophils into the CNS during EAE. We treated animals with the neutrophil-specific depleting Ab anti–Ly-6G (1A8) or corresponding isotype control (rat IgG2a) before and at the onset of clinical symptoms. This Ab allows specific targeting and depletion of neutrophils from the peripheral blood without affecting other cell populations (Supplemental Fig. 1D–G) (20). Because Ab-mediated depletion of neutrophils resulted only in a transient reduction of neutrophil numbers in the peripheral blood, immunized animals were either treated once or every other day with anti–Ly-6G. Whereas a transient depletion of neutrophils delayed the onset of disease (Fig. 2A), continuous treatment completely abrogated the development of clinical EAE (Fig. 2B). By contrast, isotype control Ab treatment had no effect on disease course in comparison with untreated EAE mice (Fig. 2A). In agreement with the early preclinical recruitment of neutrophils into the CNS during EAE, depletion of neutrophils after the onset of clinical symptoms had no effect on disease incidence or severity (Fig. 2C). A continuous treatment with anti–Ly-6G was only efficient for a maximal period of approximately 10 d (Supplemental Fig. 1H). Nevertheless, depletion of neutrophils for this time period resulted in attenuation of EAE disease course (Fig. 2D), whereas isotype control-treated animals again developed a similar disease course as untreated EAE mice. Only 2 of 8 treated animals developed severe EAE symptoms (hind limb paresis), whereas the other treated animals developed milder clinical symptoms. By contrast, 6 of 10 isotype control-treated animals developed severe EAE (hind limb paresis or paralysis) (Fig. 2D).

Neutrophil depletion does not alter the peripheral encephalitogenic T cell response

