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B Cell-Derived Interleukin 10 Does Not Regulate Spontaneous Systemic Autoimmunity in MRL.*Fas*^{lpr} mice

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Abstract

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 have been termed “Breg” and have been 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 and it is therefore pivotal to understand the significance of B cell-secreted IL-10 in spontaneous autoimmunity. By lineage specific deletion of *Il10* from B cells we demonstrate 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 *Il10* deficient B cells are unaltered. Genetic marking of cells that transcribe *Il10* illustrates 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 rather than lineage-driven phenotype. Putative Breg phenotypic subsets such as CD1d^{hi}CD5^{+} and CD21^{hi}CD23^{hi} B cells are not enriched in *Il10* transcription. These genetic studies demonstrate that in a spontaneous model of murine lupus, IL-10 dependent B cell regulation does not restrain disease and thus the pathogenic effects of B cells are not detectably counterbalanced by their IL-10 dependent regulatory functions.

INTRODUCTION

B lymphocytes are pathogenic in autoimmune diseases such as rheumatoid arthritis, 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 auto-Ab secretion, antigen presentation, and proinflammatory cytokine production, it has become apparent that B cells also exert regulatory functions (2,3).

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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. showed that mice did not recover from experimental autoimmune encephalomyelitis (EAE) when they lacked IL-10 specifically in B cells (4). Further, the adoptive transfer of IL-10 sufficient B cells but not IL-10 deficient B cells ameliorated disease in collagen induced arthritis (CIA) and an intestinal inflammation model (5,6).B cell subsets with phenotypes such as $CD1d^{hi}CD5^{+}$ (7), $CD21^{hi}CD23^{hi}$ (akin to transitional type 2 B cells) (8) or $CD23^{-}CD21^{hi}$ (marginal zone B cells) (9) have been found to be enriched in $IL\text{-}10^{+}$ B cells. Because of the causal association between IL-10 secretion and B cell regulatory function $CD1d^{hi}CD5^{+}$ B cells even have been labeled as “B10” cells (7). Recently, expression of T cell Ig domain and mucin domain protein 1 (TIM-1) has been 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, CIA and adjuvant-induced arthritis (AIA) (4,5,11). Recently, however, B10 cells have been suggested to be protective in NZB/W F_1 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 currently being investigated for SLE, including the recently-approved anti-B cell activating factor (BAFF) Ab, belimumab. Importantly, in patients with autoimmune diseases, $IL10^{+}$ B cells have been identified that can inhibit TNF-α production by monocytes in vitro (14). Hence, non-specific B cell directed therapies might be a double-edged sword.

However, as yet there is no direct evidence of a role for $IL\text{-}10^{+}$ B cells in spontaneous autoimmune syndromes such as lupus. Rather, data supporting a role in spontaneous disease comes from therapeutic cell transfer studies. Infusion of anti-CD40 treated $CD21^{hi}CD23^{hi}$ B cells into MRL.Fas^{lpr} mice, another mouse model of polygenic spontaneous lupus-like disease, ameliorated lupus (15). Analogous results were obtained by transferring wildtype B10 cells into $CD19^{-/-}$ NZB/W F_1 mice (13). Although such transfer studies demonstrate that IL-10 competent B cells have the potential to regulate disease, it is uncertain whether endogenous $IL\text{-}10^{+}$ B cells would naturally do so. Notably, depletion of B cells in 4 wk old NZB/W F_1 mice accelerated the disease course (12). Yet, whether this was a consequence of eliminating $IL\text{-}10^{+}$ B cells, as suggested by the authors, was not clear, because all B cells and not just $IL\text{-}10^{+}$ B cells were depleted. Thus, the function of native $IL\text{-}10^{+}$ B cells in 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^{lpr} model of lupus. IL-10 exerts a strong protective effect in this strain, as demonstrated by severely exacerbated disease in MRL.Fas^{lpr} 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^{lpr} 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. This work is the first direct genetic test of whether endogenous B cells via IL-10 really control a spontaneous chronic autoimmune disease. We conclude that while artificially generated and infused IL-10 secreting B cells may be a useful cellular therapy (13,15), the importance of endogenous 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*^{lpr}/2J strain for 10 generations. *Il10*^{fl} (18) and 10BiT (19) C57BL/6 mice were backcrossed to MRL-MpJ-*Fas*^{lpr}/J 8 times. MRL-MpJ-*Fas*^{lpr}/2J and MRL-MpJ-*Fas*^{lpr}/J mice were obtained from Jackson Laboratory. Homozygosity for the *lpr* mutation was verified by PCR. CD19-Cre MRL.*Fas*^{lpr} were intercrossed with *Il10*$^{fl/wt}$ MRL.*Fas*^{lpr} mice. CD19-Cre *Il10*$^{fl/wt}$ MRL.*Fas*^{lpr} mice were then crossed with *Il10*$^{fl/wt}$ MRL.*Fas*^{lpr} animals. To generate mice for the experiments, offspring CD19-Cre *Il10*$^{fl/fl}$ and *Il10*$^{fl/fl}$ MRL.*Fas*^{lpr} mice were interbred. Thus, mice in those two groups were littermates. Analogously, offspring CD19-Cre and wt mice were used to expand those two groups. Animals were analyzed at 16 wks of age if not stated otherwise. Animals were maintained under specific-pathogen-free (SPF) conditions and handled according to protocols approved by the Yale Institutional Animal Care and Use Committee (IACUC).

