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Differential Influence on Regulatory B Cells by T_H2 Cytokines Affects Protection in Allergic Airway Disease

Sophina H. Taitano,^{*,†} Luciën E. P. M. van der Vlugt,^{*} Molly M. Shea,^{*} Jennifer Yang,^{*} Nicholas W. Lukacs,^{†,‡} and Steven K. Lundy^{*,†}

The role of regulatory B cells (Bregs) in modulating immune responses and maintaining tolerance are well established. However, how cytokines present during immune responses affect Breg growth and function are not as well defined. Previously, our laboratory reported IL-5- and mCD40L-expressing fibroblast (mCD40L-Fb) stimulation induced IL-10 production from murine B cells. The current study investigated the phenotype and functional relevance of IL-10- producing B cells from this culture. We found IL-5/mCD40L-Fb stimulation induced IL-10 production exclusively from CD5⁺ splenic B cells of naive mice. After stimulation, the resulting IL-10⁺ B cells displayed markers of multiple reported Breg phenotypes. Interestingly, when investigating effects of IL-4 (a critical T_H2 cytokine) on IL-5/mCD40L-Fb-induced IL-10 production, we found IL-4 inhibited IL-10 production in a STAT6-dependent manner. Upon adoptive transfer, CD5⁺ B cells previously stimulated with IL-5/mCD40L-Fb were able to reduce development of OVA-induced allergic airway disease in mice. Using B cells from IL-10 mutant mice differentiated by IL-5/mCD40L-Fb, we found protection from allergic airway disease development was dependent on the IL-10 production from the transferred B cells. Bregs have been shown to play crucial roles in the immune tolerance network, and understanding stimuli that modulate their growth and function may be key in development of future treatments for diseases of immune dysregulation. *The Journal of Immunology*, 2018, 201: 000–000.

Allergic airway disease (AAD), also known as allergic asthma, is a chronic inflammatory disease of the lungs induced by inhalation of innocuous Ags such as pollen, pet dander, and house dust mite (1). These Ags trigger an inappropriate T_H2 cell response resulting in an influx and accumulation of lymphocytes and granulocytes into the lungs. IL-4 from T_H2 cells induces class-switching of Ag-specific B cells to IgE, which binds receptors on mast cells and activates degranulation upon Ag binding. Inflammatory components from mast cells and eosinophils induce bronchial hyper-reactivity of the smooth muscles, causing airway narrowing. Eosinophilopoiesis and efflux from the bone marrow is driven by the type 2 cytokine IL-5 and results in hyperplasia and metaplasia of mucous-producing goblet cells, airway remodeling, and obstruction of the airways. Although treatments to control symptoms are available, there is

currently no cure for AAD and patients are often on life-long medications.

IL-4 and IL-5 are products of T_H2 cells that drive protective humoral immunity against extracellular microorganisms but also mediate pathogenesis in allergic diseases. Although IL-13 is also a T_H2 cytokine, B cells do not express the IL-13 receptor and therefore will not be discussed further (2, 3). In mice, IL-4 and CD40L stimulation from T_H2 cells is well documented to induce Ab class-switching, proliferation, and differentiation of follicular B cells into Ab-secreting memory and plasma cells that produce IgG1 and IgE Ab isotypes. IL-5 supports IL-4-induced class-switching to IgG1 and IgE but is not sufficient by itself (4). IL-5 also acts on terminally differentiated IgA-producing B cells in the mucosal lymphoid tissues to increase IgA secretion (5, 6).

Beyond follicular B cells, studies in IL-5Rα-deficient mice demonstrate IL-5 as a critical growth and/or survival factor for CD5⁺ B-1a cell development (7–9). B-1a cells are also the only B cells to constitutively express the complete IL-5R heterodimer consisting of the IL-5Rα and common β-chain. B-1a cells are the major source of natural IgM, contributing 80–90% of resting serum levels and estimated to make 50% of resting serum IgA (10–12). These cells reside in the pleural and peritoneal cavities with a minority present in the spleen and arise from progenitors distinct from B-2 cells that are present in the fetal liver and omentum (13). The Abs expressed by these cells are polyreactive and self-reactive, allowing for broad protection from pathogens and a potential housekeeping role in apoptotic cell removal (14–16). In addition, B-1a cells have also been found to be as potent of APC as dendritic cells (DCs) and are able to induce naive T cell proliferation and activation directly ex vivo without additional stimulation (17–19). Their self-reactivity and APC abilities have led to speculation of their involvement in autoimmune diseases, and there is evidence in autoimmune mouse models to support this (17, 18). Conversely, their reported IL-10 production and immune suppressive abilities suggest self-reactivity, and APC functions position them as dominant regulators of the

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The online version of this article contains supplemental material.

Abbreviations used in this article: AAD, allergic airway disease; AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; Breg, regulatory B cell; DC, dendritic cell; FasL, Fas ligand; MLN, mediastinal lymph node; PI, PMA/ionomycin; SIT, specific allergen immunotherapy; Treg, regulatory T cell.

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immune system (20, 21). CD5⁺ B cells have had reported regulatory functions for over two decades (22). Multiple groups have reported regulatory function of CD5⁺ B cells in several models of allergic disease and autoimmunity (23–30).

Regulatory B cells (Bregs) are now considered important modulators of the immune response, displaying suppressive capacity in multiple mouse models of autoimmunity and allergy. They employ an array of mechanisms to alter the immune response, including surface expression of Fas ligand (FasL) and PD-L2 and the secretion of anti-inflammatory cytokines TGF- β and IL-10 (22, 31–35). Secretion of IL-10 is the most extensively studied regulatory mechanism used by Bregs in both mice and humans. Almost every subset of B cells has in part been shown to produce IL-10 after a variety of different stimuli. These include stimulation through CD40L, BCR, and multiple TLR, IL-35, IL-21, and IL-4 (36). Previously, we reported that stimulation with IL-5 and mCD40L-Fb for 5 d resulted in growth of B cells with regulatory functions (33), with increases in both FasL expression and IL-10 production. The breadth of B cells able to produce IL-10 lends evidence to the belief that there is no unifying phenotype that defines IL-10-producing Bregs. Therefore, demonstration of functional capacity defines Bregs.

In the current study, the phenotypic and functional responses of mouse B cells to stimulation through CD40 in the presence of T_H2 cytokines IL-4 and IL-5 were compared. In combination with mCD40L-Fb stimulation, IL-4 and IL-5 had independent and cooperative growth-promoting effects on mouse B cells. Stimulation with IL-5/mCD40L-Fb induced the production of IL-10 exclusively from CD5⁺ B cells, whereas the presence of IL-4 blocked IL-10 production through a STAT6-dependent signaling pathway. The functional capacity of these IL-10 producers was assessed in OVA-induced AAD model in BALB/c mice. CD5⁺ B cells from the IL-5/mCD40L-Fb cultures attenuated the development of AAD in vivo in an IL-10-dependent manner. Our findings may provide insights for the development of more effective treatments for severe allergies and other T_H2-mediated diseases through the activation of endogenous Bregs.

Materials and Methods

Mice

All experiments unless otherwise specified were performed using 8–12-wk-old female BALB/cJ (strain no. 000651) mice obtained from The Jackson Laboratory (Bar Harbor, ME). Female DBA/1 mice were compared with female C57BL/6J (strain no. 000664) mice and female BALB/cJ mice purchased from The Jackson Laboratory. STAT6 knockout (strain no. 002828) and IL-10 mutant (strain no. 004333) breeding pairs were purchased from The Jackson Laboratory on the BALB/cJ background. All mice were housed in specific pathogen-free facilities. Animal protocols and husbandry practices were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Mouse B cell culture on mCD40L-fibroblasts

NIH3T3 fibroblasts stably transduced with the gene for mouse CD40L (mCD40L-Fb) were generously provided by Dr. D. Fox and Dr. K. McDonagh (37), and a detailed description of their generation has been previously published. Fibroblasts were maintained in DMEM/10% calf serum and grown to 80–90% confluence before removal from culture flasks with 0.25% trypsin solution. Prior to coculture with B cells, fibroblast lines were exposed to 30 Gy of γ radiation to inhibit their proliferation. Purified mouse B cells were obtained from single-cell suspensions derived from the spleens of naive 8–12-wk-old mice by anti-mouse CD19 MACS magnetic bead positive selection (Miltenyi Biotec, Auburn, CA) using the manufacturer protocol. Purified B cells and mCD40L-Fb were counted using a hemocytometer, washed, then resuspended in B cell culture medium containing 1 $\times$ DMEM (SH30243.01; Hyclone), 10% heat-inactivated FCS, 5% penicillin-streptomycin, and 0.5% insulin/transferrin/selenium (51500-056; Life Technologies). All cultures in the main body of the manuscript were done using DMEM-based media, and comparison cultures using

RPMI 1640 (SH30027.01; HyClone) are shown in Supplemental Fig. 1. B cells and fibroblasts were plated at a 4:1 ratio in flat-bottom plates. For all figures, 200,000 B cells (1 million cells/ml) and 50,000 fibroblasts were used. IL-4 (50 ng/ml; R&D Systems), IL-5 (50 ng/ml; PeproTech), IL-21 (100 ng/ml; PeproTech), LPS from *Escherichia coli* strain O111:B4 (10 μ g/ml; Chemicon/Millipore), or anti-IgM (10 μ g/ml; Jackson ImmunoResearch) were added at the beginning of the cultures. Primary cultures of naive splenic B cells were stimulated for 72 h or 5 d as indicated, and secondary cultures were stimulated with fresh mCD40L-Fb and cytokines for 3 d. B cells from primary culture were counted using a hemocytometer and washed before being put into secondary culture. Control cultures were performed for all assays using nontransduced NIH3T3 fibroblasts (American Type Culture Collection, Rockville, MD), none of which yielded cytokines or viable B cells after 5 d.

