Expression of GM-CSF in T Cells Is Increased in Multiple Sclerosis and Suppressed by IFN-β Therapy

Javad Rasouli, Bogoljub Ciric, Jaime Imitola, Patricia Gonnella, Daniel Hwang, Kedar Mahajan, Elisabeth R. Mari, Farinaz Safavi, Thomas P. Leist, Guang-Xian Zhang and Abdolmohamad Rostami

J Immunol 2015; 194:5085-5093; Prepublished online 27 April 2015; doi: 10.4049/jimmunol.1403243
http://www.jimmunol.org/content/194/11/5085

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/04/25/jimmunol.1403243.DCSupplemental

Why The JI?
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication
*average

References
This article cites 31 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/194/11/5085.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Expression of GM-CSF in T Cells Is Increased in Multiple Sclerosis and Suppressed by IFN-β Therapy

Javad Rasouli, Bogoljub Ciric, Jaime Imitola, Patricia Gonnella, Daniel Hwang, Kedar Mahajan, Elisabeth R. Mari, Farinaz Safavi, Thomas P. Leist, Guang-Xian Zhang, and Abdolmohamad Rostami

Multiple sclerosis (MS) is an autoimmune disease of the CNS. Studies in animal models of MS have shown that GM-CSF produced by T cells is necessary for the development of autoimmune CNS inflammation. This suggests that GM-CSF may have a pathogenic role in MS as well, and a clinical trial testing its blockade is ongoing. However, there have been few reports on GM-CSF production by T cells in MS. The objective of this study was to characterize GM-CSF production by T cells of MS patients and to determine the effect of IFN-β therapy on its production. GM-CSF production by peripheral blood (PB) T cells and the effects of IFN-β were characterized in samples of untreated and IFN-β–treated MS patients versus healthy subjects. GM-CSF production by T cells in MS brain lesions was analyzed by immunofluorescence. Untreated MS patients had significantly greater numbers of GM-CSF+ CD4+ and CD8+ T cells in PB compared with healthy controls and IFN-β–treated MS patients. IFN-β significantly suppressed GM-CSF production by T cells in vitro. A number of CD4+ and CD8+ T cells in MS brain lesions expressed GM-CSF. Elevated GM-CSF production by PB T cells in MS is indicative of aberrant hyperactivation of the immune system. Given its essential role in animal models, abundant GM-CSF production at the sites of CNS inflammation suggests that GM-CSF contributes to MS pathogenesis. Our findings also reveal a potential mechanism of IFN-β therapy, namely suppression of GM-CSF production. The Journal of Immunology, 2015, 194: 5085–5093.
patients, 15 untreated MS patients, and 15 healthy donors at the Department of Neurology, Thomas Jefferson University. Untreated MS patients did not receive any immunosuppressive or immunomodulatory treatment before venipuncture. Information on healthy donors and MS patients is given in Table I. PBMCs were collected by Ficoll–Paque Plus density gradient centrifugation. PBMCs were washed and cultured at a density of 5 × 10^6 cells/ml in X-VIVO 15 serum–free medium. Cells were stimulated with 1 μg/ml anti-CD3 (HIT3a; BD Biosciences) and 1 μg/ml anti-CD28 (CD28.2; BD Biosciences) for 5 days in the presence of 10 μM human rIFN-β (R&D Systems). Concentration of 1000 IU/ml IFN-β was chosen based on our serial dilution curve (Supplemental Fig. 1) and the study by Ramgolam et al. (16).

CD45RA+CD4+ T cells were purified from PBMCs by negative selection with CD4 Microbeads kit, followed by negative selection with CD45RA Microbeads (Miltenyi Biotec), according to the manufacturer’s instructions. Purification of CD45RA+CD4+ T cells was checked by flow cytometry, and samples that were >95% pure were used for analysis. CD45RA+CD4+ T cells were cultured in X-VIVO 15 serum–free medium for 5 days at a density of 1 × 10^6 cells/ml. Cells were stimulated with 1 μg/ml anti-CD3 (HIT3a; BD Biosciences) and 1 μg/ml anti-CD28 (CD28.2; BD Biosciences) with or without addition of IFN-β.