Quantitative PCR

For quantitation of genomic *Il10* exon 1, DNA was extracted from FACS purified cells and qPCR was performed with the Agilent Brilliant II SYBR Green QPCR kit. *Il10* primers were: forward 5'-GCTCTTACTGACTGGCATGAG-3'; and reverse 5'-CGCAGCTCTAGGAGCATGTG-3'. The amount of *Il10* in each sample was normalized to the unaffected gene *Tlr9* (forward 5'-ACTCCGACTTCGTCCACCT-3'; and reverse 5'-GGCTCAATGGTCATGTGGCA-3'). To calculate the amount of residual *Il10* in various cell types of CD19-Cre *Il10*$^{fl/fl}$ mice, genomic DNA of the same cell type from *Il10*$^{fl/fl}$ mice was used as undeleted control. Mice were 10 wks of age. 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-BST2 (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 (Cy34.1), anti-CD23 (B3B4), anti-CD25 (PC61), anti-CD44 (1M7), anti-CD90.1 (1A14), anti-CD93 (AA4.1), anti-CD138 (281-2), anti-F4/80 (BM8), anti-I-A/I-E (M5/114), anti-IgDa (AMS15), anti-IgMa (RS3.1), anti-Ly6G/Ly6C (RB6-8C5) and anti-TCRβ (H57-597). 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, 4×10^6 splenocytes were cultured for 4 hr at 37°C in 24-well plates in 2 ml culture medium containing ionomycin (750 ng/ml) and PMA (20 ng/ml). For the last 2 hr brefeldin A (10 μg/ml) was added to the cultures. Ab clones used for intracellular staining were: anti-FoxP3 (FJK-16), anti-kappa (187.1) and anti-IFN-γ (XMG1.2). Ethidium monoazide (EMA) was used for live-dead discrimination. Cells were analyzed on a LSRII instrument (BD).

Auto-Abs

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 monoclonal Abs 400tμ23 (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 paraffin embedded and sectioned. Sections were then stained with H&E or PAS 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 0–4; 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^{lpr} mice with an $Il10^{fl}$ allele (18) with MRL.Fas^{lpr} mice carrying a CD19-Cre knock-in (17). Deletion of the $Il10^{fl}$ alleles in CD19-Cre $IL10^{fl/fl}$ mice (called B-$IL10^{-/-}$ hereafter) was measured by quantitative PCR on genomic DNA. We found that >90% of the $Il10^{fl}$ alleles were deleted in splenic B cells of the marginal zone (MZ), follicular I (FOL I) and transitional 2 (T2) type and B10 cells (Table I). Deletion in peritoneal B1a, B1b and B2 cells was similarly effective. Purity of sorted B cell populations was about 97% leading to a slight underestimation of *Il10* deletion efficiency. No deletion was observed in T cells, macrophages, neutrophils, or conventional and plasmacytoid dendritic cells. Consistent with this, supernatants of sorted B cells from B-$IL10^{-/-}$ and $IL10^{fl/fl}$ mice cultured in the presence of TLR agonists had 10-fold lower IL-10 concentrations than those from control mice (Supplemental Fig. 1). Thus, B-$IL10^{-/-}$ MRL.Fas^{lpr} 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 (GN) and interstitial nephritis (IN) in MRL.Fas^{lpr} mice is greatly enhanced by global deficiency for IL-10 (16). Severity of GN and IN in 16 week old B-$IL10^{-/-}$ and $IL10^{fl/fl}$ mice was similar, with glomeruli showing hypercellularity and collapsed capillary loops (Fig. 1*A*, 1*B*). Interstitial infiltrates were present in the perivascular, peritubular and occasionally periglomerular region in kidneys of all mice (Fig. 1*A*). Accordingly, B-$IL10^{-/-}$ mice did not have more proteinuria than $IL10^{fl/fl}$ mice (Fig. 1*C*).

