Distinct Effector Cytokine Profiles of Memory and Naive Human B Cell Subsets and Implication in Multiple Sclerosis

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Although recent animal studies have fuelled growing interest in Ab-independent functions of B cells, relatively little is known about how human B cells and their subsets may contribute to the regulation of immune responses in either health or disease. In this study, we first confirm that effector cytokine production by normal human B cells is context dependent and demonstrate that this involves the reciprocal regulation of proinflammatory and anti-inflammatory cytokines. We further report that this cytokine network is dysregulated in patients with the autoimmune disease multiple sclerosis, whose B cells exhibit a decreased average production of the down-regulatory cytokine IL-10. Treatment with the approved chemotherapeutic agent mitoxantrone reciprocally modulated B cell proinflammatory and anti-inflammatory cytokines, establishing that the B cell cytokine network can be targeted in vivo. Prospective studies of human B cells reconstituting following in vivo depletion suggested that different B cell subsets produced distinct effector cytokines. We confirmed in normal human B cell subsets that IL-10 is produced almost exclusively by naive B cells while the proinflammatory cytokines lymphotoxin and TNF-α are largely produced by memory B cells. These results point to an in vivo switch in the cytokine "program" of human B cells transitioning from the naive pool to the memory pool. We propose a model that ascribes distinct and proactive roles to memory and naive human B cell subsets in the regulation of memory immune responses and in autoimmunity. Our findings are of particular relevance at a time when B cell directed therapies are being applied to clinical trials of several autoimmune diseases. ***The Journal of Immunology,*** **2007, 178: 6092–6099.**

Studies of B lymphocytes and their roles in the normal immune response have traditionally focused on their potential to differentiate into Ab-producing cells, thereby contributing to Ag-specific humoral immunity. Similarly, studies of how defective B cell responses may contribute to pathologic states such as autoimmune disease have often considered the putative roles of self-reactive Abs. In either context, B cells are often viewed as relatively passive recipients of T cell help. However, in recent years, animal studies have generated a growing interest in the contribution of Ab-independent functions of B cells to immune responses (reviewed in Refs. 1 and 2).

Multiple reports from animal models of infection and autoimmune disease have implicated activated B cells as effective APCs (1–13). B cells may also contribute to immune responses through the secretion of effector cytokines (14, 15), and emerging animal studies have shown that the selective manipulation of B cell cytokines can impact on the expression of autoimmune disease models (16–18). The notion that abnormalities in B cell cytokines may contribute to pathologic states raises the intriguing corollary that B cell cytokines could play important roles in the regulation of normal immune responses. Relatively little is known, however, about how B cell cytokines may contribute to human autoimmune disease (13), and the potential role of B cell effector cytokines in the regulation of normal immune responses is largely unexplored.

In this study we applied a recently established paradigm of ex vivo B cell stimulation optimized to match the current prediction of the anticipated outcome of early B cell-T cell interaction, (19) to examine the regulation of human effector B cell cytokines in normal individuals and in patients with autoimmune disease. We first confirm that effector cytokine production by normal human B cells is context-dependent and demonstrate that this involves reciprocal regulation of proinflammatory and anti-inflammatory cytokines. We next show that this cytokine network is dysregulated in patients with the autoimmune disease multiple sclerosis (MS),[3] whose B cells exhibit a significantly diminished capacity to secrete the immune regulatory cytokine IL-10. Treatment with mitoxantrone, an approved chemotherapy for aggressive MS (20, 21), resulted in increased B cell production of IL-10 and decreased production of the proinflammatory cytokines TNF-α and lymphotoxin (LT). Prospective studies of reconstituting human B cells following selective and nonselective depletion allowed us to further probe the in vivo regulation of B cell effector cytokines. Together, our findings suggested that naive and memory B cell subsets preferentially produce distinct effector cytokines. We confirmed this in normal human B cell subsets, with the novel observation that IL-10 is produced almost exclusively by naive B cells while the proinflammatory cytokines LT and TNF-α are largely produced by memory B cells. Based on our findings from both normal B cells and B cells isolated from untreated and treated

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[3] Abbreviations used in this paper: MS, multiple sclerosis; CD40L, CD40 ligand; EAE, experimental autoimmune encephalitis; GC, germinal center; LT, lymphotoxin; NMO, neuromyelitis optica; RRMS, relapsing remitting MS; SPMS, secondary progressive MS.

patients with autoimmune disease, we propose a model that ascribes distinct and proactive roles to memory and naive human B cell subsets in the regulation of memory immune responses and autoimmunity. These results are discussed in the context of the emerging application of B cell-directed therapies in clinical trials of MS and other human autoimmune diseases.

