Type 2 Monocyte and Microglia Differentiation Mediated by Glatiramer Acetate Therapy in Patients with Multiple Sclerosis

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Type 2 Monocyte and Microglia Differentiation Mediated by Glatiramer Acetate Therapy in Patients with Multiple Sclerosis

Ho Jin Kim, Igal Ifergan, Jack P. Antel, Rosanne Seguin, Martin Duddy, Yves Lapierre, Farzaneh Jalili, and Amit Bar-Or

Glatiramer acetate (GA) therapy of patients with multiple sclerosis (MS) represents a unique setting in which in vivo Th2 deviation of T cells is consistently observed and associated with clinical benefit in a human autoimmune disease. We postulated that APCs are important targets of GA therapy and demonstrate that treatment of MS patients with GA reciprocally regulates the IL-10/IL-12 cytokine network of monocytes in vivo. We further show that Th1- or Th2-polarized GA-reactive T cells isolated from untreated or treated MS patients mediate type 1 and 2 APC differentiation of human monocytes, based on their ability to efficiently induce subsequent Th1 and Th2 deviation of naive T cells, respectively. These observations are extended to human microglia, providing the first demonstration of type 2 differentiation of CNS-derived APCs. Finally, we confirm that the fundamental capacity of polarized T cells to reciprocally modulate APC function is not restricted to GA-reactive T cells, thereby defining a novel and dynamic positive feedback loop between human T cell and APC responses. In the context of MS, we propose that GA therapy results in the generation of type 2 APCs, contributing to Th2 deviation both in the periphery and in the CNS of MS patients. In addition to extending insights into the therapeutic mode of action of GA, our findings revisit the concept of bystander suppression and underscore the potential of APCs as attractive targets for therapeutic immune modulation.


The peripheral activation of proinflammatory Th1 T cells has been implicated in the pathophysiology of several targeted-directed autoimmune diseases, including multiple sclerosis (MS)1 (1–3). Considerable efforts are ongoing to develop immune modulators that can be administered peripherally and either suppress or deviate the putative disease-relevant immune responses at the target site (4). In patients with MS, daily s.c. injections of the approved therapy glatiramer acetate (GA, copaxone), a copolymer comprising a random mix of the four amino acids, alanine, lysine, glutamate, and tyrosine (5), consistently induce subsequent Th1 and Th2 deviation (6–14), and have been largely attributed to the notion that the Th2 GA-reactive T cells migrate into the CNS, where they are presumed to suppress the proinflammatory Th1 CNS-reactive cells, in a process termed bystander suppression (12, 15–17). Earlier reports suggested that the GA-reactive cells were generated with GA therapy specifically cross-react with myelin basic protein (MBP) (10, 18). A more likely explanation has emerged with the recognition that significant functional degeneracy exists in the recognition of self Ags by T cells (19). The Th2 GA-reactive cells induced by GA treatment have been shown to be highly degenerate and cross-reactive with multiple peptides, including CNS Ags (7, 19). However, several questions remain: the mechanism by which GA therapy generates the increased frequency of peripheral Th2 GA-reactive cells, and, specifically, whether this results from a direct effect of GA on T cells, have not been fully elucidated. Furthermore, within the CNS, the bystander suppression has been attributed to T cells affecting neighboring T cells, yet a direct suppressive effect of Th2 on Th1 T cell responses within the CNS has not been formally established.

We hypothesized that APCs play a central role in the GA-mediated Th2 immune deviation, both in the periphery and in the CNS. It is generally accepted that the local environment during T cell activation critically determines subsequent T cell differentiation (20–23). In particular, the profiles of cytokines and costimulatory signals expressed by the APCs during initial activation can impact on whether Th1 or Th2 differentiation occurs. Reciprocal regulation of the IL-12 and IL-10 cytokine network appears to be important in this regard: APCs producing high levels of IL-12 and low levels of IL-10 have been shown to mediate Th1 differentiation, while APCs producing low levels of IL-12 and higher levels of IL-10 preferentially mediate Th2 differentiation. These can be referred to as type 1 and 2 APCs, respectively. We hypothesized that GA therapy induces type 2 APCs in the periphery of patients, which then preferentially mediate Th2 T cell differentiation. This is an attractive hypothesis because peripherally modulated APCs could subsequently migrate across the blood brain barrier (BBB), and contribute to anti-inflammation by regulating T cell responses within the CNS environment. We chose to focus on circulating
Monocytes, which are likely to be exposed to the peripheral effects of GA therapy and are also known to efficiently migrate across the endothelial cells of the BBB (24, 25). Indeed, pathological studies of MS underscore the large numbers of infiltrating monocytes present within active as well as chronic active MS lesions (26).

In the present study, we first demonstrate that GA treatment of patients with MS results in the in vivo immune modulation of circulating monocytes, which produce significantly higher amounts of IL-10 and significantly lower amounts of IL-12. We identify two distinct mechanisms by which GA therapy could mediate such monocyte modulation. Direct incubation of GA with purified human monocytes results in a dose-dependent enhancement of IL-10 and inhibition of IL-12 production. Furthermore, exposure of monocytes to the soluble products of GA-reactive T cells generated from treated patients results in similar cytokine modulation. These monocytes function as effective type 2 APCs, inducing efficient Th2 deviation of naive CD4+ T cells. We extend these observations to CNS-derived APCs, showing that GA-reactive T cells from MS patients treated with GA induce similar type 2 APC modulation of human adult CNS microglia. Our findings extend insights into the therapeutic mode of action of GA and revisit the concept of bystander suppression within the CNS environment. We identify a fundamental and novel positive feedback paradigm between T cells and APCs and underscore the potential to target APCs in human autoimmune disease.