The observed attenuation of EAE clinical severity by depletion of neutrophils could be explained with a role of neutrophils in peripheral T cell activation. Therefore, we investigated the peripheral MOG$_{35-55}$-specific T cell response and proliferation of anti–Ly-6G– or control-treated animals using ^{3}H-thymidine incorporation assay as readout. However, peripheral priming of the MOG$_{35-55}$-specific T cell responses was not altered by neutrophil depletion starting 8 d after immunization. Stimulation indices of lymphocytes isolated from anti–Ly-6G– and control-treated animals were comparable 2 d after Ab treatment, that is, 10 d after immunization (Fig. 3A) and 14 d after immunization (Fig. 3B). Furthermore, we investigated the potential importance of neutrophils for early peripheral T cell priming by starting anti–Ly-6G treatment concomitantly with EAE induction (day 0) and continued it at alternate days. Similar to a delayed treatment start, the peripheral MOG$_{35-55}$-specific T cell response was not reduced in anti–Ly-6G–treated animals compared with control-treated animals at day 10 after immunization (Supplemental Fig. 2). Additionally, differentiation of Th1
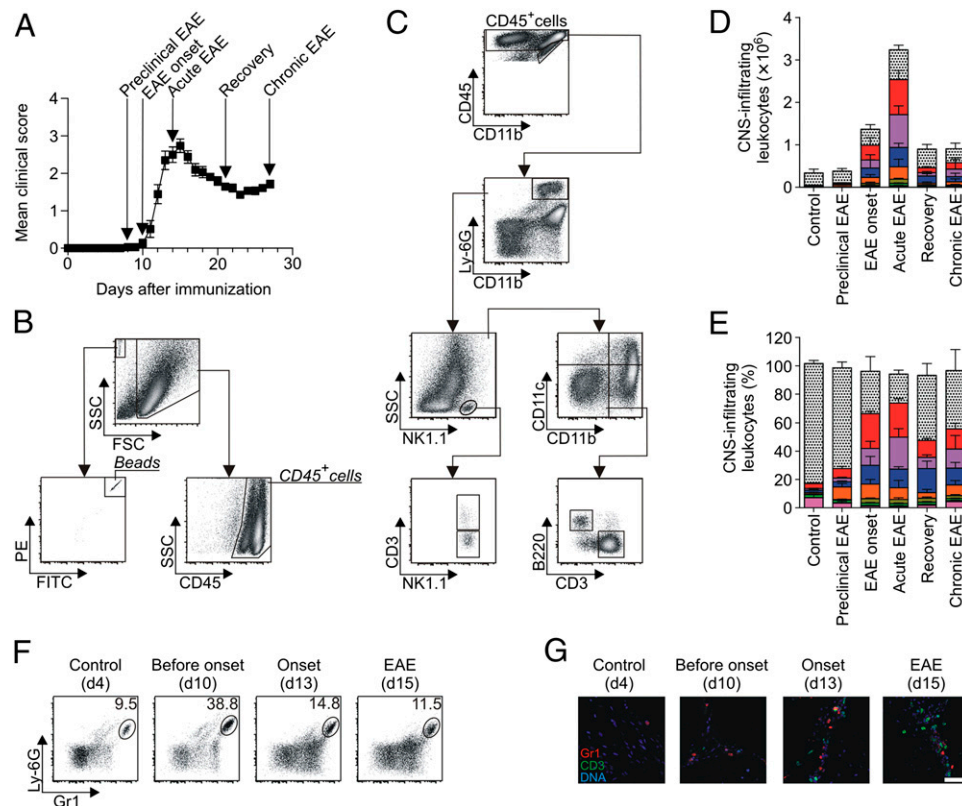
FIGURE 1. Temporal dynamics of CNS infiltration by immune cells. (**A**) Mean clinical disease course and time points of analyses are shown for animals, which completed the observation period. Mean clinical scores are mean $\pm$ SEM ($n = 14$). (**B**) Quantification of $CD45^+$ CNS-infiltrating immune cells using TruCount beads. Separation of beads and cells based on size (FSC) and granularity (SSC) is shown in the *upper panel*, and selection of fluorescent beads and $CD45^+$ cells is shown in the *lower panel*. (**C**) Identification of CNS-infiltrating immune cell types by a sequential gating strategy. Identification of microglia ($CD45^{int}CD11b^+$; dotted), neutrophils ($CD45^{high}Ly-6G^+$; orange), NK cells ($CD45^{high}Ly-6G^-NK1.1^+CD3^-$; light green), NKT cells ($CD45^{high}Ly-6G^-NK1.1^+CD3^+$; dark green), $CD11b^-$DCs ($CD45^{high}Ly-6G^-NK1.1^- CD11c^+CD11b^-$; olive), myeloid DCs ($CD45^{high}Ly-6G^-NK1.1^- CD11c^+CD11b^+$; purple), macrophages ($CD45^{high}Ly-6G^-NK1.1^-CD11c^-CD11b^+$; red), B cells ($CD45^{high}Ly-6G^-NK1.1^-CD11c^-CD11b^-B220^+$; pink), and T cells ($CD45^{high}Ly-6G^-NK1.1^-CD11c^-CD11b^-CD3^+$; blue). One representative experiment is shown. (**D**) Mean calculated numbers of CNS-infiltrating leukocytes at indicated time points over the course of EAE. Results for individual cell types are stacked as follows: microglia (dotted), macrophages (red), myeloid DCs (purple), T cells (blue), neutrophils (orange), $CD11b^-$DCs (olive), NK cells (light green), NKT cells (dark green), and B cells (pink). (**E**) Frequency of investigated immune cell types within $CD45^+$ CNS-infiltrating cells (=100%). Calculated cell numbers and frequencies at indicated time points are shown as mean $\pm$ SD ($n \geq 8$). (**F**) Frequency of neutrophils ($CD45^{high}Gr1^+Ly-6G^+$) in $CD45^{high}$ CNS-infiltrating leukocytes at indicated time points over the course of EAE. Representative results are shown ($n = 2$ or 3). (**G**) Immunohistochemical staining for neutrophils ($Gr1^+$) and T cells ($CD3^+$) in cervical spinal cord sections during the course of EAE. Scale bar, 50 μm. Representative stainings are shown ($n = 2$ or 3).

and Th17 cells was unchanged by anti–Ly-6G treatment as determined by intracellular cytokine staining (data not shown).

CNS-infiltrating neutrophils are a source of cytokines

Because T cell responses were not affected by neutrophil depletion, we investigated how neutrophils might exert a local role in mounting an immune response inside the CNS. First, we investigated the contribution of neutrophil-related enzymes such as MPO or ELANE for EAE development. MPO is involved in the regulation of endothelial barrier permeability (21) and the transmigration of neutrophils to inflamed sites (22). ELANE, together with proteinase 3, has been described to generate a proinflammatory environment by degrading anti-inflammatory progranulin, thereby producing proinflammatory granulin peptides (23). Although we detected chloric tyrosine, the reaction product of hypochloric acid that is produced by active MPO in cervical spinal cord sections during EAE (data not shown), development of EAE was not different in *Mpo* knockout mice (Fig. 4A). Because *Elane* or *Prtn3* knockout animals were not available on an EAE-susceptible background, we treated immunized animals with two different ELANE inhibitors, Sivelestat (data not shown) and GW311616A (Fig. 4B). Although inhibition of ELANE has been reported to