Materials and Methods

Patients and controls

Subject recruitment followed institutional review board approval and an informed consent process. For comparisons between MS and normal B cells, patients were recruited from the outpatient MS clinic at the Montreal Neurological Hospital (Montreal, Canada) and age- and gender-matched healthy controls were recruited from university staff personnel. We first studied 18 patients with clinically definite (Poser et al; Ref. 22) relapsing remitting MS (RRMS) ($n = 14$; 5 males, mean age 33) and secondary progressive MS (SPMS) ($n = 4$; 2 males, mean age 47). In a more comprehensive study, we further compared the responses of B cells isolated from additional patients with RRMS ($n = 24$; 6 males, mean age 29) and SPMS ($n = 15$; 5 males, mean age 42) with those from matched healthy controls. None of the patients had received disease-modifying therapies or immune suppressants and none were exposed to steroids for at least 8 wk before sampling. Because of the importance of avoiding recent immune therapy, the RRMS cohort mostly comprised newly diagnosed patients who were not yet started on therapy. The RRMS patients therefore tended to be younger as noted above and have shorter average disease duration (4.6 years, range 1.2–9.7 years vs 14.7 years, range 9.4–21 years) and lower median EDSS scores (1.8 vs 3.6) when compared with the patients in the SPMS cohort. For studies evaluating mitoxantrone effects, patients with active SPMS ($n = 8$) or RRMS ($n = 4$) were followed prospectively with sampling before and following a standard treatment protocol (23). The effects of mitoxantrone on B cell responses were similar in patients with SPMS and RRMS, and these results were pooled. For our prospective studies tracking the responses of reconstituting B cell subpopulations, we sampled patients at baseline and then serially, following either therapy with rituximab (an anti-CD20 mAb known to selectively and efficiently deplete circulating B cells) (24–28) or immune-ablative chemotherapy and autologous $CD34^+$ stem cell therapy (as part of the Canadian Collaborative MS Bone Marrow Transplant Study).

Isolation of B Cells and B cell subsets

Venous blood was drawn into EDTA-containing tubes and PBMCs were freshly separated using standard density centrifugation on Ficoll-Paque (Pharmacia Biotech). $CD19^+$ B cells were purified using magnetic cell sorting (MACS; Miltenyi Biotec), as previously described (29). Preparations were typically >98% $CD19^+$ as confirmed by flow cytometry. B cells were washed twice to remove EDTA in the separation buffers, resuspended in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/L L-glutamine (all from Sigma-Aldrich) and plated at 1.5×10^5 cells per 200 μl in U-bottom 96-well plates. For isolation of naive and memory B cell subsets, freshly isolated B cells were labeled after MACS separation with anti-CD27-PE (5 μl/10^6 cells in PBS with 5% FCS; BD Pharmingen) for 15 min at 4°C. Excess Ab was removed by washing and cells were resuspended in PBS before sorting using a BD FACSort machine. $CD27^+$ cells represented 12–40% of the total B cell population with a clear separation between the two populations. Following sorting, cells were washed in medium before culture in conditions identical with those described above for unsorted cells. In preliminary control experiments (data not shown), parallel aliquots of B cells without sorting were cultured following labeling with anti-CD27-PE. This was done to assess the potential effect on cytokine production of CD27 blockade by the labeling Ab on homotypic CD27/CD70 interactions (30). The profile of cytokines produced was identical with that of unlabelled cells.

B Cell activation and cytokine responses

We used a recently established ex vivo system of human B cell activation that underscores the importance of the strength, mode, and sequence of stimulation on human B cell cytokine production (19). In this system, optimized stimulation conditions were identified that reproduced the anticipated in vivo effects of short-term BCR- and CD40-mediated signaling in terms of B cell proliferation, Fas expression, and survival, thereby providing a model for the earliest stages of human B/T interaction. Freshly isolated B cells are stimulated in parallel by either sequential BCR engagement followed by CD40 signaling (as a mimic for B cell encountering its Ag and then receiving T cell help) or by CD40 signaling alone (as a mimic of a B cell receiving bystander T cell help in absence of specific B cell Ag recognition). For *trans*-stimulation through CD40, B cells were cocultured with a preadhered monolayer of CD154 (CD40 ligand (CD40L)) transfected L cells (donated by Y. J. Liu, DNAX Research Institute of Molecular and Cellular Biology) at an L cell to B cell ratio of 1:15. For the dual stimulation paradigm, cells were incubated with polyclonal goat anti-human IgG and IgM (Jackson ImmunoResearch Laboratories) at 0.5 μg/ml before being transferred with their medium to a preadhered monolayer of CD40L-transfected L cells. In experiments with B cell subsets, we also assessed the effects of isolated BCR engagement by using the above-mentioned anti-human IgG/IgM Ab as previously described (19). Stimulated cells were then cultured for 48 h at 37°C in 5% CO_2 in a humidified atmosphere before collecting supernatants. Aliquots of the supernatant were removed and frozen at −70°C until batched analysis for cytokines by standard ELISA, running duplicate samples from replicate wells. IL-10 and TNF-α were assayed using OptEIA ELISA kits (BD Pharmingen) following the manufacturer's protocol. LT was assayed by ELISA as previously described (19). We previously demonstrated an excellent correlation between cytokine protein secretion by ELISA and RNA expression by real time PCR (19). The ELISA approach proved capable of reproducibly capturing modulation (both induction and inhibition) of the human cytokine signals. For example, the range of IL-10 secreted by human B cells in our assays (generally between 0 and 800 pg/ml) was reliably detected and well within the linear portion of the ELISA standard curve. Proliferation was assessed by 18-h tritiated thymidine uptake, using a standard beta scintillation counter.