Materials and Methods

Monocyte and microglia isolation and culture

Venous blood samples were obtained from consenting normal donors and patients with MS, in accordance with institutional guidelines. PBMC were isolated from heparinized blood using standard Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Monocytes were purified from PBMC by CD14+ selection using MACS (Miltenyi Biotec, Toronto, Ontario, Canada). Purity and viability of isolated cells were assessed by flow cytometry and trypan blue staining, respectively. All monocyte cultures were performed in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 U/ml LPS (100 ng/ml), either without further stimulation (squares) or with the addition of LPS (100 ng/ml; circles) or IFN-γ (10 U/ml) + LPS (100 ng/ml; triangles). Cytokines were assayed by ELISA at 24 h (pg/ml). At the highest concentration of GA (100 μg/ml), monocyte production of IL-10 was significantly increased (++, p < 0.01), and IL-12 induction was significantly decreased (++, p < 0.01). Data represent the mean ± SEM of six independent experiments. Statistical significance was determined using Student’s paired t test.
penicillin, and 50 μg/ml streptomycin. Human microglia were isolated from adults undergoing surgical resection for intractable epilepsy and cultured, as previously described (27).

To study the effects of in vivo GA therapy on human monocytes, monocytes were freshly isolated (as above) from patients with MS who were either untreated (pre-GA) or treated (post-GA) with GA. Pre-GA and post-GA patients all had relapsing remitting MS (RRMS); were of similar ages (average age 29 ± 7 years); and matched for female:male ratio (3:2), disease activity (number of relapses in preceding 2 years <2), and disease severity (Expanded Disability Status Scale range 1.0–4.5). Treated patients were on GA therapy (standard dose of 20 mg/s.c. daily injections) for an average of 9 mo (range 7–14). Monocytes were cultured, unstimulated, or stimulated via CD40 by coinoculation with irradiated (3000 rad) CD40 ligand (CD40L)-transfected L cells (1:5 ratio; kind gift of Y. Liu, DNAX, Palo Alto, CA) to mimic encounter with activated T cells, or stimulated with IFN-γ (10 U/ml) and LPS (100 ng/ml), which is required for measurement of human monocyte IL-12 (28). After 24 h, monocyte production of IL-10, IL-12, and TNF-α was quantified in culture supernatants by standard ELISA kits, according to the manufacturer’s instructions. In preliminary experiments, we confirmed that the CD40L L cells expressed none of these cytokines, at either the mRNA or protein levels (data not shown).

To examine the direct effect of GA on monocytes, ex vivo purified CD14+ monocytes from normal individuals were plated at a density of 5 × 10^5 cells/ml in 24-well plates and cultured alone or in the presence of GA (4–100 μg/ml, kindly provided by Teva Neuroscience Israel), either without further stimulation, or with the addition of LPS alone (100 ng/ml) or the combination of IFN-γ and LPS, as above. Cytokine production was measured by standard ELISA kits at 24 h. Trypan blue and propidium iodide staining confirmed that direct incubation with GA was not toxic to monocytes across a broad range of concentrations (up to 200 μg/ml). Responses of monocytes were similarly assessed to whole human MBP protein (isolated as described in Ref. 29), as well as to nonoverlapping peptides of MBP, proteolipid protein, and myelin oligodendrocyte glycoprotein, and the nonmyelin Ags tetanus toxoid and hemagglutinin (kindly provided by the Immune Tolerance Network, San Francisco, CA).

To examine the indirect effects of GA on monocytes, as might occur through interaction of monocytes with Th1- or Th2-deviated GA-reactive T cells, ex vivo monocytes from normal individuals were cultured for 24 h in medium alone or in medium with added soluble products (supernatants) of Th1- or Th2-biased GA-reactive T cell lines, generated as described below. Monocytes were then washed, fresh medium was added, and their subsequent 24-h cytokine production and surface phenotype were analyzed by ELISA and flow cytometry (FACScan; BD Biosciences, San Jose, CA), respectively. Staining was performed according to standard BD PharMingen (Mississauga, Ontario, Canada) protocol with PE-conjugated Abs directed against: CD54 (ICAM-1), MHC class II/HLA-DR, CD40, CD80 (B7.1), and CD86 (B7.2), or corresponding isotype controls (all from BD PharMingen). A total of 10,000 events per sample was analyzed (CellQuest software; BD Biosciences). The identical approach was used to study the effects of GA-reactive T cell supernatants on the cytokine production and phenotype of human microglia. Additional series of experiments similarly assessed monocyte responses to supernatants of polarized Th1 (pTh1) and pTh2 T cells reactive to MBP, or T cells polyclonally activated with anti-CD3/anti-CD28. All reagents used (Ags, culture medium, and FCS), as well as supernatants from GA-reactive and other T cell lines, were routinely tested for the presence of endotoxin using the quantitative chromogenic Limulus amebocyte lysate test kit, according to the manufacturer’s instruction (QCL-1000; BioWhittaker, Walkersville, MD), and concentrations were always below the detection limit (0.1 EU/ml).

