MULTIPLE SCLEROSIS

Proinflammatory GM-CSF–producing B cells in multiple sclerosis and B cell depletion therapy

Rui Li,[1] Ayman Rezk,[1] Yusei Miyazaki,[1] Ellen Hilgenberg,[2] Hanane Touil,[1] Ping Shen,[2] Craig S. Moore,[1] Laure Michel,[3] Faisal Althekair,[1] Sathy Rajasekharan,[1] Jennifer L. Gommerman,[4] Alexandre Prat,[3] Simon Fillatreau,[2] Amit Bar-Or,[1,5]* on behalf of the Canadian B cells in MS Team

B cells are not limited to producing protective antibodies; they also perform additional functions relevant to both health and disease. However, the relative contribution of functionally distinct B cell subsets in human disease, the signals that regulate the balance between such subsets, and which of these subsets underlie the benefits of B cell depletion therapy (BCDT) are only partially elucidated. We describe a proinflammatory, granulocyte macrophage–colony stimulating factor (GM-CSF)–expressing human memory B cell subset that is increased in frequency and more readily induced in multiple sclerosis (MS) patients compared to healthy controls. In vitro, GM-CSF–expressing B cells efficiently activated myeloid cells in a GM-CSF–dependent manner, and in vivo, BCDT resulted in a GM-CSF–dependent decrease in proinflammatory myeloid responses of MS patients. A signal transducer and activator of transcription 5 (STAT5)– and STAT6-dependent mechanism was required for B cell GM-CSF production and reciprocally regulated the generation of regulatory IL-10–expressing B cells. STAT5/6 signaling was enhanced in B cells of untreated MS patients compared with healthy controls, and B cells reemerging in patients after BCDT normalized their STAT5/6 signaling as well as their GM-CSF/IL-10 cytokine secretion ratios. The diminished proinflammatory myeloid cell responses observed after BCDT persisted even as new B cells reconstituted. These data implicate a proinflammatory B cell/myeloid cell axis in disease and underscore the rationale for selective targeting of distinct B cell populations in MS and other human autoimmune diseases.

INTRODUCTION

B cell depletion therapy (BCDT) with anti-CD20 is emerging as a highly effective approach for limiting new inflammatory disease activity in several human immune-mediated diseases including multiple sclerosis (MS) (*1*–*9*). Depending on the disease, the therapeutic mode of action of BCDT is thought to involve the removal of either the antibody-related or the antibody-independent pathogenic roles of B cells. The ability of BCDT to limit new disease relapses in patients with relapsing-remitting MS (RRMS) appears to primarily reflect an impact on the antibody-independent pathogenic functions of B cells (*10*–*12*). This is thought to involve elimination of B cells with proinflammatory properties, resulting in decreased proinflammatory T helper 1 (T_H1) and T_H17 cell responses and hence decreased disease relapses (*10*, *12*). However, the particular subset(s) of pathogenic B cells involved in triggering MS relapses have not been elucidated, and it remains unknown whether such B cells contribute to aberrant T cell responses only through direct effects on T cells or also through indirect effects on a third-party cell type such as myeloid cells, which can have key roles in shaping disease-relevant T cell responses.

There has recently been a growing appreciation of the functional heterogeneity present in the B cell compartment based on expression of different cytokine profiles (*13*). This has generated considerable interest in elucidating how distinct cytokine-defined B cell subsets may contribute through anti- or proinflammatory ways to immune responses in both health and disease (*13*–*17*). The role of cytokine-producing B cells has been documented in mouse models of human autoimmune disorders, in which deficient B cell production of the regulatory cytokines interleukin-10 (IL-10) or IL-35 and exaggerated B cell production of the proinflammatory cytokine IL-6 have been associated with exacerbated disease course (*10*, *12*, *18*–*25*). Along the same lines, evidence is accumulating that dysregulated cytokine secretion by human B cells plays an important role in MS pathogenesis, a disease in which B cells exhibit deficient IL-10 production and increased provision of the proinflammatory mediators lymphotoxin-α (LTα), tumor necrosis factor–α (TNFα), and IL-6 (*10*, *12*, *19*). However, the relationship between B cells producing anti-inflammatory versus proinflammatory cytokines is poorly defined, and little is known about the molecular mechanisms that control the balance between so-called regulatory and effector B cell responses.

Here, we characterize a proinflammatory granulocyte macrophage–colony stimulating factor (GM-CSF)–producing human B cell subset that coexpresses high levels of TNFα as well as IL-6, induces proinflammatory myeloid cell activation in a GM-CSF–dependent manner, and is abnormally increased in patients with MS. We further demonstrate that induction of human GM-CSF– and IL-10–expressing B cells is reciprocally regulated through a signal transducer and activator of transcription 5 (STAT5)– and STAT6-dependent mechanism. Our studies in MS patients treated with BCDT point to a new therapeutic mechanism of action for this treatment and provide an explanation for both the rapidly diminished MS disease activity observed at the time of B cell removal and the apparent persistence of this benefit as newly reconstituting B cells with normalized STAT signaling and diminished proinflammatory responses emerge in these patients.

[1]Neuroimmunology Unit, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec H3A 2B4, Canada. [2]Deutsches Rheuma-Forschungszentrum, a Leibniz Institute, Charitéplatz 1, 10117 Berlin, Germany. [3]Centre de Recherche du CHUM (CRCHUM) and Department of Neuroscience, Université de Montréal, 900 Saint Denis Street, Montreal, Quebec H2X 0A9, Canada. [4]Department of Immunology, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S 1A8, Canada. [5]Experimental Therapeutics Program, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec H3A 2B4, Canada.
*Corresponding author. E-mail: amit.bar-or@mcgill.ca

RESULTS

Identification of human proinflammatory cytokine–producing B cells

Whereas abnormalities in pro- and anti-inflammatory cytokine–expressing B cells have been reported in patients with human autoimmune diseases such as MS, it is not known whether these cytokines are produced by distinct or identical B cell subsets. To clarify this issue, we used an ex vivo multiparametric flow cytometric approach to capture the cytokine expression profiles of individual circulating B cells. Using freshly isolated B cells from healthy donors, we detected intracellular expression of several previously described human B cell cytokines (including IL-6, TNFα, and IL-10) and also identified a subpopulation of cells expressing GM-CSF (Fig. 1A and fig. S1), a cytokine previously ascribed a key role in mediating central nervous system inflammation. Concurrent staining for multiple cytokines revealed that the GM-CSF–expressing human B cells were among the highest expressers of both TNFα (Fig. 1B) and IL-6 (Fig. 1C) while essentially not expressing IL-10 (Fig. 1D). GM-CSF–expressing B cells also expressed significantly higher levels of costimulatory molecules (CD80 and CD86) than activated B cells expressing only TNFα or IL-6 (fig. S2), a profile suggesting a robust immunostimulatory potential. GM-CSF–expressing B cells could be further characterized as $CD27^{+}$ $CD24^{high}$ $CD25^{high}$ $CD86^{high}$ $CD49d^{high}$ $CD38^{low}$ (fig. S3), a phenotype of memory B cells that is distinct from antibody-secreting cells. From these data, we conclude that circulating human GM-CSF–producing B cells represent a subset of circulating effector memory B cells equipped with a robust proinflammatory arsenal.

