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doi: 10.4049/jimmunol.174.8.5124
http://www.jimmunol.org/content/174/8/5124

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Neutrophils That Infiltrate the Central Nervous System Regulate T Cell Responses

Simone P. Zehntner, Cristina Brickman, Lyne Bourbonnière, Leah Remington, Maria Caruso, and Trevor Owens

Regulation of inflammatory responses is critical to progression of organ-specific autoimmune disease. Although many candidate cell types have been identified, immunoregulatory activity has rarely been directly assayed and never from the CNS. We have analyzed the regulatory capability of Gr-1<sup>high</sup> neutrophils isolated from the CNS of mice with experimental autoimmune encephalomyelitis. Proportions of neutrophils were markedly increased in the CNS of IFN-γ-deficient mice. Strikingly, CNS-derived neutrophils, whether or not they derived from IFN-γ-deficient mice, were potent suppressors of T cell responses to myelin or antigen. Neutrophil suppressor activity was absolutely dependent on IFN-γ production by target T cells, and suppression was abrogated by blocking NO synthase. These data identify an immunoregulatory capacity for neutrophils, and indicate that interplay between IFN-γ, NO, and activated Gr-1<sup>high</sup> neutrophils within the target organ determines the outcome of inflammatory and potentially autoimmune T cell responses. The Journal of Immunology, 2005, 174: 5124–5131.

Inflammation of the CNS occurs in response to infection, trauma, or autoimmune attack. The pathology of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), an animal model for MS, is characterized by infiltration of CD4<sup>+</sup> T lymphocytes and other leukocytes into the CNS (1). Peripheral immunization with myelin proteins leads to induction of autoimmune Th1 CD4<sup>+</sup> T cell responses, which are critical for EAE and assumed to be so for MS and other CNS inflammatory diseases such as acute disseminated encephalomyelitis and Devic’s optic neuritis (2–4).

Immune regulation is considered critical to disease progression. Earlier studies in MS indicate a role for CD8<sup>+</sup> suppressor T cells, whose mechanism was undefined (5, 6). More recent studies have pointed to CD4<sup>+</sup> T cell regulation (7–9), in particular identifying Th2 cytokines that counter inflammatory Th1 responses as potential modulators of EAE (10). Correlation of expression with remission, and/or severity of disease in knockout (KO) animals, has pointed to IL-4, IL-10, and other Th2-associated cytokines such as TGF-β as immunoregulatory mediators (11–13). Implication of Th2 cytokines as regulatory mediators would suggest the action of suppressor Th2 T cells, or IL-10-producing regulatory T cells or macrophages (14), and there is evidence that such cells are present in the CNS in EAE and MS. However correlations between Th2 cytokine expression and remission are not water tight (15, 16). Furthermore no study has directly demonstrated the immunoregulatory capacity of Th2 or any other cell isolated from the CNS. Other mechanisms need to be considered.

Interestingly, an immunoregulatory role for the proinflammatory Th1 cytokine IFN-γ in EAE has also been proposed, on the basis of exacerbated disease in gene KO mice (10, 17, 18) and on effects of administration to the CNS (19). Mice lacking IFN-γ or its receptor show lethal nonremitting disease, with disseminated infiltrates and dysregulated T cell proliferation (18, 20). The mechanism whereby IFN-γ regulates T cell responses in vitro has been shown to involve the IFN-γ inducible NO synthase (iNOS) enzyme, and the reactive nitrogen intermediate NO (21–23). These have been implicated in regulation of EAE, through observations of exacerbated disease in mice lacking iNOS (24, 25) and through inhibitor studies (reviewed in Ref. 26).

Cell population(s) that might mediate immunoregulation downstream of Th1 cells include myeloid cells. Myeloid cells, including macrophages and granulocytes, express the Gr-1 epitope on the GPI-anchored glycoproteins Ly-6G and Ly-6C (27, 28). Cells expressing Gr-1 have been implicated in suppression of T cell responses in vitro via a NO and IFN-γ-dependent mechanism (29). Gr-1<sup>+</sup> cells have also been implicated in the suppression of antitumor responses in vivo (29–33). Levels of iNOS and NO are elevated in the CNS in EAE and MS (26), and inflammatory infiltrates in the CNS contain myeloid cells. The possibility that IFN-γ-directed suppression could contribute to immunoregulation in the CNS is compelling, given that all the players are in place.