Flow cytometry

A list of fluorochrome-conjugated mAbs specific for mouse cell markers and their clone numbers and dilutions used are listed in Supplemental Table I. 7-AAD (Sigma-Aldrich) was used to label and exclude dead cells, and nonspecific binding to Fc receptors was blocked using anti-CD16/CD32 (Fc Block; BD Biosciences). Cells were stained with the indicated Abs in a suspension of PBS containing 0.5% BSA and 0.1% sodium azide for 30 min at 4°C. Staining for apoptosis was done in annexin V staining buffer (140 mM sodium chloride and 2.5 mM Calcium Chloride dehydrate) for 30 min at 4°C. Samples were analyzed with a BD LSR II flow cytometer, and the data were analyzed using FlowJo 10 software (Tree Star).

IL-10 intracellular staining on B cells was done after 5 d of culture with IL-5 or IL-4 and mCD40L-Fb. Prior to intracellular staining, B cells were further stimulated at 6 million/ml with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) (PMA/ionomycin [PI]) for 4 h at 37°C. During the last hour of PI stimulation, GolgiPlug (BD Biosciences) containing brefeldin A was added at 10 μ g/ml. After stimulation, the cells were stained using Zombie Violet Fixable Viability Dye (BioLegend) according to manufacturer's protocol to exclude dead cells. The cells were stained with surface markers as previously described, then fixed and permeabilized (Foxp3 Fixation/Permeabilization Kit; eBioscience) for IL-10 intracellular staining following the manufacturer's protocol.

To sort CD5⁺ splenic murine B cells, CD19⁺ B cells were first isolated using anti-mouse CD19 MACS magnetic bead positive selection (Miltenyi Biotec) using manufacturer's protocol. The cells were stained with CD5-PE (BioLegend) and then sorted using BD FACSAria II.

ELISA

IL-10, IL-6, and IL-4 proteins were assessed in the supernatant by sandwich ELISA kits purchased from BD Biosciences using the manufacturer protocols.

AAD model

To induce a systemic T_H2 response, BALB/cJ mice were immunized with 20 μ g of OVA protein in 2 mg of alum on day 0 and 7. On day 19, CD5⁺ or CD5[−] B cells were sorted from IL-5/mCD40L-Fb cultures of splenic BALB/c or IL-10 mutant B cells. Two million of the indicated B cell subset or PBS was tail vein injected into the OVA-sensitized mice. Starting 12 h after B cell transfer, mice were challenged with 10 μ g of OVA protein in 50 μ l 1 $\times$ PBS by forced aspiration daily for 3 d (day 20–22). Mice were anesthetized for each challenge with a 4% xylazine, 10% ketamine, and 86% PBS solution. On day 23, the mice were evaluated for airway hyperresponsiveness (AHR) and then sacrificed prior to further analysis of lung physiology and immune responses.

AHR by plethysmography

The mouse to be tested was anesthetized with sodium pentobarbital and subsequently intubated intratracheally with an 18-gauge metal tube. The mouse is attached to a Harvard pump ventilator (tidal volume, 0.4 ml; frequency, 120 breaths per min; positive end-expiratory pressure, 2.5 to 3.0 cm H₂O; Harvard Apparatus, Holliston, MA), and the plethysmograph is sealed to determine baseline resistance. The Buxco software calculates resistance by dividing the change in pressure by the change in flow (pressure/flow; U = cm H₂O/ml/s). The mouse is then challenged with 0.2 mg of methacholine (previously determined as the optimal dose) by tail vein injection. Peak resistance is recorded, and the difference between the peak and baseline resistance was reported as a measure of AHR.

Bronchoalveolar lavage fluid and lung collection

A solution of 3 mM EDTA in PBS (3 $\times$ 1 ml/mouse) was used to lavage the bronchoalveolar space for extraction of cells and cytokines. Lungs were then perfused with 10 ml 1 $\times$ PBS before collection of the tissue that was

then minced and digested with 0.7 mg/ml of collagenase IV in RPMI 1640 for 50 min at 37°C. Cells were extracted from the lung tissue by mechanical force and then stained for flow cytometry.

Statistical analysis

Data presented are from representative experiments of at least three experiments performed for each assay. Cultures were done in triplicate wells in every experiment. Asterisks signify * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and n.s., not significant in all graphs.

Results

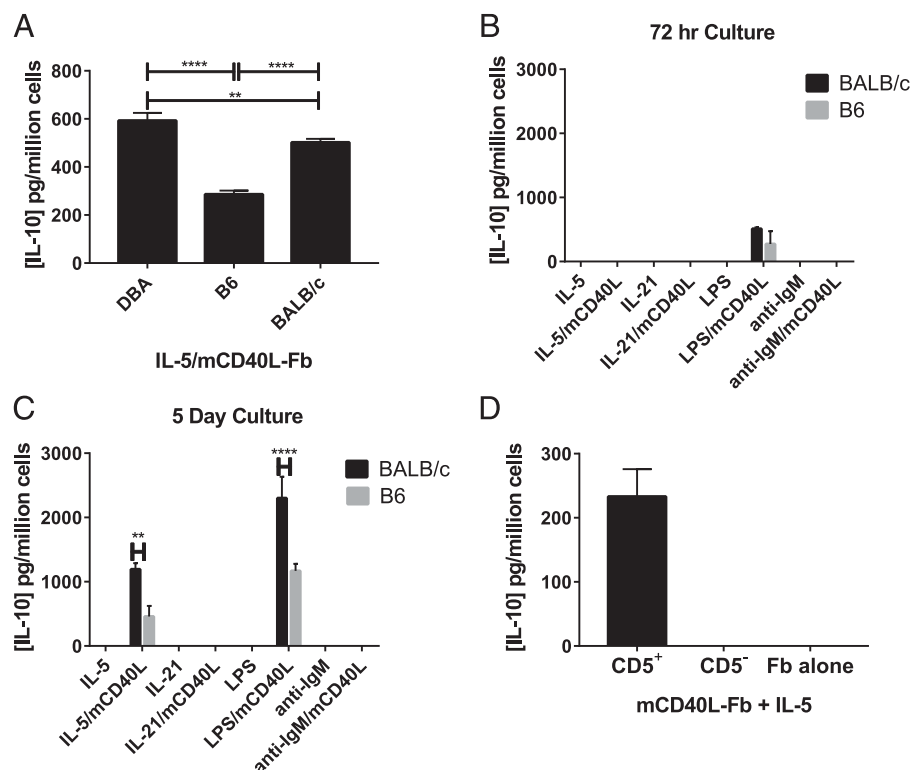
CD5⁺ B cells are induced by IL-5 to produce IL-10

We have previously shown that culture of purified mouse splenic B cells from DBA/1 mice with mCD40L-Fb in the presence of IL-5 supported the growth of FasL⁺ B cells (33). In addition to having enhanced killer function against Ag-specific T cells, these B cells also produced IL-10. The current study aimed to determine the protective ability of these Bregs in the context of AAD, which is usually modeled in C57BL/6 or BALB/c mice. To confirm IL-5-stimulated production of IL-10 from B cells was not a strain specific phenomenon, we cultured B cells from DBA/1, C57BL/6, and BALB/c mice with mCD40L-Fb and IL-5 for 5 d. IL-5/mCD40L-Fb induced IL-10 production from B cells of all three strains; however, B cells from DBA/1 mice produced slightly more IL-10 than those from BALB/c mice, and B cells from C57BL/6 mice made significantly less IL-10 than B cells from DBA/1 or BALB/c mice (Fig. 1A). Multiple types and durations of stimulations have been reported to induce IL-10 production from Bregs. To compare the levels of IL-10 produced by IL-5/mCD40L-Fb stimulation to other reported Bregs stimuli, we cultured B cells with IL-5, IL-21, LPS, and anti-IgM with or without mCD40L-Fb for 72 h or 5 d. After 72 h of culture, only B cells stimulated with LPS and mCD40L-Fb produced IL-10 (Fig. 1B). After 5 d of culture, IL-10 was detectable from the IL-5/mCD40L-Fb and LPS/mCD40L-Fb culture (Fig. 1C). Although levels of IL-10 in the LPS/mCD40L-Fb cultures were higher than IL-5/mCD40L-Fb, LPS also induced higher levels of IL-6

(Supplemental Fig. 1B). B cells from BALB/c mice secreted significantly more IL-10 than C57BL/6 B cells in response to both IL-5 or LPS and mCD40L-Fb (Fig. 1C). Interestingly, IL-10 was undetectable in cultures containing IL-21 and anti-IgM^{+/−} mCD40L-Fb (Fig. 1B, 1C). Differences in culture media used (DMEM or RPMI 1640) by other groups may contribute to these contrasting results. Therefore, these cultures were repeated to compare DMEM and RPMI 1640 media (Supplemental Fig. 1). Interestingly, B cells cultured in RPMI 1640 produced IL-10 by 72 h with LPS stimulation alone, and C57BL/6 B cells produced more IL-10 than BALB/c B cells in response to LPS. However, IL-10 was still undetectable after stimulation with IL-21 or anti-IgM^{+/−} mCD40L-Fb. In summary, IL-5/mCD40L-Fb cultures resulted in slightly less IL-10 secretion and magnitudes less IL-6 secretion compared with LPS/mCD40L. IL-10 production varied depending on the stimulation, media, and mouse strain used. To further explore the protective effects of IL-5/mCD40L-Fb-stimulated IL-10⁺ B cells in the context of AAD, B cells from BALB/c strain background were used for all subsequent experiments except where noted.