Increased frequency of GM-CSF–producing B cells in patients with MS

To evaluate the potential relevance of GM-CSF–producing B cells in MS, we quantified these cells in MS patients by flow cytometry, as described above. We discovered significantly increased frequencies of circulating GM-CSF–producing B cells in untreated patients with RRMS (9.3 ± 1.42%) compared to age- and sex-matched healthy controls

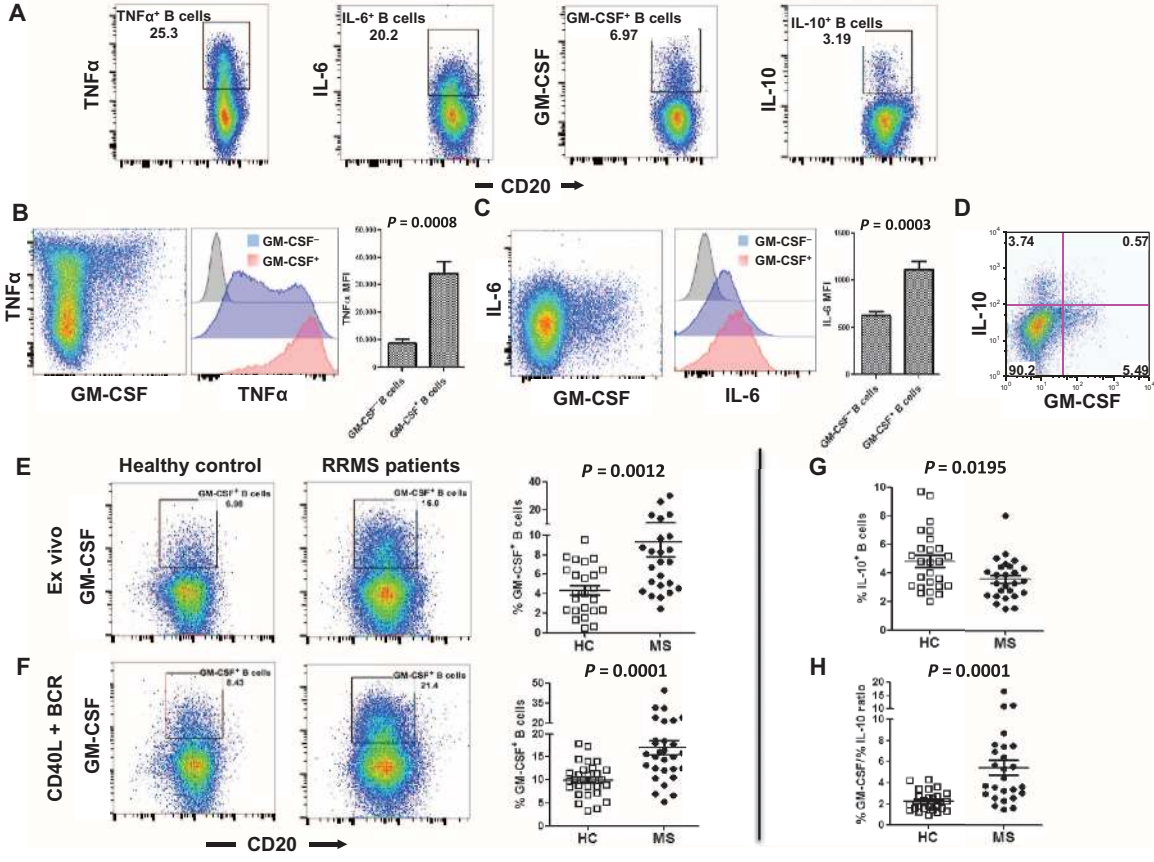

Fig. 1. Identification of human proinflammatory GM-CSF^{+} B cells ex vivo and abnormalities in patients with MS. Freshly isolated (magnetic cell sorter) human peripheral B cells were subjected to short-term stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, followed by costaining for intracellular cytokines and the B cell marker CD20. (**A** to **D**) B cell subsets were identified that expressed TNFα, IL-6, GM-CSF, and IL-10 (A). Costaining for multiple cytokines revealed that GM-CSF^{+} B cells were high producers of both TNFα (B) and IL-6 (C) but distinct from IL-10–producing B cells (D). Data represent at least seven different experiments; paired Student's *t* test. MFI, mean fluorescence intensity. (**E** to **F**) Frequencies of GM-CSF– and IL-10–expressing B cells were quantified in blood of untreated patients with RRMS and in age- and sex-matched HCs after ex vivo stimulation with PMA/ionomycin or after 24 hours of in vitro activation. MS patients were found to harbor abnormally increased frequencies of GM-CSF^{+} B cells in their circulation (E) and exhibit greater induction in the frequency of these cells after activation (F). After in vitro activation, MS B cells exhibited lower induction of IL-10 (G). Increased GM-CSF/IL-10 ratios (H) reflect the abnormal balance between B cell subset responses of MS patients compared to HCs ($n > 24$ per population, unpaired Student's *t* test).

(HCs) (4.4 ± 0.52%; n = 24, P = 0.0012; Fig. 1E). Notably, activation with the B cell receptor (BCR) and CD40 accentuated the difference in frequencies of GM-CSF–producing B cells between MS patients (17 ± 1.6%) and HCs (10 ± 0.7%; n = 29, P = 0.0001; Fig. 1F). Such activation also revealed the previously described deficit in IL-10 expression by B cells of MS patients (n = 26, P = 0.0195; Fig. 1G). These results indicated that, when activated, MS B cells exhibit a dual abnormality of (i) increased frequencies of GM-CSF–expressing B cells and (ii) diminished induction of IL-10–expressing cells. This reciprocal abnormality results in significantly increased ratios of GM-CSF/IL-10–expressing B cells in MS patients compared to HCs (n = 26, P = 0.0001; Fig. 1H) and is consistent with our observation that GM-CSF– and IL-10–expressing B cells are mutually exclusive (Fig. 1D).

Induction of GM-CSF–expressing B cells from naïve B cells

Our ex vivo staining indicated that circulating human GM-CSF$^+$ B cells were among the memory B cell pool. We wished to identify the signals inducing GM-CSF expression in naïve human B cells. Using presorted naïve (CD27$^-$) and memory (CD27$^+$) B cells from healthy donor blood, we initially confirmed that circulating GM-CSF$^+$ B cells belonged to the CD27$^+$ memory B cell pool (Fig. 2A). After a 48-hour stimulation with the BCR and CD40, GM-CSF expression was increased in memory B cells and could now be seen in the presorted naïve (CD27$^-$) B cells (Fig. 2, B and C). Because cytokines are known to affect the polarization of effector T lymphocyte subsets, we reasoned that the presence of different cytokines might influence the induction of GM-CSF expression by naïve human B cells. Transforming growth factor–β (TGFβ) and IL-4 each enhanced the induction of GM-CSF in naïve B cells, both assessed by intracellular cytokine staining (Fig. 2, D and E, and fig. S4A) and as secreted GM-CSF (Fig. 2F and fig. S4B). Selective blockade of IL-4 receptor subunits revealed that IL-4 enhanced B cell GM-CSF production through IL-4 receptor α chain (IL-4Rα) but not CD132 (fig. S5). We conclude that signaling with the BCR and CD40 in the presence of cytokines such as TGFβ or IL-4 can potently induce GM-CSF expression in naïve human B cells.