Cutaneous lupus manifestations in the MRL.Fas^{lpr} strain include facial rash, ulceration of the ears and lesions of the back and neck. In female mice dermatitis occurs more frequently than in males. The extent of dermatitis was not different between female B-$IL10^{-/-}$ and $IL10^{fl/fl}$ mice (Fig. 1*D*). 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. 1*E*) and axillary lymph node (Fig. 1*F*) weight revealed no differences between B-$IL10^{-/-}$ and $IL10^{fl/fl}$ 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 B cell receptor signaling. We therefore generated a cohort of CD19-Cre and wildtype MRL.*Fas*^{lpr} mice and performed a similar analysis as for B-IL10$^{-/-}$ and IL10$^{fl/fl}$ MRL.*Fas*^{lpr} mice. We observed no significant alterations in nephritis, dermatitis, splenomegaly or lymphadenopathy in CD19-Cre mice compared to wild type animals (Supplemental Fig. 2). Thus, *CD19* hemizygosity was not a confounding factor in our study. Taken together, the analysis demonstrates that B cell specific *Il10* deletion does not aggravate end-organ disease in lupus.

B cell homeostasis is unperturbed in B-IL10$^{-/-}$ mice

It has been 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 IL10$^{fl/fl}$ mice (Fig. 2*B*). Absence of B cell-secreted IL-10 did not affect B cell subset frequencies (Fig 2*A, C, D*). In particular we did not observe significant changes in frequencies of transitional type 2 B cells (Fig. 2*C*) and B10 cells (Fig. 2*D*), both of which have been ascribed regulatory functions (7,8). For transitional type 2 B cells there was a downtrend in frequency in B-IL10$^{-/-}$ mice ($p = 0.11$), which was also evident in absolute numbers per spleen but this did not reach significance. Splenic transitional type 2 B cell numbers were $1.63\times10^6 \pm 0.23\times10^6$ (mean ± SEM) for IL10$^{fl/fl}$ mice and $1.15\times10^6 \pm 0.17\times10^6$ for B-IL10$^{-/-}$ mice ($p = 0.10$).

Auto-Ab formation is not enhanced in mice lacking IL-10 in B cells

To ask 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 IgG antinuclear and anticytoplasmic Abs. 9 of 17 (52.9%) sera from B-IL10$^{-/-}$ mice demonstrated homogenous nuclear staining and equatorial staining of mitotic chromatin, corresponding to anti-chromatin (Fig. 3*A*, *B*). A similar fraction of sera from IL10$^{fl/fl}$ mice (6 of 13, 46.2%) produced these same staining patterns (Fig. 3*A*, *B*). A speckled nuclear staining pattern, corresponding to Abs that recognize RNA or RNA-associated proteins, was observed for 41.2% of sera from B-IL10$^{-/-}$ and 30.8% of sera from IL10$^{fl/fl}$ animals. ELISAs for serum rheumatoid factor (Fig. 3*C*) and anti-nucleosome IgG (Fig. 3*D*) demonstrated similar concentrations in B-IL10$^{-/-}$ and IL10$^{fl/fl}$ mice.

In MRL.*Fas*^{lpr} mice and other lupus-prone mouse strains, auto-Abs 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. 3*E*). Similar evaluation of CD19-Cre and wild type MRL.*Fas*^{lpr} animals revealed no confounding effect of the CD19-Cre knock-in per se (Supplemental Fig. 3). We conclude that B cell-secreted IL-10 plays no role in B cell homeostasis, activation, plasmablast differentiation, and auto-Ab formation.