Flow cytometric analysis of cell surface markers and assessment of cell viability

The following directly conjugated Abs were used for purity confirmation following each B cell and B cell subset separation (all from BD Pharmingen): anti-CD19-Cy, anti-CD3-FITC, anti-CD14-PE, and anti-CD27-FITC. The stability of CD40L expression on transfected L-cells was confirmed using anti-CD154-FITC. Cell viability and apoptosis were assessed using a commercial kit (BD Pharmingen) incorporating annexin V/propidium iodide. All staining was conducted in PBS with 2% FCS. Whole blood staining was conducted using the standard BD protocol and the same CD19 and CD27 Abs noted above, as previously described (19). Cells were analyzed without delay on a BD FACScan flow cytometer using CellQuest software.

Results

Reciprocal regulation of human B cell effector cytokines is context dependent

We first confirmed the recent report that the context of activation can define the profile of effector cytokines secreted by normal human B cells (19). Circulating $CD19^+$ B cells were freshly isolated from normal volunteers and activated ex vivo either through sequential BCR cross-linking followed by CD40 engagement (as a proxy for B cells encountering their Ag and subsequently receiving T cell help) or through CD40 alone (as a mimic for "bystander" activation of B cells receiving help from activated T cells in the absence of their specific B cell Ags). In this ex vivo paradigm, we observed reciprocal regulation of B cell secretion of LT and IL-10 (Fig. 1). B cells undergoing "dual" stimulation through BCR and CD40 engagement secreted high levels of LT and relatively low levels of IL-10. LT is a proinflammatory cytokine known to be critical for normal germinal centre (GC) formation (31) and would thus serve to promote the immune response. In contrast, B cells stimulated through the engagement of CD40 alone secreted significantly lower levels of LT ($n = 22$; $p < 0.0001$) but substantially higher levels of the down-regulatory cytokine IL-10 ($p = 0.0064$).

Abnormal B cell effector cytokine network in patients with multiple sclerosis

The context-dependent reciprocal profile of B cell effector cytokines suggested to us that B cells may contribute to the regulation

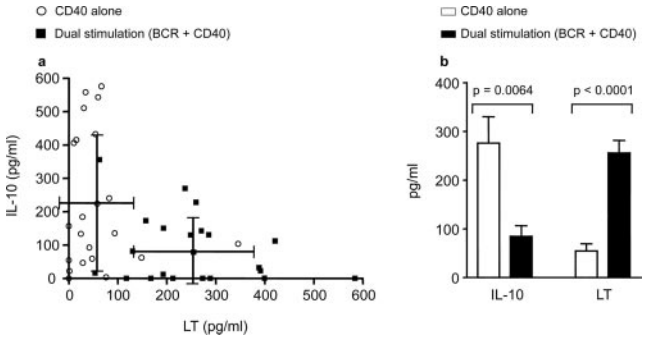

FIGURE 1. Reciprocal regulation of human B cell effector cytokines is context dependent. Freshly isolated normal human $CD19^+$ B cells were stimulated using a recently established ex vivo assay optimized to reproduce the anticipated in vivo effects of short-term BCR- and CD40-mediated signaling (see *Materials and Methods* and Ref. 19). B cells were activated in parallel by either sequential BCR engagement followed by CD40 signaling (dual stimulation, as a mimic for B cells encountering Ag and then receiving T cell help) or by CD40 signaling alone (as a mimic of a B cell receiving bystander T cell help in the absence of specific B cell Ag recognition). Culture supernatants were collected after 48 h of CD40 signaling and cytokine levels were subsequently quantified by ELISA. *a*, Data for individual patients shows the wide range of normal human B cell cytokine responses. The intersecting horizontal and vertical bars represent the mean and SD values for IL-10 production (*left*) and LT production (*right*) under the two modes of stimulation indicated. *b*, Summary of the distinct cytokine profiles. Dual stimulation resulted in relatively low levels of IL-10 but induced high levels of LT, a cytokine known to be critical in normal GC development. In contrast, bystander activation resulted in significantly less LT production ($n = 22$, $p < 0.0001$) while inducing significantly higher levels of IL-10 ($p = 0.0064$).

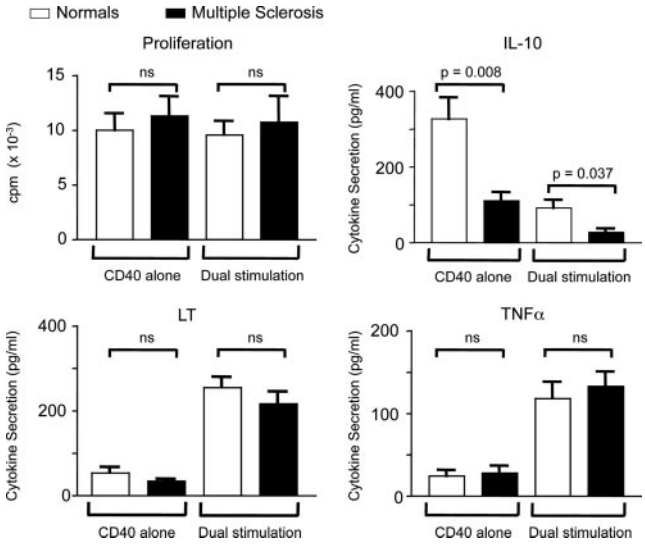

FIGURE 2. Abnormal B cell IL-10 production in patients with MS compared with controls. Using the activation paradigm described above, ex vivo B cell proliferation and cytokine responses were compared in parallel between patients with MS ($n = 18$) and age- and sex-matched normal controls. Similar proliferation was observed for B cells from MS patients and normal controls under both "dual stimulation" and "CD40-alone" conditions. Dual stimulation induced MS patient B cells to secrete similarly high levels of the proinflammatory cytokines LT and TNF-α as was seen for normal B cells. However, B cells from patients with MS exhibited a relative deficiency in their production of IL-10 in the contexts of both dual stimulation ($p = 0.037$) and, even more significantly, CD40 alone activation ($p = 0.008$).