Reciprocal regulation of GM-CSF and IL-10 expression by human B cells by STAT5 and STAT6 signaling

The near mutually exclusive expression of GM-CSF and IL-10 by human B cells raised the possibility that their induction might be reciprocally regulated. To gain further insight into the relationship between GM-CSF– and IL-10–producing B cells, we sought to identify the intracellular signals controlling the balance between the expression of these cytokines by naïve B cells. We started by investigating the phosphorylation of particular STAT molecules (because these have been implicated in the differentiation of cytokine-defined T cell subsets (*26*, *27*)] under a range of B cell–activating conditions including those promoting the expression of B cell GM-CSF. In particular, we measured the phosphorylation of STAT1, STAT3, STAT4, STAT5, and STAT6. We found that combined stimulation with CD40 and BCR together with IL-4 (which efficiently triggered GM-CSF; Fig. 2, D to F) strongly induced the phosphorylation of STAT5 (Fig. 3A) and even more so that of STAT6 (Fig. 3B). An association between STAT6 activation and GM-CSF expression by B cells was consistent with the observed correlation between the frequency of GM-CSF$^+$ B cells and phosphorylated STAT6 (pSTAT6) expression, both ex vivo (Fig. 3C) and after in vitro stimulation (Fig. 3D). Inhibition of either STAT5 or STAT6, and particularly dual inhibition of both, substantially suppressed the induction and secretion of GM-CSF from naïve B cells (Fig. 3, E to G) while markedly enhancing their IL-10 expression (Fig. 3, E, H, and I), thereby reversing the GM-CSF/IL-10 ratio (Fig. 3, J and K). Dual inhibition of STAT5 and STAT6 also decreased GM-CSF expression while increasing IL-10 expression in memory B cells (fig. S6).

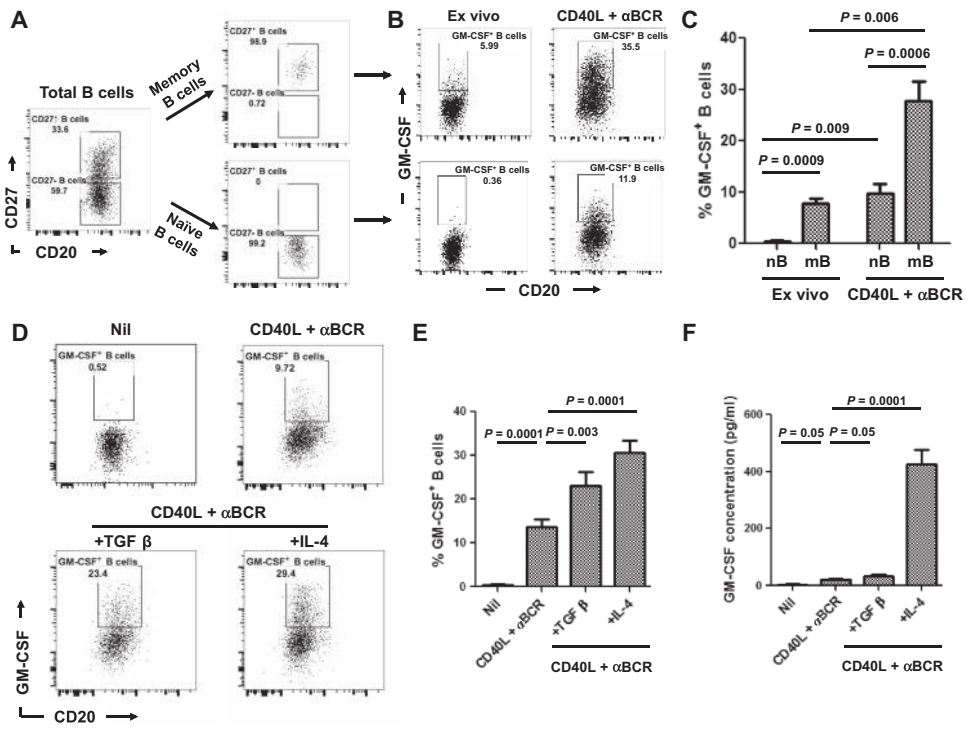

Fig. 2. Induction of GM-CSF–expressing B cells from naïve B cells. (**A**) Human naïve (CD27$^-$) and memory (CD27$^+$) B cells were isolated by cell sorting. (**B**) Cells were then either briefly stimulated with PMA/ionomycin (left panels) or activated with CD40 ligand (CD40L) and αBCR for 24 hours (right panels). (**C**) Dot plot data are representative of n = 7 independent experiments from different donors [summarized in (C), one-way analysis of variance (ANOVA)]. nB, naïve B cells; mB, memory B cells. (**D** to **F**) Naïve (CD27$^-$) B cells were either not activated (Nil) or activated by CD40L and αBCR alone or with the addition of either TGFβ or IL-4 (or other cytokines; fig. S4). (D) Representative example of intracellular GM-CSF staining detected by fluorescence-activated cell sorter (FACS) under the different conditions and summary (n = 9, one-way ANOVA) of frequency of GM-CSF–expressing cells (E) and secreted GM-CSF as quantified by enzyme-linked immunosorbent assay (ELISA) (F). We confirmed that the IL-4–mediated enhancement of GM-CSF production by B cells was mediated by IL-4 receptor α chain rather than by CD132 (fig. S5).

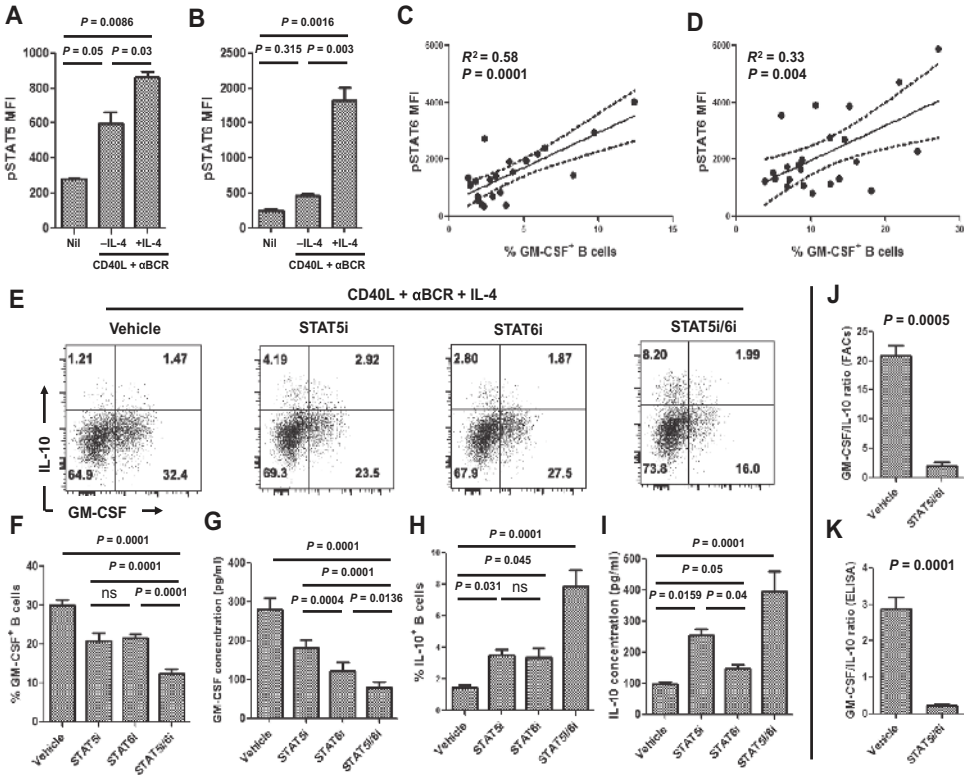

Fig. 3. STAT5- and STAT6-dependent reciprocal regulation of GM-CSF and IL-10 expression by human B cells. Human naïve B cells were isolated from healthy individuals. (**A** and **B**) pSTAT5 (A) and pSTAT6 (B) were measured after stimulation either with CD40L and anti-BCR (designated 40X) or with CD40L, anti-BCR, and IL-4 (40X + IL-4) for 48 hours. (**C** and **D**) Correlation between pSTAT6 and frequency of GM-CSF$^+$ B cells after either short-term ex vivo (C) or 48-hour stimulation with CD40L + anti-BCR + IL-4 (D). (**E** to **K**) Naïve B cells were pretreated with STAT5 and/or STAT6 inhibitors (STAT5i/6i) for 1 hour before activation with CD40L + anti-BCR + IL-4. (E) Representative example of GM-CSF and IL-10 expression assessed by intracellular staining and flow cytometry. Summary of eight independent experiments assessing frequency of GM-CSF–expressing B cells (F) and GM-CSF secretion by ELISA (G), as well as the frequency of IL-10–expressing B cells (H) and IL-10 secretion (I). Dual inhibition of STAT5 and STAT6 resulted in a significant reduction in the GM-CSF/IL-10 ratios assessed by flow cytometry (J) and ELISA (K). (n = 8, one-way ANOVA).