ameliorate Th17-induced adoptive transfer EAE (24) and a mouse model of neuromyelitis optica (25), treatment with both inhibitors did not alter the development of actively induced EAE, indicating that both enzymes are not critically involved in neutrophil recruitment and effector function necessary for the development of actively induced EAE. Among other neutrophil-produced proinflammatory soluble factors, cytokines are good candidates as early signals setting up an inflammatory milieu. Therefore, we isolated CNS-infiltrating leukocytes at onset of disease and assessed cytokine production of Ly-6G–expressing neutrophils by intracellular staining after in vitro culture in the presence of brefeldin A (Fig. 4C). A significant proportion of neutrophils produced TNF-α (15.9% $\pm$ 0.4%), IL-6 (9.6% $\pm$ 0.9%), or IL-12/23p40 (12.8% $\pm$ 1.6%), but no IL-17 or IL-10 was detectable. Furthermore, we observed that almost all neutrophils isolated produced IFN-γ (85.1% $\pm$ 2.2%). Direct ex vivo staining of BM-N proved the IFN-γ expression to be constitutive, as has previously been reported for mouse neutrophils (26). However, upon stimulation, IFN-γ secretion by BM-N was detectable in a secretion assay (Fig. 4D), indicating that neutrophil-derived IFN-γ might also be secreted by CNS-infiltrating neutrophils.
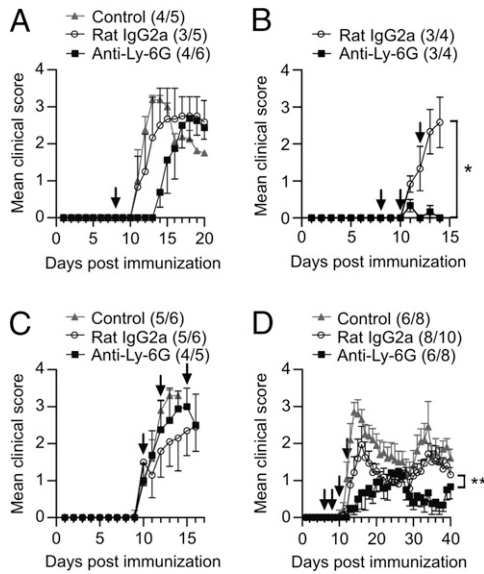
FIGURE 2. Neutrophils are critical for EAE disease initiation. (**A–D**) At indicated time points after EAE induction (black arrows), animals were treated with 100 μg anti–Ly-6G i.v. (black squares) or corresponding isotype control ratIgG2a (open circles) or left untreated (gray triangles). Animals were either treated with a single injection (A), or with repeated injections every other day (B–D). Indicated in parentheses is the number of diseased animals among immunized animals per group. Results show mean clinical score of diseased animals ± SEM. Statistical analyses were performed by repeated-measures two-way ANOVA. Asterisks indicate statistical significance with $*p < 0.05$, $**p < 0.01$.

CNS-derived neutrophils mediate maturation of professional APCs

Based on the production of proinflammatory cytokines by CNS-derived neutrophils, we hypothesized that CNS-derived neutrophils could be important for the maturation of local APCs and thereby facilitate T cell restimulation. We therefore purified neutrophils from the inflamed CNS 9–10 d after immunization and performed coculture experiments with in vitro differentiated BM-DCs. Coculture with CNS-derived neutrophils (CNS-N), but not with bone marrow–derived neutrophils (BM-N), induced the expression of MHC class II, CD80, and CD86 on BM-DCs in vitro (Fig. 5A). To see whether this effect could be mediated by neutrophil-secreted factors, we separated neutrophils and DCs in a transwell assay, which resulted in a comparable upregulation of surface markers on APCs induced by CNS-N (Fig. 5B). Likewise,
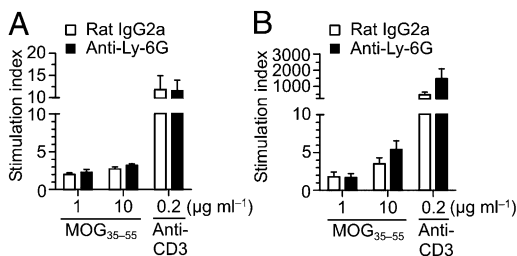

