Characterization of a rare IL-10–competent B-cell subset in humans that parallels mouse regulatory B10 cells

Characterization of a Rare IL-10-Competent B Cell Subset in Man That Parallels Mouse Regulatory B10 Cells

Running Title: Human regulatory B10 cell characterization

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Immunobiology

Abstract

Regulatory B cells control inflammation and autoimmunity in mice, including the recently identified IL-10-competent B10 cell subset that represents 1-3% of spleen B cells. In this study, a comparable IL-10-competent B10 cell subset was characterized in human blood. B10 cells were functionally identified by their ability to express cytoplasmic IL-10 following 5 h of *ex vivo* stimulation, while progenitor B10 (B10pro) cells required 48 h of *in vitro* stimulation before they acquired the ability to express IL-10. B10 and B10pro cells represented 0.6% and ~5% of blood B cells, respectively. *Ex vivo* B10 and B10pro cells were predominantly found within the $CD24^{hi}CD27^{+}$ B cell subpopulation that was able to negatively regulate monocyte cytokine production through IL-10-dependent pathways during *in vitro* functional assays. Blood B10 cells were present in ninety-one patients with rheumatoid arthritis, systemic lupus erythematosus, primary Sjögren's syndrome, autoimmune vesiculobullous skin disease, or multiple sclerosis, and were expanded in some cases as occurs in mice with autoimmune disease. Mean B10+B10pro cell frequencies were also significantly higher in patients with autoimmune disease when compared with healthy controls. The characterization of human B10 cells will facilitate their identification and the study of their regulatory activities during human disease.

Introduction

B cells are generally considered to positively regulate immune responses by producing antigen-specific Ab and helping to induce optimal $CD4^+$ T cell activation.[1] However, B cells and specific B cell subsets can also negatively regulate immune responses in mice.[2-6] The absence or loss of these regulatory B cells exacerbates disease symptoms in contact hypersensitivity, experimental autoimmune encephalomyelitis, chronic colitis, collagen-induced arthritis, and in lupus-like models of autoimmunity.[7-15] In many of these cases, B cells regulate inflammation, asthma, and T cell-mediated autoimmunity through the production of IL-10.[8-10,12-16] Both human and mouse IL-10 exhibit numerous pleiotropic activities *in vitro* and *in vivo*, including suppression of both Th1 and Th2 polarization and inhibition of antigen presentation and proinflammatory cytokine production by dendritic cells, monocytes and macrophages.[17]

In mice, a subset of IL-10-competent regulatory B cells can be functionally identified by their ability to express cytoplasmic IL-10 following 5 h of *in vitro* stimulation with LPS, PMA, and ionomycin, with monensin included in the cultures to block IL-10 secretion.[12,13] These IL-10-competent B cells have been labeled as B10 cells to identify them as the predominant, if not exclusive, source of B cell IL-10 production and to distinguish them from other regulatory B cell subsets that may also exist.[5] For example, inducible IL-12-producing B cells regulate intestinal inflammation.[18] B10 cells are found within the spleens of naïve wild type mice at frequencies of 1-3%, where they predominantly represent a subset of the phenotypically unique $CD1d^{hi}CD5^+CD19^{hi}$ B cell subpopulation that shares overlapping cell surface markers with multiple phenotypically-defined B cell subsets.[11-14,19,20] Additional B cells within the $CD1d^{hi}CD5^+$ B cell subpopulation acquire the ability to function like B10 cells during 48 h of *in vitro* stimulation with LPS or agonistic CD40 mAb.[5] These B10 progenitor (B10pro) cells are then able to express cytoplasmic IL-10 following stimulation with PMA, ionomycin and monensin for 5 h.[21] B10 cells also require diverse B cell antigen receptors for their development[21] and their regulatory functions are Ag-restricted *in vivo*.[12,13] Spleen B10 cell numbers increase significantly in diabetes- and lupus-prone mice[14,21], and the adoptive transfer of

antigen-primed CD1d^{hi}CD5^{+} B cells reduces inflammation during contact hypersensitivity and autoimmune disease.[12,13,22]

The identification and characterization of an IL-10-producing B cell subset in mice raises the issue of whether B cells with these functional properties exist in man. Studies of B cell IL-10 production in humans have yielded diverse results that are currently difficult to unify into a coherent model.[23-28] It is also unknown whether human B10 cells share overlapping physiologic triggers with mouse B10 cells that lead to IL-10 production and their expansion *in vitro*.[12,13,21] Therefore, the purpose of the current study was to enumerate and characterize the IL-10 competent B10 and B10pro cell subsets in humans.

Materials and Methods

Cells

Heparinized blood was obtained from healthy donors, ages 14-73, or from patients. Patients with rheumatoid arthritis met the American College of Rheumatology 1987 revised classification criteria.[30] Patients with systemic lupus erythematosus satisfied the 1982 classification criteria.[31] Patients with primary Sjögren's syndrome fulfilled the American-European consensus group criteria.[32] Patients with autoimmune vesiculobullous skin disease, including bullous pemphigoid, pemphigus foliaceus, pemphigus vulgaris, and dermatitis herpetiformis had typical clinical and histologic findings, with diagnostic findings on direct immunofluroesence of perilesional skin or oral mucosa.[33,34] Patients with multiple sclerosis fulfilled the 2005 McDonald criteria for relapsing remitting or primary progressive multiple sclerosis,[35] or secondary progressive multiple sclerosis as defined using the Lublin and Reingold criteria.[36] Tissues were obtained anonymously from individuals without identifiable hematologic disorders, with the purified B cells immediately cryopreserved (>90% cell viability). Cryopreserved cord blood samples were obtained from the Duke University Stem Cell Laboratory and the Carolinas Cord Blood Bank.

Blood mononuclear cells were isolated by centrifugation over a discontinuous Lymphoprep (Axis-Shield PoC As, Oslo, Norway) gradient. Cell numbers were quantified by hemocytometer, with relative lymphocyte percentages among viable cells (based on scatter properties) determined by flow cytometry. In some experiments, B cells were enriched using RosetteSep (STEMCELL Technologies, Vancouver, BC, Canada) following the manufacturer's protocols. CD19-mAb coated microbeads (Miltenyi Biotech) were used to purify blood B cells by positive selection following the manufacturer's instructions. When necessary, the cells were enriched a second time using a fresh MACS column to obtain >99% purities.

Written informed consent was obtained in all cases according to the Declaration of Helsinki. All protocols were approved by the respective ethics boards; Institutional Review Board of Duke University Medical Center, the Human Protection Committee of Dana-Farber Cancer

Institute/Harvard Medical School, University of Rochester Institutional Review Board, and the Institutional Review Board of St. Luke's–Roosevelt Hospital Center Institute for Health Sciences.