Flow cytometry

For cytokine detection, PBMCs and CD45RA+CD4+ T-cells were activated with 50 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomicyn (Sigma-Aldrich), and GolgiPlugs (1 μg/ml 100 μM human rIFN-β (R&D Systems). Cells were washed in staining buffer (1% FBS and 0.1% sodium azide in PBS) and stained with following Abs: anti-human CD4 Pacific Blue (RPA-T4); BD Biosciences), CD4 PerCP-Cy5.5 (OKT-4; eBioscience), CD8 Pacific Blue (RPA-T8; BD Biosciences), GM-CSF PE (BVD2-21C11; BD Biosciences), IL-17A Alexa Fluor 488 (eBio64DEC17; eBioscience), IFN-γ allophycocyanin (4S.B3; eBioscience), Tbet PerCP-Cy5.5 (eBioB10; eBioscience) RORγt PE (B2D; eBioscience), IL-4 FITC (ILD4-8; eBioscience), GATA3 PE (TWAJ; eBioscience), Foxp3 FITC (206D; Biolegend), IL-22 allophycocyanin (IL22JOP; eBioscience), CCR10 PE (1B5; BD Biosciences), CD45RA PE (HI100; BD Biosciences), and CD45RO PE-Cy7 (UCHL1; BD Biosciences). Cells were fixed and permeabilized with Caltag Fix/Perm reagents (Invitrogen) following the manufacturer’s instructions. Data were acquired on a FACSARia (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cytokine quantification

PBMCs and purified CD4+ T cells were cultured for 5 days, and supernatants were collected and stored at −20°C until the day of analysis. GM-CSF, IFN-γ, IL-10, IL-17A, and IFN-γ concentrations were quantified by ELISA (R&D Systems), according to the manufacturer’s instructions.

Immunohistochemistry

MS brain tissues were obtained from the Rocky Mountain Multiple Sclerosis Center Tissue Bank (Aurora, CO). Tissues were cryosectioned (10 μm thick) for immunohistochemistry at −25°C (Cryotome SME; Thermo Fisher Scientific, Pittsburgh, PA) and stored at −80°C.

Brain sections were fixed in acetone, washed in PBS, and blocked with 8% horse serum and 3% BSA in PBS. Subsequently, sections were incubated with primary Abs (5 μg/ml in 8% horse serum, 3% BSA, and 0.03% Triton X-100 in PBS) overnight at 4°C. Mouse monoclonal anti-human CD4 (BC1/F6), rat monoclonal anti-human CD4 (YTC182.20), rat monoclonal anti-human GM-CSF (BVD2-21C11), mouse monoclonal anti-human CD4 (BC/1F6), rat monoclonal anti-human CD4 (YNB46.1.8), mouse monoclonal anti-human CD8 (YTC182.20), rat monoclonal anti-human GM-CSF (BVD2-21C11), rat monoclonal anti-human IL-10, IL-17A, and IFN-γ Abs were purchased from Abcam (Cambridge, MA). Sections were then washed in PBS and incubated with secondary Abs at room temperature for 1 h. Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 594 donkey anti-mouse IgG, Alexa Fluor 594 donkey anti-rat IgG, and Alexa Fluor 647 donkey anti-rabbit IgG were purchased from Life Technologies (Philadelphia, PA). To quench autofluorescence, sections were washed in PBS, rinsed in distilled water, and incubated for 10 min in 10 mM CuSO4 in 50 mM ammonium acetate (pH 5). Sections were then rinsed in distilled water and mounted with Prolong Diamond Antifade Mountant with DAPI (Life Technologies). Sections serving as controls for specificity of staining (negative controls) were generated from serial sections of each sample by staining with secondary Abs only. Immunofluorescent images were captured using a Zeiss LSM 510 UV META confocal microscope with a 20x Plan-Apochromat ×0.75 NA objective or a Plan-Neofluar ×40/1.3 NA objective. Z-stacks were acquired at a thickness of 1 μm, and images were analyzed using the ImageJ software (National Institutes of Health). Representative confocal images were generated by maximum or average projection through the z-axis of the image stack. Resulting projections of each channel were then overlaid in Photoshop CS6 software (Adobe System). For quantification, lesion sizes from three MS brains were examined. Nine sections (three per lesion) were randomly selected and quantitated. The number of stained cells per section was counted under ×20 magnification. Data are expressed as mean ± SEM.

Statistics

Statistical analysis was performed by GraphPad Prism 6 software. One-way ANOVA was used to determine significance between multiple groups. The paired, two-tailed Student t test was used to analyze datasets before and after in vitro treatment with IFN-β. Parametric data were analyzed using an unpaired, two-tailed student t test. The Bonferroni correction was applied for adjustment of the significance values for multiple comparisons. Adjusted p ≤ 0.05 was considered significant. Data represent mean ± SEM.