of immune responses through differential cytokine production. This concept is supported by recent observations from animal models of several autoimmune diseases including experimental autoimmune encephalomyelitis (EAE), where manipulation of B cell cytokines impacted disease severity (16). We therefore wished to examine whether defects in the regulation of the human B cell effector cytokine network are implicated in the autoimmune disease multiple sclerosis. Using the established activation paradigm, we compared in parallel the proliferation and effector cytokine responses of ex vivo B cells obtained from patients with MS and from age and sex-matched normal controls (Fig. 2; $n = 18$). The proliferative responses of MS patient and normal B cells were no different, whether stimulated through CD40 alone or through dual BCR and CD40 engagement. B cells from MS patients and normal controls exhibited the same capacity to produce high levels of LT as well as the additional proinflammatory cytokine TNF-α, (31) under the dual stimulation paradigm. In contrast, compared with normal B cells, MS patient B cells were relatively deficient in their capacity to produce IL-10 under both dual stimulation and, more significantly, under the bystander activation paradigm ($p = 0.037$ and $p = 0.008$, respectively). This abnormality of B cell cytokine production in patients with MS is of particular interest because the depletion of IL-10 selectively from B cells resulted in more severe phenotypes of EAE and experimental colitis as well as an increased susceptibility to experimental arthritis (16–18). We next considered whether the observed abnormality in the secretion of IL-10 from B cells of MS patients was shared by patients with RRMS and SPMS. Fig. 3 depicts the levels of IL-10 secreted by B cells freshly isolated from a series of untreated patients with RRMS ($n = 24$) and SPMS ($n = 15$) as well as age- and gender-matched controls (see *Materials and Methods*). There were no significant differences in the IL-10 production by B cells from patients with the different clinical subtypes under either mode of stimulation. We confirmed the finding of significantly decreased levels of secreted B cell IL-10 in the pooled MS patient cohort ($n = 39$) compared with the matched healthy controls ($p = 0.028$ in the dual stimulation condition and $p = 0.006$ in the bystander activation paradigm).

Treatment with mitoxantrone results in reciprocal modulation of the effector B cell cytokine network in MS patients

We next asked whether the abnormal cytokine profile seen in patients with MS could be targeted therapeutically. Mitoxantrone, recently approved for the therapy of MS, is a relatively nonselective anti-metabolite chemotherapeutic drug thought to impact both T cell and B cell responses (20, 21). We applied our ex vivo assay to B cells freshly isolated from a sequential cohort of MS patients before and during standard mitoxantrone treatment (23). As shown in Fig. 4, in vivo therapy with mitoxantrone resulted in reciprocal regulation of the B cell cytokine network. Although proliferative responses were not significantly altered (data not shown), therapy significantly decreased B cell secretion of the proinflammatory cytokine LT (Fig. 4*a*; $n = 12$; $p = 0.008$). TNF-α levels were also significantly reduced under dual stimulation ($p = 0.006$; data not shown). In contrast, the production of the anti-inflammatory cytokine IL-10 was significantly increased in both the bystander and dual stimulation paradigms (Fig. 4*b*; $p = 0.007$ and $p = 0.004$, respectively).

Although one possible explanation for this reciprocal effect of mitoxantrone was that the drug modulated the cytokine expression profile of individual B cells, we favored the alternate hypothesis that mitoxantrone induced shifts in the subpopulations of circulating B cells. The latter hypothesis raised the intriguing corollary that different subsets of human B cells may be responsible for the

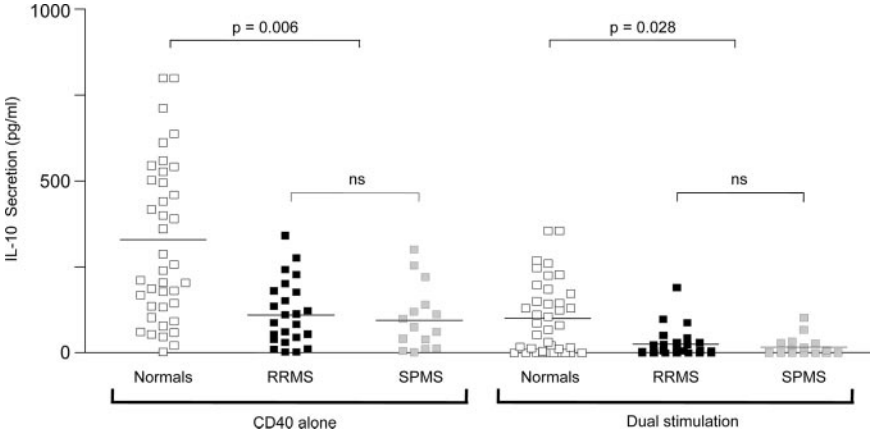

FIGURE 3. Similar profiles of IL-10 production by B cells from patients with relapsing remitting and secondary progressive MS. IL-10 production was evaluated in B cells activated ex vivo as described above following isolation from untreated patients with RRMS ($n = 24$) and SPMS ($n = 15$) as well as from age- and gender-matched healthy controls (Normals). No significant differences were observed in the average levels of IL-10 production by B cells from patients with the two different clinical subtypes under either mode of stimulation. Decreased production of B cell IL-10 was seen in the pooled patient cohorts ($n = 39$) compared with the matched healthy controls ($p = 0.028$ in the dual stimulation condition and $p = 0.006$ in the bystander activation paradigm).

