B Cell-Derived IL-10 Does Not Regulate Spontaneous Systemic Autoimmunity in MRL.Fas<sup>lpr</sup> Mice

Lino L. Teichmann, Michael Kashgarian, Casey T. Weaver, Axel Roers, Werner Müller and Mark J. Shlomchik

*J Immunol* 2012; 188:678-685; Prepublished online 9 December 2011;
doi: 10.4049/jimmunol.1102456
http://www.jimmunol.org/content/188/2/678

---

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/12/09/jimmunol.1102456.DC1

References
This article cites 46 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/188/2/678.full#ref-list-1

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

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

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
B Cell-Derived IL-10 Does Not Regulate Spontaneous Systemic Autoimmunity in MRL.Fas<sup>lpr</sup> Mice

Lino L. Teichmann,* Michael Kashgarian,† Casey T. Weaver,‡ Axel Roers,§ Werner Müller,‖ and Mark J. Shlomchik*,*‖

B cells contribute to the pathogenesis of chronic autoimmune disorders, like systemic lupus erythematosus (SLE), via multiple effector functions. However, B cells are also implicated in regulating SLE and other autoimmune syndromes via release of IL-10. B cells secreting IL-10 were termed “Bregs” and were proposed as a separate subset of cells, a concept that remains controversial. The balance between pro- and anti-inflammatory effects could determine the success of B cell-targeted therapies for autoimmune disorders; therefore, it is pivotal to understand the significance of B cell-secreted IL-10 in spontaneous autoimmunity. By lineage-specific deletion of II10 from B cells, we demonstrated that B cell-derived IL-10 is ineffective in suppressing the spontaneous activation of self-reactive B and T cells during lupus. Correspondingly, severity of organ disease and survival rates in mice harboring II10-deficient B cells are unaltered. Genetic marking of cells that transcribe II10 illustrated that the pool of IL-10–competent cells is dominated by CD4 T cells and macrophages. IL-10–competent cells of the B lineage are rare in vivo and, among them, short-lived plasmablasts have the highest frequency, suggesting an activation-driven, rather than lineage-driven, phenotype. Putative Breg phenotypic subsets, such as CD1d<sup>hi</sup>CD5<sup>+</sup> and CD21<sup>hi</sup>CD23<sup>hi</sup> B cells, are not enriched in IL-10–competent cells. B cells secreting IL-10 were termed “Bregs” and were proposed as a separate subset of cells, a concept that remains controversial. The documented mechanism by which B cells inhibit an immune response is through secretion of the anti-inflammatory cytokine IL-10. Using mixed bone marrow chimeras, Fillatreau et al. (4) showed that mice did not recover from experimental autoimmune encephalomyelitis (EAE) when they lacked IL-10 specifically in B cells but not IL-10–deficient B cells, ameliorated disease in collagen-induced arthritis and an intestinal inflammation model (5, 6). B cell subsets with phenotypes such as CD1d<sup>hi</sup>CD5<sup>+</sup> (7), CD21<sup>hi</sup>CD23<sup>hi</sup> (akin to transitional type 2 [T2] B cells) (8), or CD23<sup>−</sup>CD21<sup>hi</sup> (marginal zone [MZ] B cells) (9) have been found enriched in IL-10–deficient B cells. Because of the causal association between IL-10 secretion and B cell regulatory function, CD1d<sup>hi</sup>CD23<sup>−</sup>CD5<sup>+</sup> B cells even have been labeled as “B10” cells (7). Recently, expression of T cell Ig domain and mucin domain protein 1 was described to identify IL-10–producing B cells across diverse B cell phenotypes (10).

B cells contribute to the pathogenesis of chronic autoimmune disorders, like systemic lupus erythematosus (SLE), multiple sclerosis, and type I diabetes, and are a major clinical target for the treatment of these disorders (1). Notwithstanding their capacity to promote autoimmunity by autoantibody secretion, Ag presentation, and proinflammatory cytokine production, it has become apparent that B cells also exert regulatory functions (2, 3).

The documented mechanism by which B cells inhibit an immune response is through secretion of the anti-inflammatory cytokine IL-10. Using mixed bone marrow chimeras, Fillatreau et al. (4) showed that mice did not recover from experimental autoimmune encephalomyelitis (EAE) when they lacked IL-10 specifically in B cells. Further, the adoptive transfer of IL-10–deficient B cells, but not IL-10–deficient B cells, ameliorated disease in collagen-induced arthritis and an intestinal inflammation model (5, 6). B cell subsets with phenotypes such as CD1d<sup>hi</sup>CD5<sup>+</sup> (7), CD21<sup>hi</sup>CD23<sup>hi</sup> (akin to transitional type 2 [T2] B cells) (8), or CD23<sup>−</sup>CD21<sup>hi</sup> (marginal zone [MZ] B cells) (9) have been found enriched in IL-10–deficient B cells. Because of the causal association between IL-10 secretion and B cell regulatory function, CD1d<sup>hi</sup>CD23<sup>−</sup>CD5<sup>+</sup> B cells even have been labeled as “B10” cells (7). Recently, expression of T cell Ig domain and mucin domain protein 1 was described to identify IL-10–producing B cells across diverse B cell phenotypes (10).