In this study we show that Gr-1<sup>+</sup> cells isolated from the CNS of mice with EAE suppress T cell responses to myelin and other Ags. Gr-1<sup>high</sup> populations are dominated by neutrophils. These neutrophils, which infiltrate the target organ in EAE, are profoundly suppressive. We further show that suppression of T cells is absolutely dependent on IFN-γ production by those T cells, and on NO. This direct analysis of regulatory cells isolated from the CNS identifies a novel role for Gr-1<sup>high</sup> neutrophils in immune regulation.

Materials and Methods

Mice

Specific pathogen-free female SJL/J mice (8–to 10-wk-old) were purchased from Charles River Breeding Laboratories. Heterozygous BALB/c backcrossed mice with the disrupted IFN-γ gene (GKO) were originally obtained from Genentech and bred in our facility (20, 34). Homozygous
IFN-γ KO mice were backcrossed onto the SJL/J background. Homozygous SJL/J backcrossed IFN-γ KO mice at F2 to F7 (IFN-γ KO) were used in this study. Controls were wild-type (WT) F2 to F7 littermates.

Induction of EAE
EAE was actively induced by s.c. immunization with an emulsified emulsion of 400 μg of bovine myelin basic protein (MBP; Sigma-Aldrich) in CFA (Difco), initially at two sites at the base of tail, followed 7 days later by s.c. injection in the flanks. Each injection site contained 25 μg of heat-killed Mycobacterium tuberculosis H37RA (Difco). Mice were weighed and monitored daily for clinical signs of EAE. Disease severity was scored as follows: 1, Limp tail, trembling; 2, 1 plus hind limb paresis (assessed by the animal's slowness or inability to regain upright status when overturned, sprawling limbs when walking); 3, 2 plus unilateral hind limb paralysis; 4, 3 plus paralysis of both hind limbs or a forelimb; and 5, moribund or dead. Animal breeding and maintenance, and all experimental protocols were in accordance with Canadian Council for Animal Care guidelines and approved by McGill University Animal Care Committee (Montreal, Quebec, Canada).

Isolation of CNS infiltrate
Animals were anesthetized and perfused intracardially with ice-cold PBS. Brain and spinal cord tissue was collected, mechanically dissociated, and centrifuged at 400 × g for 10 min at 4°C. The cell pellet was resuspended in 37% isotonic Percoll (Amerham Pharmacia Biotech) as previously described (35). Cells were centrifuged at 2800 × g with no brake and moderated acceleration for 20 min at room temperature. Mononuclear cells were collected from the pellet and washed twice in 10% RPMI 1640 (Invitrogen Life Technologies).

Flow cytometry analysis
Cells were blocked with normal rat Ig (Cedarlane Laboratories) in 2.4% (anti-FcγRIIb/FcRII) hybridoma supernatant, followed by staining for 15 min at 4°C with the appropriate conjugated mAb. The following mAbs were obtained from BD Pharmingen: anti-Ly-6G biotin (RB6–8C5), anti-Ly-6G FITC (RB6–8C5), anti-CD45 CyChrome (30-F11), and anti-CD45 (16-2-8C5). Biotinylated mAbs were visualized using Streptavidin-PE (BD Pharmingen) or Streptavidin–FITC (Jackson ImmunoResearch Laboratories). Staining was analyzed using a FACScan (BD Biosciences). Propidium iodide staining and forward/side scatter gating were used to exclude dead cells.

FACS analysis
Cells were immunostained as previously described and sorted using a FACS Vantage (BD Biosciences) to isolate CD45− GR-1high cells. Alternatively GR-1high cells were isolated using MACS beads (Miltenyi Biotec). Briefly, cells were blocked with rat Ig and anti-FcRII and incubated with biotinylated anti-GR-1. Streptavidin-conjugated magnetic beads (Miltenyi Biotec) were added and incubated for 15 min on ice. The samples were then performed on a magnetized column. Even flow cytometry and cytospins were used to confirm success of the isolation procedure. Cytospins of sorted cells were stained using Diff-Quik (VWR).