FIGURE 3. Neutrophil depletion does not impair peripheral T cell priming. (**A, B**) Animals were treated with 100 μg anti–Ly-6G or isotype control from day 8 after immunization. Single-cell suspensions were prepared from draining lymph nodes of anti–Ly-6G (black bars) or isotype control-treated (white bars) animals during preclinical EAE (day 10) (A) and during acute disease (day 14) (B). Cells were restimulated in vitro with either MOG_{35-55} peptide or anti-CD3 (2C11) at indicated concentrations. Resulting T cell proliferation was assessed by ^{3}H-thymidine incorporation.
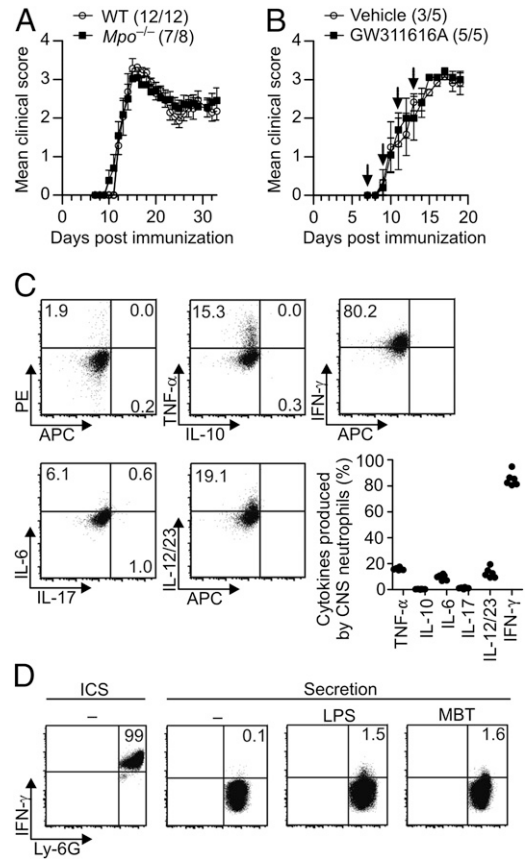

FIGURE 4. CNS-infiltrating neutrophils are a source of proinflammatory cytokines. (**A**) Course of EAE in myeloperoxidase knockout ($Mpo^{-/-}$; black squares) and WT control animals (open circles). (**B**) Treatment of immunized mice with neutrophil-elastase inhibitor GW311616A (black squares) in comparison with PBS-treated vehicle control (open circles). One representative experiment of two experiments is shown. Disease incidences are shown in the legend. (**C**) Intracellular cytokine staining of infiltrating cells from the inflamed CNS at disease onset (day 9 after immunization) without further stimulation. Representative stainings of gated neutrophils ($CD45^{high}Ly$-6G) and statistics of analyzed animals ($n = 5$) are shown. (**D**) BM-N were either stained directly for intracellular IFN-γ ex vivo (*left panel*, ICS) or cultured for 3 h in presence of brefeldin A together with the indicated stimuli. IFN-γ secretion was analyzed by Miltenyi cytokine secretion assay. Representative stainings of two independent experiments are shown.