Results

IFN-γ- and IFN-γ−CD4+ and CD8+ T cells in PB are major producers of GM-CSF

We first characterized the phenotype of GM-CSF–producing T cells in PB of healthy individuals. Subsets of both CD4+ and CD8+ T cells produced GM-CSF upon stimulation with PMA and ionomycin (Fig. 1A, Table I). GM-CSF was produced by both IFN-γ− and IFN-γ+ T cells, whereas the percentages of IL-17A+ and IL-22+ T cells were low (Fig. 1B, Supplemental Table I). A negligible number of CD4+ and CD8+ GM-CSF+ T cells expressed RORγt, IL-4, GATA3, and Foxp3 (data not shown). GM-CSF−IFN-γ− T cells did not express lineage-specific cytokines or transcription factors (Supplemental Table I), and we designated them “GM-CSF−only” T cells. These GM-CSF−only producing T cells either did not express T-bet or expressed it at a low level, whereas the majority of GM-CSF−/IFN-γ−/IL-17A−/IL-22− T cells were clearly T-bet+ (Fig. 1C, Supplemental Table I), a phenotype consistent with CD4+ T cells. CCR6 was expressed on a small number of cells, and we discontinued staining for this marker, deeming it uninformative in this context. Both CXCR3 and CCR4 were present on substantial numbers of CD4+ and CD8+ GM-CSF−producing T cells. CCR6 was expressed on a small number of cells, and we discontinued staining for this marker, deeming it uninformative in this context. Both CXCR3 and CCR4 were present on substantial numbers of CD4+ and CD8+ GM-CSF+ T cells. CCR4 was clearly T-bet+ (Fig. 1C, Supplemental Table I). We then characterized GM-CSF−producing T cells in naive CD45RA+CD45RO− effectors CD45RA−CD45RO+ populations, which were similar in size (45–50% each) (Supplemental Fig. 2A, 2C). The majority (>75%) of CD8+ T cells were CD45RA−CD45RO− (Supplemental Fig. 2B, 2D). Among both CD4+ and CD8+ T cells, CD45RA−CD45RO+ populations were predominant producers of GM-CSF and IFN-γ, whereas a small number of CD45RA+CD45RO− produced these cytokines (Supplemental Fig. 2). In a recent publication, Noster et al. (17) sorted CD45RA+CD4+ T cells based on surface expression of chemokine receptors and analyzed their cytokine production. CXCR3−CCR4+CCR6−CCR10+ cells produced GM-CSF but not IFN-γ, IL-17A, or IL-22. The authors concluded that the above combination of chemokine receptors is characteristic of GM-CSF−only producing cells. We attempted to determine expression of the chemokine receptors on all GM-CSF−only CD4+ T cells. CCR6 was expressed on a small number of cells, and we discontinued staining for this marker, deeming it uninformative in this context. Both CXCR3 and CCR4 were present on substantial portions of CD4+ T cells before they had been exposed to PMA and ionomycin, but after exposure, the percentage of CXCR3+ and CCR4+ cells dropped several fold, precluding reliable correlation between cytokine and the chemokine receptors expression. However, the numbers of CCR10−/CD4+ T cells were not altered by PMA and ionomycin, and we continued analyses using this marker. Only one-third of CD45RA−GM-CSF−/IFN-γ− CD4+ T cells stained for CCR10 (Fig. 1D). Hence, CXCR3−CCR4−CCR6−CCR10− phenotype identifies a minority of GM-CSF−only CD4+ T cells.
Untreated MS patients have increased numbers of GM-CSF+ T cells in PB, whereas patients undergoing IFN-β therapy have normal numbers

We then compared GM-CSF production by T cells of untreated and IFN-β–treated MS patients and of healthy donors. Untreated MS patients had on average twice as many GM-CSF+CD4+ and three times more GM-CSF+CD8+ T cells compared with both healthy donors and IFN-β–treated MS patients, whereas the latter two did not differ (Fig. 2A). Untreated MS patients also had significantly increased numbers of IFN-γ+CD4+ T cells compared with healthy donors and IFN-β–treated patients, but the increase was less pronounced than in the case of GM-CSF+ cells (Fig. 2B). There was a trend toward a higher percentage of IFN-γ+CD8+ T cells in samples of untreated MS patients, but the difference did not reach statistical significance (Fig. 2B). IL-17A+ and IL-22+ T cells were present in low numbers in all three tested groups and did not contribute substantially to the pool of GM-CSF+ T cells (data not shown). Frequencies of both GM-CSF+IFN-γ+ and GM-CSF–only CD4+ and CD8+ T cells were significantly greater in the case of untreated MS patients when compared with healthy donors and IFN-β–treated patients (Fig. 2C, 2D). In addition, the numbers of GM-CSF+IFN-γ+ and GM-CSF–only CD4+ T cells were similar, whereas among CD8+ T cells, GM-CSF+IFN-γ+ cells were approximately twice as abundant as GM-CSF–only cells. These data demonstrate that untreated MS patients have increased numbers of GM-CSF–producing T cells in their PB and that IFN-β therapy normalized their numbers.