In vitro proliferation assay of lymph node cells
A single cell suspension was prepared from the draining lymph nodes, and lymph node cells (4 × 106 cells) were cultured in the presence of sorted GR-1high cells for 3 days in 200 μl of culture, with or without Ag (50 μg/ml purified protein derivative (PPD) or 50 μg/ml MBP). Some cultures were also supplemented with IFN-γ (10 U/ml), anti-IFN-γ (XMG1.2, 2 μg/ml), or l-NMMA (Nω-nitro-L-arginine monooctate, 0.5 mM; Calbiochem). Cultures were pulsed with 0.5 μCi [3H]thymidine (ICN Pharmaceuticals) during the last 18 h of incubation before harvesting onto glass fiber filters. [3H]Thymidine uptake was measured as cpm.

TNF-α measurements
Supernatants were harvested from mixed cultures before the [3H]thymidine pulse, and levels of TNF-α were measured using a mouse TNF-α ELISA (RayBiotech). The assay was performed according to the manufacturer’s protocol. Briefly, culture supernatants were incubated overnight on precoated plates, subsequently incubated with biotinylated anti-TNF-α detection Ab and streptavidin–HRP. Samples were washed with sodium phosphate buffered saline containing tetramethyl benzidine substrate and read on a microplate reader at 450 nm. The concentration was determined by comparison to a standard curve generated from serial dilution of recombinant TNF-α.

Nitrate/nitrite measurements
Supernatants were harvested from mixed cultures before the [3H]thymidine pulse, and levels of nitrates and nitrites were measured as an indirect measure of the NO being produced in culture. Nitrates were first converted to nitrite by the enzyme nitrate reductase in the presence of NADPH (Boehringer Mannheim), and then total nitrates were measured by addition of Griess reagent. Samples were read on a microplate reader at 540 nm and the concentration determined by comparison to a standard curve generated from serial dilution of sodium nitrate.

RNAse protection assay
RNA was purified from PBS-perfused CNS using Trizol (Invitrogen Life Technologies). Multiprobe DNA templates for cytokines and for L32 and GAPDH were purchased from BD Pharmingen. RNAse protection assay was performed according to the manufacturer’s protocol. Briefly, T7 poly-adenylated templates were cloned into T7 compatible pBluescript SK plus vector. RNAse protection probes were labeled with α-32P[UTP (DuPont NEN Research Products). Labeled probes were hybridized with 10–15 μg of total RNA, digested with RNAse, then treated with proteinase K. RNAse-protected RNA duplexes were extracted with phenol/chloroform/isoamyl alcohol (Invitrogen Life Technologies) and resolved on 5% denaturing polyacrylamide gels using undigested labeled probes as size markers. Gels were visualized by PhosphorImager (Molecular Dynamics), and then subsequently analyzed using ImageQuant software.

Histology and immunohistochemistry
Mice were anesthetized and perfused intracardially with ice-cold PBS. Tissue samples were collected and embedded in OCT, or fixed in 4% paraformaldehyde (Sigma-Aldrich) and paraffin-embedded. Immunohistochemical staining was performed on 10-μm cryostat sections. Frozen sections were blocked in 5% normal rabbit serum (Vector Laboratories), then treated with 0.3% H2O2. Sections were incubated with anti-Gr-1 (BD Pharmingen), overnight at 4°C, followed by the appropriate biotinylated secondary Ab (Vector Laboratories) for 1 h at room temperature. The signal was amplified using an avidin-HRP complex (Vectastain ABC kit;Vector Laboratories) and detected with diaminobenzidine (Medicorp). Control sections were incubated with isotype-matched primary and secondary Abs. Five-micrometer paraffin sections were stained with H&E.