**APC assays**

To examine the functional effects of differentially modulated monocytes or microglia on subsequent T cell responses, we examined their APC potential in allogeneic MLR. Monocytes or microglia were either cultured in medium alone or exposed for 24 h to the soluble products of the indicated GA-reactive T cells, as above. They were then carefully washed and used as APCs for naive (CD45RA−CD45RO+) CD4+ T cells, which were freshly isolated from normal donors by CD4-negative selection, followed by CD45RA-positive selection of MACS. T cell proliferation and cytokine responses were measured after 72 h using thymidine incorporation and standard ELISA, respectively. To define the functional profile of responding T cells, IFN-γ and IL-5 were used as prototype Th1 and Th2 cytokines, respectively.

**Generation of GA- and MBP-reactive T cell lines**

GA- and MBP-reactive T cell lines with confirmed specificity were generated from normal individuals, or age- and sex-matched patients with RRMS who were either untreated (pre-GA) or GA treated (post-GA) by combining an IL-7-based assay with a traditional split-well paradigm, as previously described (29): PBMC were isolated as above and plated at 2 × 10^5 per well in 96-well plates in medium containing 10 ng/ml IL-7 (PeproTech, Rocky Hill, NJ) without Ag, or with GA or MBP at a final concentration of 20 μg/ml. After 7 days, 100 μl of supernatant was removed from each well and replaced with fresh medium enriched with human rIL-2 (20 U/ml final concentration). On day 11, each well was evaluated for specificity to Ag in a split-well assay: equal aliquots of cells from each well were restimulated with or without Ag, with freshly isolated autologous irradiated PBMC (3000 rad). Three days after restimulation (day 14), supernatants were removed and 18-h [³H]thymidine uptake was measured...
same levels of IFN-γ/tnanants (pTh1 sups) containing the compared with the effects of pTh1 supposure to matched levels of rIFN-γ/tnatants from normal individuals were generated as above, with the concurrent addition of either IL-12 (2.5 ng/ml)- and anti-IL-4-blocking Ab (5 mg/ml; all from R&D Systems, Minneapolis, MN), respectively.

Roles of the T cell cy-

Statistical analysis
Statistical analysis was conducted using PRISM software (GraphPad, San Diego, CA). The statistical significance (p values of <0.05) was determined by unpaired or paired Student’s t test, as appropriate.

Results
In vivo therapy with GA modulates monocytes to produce higher amounts of IL-10 and lower amounts of IL-12

Initially, we addressed whether GA therapy of patients with MS results in the in vivo modulation of the cytokine profile of circulating monocytes. Ex vivo isolated CD14+ monocytes were typically >98% pure (Fig. 1a) with minimal T cell (CD3+) or B cell (CD19+) contamination. When cultured without further stimulation (unstimulated), monocytes isolated from the circulation of MS patients treated with GA (post-GA) produced significantly higher amounts of IL-10 than monocytes from untreated (pre-GA) patients (Fig. 1b; p < 0.01). This difference was further accentuated when monocytes were stimulated via CD40/CD40L engagement, as a mimic for encounter with activated T cells (Fig. 1c; p < 0.001). IL-12 induction was only measured when monocytes were stimulated with IFN-γ/LPS. Monocytes from GA-treated (post-GA) patients produced significantly lower amounts of IL-12 than monocytes from untreated (pre-GA) patients (Fig. 1d; p < 0.01).

There were no differences in TNF-α secretion under any of the stimulation conditions (data not shown).

Reciprocal regulation of IL-10 and IL-12 production by monocytes via both direct and indirect mechanisms

Fig. 2 demonstrates the effects of direct incubation of GA on ex vivo purified normal human monocytes. In the absence of GA, unexposed (squares) normal monocytes produce low levels of IL-10 (32 ± 15 pg/ml), and are induced by IFN-γ/LPS (triangles) to produce moderate levels of IL-12 (165 ± 32 pg/ml). These are similar to the IL-10 and IL-12 levels produced by monocytes isolated from untreated patients with RRMS, as previously depicted in Fig. 1, b and d, respectively. Fig. 2 further shows that direct addition of GA to normal monocytes resulted in significantly increased IL-10 production (p < 0.05), while IL-12 induction was significantly decreased (p < 0.01). We detected no significant induction of monocyte IL-10 to the whole human MBP, to any of the myelin peptides (individually or mixed), or to the nonmyelin-related Ags, such as tetanus toxoid and HA. Of note, the reciprocal regulation of monocyte IL-10 and IL-12 production observed with GA was only seen at the highest GA concentration (100 μg/ml).

There was marginal up-regulation of monocyte expression of the costimulatory molecules CD80, CD86, and of HLA-DR, but not of ICAM-1 and CD40, at the highest GA concentration (data not shown).