overnight stimulation of BM-DCs with supernatants from CNS-N, but not from BM-N, resulted in an increase of MHC class II, CD80, and CD86 on the cell surface, which could be reduced to control levels by heat denaturation of CNS-N–derived supernatant (Fig. 5C). To identify the effector mechanism responsible for this effect, we found an increased ROS production by CNS-N compared with BM-N (Supplemental Fig. 3A). However, blocking of ROS-induced oxidization by *N*-acetylcysteine was not sufficient to reduce CNS-N–mediated maturation of BM-DCs (Supplemental Fig. 3B, 3C). Having thus demonstrated that one or several heat-sensitive protein components are of key importance for the APC maturation capacity of CNS-N, instead of ROS, we next sought to dissect the contribution of the individual proinflammatory cytokines produced by CNS-N. However, Ab-mediated blockade of single cytokines (Supplemental Fig. 3D, 3E), as well as using DCs derived from IFN-γ receptor or TNFR knockout mice (data not shown), could not impair DC maturation induced by CNS neutrophils, implying a redundant function of the different secreted cytokines. Even a combined blockade of IL-6, IL-12/23p40, IFN-γ,
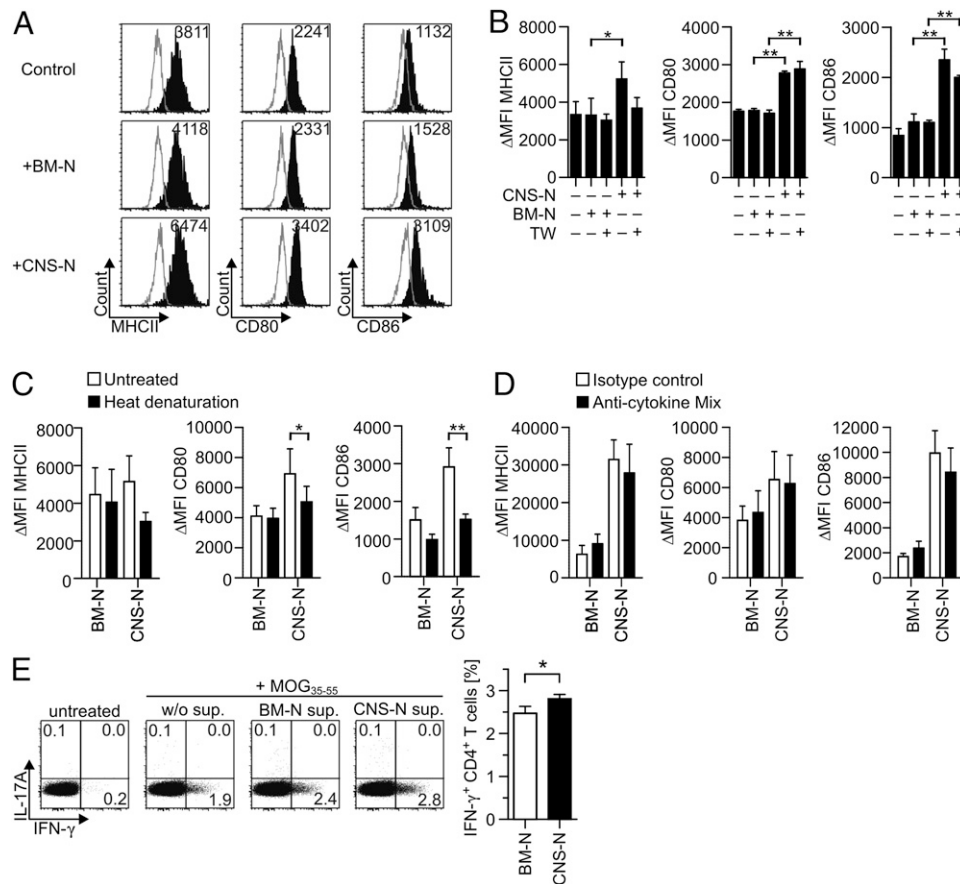
FIGURE 5. CNS-derived neutrophils mature APCs in vitro. (**A**) Neutrophils were isolated from the CNS at disease onset (day 10 after immunization; CNS-N) or from bone marrow of control animals (BM-N) and cocultured with BM-DCs for 16 h. Analysis of MHC class II, CD80, and CD86 surface expression by flow cytometry of gated BM-DCs (CD11c$^+$Ly-6G$^-$). Representative histogram overlays of respective surface stainings (black curves) with control stainings (gray curves) of three independent experiments are shown. (**B**) BM-DCs and neutrophils were either cocultured or separated by a transwell (TW), as indicated. Statistical analyses were performed by one-way ANOVA with Bonferroni post hoc test. Asterisks indicate statistical significance of post hoc tests with *$p < 0.05$, **$p < 0.01$. (**C**) CNS-N were isolated from the inflamed CNS at day 10 after immunization, and BM-N from healthy mice were purified and cultivated for 16 h. BM-DCs were stimulated with supernatants from CNS-N or BM-N cultured for 16 h. Where indicated, supernatants were heat denatured. Statistical analyses were performed by two-way ANOVA with Bonferroni post hoc test. Asterisks indicate statistical significance of post hoc tests with *$p < 0.05$, **$p < 0.01$. (**D**) BM-DCs were stimulated with supernatants from CNS-N or BM-N for 16 h. Where indicated, a pool of blocking Abs against IFN-γ, TNF-α, IL-6, and IL-12/23p40 at 10 μg ml^{-1} each or corresponding isotype controls was added. (**E**) 2D2-transgenic T cells and MOG$_{35-55}$ peptide were added to BM-DCs after 16 h of stimulation with CNS-N– or BM-N–derived supernatant. Intracellular cytokine staining was performed after 6 h of in vitro culture. Representative stainings of gated 2D2 T cells (CD4$^+$ TCRVα3.2$^+$) are shown. All results represent mean ± SEM of at least three independent experiments. Statistical analysis of IFN-γ secretion was performed by unpaired Student's t test with *$p < 0.05$.

and TNF-α showed only slight and nonsignificant reduction of MHC class II, CD80, and CD86 expression on BM-DCs stimulated with CNS-N–derived supernatant (Fig. 5D). Therefore, besides proinflammatory cytokines, CNS-derived neutrophils provide currently unknown additional soluble proteins, which mediate the recorded effects.