IFN-β suppresses GM-CSF production by T cells of healthy individuals in vitro

IFN-β–treated MS patients had fewer GM-CSF+ T cells compared with untreated patients, which prompted us to examine the effect of IFN-β on GM-CSF production by T cells. PBMCs from healthy donors were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IFN-β, and their cytokine production was analyzed.

Table I. Clinical characteristics of MS patients

<table>
<thead>
<tr>
<th></th>
<th>Healthy Donors</th>
<th>Untreated MS</th>
<th>IFN-β-Treated MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>15</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Female/male</td>
<td>9/6</td>
<td>11/4</td>
<td>9/1</td>
</tr>
<tr>
<td>Age</td>
<td>43.0 ± 3.5</td>
<td>49.5 ± 3.3</td>
<td>51.7 ± 3.9</td>
</tr>
<tr>
<td>Rebif/avonex</td>
<td>—</td>
<td>—</td>
<td>4/6</td>
</tr>
<tr>
<td>MS type</td>
<td>—</td>
<td>RRMS</td>
<td>RRMS</td>
</tr>
<tr>
<td>Years since diagnosis</td>
<td>—</td>
<td>10.6 ± 2.5</td>
<td>12.7 ± 2.5</td>
</tr>
</tbody>
</table>

RRMS, relapse-remitting MS.
after 5 d. IFN-β significantly reduced GM-CSF and increased IL-10 concentrations in cell culture supernatants, whereas it caused no change in concentrations of IFN-γ (Fig. 3A). Concentrations of IL-17A were also significantly reduced but only to a small extent. IFN-β reduced frequency of GM-CSF⁺ CD4⁺ and CD8⁺ T cells, whereas numbers of IFN-γ⁺ T cells remained unchanged (Fig. 3B). These data show that IFN-β inhibits GM-CSF production by T cells in vitro, whereas IFN-γ production remains unaffected.

To test whether IFN-β suppresses GM-CSF production by directly acting on T cells, we purified CD45RA⁺ CD4⁺ T cells and activated them with anti-CD3 and anti-CD28 Abs in the presence of IFN-β. IFN-β significantly decreased the percentage of GM-CSF⁺ cells in cultures of CD45RA⁺ CD4⁺ T cells, similar to PBMCs (Fig. 3C). IFN-β also reduced the percentage of both GM-CSF-only and GM-CSF⁺ IFN-γ⁺ cells in purified CD45RA⁺ CD4⁺ T cells, whereas it had no significant effect on GM-CSF⁺ IFN-γ⁺ CD4⁺ T cells in PBMC cultures. Furthermore, IFN-β reduced GM-CSF concentrations in culture supernatants of CD45RA⁺ CD4⁺ T cells and PBMCs (Fig. 3C). These data show that IFN-β suppresses GM-CSF production by CD4⁺ T cells by acting directly on them.

**IFN-β reduces GM-CSF production but not numbers of GM-CSF⁺ T cells of MS patients in vitro**

We then investigated the effect of IFN-β on GM-CSF production by PBMCs of MS patients. IFN-β significantly reduced GM-CSF concentrations in samples of both untreated and IFN-β-treated MS patients while increasing IL-10 and IFN-γ concentrations (Fig. 4A). IL-17A production remained unchanged. In contrast to reduced GM-CSF concentrations in culture supernatants, IFN-β did not reduce numbers of GM-CSF⁺ CD4⁺ and CD8⁺ T cells (Fig. 4B). This was also in contrast to its suppressive effect on frequency of GM-CSF⁺ T cells from healthy donors. IFN-β increased the percentages of IFN-γ⁺CD4⁺ and CD8⁺ T cells from MS patients in vitro (Fig. 4B), and the vast majority of these IFN-γ⁺ T cells simultaneously expressed GM-CSF.