The actual concentrations of GA that monocytes are likely to be exposed to in vivo are unknown. Furthermore, CNS-infiltrating monocytes are unlikely to encounter appreciable concentrations of GA within MS lesions. We therefore asked whether monocytes could also be modulated indirectly, through interaction with GA-reactive T cells that underwent Th2 deviation in GA-treated patients, an encounter that could take place both in the periphery and within CNS lesions. Specifically, we wished to test whether soluble products (supernatants) from pTh1 and pTh2 GA-reactive T cells had differential effects on the functional profiles of monocytes. To carry out these experiments, we first used normal PBMC to generate GA-reactive T cell lines polarized in vitro into Th1 or Th2 phenotypes (see Materials and Methods). Using IFN-γ as the prototype Th1 cytokine and IL-5 as a prototype Th2 cytokine, we confirmed that these in vitro pTh1 and pTh2 T cell lines produced...
exclusively IFN-γ or IL-5, respectively (Fig. 3a). We then compared the responses of normal ex vivo purified monocytes, cultured in medium only, or cultured with added supernatants from the pTh1 or pTh2 T cell lines. We found that pTh2 GA-reactive T cell supernatants induced monocytes to produce significantly more IL-10 (Fig. 3b; p < 0.01), and significantly less IL-12 (Fig. 3c; p < 0.01) and TNF-α (Fig. 3d; p < 0.001), compared with soluble products from pTh1 GA-reactive T cells. To examine whether the effects of the T cell supernatants on monocyte responses are mediated through IFN-γ and IL-5, we conducted additional experiments in which rIFN-γ or rIL-5 was added to monocytes in parallel to the pTh1 and pTh2 supernatant exposures. In each experiment, we used the same concentrations of rIFN-γ as present in the pTh1 supernatants, and the same concentrations of IL-5 as present in the pTh2 supernatants. Fig. 4a illustrates that, compared with medium only, the addition of rIL-5 did not contribute to monocyte production of IL-10. The presence of rIFN-γ induced both IL-12p70 (Fig. 4b) and TNF-α (Fig. 4c) production from monocytes, but only partially compared with pTh1 supernatants that contained the same levels of IFN-γ. Thus, the presence of rIL-5 did not reproduce the effects of the pTh2 supernatants on monocyte IL-10, and the presence of rIFN-γ only partially reproduced the effects of pTh1 supernatants on monocyte IL-12 and TNF-α. We confirmed the above observations with an additional approach using mAbs to block the effects of IFN-γ or IL-5. Blockade of IL-5 did not affect the production of IL-10 induced by the pTh2 supernatants (n = 4; p > 0.4), and blockade of IFN-γ significantly (p < 0.03), but only partially, reduced the monocyte TNF-α production that was induced by the pTh1 supernatants (data not shown). To confirm that the phenomenon of differential regulation of monocyte responses by soluble products of in vitro polarized T cells also occurs with soluble products of in vivo polarized T cells, we next generated GA-reactive T cell lines of confirmed specificity from MS patients before (pre-GA) and following (post-GA) initiation of GA therapy. The cytokine profiles of these GA-reactive lines are depicted in Fig. 5a, which demonstrates the Th2 immune deviation mediated by GA therapy in vivo. Compared with the pre-GA lines that produced considerably more IFN-γ than IL-5, significant increases in IL-5 production are noted in all post-GA T cell lines, with a proportion of these completely polarized toward a Th2 phenotype. The differential effects of medium only or the supernatant of these in vivo generated pre-GA (Th1)- and post-GA (Th2)-polarized lines, on normal ex vivo purified monocytes, are depicted in Fig. 5, b–d. Treatment of MS patients with GA results in Th2-deviated GA-reactive T cell lines capable of inducing monocytes to produce significantly higher levels of IL-10 (Fig. 5b; p < 0.01), and significantly lower levels of IL-12 (Fig. 5c; p < 0.001) and TNF-α (Fig. 5d; p < 0.01), compared with soluble products from corresponding lines generated from untreated patients.

To determine whether the modulation of monocyte function by GA-reactive T cells is a phenomenon specific to GA-reactive T cells, we conducted similar experiments in which monocytes were exposed to supernatants of pTh1 and pTh2 T cells reactive to MBP, or polyclonally activated (anti-CD3/anti-CD28). We found that regardless of their specificity, pTh1 supernatants induced naive monocytes to produce higher levels of IL-12 and lower levels of IL-10 (data not shown), similar to the monocytes exposed to pre-GA supernatants in Fig. 5. In turn, the MBP-reactive or CD3/CD28-stimulated pTh2 supernatants induced naive monocytes to produce higher levels of IL-10 and lower levels of IL-12 (data not shown), similar to the monocytes exposed to the post-GA supernatants in Fig. 5.