Quantitative real-time PCR
Quantitative PCR using the ABI Prism 7000 Sequence Detection System was performed for IL-10 expression (primer and probe sequences in Ref. 36). An 18 S rRNA (primers and probes from Applied Biosystems) was used as an endogenous control. Each reaction was performed in 25 μl with 50 pg TaqMan 2× PCR Master Mix (Applied Biosystems), 900 nM each of the forward and reverse primer, and 200 nM probe. cDNA was diluted 1:1000 for 18 S rRNA analysis. IL-10 primers were synthesized by Sigma-Aldrich. Corresponding probes were synthesized at Applied Biosystems. Conditions for the PCR were 2 min at 50°C, 10 min at 95°C, and then 40 cycles, each consisting of 15 s at 95°C and 1 min at 60°C. To determine the relative RNA levels within the samples, standard curves for the PCR were prepared by using the cDNA from a pooled sample. Standard curves of cycle threshold vs arbitrary levels of input RNA were prepared, and relative levels of mRNA in each sample were determined.

Results
IFN-γ KO mice develop exacerbated disease with polymorphonuclear leukocyte infiltration
To ask whether the removal of IFN-γ would exacerbate EAE in the susceptible SJL/J strain, as was previously demonstrated in BALB/c mice (17, 20), we backcrossed IFN-γ KO mice to SJL/J. Disease incidence and severity were equivalent in actively immunized IFN-γ KO SJL/J and IFN-γ KO BALB/c mice (Fig. 1A). Disease in IFN-γ KO mice was invariably nonremitting and lethal. By contrast, control SJL/J mice and WT littermates developed milder EAE, from which they recovered. Histopathology showed discrete multifocal perivascular infiltration in white matter within the spinal cord of SJL/J mice and WT littermates. In contrast, extensive disseminated infiltration by Gr-1high leukocytes was observed in SJL/J IFN-γ KO mice (data not shown). Chemokine expression profiles in the CNS of IFN-γ KO mice with EAE...
FIGURE 1. IFN-γKO mice develop exacerbated disease, independent of strain background. A, Clinical disease course in mice with EAE, induced by MBP/CFA immunization. Clinical scores are presented as mean scores of all mice presented: IFN-γKO SJL/J ( ), IFN-γKO BALB/c ( ), and SJL/J ( ). ≥ indicates death. One representative experiment is shown. Results were confirmed in at least five replicate experiments with similar outcomes. B, Representative lanes of RNase protection assay analysis of chemokine expression in the CNS of SJL/J- and IFN-γ-deficient unmanipulated mice, and mice with EAE. RNA was isolated from perfused spinal cords, chemokine mRNA was detected by RNase protection using the BD Pharmingen probe set. Bands were quantitated by densitometry and analyzed using ImageQuant software. Three replicate experiments are graphed. CCL5/RANTES and CCL2/MCP-1 were significantly elevated in SJL/J mice with EAE, whereas CXCL1/MIP-2 and CXCL1/TCA-3 were significantly elevated in IFN-γKO mice with EAE.

FIGURE 2. Neutrophils are present in the CNS of WT and IFN-γ-deficient mice with EAE. A, Flow cytometric analysis of Gr-1 expression on CD45<sup>high</sup> cells isolated from the CNS of WT and IFN-γ-deficient mice with EAE shows a larger proportion of Gr-1<sup>high</sup> cells in the CNS of mice lacking IFN-γ than in WT CNS. B, Cumulative results of flow cytometric analyses are shown as the percentage of CD45<sup>high</sup> cells expressing Gr-1<sup>high</sup> to total number of CD45<sup>high</sup> cells. Animals with mild EAE (grades I and II; open symbols) and animals with severe EAE (grades III, IV, and V; closed symbols) are illustrated. A significant increase in proportions and total Gr-1<sup>high</sup> cells was observed in the CNS of IFN-γ-deficient mice. Statistical significance was analyzed by Student’s t test. C, The majority of Gr-1<sup>high</sup> cells colabeled with CD11b/Mac-1 and the neutrophil marker clone 7/4 as analyzed by flow cytometry. Cytospins of sorted CD45<sup>high</sup> Gr-1<sup>high</sup> (D) and Gr-1<sup>low</sup> (E) cells stained with DiffQuick illustrate that the Gr-1<sup>high</sup> population have the characteristic polymorphonuclear morphology of neutrophils. CD45<sup>high</sup> Gr-1<sup>low</sup> cells contained very few polymorphonuclear leukocytes, and were predominantly foamy macrophages. Cumulative results of flow cytometric analyses of infiltrating CD45<sup>high</sup> Gr-1<sup>low</sup> are shown as the percentage of CD45<sup>high</sup> cells expressing Mac-1 (F) or CD3 (G).
matched those previously observed in IFN-γ-deficient BALB/c mice (20), showing elevated expression of CXCL2/MIP-2 and CCL1/TCA-3, reduction in CCL2/MCP-1 expression, and barely detectable CCL5/RANTES (Fig. 1B). Thus the clinical phenotype of EAE that has been described in BALB/c IFN-γ-KO mice is maintained on the SJL/J strain background. Disease progression, leukocyte dynamics and functional responses were indistinguishable between SJL/J and WT littermates.