CD5⁺ B cells are one of the subsets extensively reported to produce IL-10 after a variety of stimulations (38). To determine if CD5⁺ B cells were the IL-10 producers, we sorted CD5⁺ and CD5[−] splenic B cells from naive BALB/c mice by fluorescence-activated cell sorting and cultured them with IL-5/mCD40L-Fb. Only CD5⁺ B cells but not CD5[−] B cells produced detectable levels of IL-10 in culture supernatants (Fig. 1D). Similar results were found using DBA mice (data not shown). Intracellular staining for IL-10 was assessed after IL-5/mCD40L-Fb culture to further investigate phenotypic similarities to other reported Bregs. IL-10⁺ B cells were highly enriched for expression of CD5, CD9, IgM, CD43, and CD80 and rarely expressed CD23 (Fig. 2, Supplemental Fig. 2). IL-10⁺ B cells were also enriched in CD1d^{hi} B cells, a marker of a CD5⁺IL-10⁺ subset termed B10 cells. However, CD1d^{hi} B cells made up merely 6% of IL-10⁺ B cells. The IL-10-producing B cells after culture were moderately enriched for CD5, and they

FIGURE 1. IL-5/mCD40L-Fb stimulation induces IL-10 production from CD5⁺ B cells. (A–C) Total splenic B cells or (D) sorted CD5⁺ or CD5[−] B cells were cultured with IL-5/mCD40L-Fb or (B and C) the indicated stimulation for (B) 72 h or (A, C, and D) 5 d. (A–D) IL-10 in culture supernatant was assessed by ELISA. Data are from a single representative experiment of three independent experiments (mean ± SEM) with at least three mice per experiment. (A–D) Tukey multiple comparison test was used for statistics. ** $p < 0.01$, *** $p < 0.0001$.



expressed very high levels of CD9 and CD80, which are surface molecules that have been suggested as universal Breg markers (39). The phenotype of IL-10⁺ BALB/c and C57BL/6B cells after IL-5/mCD40L-Fb culture was similar for all markers except CD1d^{hi}, which was expressed on more C57BL/6 IL-10⁺ B cells (Supplemental Fig. 3). In conclusion, CD5⁺ B cells from naive mice produce IL-10 after IL-5/mCD40L-Fb stimulation and express additional Breg markers after stimulation.

IL-4 inhibits IL-10 production from CD5⁺ B cells via the STAT6 signaling pathway

IL-5 is a major product of T_H2 cells activated during allergic reactions, and it was not clear why the stimulation of Breg functions by IL-5 is not sufficient to prevent development of AAD endogenously. In addition to IL-5, IL-4 is also present at high levels during T_H2 responses, and therefore, we investigated the effects of IL-4 on IL-5-induced IL-10 production by B cells. Sorted CD5⁺ splenic B cells were cultured with mCD40L-Fb alone with no cytokine or with IL-5, IL-4, or both cytokines. Higher cell numbers were observed when CD5⁺ B cells were stimulated with IL-5/mCD40L-Fb but not IL-4/mCD40L-Fb compared with mCD40L-Fb alone (Fig. 3A), suggesting IL-5 was used as a

growth factor, whereas IL-4 was not. IL-4 stimulation blocked IL-10 production irrespective of the presence of IL-5 (Fig. 3B). Further, IL-4 resulted in suppression of IL-10 mRNA similar to protein levels (data not shown). The release of IL-10 did not occur until day 4 of B cell stimulation with IL-5/mCD40L-Fb (Fig. 1C).

Based on our findings, we questioned whether IL-4 blockade of the generation of IL-10⁺ B cells would also affect existing IL-10⁺ B cells and if so, whether this inhibition could be overcome. To address these questions, we sorted splenic CD5⁺ B cells and cultured them first with IL-5/mCD40L-Fb to generate IL-10⁺ B cells or IL-4/mCD40L-Fb to determine if effects of IL-4 could be circumvented. These first cultures will be referred to as the primary culture. After primary culture, B cells were recultured in a secondary culture of mCD40L-Fb alone or in the presence of IL-5, IL-4, or both cytokines. Only B cells from the IL-5/mCD40L-Fb primary culture secreted IL-10 in the secondary culture, suggesting the effects of IL-4 in the primary culture are long lasting (Fig. 3D). IL-10 production induced by mCD40L-Fb stimulation alone was not inhibited in the presence of IL-4 in the secondary culture. However, the addition of IL-5 enhanced mCD40L-induced IL-10 production. This enhancement by IL-5 was significantly reduced by the presence of IL-4 (Fig. 3D).

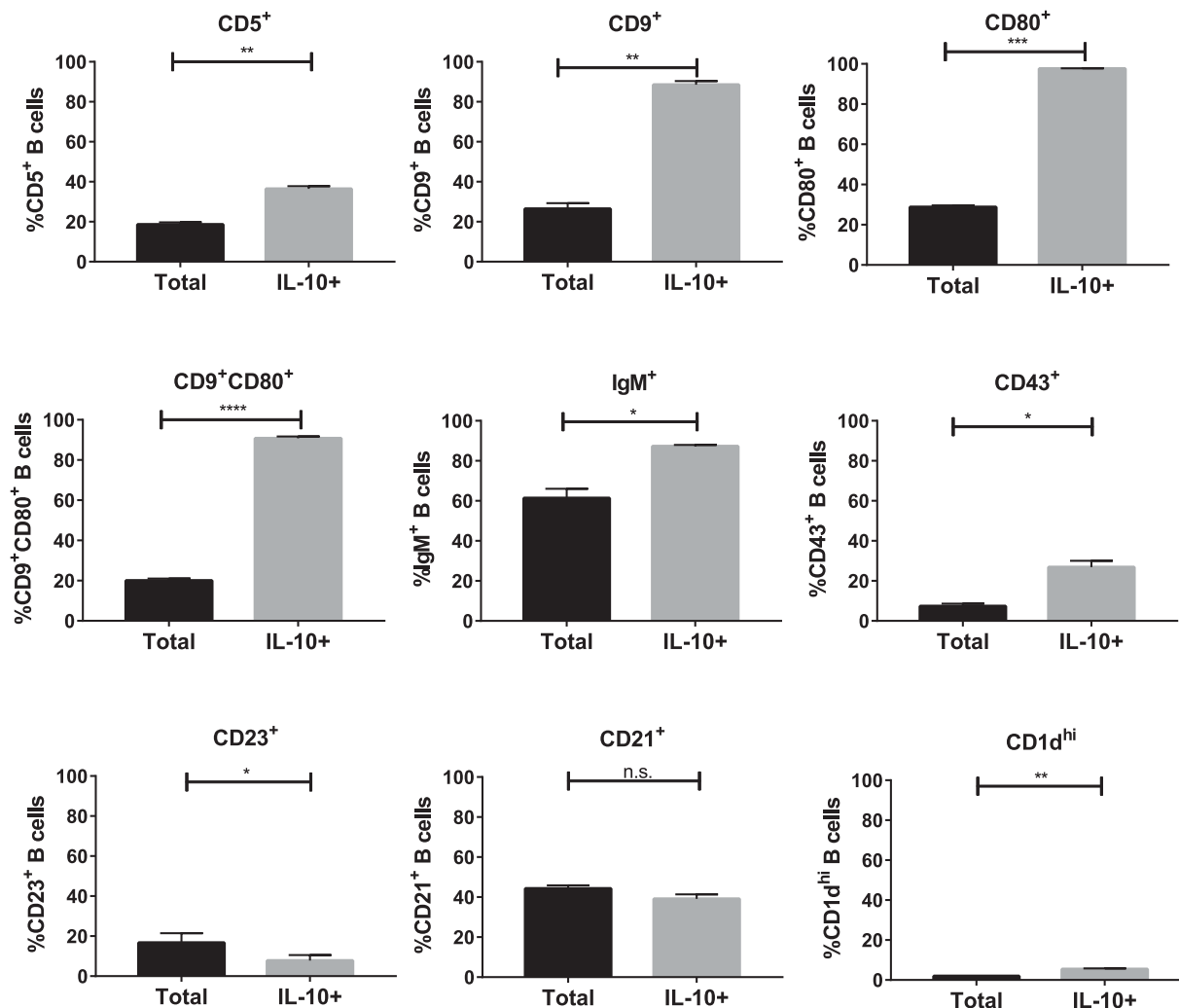


FIGURE 2. Phenotype of IL-10⁺ B cells after IL-5/mCD40L-Fb stimulation. Total splenic BALB/c B cells were cultured for 5 d with IL-5/mCD40L-Fb. IL-10 intracellular staining was performed on cells from the culture after PI/brefeldin A restimulation. Flow cytometry data on B cell markers and their expression in the total B cell population or IL-10⁺ B cells is summarized in bar graphs. Data are from a single representative experiment of three independent experiments (mean ± SEM) with at least three mice per experiment. Two-tailed paired *t* test were used for statistics. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. n.s., not significant.