Reciprocally modulated monocytes acquire distinct phenotypes and function as type 1 and 2 APCs, inducing efficient Th1 and Th2 T cell responses, respectively. Fig. 6a and Table I depict the effects of the pre-GA (Th1) and post-GA (Th2) T cell supernatants described above on ex vivo purified monocyte expression of surface molecules involved in adhesion and Ag presentation. Compared with monocytes incubated in medium only, exposure to the pre-GA supernatants significantly increased monocyte expression of ICAM-1 (p < 0.05), HLA-DR/MHC class II (p < 0.01), CD40 (p < 0.01), CD80/B7.1 (p < 0.01), and CD86/B7.2 (p < 0.05). Post-GA supernatants (from GA-reactive T cells generated from GA-treated patients) significantly induced expression of ICAM-1 and HLA-DR (p < 0.05),...
induced similar proliferative responses from the naive T cells. However, pre-GA-exposed monocytes induced Th1 cells producing high amounts of IFN-γ by tritiated thymidine incorporation (cpm, \( \times 10^3 \)) and by ELISA (pg/ml), respectively. b. Monocytes transiently exposed to pre-GA or post-GA supernatants induced similar proliferative responses from the naive T cells. However, pre-GA-exposed monocytes induced Th1 cells producing high amounts of IFN-γ (c) and essentially no IL-5 (d), while post-GA-exposed monocytes induced efficient Th2 deviation of the responding T cells, marked by significantly lower production of IFN-γ (c) and significantly higher production of IL-5 (d). Data shown represent the mean \( \pm \) SEM of six independent experiments. Statistical significance was determined using Student’s paired t test (*, \( p < 0.05 \); **, \( p < 0.01 \); ns, not significant).

![Figure 6](https://www.jimmunol.org/)

**FIGURE 6.** Reciprocally modulated monocytes acquire distinct phenotypes and function as type 1 and 2 APCs, inducing Th1 and Th2 T cell responses, respectively. a. Monocyte surface expression of molecules involved in adhesion (ICAM-1) and Ag presentation (HLA-DR, CD40, CD80/B7.1, and CD86/B7.2) was measured following 24-h exposure of ex vivo purified CD14+ monocytes to culture medium (medium only), or to soluble products of GA-reactive T cells generated from MS patients before (pre-GA) or after (post-GA) initiation of GA therapy. Shown is a representative histogram plot of three independent experiments (summarized in Table I). Monocytes pre-exposed (24 h) to the medium only, or to the pre-GA or post-GA T cell supernatants were then washed and used in MLR with naive (CD45RA+RO-) CD4+ T cells. T cell proliferation and cytokine responses were measured by tritiated thymidine incorporation (cpm, \( \times 10^3 \)) and by ELISA (pg/ml), respectively. b. Monocytes transiently exposed to pre-GA or post-GA supernatants induced similar proliferative responses from the naive T cells. However, pre-GA-exposed monocytes induced Th1 cells producing high amounts of IFN-γ (c) and essentially no IL-5 (d), while post-GA-exposed monocytes induced efficient Th2 deviation of the responding T cells, marked by significantly lower production of IFN-γ (c) and significantly higher production of IL-5 (d). Data shown represent the mean \( \pm \) SEM of six independent experiments. Statistical significance was determined using Student’s paired t test (*, \( p < 0.05 \); **, \( p < 0.01 \); ns, not significant).

although to lesser levels than those induced by pre-GA supernatants. In contrast, post-GA supernatants induced significantly higher levels of expression of CD86 on monocytes (\( p < 0.05 \)) than did the pre-GA supernatants.

To examine the functional consequences of the differential modulation of monocytes, we used them as APCs to freshly sorted naive (CD45RA+RO-) CD4+ T cells, in an allogeneic MLR. As shown in Fig. 6b, monocytes transiently exposed to T cell supernatants induced greater proliferation from naive T cells than did monocytes exposed to medium alone. There was no difference in the naive T cell proliferation induced by monocytes previously exposed to pre-GA or post-GA T cell supernatants. However, pre-GA-exposed monocytes functioned as effective type 1 APCs and induced Th1 cells producing high amounts of IFN-γ (Fig. 6c) and essentially no IL-5 (Fig. 6d), while post-GA-exposed monocytes functioned as effective type 2 APCs and induced marked Th2 deviation of the responding T cells, notable for significantly lower production of IFN-γ (Fig. 6c; \( p < 0.01 \)) and significantly higher production of IL-5 (Fig. 6d; \( p < 0.01 \)).

**Human microglia can be differentially modulated to become type 1 or 2 APCs, following exposure to pre-GA or post-GA T cell supernatants, respectively**