Elevated proportions of neutrophils in IFN-γKO CNS

Leukocytes were isolated from the perfused CNS of mice with EAE. The total number of cells recovered from IFN-γKO CNS was 1.5-fold higher than from WT animals (2.30 ± 0.85 million vs 1.56 ± 0.52 million). We used flow cytometry to analyze cellular phenotype in the CNS. Levels of CD45 were used to discriminate neutrophils in the CNS of IFN-γ-deficient mice from WT animals (2.30 ± 0.52 million). We used flow cytometry to analyze cellular phenotype in the CNS. Levels of CD45 were used to discriminate neutrophils in the CNS of IFN-γ-deficient mice from WT animals (2.30 ± 0.52 million). We used flow cytometry to analyze cellular phenotype in the CNS. Levels of CD45 were used to discriminate neutrophils in the CNS of IFN-γ-deficient mice from WT animals (2.30 ± 0.52 million).

Increasing equivalently upon immunization with MBP plus CFA (Fig. 2A) and WT (Fig. 2B), in accordance with what was observed by immunohistochemistry. There was no obvious correlation between disease severity and the proportions of Gr-1high cells in either WT or IFN-γ-deficient CNS (Fig. 2B, compare mild with severe disease, open and closed symbols). With the exception of one outlier, the proportion of Gr-1high cells in WT CNS was never higher than 10%, even with severe disease. By contrast, even IFN-γ-deficient mice with mild disease had greater than 20% Gr-1high cells in CNS (Fig. 2B). Almost all (≥98.5 ± 0.2%) of the Gr-1high cells colabeled with CD11b/Mac-1 and the neutrophil marker clone 7/4 (Fig. 2C). Very few (1.6 ± 0.72%) of the Gr-1high cells expressed high levels of the macrophage marker F4/80low, although a small proportion (9.8 ± 1.9%) of 7/4high F4/80low cells was observed among Gr-1high cells (data not shown).

Analysis of cytospins of sorted cells showed that Gr-1high cells almost exclusively exhibited the polymorphonuclear morphology that is characteristic of neutrophils (Fig. 2D). Intracellular eosinophilic granules or other large granules were not visible, excluding a major contribution from basophils and eosinophils. By contrast, Gr-1low cells were very heterogeneous, and included foamy macrophages as well as granulocytes (Fig. 2E). The proportions of Gr-1lowMac-1+ macrophages in IFN-γKO and WT CNS were similar (Fig. 2F), whereas proportions of CD3+ T cells (Fig. 2G) were higher (although not to statistical significance) than those in WT mice.

In contrast to findings in the CNS, there was no significant difference between the proportions of Gr-1high cells in the blood and spleen of WT and IFN-γKO mice (data not shown). Proportions of Gr-1high cells in blood or spleen of WT or IFN-γ-deficient animals increased equivalently upon immunization with MBP plus CFA (data not shown). As in CNS, Gr-1high cells sorted from blood were predominantly polymorphonuclear, whereas Gr-1low cells included monocytes and lymphocytes (data not shown). The increase in neutrophils in the CNS of IFN-γ-deficient mice therefore did not reflect a peripheral neutrophilia.