Inhibition of STAT5 and STAT6 did not appear to affect B cell survival (fig. S7). From these results, we conclude that STAT5 and STAT6 reciprocally regulate IL-10 and GM-CSF production by human B cells, results that also offer a coherent explanation for the observations that GM-CSF– and IL-10–expressing B cells, when examined ex vivo, appear as distinct cell subsets. Our results further indicate that STAT6 may be particularly important for the de novo induction of GM-CSF in naïve B cells (Fig. 3), whereas STAT5 may play a more important role in maintaining GM-CSF expression in memory B cells (fig. S6).

In keeping with the enhanced propensity of MS B cells to produce GM-CSF, yet their decreased induction of IL-10, we found that B cells from MS patients exhibited higher pSTAT6 levels as compared to age- and sex-matched HCs (fig. S8). Together, these findings highlight the role of STAT5 and STAT6 signaling in reciprocal regulation of pro- and anti-inflammatory cytokines from human B cells and implicate in vivo B cell STAT signaling as a potential mechanism underlying the overrepresentation of GM-CSF$^+$ B cells in the face of deficient induction of IL-10–expressing B cells in MS patients.

Induction of proinflammatory myeloid cell responses by GM-CSF–expressing B cells

The overabundance of GM-CSF–expressing B cells in MS patients, together with their proinflammatory cytokine profile, suggested that these cells might contribute to the pathogenic activity of B cells in this disease. To assess this possibility, we analyzed the function of these cells. We considered that GM-CSF$^+$ B cells might directly affect the innate immune system and, more specifically, myeloid cells, which are known to contribute to shaping disease-relevant T cell responses.

We first generated CD14$^+$ monocyte–derived macrophages from healthy donors and exposed them to supernatants of autologous B cells activated in different ways, including under conditions inducing GM-CSF production from B cells. Preexposure to supernatants from cultures enriched in GM-CSF–expressing B cells strongly enhanced macrophage secretion of IL-12 (Fig. 4A), IL-6 (Fig. 4B), and IL-1β (fig. S9). This proinflammatory effect was GM-CSF–dependent because selectively blocking GM-CSF in the B cell supernatants before their addition to the macrophages completely abrogated the enhanced proinflammatory myeloid cell responses. Notably, exposure to supernatants of the GM-CSF–expressing B cell–enriched cultures also resulted in decreased myeloid cell IL-10 secretion, although this effect was not GM-CSF–dependent but rather appeared to be mediated by IL-6 (Fig. 4C and fig. S10). We conclude that GM-CSF$^+$ B cells can stimulate the proinflammatory function of macrophages in a GM-CSF–dependent manner.

We next compared B cells from MS patients and matched HCs for their capacity to induce proinflammatory macrophage responses. Using the same culture system, we found that GM-CSF–expressing B cells from MS patients efficiently induced GM-CSF–dependent proinflammatory responses of autologous myeloid cells, in fact at a higher level compared to the effects of GM-CSF$^+$ B cells induced in parallel from matched HCs (Fig. 4, D to F). Using a crossover approach to compare the impact of GM-CSF–expressing B cells derived from MS patients and matched HCs on the responses of myeloid cells obtained from both cohorts, we found that MS-derived GM-CSF–expressing B cells induced higher proinflammatory cytokine responses in macrophages of both patients and controls (Fig. 4, G and H). In contrast, the macrophages of MS patients and HCs responded similarly to the same B cell supernatants (fig. S11), indicating that the enhanced proinflammatory myeloid responses seen with samples from the MS patients reflected an abnormality in the patient-derived GM-CSF–expressing B cells but not in their myeloid cells. This is in keeping with our observation that B cells

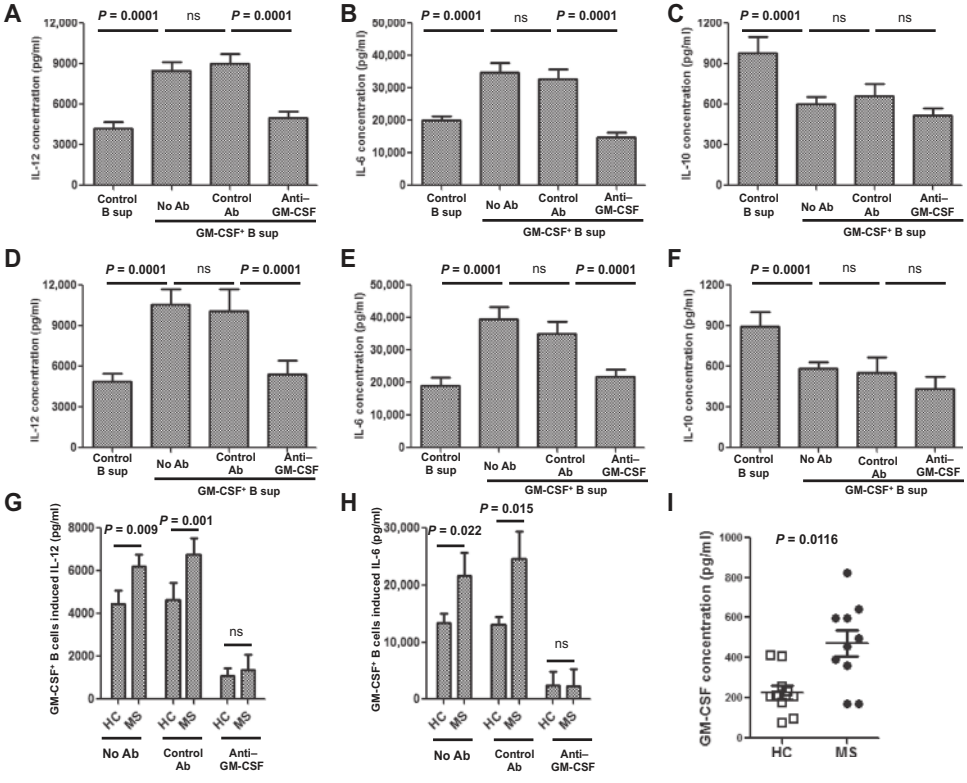

Fig. 4. Induction of myeloid cell proinflammatory responses by GM-CSF$^+$ B cells. (**A** to **F**) Macrophages were generated by M-CSF treatment of freshly isolated CD14$^+$ peripheral monocytes obtained from HCs (A to C) or matched untreated patients with RRMS (D to F). Macrophages were then exposed to supernatants of either enriched GM-CSF$^+$ B cell cultures or control B cell cultures (B sup) for 24 hours, thoroughly washed, and then stimulated with LPS for an additional 24 hours. Anti–GM-CSF function-blocking antibody (anti–GM-CSF) or control antibody (control Ab) were used to selectively neutralize GM-CSF in B cell supernatants. ELISA was used to quantify macrophage secretion of IL-12 (A and D), IL-6 (B and E), and IL-10 (C and F) (n = 10, one-way ANOVA). Monocyte-derived macrophages of healthy donors were preexposed to supernatants of enriched GM-CSF$^+$ B cell cultures generated from either HCs or MS patients, washed, and then stimulated in fresh medium with LPS with subsequent ELISA measurement of secreted macrophage cytokines. (**G** and **H**) The GM-CSF$^+$ B cell–induced cytokine production from macrophages was calculated as the difference in macrophage cytokine secretion observed after GM-CSF$^+$ B cell supernatant exposure and control B sup exposure, shown for IL-12 (G) and IL-6 (H) (n = 12). These differences in myeloid cell responses to supernatants of MS versus HC GM-CSF$^+$ B cell–enriched cultures were GM-CSF–dependent, as seen when using GM-CSF–neutralizing antibody in the effector B cell supernatants (anti–GM-CSF). No such effect was seen with the control antibody (fig. S11). (**I**) B cells from either HCs or MS patients were stimulated with CD40L + αBCR + IL-4. GM-CSF level was measured by ELISA. In keeping with the observed functional effects, GM-CSF$^+$ B cell–enriched cultures of MS patients showed abnormally increased levels of GM-CSF compared to similarly activated GM-CSF$^+$ B cell–enriched cultures of HCs. ns, nonsignificant.