To examine whether CNS resident APCs can be the target of immune modulation by CNS-infiltrating GA-reactive T cells, we isolated human adult brain-derived microglia and transiently exposed them to supernatants of GA-reactive T cells generated from MS patients before (pre-GA) or after (post-GA) initiation of GA therapy. We measured the cytokine profiles subsequently produced by the microglia, and observed that pre-GA-exposed microglia produced significantly lower amounts of IL-10 (Fig. 7a; \( p < 0.05 \)) and significantly higher amounts of TNF-α (Fig. 7b; \( p < 0.01 \)) than post-GA-exposed microglia. No IL-12p70 production by these microglia was detected in any of the above conditions. We next assessed the phenotype and APC function of these differentially modulated microglia, as described above for monocytes. Fig. 8a and Table I show that exposure of microglia to the pre-GA supernatants significantly induced their expression of ICAM-1 (\( p < 0.05 \)), HLA-DR (\( p < 0.05 \)), CD40 (\( p < 0.05 \)), CD80 (\( p < 0.01 \)), and CD86 (\( p < 0.05 \)), while post-GA supernatants may have induced a slight (but not statistically significant) increase only in CD40 expression of microglia. This contrasts with the effects of the same post-GA supernatants on monocytes, which significantly up-regulated ICAM-1, HLA-DR, and CD86 expression (Fig. 6a and Table I). The pre-GA and post-GA T cell supernatants had opposite effects on CD86 expression of monocytes and microglia: CD86 was significantly induced in microglia by pre-GA (\( p < 0.05 \)), but not by post-GA supernatants. In contrast, significantly more CD86 was induced in monocytes by post-GA (\( p < 0.01 \))
APCs is a requirement for achieving Th2 priming of effector T cells, in animal models, in which it has been suggested that the presence of APCs within the peripheral and central nervous system (CNS) underscores the abundance of invading monocytes/macrophages (25, 26) consistent with pathologic studies of MS and experimental autoimmune encephalomyelitis (EAE) (25, 26). Monocytes are significantly more abundant in infiltrating the CNS parenchyma (35). Monocytes as well as resident CNS microglia can produce proinflammatory cytokines and free radicals, and the axonal injury observed in the brains of patients with MS is correlated with the presence of invading monocytes/macrophages and activated microglia (36, 37). Conversely, invading monocytes are thought to play a role in clearing myelin debris at sites of CNS injury, contributing to a more permissive repair environment, as remyelination in injury models is limited in animals depleted of monocytes (38). Understanding how the biology of circulating and resident CNS APCs can be modulated and how this may impact on the CNS inflammatory environment is thus of considerable interest.

By comparing ex vivo isolated monocytes from untreated and GA-treated patients with MS, we provide the first demonstration that in vivo therapy with GA results in the modulation of human APCs, notable for reciprocal regulation of the IL-10/IL-12 regulatory cytokine network by circulating monocytes. Our findings extend a preliminary report of altered cytokine levels in the serum of GA-treated patients (39) and identify a likely cellular source for differential cytokine expression. Compared with untreated MS patients, ex vivo monocytes from GA-treated patients produced significantly less IL-12 and significantly more IL-10. The difference in IL-10 production was accentuated when monocytes were stimulated via CD40L/CD40 engagement, mimicking encounter with activated T cells. We considered whether the mechanism of in vivo modulation of human APCs by GA could be mediated by direct exposure of the monocytes to GA. At higher concentrations, short-term incubation of GA with ex vivo purified monocytes resulted in increased IL-10 production and decreased IL-12 production. This was not seen when monocytes were exposed to a range of other Ags (including human MBP and other myelin and nonmyelin Ags), suggesting that the ability of GA to reciprocally regulate monocyte IL-10 and IL-12 is not simply related to Ag processing nor to the resemblance of GA to myelin Ags. We suspect that this quality of GA may relate to its random copolymer nature. Consistent with this, recent findings of the Strominger laboratory (40) show that other novel synthetic amino acid copolymers (not based on G-L-A-T) can suppress EAE and inhibit autoantigen-specific T cell responses.

Although our results confirm recent reports that GA can directly modulate human APC responses in vitro (41, 42) and extend this observation to ex vivo human monocytes, it is not known whether APCs actually encounter such concentrations of GA in vivo (43). Higher levels of GA may be present at sites of injection or in

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**Table 1. Surface molecule expression on monocytes and microglia exposed to medium only, or to either pre-GA or post-GA T cell supernatants**

<table>
<thead>
<tr>
<th>APC</th>
<th>Molecule</th>
<th>Medium Only (1)</th>
<th>Pre-GA (2) Supernatants</th>
<th>Post-GA (3) Supernatants</th>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>695.3 ± 289</td>
<td>1615.0 ± 638</td>
<td>1274.0 ± 489</td>
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<tr>
<td></td>
<td></td>
<td>434.9 ± 91</td>
<td>878.2 ± 74</td>
<td>583.3 ± 179</td>
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<td></td>
<td>13.4 ± 3.9</td>
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<td></td>
<td>7.8 ± 1.1</td>
<td>23.2 ± 5.1</td>
<td>11.4 ± 2.2</td>
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<tr>
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<td></td>
<td>27.8 ± 2.1</td>
<td>42.2 ± 7.3</td>
<td>69.8 ± 19</td>
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<tr>
<td>Microglia</td>
<td>ICAM-1</td>
<td>282.4 ± 55</td>
<td>678.4 ± 133</td>
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<td></td>
<td>HLA-DR</td>
<td>326.7 ± 10.9</td>
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<td>13.2 ± 2.2</td>
<td>9.4 ± 1.5</td>
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<td>CD80</td>
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<td>10.9 ± 1.5</td>
<td>7.3 ± 3.2</td>
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<td></td>
<td>CD86</td>
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<td>86.3 ± 26</td>
<td>35.0 ± 13</td>
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</table>

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*a* Represented as the average mean fluorescent intensity ± SD; *n* = 3–5 independent experiments.

*b* p < 0.05; **, p < 0.01.