These data demonstrate that IFN-β has different effects on T cells of healthy individuals and MS patients, irrespective of whether they come from IFN-β–treated or untreated patients. Most notably, in contrast to healthy individuals, IFN-β did not reduce numbers of GM-CSF⁺ T cells in the samples from MS patients, but it increased numbers of IFN-γ⁺ T cells. However, even though IFN-β did not reduce numbers of T cells having the capacity to produce GM-CSF, it reduced GM-CSF concentrations in cell culture supernatants.

To identify subpopulations of GM-CSF⁺ T cells affected by IFN-β treatment in vitro, PBMCs were cultured in the presence of IFN-β for 5 d and analyzed by flow cytometry. IFN-β significantly decreased the frequency of GM-CSF⁺ CD4⁺ and CD8⁺ T cells from healthy donors and from untreated and IFN-β–treated MS patients (Fig. 5). However, IFN-β either had no effect on or it increased the numbers of GM-CSF⁺ IFN-γ⁺ CD4⁺ and CD8⁺ T cells in all three groups tested. These data suggest that only IFN-γ⁺ T cells are susceptible to suppression by IFN-β and that reduction in total numbers of GM-CSF⁺ T cells in samples from healthy donors (Fig. 3) is due to reduction in numbers of GM-CSF⁺ T cells. However, in samples from MS patients, a similar reduction in GM-CSF⁺ T cells was compensated for by a pronounced increase in GM-CSF⁺ IFN-γ⁺ T cells, resulting in a lack of effect of IFN-β on total numbers of GM-CSF⁺ T cells (Fig. 4B). These data also suggest that reduced concentrations of GM-CSF in cell culture supernatants of IFN-β–treated samples are mostly due to inhibition of GM-CSF⁺ T cells.

**CD4⁺ and CD8⁺ T cells express GM-CSF in MS lesions**

To expand the relevance of our findings obtained with PB T cells, we investigated GM-CSF expression by T cells in active MS lesions.
Approximately 15–20% of both CD4+ and CD8+ T cells expressed GM-CSF, and the majority coexpressed IL-17A or IFN-γ (Figs. 6, 7). Although the frequencies of GM-CSF+ T cells in MS lesions appeared to be similar to those in PB, it is likely that a substantially higher proportion of T cells in MS lesions had the capacity to express GM-CSF given that they had not been stimulated with PMA and ionomycin prior to tissue staining, as was the case with PBMCs. Hence, because of important methodological differences in analyses, a direct comparison of frequencies of cytokine-producing T cells in PB and MS lesions would be false. Nonetheless, it is obvious that higher proportions of T cells in MS lesions express or have the capacity to express cytokines compared with T cells in PB. This is especially notable in the case of IL-17A. Typically <1% of PB T cells expressed IL-17A, whereas in MS lesions, >30% of T cells expressed this cytokine even without prior stimulation with PMA and ionomycin. Taken together, these data show expression of GM-CSF by T cells at CNS inflammation sites, supporting the hypothesis that GM-CSF contributes to MS pathogenesis.

Discussion
Although GM-CSF was discovered >40 y ago (18), and its essential role in EAE was demonstrated 14 y ago (6), this cytokine has been little studied in MS. It is likely that in MS, as in EAE, myelin-specific T cells are the principal source of pathogenic GM-CSF. We found that CD4+ and CD8+ T cells in MS lesions produce GM-CSF, which likely facilitates disease pathogenesis. Treatment-naïve MS patients had greater numbers of GM-CSF+ T cells in PB compared with healthy individuals and patients undergoing IFN-β therapy. Notably, IFN-β therapy reduced numbers of GM-CSF+ T cells in MS lesions, an effect that is consistent with our in vitro data. Possibly, IFN-β also suppresses GM-CSF production by other cell types, which may add to its therapeutic efficacy in MS.
Our data show that a number of CD4+ and CD8+ T cells in MS brain lesions express GM-CSF, whereas the frequency of GM-CSF+ T cells among PBMCs was lower. In addition, a large proportion of GM-CSF+ T cells in MS lesions also expressed IFN-γ and IL-17A, whereas their frequency at the periphery was smaller, especially in the case of IL-17A+ cells. The enrichment in cytokine-producing T cells in MS lesions has been described previously, as the majority (∼80%) of T cells, both CD4+ and CD8+, in MS lesions expressed IL-17A, whereas in the periphery only a fraction of T cells expressed this cytokine (19). Given that a variety of cell types, both resident CNS cells (20–23) and peripheral myeloid cells that infiltrate the CNS, express GM-CSF receptor, the abundant GM-CSF production in MS lesions suggests its contribution to pathogenesis.