from MS patients produce higher amounts of GM-CSF compared to B cells from HCs in these experiments (Fig. 4I), as previously found (Fig. 1). From these results, we conclude that GM-CSF from B cells can promote inflammation by stimulating the secretion of proinflammatory cytokines while inhibiting anti-inflammatory cytokine production by myeloid cells, and that this capacity is enhanced in MS.

Diminished proinflammatory responses of macrophages in patients with MS after anti-CD20 BCDT

On the basis of our in vitro data, we predicted that if GM-CSF from B cells does indeed increase the proinflammatory responses of myeloid cells in MS patients, then BCDT should lead to a reduction of these macrophage responses in treated patients. To test this prediction, we studied a cohort of patients with RRMS who underwent B cell depletion with the anti-CD20 monoclonal antibody rituximab and compared the responses of monocyte-derived macrophages in samples collected from the same patients before B cell depletion and after anti-CD20 treatment (at a time when B cells were depleted from the circulation). As predicted, MS patient macrophages obtained after B cell removal exhibited reduced secretion of IL-12 (Fig. 5A) and IL-6 (Fig. 5B) yet produced more IL-10 (Fig. 5C) than pretreatment macrophages from the same patients. These differences were reversed by adding back B cell supernatants (generated by isolating and activating B cells from the same patients before BCDT) in a GM-CSF–dependent manner (Fig. 5, A to C). We conclude from these results that GM-CSF$^+$ B cells can contribute to proinflammatory macrophage responses in vivo in MS patients, and infer that the mode of action by which BCDT limits new MS disease activity may reflect, at least in part, diminished macrophage proinflammatory responses in these treated patients due to the removal of GM-CSF$^+$ B cells.

Diminished myeloid cell proinflammatory responses after BCDT in MS patients persist during B cell reconstitution

In addition to limiting the development of new MS disease activity during the period of B cell depletion, it seems that for many patients treated with BCDT, disease activity is maintained below pretreatment levels, even after new B cells reconstitute (*3*, *4*, *6*). This suggests that the reconstituting B cells differ from pretreatment B cells in ways relevant to the mechanism(s) by which B cells contribute to new MS disease activity. To determine whether a persistent reduction of GM-CSF–expressing B cells and, subsequently, a long-lasting attenuation of macrophage activation may contribute to this observation, we compared the frequency of GM-CSF–expressing B cells and the functional response profiles of myeloid cells in samples obtained from MS patients before and after rituximab treatment—at a time when B cells were reconstituted to pretreatment levels (Fig. 6A and fig. S12). Both the frequencies of circulating GM-CSF$^+$ B cells (Fig. 6B) and the amounts of GM-CSF secreted by B cells (Fig. 6C) were diminished in the reconstituted B cell population as compared to pretreatment levels in the same patients. At the same time, and consistent with previous reports, IL-10 production was

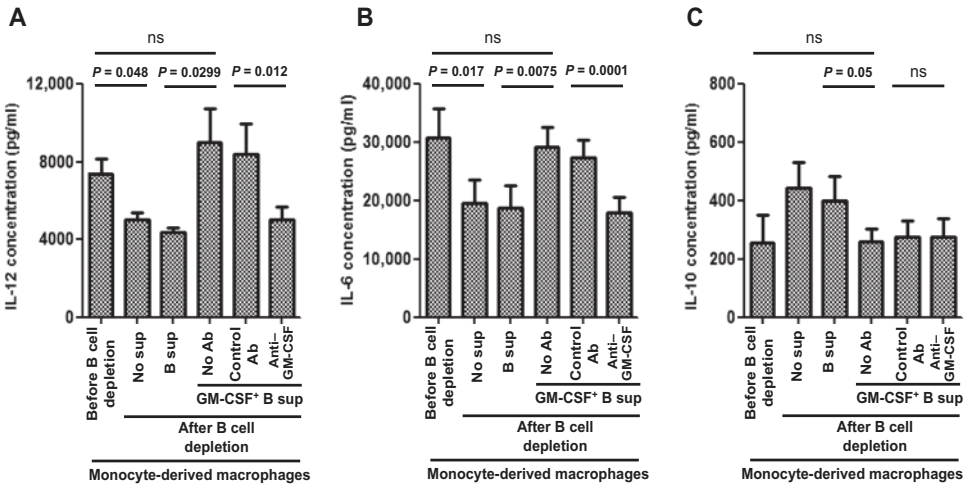

Fig. 5. Diminished proinflammatory responses of macrophages in patients with MS after anti-CD20 BCDT. (**A** to **C**) Macrophages were induced from purified CD14$^+$ peripheral monocytes isolated from MS patients, either before or after B cell depletion. Postdepletion macrophages were preexposed in parallel for 24 hours to supernatants of either enriched GM-CSF$^+$ B cell cultures, control B cell cultures (B sup), or no supernatant (no sup) that were generated from B cells obtained from the same patients before undergoing B cell depletion. After the 24-hour preexposure, the macrophages were thoroughly washed and stimulated in fresh medium with LPS for an additional 24 hours. Macrophage secretion of IL-12 (A), IL-6 (B), and IL-10 (C) was assessed by ELISA. The effects of anti–GM-CSF compared to the appropriate control antibody (control Ab) were also assessed in parallel cultures. Data from n = 5 independent patients who underwent B cell depletion.

significantly increased (Fig. 6D), resulting in substantially diminished (essentially normalized) GM-CSF/IL-10 ratios in the reconstituting B cells (Fig. 6E). These decreased B cell GM-CSF/IL-10 ratios were associated with lower levels of B cell pSTAT5 (Fig. 6F) and pSTAT6 (Fig. 6G), providing an ex vivo correlate for our in vitro data (Fig. 3).

We next examined the macrophage responses in the same patients. We observed that the reduced secretion of IL-12 (Fig. 6H) and IL-6 (Fig. 6I) by myeloid cells isolated from MS patients during the B cell–depleted state persisted in myeloid cells isolated from the same patients after B cell reconstitution. Adding back B cell supernatants (generated from the same patients before B cell depletion) reconstituted the myeloid cell production of both IL-12 and IL-6 in a GM-CSF–dependent manner. The myeloid cell IL-10 response that was enhanced upon depletion of B cells also persisted after B cell reconstitution (Fig. 6J). Together, these findings demonstrate that the decreased myeloid cell proinflammatory responses, which manifest in MS patients after in vivo B cell depletion as diminished ratios of IL-12/IL-10 (Fig. 6K) and IL-6/IL-10 (Fig. 6L) secretion, persist even as B cells reconstitute in these patients, in keeping with the diminished proinflammatory response profile of the reconstituted B cells. Collectively, these data corroborate the concept that the GM-CSF$^+$ B cell macrophage axis is important to B cell–mediated pathogenesis in RRMS and relevant to the therapeutic mode of action of BCDT in this disease.