**CD80 expressed as "percent (%) +" monocytes: medium only = 5.6%; pre-GA = 85%; post-GA = 37%.

**CD80 expressed as "percent (%) +" microglia: medium only = 0%; pre-GA = 93%; post-GA = 12%.

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Discussion

The use of GA in patients with MS represents one of the few settings in which in vivo Th2 deviation of human CD4+ T cells is consistently observed (7–11) and is associated with significant decreases in clinical and imaging parameters of inflammatory disease activity (30, 31). This provides a unique system for elucidating the mechanisms by which peripherally administered immune-modulating agents may result in therapeutic immune deviation and suppression of target tissue injury. How GA immunization results in the observed peripheral Th2 shifts and how such modulation relates to the established therapeutic benefit of GA in MS have not been fully elucidated. In this study, we investigated a model in which the modulation of APCs by GA therapy plays an important role in promoting an anti-inflammatory environment both in the periphery and within the CNS.

The importance of APCs in regulating Th1 and Th2 differentiation has been well established in vitro and, more recently, in animal models, in which it has been suggested that the presence of APCs is a requirement for achieving Th2 priming of effector T cells in vivo (32). We chose to study circulating CD14+ monocytes, in part because they represent a mobile compartment of potential APCs that could be exposed to the GA in the periphery and then migrate to the target tissue. Our lab recently showed that monocytes are significantly more efficient than T cells in migrating across an in vitro model of the BBB comprised of human brain-derived endothelial cells (25, 26) consistent with pathologic studies of MS and experimental autoimmune encephalomyelitis (EAE) that underscore the abundance of invading monocytes/macrophages within the inflammatory CNS lesions (33). Another reason to focus on monocytes is that, in addition to their potential as APCs, they have also been implicated as potential mediators of both CNS injury and repair. In vivo depletion of monocytes is associated with a marked suppression of EAE (34), and, in such animals, T cells tend to accumulate in the leptomeninges without infiltrating the CNS parenchyma (35). Monocytes as well as resident CNS microglia can produce proinflammatory cytokines and free radicals, and the axonal injury observed in the brains of patients with MS is correlated with the presence of invading monocytes/macrophages and activated microglia (36, 37). Conversely, invading monocytes are thought to play a role in clearing myelin debris at sites of CNS injury, contributing to a more permissive repair environment, as remyelination in injury models is limited in animals depleted of monocytes (38). Understanding how the biology of circulating and resident CNS APCs can be modulated and how this may impact on the CNS inflammatory environment is thus of considerable interest.

By comparing ex vivo isolated monocytes from untreated and GA-treated patients with MS, we provide the first demonstration that in vivo therapy with GA results in the modulation of human APCs, notable for reciprocal regulation of the IL-10/IL-12 regulatory cytokine network by circulating monocytes. Our findings extend a preliminary report of altered cytokine levels in the serum of GA-treated patients (39) and identify a likely cellular source for differential cytokine expression. Compared with untreated MS patients, ex vivo monocytes from GA-treated patients produced significantly less IL-12 and significantly more IL-10. The difference in IL-10 production was accentuated when monocytes were stimulated via CD40L/CD40 engagement, mimicking encounter with activated T cells. We considered whether the mechanism of in vivo modulation of human APCs by GA could be mediated by direct exposure of the monocytes to GA. At higher concentrations, short-term incubation of GA with ex vivo purified monocytes resulted in increased IL-10 production and decreased IL-12 production. This was not seen when monocytes were exposed to a range of other Ags (including human MBP and other myelin and nonmyelin Ags), suggesting that the ability of GA to reciprocally regulate monocyte IL-10 and IL-12 is not simply related to Ag processing nor to the resemblance of GA to myelin Ags. We suspect that this quality of GA may relate to its random copolymer nature. Consistent with this, recent findings of the Strominger laboratory (40) show that other novel synthetic amino acid copolymers (not based on G-L-A-T) can suppress EAE and inhibit autoantigen-specific T cell responses.

Although our results confirm recent reports that GA can directly modulate human APC responses in vitro (41, 42) and extend this observation to ex vivo human monocytes, it is not known whether APCs actually encounter such concentrations of GA in vivo (43). Higher levels of GA may be present at sites of injection or in...
treated patients, as well as within active MS lesions, where in cells. Such an encounter is likely to occur both in the periphery of responses through interaction of naive APCs with GA-modulated T

We therefore considered an alternate mechanism in which GA therapy may indirectly modulate APC responses through interaction of naïve APCs with GA-modulated T cells. Such an encounter is likely to occur both in the periphery of treated patients, as well as within active MS lesions, where infiltrating monocytes and activated T cells are abundant. To avoid the issue of allogeneic reactions, our initial studies focused on APC responses to soluble products of GA-reactive T cells. We show that, compared with the effects of Th1 supernatants, ex vivo human monocytes exposed to supernatants of pTh2 GA-reactive T cells consistently produce higher levels of IL-10 and lower levels of IL-12 and TNF-α. This reciprocal regulation of monocyte IL-10 and IL-12 production was reproduced whether the GA-reactive T cells were generated in vitro or in vivo, under in vitro polarizing conditions, or through in vivo deviation in MS patients immunized with GA.