IL-10–secreting B cells have mainly been studied in infections and autoimmune syndromes induced by immunization, such as EAE, collagen-induced arthritis, and adjuvant-induced arthritis (AIA) (4, 5, 11). Recently, however, B10 cells were suggested to be protective in NZB/W F<sub>1</sub> mice, a mouse model of spontaneous lupus-like disease with polygenic inheritance (12, 13). This is of particular importance, because such disease models are strongly reflective of human autoimmune conditions, and several B cell-targeted therapies are being investigated for SLE, including the recently approved anti-BAFF Ab, belimumab. Importantly, in patients with autoimmune diseases, IL-10–dependent B cells have been identified that can inhibit TNF-α production by monocytes in vitro (14). Hence, nonspecific B cell-directed therapies might be a double-edged sword.

However, there is no direct evidence of a role for IL-10–dependent B cells in spontaneous autoimmune disorders, such as lupus. Rather, data supporting a role in spontaneous disease comes from therapeutic cell-transfer studies. Infusion of anti-CD40–treated CD21<sup>hi</sup>CD23<sup>−</sup> B cells into MRL.Fas<sup>lpr</sup> mice, another mouse model of polygenic spontaneous lupus-like disease, ameliorated lupus (15). Analogous results were obtained by transferring wild-type B10 cells into CD19<sup>−/−</sup> NZB/W F<sub>1</sub> mice (13). Although such transfer studies demonstrated that IL-10–competent B cells have the potential to regulate disease, it is uncertain whether endogenous
IL-10\(^{+}\) B cells would naturally do so. Notably, depletion of B cells in 4-wk-old N2ZW/W F1 mice accelerated the disease course (12). Yet, it was not clear whether this was a consequence of eliminating IL-10\(^{+}\) B cells, as suggested by the investigators, because all B cells, and not just IL-10\(^{+}\) B cells, were depleted. Thus, the function of native IL-10\(^{+}\) B cells in the context of this disease remains unknown.

In this study, we sought to determine the effect of IL-10 secreted by B cells on murine lupus and what aspects of the disease it modulates. To answer these questions, we deleted the Il10 gene in cells of the B lineage in the MRL.Fas\(^{gr}\) model of lupus. IL-10 exerts a strong protective effect in this strain, as demonstrated by severely exacerbated disease in MRL.Fas\(^{gr}\) mice globally lacking in IL-10 (16). The finding that transfer of IL-10–secreting CD21\(^{hi}\) CD23\(^{hi}\) B cells mitigates disease in MRL.Fas\(^{gr}\) mice (15) further suggests that IL-10 derived from B cells restrains disease in this strain.

Surprisingly, despite efficient Il10 gene deletion in the B cell lineage, we discerned no appreciable effect of B cell-derived IL-10 on anti-self B and T cell responses and, consequently, organ manifestations. To our knowledge, this work is the first direct genetic test of whether endogenous B cells via IL-10 really control a spontaneous chronic autoimmune disease. We concluded that, although artificially generated and infused IL-10–secreting B cells may be a useful cellular therapy (13, 15), the importance of endogenous regulatory B cells (Bregs) may have been overestimated in lupus and possibly other spontaneous chronic autoimmune syndromes.

Materials and Methods

Mice

CD19-Cre C57BL/6 mice (17) were backcrossed to the MRL-MpJ-Fas\(^{gr}\) 2J strain for 10 generations. Il10\(^{fl/wt}\) (18) and Il10\(^{fl/wt}\) mice were backcrossed to MRL-MpJ-Fas\(^{gr}\) mice eight times. MRL-MpJ-Fas\(^{gr}\) and MRL-MpJ-Fas\(^{gr}\) mice were obtained from The Jackson Laboratory. Homozygosity for the lpr mutation was verified by PCR. CD19-Cre MRL.Fas\(^{gr}\) mice were intercrossed with Il10\(^{fl/wt}\) Faslpr mice. CD19-Cre Il10\(^{fl/wt}\) MRL.Fas\(^{gr}\) mice were then crossed with Il10\(^{fl/wt}\) MRL.Fas\(^{gr}\) animals. To generate mice for the experiments, offspring CD19-Cre Il10\(^{fl/wt}\) and Il10\(^{fl/wt}\) MRL.Fas\(^{gr}\) mice were interbred. Thus, mice in those two groups were littermates. Analogously, offspring CD19-Cre and wild-type mice were used to expand those two groups. Animals were maintained under specific pathogen-free conditions and handled according to protocols approved by the Yale Institutional Animal Care and Use Committee.