Although healthy individuals and MS patients have similar overall frequency of myelin-specific T cells in PB (24–27), more T cells of MS patients have activated effector/memory phenotype (28–30). Our ex vivo analysis showed that IFN-γ+CD4+ and CD8+ T cells represent a majority of GM-CSF-producing cells, but a substantial proportion of IFN-γ- T cells also expresses GM-CSF. Both IL-22+ and IL-17A+ T cells were present in much smaller numbers than IFN-γ+ and GM-CSF+ cells. This was true for healthy controls and treated and untreated MS patients. Consistent with previous findings that T-bet is not required for GM-CSF expression (4), both T-bet+ and T-bet− T cells expressed GM-CSF. GM-CSF–only T cells either did not express T-bet or its levels were low, whereas among GM-CSF+IFN-γ+ T cells, most cells were T-bet+, which is typical Th1/Tc1 phenotype. We could not classify GM-CSF–only T cells into any conventional lineage of effector T cells because they did not express hallmark cytokines or transcription factors of those lineages.

Noster et al. (17) have recently described a CD4+ T cell subpopulation from PB that produces GM-CSF but does not express any of the Th lineage–specific cytokines or transcription factors. These GM-CSF–only expressing CD4+ T cells had CD45RA−CCR7− effector memory phenotype and the characteristic combination of chemokine receptors expressed on their surface (CXCR3−CCR4−CCR6+CCR10+). However, our analyses, based

**FIGURE 4.** IFN-β reduces GM-CSF production by T cells from MS patients. PBMCs of IFN-β–treated and untreated MS patients were cultured with anti-CD3 and anti-CD28 Abs for 5 d with or without addition of IFN-β. (A) GM-CSF, IL-10, IL-17A, and IFN-γ concentrations in above cell culture supernatants. (B) Percentages of GM-CSF+ and IFN-γ+CD4+ and CD8+ cells in total CD4+ and CD8+ T cells from above PBMC cultures. *p < 0.05, **p < 0.01, ***p < 0.001 paired, two-tailed Student t test.
on staining for CCR10, showed that only a portion of GM-CSF–only cells have the above combination of surface markers. Our data showing that this surface phenotype is not characteristic of GM-CSF–only CD4+ T cells is consistent with those of Noster et al. showing that the majority of sorted CXCR3+ CCR4+CCR6+ CCR10+ CD4+ T cells did not produce GM-CSF upon activation. The same authors also described that MS patients had normal numbers of GM-CSF+CD4+ T cells in PB. Information on whether the patients were treated with IFN-β has not been provided, which precludes determining whether this result is in agreement with our findings that IFN-β–treated MS patients have normal numbers of GM-CSF+CD4+ T cells in their PB (17). Consistent with our data, a recent publication describes that MS patients have significantly greater frequencies of GM-CSF+ and IFN-γ+ CD4+ T cell in PB compared with patients with other neurological diseases (31). Furthermore, MS patients treated with one of the immunomodulatory therapies (IFN-β, methylprednisolone, Natalizumab, and Glatiramer acetate) had a significantly lower percentage of GM-CSF+CD4+ T cells compared with untreated patients. The authors compared data from treated MS patients, irrespective of therapy, as a single group, with untreated MS patients, which precludes determining whether IFN-β–treated MS patients have reduced frequencies of GM-CSF+CD4+ T cells compared with untreated patients. The authors compared data from treated MS patients, irrespective of therapy, as a single group, with untreated MS patients, which precludes determining whether IFN-β–treated MS patients have reduced frequencies of GM-CSF+CD4+ T cells compared with untreated patients. In contrast to our data, they did not find an increased frequency of GM-CSF+CD8+ T cells in PB of MS patients (31). The reason for this discrepancy between our data is unclear.