production of distinct proinflammatory and anti-inflammatory cytokines. Using flow cytometry of whole blood samples prospectively obtained from the same patients before and during mitoxantrone therapy, we found that treatment with mitoxantrone resulted in a significantly diminished proportion of circulating $CD19^+$ B cells expressing the memory marker CD27 (Fig. 4*c*, a representative patient; Fig. 4*d*, a pooled cohort). At the time that blood was obtained for B cell cytokine measurements in the mitoxantrone-treated patients (week 4), the average percentage of circulating $CD19^+CD27^+$ B cells dropped to 14% from a pretreatment level of ~30% (Fig. 4*d*; $n = 9$, $p = 0.004$). An evaluation of absolute B cell counts (data not shown) identified two phases to the changes in circulating B cell phenotypes: an early effect of mitoxantrone with a rapid decrease over 24 h in the numbers of both $CD27^-$ and $CD27^+$ B cells and a more delayed further decrease particularly of $CD27^+$ B cells over the subsequent weeks. These results extend recent reports that higher concentrations of mitoxantrone (as achieved soon after i.v. infusion) can mediate immune cell necrosis, whereas at lower concentrations mitoxantrone can trigger immune cell apoptosis (20, 21). Together, these findings support the notion that mitoxantrone induces the preferential death of $CD19^+CD27^+$ memory B cells, resulting in a relatively higher proportion of circulating $CD19^+CD27^-$ naive B cells. However, we considered it premature to conclude that mitoxantrone treatment resulted in the reciprocal modulation of B cell cytokines by altering the balance of memory vs naive circulating B cells because one cannot exclude the possibility that the decreased proportion of $CD27^+$ B cells might reflect a down-regulation of CD27 on the surface of memory B

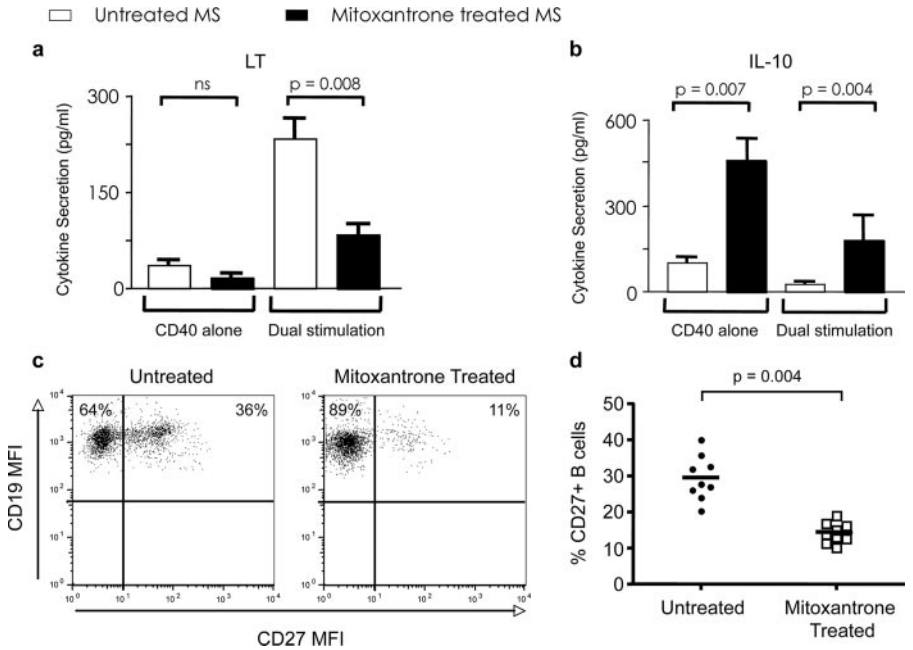

FIGURE 4. Mitoxantrone therapy reciprocally regulates B cell cytokine production in association with decreased frequencies of circulating $CD2^+$ B cells. Ex vivo B cell responses were studied prospectively in a sequential cohort of patients with MS ($n = 12$) before and during treatment with a standard regimen of mitoxantrone, an approved chemotherapeutic agent for active MS. In vivo therapy resulted in reciprocal regulation of the B cell cytokine network. *a*, Although proliferative responses were not significantly altered by treatment (data not shown), B cell production of the proinflammatory cytokine LT was significantly reduced under the dual stimulation paradigm ($n = 12$; $p = 0.008$). TNF-α levels were also significantly reduced under dual stimulation ($p = 0.006$; data not shown). *b*, In contrast, the production of the anti-inflammatory cytokine IL-10 was significantly increased in both the bystander and dual stimulation paradigms ($p = 0.007$ and $p = 0.004$, respectively). *c* and *d*, Flow cytometry (FACS) was used to assess the frequency of CD27-expressing cells within circulating $CD19^+$ B cells before (Untreated) and 4 wk after the initiation of mitoxantrone (Mitoxantrone Treated). Therapy significantly reduced the proportion of CD27-expressing B cells as shown for a representative patient (*c*) and as pooled data for the cohort (*d*) ($p = 0.004$). MFI, Mean fluorescence intensity.