Antibodies, immunofluorescence analysis and cell sorting

Anti-human mAbs included: IgD (IA6-2) from BD PharMingen (San Diego, CA); CD21 (BU33), CD22 (RFB4), CD23 (D.6) from Ancell (Bayport, MN); IgM (MHM-88), CD1d (51.1), CD5 (UCHT2), CD19 (HIB19), CD24 (ML5), CD25 (BC96), CD27 (O323), CD38 (HIT2), CD40 (HB14), CD48 (BJ40), and CD148 (A3), TNFα (MAb11), functional grade CD3 (HIT3a), and phycoerythrin-conjugated or unconjugated anti-IL-10 mAb (JES3-19F1) mAbs from BioLegend (San Diego, CA). Anti-human IgM Ab was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Single cell suspensions were stained on ice using predetermined optimal concentrations of each Ab for 20-60 min, and fixed as described.[29] Cells with the light scatter properties of lymphocytes were analyzed by 2-6 color immunofluorescence staining and FACScan or FACSCalibur flow cytometers (Becton Dickinson, San Jose, CA). Dead cells were excluded from the analysis based on their forward- and side-light scatter properties and the use of LIVE/DEAD® Fixable Violet Dead Cell Stain Kits (Invitrogen-Molecular Probes, Carlsbad, CA). All histograms are shown on a 4-decade logarithmic scale, with gates shown to indicate background isotype-matched control mAb staining set with <2% of the cells being positive. Blood $CD24^{hi}CD27^{+}$ and $CD24^{low}CD27^{-}$ B cells were isolated using a FACSVantage SE flow cytometer (Becton Dickinson) with 90-95% purities.

Analysis of IL-10 production

Intracellular IL-10 analysis by flow cytometry was as described.[12] Briefly, cells were resuspended (2×10^6 cells/ml) in medium [RPMI 1640 media containing 10% FCS, 200 μg/ml penicillin, 200 U/ml streptomycin, and 4 mM L-Glutamine (all from Gibco, Carlsbad, CA)] and stimulated with LPS (10 μg/ml, *Escherichia coli* serotype 0111: B4; Sigma), CpG (ODN 2006, 10 μg/ml;

Invivogen), or other TLR agonists (TLR1, Pam3CSK4, 1 μg/ml; TLR2, heat-killed *Listeria monocytogenes*, 10^8 cells/ml; TLR3, Poly(I:C), 10 μg/ml; TLR5, *S. typhimurium* flagellin, 1 μg/ml; TLR6, Pam2CGDPKHPKSF, 1 μg/ml; TLR7, Imiquimod, 1 μg/ml; TLR8, ssRNA40, 1 μg/ml; Invivogen), CD40L (1 μg/ml; R&D Systems, Minneapolis, MN), anti-human CD40 mAb (1 μg/ml; BioLegend), PMA (50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma), Brefeldin A (1X solution/ml; BioLegend), monensin (2 mM; eBioscience), and anti-IgM antibody (10 μg/ml) as indicated in 48-well flat-bottom plates before staining and flow cytometry analysis. For analysis of cell proliferation, lymphocytes were stained with CFSE Vybrant™ CFDA SE fluorescent dye (5 μM; Invitrogen-Molecular Probes) according to the manufacturer's instructions. For IL-10 detection, Fc receptors were blocked using FcγR-Binding inhibitor (eBioscience). Stained cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD PharMingen) according to the manufacturer's instructions and stained with anti-IL-10 mAb.

Secreted IL-10 was quantified by ELISA. Purified B cells (4×10^5) were cultured in 0.2 ml of complete medium in a 96-well flat-bottom tissue culture plates. Culture supernatant fluid IL-10 concentrations for triplicate samples were quantified using IL-10 OptEIA ELISA kits (BD PharMingen) following the manufacturer's protocols.

Il10 transcript expression

In some experiments, IL-10-secreting blood B cells were identified after 4 h of *in vitro* stimulation using an IL-10 secretion detection kit (Miltenyi Biotech, Auburn, CA) with subsequent staining for CD19 expression before cell sorting into IL-10^+CD19^+ and IL-10^-CD19^+ populations. RNA extraction, cDNA generation, and real-time PCR analysis was as described.[13] IL-10 primers were: sense 5'-CTTCGAGATC TCCGAGATGC CTTC-3', antisense 5'-ATTCTTCACC TGCTCCACGG CCTT-3'. GAPDH primers were: sense 5'-GCCACCCAGA AGACTGTGGA TGGC-3' and antisense 5'-CATGTAGGCC ATGAGGTCCA CCAC-3'.

***In vitro* functional assays**

Blood $CD24^{hi}CD27^{+}CD19^{+}$ and $CD24^{low}CD27^{-}CD19^{+}$ B cells from healthy controls were purified by cell sorting and stimulated with CpG (10 μg/ml) plus CD40L (1 μg/ml) for 24 h. Naïve $CD4^{+}CD25^{-}$ T cells were purified by MACS (Miltenyi Biotech), and cultured in replicate wells either alone ($1x10^6$/ml) or with CD40/CpG-stimulated B cells ($1x10^6$/ml) in tissue culture plates coated with CD3 mAb (0.5 μg/ml) for 72 h with either BFA or PIB added during the final 5 hours. The cells were stained for cell surface CD4 and cytoplasmic TNF-α expression before analysis by flow cytometry. Alternatively, CD40/CpG-stimulated sorted B cell populations ($1x10^6$/ml) were cultured for 20 h with equal numbers of blood monocytes that had been purified by plastic adherence or MACS, with anti-IL-10 mAb (10 μg/ml) added to some cultures. The cultured cells were then washed, stimulated with LPS (1 μg/ml) for 4 h in the presence of BFA, and stained for cell surface CD14 and cytoplasmic TNF-α or IFN-γ expression before analysis by flow cytometry.

Statistical analysis

All data are shown as means (±SEM). Significant differences between sample means were determined using the Student's *t* test.

Results

Enumeration of human IL-10-producing B cells

Mouse B10 cell frequencies have been described, with background cytoplasmic IL-10 staining of ≤0.2% commonly observed with B cells from IL-$10^{-/-}$ mice.[12,13,21] A similar strategy was therefore optimized to enumerate human blood IL-10-competent B cells. B cells spontaneously expressing IL-10 were below the threshold for reliable quantification by immunofluorescence staining, even when the cells were cultured with Brefeldin A to stop Golgi transport (Fig. 1A). However, a distinct subset of cytoplasmic IL-10^{+} B cell was observed at low 0.25-2% frequencies after *ex vivo* stimulation using phorbol ester and ionomycin plus Brefeldin A (PIB) (Fig. 1A-B). Stimulation with PIB for 5 h induced 0.8±0.1% of blood B cells on average to express IL-10 (n=14, 1.9±0.3 x10^{-3} B10 cells/ml, Fig. 1B-C). B cell stimulation with TLR agonists did not substantially alter mean B10 cell numbers, although adding either CpG oligonucleotides (CpG, TLR9 agonist) or LPS to the PIB cultures increased IL-10^{+} B cell frequencies in some individuals (Fig. 1A-C). Stimulation beyond 5 h or using 10-fold higher PMA or ionomycin concentrations resulted in extensive B cell death, which complicated B10 cell enumeration. Background IL-10 mAb staining was also reduced by the exclusion of cell doublets and dead cells from the analysis. Brefeldin A was also used to block IL-10 secretion rather than monensin since it optimized human B cell cytoplasmic IL-10 expression (Fig. 1A). B cells cultured in brefeldin A served as negative controls since they gave results similar to isotype control mAb staining. Thus, blood B10 cells were rare but readily quantified in healthy humans.