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 antigen 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$^{-/-}$ mice compared to IL10$^{fl/fl}$ mice (Fig. 4*A*). CD4 and CD8 staining did not reveal any changes in T cell composition in the absence of B cell-secreted IL-10 (Fig. 4*B*), including CD4$^-$CD8$^-$ cells that typically accumulate in *Fas*^{lpr} animals. In MRL.*Fas*^{lpr} mice there is a paucity of

phenotypically naïve T cells; this compartment amounted to less than 4% of all $CD4^+$ T cells in both B-$IL10^{-/-}$ and $IL10^{fl/fl}$ mice (Fig. 4*C*).

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 T helper and cytotoxic cells into IFN-γ secreting effectors. Excessive production of IFN-γ has been linked to SLE pathogenesis (28). In MRL.*Fas*^{lpr} 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 hr culture with phorbol myristate acetate (PMA) and ionomycin. The frequency of IFN-γ producing cells was decreased among $CD4^+$ (Fig. 4*D*) but not $CD8^+$ (Fig. 4*E*) T cells in B-$IL10^{-/-}$ mice. However, the effect was small. B cells can expand T_{regs} (31–33), prompting us to test whether B cells promote differentiation into T_{regs} via IL-10 secretion. We found similar percentages of $CD4^+$ T cells from B-$IL10^{-/-}$ and $IL10^{fl/fl}$ mice to be $FoxP3^+CD25^+$ (Fig. 4*F*). 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. We therefore conducted a survival analysis. The median survival of B-$IL10^{-/-}$ and $IL10^{fl/fl}$ mice was 139 and 142 days, respectively (Fig. 5). A logrank 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.*Fas*^{lpr} 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.*Fas*^{lpr} background. In the spleen of aged 10BiT MRL.*Fas*^{lpr} mice the percentage of Thy-1.1^+ cells was highest in $CD4^+FoxP3^-$ T cells (15.8%), macrophages (12.7%) and T_{regs} (11.9%) (Fig. 6*A*, *Upper*). The same cell types most frequently upregulated Thy-1.1 in axillary lymph nodes (Fig. 6*A*, *Lower*). Only very few B cells were Thy-1.1^+ (less than 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 $CD138^{hi}CD19^{lo}CD44^+$intracellular-kappa^{hi} 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. 6*B*, *Upper*) and 0.7% in axillary lymph nodes (Fig. 6*B*, *Lower*). In contrast, 78.8% of Thy1.1^+ 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. 6*C*). In young, pre-autoimmune 10BiT MRL.*Fas*^{lpr} mice, most analyzed cell subsets contained lower percentages of 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 $CD4^+FoxP3^-$ T cells and macrophages. The fraction of Thy-1.1^+ cells in the Treg compartment remained essentially unchanged (11.9% in aged mice vs. 9.7% in young mice).

In conclusion, T cells and macrophages are the predominant cell types that express *Il10* in MRL.*Fas*^{lpr} mice. IL-10 competent B cells (Thy-1.1^{+}), on the contrary, 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 have 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 demonstrate 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.*Fas*^{lpr} mice that 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 this model we maximized the opportunity for IL-10^{+} B cells to exert regulatory effects without making prior assumptions at what stage of disease those might ensue. The extent of deletion, though 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-secreted IL-10 were truly regulating spontaneous lupus in a biologically significant fashion. Thus, our work indicates 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.*Fas*^{lpr} mice (15,16), our findings are 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.*Fas*^{lpr} 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 antigen in combination with an adjuvant, resulting in 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 has been described that CD24^{hi}CD38^{hi} B cells from healthy individuals suppress T helper 1 cell differentiation in vitro in a partially IL-10 dependent manner (38). In contrast, CD24^{hi}CD38^{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*^{lpr} 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^{hi}CD5^{+} or CD21^{hi}CD23^{hi} phenotype did not spontaneously synthesize IL-10 mRNA in vivo. From these data, we cannot 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). Whether the

B cells that gave rise to IL-10 competent plasmablasts had a specific phenotype is unclear. 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. Signals that have repeatedly been found to induce IL-10 production in B cells are Toll-like receptor activation, particularly in combination with B cell receptor ligation, and CD40 stimulation (41). In MRL.*Fas*^{lpr} mice self-reactive B cells are spontaneously activated via TLR7/9 and B cell receptor cross-linking by immune complexes (42). Further CD40L deficient MRl.*Fas*^{lpr} 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. Chronic exposure to those signals, however, might have a different outcome than acute stimulation or other factors might impede a regulatory B cell phenotype in MRL.Fas^{lpr} mice.