Neutrophil suppression of T cell proliferation is dependent on IFN-γ

To assess the role of the Gr-1+ neutrophils, we assayed their effect on T cell responses. Gr-1high cells were sorted from CNS or spleen of IFN-γ-deficient mice or WT littermates, and titrated into syngeneic T cell cultures. As previously published for BALB/c T cells, background proliferation of IFN-γ-deficient T cells was elevated, consistent with an immunosuppressive role for IFN-γ.

Gr-1high cells sorted from the CNS of WT and IFN-γKO mice with EAE, suppressed WT T cell responses to both PPD and MBP (Fig. 3A). This suppression was maintained in the face of a 4-fold dilution in the number of either population of CNS-derived neutrophils, to numbers that were not suppressive at all in the case of splenic neutrophils (see below), illustrating potent suppression of T cell proliferation (Fig. 3A). Strikingly, responses of IFN-γ-deficient T cells were not suppressed by coculture with Gr-1high cells sorted from either WT or IFN-γ-deficient mice (Fig. 3B). Thus, neutrophils derived from the CNS have potent suppressor capability, whether or not they originate from an IFN-γ-deficient mouse, and the ability to suppress T cells within the target organ is dependent on IFN-γ production by those T cells.

In contrast, Gr-1high cells from the spleen of WT and IFN-γ-deficient mice showed differential suppressive ability. Although Gr-1high cells sorted from the spleen of WT mice convincingly suppressed WT T cell responses to both PPD and MBP (Fig. 3C), splenic Gr-1high cells from IFN-γ-deficient mice were much less effective (Fig. 3C). As for CNS-derived Gr-1high cells, the suppressive effect of splenic neutrophils was abrogated when target T cells could not produce IFN-γ (Fig. 3D).

![FIGURE 3. Neutrophil suppression of T cell proliferation is dependent on IFN-γ production by T cells.](http://www.jimmunol.org/DownloadedFrom)
To further assess the role of IFN-γ in T cell suppression, an anti-IFN-γ mAb (XM1G12), was added to cocultures of WT T cells and Gr-1<sup>−/−</sup> neutrophils, or IFN-γKO T cell cocultures, were supplemented with exogenous IFN-γ. IFN-γ blockade completely abrogated the suppression of WT T cells by neutrophils derived from the CNS (Fig. 4A) or spleen (Fig. 4D). Conversely when IFN-γKO T cell cocultures were supplemented with IFN-γ, the ability of neutrophils to suppress T cell proliferation was restored (Fig. 4, A and E). Although the addition of IFN-γ reduced both Ag-specific proliferation and baseline responses, significant suppression was still observed. These data confirm that neutrophil suppressor function is controlled by IFN-γ. TNF-α production by T cells, an effector function associated with EAE and MS, was equivalently suppressed by neutrophils (Fig. 4C). This suppression was also dependent on IFN-γ (data not shown).

NO has been identified as a mediator of IFN-γ-induced T cell suppression (21, 23, 38). We assayed NO production in cocultures using a modified Griess reaction to measure nitrates in supernatants. High levels of NO (44 ± 10 μM nitrates) were produced in the Ag-stimulated cultures containing WT T cells and neutrophils, irrespective of the source of the neutrophils. IFN-γ blockade significantly reduced NO levels (to close to or below detection limit), and little or no NO was produced in cocultures with IFN-γKO T cells (at close to or below detection limit). Supplementation of IFN-γ-deficient T cell cocultures with IFN-γ restored the NO levels (37 ± 5 μM nitrates). Consistent with IFN-γ stimulation of neutrophils leading to the production of NO, high levels (29 ± 4 μM nitrates) were observed in all cultures in which exogenous IFN-γ was added to neutrophils, irrespective of the source of the neutrophils. Suppression of WT T cell responses due to culture with Gr-1<sup>high</sup> cells isolated from the CNS (Fig. 4B) or spleen (data not shown) was reversed in the presence of L-NMMA, a competitive inhibitor of NO synthase. L-NMMA also reversed the suppression observed in cocultures of IFN-γKO T cells in the presence of IFN-γ (data not shown).