Human B10pro cell identification

In mice, B10pro cell maturation into IL-10-competent B10 cells is induced by 48 h stimulation with either LPS or agonistic CD40 mAb.[21] Human blood B10pro cells capable of maturing into IL-10 competent cells after *in vitro* culture were also identified. The total frequency of B10 and B10pro cells (B10+B10pro) is quantified in this assay, as the B cells that acquire IL-10 competence *in vitro* (e.g. matured B10pro cells) cannot be differentiated from preexisting B10

cells that inherently express cytoplasmic IL-10 after 5 h PIB stimulation. Culturing human B cells in media alone for 48 h resulted in ~0.2% of B cells expressing cytoplasmic IL-10 following PIB stimulation during the last 5 h of culture (Fig. 1B). Adding LPS, CpG, or recombinant CD40 ligand (CD40L, CD154) alone, together, or in combination with brefeldin A to the 48 h cultures did not increase IL-10-producing B10 cell frequencies. However, following PIB stimulation during the last 5 h of culture, B10+B10pro cell frequencies increased to 0.6±0.1, 1.9±0.4, 0.8±0.1, 1.2±0.2, and 4.1±1.0% following 48 h of TLR1 agonist, LPS, TLR6 agonist, TLR7 agonist, or CpG stimulation, respectively (Fig. 1E, left panel). When compared with media or most TLR agonists alone, the addition of CD40L to the cultures significantly enhanced mean B10+B10pro cell frequencies (Fig. 1E, right panel). CD40L induced higher IL-10^+ B cell frequencies than agonistic CD40 mAb (Fig. 1D). Thus, dual CD40 and TLR stimulation induced the highest frequencies of B10pro cells to become IL-10 competent B10 cells, with the highest frequencies (7.0±1.4%) and numbers of IL-10^+ cells (1.6±0.3 x 10^4 cells/ml, n=14) induced after 48 h of CD40L plus CpG stimulation. B10 cell percentages relative to all B cells decreased with age among 60 healthy individuals, with a significant inverse correlation between B10+B10pro cell frequencies and age ($p<0.05$, data not shown). Thus, B10pro cell maturation *in vitro* was required to optimally enumerate IL-10-competent B cell frequencies.

B10 cell numbers in newborn blood and adult tissues

Newborn blood contained both B10 and B10pro cells. Mean B10 cell frequencies in cord blood after 5 h of CpG+PIB stimulation were 0.45±0.14% (Fig. 2A). B10+B10pro cell frequencies were similar or higher in cord blood relative to adult blood after culture with CD40L and TLR agonists; TLR1 (2.6±0.6%), LPS (7.6±1.8%), TLR6 (4.2±1.4%), or TLR9 (CpG, 9.6±2.3%) agonists with PIB added during the final 5 h of culture. B10 cells were also found within spleens (0.31±0.06%, n=4, CpG+PIB) and tonsils (0.31±0.11%, n=3, CpG+PIB) of adults without known disease (Fig. 2B). Thus, newborn and adult blood and tissues contain quantifiable numbers of B10 and B10pro cells.

Regulation of B10 cell IL-10 production and secretion *in vitro*

The time course of blood B cell IL-10 induction was assessed *in vitro* by quantifying IL-10 transcripts. By 12, 24, and 48 h, B cell stimulation with CD40L plus CpG induced 6.8-, 24-, and 5.9-fold higher *Il10* transcript levels, respectively, than was observed for unstimulated B cells ($p<0.05$; Fig. 3A). Blood B cells that were actively secreting IL-10 after *in vitro* stimulation expressed *Il10* transcripts at 19-fold higher levels than were detected in IL-10$^-$ B cells (Fig. 3B). Thus, *Il10* gene transcription parallels the induction of B10 cell cytoplasmic IL-10 expression.

The response of human B10+B10pro cells to CD40L, CpG, and antigen receptor generated signals was also examined. In comparison with CD40L alone, CpG induced the highest levels of B10pro cell maturation into IL-10-competent B10 cells, which was further increased when both CD40L and CpG were added to the cultures (Fig. 3C). By contrast, IgM ligation using mitogenic antibody did not induce cytoplasmic IL-10 expression, but actually inhibited the B10pro cell maturation effects of CpG+CD40L stimulation. *In vitro* IgM signals also inhibit mouse B10pro cell maturation and cytoplasmic IL-10 induction.[21] Among TLR agonists, LPS and CpG were also the most potent stimuli for inducing IL-10 secretion by human blood B cells (Fig. 3D). The addition of CD40L to the cultures was not required, but enhanced LPS- and CpG-induced IL-10 secretion. Thus, similar signals induce human and mouse B10pro and B10 cells to mature and express cytoplasmic IL-10 *in vitro*.

Phenotypic characterization of IL-10-competent B cells

Whether human B10 cells represent a unique or known B cell subset was determined by analyzing their cell surface phenotype. Cell surface IgM, IgD, CD1d, CD5, CD10, CD19, CD21, CD22, CD23, CD24, CD25, CD27, CD38, and CD40 densities did not change when B cells were stimulated with PIB, LPS+PIB, or CpG+PIB for 5 h, and/or permeabilized. Furthermore, the transport of newly synthesized proteins to the cell surface is inhibited by the addition of brefeldin A to the cultures. Therefore, these markers were used to phenotype freshly isolated B10 cells.

Half of blood B10 cells expressed high IgM levels and low IgD levels (Fig. 4A). Both CD24 and CD27 expression were high on the majority of B10 cells, while IL-10^{-} B cells expressed either high or low density CD24 and CD27. CD19 and CD25 expression were also higher on B10 cells than IL-10^{-} B cells. Otherwise, the remaining cell surface markers were absent or expressed similarly by both B10 cells and IL-10^{-} B cells. The same results were obtained following PIB, LPS+PIB, or CpG+PIB stimulation (data not shown). Thereby, freshly isolated blood IL-10^{+} B10 cells were predominantly found within the CD24^{hi} or CD27^{+} B cell subpopulations.

The phenotype of blood B10+B10pro cells was also assessed following 48 h of culture with CD40L+CpG, and 5 h of LPS+PIB stimulation. Prolonged B cell stimulation induced significant changes in the cell surface phenotype of both IL-10^{+} and IL-10^{-} B cells, with most B cells induced to express CD25 and CD38 (Fig. 4B). Nonetheless, B10+B10pro cells on average expressed higher densities of CD1d, CD19, CD20, CD21, CD23, CD24, CD25, CD27, and CD38 when compared with IL-10^{-} B cells, consistent with an activated phenotype. Spleen B10 cells were also predominantly CD27^{+}, although the expression of most cell surface molecules was similar if not identical for B10 cells and IL-10^{-} B cells (Fig. 4C). Spleen B10+B10pro cells and IL-10^{-} B cells also had similar phenotypes after 48 h of stimulation *in vitro* (Fig. 4D). Thereby, blood B10 cells represented a subset of the CD24^{hi}CD27^{+} subpopulation, while IL-10 expression remained the best marker for categorizing B10 cells and B10+B10pro cells.

Blood B10 cells are enriched within the CD24^{hi}CD27^{+} B cell subpopulation

In addition to being predominantly CD24^{hi} and CD27^{+}, most B10 cells also expressed additional cell surface markers of activation (CD48^{hi}) and memory (CD148^{hi}) that were not affected by the 5 h culture conditions used to induce B cell cytoplasmic IL-10 expression (Fig. 5A-B). Even when the spectrum of blood donors was compared, B10 cells were always predominantly CD24^{hi} and CD27^{+} (Fig. 5C). Cell surface IgD, CD27, and CD38 expression profiles have also been used frequently to define human B cell subsets.[37,38] However, when blood B10 cells were analyzed

based on their CD38 versus IgD, or CD27 versus IgD expression profiles, IL-10^{+} B10 cells from representative blood donors did not fall into distinct subpopulations (Fig. 5C).