Quantitative PCR

For quantification of genomic Il10 exon 1, DNA was extracted from FACS-purified cells, and quantitative PCR was performed with the Agilent Brilliant II SYBR Green QPCR kit. Il10 primers were forward 5'-GCTCTTACTGACTGCTAGATGCA-3' and reverse 5'-GGCGGACTCTGAGAGTTG-3'. The amount of Il10 in each sample was normalized to the unaffected gene Th9 (forward 5'-ACTCCGGATCTTGGACCA-3' and reverse 5'-GGCTCATGCTATGTTGCA-3'). To calculate the amount of residual Il10 in various cell types of CD19-Cre IL10\(^{fl/wt}\) mice, genomic DNA of the same cell type from Il10\(^{fl/wt}\) mice was used as undeleted control. Mice were 10 wk old. Samples were run on a Stratagene MX3000P instrument.

Flow cytometry

Surface staining was performed in ice-cold PBS with 3% calf serum in the presence of FcR blocking Ab 2.4G2. Ab clones used for surface staining were anti-B220 (927), anti-CD1d (1B1), anti-CD4 (GK1.5), anti-CD5 (53-7.3), anti-CD8 (TIB 105), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (1D3), anti-CD21/35 (7G6), anti-CD22 (Cy3.1), anti-CD23 (B3B4), anti-CD25 (PC61), anti-CD44 (1M7), anti-CD90.1 (1A14), anti-CD93 (AA4.1), anti-CD138 (281-2), anti-CD14 (B2B8), anti-CD16 (53-7.3), anti-CD23 (B3B4), anti-IgA (AMS15), anti-IgM (RS1.1), anti-IgG (AMS15), and anti-IgG (AMS15). Intracellular staining was performed using the BD Cytofix/Cytoperm and Perm/Wash buffers or, for intracellular Foxp3 staining, the eBioscience Foxp3 staining buffer set. For intracellular cytokine staining, 4 × 10\(^5\) splenocytes were cultured for 4 h at 37°C in 24-well plates in 2 ml culture medium containing ionomycin (500 ng/ml) and PMA (20 ng/ml). Brefeldin A (10 μg/ml) was added to the cultures for the last 2 h. Ab clones used for intracellular staining were anti-Foxp3 (FJK-16), anti-κ (187.1), and anti-IFN-γ (XM1G.1). Ethidium monoazide was used for live-dead discrimination. Cells were analyzed on an LSR II instrument (BD).

Autoantibodies

HEp-2 immunofluorescence assays (Antibodies, Inc.) were performed, as previously described (20), with serum dilutions of 1:100. Stained slides were read on a Olympus BX-40 microscope. Anti-IgG2a rheumatoid factor and anti-nucleosome IgG serum concentrations were determined by ELISA, as previously described (21). The mAbs 400μg/23 (IgM rheumatoid factor) and PL2-3 (IgG2a anti-nucleosome) were used as standards.

Luminex

IL-10 concentrations in the supernatants of B cell cultures were measured by Luminex assay (Bio-Rad), according to the manufacturer’s protocol.

Evaluation of clinical disease

To assess kidney disease, formalin-fixed kidneys were embedded in paraffin and sectioned. Sections were stained with H&E or periodic acid-Schiff and scored for glomerular and interstitial nephritis by a pathologist (M.K.) who was blinded to the genotype of the mice. Proteinuria was measured with Bayer Albustix reagent strips. For dermatitis, the size of lesions on the dorsum of the neck and back was scored from 0 to 4; additionally, 0.5 points were given for dermatitis of each ear and the face. For survival analysis, mice were aged until they succumbed to terminal autoimmune disease or deemed moribund by Yale Veterinary Clinical Services.

Results

B cell-specific Il10 deletion in lupus-prone mice

We deleted the Il10 gene in B cells by intercrossing MRL.Fas\(^{gr}\) mice with an Il10\(^{fl/wt}\) allele (18) with MRL.Fas\(^{gr}\) mice carrying a CD19-Cre knock-in (17). Deletion of the Il10\(^{fl/wt}\) alleles in CD19-Cre IL10\(^{fl/wt}\) mice (called B-Il10\(^{−/−}\) mice hereafter) was measured by quantitative PCR on genomic DNA. We found that >90% of the Il10\(^{fl/wt}\) alleles were deleted in splenic B cells of the MZ, follicular I type (FOL 1), and T2 and B10 cells (Table I). Deletion in peritoneal B1a, B1b, and B2 cells was similarly effective. Purity of sorted B cell populations was ~97%, leading to a slight underestimation of Il10-deletion efficiency. No deletion was observed in T cells, macrophages, neutrophils, or conventional and peripheral macrophages*.