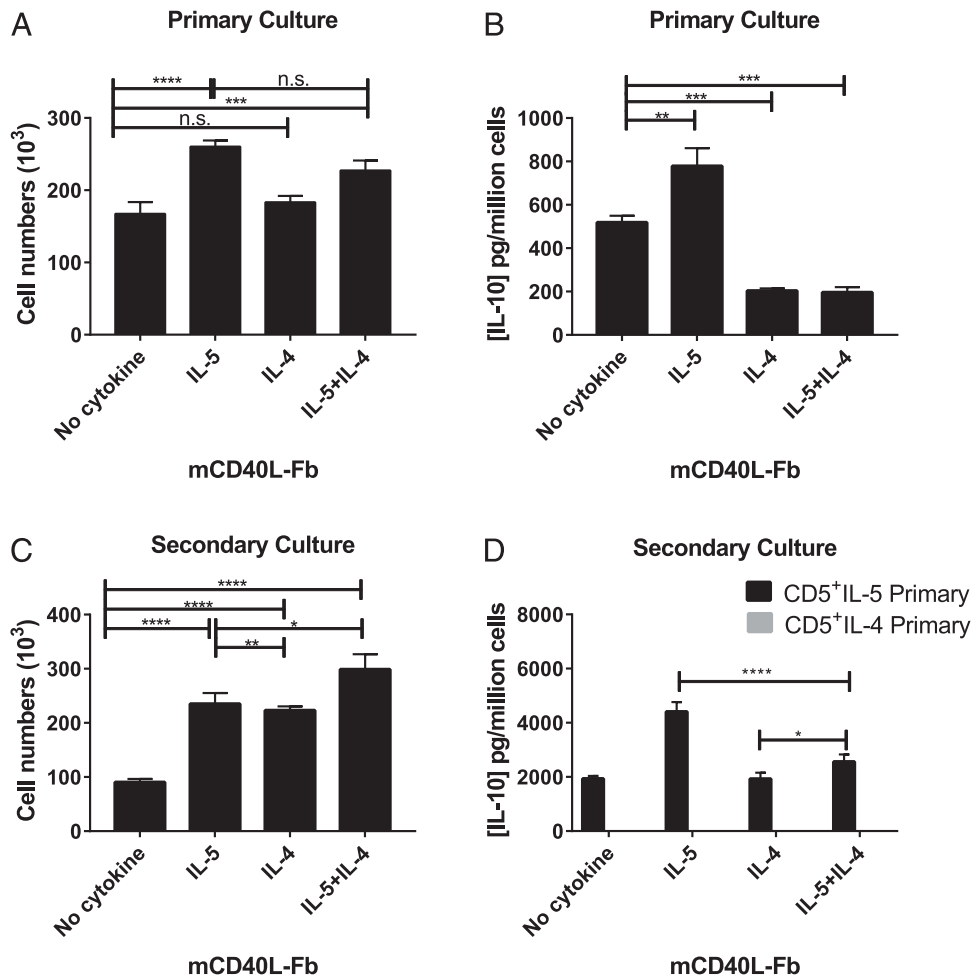


FIGURE 3. IL-4 inhibits IL-10 production from CD5⁺ B cells. (A–D) Sorted CD5⁺ B cells were cultured with mCD40L-Fb and the indicated cytokines in primary or secondary culture. (A and C) Cells were counted after culture, and (B and D) supernatants were collected for IL-10 ELISA. (C and D) CD5⁺ B cells from primary culture with IL-5/mCD40L-Fb were washed, counted, and then recultured with mCD40L-Fb and the indicated cytokines. Data are from a single representative experiment of three independent experiments (mean $\pm$ SEM). (A–D) Tukey multiple comparison test was used for statistics. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n.s., not significant.

To further explore the mechanism of IL-4 inhibition of IL-10 production in primary cultures, CD5⁺ splenic B cells from STAT6^{-/-} mice were used to determine whether the effects of IL-4 were dependent on activation of this major signaling pathway. The frequency and number of CD5⁺ B cells from naive STAT6^{-/-} mice were similar to BALB/c, therefore STAT6 deficiency did not affect CD5⁺ B cell development (Fig. 4A, 4B). In contrast to effects on BALB/c B cells, IL-4 failed to inhibit IL-10 production induced by IL-5/mCD40L-Fb stimulation in STAT6^{-/-} B cells (Fig. 4C) in primary culture. In conclusion, the data suggest that in the primary culture, IL-4 blocks the initiation of IL-10 production in a STAT6-dependent manner.

IL-5/mCD40L-Fb-stimulated CD5⁺ B cells reduced AAD

The finding that IL-4 did not significantly inhibit baseline mCD40L-stimulated Breg production of IL-10 in vitro in secondary culture suggested that these established Bregs might be resistant to the effects of IL-4 in vivo. To test this, an OVA-induced allergy model was used to determine the in vivo suppressive abilities of the IL-5/mCD40L-Fb-stimulated CD5⁺ B cells on OVA-sensitized mice following adoptive transfer. CD5⁺ and CD5⁻ B cells sorted from IL-5/mCD40L-Fb cultures were i.v. injected into OVA-sensitized mice, followed by three daily airway challenges by forced aspiration. Using plethysmography, a decrease in airway

resistance was observed only in CD5⁺ B cell recipient mice compared with allergic controls (Fig. 5A). CD5⁺ B cell transfer significantly reduced the amount of IL-4 detected in the bronchoalveolar lavage (BAL) fluid (Fig. 5B), whereas IL-5 levels were unchanged and IL-10 was undetectable (data not shown). CD5⁺ B cell recipients also had significant reduction in total cells and eosinophils in the BAL fluid (Fig. 5C, 5D). In contrast, transfer of CD5⁻ B cells from IL-5/mCD40L-Fb culture resulted in similar airway resistance, cytokine levels, and cell numbers in the BAL as allergic controls, conferring no protection. Induction of Foxp3⁺ regulatory T cells (Tregs) in the lungs and mediastinal lymph nodes (MLNs) were assessed; however, no differences were found between the treatment groups (Fig. 5E, 5F). To summarize, transfer of CD5⁺ but not CD5⁻ B cells from the IL-5/mCD40L-Fb cultures reduced the development of airway hyper-responsiveness of the lungs and cellular infiltration and IL-4 production in the BAL fluid of mice in this model of acute AAD.

Protective effects of CD5⁺ B cells are IL-10 dependent

Last, we investigated the relative importance of IL-10 production from CD5⁺ B cells for protection against AAD development. B cells from IL-10 mutant (IL-10^{mut}) and wild-type BALB/c mice were cultured with IL-5/mCD40L-Fb, and CD5⁺ B cells were subsequently sorted and adoptively transferred into OVA-sensitized

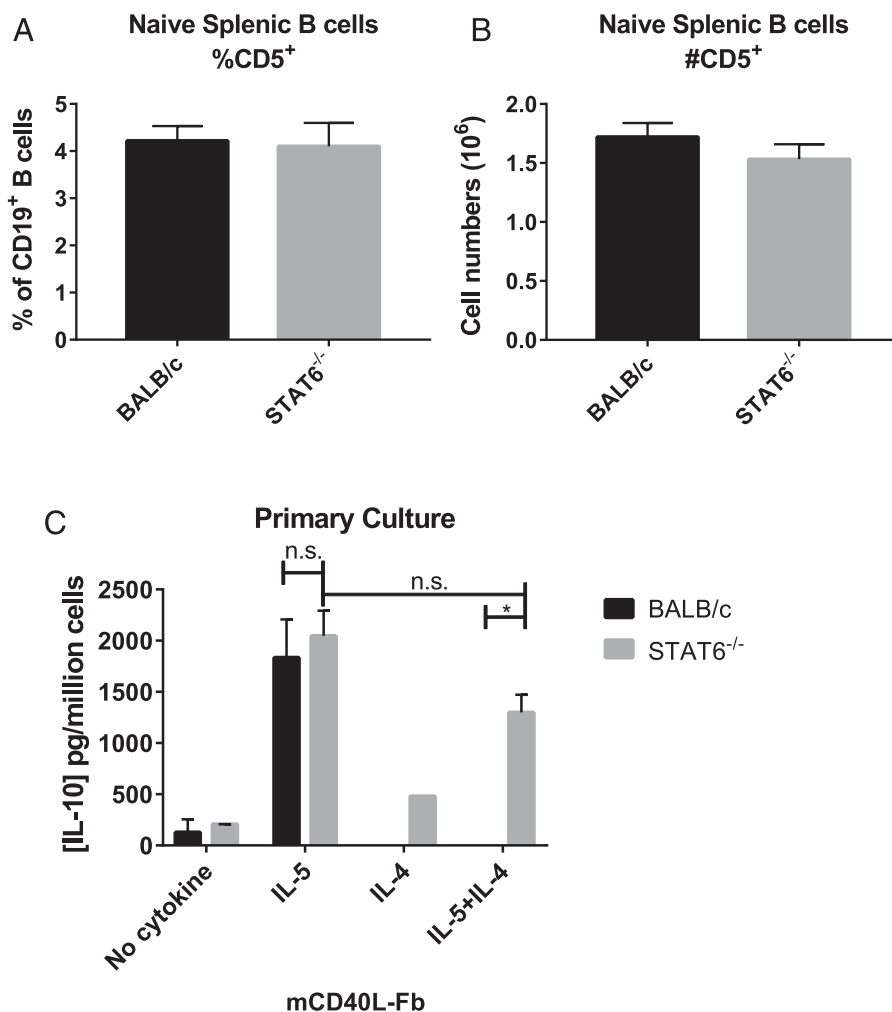


FIGURE 4. IL-4 inhibition of IL-10 is mediated through STAT6. **(A and B)** Percentage and absolute numbers of CD5⁺ B cells ex vivo from spleens of naive BALB/c and STAT6^{-/-} mice were assessed by flow cytometry. Data were obtained from 10 mice from three independent experiments (mean $\pm$ SEM). **(C)** Sorted CD5⁺ B cells from these mice were stimulated with mCD40L-Fb and the indicated cytokines for 5 d in primary culture. Culture supernatants were collected for IL-10 ELISA. Data are from one representative experiment of three independent experiments (mean $\pm$ SEM) with at least three mice of each strain in each experiment. Statistics were done using an unpaired Mann–Whitney *U* test (A and B) or Tukey multiple comparison test (C). **p* < 0.05. n.s., not significant.

mice. Protection of lung function and reduced airway resistance were lost compared with WT CD5⁺ B cell recipients (Fig. 6A). Furthermore, the reductions in IL-4 levels and total cell and eosinophils numbers in the BAL were abolished in the absence of IL-10 secretion by the transferred B cells (Fig. 6B–D). These data showed protection conferred by CD5⁺ B cells from the IL-5/mCD40L-Fb cultures in these experiments was only dependent on their IL-10 secretion and could not be attributed to other suppressive mechanisms.