Recently, it was reported that deletion of all mature B cells, including B10 cells, in young preautoimmune NZB/W F_1 mice accelerates disease onset and decreases survival time (12). CD19$^{-/-}$NZB/W F_1 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 employ IL-10 independent mechanisms to suppress an immune response that are potent enough to compensate for *Il10* deficiency. However, in vivo evidence for such mechanisms has yet to be presented. Rather, in essentially all papers on Bregs in which a mechanism of regulation has been demonstrated, IL-10 is implicated (4–7). Of greatest relevance to the present data, in MRL.*Fas*^{lpr} mice regulatory effects of infused B cells are clearly IL-10 dependent (15). Even if Bregs were to possess inhibitory means apart from IL-10, published studies indicate that *Il10* transcription would at least identify most regulatory B cells. Yet, the fraction of *Il10* transcribing B cells in MRL.*Fas*^{lpr} mice was very small. Our data therefore does not favor the interpretation that other factors 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), suggest 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 are enriched for *Il10* transcription (other than plasmablasts), calling into question the existence of specific Breg populations, at least in the context of ongoing lupus. Further, 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 B cell regulatory capacity in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

AIA	adjuvant-induced arthritis
ANA	anti-nuclear Ab
Breg	regulatory B cell
CIA	collagen induced arthritis
EAE	experimental autoimmune encephalomyelitis
FOL 1	follicular 1 type
MZ	marginal zone
SLE	systemic lupus erythematosus
T2	transitional 2 type

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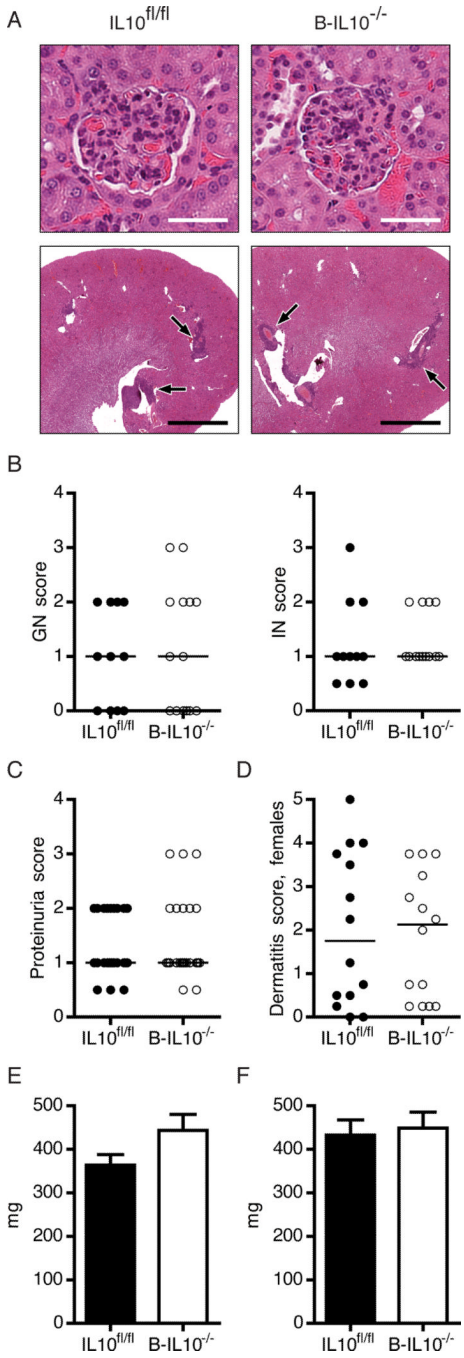

FIGURE 1.

Deletion of *Il10* in B cells does not exacerbate organ disease. *A*, Representative H&E stained kidney sections showing glomeruli (upper images) and perivascular infiltrates (lower images, black arrows). Scale bars represent 50 μm in upper and 2 mm in lower images. *B–D*, Glomerular and interstitial renal disease (*B*, n ≥ 12), proteinuria (*C*, n ≥ 25) and dermatitis severity (*D*, n = 14) were scored for B-IL10$^{-/-}$ and IL10$^{fl/fl}$ mice. Each dot represents an individu al mouse. Horizontal lines indicate the median. *E* and *F*, Weight of spleens (*E*) and the two largest axillary lymph nodes (*F*) (n ≥ 25). Data are represented as mean ± SEM in bar graphs. Data shown are combined from 5 experiments.