Table I. Deletion efficiency in B-Il10\(^{−/−}\) mice

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>n</th>
<th>% Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>MZ B</td>
<td>3</td>
<td>94.1</td>
</tr>
<tr>
<td>FOL I B</td>
<td>3</td>
<td>93.2</td>
</tr>
<tr>
<td>T2 B</td>
<td>3</td>
<td>94.0</td>
</tr>
<tr>
<td>B10</td>
<td>3</td>
<td>91.1</td>
</tr>
<tr>
<td>T</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Conventional dendritic cells</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Plasmacytoid dendritic cells</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>B1a(^{Δ}A)</td>
<td>2</td>
<td>95.0</td>
</tr>
<tr>
<td>B1b(^{Δ}A)</td>
<td>3</td>
<td>90.9</td>
</tr>
<tr>
<td>B2(^{Δ}A)</td>
<td>3</td>
<td>88.6</td>
</tr>
<tr>
<td>Peritonal macrophages*</td>
<td>3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Measurement of the amount of residual Il10 by quantitative PCR in various cell types of B-Il10\(^{−/−}\) mice in the spleen or peritoneal lavage, as indicated. Cell populations were purified by FACS. MZ, FOL I, and T2 B cells were gated as illustrated in Fig. 2A. B10 cells were identified as shown in Fig. 2D. The following equation was used to calculate the percentage deletion value: (1 − residual Il10 \(\times 100\). Residual Il10 values were calculated as 2\(^{−∆∆Ct}\) comparing B-Il10\(^{−/−}\) and Il10\(^{fl/wt}\) mice. Negative percentage deletion values were set to 0.

T cells, TCR\(\beta\)CD19\(^{-}\); macrophages, CD11b\(^{F4/80}\)Gr1\(^{−}\); neutrophils, CD11b\(^{Gr1}\); conventional dendritic cells, CD11c\(^{B2}\)B220\(^{−}\); B1a cells, CD19\(^{B2}\)CD11b\(^{−}\); B1b cells, CD19\(^{B2}\)CD11b\(^{−}\); B2 cells, CD19\(^{B2}\)CD11b\(^{−}\); peritonal macrophages, CD11b\(^{F4/80}\).

*Peripheral cells.
plasmacytoid dendritic cells. Consistent with this, supernatants of sorted B cells from B-IL10−/− and IL10ββ mice cultured in the presence of TLR agonists had 10-fold lower IL-10 concentrations than did those from control mice (Supplemental Fig. 1). Thus, B-IL10−/− MRL.Fasββ mice are a suitable tool to investigate the function of IL-10-secreting B cells in systemic autoimmunity.

**Deficiency for IL-10 in B cells does not exacerbate organ disease**

Glomerulonephritis and interstitial nephritis in MRL.Fasββ mice are greatly enhanced by global deficiency for IL-10 (16). Severity of glomerulonephritis and interstitial nephritis in 16-wk-old B-IL10−/− and IL10ββ mice was similar, with glomeruli showing hypercellularity and collapsed capillary loops (Fig. 1A, 1B). Interstitial infiltrates were present in the perivascular, peritubular, and, and, occasionally, periglomerular region in kidneys of all mice (Fig. 1A). Accordingly, B-IL10−/− mice did not have more proteinuria than IL10ββ mice (Fig. 1C).

Cutaneous lupus manifestations in the MRL.Fasββ strain include facial rash, ulceration of the ears, and lesions of the back and neck. Dermatitis occurs more frequently in female mice than in males. The extent of dermatitis was not different between female B-IL10−/− and IL10ββ mice (Fig. 1D). Likewise, male mice in both groups had equally severe dermatitis (Supplemental Fig. 2).

MRL mice spontaneously develop splenomegaly and lymphadenopathy, as do many SLE patients during active disease. Measurement of spleen (Fig. 1E) and axillary lymph node (Fig. 1F) weight revealed no differences between B-IL10−/− and IL10ββ mice.

The inability of B cell-secreted IL-10 to modulate lupus-like organ manifestations prompted us to ask whether disruption of one copy of the CD19 gene by the CD19-Cre allele might influence disease expression, countering the effect of Il10 deletion in B cells. Hemizygosity of CD19 results in lower expression levels on B cells, possibly changing the threshold for BCR signaling. Therefore, we generated a cohort of CD19-Cre and wild-type MRL.Fasββ mice and performed a similar analysis as for B-IL10−/− and IL10ββ MRL.Fasββ mice. We observed no significant alterations in nephritis, dermatitis, splenomegaly, or lymphadenopathy in CD19-Cre mice compared with wild-type animals (Supplemental Fig. 2). Thus, CD19 hemizygosity was not a confounding factor in our study. Taken together, the analysis demonstrated that B cell-specific Il10 deletion does not aggravate organ disease in lupus.

**B cell homeostasis is unperturbed in B-IL10−/− mice**

It was proposed that IL-10 regulates B cell differentiation and survival (22). We examined whether IL-10 secreted by B cells influences B cell homeostasis in an autocrine or paracrine manner. Splenic B cell numbers in B-IL10−/− mice were not different from those in IL10ββ mice (Fig. 2B). Absence of B cell-secreted IL-10 did not affect B cell subset frequencies (Fig. 2A, 2C, 2D). In particular, we did not observe significant changes in frequencies of T2 B cells (Fig. 2C) and B10 cells (Fig. 2D), both of which have been ascribed regulatory functions (7, 8). For T2 B cells, there was a downtrend in frequency in B-IL10−/− mice (p = 0.11), which was also evident in absolute numbers/spleen; however, this did not reach statistical significance. Splenic T2 B cell numbers were 1.63 × 106 ± 0.23 × 106 (mean ± SEM) for IL10ββ mice and 1.15 × 106 ± 0.17 × 106 for B-IL10−/− mice (p = 0.10).