Even though samples of untreated MS patients had two to three times more GM-CSF+ T cells than those from healthy donors, activated PBMCs of all three test groups produced similar quantities of GM-CSF after 5 d of culture, and IFN-β reduced concentrations of GM-CSF in culture supernatants to a similar extent. However, IFN-β reduced the percentage of GM-CSF+ CD4+ and CD8+ T cells only in the case of healthy individuals, whereas this percentage remained unchanged in the case of MS patients, both untreated and undergoing IFN-β therapy. It appears that the main reason for this differential effect is an increase in GM-CSF+IFN-γ+ T cells in samples from MS patients treated with IFN-β, whereas in samples from healthy donors, there was no such increase. This increase in MS patient samples canceled out the decrease in percentage of GM-CSF–only T cells, so the net effect was no change in overall number of GM-CSF+ T cells. Concentrations of IFN-γ in cell culture supernatants were in agreement with these observations because they remained unchanged in the case of healthy donors, whereas in MS patient samples they were increased. It should be noted that the samples from healthy donors also showed a trend toward an increase in numbers of GM-CSF+IFN-γ+ T cells, but the increase was smaller than in the case of MS samples and did not reach statistical difference. Hence, it appears that the difference between healthy individuals and MS patients in this regard is not substantial.

Others have found that IFN-β has no effect on IFN-γ production by naïve human CD4+ T cells cultured in nonpolarizing conditions (14). In agreement with this, we found that IFN-β has no effect on IFN-γ production by T cells/PBMCs of healthy donors; however, in the case of MS patients, both treatment-naive and under IFN-β

---

**FIGURE 5.** IFN-β reduces numbers of GM-CSF only CD4+ and CD8+ T cells. PBMCs from healthy donors (n = 15) and MS patients (untreated MS, n = 13; IFN-β–treated, n = 9) were stimulated with anti-CD3/anti-CD28 Abs in the presence or absence of IFN-β. Percentages of GM-CSF IFN-γ+ and GM-CSF–only CD4+ and CD8+ T cells in total CD4+ and CD8+ cells are shown. *p < 0.05, **p < 0.01, ***p < 0.001 paired, two-tailed Student t test.
therapy, there was a significant increase in percentage of IFN-γ+ CD4+ and CD8+ T cells as well as an increase in IFN-γ concentrations in cell culture supernatants. Moreover, the same authors showed that IFN-β suppresses Th1 cell–mediated EAE, whereas it has the opposite effect on Th17 cell–mediated disease. Our data showing that IFN-β therapy reduced numbers of IFN-γ+CD4+ T cells in PB of MS patients support the hypothesis that in MS, similar to EAE, IFN-β has beneficial effects by acting on Th1 responses.

MS patients under IFN-β therapy have normal levels of IFN-γ+GM-CSF+ and GM-CSF–only T cells in PB, whereas they are both increased in untreated MS patients, demonstrating that the therapy reduces numbers of both IFN-γ/GM-CSF+ and GM-CSF–only T cells. This is inconsistent with our in vitro data, where IFN-β reduced only numbers of GM-CSF–only T cells, indicating that only this T cell subpopulation is susceptible to IFN-β suppression of GM-CSF production. However, it is also possible that in vitro a portion of GM-CSF–only T cells did not stop GM-CSF production, but rather IFN-β induced IFN-γ expression in them, resulting in smaller GM-CSF–only and greater GM-CSF+IFN-γ+ cell numbers, whereas the total number of GM-CSF+ cells remained unchanged. The possibility that GM-CSF–only T cells upregulated IFN-γ production is strengthened by findings of Noster et al. (17) that these cells start expressing IFN-γ upon exposure to IL-12.

In summary, our findings demonstrate that untreated MS patients have significantly increased numbers of GM-CSF–producing CD4+ and CD8+ T cells. Whether myelin-specific T cells of MS patients produce GM-CSF and to which extent they contribute to the overall number of GM-CSF+ T cells remains to be addressed in future studies. Likewise, whether a greater frequency of GM-CSF–producing T cells contributes to MS immunopathogenesis or whether this is only an epiphenomenon of an abnormally activated immune system requires further studying.

The suppression of GM-CSF by IFN-β might be one of the mechanisms underlying its beneficial effects. We did not study whether MS patients that are not responsive to IFN-β therapy maintain elevated numbers of GM-CSF–producing T cells despite IFN-β treatment or whether their PBMC GM-CSF production in vitro remains unaffected by IFN-β. If shown to be the case, analysis of the effects of IFN-β on GM-CSF production by T cells can potentially be predictive of a patient’s likelihood to respond to IFN-β therapy.

**FIGURE 6.** CD4+ and CD8+ T cells coexpress GM-CSF and IL-17A in MS brain lesions. Serial sections from MS brain were stained with (A) H&E and Luxol fast blue to demonstrate the infiltration of T cells and demyelination in the MS lesion. Original magnification ×40. CD4+ (B) and CD8+ (C) T cells were stained for GM-CSF and IL-17A and analyzed by confocal microscopy. Merged images show colocalization of GM-CSF and IL-17A in CD4+ and CD8+ T cells. Negative controls are virtually devoid of staining. Scale bars, 10 μm.