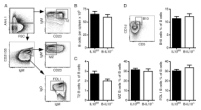

FIGURE 2.

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 $CD19^{+}TCR^{-}$ cells. Data shown are combined from 3 experiments (mean ± SEM).

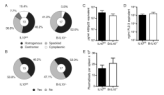

FIGURE 3.

Auto-Ab formation is not enhanced in mice lacking IL-10 in B cells. *A* and *B*, Hep-2 ANA 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 IL10$^{fl/fl}$ mice. The numbers in the circles indicate the numbers of mice analyzed in each group. *C* and *D*, 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^{lo}CD22^{lo}CD44^{hi}CD138^{hi}SSC^{int} cells. Plasmablast data are pooled from 3 experiments. Hep-2 assay and ELISAs were performed once with mouse sera prepared in 3 experiments. Data are represented as mean ± SEM in bar graphs.

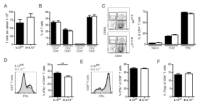

FIGURE 4.

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 $IL10^{fl/fl}$ (black bars) and B-$IL10^{-/-}$ (white bars) mice. *C*, CD44 and CD62L staining of $CD4^+$ T cells of $IL10^{fl/fl}$ (black bars) and $BIL10^{-/-}$ (white bars) mice to identify naïve ($CD44^-CD62L^+$), central-memory ($CD44^+CD62L^+$, TCM) and effector memory ($CD44^+CD62L^-$, TEM) populations. Representative flow cytometric data on gated $CD4^+$ T cells is shown on the left. *D* and *E*, Intracellular IFN-γ staining of PMA/ionomycin-stimulated splenocytes gated on $CD4^+$ (*D*) or $CD8^+$ *E*, T cells. Histograms show representative flow cytometric data. Statistics were calculated by two-tailed Mann-Whitney U test. $^{**}P < 0.01$. (*F*) Frequency of Tregs ($FoxP3^+CD25^+$) as a percentage of $CD4^+$ T cells. n ≥ 15. Data shown are combined from 3 experiments (mean ± SEM).

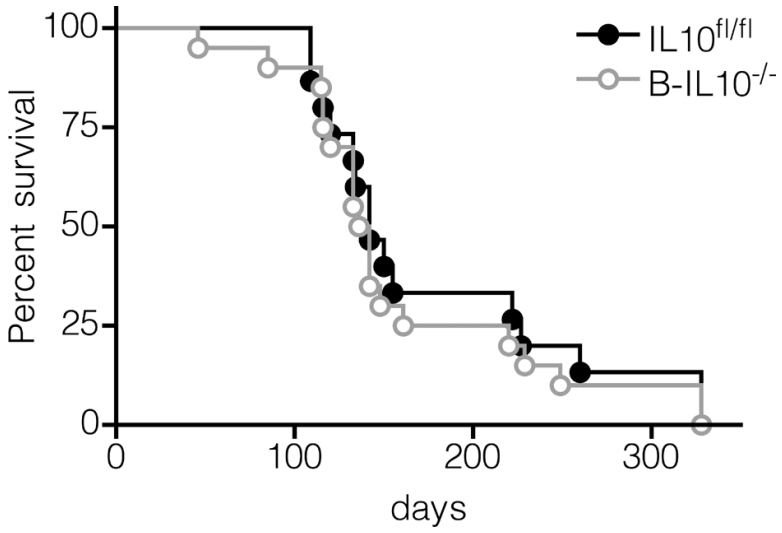

FIGURE 5.

IL-10 produced by B cells confers no survival advantage. Kaplan-Meier survival curves of $IL10^{fl/fl}$ (n = 15, 9 females and 6 males) and B-$IL10^{-/-}$ (n = 20, 12 females and 8 males) mice.