**Autoantibody formation is not enhanced in mice lacking IL-10 in B cells**

To determine whether Il10 expression in B cells regulates the humoral response against self in lupus, we used the HEp-2 cell-based immunofluorescent microscopy assay to detect serum anti-nuclear and anti-cytoplasmic IgG. Nine of 17 (52.9%) sera from B-IL10−/− mice demonstrated homogenous nuclear staining and equatorial staining of mitotic chromatin, corresponding to antichromatin (Fig. 3A, 3B). A similar fraction of sera from IL10ββ mice (6 of 13, 46.2%) produced these same staining patterns (Fig. 3A, 3B). A speckled nuclear staining pattern, corresponding to Abs that recognize RNA or RNA-associated proteins, was ob-
served for 41.2% of sera from B-IL10/2 animals and 30.8% of sera from IL10fl/fl animals. ELISAs for serum rheumatoid factor (Fig. 3C) and antinucleosome IgG (Fig. 3D) demonstrated similar concentrations in B-IL10/2 and IL10fl/fl mice.

In MRL.Faslpr mice and other lupus-prone mouse strains, autoantibodies derive, in large part, from short-lived plasmablasts in the spleen (23). As expected, B cell-specific IL-10 deficiency did not alter splenic plasmablast numbers as determined by flow cytometry (Fig. 3E). Similar evaluation of CD19-Cre and wild-type MRL.Faslpr animals revealed no confounding effect of the CD19-Cre knock-in per se (Supplemental Fig. 3). We concluded that B cell-secreted IL-10 plays no role in B cell homeostasis, activation, plasmablast differentiation, or autoantibody formation.

**FIGURE 2.** B cell-derived IL-10 does not affect B cell homeostasis. A, Contour plots show gating of T2, MZ, and FOL I B cells after exclusion of IgM− IgD− cells and gating on CD19+ cells. B, Numbers of B cells (CD19+CD22+) per spleen (n = 15). C, Frequencies of T2, MZ, and FOL I B cells as a percentage of total B cells (n ≥ 8). B cell subsets were gated as depicted in A. D, Frequency of B10 cells as a percentage of total B cells (n ≥ 11). The contour plot illustrates how B10 cells were identified after gating on CD19+TCRβ− cells. Data shown are combined from three experiments (mean ± SEM).

B cell-derived IL-10 does not affect T cell activation or differentiation

T cells contribute considerably to the pathogenesis of SLE (24). B cells affect T cells in autoimmunity in Ab-independent ways that probably involve both Ag presentation and cytokine secretion (25, 26). We explored whether T cell autoimmunity is tempered by B cell-derived IL-10. T cell numbers in the spleen were unaltered in B-IL10/2 mice compared with IL10fl/fl mice (Fig. 4A). CD4 and CD8 staining did not reveal any changes in T cell composition in the absence of B cell-secreted IL-10 (Fig. 4B), including CD4− CD8− cells that typically accumulate in Faslpr animals. In MRL.Faslpr mice, there is a paucity of phenotypically naive (CD44− CD62L+) T cells; this compartment amounted to 4% of all CD4+ T cells in both B-IL10/2 and IL10fl/fl mice (Fig. 4C).

**FIGURE 3.** Autoantibody formation is not enhanced in mice lacking IL-10 in B cells. HEp-2 anti-nuclear Ab-staining patterns classified as homogenous, speckled, centromere, or cytoplasmic (A) and mitotic chromatin staining classified as positive or negative (B) produced by sera from B-IL10/−− and IL10fl/fl mice. The numbers in the circles indicate the numbers of mice analyzed in each group. ELISAs showing serum concentrations of anti-IgG2a rheumatoid factor (C) and anti-nucleosome IgG (D) (n = 13). E, Plasmablasts were enumerated in spleens (n = 15). After exclusion of T cells, plasmablasts were gated as CD19+CD22+CD44+CD138+SSCint cells. Plasmablast data are pooled from three experiments. HEp-2 assay and ELISAs were performed once with mouse sera prepared in three experiments. Data in C–E are mean ± SEM.
In myeloid cells, IL-10 inhibits the transcription of p35 and p40, the subunits of IL-12 (27). IL-12 release by dendritic cells and macrophages induces differentiation of Th and cytotoxic cells into IFN-γ-secreting effectors. Excessive production of IFN-γ has been linked to SLE pathogenesis (28). In MRL.Faslpr mice, deletion of Ifng or Ifngr1 dramatically ameliorates disease (29, 30). To determine whether B cell-derived IL-10 suppresses differentiation of CD4+ and CD8+ T cells into IFN-γ-secreting effectors, we stained splenocytes for intracellular IFN-γ after 4 h of culture with PMA/ionomycin-stimulated splenocytes gated on CD4+ (Fig. 4D), but not CD8+ (Fig. 4E), T cells. Frequencies of CD4+ T cells in B-IL10−/− mice; however, the effect was small. B cells can expand regulatory T cells (Tregs) (31–33), prompting us to test whether B cells promote differentiation of CD4+ T cells into IFN-γ–secreting effectors, we stained splenocytes for intracellular IFN-γ after 4 h of culture with PMA and ionomycin. The frequency of IFN-γ–producing cells was decreased among CD4+ (Fig. 4D), but not CD8+ (Fig. 4E), T cells in B-IL10−/− mice; however, the effect was small. B cells can expand regulatory T cells (Tregs) (31–33), prompting us to test whether B cells promote differentiation of CD4+ T cells into IFN-γ–secreting effectors. We found similar percentages of CD4+ T cells from B-IL10−/− and IL10+ mice to be Foxp3+CD25+ (Fig. 4F). The CD19-Cre knock-in itself did not affect any of these T cell phenotypes (Supplemental Fig. 4). Thus, Il10 deletion in B cells has no effect on the activation, expansion, or differentiation of T cells.