Since the CD24^{hi}CD27^{+} B cell subpopulation represented 24±5% (n=7) of blood B cells on average, it was assessed whether CD24 and CD27 could be used as overlapping markers to enrich for B10 cells. When purified CD24^{hi}CD27^{+} and CD24^{low}CD27^{-} B cells were cultured individually with LPS+PIB for 5 h to induce IL-10 expression, B10 cell frequencies were >10-fold higher within the CD24^{hi}CD27^{+} subpopulation when compared with CD24^{low}CD27^{-} B cells (Fig. 5D). To determine whether *ex vivo* B10pro cells also localize within the CD24^{hi}CD27^{+} subpopulation, purified CD24^{hi}CD27^{+} and CD24^{low}CD27^{-} B cells were cultured individually to induce B10pro cell maturation. Again, the frequency of IL-10^{+} B10+B10pro cells was >10-fold higher within the CD24^{hi}CD27^{+} subpopulation when compared with CD24^{low}CD27^{-} cells (Fig. 5E). The capacity of freshly isolated CD24^{hi}CD27^{+} and CD24^{low}CD27^{-} B cells to secrete IL-10 was also assessed. Again, IL-10 was predominantly produced by the CD24^{hi}CD27^{+} B cell subpopulation in response to CD40L, LPS, LPS+CD40L, CpG, or CpG+CD40L stimulation for 72 h (Fig. 5F). Thus, blood B10 and B10pro cells predominantly represented a small subset of cells within the CD24^{hi}CD27^{+} B cell subpopulation.

Since B10 and B10pro cells express markers for activated and memory B cells, their ability to proliferate in response to mitogens was assessed. Purified blood B cells were labeled with CFSE before *in vitro* culture. There was little if any IL-10^{+} or IL-10^{-} B cell proliferation during 48 h cultures regardless of whether the cells were stimulated with LPS or CpG. However, IL-10^{+} B cells exhibited a significant proliferative capacity in response to CpG at 96 h, while IL-10^{-} B cell proliferation was modest (Fig. 5G). Thus, blood B10 and B10+B10pro cells preferentially proliferated in response to mitogen stimulation, consistent with their CD24^{hi}CD27^{+} phenotype.

B10 cells regulate innate immunity

Resting or activated mouse B10 cells do not significantly influence mitogen-driven T cell activation or proliferation *in vitro*.[22] However, activated B10 cells do inhibit TNF-α production

by antigen-specific $CD4^+$ T cells during antigen-driven *in vitro* assays. *In vitro* T cell-B cell co-culture systems were therefore assessed to determine whether human B10 cells regulate mitogen-driven T cell function. First, purified blood $CD24^{hi}CD27^+$ B cells or $CD24^{low}CD27^-$ B cells were stimulated with CD40L and CpG for 24 h, washed extensively, and added to cultures of purified $CD4^+$ T cells that were stimulated with plate-bound CD3 mAb for 72 h. In these assays, both $CD24^{hi}CD27^+$ B cells and $CD24^{low}CD27^-$ B cells inhibited $CD4^+$ T cell expression of TNF-α equally (Fig. 6A). Unstimulated B cells did not affect $CD4^+$ T cell expression of TNF-α in these assays, and the addition of anti-IL-10 mAb to cultures containing activated $CD24^{hi}CD27^+$ or $CD24^{low}CD27^-$ B cells did not rescue TNF-α expression by $CD4^+$ T cells (data not shown). Thus, activated human B cells can inhibit mitogen-induced TNF-α production by $CD4^+$ T cells through IL-10-independent pathways that are not unique to $CD24^{hi}CD27^+$ B10 cells.

Even though mouse B10 cell regulation of T cell function requires antigen-specific *in vitro* assays that are not amenable to human studies, we have found that mouse monocyte cytokine production is directly regulated by B10 cells through IL-10-dependent pathways that are not antigen-specific (M. Horikawa, et al., manuscript in preparation). The ability of human B10 cells to regulate innate monocyte responses was therefore assessed. Remarkably, TNF-α production was significantly reduced when monocytes were cultured with activated blood $CD24^{hi}CD27^+$ B cells and this effect was blocked by anti-IL-10 mAb (Fig. 6B). The addition of anti-IL-10 mAb to monocytes cultured alone did not affect TNF-α production (data not shown), while $CD24^{low}CD27^-$ B cells variably affected monocyte TNF-α production through IL-10-independent pathways. Thus, B10 cell production of IL-10 regulate monocyte cytokine production.

B10 cells in patients with autoimmune disease

To determine whether blood B10 cell numbers are altered during inflammation and autoimmune disease, B10 and B10pro cells were examined in 91 patients with systemic lupus erythematosis, rheumatoid arthritis, primary Sjögren's syndrome, autoimmune vesiculobullous skin disease, or multiple sclerosis (Fig. 7A-B). Most patients were undergoing active treatment with

immunomodulatory agents and/or low doses of prednisone (Table 1). B10 cell frequencies were significantly higher in one pemphigus vulgaris patient (PV07) and one dermatitis herpetiformis patient (DH02) not undergoing immunosuppressive therapy. Two lupus patients (SLE02, SLE06) and one rheumatoid arthritis patient (RA03) also had significantly higher B10 cell frequencies, but retrospective evaluation of their disease status, autoantibody profile, and treatment regimen did not indicate why these individuals had higher B10 cell frequencies. No patients expressed significantly lower B10 cell frequencies than age-matched controls. Mean B10 cell frequencies were significantly higher for rheumatoid arthritis patients after culture with CpG but not for other patient groups (Fig 7B) even though B10 cell frequencies increased similarly in most cases after either LPS or ~~CPG~~ CpG stimulation (Fig. 7C, left panel).

Mean B10+B10pro cell frequencies from patients with autoimmune disease were significantly higher than controls following either CD40L+LPS or CD40L+CpG stimulation (Fig. 7A-B). Multiple patients had significantly higher B10+B10pro cell frequencies, including two patients not undergoing therapy (SLE04, PF03; Table 1). No patients expressed significantly lower B10+B10pro cell frequencies relative to age-matched controls. B10+B10pro cell frequencies increased following either LPS or CPG stimulation, but the scatter of the results was broad, suggesting inherently different patient sensitivities to LPS and CpG stimulation (Fig. 7C, right panel). Patients with high blood B10 cell frequencies did not necessarily have high B10+B10pro cell frequencies after either LPS or CpG stimulation (data not shown). Likewise, B10 or B10+B10pro cell frequencies did not correlate with $CD27^+$ B cell frequencies (data not shown). Relative B10 and B10+B10pro cell frequencies did correlate with the intensity of cytoplasmic IL-10 expression, but only one patient generated significantly higher ($p<0.05$) cytoplasmic IL-10 expression levels on a per cell basis relative to controls and other patients (Fig. 7D). Thus, blood B10 and B10pro cell numbers were not decreased in patients with systemic or organ-specific autoimmune disease when compared with healthy controls, but were significantly increased in some patients.

Discussion

These studies demonstrate the existence of human IL-10-competent B10 cells, which were readily identified by their ability to express cytoplasmic IL-10 after *in vitro* stimulation for 5 h (Fig 1). Moreover, human B10 cells were able to regulate cytokine production by monocytes *in vitro*, demonstrating a functional link between regulatory B cells and the innate immune system (Fig. 6B). Peripheral blood B10 cell frequencies were characteristically low in most individuals, consistent with their low frequencies in mice. Human B10pro cells were also identified at low frequencies by their ability to express IL-10 after *in vitro* maturation during 48 h cultures. Remarkably, the adaptive and innate activation pathways that induced human B10 and B10pro cell generation, maturation, cytoplasmic IL-10 expression, and IL-10 secretion were similar to those used to characterize mouse regulatory B10 cells. Specifically, human B10 cells responded to phorbol ester/ionomycin, LPS and CpG stimulation, with B10pro cell maturation in response to CD40, LPS, and CpG induced signals. Previous studies of IL-10 production by human B cells have predominantly studied bulk B cell populations using stimulation and assay conditions that were not optimized for quantifying or characterizing individual B cells that were competent to express IL-10.[25,26,39-43] Nonetheless, the results herein demonstrate that rare B10 and B10pro cells that are competent to express IL-10 exist in human blood and can be quantified *in vitro*.