Discussion

Bregs are essential modulators of the immune system. A fraction of B cells within most subsets have reported regulatory capacity in response to a variety of stimulations (36). Previously, we reported that IL-5/mCD40L-Fb costimulation induced B cell IL-10 production (33). CD40L stimulation is known to induce IL-10 production from B cells; however, that the addition of IL-5 enhanced mCD40L-Fb is a novel finding. The current study identified the phenotype of the IL-10 producers and defined the effects of individual and combined stimulation by T_H2 cytokines IL-5 and IL-4 on mCD40L-Fb-induced IL-10 production from B cells. CD5⁺ B cells stimulated with IL-5/mCD40L-Fb in vitro displayed a potent suppressive capacity in vivo that was dependent on IL-10 as assessed in a murine AAD model.

IL-5 enhanced mCD40L-Fb-induced IL-10 production exclusively from splenic CD5⁺ B cells of naive mice. Confinement of IL-10 producers to the CD5⁺ B cell compartment suggests they are B-1a lymphocytes. Strain differences in amounts of IL-10 produced were

observed. Others also have reported reduced CD5⁺ B-1a lymphocyte frequencies and IL-10-producing capabilities in C57BL/6 mice compared with BALB/c mice (30, 40). Multiple studies have shown murine and human B-1a cells produce IL-10 in a regulatory capacity (28–30, 41). Their BCRs recognize microbial pathogens, self-antigens, and potentially allergens (16, 42, 43), and they are potent APCs (17, 18, 44). The combination of their BCR specificity, regulatory capacity, and APC function position B-1a cells to be an effective immune regulatory subset in allergy and autoimmunity.

A comparison was made between B cells from BALB/c and C57BL/6 mice and various culture stimulations reported to induce IL-10 secretion. Only stimulation with LPS/mCD40L and IL-5/mCD40L induced detectable IL-10 in the supernatants in DMEM media, with BALB/c B cells secreting more than C57BL/6. Interestingly, when the media was made with RPMI 1640, IL-10 was also detectable from cultures with only LPS, which induced greater IL-10 secretion from C57BL/6 than BALB/c B cells. The Breg field is potentially confusing because of the variety of phenotypes and stimulations used by different investigators. Our data further illustrate that differences are introduced depending on the strain of mouse and type of media used. Therefore, differences in methods may explain why our study did not find IL-10 produced in response to IL-21 or anti-IgM as others have and highlight the necessity for publication of detailed methods.

After IL-5/mCD40L-Fb stimulation, CD9 was expressed on the majority of IL-10⁺ B cells. CD9 is a tetraspanin family transmembrane protein found on B-1a, marginal zone, and transitional 2 marginal zone precursor B cells but not on follicular B cells

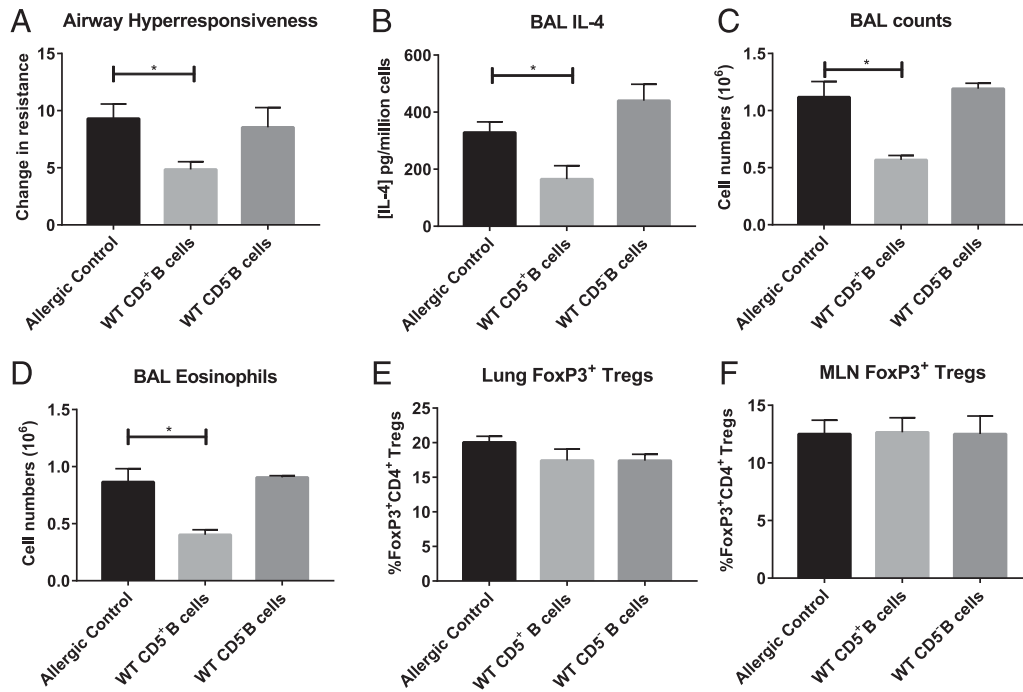
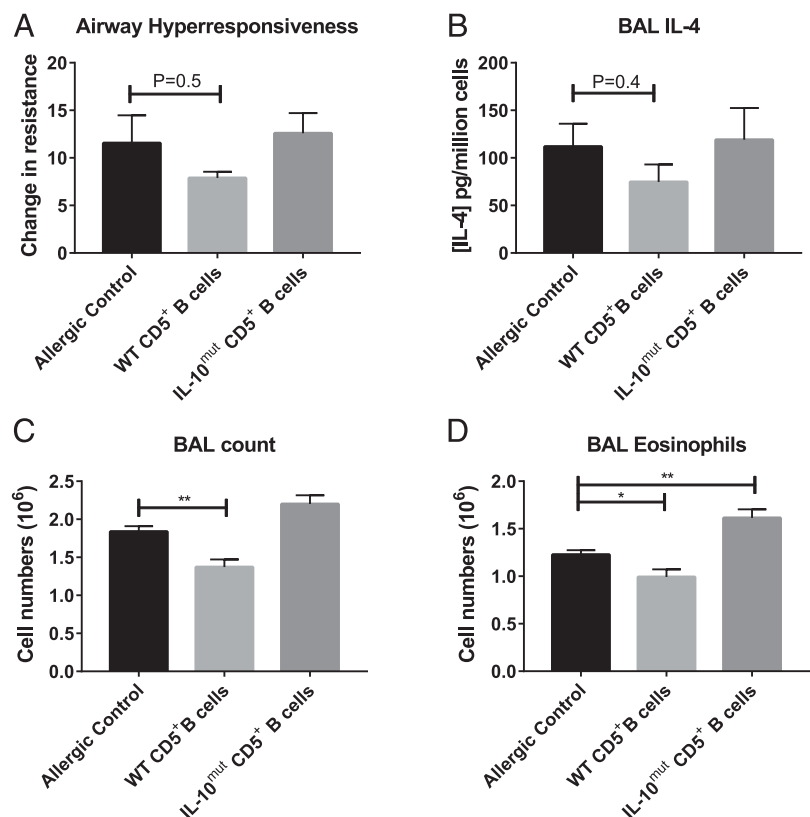


FIGURE 5. IL-5/mCD40L-Fb-stimulated CD5⁺ B cells prevent AAD. Mice were sensitized to OVA in alum twice by i.p. injection. Purified splenic B cells from BALB/c mice were cultured with IL-5/mCD40L-Fb for 5 d and then FACS sorted into CD5⁺ and CD5⁻ subsets. Sensitized mice received i.v. adoptive transfers of 2×10^6 sorted B cells per mouse prior to intratracheal challenge daily with 10 μ g OVA for 3 d. (A) AHR was assessed by plethysmography. (B) BAL fluid was collected and assessed by ELISA for IL-4. (C and D) Cells in the BAL fluid were counted and assessed by flow cytometry for eosinophils (CD45⁺CD4⁻CD19⁻Ly6G⁻SiglecF⁺MHCII⁺). (E and F) Tregs in the lung and MLNs (CD45⁺CD3⁺CD4⁺CD25⁺Foxp3⁺) were determined by intracellular flow cytometry. Data are a single representative experiment of three independent experiments (mean $\pm$ SEM); three to five mice per group. Statistics are from Mann-Whitney *U* test comparing treatment groups to allergic control. **p* < 0.05.

(45). All B cell subsets found to express CD9 can produce IL-10 in a regulatory capacity (36), making it a more all-encompassing marker for IL-10⁺ Bregs (46). Moreover, adoptive transfers of

CD9⁺ B cells from naive mice were protective in a murine model of AAD in an IL-10-dependent manner and have recently been implicated in the immunosuppressive activity of IL-10⁺ Bregs (45,

FIGURE 6. CD5⁺ B cell AAD protection is IL-10 dependent. OVA-sensitized mice received 2×10^6 sorted CD5⁺ B cells per mouse obtained from IL-5/mCD40L-Fb-stimulated cultures of splenic B cells from wild-type BALB/c or IL-10^{-/-} mice. Transfer recipients and untreated controls were then challenged daily with 10 μ g OVA for 3 d. (A) AHR was assessed by plethysmography. (B) BAL fluid was collected and assessed by ELISA for IL-4. (C and D) Cells in the BAL fluid were counted and assessed by flow cytometry for eosinophils (CD45⁺CD4⁻CD19⁻Ly6G⁻SiglecF⁺MHCII⁺). Data is from one experiment representative of three independent experiments (mean $\pm$ SEM) with at least six mice per group. Statistics were done using Mann-Whitney tests comparing each treatment group to allergic control. **p* < 0.05, ***p* < 0.01.