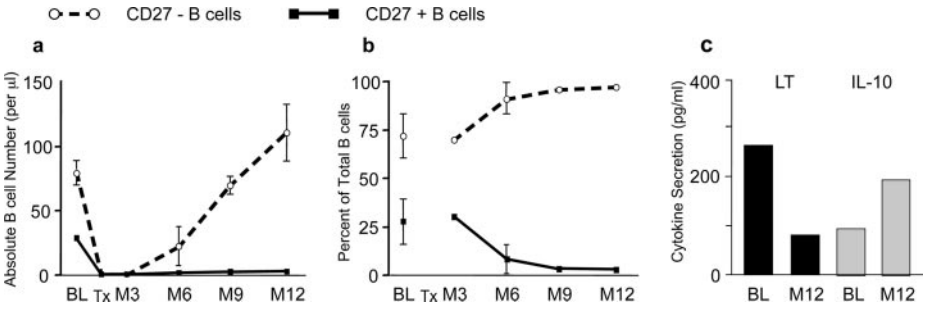

FIGURE 5. B cell cytokine profiles following selective in vivo depletion and reconstitution. *a* and *b*, Whole blood samples for FACS analysis of B cell subsets and freshly isolated $CD19^+$ B cells for activation assays were obtained prospectively from patients with NMO before treatment (Tx) with B cell-depleting rituximab (anti-CD20 Ab), and during 12 mo of B cell reconstitution. Absolute counts of $CD27^-$ and $CD27^+$ B cells (*a*), and the percentages of these subsets are shown (*b*) starting at baseline (BL). No B cells were detectable by FACS immediately after depletion; therefore, the percentages of B cell subsets could not be assessed at this time. *c*, At month 12 (M12) of reconstitution, the $CD27^-$ B cell subpopulation comprised >95% of the total $CD19^+$ B cell population. Using the established B cell activation paradigm (as described above), the B cell cytokine profile of $CD19^+$ B cells was compared between baseline (BL) and month 12 (M12) of reconstitution (representative patient). The decreased proportion of $CD27^+$ B cells and concomitant increase in $CD27^-$ B cells observed at M12 was associated with decreased B cell production of LT (and TNF-α, not shown), in the face of increased B cell production of IL-10.

cells rather than the actual loss of memory B cells. To better address this question, we sought an in vivo human paradigm where shifts in the proportion of B cell memory and naive subsets, as well as their effector cytokine networks, could be examined in parallel.

Altered cytokine profiles of human B cells following selective B cell depletion

We took advantage of an opportunity to prospectively track in vivo changes of B cell cytokine profiles in the context of human B cell reconstitution following selective B cell depletion. Rituximab, a monoclonal anti-CD20 Ab, has been shown to efficiently deplete circulating B cells in patients with lymphoma and is currently under investigation as a potential therapy for several autoimmune diseases including CNS inflammatory diseases such as MS and neuromyelitis optica (NMO or Devic's disease) (24–28). We were able to study the effects of rituximab in a small open label study of patients with NMO. Absolute counts (Fig. 5*a*) and percentages (Fig. 5*b*) of circulating CD19+CD27- B cells and CD19+CD27+ B cells were analyzed at baseline and at 3-mo intervals for 12 mo following selective B cell depletion (months 3, 6, 9, and 12). Cytokine production by stimulated ex vivo B cells was compared between baseline and month 12 samples (Fig. 5*c*, representative patient). At baseline, the naive CD27- B cell subset comprised an average of 73% of the total $CD19^+$ B cell population (Fig. 5*b*). As expected, rituximab treatment depleted the great majority of circulating B cells (Fig. 5*a*; Tx). Re-emergence of B cells was noted by month 6 following depletion in all patients, consistent with prior reports in other cohorts (24–28), and the great majority of these reconstituting B cells were of the naive $CD27^-$ phenotype. An examination of the profiles of B cell cytokines at baseline and 12 mo after the selective B cell depletion (Fig. 5*c*) revealed that newly developed B cells secreted higher levels of IL-10 and decreased levels of LT. We obtained similar reciprocal changes in B cell cytokine production when we prospectively followed the responses of reconstituting B cells in a cohort of MS patients treated with nonselective chemoablation and autologous $CD34^+$ stem cell therapy as part of the Canadian Collaborative Bone Marrow Transplant Study (data not shown). These studies of nonselective and selective B cell reconstitution point to an in vivo switch in the cytokine program of human B cells, from their development as naive B cells through their transition into memory B cells. Our findings further support the notion that newly developing naive $CD27^-$ B cells produce IL-10, while the LT is preferentially produced by the memory $CD27^+$ B cells. We therefore decided to revisit the fundamental paradigm of reciprocal regulation of human B cell effector cytokines in B cell subsets isolated from normal individuals.