**IL-10 produced by B cells confers no survival advantage**

The question remained whether B cell-produced IL-10 might have many subtle effects, each of which individually is not detectable, but together would have an impact on disease outcome. Therefore, we conducted a survival analysis. The median survival of B-IL10−/− and IL10+ mice was 139 and 142 d, respectively (Fig. 5). A log-rank test showed no difference between the survival curves of both groups (p = 0.57).

**B cells are only a minor source of IL-10 in MRL.Faslpr mice**

We sought to investigate why, in contrast to the proposed theory that IL-10–competent B cells have a protective role in lupus (12, 13), Il10 deletion in B cells had no appreciable effect on disease expression. For this purpose, we crossed the IL-10 reporter locus 10BiT (19) that encodes Thy-1.1 under the control of an Il10 promoter, thus marking cells that transcribe Il10, on the MRL. Faslpr background. In the spleen of aged 10BiT MRL.Faslpr mice, the percentage of Thy-1.1+ cells was highest in CD4+Foxp3− T cells (15.8%), macrophages (12.7%), and Tregs (11.9%) (Fig. 6A, upper panel). The same cell types most frequently upregulated Thy-1.1 in axillary lymph nodes (Fig. 6A, lower panel). Only very few B cells were Thy-1.1+ (<1%) in both tissues. Importantly, we did not observe an enrichment of Thy-1.1–expressing B cells in putative Breg subsets, such as B10 and T2. In fact, almost all splenic B cells in the FACS gates that delineate the B10 and T2 subsets lacked Thy-1.1 expression. In the axillary lymph nodes, B10 phenotype cells expressing Thy-1.1 were undetectable. Notably, a substantial fraction of CD138hiCD19−CD44+ intracellular-$\alpha$2hi cells (2.1% in the spleen and 5.5% in the axillary lymph nodes), which are mainly short-lived plasmablasts (34), had detectable Thy-1.1 expression, indicating IL-10 mRNA synthesis. However, B cells and plasmablasts together constituted only 3.9% of the total Thy-1.1+ population in the spleen (Fig. 6B, upper panel) and 0.7% in axillary lymph nodes (Fig. 6B, lower panel). In contrast, 78.8% of Thy-1.1+ cells in the spleen and 92.4% in axillary lymph nodes were T cells. Expression levels of Thy-1.1 were markedly higher in T cells than in all other cell types, likely reflecting a capacity of T cells to produce greater amounts of IL-10 (Fig. 6C). In young, preautoimmune 10BiT MRL.Faslpr mice, most analyzed cell subsets contained lower percentages of

**FIGURE 4.** B cell-derived IL-10 does not constrain T cell activation, expansion, or differentiation into effectors. A, T cell numbers in the spleen. B, Frequencies of CD4+, CD8+, CD4+CD8−, and double-negative T cells as a percentage of total T cells of IL10+ (black bars) and B-IL10−/− (white bars) mice. C, CD44 and CD62L staining of CD4+ T cells of IL10+ (black bars) and B-IL10−/− (white bars) mice to identify naive (CD44+CD62L−), CD44+CD62L+ and CD44−CD62L− populations. Representative contour plots of gated CD4+ T cells are shown (left panels). Intracellular IFN-γ staining of PMA/ionomycin-stimulated splenocytes gated on CD4+ (D) or CD8+ (E) T cells. Histograms to the left of the bar graphs show representative examples of flow cytometric data. **p < 0.01, two-tailed Mann–Whitney U test. F, Frequency of Tregs (Foxp3+CD25+) as a percentage of CD4+ T cells. A–F, n ≥ 15. Data shown are combined from three experiments (mean ± SEM).

**FIGURE 5.** IL-10 produced by B cells confers no survival advantage. Kaplan–Meier survival curves of IL10+ (n = 15; 9 females and 6 males) and B-IL10−/− (n = 20; 12 females and 8 males) mice.
Thy-1.1+ cells than in aged animals (Table II), indicating that Il10 transcription is induced over the disease course. Induction was particularly strong in CD4+Foxp32 T cells and macrophages. The fraction of Thy-1.1+ cells in the Treg compartment remained essentially unchanged (11.9% in aged mice versus 9.7% in young mice). In conclusion, T cells and macrophages are the predominant cell types that express Il10 in MRL.