To investigate the consequence of BM-DC exposure to CNS-N–derived proinflammatory proteins, we next investigated the capacity of BM-DCs to restimulate effector T cells. In concordance with the elevated expression of MHC class II and costimulatory molecules, IFN-γ production of in vitro differentiated MOG$_{35-55}$-specific 2D2-TCR transgenic Th1 cells was significantly increased after short-term restimulation with peptide-pulsed BM-DCs conditioned with CNS-N–derived supernatant in comparison with BM-DCs conditioned in BM-N–derived supernatant (Fig. 5E). Notably, although low levels of MHC class II expression could be detected on CNS-N by flow cytometry (Supplemental Fig. 4A), an in situ detection by immunohistochemistry failed (Supplemental Fig. 4B). Furthermore, peptide-pulsed CNS-N were not able to induce IFN-γ production in 2D2 TCR-transgenic Th1 cells (Supplemental Fig. 4C),

corroborating that CNS-derived neutrophils most likely do not directly restimulate T cells in the CNS.

Neutrophils are essential for recruitment and maturation of professional APCs

Finally, we investigated whether the absence of neutrophil-secreted cytokines in the CNS inflammatory infiltrate resulted in an equally impaired maturation of CNS-infiltrating APCs in EAE as we observed in vitro. Therefore, we characterized leukocyte infiltration in the CNS of neutrophil-depleted animals during preclinical EAE (day 10) and during acute EAE (day 14) in comparison with control animals treated with rat IgG2a. Corresponding to the depletion of neutrophils from peripheral blood (see Supplemental Fig. 1), the recruitment of neutrophils to the CNS was completely abrogated (Fig. 6A). This absence of neutrophils in the CNS resulted in a significant impairment of overall leukocyte recruitment by reducing the total number of infiltrating CD45^{high} cells during acute EAE (Fig. 6B). To detect a specific effect of neutrophil depletion on the inflammatory response in the CNS, we performed a detailed characterization of CNS-infiltrating leukocyte subsets in anti–Ly-6G–