Memory and naive human B cells express distinct profiles of effector cytokines

Freshly isolated $CD19^+$ B cells from normal volunteers were further sorted into $CD27^-$ naive and $CD27^+$ memory subsets, which were then activated by BCR cross-linking alone, CD40 engagement alone, or dual stimulation as described above. The isolated B cell subsets produced essentially no LT or TNF-α when stimulated with BCR cross-linking alone (data not shown) in keeping with previously published results using whole $CD19^+$ cells (19). Similarly, the isolated subsets produced no IL-10 when stimulated with the anti-BCR Ab alone at the concentration routinely used in our system. We did find that when using the anti-BCR Ab at very high concentrations (which may be more in keeping with T cell-independent activation), small amounts of B cell IL-10 could be induced from the naive B cell subset (data not shown) as we had previously observed in the context of whole $CD19^+$ B cell stimulation (19). When comparing the effects of the "bystander" vs "dual" stimulation' paradigms (Fig. 6) on the responses of naive and memory B cell subsets, we found no significant differences in B cell proliferative responses yet observed striking differences in the cytokine production profiles of the different subsets. With CD40 stimulation alone, neither memory nor naive B cells produced significant amounts of LT or TNF-α in keeping with the results seen in unsorted $CD19^+$ cells. There was, however, a marked difference in the production of the down-regulatory cytokine IL-10 ($n = 16$; $p = 0.004$), establishing that the IL-10 previously seen in this "bystander" activation setting was almost exclusively produced by the naive $CD27^-$ B cell subset. Upon dual stimulation, IL-10 production by the naive B cells was significantly abrogated and was essentially absent from memory B cells. However, markedly different LT and TNF-α responses were observed from naive and memory B cells ($p = 0.001$ for both), establishing that the majority of these proinflammatory cytokines were secreted from the memory B cell subset when stimulated by both BCR and CD40 engagement. We considered whether the reduced production of IL-10 by B cells from MS patients might have reflected a lower proportion of circulating $CD27^-$ naive B cells. However, using whole

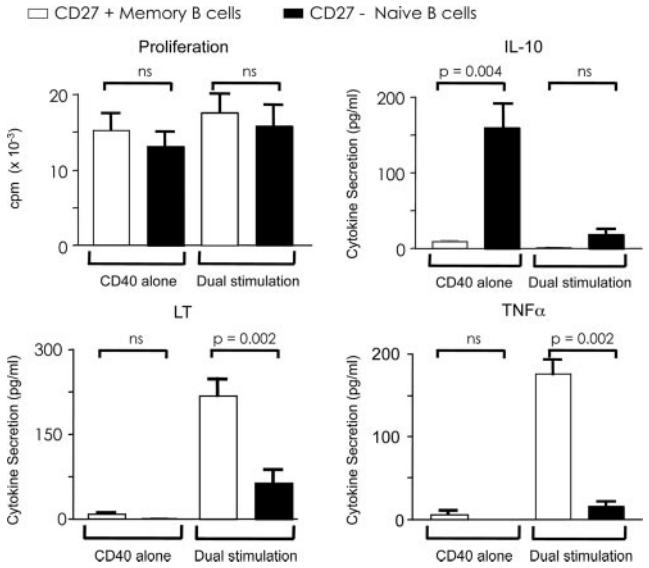

FIGURE 6. Memory and naive human B cells express distinct patterns of effector cytokines. Freshly isolated CD19 B cells from normal volunteers ($n = 16$) were further sorted into naive $CD27^-$ and memory $CD27^+$ subsets using flow cytometry. Naive and memory subsets were then activated in parallel by either CD40 signaling alone or by dual stimulation (as described above). Although the proliferative responses of naive and memory B cells were similar, these subsets produced distinct profiles of cytokines that were dependent on the mode of activation. Memory B cells activated sequentially by BCR engagement followed by CD40-mediated signaling, secreted high levels of the proinflammatory cytokines LT and TNF-α and essentially none of the regulatory cytokine IL-10, an overall cytokine profile that would be expected to efficiently promote a desired memory immune response. In contrast, naive B cells produced very limited amounts of the proinflammatory cytokines LT and TNF-α ($p = 0.002$ for both) under the identical dual stimulation condition. In the absence of Ag engagement, memory B cells stimulated by CD40 signaling alone failed to produce effector cytokines. In contrast, naive B cells stimulated by CD40 signaling alone were induced to secrete high levels of the regulatory cytokine IL-10 ($p = 0.004$) and essentially none of the proinflammatory cytokines LT and TNF-α, an overall cytokine profile that would be expected to acquiesce undesired immune responses.

blood flow cytometry we found similar proportions of naive $CD27^-$B cells in patients with MS and normal controls ($28.2 \pm 14.5\%$ vs $29.7 \pm 13.3\%$, respectively; $n = 36$, $p = 0.735$).

Discussion

The early interaction between T cells and B cells is known to be pivotal in regulating adaptive immune responses relevant to both health and disease (32). Studies of this process have considered how distinct T cell subsets and T cell cytokines can impact the differentiation of B cells and subsequent Ab production. However, relatively little attention has been paid to the potential contribution of B cell cytokines during the early stages of encounter with the T cell. Our findings confirm that the mode of stimulation critically determines the profile of cytokine secretion by human B cells and highlight the potential for these cells to contribute in vivo with either proinflammatory or anti-inflammatory cytokine responses, depending on the context of their activation. By examining in parallel the responses of adult B cell subsets, we discovered that the production of different effector cytokine profiles is a fundamental characteristic distinguishing naive ($CD27^-$) vs memory ($CD27^+$) human B cells and is dependent on the mode of stimulation. Based on these findings, we attribute distinct and context-dependent roles to memory and naive B cell subsets in the regulation of normal immune responses.