On the contrary, IL-10–competent B cells (Thy-1.1+) are rare and are not restricted to a specific B cell phenotypic subset, although they are more common among plasmablasts.

Discussion

In this study, we addressed the role of IL-10 derived from endogenous, unmanipulated B cells in spontaneous chronic autoimmune disease. By deleting Il10 specifically in B cells in lupus-prone mice, we demonstrated that B cell-secreted IL-10 has no protective effect in lupus. This was reflected in equally severe organ disease, similar degrees of immune system activation, and indistinguishable survival rates in MRL.Faslpr mice that do or do not lack IL-10 specifically in B cells.

Consistent with those results, using reporter mice, we found that B cells were only a minor source of IL-10 in vivo. By deleting Il10 in B cells from birth in MRL.Faslpr mice we maximized the opportunity for IL-10+ B cells to exert regulatory effects without making prior assumptions about at what stage of disease those might occur. The extent of deletion, although not 100%, as with every Cre-loxP system, was 10–20 fold, which we believe should have been more than enough to reveal a phenotype if B cell-derived IL-10 were truly regulating spontaneous lupus in a biologically significant fashion.

Thus, our work indicated that B cell-derived IL-10 is not a principal regulator of disease in murine lupus.

Based on the existing paradigm (12, 13, 15) and prior results in MRL.Faslpr mice (15, 16), our findings were unexpected. Endogenous IL-10+ B cells do regulate the immune response in infections with bacteria, viruses, and parasites (35–37). Directly pertinent to autoimmunity, IL-10–producing B cells contained disease in EAE (4) and AIA (11). An important difference between these diseases and lupus in MRL.Faslpr mice could be the nature and kinetics of the initiating stimulus. In infectious disease models, as well as induced “autoimmunity” models, such as EAE and AIA, the immune response is incited by inoculation with a pathogen or an Ag in combination with an adjuvant, resulting in the sudden onset of immune response and pathology. In contrast, spontaneous chronic autoimmune diseases, like lupus, have a gradual onset. B cells might require abrupt and strong stimulation for robust IL-10 production. Thus B cell-derived IL-10 could be...
critical in pathogen responses and induced models of autoimmunity, whereas syndromes of chronic autoimmunity in humans, such as SLE, rheumatoid arthritis, type 1 diabetes, and multiple sclerosis, and mice may not be regulated by B cell-secreted IL-10, even if IL-10^+ B cells might be therapeutic when infused.

It was described that CD24^hiCD38^hi B cells from healthy individuals suppress Th1 cell differentiation in vitro in a partially IL-10–dependent manner (38). In contrast, CD24^hiCD38^hi B cells from SLE patients lacked an equivalent suppressive capacity and expressed less IL-10 after stimulation. These results, although in vitro, are consistent with our findings that, in lupus, B cells do not bring their regulatory potential to bear.

To define the cells that produce IL-10 in the context of ongoing autoimmunity, we used 10BiT reporter mice on the MRL.Fas^byr background. B cells represented only a minor fraction of IL10-transcribing cells and had low expression levels. Importantly, the vast majority of B cells with a CD1d^hiCD5^+ or CD21^hiCD23^hi phenotype did not spontaneously synthesize IL-10 mRNA in vivo. From these data, we could not confirm that there are bona fide, discrete, IL-10–producing Breg subsets at least during active murine lupus. However, there was a considerable plasmablast population that transcribed IL10. This argues that the stimuli that lead to the acquisition of IL-10 competence frequently induce plasmablast differentiation at the same time. Similar findings were reported for Vert-X C57BL/6 mice, another IL-10 reporter mouse, after challenge with different immunogens (35). It is unclear whether the B cells that gave rise to IL-10–competent plasmablasts had a specific phenotype. Because plasmablasts are short-lived, our results imply that, in lupus, IL-10–producing B cell progeny represent a transient activation state and not a stable cell lineage with homeostatic regulation.

Both resting (39, 40) and activated (3) B cells can suppress immune responses. TLR activation, particularly in combination with BCR ligation, and CD40 stimulation are signals that have repeatedly been found to induce IL-10 production in B cells (41). In MRL.Fas^byr mice, self-reactive B cells are spontaneously activated via TLR7/9 and BC2 cross-linking by immune complexes (42). Further, CD40L-deficient MRL.Fas^byr mice do not develop nephritis or make rheumatoid factor and anti-dsDNA autoantibodies (43), arguing that CD40–CD40L interactions occur in this strain. Hence, it is reasonable to assume that B cells receive signals in vivo that are known to induce IL-10. However, chronic exposure to those signals might have a different outcome than acute stimulation, or other factors might impede a Breg phenotype in MRL.Fas^byr mice.