46). A human IL-10⁺ Breg population, CD27⁺CD24^{hi}CD38^{hi}, is also reported to express CD9. Our data supports CD9 expression as a marker of activated Bregs, as 95% of IL-10⁺ B cells after IL-5/mCD40L-Fb stimulation expressed CD9 on their cell surface. Moreover, a recent paper suggested the combined expression of CD9 and CD80 more specifically marked IL-10⁺ B cells (39). Our data found IL-10⁺ B cells enriched in both CD9⁺ and CD80⁺ B cells and support the concept that CD9⁺CD80⁺ B cells are enriched for IL-10 producers. However, stimulation of sorted CD9⁺CD5⁺ B cells from naive mice with IL-5/mCD40L-Fb did not result in IL-10 production, suggesting that CD9 expression may identify B cells that acquired IL-10 production rather than an *in vivo* population that is sensitive to IL-5/mCD40L stimulation (data not shown).

T_H2 cytokines IL-5 and IL-4 differentially affected CD5⁺ B cell growth and IL-10 production. CD5⁺ B cells constitutively express the IL-5R α and use IL-5 as a growth or survival factor (7). In primary culture, IL-5 stimulated growth of CD5⁺ B cells, whereas IL-4 did not. IL-5-induced proliferation of CD5⁺ B cells likely accounts for increased IL-10 production induced by mCD40L-Fb, as a higher percentage of IL-10⁺ B cells are present in IL-5/mCD40L-Fb compared with mCD40L-Fb cultures (data not shown). Primary culture with IL-4/mCD40L-Fb led to growth of B cells that could not be induced to produce IL-10 in secondary culture. IL-4 inhibition of IL-10 is not unique to IL-5/mCD40L-Fb culture system, as LPS stimulation, another inducer of IL-10⁺ Bregs, was also inhibited in the presence of IL-4 (data not shown). Another group showed that IL-4 blocks LPS-stimulated IL-10 production from bone marrow-derived DCs (47). We speculate IL-4 rendered the IL-10 gene locus inaccessible in the B cells, as IL-4-induced STAT6 signaling is reported to compromise histone acetylation of the IL-10 promoter in LPS-stimulated DCs (47). STAT6 may also compete with other transcription factors and/or cofactors essential for IL-10 gene expression. Additionally, IL-4 suppression of the common β -chain, which is essential for IL-5 signaling, may explain the inhibition of IL-5 enhancement of IL-10 in the secondary culture found in this study (48). Although IL-13 is also a T_H2 cytokine, murine B cells do not express the IL-13 receptor (2, 3). Cultures including IL-13 had no effect on IL-5/mCD40L-Fb-induced IL-10 (data not shown). Ongoing and future studies are focused on understanding the molecular mechanisms of IL-4 inhibition of IL-10 production in our culture system.

Transfer of IL-5/mCD40L-Fb-stimulated CD5⁺ B cells reduced development of OVA-induced AAD, reducing airway resistance and total cellular infiltration and eosinophil numbers in the BAL. The activity of the transferred cells despite the increased presence of IL-4 systemically in these mice suggests that previously activated Bregs are resistant to IL-4 *in vivo*. These results correlate with what was found in the secondary cultures of IL-5/mCD40L-Fb-stimulated B cells with IL-4 in the transfer recipient mice.

The adoptive transfer data serve as evidence that enhancing Bregs *in vivo* may provide therapeutic benefits in allergic diseases. Adoptive transfers of Bregs may not prove to be a practical approach clinically; however, a better understanding of how these cells are stimulated could lead to identification of novel pharmacological targets. Further investigations will be focused on modulating the regulatory functions of endogenous lung CD5⁺ B cells as a potential treatment after the establishment of AAD.

Although B cells possess several mechanisms of immune suppression (20), protection in this study appeared to be dependent on IL-10, as transfer of IL-10^{mut} CD5⁺ B cells fully reversed protective trends mediated by wild-type IL-5/mCD40L-Fb-stimulated CD5⁺ B cells in the AAD model. Our laboratory previously reported that CD5⁺ B cells from the IL-5/mCD40L-Fb

culture expressed FasL and could kill CD4⁺ T cells *in vitro* (33). Apoptotic CD4⁺ T cells were assessed in the lungs of the mice in the current study; however, no significant differences were detected following transfers (data not shown). Thus, IL-10 but not FasL expression or other regulatory functions of the transferred cells appeared to play a role in the protection afforded in this study. Many groups have reported that B cells are central in maintaining the Foxp3⁺ Treg compartment, and induction of Foxp3⁺ Tregs and IL-10-producing Tregs are thought to be critical mechanisms used by IL-10⁺ Bregs to regulate immune responses (49–54). However, no differences were found in percentages or numbers of Foxp3⁺ Tregs in the lungs or MLNs in our adoptive transfer experiments.

Our results suggest that regulatory CD5⁺ B cells may be preferentially active in an environment where IL-5 and CD40L are present but where IL-4 production is minimal. A potential cellular source of IL-5 without IL-4 is ILC2 cells (55). In the early stages of an allergic response, ILC2s are the initial IL-5 producers and produce minimal IL-4; however, their CD40L expression is unknown. Human ILC3s are known to express CD40L and interact with B cells, enhancing B cell survival, proliferation, and induction of IL-10-producing Bregs (56). ILC3s and Bregs were found in close contact within regulatory niches of the palatine tonsils in healthy controls. ILC3 frequencies were reduced in tonsil tissue, and both ILC3s and Bregs were reduced in peripheral blood of allergic patients (56). The ability of ILC2s to produce IL-5 and minimal IL-4 and reported interactions between other subsets of ILCs suggests ILC2s could be a natural source of IL-5 and CD40L for endogenous Bregs. Interactions between ILCs and Bregs may be an important mechanism in the maintenance of immune tolerance.

Most studies suggest IL-4 and IL-5 are produced simultaneously by T_H2 cells; however, T_H cells that primarily produce IL-5 have been described during chronic T_H2 responses. Chronically activated T_H2 cells could potentially provide conditions mimicking our *in vitro* cultures and allow for IL-5/mCD40L-Fb-mediated induction of IL-10⁺ Bregs. Murine T cells that had undergone multiple rounds of T_H2 polarization *in vitro* expressed CCR8 and produced abundant IL-5 relative to IL-4 (57). The existence of T_H2 cells producing IL-5 and not IL-4 was confirmed *in vivo* in a chronic atopic dermatitis model. Intracellular staining of human peripheral T cells for IL-5 and IL-4 paralleled murine results, as CCR8⁺ CD4⁺ T cells produced abundant IL-5 and minimal IL-4 (57). T cells producing IL-5 dominantly over IL-4 have also been observed during murine and human IL-33-induced T_H2 polarization *in vitro* (58) and murine schistosome infection *in vivo*, another chronic T_H2 response (59). Bregs are induced during murine chronic helminth infections, and adoptive transfer of helminth-induced CD5⁺ Bregs can prevent and reverse AAD (27). Although Bregs can be induced from worm-secreted proteins, this may not be the only mechanism of induction during helminth infection. CD5⁺ B cells have also been shown to increase in number during chronic house dust mite-induced AAD in mice. Moreover, absence of CD5⁺ B cells in *xid* mice resulted in more severe allergic symptoms (26). Chronic Ag stimulation in mice can eventually result in resolution of pulmonary AAD response (60–62). CD5⁺ Bregs were induced after 42 d of OVA challenge in hilar lymph nodes of tolerant mice. Adoptive transfer of these CD5⁺ hilar lymph node B cells into OVA-sensitized mice prevented development of AAD in a TGF- β -dependent manner (29, 60).

Similar studies have led to use of chronic Ag stimulation as a strategy for treating allergic diseases. Specific allergen immunotherapy (SIT) is the only current disease-modifying treatment for allergic diseases that has curative potential (63). Multiple high doses of disease-causing allergen are received *s.c.* or sublingually to induce permanent tolerance. Studies in mice and humans both

suggest induction of tolerance through SIT involves the expansion and function of Bregs (63, 64). Chronic stimulation induces B cells with multiple regulatory abilities, including the production of IL-10. Our results suggest emergence of IL-5 dominant T_H2 cells after chronic stimulation may contribute to induction and expansion of Bregs and tolerance seen during helminth infections and SIT. Further investigation is necessary to understand temporal and spatial activation of Bregs.

Future studies are planned to better understand the IL-10–dependent mechanisms of IL-5/mCD40L-Fc-induced B cells and the *in vivo* inhibitory effects of IL-4 on the induction of Breg growth and function. It is hoped that understanding the pathways regulating suppressive functions of endogenous Bregs can lead to new and possibly more specific therapeutic strategies for treatment of severe AAD.

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Disclosures

The authors have no financial conflicts of interest.