Previous studies had shown IL-10 was not detectable by RT-PCR in IFN-γ<sup>−/−</sup> mice with EAE (20). To ask whether IL-10 might contribute to neutrophil suppression we used quantitative real-time PCR to assess mRNA. Levels in whole CNS were lower in IFN-γ<sup>−/−</sup> than in WT mice (Fig. 5A). However, sorted Gr-1<sup>high</sup> cells from CNS of either WT or IFN-γ<sup>−/−</sup> mice with EAE expressed very low to undetectable levels of IL-10 message, whereas Gr-1<sup>−/−</sup> cells did express IL-10 (Fig. 5B). Splenic neutrophils also did not express IL-10 mRNA (data not shown). This argues against a role for IL-10 in suppression of T cell responses in vitro, which was confirmed by lack of effect of addition of either anti-IL-10 or anti-IL-10R mAbs to cocultures (data not shown). These data suggest that IFN-γ produced by activated T cells stimulates NO production by activated Gr-1<sup>high</sup> neutrophils within the CNS during inflammation. A model for IFN-γ-induced immune regulation in the CNS is shown in Fig. 6.

**Discussion**

We demonstrate a significant population of Gr-1<sup>high</sup> neutrophils within the CNS during EAE, whose proportions increase in the absence of IFN-γ. In addition we show potent T cell suppressor capability for those neutrophils, whether or not they derive from IFN-γ-deficient mice. The ability to suppress is absolutely dependent on IFN-γ production by T cells, and the mechanism of suppression involves IFN-γ stimulation of NO. Our findings identify interplay between regulatory neutrophils and T cells, which may explain the aggressive and lethal encephalomyelitis in IFN-γ-deficient mice.

The Gr-1 epitope defines the Ly6G and Ly6C molecules, which are expressed by myeloid lineage cells, including granulocytes and some macrophages (27, 28). Cells expressing Gr-1 from spleen, peritoneum, and bone marrow have been reported to suppress T cell responses (23, 29, 30, 32, 33, 39–41), but have generally been identified as macrophages. Bronte et al. (40) identified a splenic Mac1<sup>−/−</sup>/Gr-1<sup>−/−</sup> population, which suppressed Ag-specific CD8<sup>+</sup> T cell responses that they identified as macrophages. Similarly, Cau-ley et al. (23) demonstrated that Gr-1 depletion by Abs ameliorated the suppression of superantigen T cell responses in spleen, and further identified the Gr-1<sup>−/−</sup> cells as including both macrophages and neutrophils. Dupuis et al. (41) depleted suppressor populations by treatment with i-leucine methyl ester, which kills cells with a high lysosomal content, and identified the suppressive cells as Gr-1<sup>−/−</sup> Mac-1<sup>−/−</sup> macrophages. i-leucine methyl ester is also capable of removing neutrophils (42). We demonstrate that the vast majority of Gr-1<sup>high</sup> cells were neutrophils. We believe it was these Gr-1<sup>high</sup> neutrophils that effected suppression. Functional heterogeneity within the Gr-1<sup>high</sup> population is impossible to evaluate due to the small size of the subpopulations. Our data do not exclude that macrophages may also effect suppression within the CNS, although they represent at most a minor contaminant of the Gr-1<sup>high</sup> populations in vitro. Gr-1<sup>low</sup> cells within the CNS were...
preponderantly macrophages and it may be such cells that form the basis for reports of suppressor Gr-1$^{+}$ macrophages.