FIGURE 6. CNS-infiltrating neutrophils contribute to local leukocyte recruitment and APC maturation. Animals were treated with 100 μg anti–Ly-6G or isotype control from day 8 after immunization. CD45$^+$ CNS-infiltrating cells were isolated during preclinical EAE (day 10) or acute EAE (day 14) and analyzed by flow cytometry. (**A**) Quantification of CNS-infiltrating neutrophils (Gr1$^+$Ly-6C^{int}) from anti–Ly-6G (black bars) or isotype control-treated animals (white bars). (**B**) Quantification of CD45^{high} infiltrating cells and CD45^{int} CNS-resident microglia. (**C**) Mean calculated frequency of leukocyte subsets within CD45^{high} CNS-infiltrating cells 14 d after immunization. All data are shown as mean ± SEM ($n \geq 3$). (**D**, **E**) Frequencies of CD4$^+$FoxP3$^+$ T regulatory cells, and expression of activation markers CD69 and CD25 on effector T cells (D) as well as IFN-γ–producing Th1 cells and IL-17A–producing Th17 cells (E) were analyzed by combined surface and intracellular cytokine stainings. Representative stainings are shown. Results represent mean ± SEM ($n \geq 3$). (**F–H**) Maturation of microglia and macrophages into MHC class II$^+$CD11c$^+$ professional APCs (F) and expression of the costimulatory molecules CD80 (G) and CD86 (H) are shown from representative stainings. Histograms represent mean ± SEM pooled from two independent experiments ($n \geq 5$). Statistical analyses were performed by two-way ANOVA. Asterisks indicate statistical significance with *$p < 0.05$, **$p < 0.01$.
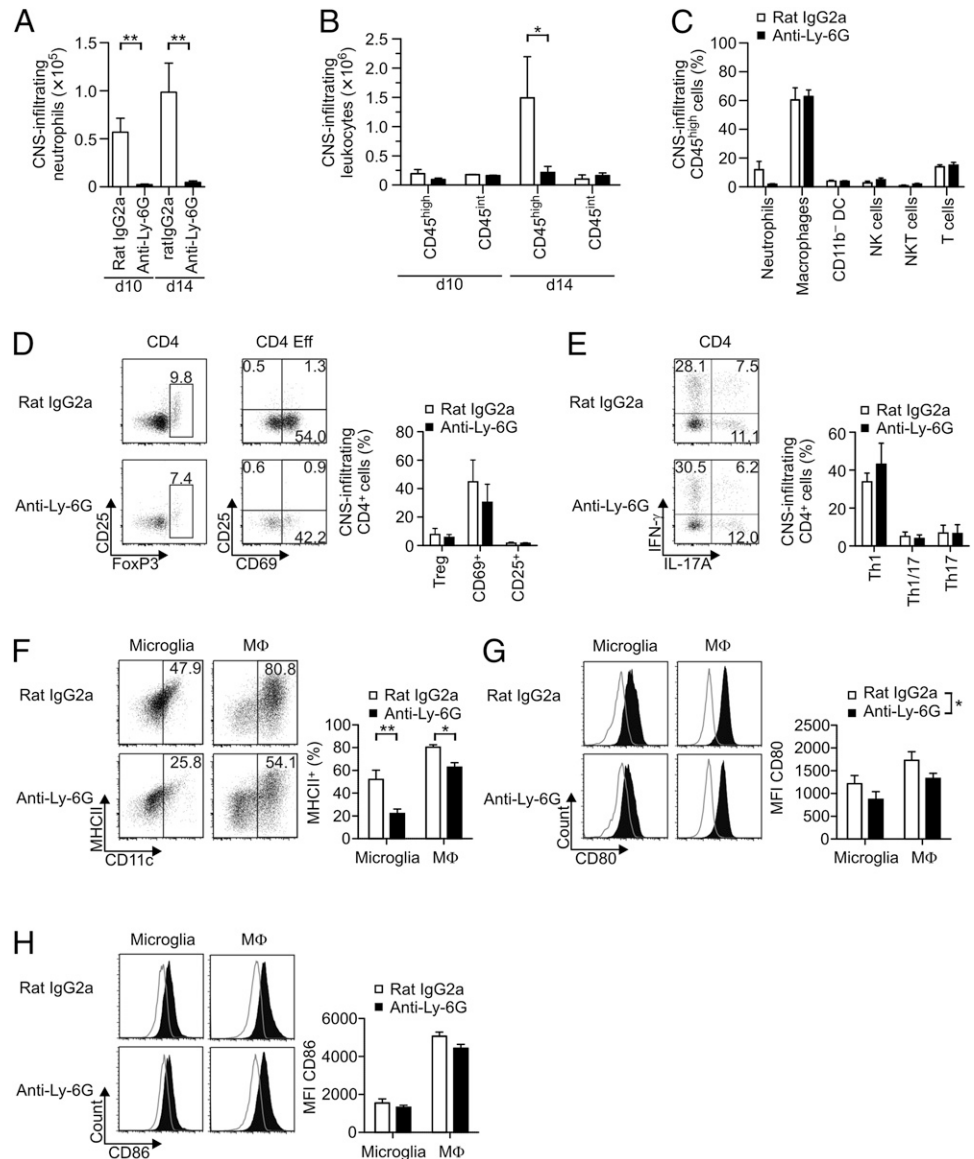
and control-treated animals during acute EAE (Fig. 6C–E). Of note, although a diminished number of leukocytes infiltrated into the CNS of anti–Ly-6G–treated animals in comparison with control-treated animals during acute EAE, we did not observe any difference in the frequencies of infiltrating leukocyte subsets besides a reduced frequency of neutrophils (Fig. 6C). The absence of neutrophils in the CNS led to a slightly reduced proportion of CD69-expressing CD4$^+$ T cells, but affected neither the ratio between CD4$^+$FoxP3$^+$ regulatory T cells and effector T cells, nor the differentiation of CD4$^+$ T cells into IFN-γ–producing Th1 or IL-17A–producing Th17 cells (Fig. 6D, 6E), again indicating that neutrophils do not exert a direct effect on T cells. However, in agreement with our in vitro findings, we detected a strongly impaired differentiation of microglia and monocytes/macrophages into MHC class II–expressing professional CD11c$^+$ APCs (Fig. 6F) and an impaired upregulation of the costimulatory molecule CD80 (Fig. 6G), whereas the expression of CD86 was only mildly affected by neutrophil depletion (Fig. 6H).

Discussion

In this study, we provide evidence that neutrophils are functionally important for the development of adaptive CNS inflammation and initiation of clinical disease in MOG$_{35-55}$-induced EAE in C57BL/6

mice, which is dependent on neutrophils infiltrating the CNS and secreting soluble factors—most likely several proinflammatory proteins—that contribute to the maturation of professional APCs during early CNS inflammation. This in turn affects the reactivation of myelin Ag-specific T cells as shown in vitro and most likely also inside the CNS, as their infiltrating numbers are greatly reduced.

In our experimental model, neutrophils start to infiltrate the CNS already 2–3 d before the onset of disease where they first primarily locate to the meninges, indicating that neutrophils contribute to early processes during CNS inflammation in EAE. Accordingly, significantly increased levels of neutrophil-attracting chemokines such as CXCL1 and CXCL2 have been shown to be detectable already several days before onset of disease in C57BL/6 mice (27) similar to other EAE models (11). Neutrophil recruitment to the CNS in MOG$_{35-55}$-induced EAE in C57BL/6 mice is probably mediated via CXCR2 signaling, as described for EAE models in other mouse strains (11), because neutrophil infiltration into the CNS is also dependent on CXCR2 expression in a cuprizone-mediated demyelinating disease model in C57BL/6 mice (28). The same pathway could target neutrophils in humans, because the human ortholog CXCL8 (IL-8) can be detected in the cere-

brospinal fluid of MS patients (14), and neutrophils in the peripheral blood of MS patients are characterized by a primed phenotype, including elevated expression of IL-8R in comparison with healthy individuals (29).