Recently, it was reported that deletion of all mature B cells, including B10 cells, in young preautoimmune NZB/W F1 mice accelerates disease onset and decreases survival time (12). CD19^−/− NZB/W F1 mice had exacerbated nephritis, paralleled by a reduction of B10 cells (13). Both studies were interpreted to support a protective effect of B10 cells in lupus. However, in these studies, the total mature B cell population was either depleted or genetically impaired, but it was not directly tested whether the observed effects were actually caused by the lack of B10 cells or any other IL-10–producing B cell population. Many other mechanisms could explain the observed effects. Altered activation state of macrophages after uptake of Ab-coated B cells during the depletion process, indirect effects on the T cell compartment owing to lack of global B cell interactions, structural changes in lymphoid architecture, or skewing of residual or regenerating B cell compartments could all potentially account for these earlier findings.

Hypothetically, it is possible that B cells can use IL-10–independent mechanisms to suppress an immune response that is potent enough to compensate for IL10 deficiency. However, in vivo evidence for such mechanisms has yet to be presented. Rather, in essentially all articles on Bregs in which a mechanism of regulation was demonstrated, IL-10 was implicated (4–7). Of greatest relevance to the present data, regulatory effects of infused B cells are clearly IL-10 dependent in MRL.Fas^byr mice (15). Even if Bregs were to possess inhibitory means apart from IL-10, published studies indicated that IL10 transcription would at least identify most Bregs. Yet, the fraction of IL10-transcribing B cells in MRL.Fas^byr mice was very small. Therefore, our data do not favor the interpretation that factors other than IL-10 account for suppressive effects of endogenous Bregs in lupus. In any case, it is important to emphasize that the previously implicated mechanism of B cell regulation in this instance was not validated when directly tested.

Our results, along with studies of B cell-targeted therapies in humans (1, 44) and mice (45, 46), suggested that B cells have a net pathogenic role in lupus that is not substantially counterbalanced by their IL-10–dependent regulatory functions. Using lupus-prone mice bearing an IL-10 reporter transgenic locus, we did not identify distinct B cell subsets that were enriched for IL10 transcription (other than plasmablasts), calling into question the existence of discrete Breg populations, at least in the context of ongoing lupus. Our findings should precipitate a rethinking of whether endogenous B cells exist that regulate spontaneous chronic autoimmunity and emphasize the need to define the variables that govern Breg capacity in vivo.

Acknowledgments
We thank the Yale Animal Resources Center for outstanding animal husbandry.

Disclosures
The authors have no financial conflicts of interest.

References


SUPPLEMENTAL FIGURE S1. Efficient deletion of *Il10* in B cells of B-IL10−/− mice. B cells were purified by FACS from spleens of IL10fl/fl (black bars) and B-IL10−/− (white bars) mice and stimulated with 10 µg/ml LPS E. coli 0111:B4, 5 µg/ml CpG ODN 2395 or medium for 48 hrs. IL-10 was measured in the supernatants of B cell cultures by Luminex assay (n = 3). Data are from a single experiment (mean ± SEM).
SUPPLEMENTAL FIGURE S2. Organ disease in MRL.Fas<sup>lpr</sup> mice is not affected by the CD19-Cre knock-in. A, Dermatitis severity was scored for male B-IL10<sup>−/−</sup> and IL10<sup>fl/fl</sup> mice (n ≥ 13). B and C, Proteinuria (B, n ≥ 14) and dermatitis severity (C, n = 5 — 9) were scored for wild type (WT) and CD19-Cre mice. Each dot represents an individual mouse. Horizontal lines indicate the median. D and E, Weight of spleens (D) and the two largest axillary lymph nodes (E) (n ≥ 14). Data are represented as mean ± SEM in bar graphs. Data shown are combined from 5 experiments.
SUPPLEMENTAL FIGURE S3. CD19-Cre does not confound analysis of the B cell system. A and B, Numbers of B cells (A) and plasmablasts (B) per spleen (n ≥ 6). C and D, HEp-2 ANA staining patterns classified as homogenous, speckled, or cytoplasmic (C) and mitotic chromatin staining classified as positive or negative (D) produced by sera from wild type (WT) and CD19-Cre mice. The numbers in the circles indicate the numbers of mice analyzed in each group. E and F, ELISAs showing serum concentrations of anti-IgG2a rheumatoid factor (E) and anti-nuclesome IgG (F) (n ≥ 10). B cell and plasmablast data are pooled from 3 experiments. HEp-2a assay and ELISAs were performed once with mouse sera prepared in 3 experiments. Data are represented as mean ± SEM in bar graphs.
SUPPLEMENTAL FIGURE S4. CD19-Cre has no effect on the anti-self T cell response. A, T cell numbers in the spleen. B, Frequencies of CD4+, CD8+, CD4+CD8+ and double-negative T cells as a percentage of total T cells of wild type (WT, black bars) and CD19-Cre (white bars) mice. C, CD44 and CD62L staining of CD4+ T cells of wild type (WT, black bars) and CD19-Cre (white bars) mice to identify naïve (CD44−CD62L+) and activated/memory populations. D and E, Intracellular IFN-γ staining of PMA/ionomycin-stimulated splenocytes gated on CD4+ (D) or CD8+ (E) T cells. F, Frequency of Tregs (Foxp3+CD25+) as a percentage of CD4+ T cells. A−F, n ≥ 6. Data shown are combined from 3 experiments (mean ± SEM).