Neutrophils can produce cytokines, chemokines, and other inflammatory mediators (43, 44). Rodent neutrophils produce NO, which has been implicated both in cytopathology and in immune suppression (18, 21, 26). Increased granulocyte/neutrophil counts in cerebrospinal fluid have been described in Devic’s optic neuritis, in severe cases of acute disseminated encephalomyelitis, as well as other CNS inflammatory pathologies (3, 4). We and others have demonstrated the presence of neutrophils in several animal models of CNS demyelinating disease, and that neutrophils become more apparent in exacerbated CNS inflammatory disease in transgenic or KO animals in which immune regulation is perturbed (18, 20, 45–51). Neutrophils have been demonstrated in MBP-induced EAE, in SJL/J, and in WT and IFN-γ/KO BALB/c (20, 45, 48); in EAE induced by spinal cord homogenate in B6/H mice (46); in myelin oligodendrocyte glycoprotein (MOG)p35–55-induced EAE in IFN-γR-deficient and WT C57BL/6 mice (18, 50); as well as in MOGp92–106-induced EAE in A.SW mice (51). McColl et al. (45) demonstrated that systemic administration of anti-Gr-1 Abs blocked the effector phase of EAE. It is likely that the Rb6 anti-Gr-1 Ab acted to deplete peripheral Gr-1$^{+}$ cells, which our cytopsins and other studies (23, 29, 30, 32, 33, 39–41) show to include Gr-1$^{low}$ macrophages. Peripheral macrophage depletion prevents EAE (52, 53), and this may explain the apparently paradoxical effect described by McColl et al. (45).

The elevated suppressor capacity of CNS-derived neutrophils is consistent with selection of a suppressor/effector neutrophil subset. This may be due to selective migration of neutrophil populations from the periphery by chemoattraction to the site of inflammation. Recruitment and activation of neutrophils can be regulated by both chemokines and cytokines (44, 49), and their increased proportions in the CNS in certain EAE models and in absence of IFN-γ may reflect a dysregulated chemokine environment (20). Alternatively, the increased proportion of neutrophils in the CNS of IFN-γ/KO mice with EAE may reflect IFN-γ inhibition of neutrophil migration across blood-brain barrier endothelia (54, 55). The fact that the suppressive capability of splenic neutrophil populations from IFN-γ-deficient mice was markedly reduced could indicate an effect of IFN-γ on neutrophil traffic, or on their development and activation. It is significant that the suppressive potential of CNS-derived neutrophils in IFN-γ-deficient mice was equivalent to that of WT CNS neutrophils, suggesting that IFN-γ controls neutrophil effector function, as opposed to regulating their development.

Our results bear on the role of IFN-γ as a regulatory cytokine in the CNS. The fact that enhanced T cell proliferation in IFN-γ$^{-/-}$ mice with EAE was observed suggested lack of immunoregulation (20). In this study we demonstrate that neutrophils in the CNS have immunoregulatory capabilities, involving production of NO. We also show that NO is essential for suppression by blockade of its effect. NO has been demonstrated to be an important mediator of T cell suppression (26, 56). Neutrophil production of NO is induced by IFN-γ (57, 58). The mechanism of NO-mediated T cell suppression was shown to involve apoptosis in some (19, 22) but not all studies (59). Dupuis et al. (41) showed that IFN-γ production by T cells was required for NO-mediated suppression by splenic Gr-1$^{+}$ cells. Splenic Gr-1$^{+}$ cells isolated from tumor-bearing mice were demonstrated to suppress tumor-specific T cell responses in an IFN-γ and peroxynitrite-dependent manner (29). Production of NO by the IFN-γ-regulated enzyme iNOS was shown to suppress EAE (60), and iNOS KO mice show exacerbated EAE (25, 26). Our data exclude IL-10 as a mediator of neutrophil suppression in vitro, although it clearly can play a role in immunoregulation in the CNS (10).

Consideration of relative levels of IFN-γ and NO within the CNS may help to reconcile the observations that neutrophils are associated with more severe disease, with our finding that neutrophils isolated from the CNS are effective T cell suppressors. Increased neutrophil and possibly macrophage-mediated T cell suppression may serve as a feedback control for excessive T cell proliferation. This interpretation is consistent with a body of data suggesting that IFN-γ plays a disease-modulating role in EAE. Our
results clearly identify a suppressive role for CNS-derived neutrophils.

Acknowledgments
We thank Jason Millward and Alice Han for preliminary flow cytometric analyses.

Disclosures
The authors have no financial interest of conflict.

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dendritic cells after immunization: induction of a suppressive population of Mac-1

macrophages that suppress naive CD4


dendritic cells after immunization: induction of a suppressive population of Mac-1

macrophages that suppress naive CD4