DISCUSSION

The emerging ability of anti-CD20–mediated B cell depletion to substantially limit new disease activity in patients with MS (*3*–*6*, *9*) was a surprise to many, given the traditional view that MS is a T cell–mediated disease. Moreover, the therapeutic mode of action of B cell depletion in MS appears unrelated to antibodies because the abnormal antibody profiles in the cerebrospinal fluid of patients remain largely unaffected after depletion (*11*, *28*). This has pointed to important antibody-independent contributions of B cells to new MS disease activity. Indeed, previous studies have separately reported that B cells of MS patients can exhibit abnormally increased secretion of LTα, TNFα, or IL-6 (*10*, *12*, *19*, *23*). It has been suggested that removal of such proinflammatory cytokine–secreting B cells may explain the decreased proinflammatory (T_H1 and T_H17) T cell responses observed after B cell depletion (*10*, *12*), although whether this reflects a direct effect of B cell removal on T cell responses or an indirect effect through a third-party cell type has not been elucidated. Whereas the multiple reported cytokine abnormalities of MS B cells could be in keeping with a broad abnormality affecting large populations of proinflammatory B cells in patients, we postulated here that a more likely explanation would involve an abnormality of a relatively small subset of B cells that co-express high levels of multiple proinflammatory cytokines. Previous work has also established that B cell expression of major histocompatibility complex II, as well as the costimulatory molecules CD80 and CD86, is important for aberrant T cell activation in autoimmune conditions (*29*, *30*).

Here, we describe GM-CSF–expressing human B cells with substantial proinflammatory features underscored by coexpression of particularly high levels of TNFα and IL-6, as well as the propensity to efficiently up-regulate surface expression of CD80 and CD86 costimulatory molecules after activation. Our findings reveal a proinflammatory B cell/myeloid cell axis, wherein GM-CSF from B cells is capable of inducing GM-CSF–dependent proinflammatory myeloid cell responses, including enhanced secretion of IL-12 and IL-6, known to be involved in T_H1 and T_H17 cell differentiation, respectively. These GM-CSF$^+$ B cells are increased and also more readily induced in untreated patients with MS, consistent with a potential contribution to disease activity. We further demonstrate that removal of these GM-CSF$^+$ B cells as part of highly effective BCDT in MS patients is associated with a significant GM-CSF–dependent reduction of myeloid cell proinflammatory responses, matching our in vitro observations. Together, our results indicate that previous reports of diminished T_H1 and T_H17 responses in MS patients after B cell depletion may in fact reflect, at least in part, removal of abnormal GM-CSF$^+$ B cells, leading to decreased in vivo proinflammatory myeloid cell cytokine responses, in turn contributing to diminished responses of disease-relevant proinflammatory T cells. These insights extend our understanding of the therapeutic mechanisms of action of B cell depletion in MS and potentially in other autoimmune diseases.

Our results, which further underscore the functional diversity that exists among human B cells, also provide insights into molecular