IL-10-competence remains the best phenotypic marker for defining human B10 cells. However, freshly isolated blood B10 and B10pro cells were also predominantly $CD24^{hi}CD27^{+}$, with ~60% also expressing CD38 (Fig. 5A, C). Others have found similar total numbers of $IL\text{-}10^{+}$ B cells in the $CD24^{hi}CD38^{hi}$ and $CD24^{int}CD38^{int}$ B cell fractions[28], in agreement with the current findings (Fig. 5C). B10 cells also expressed CD48 and CD148 at high levels (Fig. 5A). CD48 is upregulated on activated B cells[44] and CD148 is considered a marker for human memory B cells.[45] CD27 expression is also a well-characterized marker for memory B cells, although some memory B cells may be $CD27^{-}$.[38,46,47] The $CD27^{+}$ B cell subpopulation can also expand during the course of autoimmunity and may serve as a marker for disease activity.[38,47] However, B10 cell frequencies did not parallel the size of the blood $CD27^{+}$ memory B cell pool

in normal donors or patients (data not shown). Thus, the $CD24^{hi}CD27^{+}$ phenotype of B10 and B10pro cells may indicate their selection into the memory B cell pool during development or they may represent a distinct B cell subset that shares common cell surface markers with memory B cells. Consistent with a memory phenotype, the proliferative capacity of blood B10 cells in response to mitogen stimulation was higher than that for other B cells (Fig. 5F), as is seen for mouse B10 cells.[21] Moreover, IL-10 was also predominantly secreted by *ex vivo* $CD24^{hi}CD27^{+}$ B cells (Fig. 5G). However, most $CD24^{lo}CD27^{-}$ B cells were not IL-10 competent, even after 48 h of LPS or CpG stimulation along with CD40 ligation. Human transitional B cells are also rare (2-3% of B cells) in adult blood, and are generally $CD10^{+}CD24^{hi}CD38^{hi}$ cells that are also $IgD^{+}CD27^{-}$.[48,49] Given that CD10 expression is a well-accepted marker for most cells within the transitional B cell pool.[50], its absence on B10 cells suggests that these cells are not recent emigrants from the bone marrow. Thereby, in addition to inherent IL-10 competence, B10 cells demonstrate elevated proliferative responses that may reflect prior antigen stimulation.

Since IL-10 is critical for B cell regulatory activity in mice, the current studies demonstrate that B10 cells were functionally competent to express IL-10 in healthy individuals and 91 autoimmune disease patients (Fig. 7A-B). Blood B10 cell frequencies in most patients with lupus, rheumatoid arthritis, Sjögren's syndrome, autoimmune skin disease, and multiple sclerosis were not significantly different from those observed in healthy controls, although mean B10+B10pro cell frequencies were significantly increased. Multiple patients also had significantly higher B10 and/or B10+B10pro cell frequencies, including systemic lupus and pemphigus patients with either untreated or severe disease (Table 1). Consistent with this, IL-10 production by blood B cells is reported to be higher in patients with rheumatoid arthritis, lupus, and systemic sclerosis.[23,24,26] Moreover, elevated B10/B10pro cell frequencies in humans parallels what has been found during inflammation[12,13] and autoimmunity in mice.[14,21] Although patient cohorts with recent-onset disease and clinically active disease across multiple organ systems will be needed to fully assess the relationship of blood B10 cell numbers with clinical, laboratory, and treatment status, none of the patients or patient groups had significantly lower blood B10 cell

numbers than age-matched healthy controls. Since multiple sclerosis and lupus patient's B cells are reported to produce decreased amounts of IL-10,[27,28] the current studies demonstrate that a careful enumeration of IL-10 competent B10 cell frequencies within patients will be required in order to interpret experimental results, particularly when mixed populations of B cells are assayed functionally. Blair et al. have also functionally characterized human blood $CD24^{hi}CD38^{hi}$ B cells stimulated *in vitro* with CD40L expressing CHO cells and shown that they reduce $CD4^+$ T cell expression of IFN-γ and TNF-α following T cell stimulation with CD3 mAb.[28] By contrast, we find that both activated B10 and non-B10 cells reduce TNF-α expression following $CD4^+$ T cell stimulation with CD3 mAb through IL-10-independent pathways (Fig. 6A). Nonetheless, we have found that mouse spleen $CD1d^{hi}CD5^+$ B cells can inhibit $CD4^+$ T cell IFN-γ and TNF-α expression, which is completely dependent on IL-10 expression, but this requires B10 cell activation and is only observed with antigen-specific T cell activation.[22] Furthermore, mouse spleen $CD1d^{hi}CD5^+$ B cells regulate the antigen-presenting capacity of dendritic cells *in vitro*, and can thereby also regulate $CD4^+$ T cell activation indirectly.[22] Moreover, the ability of human B10 cells to influence innate monocyte function (Fig. 6B) expands their regulatory role during immunity and disease. Thus, it is likely that IL-10 produced by human B10 cells will have pleiotropic regulatory effects on the immune system as occurs in mice.

In summary, the current findings demonstrate the existence of a small but significant subset of $CD24^{hi}CD27^+$ B cells that is pre-programmed *in vivo* to express IL-10 after *ex vivo* maturation/stimulation. Monitoring the numbers and ability of individual B10 cells to produce IL-10 will become even more informative once the *in vivo* physiologic triggers of B10 cell regulatory activity are identified. The identification of antigen-specific B10 cells may also facilitate a further understanding of their relevance to immune responsiveness since antigen receptor specificity is important for mouse B10 cell development and *in vivo* functional activity in the regulation of inflammation and autoimmunity.[8,12,13]

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Authorship Contributions

Y.I., T.M., M.H., D.D., K.Y., G.V., and T.T. made substantial contributions to conception and design of the study, acquisition of the data, and drafted the article. P.S. S.B., C.M, A.W., R.H. and E.St.C. provided critical patient materials, information, and input into the design of the study. All authors reviewed the data and manuscript critically for intellectual content, analysis and interpretation of the data, and have given final approval of the version to be published.

Conflict of Interest Disclosure

The authors have no financial conflicts of interest, but T.F.T. is a paid consultant for MedImmune Inc. and a paid consultant and shareholder for Angelica Therapeutics, Inc.

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Table 1. Patient characteristics.