Reciprocal regulation of APC IL-10 and IL-12 production has been strongly implicated in T cell differentiation. Recent animal studies have shown that exogenous exposures can modulate in vivo APC production of IL-10 and IL-12 that, in turn, regulate Th1/Th2 commitment (45–48). For example, parasitic infection has been shown to generate activated macrophages that produce low IL-12 levels and drive Th2 responses (47). Similarly, preferential IL-10 induction in APCs by low-titer viral infection induces Th1 to Th2 shifts in T cell responses (48). In the present study, we extend the animal model work and demonstrate that exposure to GA treatment induces reciprocal regulation of IL-10 and IL-12 production by human monocytes that is associated with functional immune deviation toward type 2 APCs. Monocytes exposed to pretreatment Th1 GA-reactive cell products produced high levels of IL-12 and low levels of IL-10, and efficiently drove subsequent Th1 responses of naïve T cells. In contrast, monocytes exposed to posttreatment Th2 GA-reactive cell products produced low levels of IL-12 and high levels of IL-10, and efficiently drove subsequent Th2 differentiation of naïve T cells.

We subsequently found that this novel paradigm, wherein T cell polarity can modulate APC responses, is a more generalizable phenomenon that is not restricted to GA-reactive T cells. Regardless of the T cell specificity, pTh1 supernatants induced naïve monocytes to produce higher levels of IL-12 and lower levels of IL-10, while pTh2 supernatants induced naïve monocytes to produce higher levels of IL-10 and lower levels of IL-12. Therefore, in addition to furthering our insights into the therapeutic mechanism of action of GA in patients with MS, our findings identify a novel positive feedback loop that is likely to exist between human T cells and APCs in vivo. APCs exposed to Th1 cells would adopt a type 1 APC phenotype and subsequently mediate further Th1 differentiation. In contrast, exposure to Th2 cells would induce type 2 APC differentiation that would, in turn, promote further Th2 deviation. Given the ability of monocytes to efficiently migrate across the BBB (25, 26), type 1 and 2 monocytes induced in the periphery would be expected to migrate into the CNS of patients with MS and contribute to local Th1 or Th2 immune deviation, respectively.

Based on our findings with monocytes, we further considered the possibility that invading Th1 and Th2 T cells could have distinct effects on the responses of resident CNS APCs, and that these may, in turn, contribute to shaping the local inflammatory environment. In this context, IL-10 has been implicated as having a critical role in regulating EAE (49). The notion that resident tissue APCs can promote local T cell differentiation without the need to first migrate into secondary lymphoid organs has been recently suggested in studies of Ag exposure within the lung microenvironment (50). Among CNS resident cells, microglia have previously been identified as efficient APCs (51–53) capable of determining the fate of CD4+ T cell responses. Our results confirm that the profile of effector cytokines of human microglia (IL-10 and TNF-α) is differentially regulated by Th1 and Th2 GA-reactive T cells, extending recent observations of the effects of non-Ag-specific T cell supernatants on APC responses (54). We provide, to our knowledge, the first demonstration that CNS-derived microglia can be modulated into type 1 and 2 APCs capable of inducing efficient Th1 and Th2 T cell differentiation, respectively.

Finally, we observed some differences in the inflammatory response profiles of peripheral monocytes and resident CNS microglia, exposed to the identical T cell supernatants. Compared with monocytes, the phenotype of microglia appeared less sensitive to the effects of T cell soluble products and particularly to the post-GA supernatants (Table I). Also, the pre-GA and post-GA T cell supernatants had opposite effects on CD86 expression of monocytes and microglia: CD86 was significantly induced in microglia by pre-GA, but not by post-GA supernatants. In contrast, significantly more CD86 was induced in monocytes by post-GA compared with pre-GA supernatants. Despite these phenotypic differences, our findings demonstrate that both monocytes and microglia are capable of being functionally modulated by pre-GA (Th1) and post-GA (Th2) supernatants, and that both peripheral and CNS APCs can be differentiated by these soluble T cell products into type 1 and 2 APCs, respectively.
In summary, our results provide the first demonstration that treatment with GA reciprocally regulates the IL-10 and IL-12 cytokine network of APCs in vivo, and that the associated type 2 APC deviation may be induced by both direct and indirect mechanisms. We also demonstrate for the first time the potential for type 2 APC modulation of human CNS-derived microglia. The resultant peripheral and CNS-derived type 2 APCs can both efficiently induce Th2 T cell deviation. The present study extends our understanding of the therapeutic mode of action of GA in MS at a time that immune-modulatory capacities of synthetic copolymers are of particular interest (54). In addition to identifying APCs as relevant targets of GA therapy, our findings revisit the concept of bystander suppression, and suggest that shifts within the CNS toward Th2/anti-inflammatory immune responses may not be predicated solely on peripheral generation of Th2 T cells and their subsequent anti-inflammatory effects within the CNS. We propose that GA therapy also generates type 2 APCs that may contribute to Th2 T cell deviation and an anti-inflammatory environment both in the periphery and in the CNS of patients. The latter may be mediated by both invading peripheral APCs (e.g., monocytes) as well as resident CNS APCs (e.g., microglia). We underscore the dynamic involvement of peripheral and CNS APCs in modulating immune responses within the CNS. The fundamental observation that differentially polarized T cells can reciprocally regulate the APC IL-10/IL-12 regulatory cytokine network defines a novel positive feedback loop between human T cells and APCs, and identifies that in vivo modulation of peripheral APCs may represent an attractive therapeutic approach for human target-directed inflammatory diseases.

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References