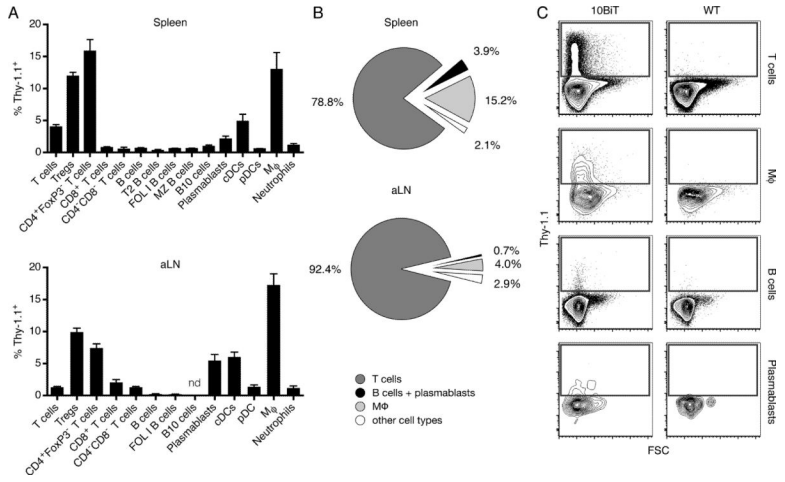

FIGURE 6.

T cells and macrophages are the main source of IL-10 in MRL.*Fas*^{lpr} mice. *A* and *B*, Flow cytometry of spleen and axillary lymph node single-cell suspensions of 16 wk old 10BiT MRL.*Fas*^{lpr} mice (n = 4). Bar graphs in (*A*) show the frequency of Thy-1.1^{+} cells in various cell populations. Pie charts in (*B*) illustrate the composition of the total Thy-1.1^{+} cell pool. *C*, Representative contour plots showing Thy-1.1 expression of T cells, macrophages, B cells and plasmablasts (CD138^{hi}CD19^{lo}CD44^{+}intracellular-kappa^{hi}). Data are combined from two experiments (mean ± SEM in bar graphs).

Table I

Deletion efficiency in B-IL10$^{-/-}$ mice.

Cell population	n	% Deletion
MZ B	3	94.1
FOL I B	3	93.2
T2 B	3	94.0
B10	3	91.1
T	2	0
MΠ	2	0
Neutrophils	3	0
cDC	2	0
pDC	2	0
B1a*	2	95.0
B1b*	3	90.9
B2*	3	88.6
PerMΠ*	3	1.4

Cell populations were purified by FACS. MZ, FOL I and T2 B cells were gated as illustrated in Fig. 2*A*. B10 cells were identified as shown in Fig. 2*D*. T cells, TCRβ^{+}CD19^{-}; macrophages (MΠ), CD11b^{+}F4/80^{hi}Gr1$^{lo\text{-}int}$; neutrophils, CD11b^{+}Gr1^{hi}; c D C s, C D 1 1 c^{hi}IA/IE^{+}CD19^{-}; pDCs, CD11c^{int}Bst2^{+}; B1a cells, CD19^{+}CD11b^{+}CD5^{+}; B1b cells, CD19^{+}CD11b^{+}CD5^{-}; B2 cells, CD19^{+}CD11b^{-}. peritoneal MΠ: CD11b^{+}F4/80^{hi}. To calculate % deletion values the equation (1 − residual *Il10*) × 100 was used. Residual *Il10* values were calculated as $2^{-\Delta\Delta Ct}$ comparing B-IL10$^{-/-}$ and IL10$^{fl/fl}$ mice. Negative % deletion values were set to 0.

*Measurement of the amount of residual *Il10* by quantitative PCR in various cell types of B-IL10$^{-/-}$ mice in the spleen and peritoneal lavage.

Table II

Il10 transcription is strongly induced in $CD4^+FoxP3^-$ T cells and macrophages during lupus.

Cell population	n	% Thy-1.1$^+$	
		16 wk	5 wk
T cells	4	3.97	2.02
Tregs	4	11.92	9.74
$CD4^+FoxP3^-$ T cells	4	15.81	0.89
$CD8^+$ T cells	4	0.75	1.08
$CD4^-CD8^-$ T cells	4	0.52	2.27
B cells	4	0.63	0.10
T2 B cells	4	0.30	nd
FOL I B cells	4	0.59	0.03
MZ B cells	4	0.61	nd
B10 cells	4	0.94	0.28
Plasmablasts	4	2.09	
cDCs	4	4.86	0.13
pDCs	4	0.55	0.87
MΠ	4	12.96	0.53
Neutrophils	4	1.13	0.10

Flow cytometry of spleen single cell suspensions of 16 and 5 wk old 10BiT MRL.Fas^{lpr} mice. The data for the 16 wk old mice is the same as in bar graph Fig. 6*A* and is shown here in tabular form for easier comparison to the results obtained from 5 wk old animals. nd, no detectable Thy-1.1 expression.