Diagnosis Number	Sex	Age	Disease Duration (y)	Autoantibody/Clinical Status	Immunosuppressive Therapy
RA01[1]	M	54	11	RF=600 IU/ml; anti-CCP=68.5 U/ml	MTX, ADA, Pred 5 mg/d
RA02	F	44	4	RF=352 IU/ml; anti-CCP>100 U/ml	MTX
RA03	F	54	14	RF=146 IU/mL	MTX, LEF
RA04	F	85	18	RF=neg	MTX, IFX
RA05	M	69	19	RF=208 IU/ml; anti-CCP>100 U/ml	MTX, Pred 5 mg/d
RA06	F	71	8	RF=neg	MTX
RA07	F	67	13	RF=333 IU/ml	ETN
RA08	F	58	25	RF=53 IU/ml	MTX, ETN, Pred 3 mg/d
RA09	F	68	7	RF=339 IU/ml	MTX, Pred 3 mg/d
RA10	M	75	13	RF=420 IU/ml; anti-CCP=18.9 U/ml	MTX, LEF, Pred 10 mg/d
RA11	F	73	7	RF and anti-CCP=neg	MTX, LEF
RA12	M	61	27	RF=107 IU/ml	MTX, LEF
RA13	F	66	6	RF=pos	ADA
RA14	F	84	11	RF=275 IU/ml; anti-CCP=28 U/ml	ETN
RA15	F	52	3	RF=neg; anti-CCP>100 U/ml	ETN
RA16	F	76	18	RF=pos	MTX
RA17	M	63	11	RF=neg; anti-CCP>100 U/ml	MTX, SSZ, Pred 1 mg/d
RA18	F	43	15	RF=23 IU/ml	MTX, IFX
RA19	F	62	30	RF=148 IU/ml; anti-CCP>100 U/ml	LEF; Pred 5 mg/d
SLE01	F	65	11	ANA=1:2560; IgG CL and anti-dsDNA= pos	HCQ, LEF, Pred 5 mg/d
SLE02	M	31	3	ANA=1:640; anti-RNP, anti-Sm, and anti-Ro=pos	HCQ, Pred 3 mg/d
SLE03	M	63	32	ANA=1:640; anti-dsDNA and IgG anti-CL= pos	HCQ, Pred 5 mg/d
SLE04	F	37	5	ANA=1:2560; anti-Ro=pos	None
SLE05	F	43	15	ANA=1:160; RF=36 IU/ml	HCQ, MMF
SLE06	F	46	8	ANA=pos; anti-Ro=pos	MMF 2 g/d, Pred 10 mg/d
SLE07	M	31	23	ANA=1:160; anti-dsDNA and IgG anti-CL=pos	HCQ
SLE08	F	47	10	ANA=1:2560; anti-dsDNA, anti-Ro and anti-La=pos; RF=103 IU/ml	None
SLE09	F	37	16	ANA=pos; anti-dsDNA, anti-IgM and IgG CL=pos	HCQ; Pred 10 mg/d
SLE10	F	48	7	ANA=1:2560; anti-dsDNA, anti-Ro and anti-La =pos	Pred 5 mg/d
SLE11	F	37	25	ANA=1:640	HCQ
SLE12	F	49	6	ANA=1:640; anti-dsDNA and anti-Ro=pos	HCQ
SLE13	F	48	20	ANA=1:640; anti-dsDNA and anti-Ro=pos	Pred 5 mg/d
SLE14	F	58	13	ANA=pos; anti-dsDNA, anti-Ro and anti-La=pos	MMF, HCQ, Pred 10 mg/d
SjS01	F	52	1	ANA=1:2560; RF=354 IU/ml; anti-Ro and anti-La=pos	None
SjS02	F	65	15	ANA=1:2560; RF=22 IU/ml; anti-Ro= pos	HCQ
SjS03	F	57	37	ANA=1:160; anti-Ro=pos	MMF, Pred 40 mg/d
SjS04	F	67	22	ANA=1:2560; RF 110 IU/ml, anti-Ro and anti-La=pos	HCQ
SjS05	F	60	9	ANA=1:2560; RF=126 IU/ml, anti-Ro=pos	HCQ
SjS06	F	58	21	ANA=1:2560; anti-Ro and anti-La=pos	None

SjS07	F	41	13	ANA=1:2560; anti-Ro and anti-La=pos	HCQ
SjS08	F	59	8	ANA=1:2560; RF=508 IU/ml; anti-Ro=pos	None
SjS09	F	42	4	ANA=1:2560; RF=110 IU/ml; anti-Ro and anti-La=pos	HCQ
SjS10	F	58	5	ANA=1:2560; anti-Ro and anti-La=pos	HCQ, Pred 3 mg/d
SjS11	M	66	5	ANA=1:2560; anti-Ro and anti-La=pos	None
SjS12	F	76	13	ANA=1:2560; anti-Ro=pos; RF=32 IU/ml	None
SjS13	M	51	1	ANA=1:160	HCQ
SjS14	F	68	28	ANA=1:2560; anti-Ro=pos	HCQ
SjS15	F	66	12	ANA=1:2560	None
SjS16	F	64	8	ANA=1:2560; anti-Ro=pos	None
SjS17	F	78	13	ANA=pos; anti-Ro=pos	HCQ
BP01	M	72	0.3	Anti-BP180=84 U/ml; anti-BP230=115 U/ml, no clinical disease	Pred 60 mg/d
BP02	M	54	1.2	Anti-BP180=72 U/ml; anti-BP230=neg, no clinical disease	MMF, Pred 12 mg/d
BP03	F	56	2	Anti-BP180=51 U/ml; anti-BP230=neg, no clinical disease	Pred 20 mg/d
BP04	M	75	4.3	Anti-BP180=45 U/ml; anti-BP230=3, mild disease	None
BP05	F	66	1.8	Anti-BP180=96 U/ml; anti-BP230=131, severe disease	None
BP06	M	77	0.5	Anti-BP180=5 U/ml; anti-BP230=95, mild disease	None
BP07	F	67	17	Anti-BP180=46 U/ml; anti-BP230=neg, mild disease	RTX (20 mos earlier)
BP08	F	54	2	Anti-BP180=22 U/ml; anti-BP230=7, no clinical disease	RTX (13 mos earlier), Pred 15 mg/d
BP09	F	62	5.4	Anti-BP180=30 U/ml; anti-BP230=3, mild disease	RTX (52 mos earlier)
BP10	M	73	0.25	Anti-BP180=222 U/ml; anti-BP230=7, no clinical disease	Pred 50 mg/d
PF01	M	54	8.6	Anti-DSG1=134 U/ml; anti-DSG3=neg, mild disease activity	AZA
PF02	M	55	9.8	Anti-DSG1=neg; anti-DSG3=neg, mild disease activity	RTX (30 mos earlier)
PF03	M	46	6.6	Anti-DSG1=416; anti-DSG3=neg, moderate disease activity	None
PF04	M	50	5.3	Anti-DSG1=1906; anti-DSG3=neg, moderate disease activity	MMF
PF05	M	72	2.8	Anti-DSG1=118; anti-DSG3=neg, no clinical disease	Dapsone 100 mg/d
PF06	F	47	0.4	Anti-DSG1=211; anti-DSG3=neg, moderate clinical disease activity	Pred 40 mg/d
PV02	M	43	3	Anti-DSG1=neg; anti-DSG3=213 U/ml, moderate oral disease activity	MMF, Pred 20 mg/d
PV03	M	73	3.3	Anti-DSG1=neg; anti-DSG3=948 U/ml, mild oral disease	Pred 12 mg/d
PV04	F	55	4.7	Anti-DSG1=neg; anti-DSG3=406 U/ml, moderate oral disease activity	RTX (15 mos earlier)
PV05	M	59	8.3	Anti-DSG1=neg; anti-DSG3=50 U/ml, no disease activity	AZA
PV06	F	48	8.6	Anti-DSG1=neg; anti-DSG3=25 U/ml, no disease activity	None
PV07	M	45	0.25	Anti-DSG1=968; anti-DSG3=735, severe disease involving 20% of the skin	MMF; Pred 80 mg/d
PV08	M	84	0.2	Anti-DSG1=75; anti-DSG3=146 U/ml, moderate disease	None