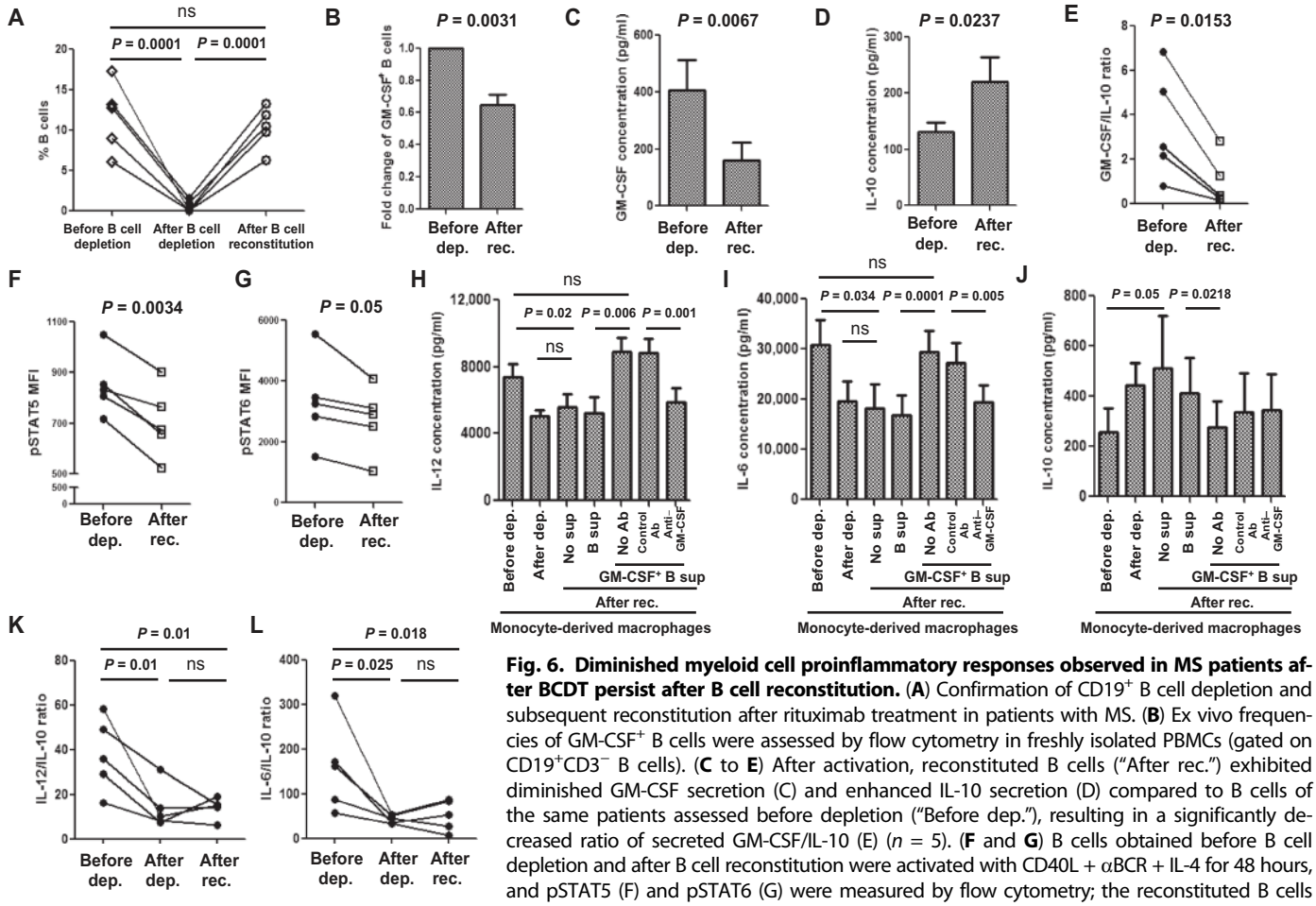

Fig. 6. Diminished myeloid cell proinflammatory responses observed in MS patients after BCDT persist after B cell reconstitution. (**A**) Confirmation of $CD19^+$ B cell depletion and subsequent reconstitution after rituximab treatment in patients with MS. (**B**) Ex vivo frequencies of GM-CSF^+ B cells were assessed by flow cytometry in freshly isolated PBMCs (gated on $CD19^+CD3^-$ B cells). (**C** to **E**) After activation, reconstituted B cells ("After rec.") exhibited diminished GM-CSF secretion (C) and enhanced IL-10 secretion (D) compared to B cells of the same patients assessed before depletion ("Before dep."), resulting in a significantly decreased ratio of secreted GM-CSF/IL-10 (E) (n = 5). (**F** and **G**) B cells obtained before B cell depletion and after B cell reconstitution were activated with CD40L + αBCR + IL-4 for 48 hours, and pSTAT5 (F) and pSTAT6 (G) were measured by flow cytometry; the reconstituted B cells exhibited lower levels of both pSTAT5 and pSTAT6 compared to pretreatment B cells of the same patients. (**H** to **J**) Reconstituting macrophages were preexposed in parallel for 24 hours to supernatants of either enriched GM-CSF^+ B cell cultures, control B cell cultures (B sup), or no supernatant (no sup) that were generated from B cells obtained from the same patients before undergoing B cell depletion. After the 24-hour preexposure, the macrophages were thoroughly washed and stimulated in fresh medium with LPS for an additional 24 hours. Macrophage secretion of IL-12 (H), IL-6 (I), and IL-10 (J) was assessed by ELISA. The effects of anti–GM-CSF compared to the appropriate control antibody were also assessed in parallel cultures. (**K** and **L**) The anti-inflammatory effect of B cell depletion on myeloid cell responses, seen as reduced ratios of IL-12/IL-10 (K) and IL-6/IL-10 (L) after B cell depletion, persisted in macrophages isolated later from the same patients at a time that their B cells reconstituted. Data are from five different MS patients undergoing BCDT.

mechanisms involved in the reciprocal regulation of human pro- and anti-inflammatory B cell responses. Relatively little has been known about the mechanisms that induce proinflammatory B cell subsets, and the mechanisms that influence the balance between effector and regulatory B cells have not been elucidated. We demonstrate that the responses of human GM-CSF^+ B cells are context-dependent and regulated by exogenous signals, including specific cytokines that are integrated during B cell activation. We further report that pSTAT5 and particularly pSTAT6 substantially induce GM-CSF from naïve B cells. These results also indicate that pSTAT regulation of GM-CSF expression may be cell subset–dependent because recent studies have shown that T cell expression of GM-CSF is induced by phosphorylation of STAT4 and STAT5 (and decreased by phosphorylation of STAT3) (*26*, *27*). Although we find that human B cell GM-CSF production can also be controlled by STAT5, we noted that classic pSTAT5 inducers such as IL-2, IL-7, and IL-15 did not induce GM-CSF from B cells, indicating that pSTAT5 alone may be insufficient. Our data further indicate that IL-4 induction of pSTAT6 provides additional help for pSTAT5 to efficiently induce GM-CSF from B cells. We were particularly intrigued to discover that phosphorylation of STAT5 and STAT6, which substantially induces B cell expression of GM-CSF, reciprocally down-regulates THE responses of IL-10–expressing B cells. The discovery that particular signals can reciprocally regulate pro- and anti-inflammatory human B cell responses is of considerable interest because restoring an abnormal balance between these subsets may have important therapeutic implications for both autoimmune disease and host defenses. In this regard, we documented increased pSTAT6 levels together with abnormally increased ratios of GM-CSF/IL-10 cytokine secretion in B cells of untreated MS patients, whereas B cells that reconstituted in the patients after B cell depletion exhibited diminished pSTAT5 and pSTAT6 levels, as well as reversal of the previously abnormal GM-CSF/IL-10 ratio. These results are in keeping with our observation that the

(GM-CSF–dependent) diminished proinflammatory myeloid cell responses documented after B cell depletion persist even as new B cells reconstitute in these patients. Together, our results provide explanations for both the diminished MS disease activity observed with B cell removal and the apparent persistence of this benefit in patients even as their B cells reconstitute after depletion.

GM-CSF–expressing B cells have been described in mice as a B1a-derived subset named innate response activated (IRA) B cells (*17*). In several murine disease models, these IRA B cells are attributed to pathogenic roles (*31*), except in sepsis where they appear protective (*17*, *32*). Human GM-CSF–secreting B cells have been described as immunoglobulin M (IgM)–positive, GM-CSF$^+$ B cells that appear abnormally expanded in the spleen of patients with active cardiovascular disease, although their detailed characteristics and functional relevance have not been examined. We note several differences between murine IRA B cells and human GM-CSF–expressing B cells that we describe in detail here: First, whereas the mouse IRA B cells are derived from B1a lineage cells and have a surface phenotype similar to plasmablasts, the human GM-CSF–expressing B cells we describe belong to the B2 lineage and have the surface phenotype of memory B cells. Second, mouse IRA B cells are induced by innate stimuli such as lipopolysaccharide (LPS), whereas the human GM-CSF–expressing B cells are mainly induced by adaptive stimuli such as CD40L and T cell cytokines; in fact, we found that CpG exposure, as previously reported (*33*), induces IL-10–expressing B cells yet significantly diminished the human GM-CSF–expressing B cell responses (fig. S13). Third, mouse IRA B cells do not appear to produce other cytokines such as IL-6 or TNFα, whereas the human GM-CSF–expressing B cells are notable for being among the highest producers of both TNFα and IL-6. In both mice and humans, the GM-CSF–expressing B cells appear distinct from IL-10–expressing B10/regulatory B cells and are functionally able to enhance myeloid lineage cell responses, which may underlie their contributions to both pathogen-induced responses and autoimmune diseases.

There are a number of inherent limitations and challenges involved when working with human samples and particularly in studies of serial immune monitoring. Our studies in MS patients and controls focus on fresh or carefully cryopreserved peripheral blood cells, as opposed to lymphoid tissues or the diseased target organ, which reflects an ongoing constraint in our field. From a conceptual standpoint, our studies comparing post–B cell depletion samples to pre–B cell depletion samples focus on time points within 1 year of depletion. Whereas our results indicate that the GM-CSF–producing B cells do not reconstitute to pretreatment levels during this follow-up period of up to 1 year, most of the reconstituting B cells over this time frame are naïve (whereas the GM-CSF–expressing B cells evaluated ex vivo are largely memory B cells). Of interest in future work would be to serially monitor patients to establish whether the abnormalities in GM-CSF–expressing B cells are persistent in a subset of individual patients or fluctuate with disease activity. This could include examining patients at later time points after B cell depletion to assess whether reemergence of GM-CSF B cells will be associated with reemergence of disease activity in particular patients.

In conclusion, we identified human proinflammatory cytokine–producing effector B cells. The abnormal increases in GM-CSF$^+$ B cell frequencies and responses in patients with MS, their enhanced capacity to activate proinflammatory myeloid cells, and the observations from BCDT in patients all point to a pathogenic role for this effector B cell subset in MS and implicate a therapeutic mode of action of B cell depletion involving B cell/myeloid cell interaction. Our results also underscore the important functional heterogeneity that exists within the human B cell population and provide insights into the molecular mechanisms that can cross-regulate pro- versus anti-inflammatory B cell responses. Together, our findings contribute to the rationale for developing more selective strategies targeting distinct disease-implicated B cell subsets and/or restoring the balance between particular effector and regulatory B cells in patients with autoimmune disease such as MS.

MATERIALS AND METHODS

Study design

This is an experimental laboratory study performed with human peripheral blood samples. The study was designed to better define and understand the roles of proinflammatory cytokine–producing B cells in the pathogenesis of MS. Well-characterized patients with confirmed RRMS (table S1) were recruited at the MS Clinic of the Montreal Neurological Institute and Hospital (MNI/H). Untreated patients had never received immune-suppressive agents, and they received no immune-modulating treatments within at least 6 months of sampling, nor steroid treatment within at least 90 days. Age- and sex-matched control subjects (table S2) were recruited among demographically similar healthy volunteers. Samples were also examined from patients with MS undergoing BCDT (table S3). All subjects provided informed consent as approved by the MNI/H ethics review board. Study components were not predefined. The number of replicates for each experiment is indicated in Results and in the figure legends. Ex vivo comparisons of GM-CSF–producing B cells between MS and HC were performed blindly. All mechanistic studies using immune cells from healthy donors were performed without randomization or blinding.