As part of this paradigm, we suggest that normal human B cells can contribute to the maintenance of tolerance thorough the production of IL-10. This concept is supported by our novel observation that B cells from MS patients exhibit a relative deficiency in their capacity to produce this down-regulatory cytokine, a finding that also extends results from recent animal model studies reporting that IL-10 produced by B cells can protect against the development of autoimmune arthritis (18) and promote disease resolution in experimental colitis and EAE (16, 17). Of note, IL-10 is also an established growth factor for B cells (33), and B cells isolated from patients with systemic lupus erythematosus and possibly rheumatoid arthritis and Sjogren's syndrome were found to produce more IL-10 than normal B cells (34, 35). This points to the multiple roles and complex biology of IL-10, and it is interesting to speculate that hyperactive B cell production of IL-10 may contribute to systemic lupus erythematosus pathogenesis whereas a relative deficiency of B cell IL-10 may be associated with inadequate immune regulation in the context of other autoimmune diseases including MS. It is of interest that in MS recent reports have also identified reduced IL-10 production by type-1 regulatory T cells (36), as well as by natural regulatory T cells (37). Although the mechanism underlying the IL-10 deficiencies in these studies remains to be elucidated, it is of interest to consider the possibility that an abnormality in IL-10 regulation may be common to different cell subsets in patients with MS (or possibly in different patient subsets). It is important to keep in mind that in all of these reports on MS T cells, as well as in our novel report on MS B cells, the identified abnormality in IL-10 production represents an "average" difference between cohorts; there remains considerable overlap between levels of IL-10 produced by cells from patients and normal controls. As such, our findings should not be taken to imply that a deficiency of B cell IL-10 production is a fundamental feature of all patients with MS. Although we found no significant differences in the IL-10 production by B cells from patients with the RRMS or SPMS clinical subtypes, our results do not preclude the existence of different patient subsets that may be distinguishable on the basis of their B cell capacity to produce IL-10. These considerations highlight an ongoing challenge in the field and the need to develop measures that will distinguish patient subsets on the basis of shared or distinct mechanisms of disease rather than reliance on clinical phenotyping alone.

Effector cytokine functions and other Ab-independent roles of B cells are of increasing interest in MS (38), as chronically activated B cells have been described within the meninges of patients with an apparent recapitulation of GC-like architecture (39–42). Such ectopic lymph node structures have been described in the target organs of several other human autoimmune diseases, including Sjogren's syndrome, diabetes, gastritis, and rheumatoid arthritis (43–46). Our present findings extend these reports and highlight the potential contribution of distinct subsets of B cells to ongoing inflammation, both within the target organs of human autoimmune diseases and in the periphery, where they may elaborate chemokines, produce factors that promote lymphoneogenesis, act as APCs, and regulate the local immune response through the expression of effector cytokines. Given these newly recognized roles of B cells, it becomes important to consider how currently approved treatments as well as future therapies may impact B cell biology. Although it is premature to suggest that the clinical benefit of mitoxantrone in patients with MS relates, even in part, to the drug's impact on B cells, our observations establish that the human B cell effector cytokine network can be targeted in vivo.

Our prospective studies of B cell reconstitution following both nonselective and selective depletion allowed us to examine shifts in the proportion of memory and naive B cell subsets, as well as

their effector cytokine networks, in parallel. In both settings, newly developing naive B cells were the main producers of IL-10, while the mature memory B cells were the main contributors of LT and TNF-α. Collectively, these results point to an in vivo switch in the cytokine "program" of human B cells transitioning from the naive to the memory pool. Based on our findings, we propose a model in which naive and memory B cells are programmed to play distinct roles in the regulation of normal immune responses through differential release of effector cytokines under different physiological contexts of activation. When appropriately stimulated by the engagement of their BCRs with cognate Ag followed by T cell help through CD40 signaling, memory B cells actively contribute to the efficiency of a desired memory immune response by elaborating TNF-α and LT, which would promote local follicular dendritic cell activation, GC formation, and lymphoneogenesis (47–52). In contrast, naive B cells that in the normal state are likely to harbor the autoreactive B cell pool are particularly capable of producing IL-10 in the context of "bystander" activation, which would serve to acquiesce otherwise undesired immune responses. Hence, the B cell cytokine output during early B cell-T cell encounter provides a "checkpoint" in the activation of both cells.

In summary, our study defines a novel concept for the immune functions of human B cell subsets. We ascribe distinct and active roles for naive vs memory B cell subsets in the regulation of normal immune responses and implicate an abnormality in this function as a contributor to human autoimmune disease. Dysregulation of B cell cytokine responses may represent a shared "checkpoint" contributing to several human autoimmune diseases, though possibly in different ways. These considerations are of particular relevance at a time when B cell directed therapies are being increasingly applied in human trials of distinct autoimmune diseases.

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Disclosures

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