Due to their close relationship with other phagocytes such as macrophages, a specific genetic targeting or depletion of neutrophils was to date not possible. Therefore, we used the mAb 1A8 that binds the neutrophil-specific surface marker Ly-6G and depletes these cells from peripheral blood. This Ab is superior to other neutrophil-depleting Abs such as the mAb RB6-8C5, because it is highly specific for neutrophils and spares other immune cell populations, as shown by our data and by others (16, 20). Depletion from peripheral blood starting approximately 2 d before onset of EAE resulted in strikingly reduced numbers of CNS-infiltrating neutrophils and a concomitant reduction of CNS inflammation and disease severity. This was unrelated to an impaired priming of the peripheral T cell response or an altered differentiation of Th1 and Th17 cells in the absence of neutrophils. Instead, our in vitro and in vivo data suggest that soluble proteins derived from CNS-infiltrating neutrophils promote the maturation of myeloid (CD11b$^+$) precursors into CD11c$^+$MHC class II$^+$ professional APCs inside the CNS and enhance their capacity to restimulate myelin-specific effector T cells. These APCs, which have been shown to derive from Ly-6C^{high}–expressing inflammatory monocytes in the peripheral blood in EAE induced in C57BL/6 and SJL mice (9, 10), are very potent T cell stimulators and have been reported to drive Th17 differentiation as well as epitope spreading in the CNS during EAE in SJL mice (8, 30). Notably, Ly-6C^{high}–expressing cells are specifically spared by the Ab used in our study, in contrast to the widely used clone RB6-8C5 (20).

Whereas neutrophils have been described to influence the recruitment and maturation of professional APCs such as DCs in cell contact–dependent and independent manners (32), also by secreting cytokines such as TNF-α (31), we were able to exclude the proinflammatory cytokines TNF-α, IFN-γ, IL-6, and IL-12/23, as well as MPO, ELANE, and ROS production, although we found evidence of increased enzyme activity in EAE, in promoting the maturation of professional APCs by CNS-infiltrating neutrophils.

In defense against pathogens, neutrophils have recently been attributed to possess a considerable amount of plasticity. Depending on the inflammatory environment and infectious challenge, they can exert a plethora of different effector mechanisms and secrete a diverse and partly redundant array of inflammatory mediators, which regulate innate as well as adaptive immune cells (15). This most likely explains why we could not pinpoint a key factor, such as proinflammatory cytokines, enzymes, or ROS, mediating the disease-promoting role of neutrophils. Therefore, other soluble alarmins, such as bactericidal agents, most likely contribute to APC maturation in our setting (33). The fact that other phagocytes can also secrete almost all of the proinflammatory mediators produced by neutrophils poses another challenge to the identification of a specific neutrophil function involved in the development of CNS inflammation. The observed necessity of neutrophil recruitment for the development of CNS inflammation could also be related to their terminally differentiated phenotype, which specifically delineates them from other myeloid phagocytes. By their rapid recruitment and immediate action, neutrophils may provide an initial proinflammatory cytokine milieu, setting up the scene for further immune cell recruitment and activation. In this scenario, neutrophils would thus be of critical but transient importance for the generation of autoimmune CNS inflammation, something that is reflected by neutrophils becoming less abundant in inflammatory EAE lesions after disease onset (see Fig. 1) as well as less important for disease

progression, as shown by the inefficacy of neutrophil depletion after disease onset (see Fig. 2C).

Reports describing early pathological changes in MS brains are scarce, and the sequence of events involved in the formation of inflammatory lesions in the human CNS has still to be clarified. The limited availability of tissue samples from early or even preactive lesions poses a major challenge to the investigation of such a mechanism in humans. Furthermore, the studies describing the existence of neutrophil-attracting chemokines in the CNS of human MS patients mainly involve Asian patient cohorts (14); therefore, a functional role of neutrophils on Asian types of MS might be more likely to emerge.

By showing that early CNS-infiltrating neutrophils contribute to the generation of a proinflammatory milieu and maturation of professional APCs, our study adds an additional facet to how inflammatory lesions in MS could develop in the CNS. Furthermore, our study emphasizes that innate immune cells, although not directly involved in Ag presentation, play an essential role for the initiation and shaping of adaptive immune responses also in autoimmune disease.

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Disclosures

The authors have no financial conflicts of interest.

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