Cell culture

Human peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation using Ficoll (GE Healthcare). CD19 and CD14 beads (Miltenyi Biotec) were used to positively select B cells and monocytes, respectively, according to the manufacturer's protocol. The purity of the cells was checked by flow cytometry after each isolation, and the typical purity for both B cells and monocytes is >98%. For the experiments that required further cell isolation, purified B cells were further sorted using FACSAria II (BD Biosciences). All cells were cultured in serum-free ex vivo medium (Gibco, Life Technologies). B cells were than plated in U-bottom 96-well plates at 3×10^5 per well in a total volume of 200 μl. M-CSF (20 ng/ml, R&D Systems) was used to induce macrophages from CD14$^+$ monocytes and cultured in flat-bottom 96-well plates at 1×10^5 per well in a total volume of 200 μl.

Reagents to stimulate B cell and functional blocking antibodies

The reagents to stimulate B cells included soluble CD40L (1 μg/ml, Enzo Life Sciences), goat anti-human IgM BCR cross-linking antibody (XAb) (10 μg/ml, Jackson ImmunoResearch), CpG DNA (1 μM; ODN2006, InvivoGen), Pam3CSK4 (1 μg/ml, InvivoGen), LPS (1 μg/ml; 0111:B4, Sigma-Aldrich), IL-4 (20 ng/ml, R&D Systems), interferon-γ (IFN-γ) (10 ng/ml, R&D Systems), IL-17 (1 ng/ml, R&D Systems), TGFβ (20 ng/ml, R&D Systems), IL-12 (20 ng/ml, R&D Systems), IL-21 (20 ng/ml, R&D Systems), and IL-13 (20 ng/ml, R&D Systems). Functional blocking antibody for IL-10 (JES3-9D7) and matched control antibody were purchased

from BioLegend and used at 1 μg/ml concentration. Functional blocking antibody for GM-CSF and matched control antibody were purchased from R&D Systems (ImmunoGen: human GM-CSF: Ala18-Glu144, 1 μg/ml).

Antibodies for flow cytometry

Antibodies to phenotype the effector B cells included CD1d (CD1d42), CD5 (UCHT2), CD11c (B-ly6), CD14 (MφP9), CD20 (L27), CD20 (2H7), CD24 (ML5), CD25 (M-A251), CD27 (L128), CD38 (HB7), CD40 (5C3), CD43 (1G10), CD69 (L78), CD80 (BB1), CD83 (HB15e), CD86 [2331(Fun-1)], CD196 (CCR6, 11A9), CD197 (CCR7, 150503), and HLA-DR (G46-6) (BD Biosciences); CD267 (TACI, 11H3) and CD268 (BAFF-R, 8A7) (eBioscience); CD269 (BCMA, FAB193P) (R&D Systems). Antibodies to detect intracellular cytokines included IL-4 (8D4-8), IL-6 (MQ2-6A3), IL-9 (MH9A3), IL-10 (JEF3-19F1), IL-17A (SCPL1362), IFN-γ (B27), TNFα (MAb11), and GM-CSF (BVD2-21C11) (BD Biosciences).

Intracellular cytokine staining

PMA (20 ng/ml, Sigma-Aldrich), ionomycin (500 ng/ml, Sigma-Aldrich), and GolgiStop (Monensin, BD Biosciences) were added 5 hours before staining. Cell surface staining was performed before intracellular cytokine staining (ICS). After washing two times, fixation/permeabilization buffer (BD Biosciences) was added to fix the cells. Cells were then washed twice with ICS washing buffer (BD Biosciences). ICS antibodies were added to the cell suspension. After washing two times with ICS washing buffer, cells were then analyzed by FACSCalibur (BD Biosciences).

Enzyme-linked immunosorbent assay

Purified B cells were stimulated for 48 hours, and the supernatant was collected afterward. Cytokine (GM-CSF, TNFα, IL-6, and IL-10) level in the cell-cultured supernatant was measured with OptEIA ELISA kit (BD Biosciences) according to the manufacturer's protocol. Briefly, ELISA plates were coated with capture antibody at least 12 hours ahead. After 1-hour blocking with blocking buffer [10% fetal calf serum and phosphate-buffered saline (PBS)], samples were added to the plate and incubated for 2 hours at room temperature. Detection antibody was then added and incubated for 1 hour at room temperature. The color of the plate was developed by trimethylboron (BD Biosciences), and the reaction was stopped by 0.005 M H_2SO_4. Finally, the plate was read by a Bio-Rad microplate reader (Model 550, Bio-Rad). The plate was washed with ELISA washing buffer (0.05% Tween 20 and PBS) between each step.

Statistic analysis

GraphPad Prism 6 was used for all statistic analysis. Student's unpaired *t* test was used for statistical comparisons between two groups. One-way ANOVA was used for statistical comparisons among more than two groups. All statistical tests have been indicated in the figure legends. *P* values of ≤0.05 were considered significant.

SUPPLEMENTARY MATERIALS

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Fig. S1. Gating strategy and isotype controls.
Fig. S2. Enhanced up-regulation of CD80 and CD86 by GM-CSF$^+$ B cells.
Fig. S3. Ex vivo surface phenotype of GM-CSF$^+$ B cells.
Fig. S4. Regulation of B cell GM-CSF production by cytokines.
Fig. S5. Induction of B cell GM-CSF by IL-4 through IL-4Rα (but not CD132).
Fig. S6. Regulation of memory B cell GM-CSF and IL-10 production by STAT5 and STAT6.
Fig. S7. Inhibition of STATs did not alter B cell survival.
Fig. S8. Enhanced induction of STAT6 phosphorylation in B cells of MS patients.
Fig. S9. GM-CSF$^+$ B cells induce IL-1β but not TNFα production from myeloid cells.
Fig. S10. Suppression of myeloid cell IL-10 by IL-6 secreted from GM-CSF$^+$ B cells.
Fig. S11. Similar capacity of myeloid cells from HCs and MS patients to be regulated by GM-CSF$^+$ B cell soluble products from HCs.
Fig. S12. Percentage of naïve and memory B cell subsets in reconstituted B cells.
Fig. S13. CpG decreased the frequency of GM-CSF$^+$ B cells but increased that of IL-10$^+$ B cells.
Table S1. Demography of MS patients.
Table S2. Information of HCs.
Table S3. Information of anti-CD20–treated MS patients.

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Proinflammatory GM-CSF–producing B cells in multiple sclerosis and B cell depletion therapy

Rui Li, Ayman Rezk, Yusei Miyazaki, Ellen Hilgenberg, Hanane Touil, Ping Shen, Craig S. Moore, Laure Michel, Faisal Althekair, Sathy Rajasekharan, Jennifer L. Gommerman, Alexandre Prat, Simon Fillatreau, Amit Bar-Or and on behalf of the Canadian B cells in MS Team (October 21, 2015)

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Editor's Summary

Eclipsing multiple sclerosis

B cell depletion therapy (BCDT) has been shown to limit inflammation in some cases of multiple sclerosis (MS); however, how exactly BCDT works has remained unclear. Now, Li *et al*. report that a subset of B cells that produce the cytokine granulocyte macrophage–colony stimulating factor (GM-CSF) contributes to MS pathogenesis. These cells are more frequent in MS patients than in healthy controls and increase proinflammatory myeloid responses. Moreover, production of these cells counterbalances the generation of interleukin-10 (IL-10)–producing regulatory B cells, which are thought to be protective in disease. After BCDT, the ratio of GM-CSF/IL-10–producing B cells is normalized, suggesting that BCDT may work in part by decreasing the number of pathogenic GM-CSF –producing B cells.