				activity	
PV09	M	64	9.8	Anti-DSG1=14; anti-DSG3=115 U/ml, mild disease activity	Pred 20 mg/d; IFX
PV10	M	59	3.8	Anti-DSG1=1; anti-DSG3=49 U/ml, no disease activity	AZA
PV11	M	55	6	Anti-DSG1=34; anti-DSG3=35 U/ml, mild oral disease activity	Pred 20 mg/d; MMF
PV12	F	58	9.5	Anti-DSG1=1; anti-DSG3=43 U/ml, mild oral disease activity	AZA
PV13	M	75	5	Anti-DSG1=1; anti-DSG3=42 U/ml, mild disease	RTX (6 mos earlier), Pred 1 mg/d
PV14	M	70	8.4	Anti-DSG1=110; anti-DSG3=113 U/ml, no clinical disease	AZA
PV15	F	37	0.3	Anti-DSG1=104; anti-DSG3=810 U/ml, moderate disease activity	Pred 40 mg/d
PV16	M	78	15	Anti-DSG1=297; anti-DSG3=447 U/ml, moderate disease activity	None
DH1	F	65	8.4	Gluten-free diet, no clinical disease	Dapsone 25 mg/d
DH2	M	40	30	normal diet, no clinical disease	Dapsone175 mg/d
MS01	F	72	54	SPMS, EDSS 6.5, not clinically active	None
MS02	M	62	24	RRMS, EDSS 6.5, clinically active	BIFN
MS03	M	33	2	RRMS, EDSS 1.0, disease not clinically active	BIFN
MS04	M	75	29	SPMS, EDSS 8.0, disease not clinically active	ITMTX
MS05	M	52	24	PPMS, EDSS 6.5, disease clinically active	MMF, pulse steroids
MS06	M	55	25	PPMS, EDSS 7.5, disease clinically active	ITMTX
MS07	F	39	16	SPMS, EDSS 7.0, disease not clinically active	Natalizumab (2 mos prior)
MS08	F	51	7	SPMS, EDSS 5.5, disease not clinically active	BIFN

[1]Abbreviations: ANA, antinuclear Ab; ADA, adalimumab; AZA, azathioprine; BIFN, beta interferon; BP, bullous pemphigoid; CCP, cyclic citrullinated peptide; CL, cardiolipin; DH, dermatitis herpetiformis; dsDNA, double stranded DNA; DSG, desmoglein; EDSS, disability scale from 0=normal to 10=death; ETN, etanercept; HCQ, hydroxychloroquine; IFX, infliximab; ITMTX, intrathecal methotrexate; LEF, leflunomide; MMF, mycophenolate mofetil; MTX, methotrexate; PF, pemphigus foliaceus; PPMS, primary progressive multiple sclerosis; Pred, Prednisone; PV, pemphigus vulgaris; RA, rheumatoid arthritis; RF, rheumatoid factor; RRMS, relapsing remitting multiple sclerosis; RTX, rituximab; SjS, primary Sjögren's syndrome; SLE, lupus; SPMS, secondary progressive multiple sclerosis; SSZ, sulfasalazine; y, year.

[2]Normal values: anti-BP=180, anti-BP=230, anti-DSG1 and anti-DSG3 antibodies <9 IU/ml.

Figure Legends

Figure 1. Enumeration of human blood IL-10-competent B10 and B10pro cells. (**A**) Visualizing IL-10$^+$ B cells. Purified blood mononuclear cells were cultured with Brefeldin A (BFA), LPS plus PMA, ionomycin and Brefeldin A (PIB), or LPS plus PMA, ionomycin and monensin (PIM) for 5 h and stained for cell viability, cell surface CD19 expression, and cytoplasmic IL-10. Representative cytoplasmic IL-10 staining by viable, single CD19$^+$ B cells is shown in the flow cytometry dot-plots, with percentages indicating cytoplasmic IL-10$^+$ B cell frequencies within the indicated gates. Blood mononuclear cells that were cultured with Brefeldin A alone before immunofluorescence staining served as negative controls, with background staining similar to that obtained using isotype-matched control mAbs. Bar graphs represent mean (±SEM) B10 cell frequencies from 3 individuals. (**B**) Representative IL-10 production by B cells from an individual with relatively high B10 cell frequencies. B10 cells were identified after *in vitro* stimulation for 5 h as in (A). Alternatively, IL-10$^+$ B cell frequencies were determined after *in vitro* B10pro cell maturation by stimulation with LPS, CD40L+LPS, CpG, or CD40L+CpG, with PIB added during the final 5 h of 48 h cultures. As negative controls for IL-10 staining, only BFA was added to some cultures during the final 5 h. Percentages indicate the frequencies of cytoplasmic IL-10$^+$ B cells within the indicated gates among total CD19$^+$ B cells. (**C**) B10 cell frequencies in individuals after with TLR agonist stimulation as in (A-B). Dots represent results from single individuals after 5 h culture with BFA alone, PIB, or the indicated TLR agonist+PIB. Horizontal bars indicate means. (**D**) CD40L induced optimal B10+B10pro cell maturation during 48 h *in vitro* cultures with either recombinant CD40L or CD40 mAb, plus LPS for 48 h, with PIB added during the final 5 h. Bar graphs represent means (±SEM) from 5 individuals. Similar results were obtained in 2 independent experiments. (**E**) Representative B10+B10pro cell frequencies after *in vitro* maturation and stimulation. Blood mononuclear cells were cultured for 48 h with media alone or media containing CD40L, along with the indicated TLR agonists, with PIB added during the last 5 h of culture. Significant differences between cultures with or without

CD40L are indicated: $^{\#}p<0.05$, $^{\#\#}p<0.01$. (C-E) Significant differences between means of controls and individual stimuli are indicated: *p<0.05, **p<0.01.

Figure 2. Human B10 and B10pro cells in (**A**) cord blood, and (**B**) spleen and tonsil. B10 cells and B10+B10pro cells were identified after *in vitro* stimulation for 5 h and 48 h, respectively, as in figure 1. Representative results are shown along with graphs indicating IL-10^+ B cell frequencies within individuals. Cells cultured with BFA alone served as negative controls for background IL-10 staining. Significant differences between means of BFA controls and individual stimuli are indicated: *p<0.05, **p<0.01.

Figure 3. Blood B cell stimulation induces IL-10 transcription and secretion *in vitro*. (**A**) Time course of *Il10* transcript induction. Purified $CD19^+$ B cells were cultured with media alone or CD40L+CpG for the times indicated, with *Il10* transcripts quantified by real-time RT-PCR analysis. Bar graphs indicate mean relative *Il10* transcript (±SEM) levels in six individuals. (**B**) B cells secreting IL-10 express *Il10* transcripts. Purified blood B cells were cultured with PMA and ionomycin for 4 h before CD19 staining and secreted IL-10 capture (left panel). Cell surface IL-10^+ and IL-10^- B cells were isolated using the indicated gates and subsequently reassessed for IL-10 secretion (right panels) before relative *Il10* transcript levels were quantified by real-time RT-PCR analysis. Mean fold-differences (±SEM) for *Il10* transcript levels from 3 different individuals are shown, with transcript levels normalized so that the relative mean IL-10^- B cell value is 1.0. (**C**) Cell surface signals that regulate cytoplasmic IL-10 expression. Blood B cells were cultured with CpG, CD40L, and anti-IgM Ab (IgM) as indicated for 48 h with PIB added during the final 5 h of culture. Representative frequencies of IL-10-producing cells are shown, with bar graphs indicating mean (±SEM) percentages in 5 individuals. (**D**) TLR agonists that induce IL-10 secretion. Purified $CD19^+$ B cells were cultured with media alone, CD40L, or with TLR agonists and CD40L as indicated for 48 or 72 h. IL-10 secreted into the culture supernatant fluid was quantified by ELISA. Bar graphs indicate mean IL-10 (±SEM) concentrations from ≥4

different individuals. (A-D) Similar results were obtained in 2 independent experiments. Significant differences between means of cells cultured in media alone and stimulated cultures are indicated: *$p<0.05$, **, $p<0.01$.