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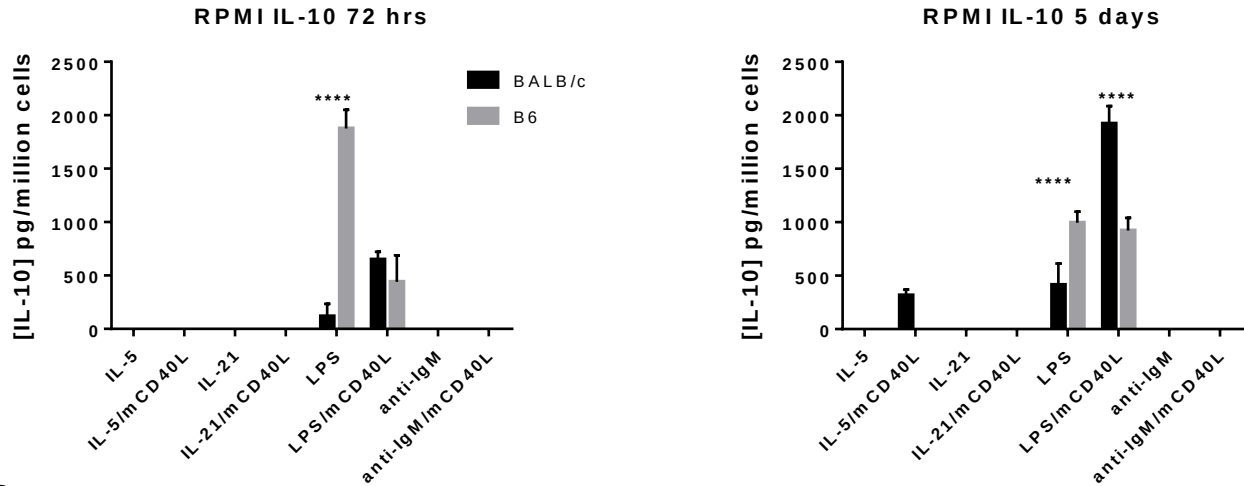
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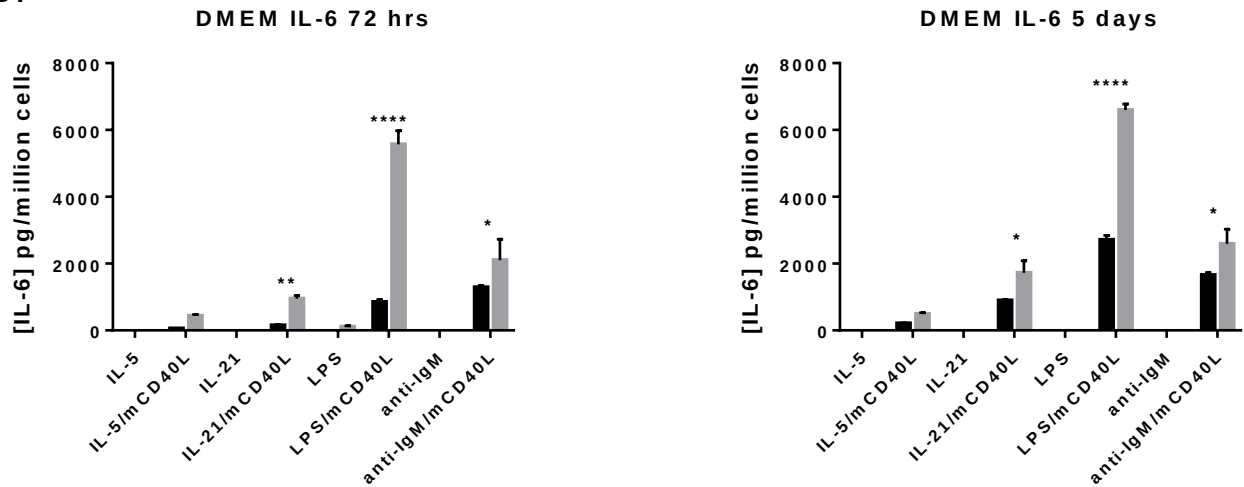
Supplemental Table 1. Antibodies Used for Flow Cytometry

IL-10 Intracellular staining of B cells				
Marker	Clone	Color	Dilution	Supplier
CD1d	1B1	FITC	1:200	BD Bioscience
IL-10	JES5-16E3	PE	1:100	Biolegend
CD11b	M1/70	PEDazzle	1:800	Biolegend
CD43	S7	PE-Cy7	1:1600	BD Bioscience
CD9	MZ3	APC	1:100	eBioscience
IgM	II/41	APC-eFluor780	1:100	eBioscience
Zombie Violet		PacificBlue	1:400	Biolegend
CD23	B3B4	BV510	1:200	Biolegend
CD21	7G6	BV605	1:1600	BD Bioscience
CD16/32	2.4G2	-----	1:100	BD Bioscience
IL-10 Intracellular staining of B cells				
Marker	Clone	Color	Dilution	Supplier
CD9	MZ3	FITC	1:100	Biolegend
IL-10	JES5-16E3	PE	1:100	Biolegend
CD80	16-10A1	PE Dazzle	1:200	Biolegend
CD5	53-7.3	PE-Cy5	1:800	BD Bioscience
Zombie Violet		PacificBlue	1:400	Biolegend
CD16/32	2.4G2	-----	1:100	BD Bioscience
Regulatory T cell panel				
Marker	Clone	Color	Dilution	Supplier
CD73	TY/23	AlexaFluor 488	1:100	BD Bioscience
FoxP3	NRRF-30	PE	1:150	eBioscience
CD127	A7R34	PeDazzle	1:200	Biolegend
CD25	PC61	PECy7	1:200	Biolegend
CD39	Duha59	Alexa647	1:100	Biolegend
CD45	30-F11	AlexaFluor700	1:400	Biolegend
CD3	17A2	APC-eFluor780	1:200	BD Bioscience
CD4	GK1.5	BV510	1:400	Biolegend
CD16/32	2.4G2	-----	1:100	BD Bioscience
Cell types in BALF/lung tissue for AAD				
Marker	Clone	Color	Dilution	Supplier
CD11b	M1/70	FITC	1:200	BD Bioscience
CD11c	HL3	PE	1:400	BD Bioscience
Ly6G	1A8	PE-Dazzle594	1:800	Biolegend
7AAD		PerCP	1:50	BD Bioscience
CD4	GK1.5	PE-Cy7	1:400	Biolegend
MHC II (I-A/I-E)	53-7.3	APC	1:1600	eBioscience
CD45	30-F11	AlexaFluor700	1:400	Biolegend
F4/80	BM8	APCeFluor780	1:100	eBioscience
CD19	6D5	PacificBlue	1:400	Biolegend
Siglec F	E50-2440	BV510	1:100	BD Bioscience
CD335/NKp46	29A1.4	BV605	1:200	Biolegend
CD16/32	2.4G2	-----	1:100	BD Bioscience

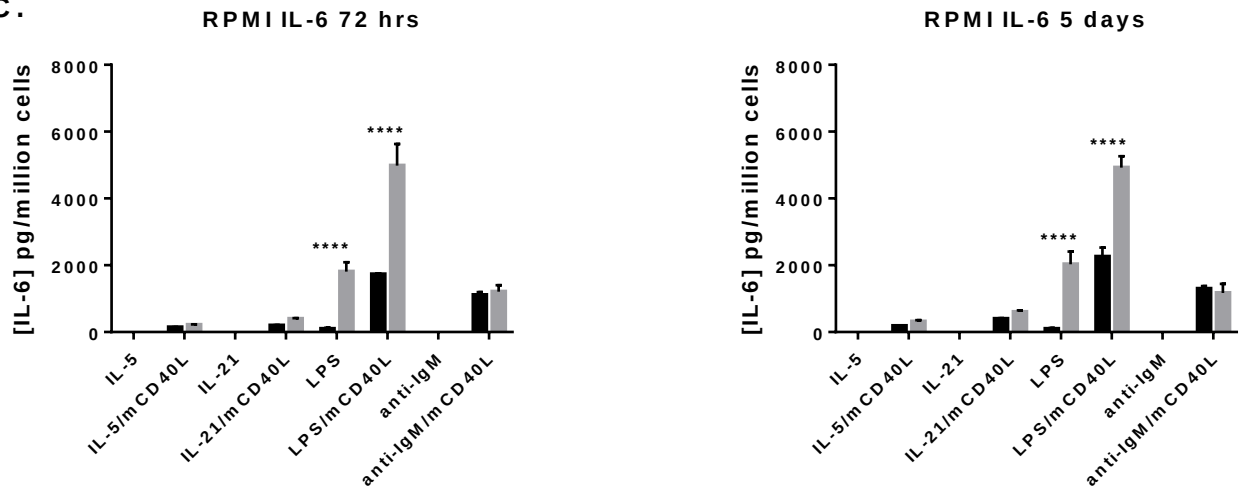
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B.