**FIGURE 7.** CD4+ and CD8+ T cells coexpress GM-CSF and IFN-γ in MS brain lesions. Stained sections from three MS patients were analyzed by confocal microscopy. CD4+ (A) and CD8+ (B) T cells in brain lesions were stained for GM-CSF and IFN-γ. Merged images show colocalization of GM-CSF and IFN-γ in CD4+ and CD8+ T cells. Negative controls are virtually devoid of staining. (C) Quantification of CD4+ and CD8+ T cells performed by counting average of 200 cells/group from three MS brains at original magnification ×20. Scale bars, 10 μm.
Acknowledgments
We thank K. Regan for editorial assistance. We especially thank Dr. Mark Curtis for help with histopathology of the MS brain tissue, Dr. Benjamin Leiby for input on statistical analyses, and Rocky Mountain Multiple Sclerosis Center Tissue Bank for providing MS brain tissue.

Disclosures
The authors have no financial conflicts of interest.

References
**Supplementary Table I.** Frequency (%) of T-bet, IL-22, and IL-17A expression in GM-CSF$^+$ T cells of healthy donors

<table>
<thead>
<tr>
<th></th>
<th>CD4$^+$</th>
<th>CD8$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total T-bet$^+$ (%)</td>
<td>10.6 ± 2.9</td>
<td>23.6 ± 3.3</td>
</tr>
<tr>
<td>Total GM-CSF$^+$ T-bet$^+$ (%)</td>
<td>5.1 ± 2.3</td>
<td>8.9 ± 1.6</td>
</tr>
<tr>
<td>T-bet$^+$ in GM-CSF$^+$ IFN-γ$^+$ (%)</td>
<td>52.9 ± 11.9</td>
<td>77.1 ± 4.2</td>
</tr>
<tr>
<td>T-bet$^+$ in GM-CSF$^+$ IFN-γ$^-$ (%)</td>
<td>6.1 ± 1.4</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>T-bet$^+$ in GM-CSF$^-$ IFN-γ$^+$ (%)</td>
<td>36.8 ± 5.7</td>
<td>63.4 ± 8.2</td>
</tr>
<tr>
<td>Total IL-22$^+$ (%)</td>
<td>1.6 ± 0.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>GM-CSF$^+$ IL-22$^+$ (%)</td>
<td>1.1 ± 0.4</td>
<td>0.27 ± 0.1</td>
</tr>
<tr>
<td>IL-22$^+$ in GM-CSF$^+$ IFN-γ$^+$ (%)</td>
<td>6.4 ± 0.4</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>IL-22$^+$ in GM-CSF$^+$ IFN-γ$^-$ (%)</td>
<td>11.6 ± 3.4</td>
<td>4.6 ± 1.8</td>
</tr>
<tr>
<td>Total IL-17A$^+$ (%)</td>
<td>1.44 ± 0.2</td>
<td>0.52 ± 0.1</td>
</tr>
<tr>
<td>GM-CSF$^+$ IL-17A$^+$ (%)</td>
<td>0.37 ± 0.1</td>
<td>0.26 ± 0.1</td>
</tr>
</tbody>
</table>
Supplementary Figure 1. IFN-β suppresses GM-CSF production by T cells in a dose-dependent manner. PBMCs from healthy donors (n=4) were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IFN-β (0-10000 IU/ml) for five days. Concentrations of GM-CSF in cell culture supernatants were measured by ELISA. ** p<0.01, *** p<0.001 (Paired, two-tailed Student t-test).
Supplementary Figure 2. PB CD45RA⁺RO⁻ CD4⁺ and CD8⁺ cells express GM-CSF and IFN-γ. PBMCs from healthy donors (n=10) were activated with PMA/iono/GP, stained for CD4, CD8, CD45RA, CD45RO, GM-CSF, and IFN-γ, and analyzed by flow cytometry. Representative dot-plots of GM-CSF- and IFN-γ–expressing CD4⁺ (A) and CD8⁺ (B) T cells within CD45RA⁺RO⁻ and CD45RA⁺RO⁺ T cell subpopulations of one donor. Summary (%) of CD4⁺ (C) and CD8⁺ T cells (D) that express GM-CSF and IFN-γ among total CD45RA⁺RO⁻ and CD45RA⁺RO⁺ T cells. Mean ± SEM, *** p< 0.001, NS: not significant (Unpaired, two-tailed Student t-test).