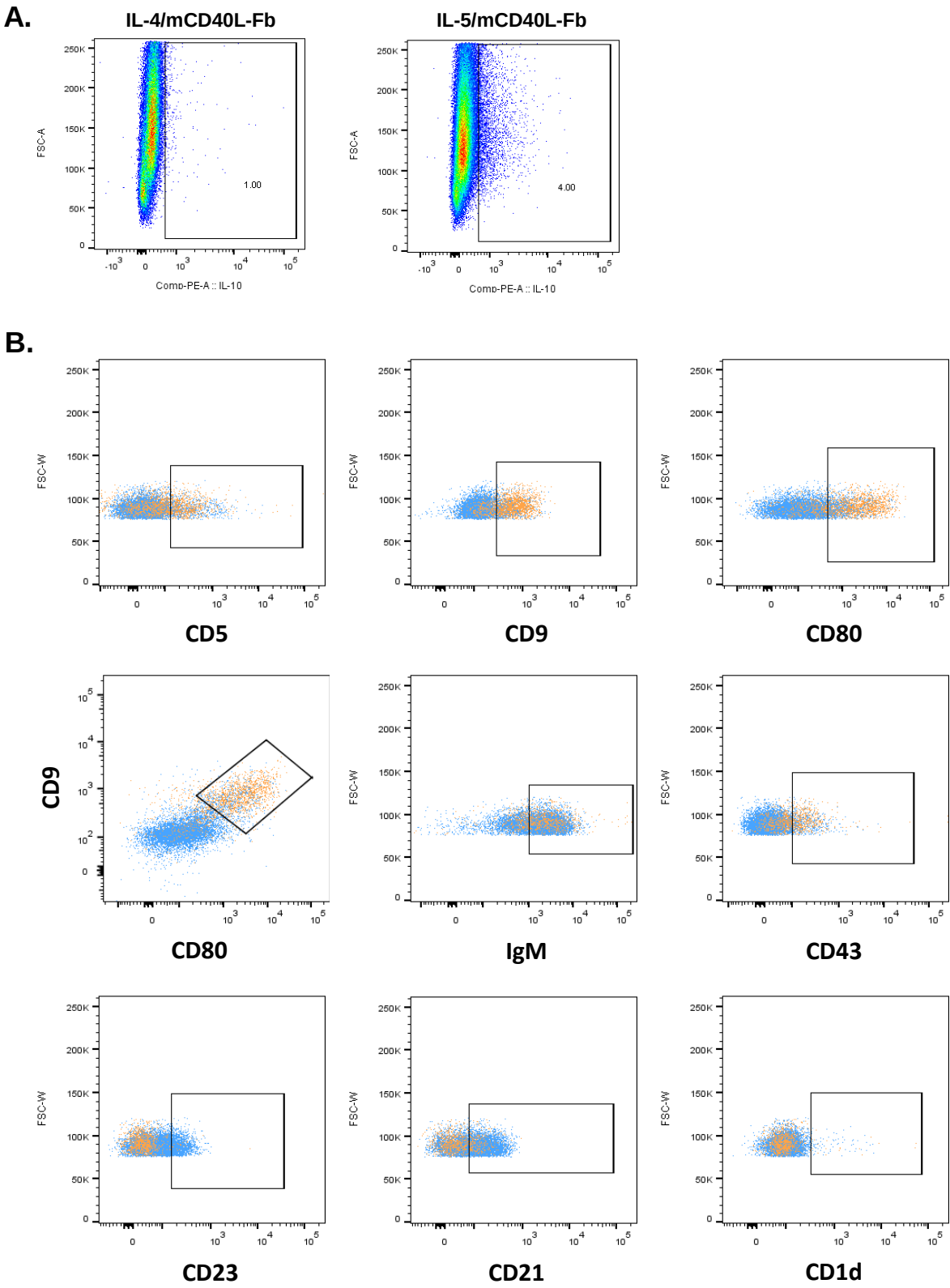


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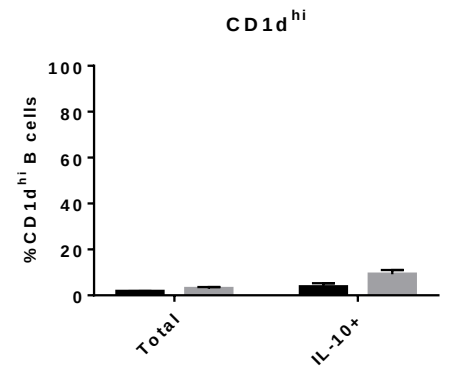
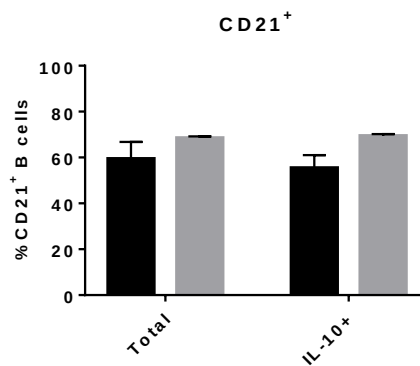
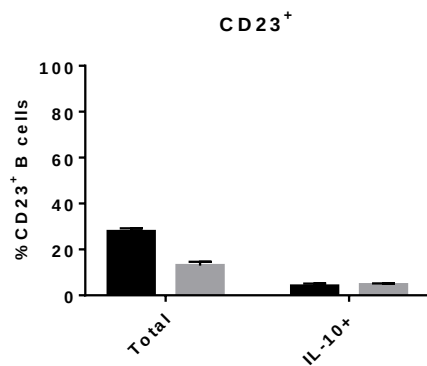
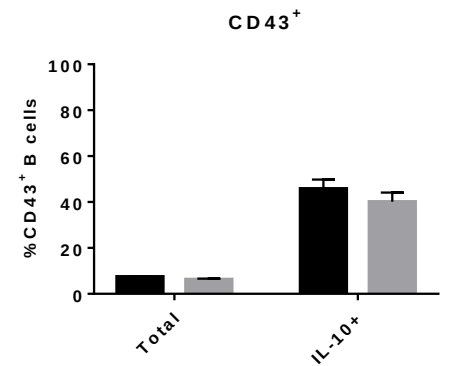
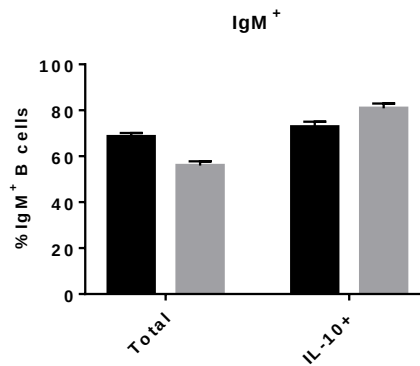
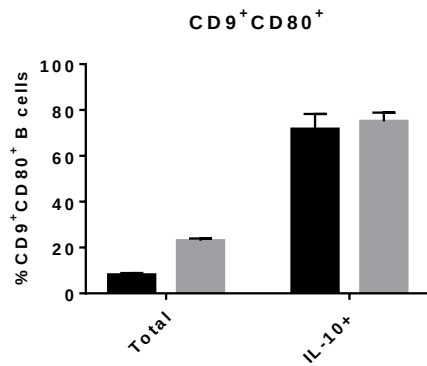
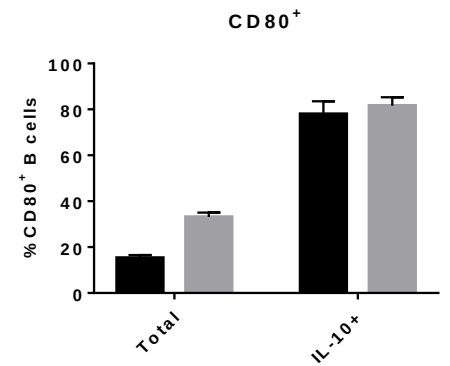
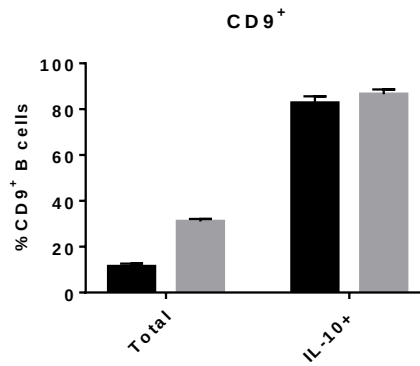
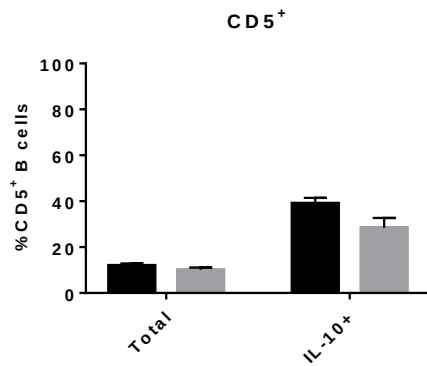
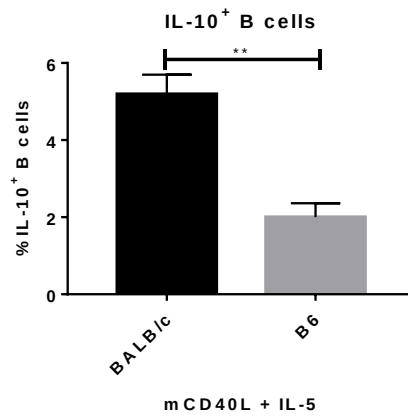


Supplemental Figure 1. Comparison of stimuli and media for induction of IL-10 secretion. (A-C) Total splenic B cells were cultured with the indicated stimulation for 72 hours or 5 days. IL-10 and IL-6 in culture supernatants were assessed by ELISA. Data are from a single representative experiment of 4 independent experiments (mean $\pm$ SEM); at least 3 mice/experiment. (A-D) Sidak's multiple comparison test was used for statistics. *P* values are indicated by: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Supplemental Figure 2



Supplemental Figure 2. Representative flow plots of IL-10 intracellular staining. IL-10⁺ gate was set according to fluorescence minus one. **(A)** IL-10 intracellular staining was performed on splenic B cells from IL-5 or IL-4/mCD40L-Fb cultures after PMA/ionomycin/Brefeldin A restimulation. **(B)** IL-10⁺ B cells and total B cells are plotted comparing single markers against forward scatter area. IL-10⁺ B cells (orange) are overlaid on total B cells (blue).



Supplemental Figure 3. Comparison of phenotype of IL-10⁺ B cells between BALB/c and C57BL/6. (A) Total splenic BALB/c (black) or C57BL/6 (gray) B cells were cultured for 5 days with IL-5/mCD40L-Fb. IL-10 intracellular staining was performed on cells from the culture after PMA/ionomycin/Brefeldin A restimulation. Flow cytometry data on B cell markers and their expression in the total B cell population or IL-10⁺ B cells is summarized in bar graphs. Data are from a single representative experiment of 5 independent experiments (mean $\pm$ SEM) with at least 3 mice/experiment. Two-tailed paired Student's *t* tests were used for statistics. ***P* < 0.01.