Figure 4. Phenotypes of blood and tissue B10 cells *ex vivo* and B10+B10pro cells after *in vitro* culture. (**A**) Representative cell surface phenotype of blood B cells cultured with LPS+PIB for 5 h. (**B**) Representative cell surface phenotype of blood B10+B10pro cells after stimulation with CD40L+LPS for 48 h with PIB added during the final 5 h of culture. (**C**) Representative cell surface phenotype of spleen B10 cells cultured with CpG+PIB for 5 h. (**D**) Representative cell surface phenotype of spleen B10+B10pro cells after stimulation with CD40L+CpG for 48 h with PIB added during the final 5 h of culture. (A-D) Cultured cells were stained for viability and cell surface molecule expression, permeabilized, stained with anti-IL-10 mAb, and analyzed by flow cytometry. Representative cell surface molecule expression by IL-10^+ (thick line) and IL10^- (thin line) $CD19^+$ B cells from three individuals is shown. Shaded histograms represent isotype-matched control mAb staining.

Figure 5. *Ex vivo* blood B10 and B10pro cells share cell surface markers with memory B cells. (**A**) Blood B10 cells predominantly exhibit a $CD24^{hi}CD27^+CD48^{hi}CD148^{hi}$ phenotype. Purified blood B cells were cultured with CpG+PIB for 5 h before immunofluorescence staining for viability, cell surface molecule expression, and cytoplasmic IL-10. Cell surface CD24, CD27, CD38, CD48, and CD148 expression by IL-10^+ (thick line) and IL-10^- (thin line) $CD19^+$ cells was assessed by flow cytometry. (**B**) Cytoplasmic IL-10 induction does not affect the cell surface phenotype of B cells. $CD19^+$ blood B cells were cultured with media on ice (thin line) or with CpG+PIB (thick line) for 5 h before immunofluorescence staining and flow cytometry analysis as in (A). (A-B) Shaded histograms represent isotype-matched control mAb staining. Results represent those obtained for 3 individuals. (**C**) Distributions of B10 cells within B cell subsets defined by CD24, CD27, IgD/CD38, and IgD/CD27 expression. Purified blood B cells were

cultured with LPS+PIB for 5 h before immunofluorescence staining and flow cytometry analysis as in (A). The horizontal and vertical lines on each contour plot are shown for reference, with the lower left quadrants delineating the $IgD^{-}CD38^{-}$ and $IgD^{-}CD27^{-}$ subsets determined by control mAb staining. Results represent those obtained for 5 individuals. (**D**) The *ex vivo* $CD24^{hi}CD27^{+}$ B cell subset includes the majority of B10 cells. Purified B cells were cultured with LPS+PIB for 5 h before immunofluorescence staining for cell surface CD19, CD24, and CD27 expression and cytoplasmic IL-10 expression, with subsequent flow cytometry analysis. (**E**) B10pro cells derive from the $CD24^{hi}CD27^{+}$ B cell subset. Purified blood B cells were sorted into the $CD24^{hi}CD27^{+}$ and $CD24^{low}CD27^{-}$ B cell subsets as indicated by the gates shown with purities >90% when reanalyzed by flow cytometry. The purified B cells were cultured with CD40L plus either LPS or CpG for 48 h, with PIB added during the final 5 h of culture before the relative percentages of IL-10^{+} B cells within the indicated gates was determined. Similar results were obtained in 2 independent experiments. (**F**) *Ex vivo* $CD24^{hi}CD27^{+}$ B cells are the predominant source of secreted IL-10. Purified blood B cells were sorted into the $CD24^{hi}CD27^{+}$ and $CD24^{low}CD27^{-}$ B cell subsets as in (E) and cultured with the indicated stimuli for 72 h. IL-10 secreted into the culture supernatant fluid was quantified by ELISA. Bar graphs indicate mean IL-10 (±SEM) concentrations from triplicate ELISA determinations. Significant differences between means from $CD24^{hi}CD27^{+}$ and $CD24^{low}CD27^{-}$ B cells are indicated: **, $p<0.01$. Differences between means from cells in media or with stimuli are indicated: $^{\#\#}$, $p<0.01$. (**G**) B10 cell proliferation *in vitro*. Blood mononuclear cells were labeled with CFSE and cultured with CD40L and CPG (top panels) or CD40L and LPS (top panels) for 48-96 h, with PIB added for the last 5 h of culture. Histograms (right) represent CFSE expression by the IL-10^{+} (thick line) or IL-10^{-} (thin line) B cell subsets. Results are representative of two independent experiments.

Figure 6. B10 cell regulation of innate immunity. (**A**) B10 cell effects on mitogen-stimulated T cell cytokine production. Purified blood $CD24^{hi}CD27^{+}$ or $CD24^{low}CD27^{-}$ B cells were stimulated with CD40L plus CpG for 24 h, isolated, and then cultured with CD3 mAb-stimulated $CD4^{+}$ T

cells for 72 h. After PMA plus ionomycin stimulation, $CD4^+$ T cell TNF-α expression was assessed by flow cytometry (heavy lines). $CD4^+$ T cells cultured alone are shown as positive controls (thin lines). Background cell staining using unstimulated T cells is shown (shaded lines) (**B**) B10 cells regulate monocyte cytokine production. Purified blood $CD24^{hi}CD27^+$ or $CD24^+CD27^-$ B cells were stimulated with CD40L plus CpG for 24 h, and were cultured with blood monocytes for 20 h before cytoplasmic TNF-α expression by $CD14^+$ monocytes was assessed after 4 h of LPS stimulation (heavy lines). Anti-IL-10 mAb was added to some cultures as indicated (dashed lines). Monocytes cultured alone are shown as positive controls (thin lines), with background cell staining using unstimulated monocytes shown (shaded lines). (A, B) Results represent those obtained in ≥2 independent experiments.

Figure 7. Blood B10 cell frequencies in patients with autoimmune disease. **(A)** Representative B cell cytoplasmic IL-10 expression by control (Ctrl) individuals, and lupus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SjS), blistering skin disease (BD), and multiple sclerosis (MS) patients with relatively high B10 cell frequencies after *in vitro* CpG+PIB stimulation for 5 h. B10+B10pro cell maturation was induced by 48 h CD40L+CpG stimulation, with PIB added during the final 5 h of culture. Percentages indicate $IL\text{-}10^+$ B cell frequencies among $CD19^+$ B cells. (**B**) $IL\text{-}10^+$ B cell frequencies as in (A) with each dot representing single individuals. Horizontal bars indicate group means. The solid horizontal lines indicate means plus 2 SD (95% confidence interval) for controls, while dashed lines represent means plus 2 SD for all values. The patients are described in Table 1. Significant differences between means of patient groups and healthy controls are indicated: *$p<0.05$, **, $p<0.01$. (**C**) Relative frequencies of B10 cells and B10+B10pro cells identified for control individuals and patients with autoimmune disease as in (B) compared after CpG or LPS stimulation with each dot representing an individual. (**D**) Relationship between cytoplasmic IL-10 expression levels and B10+B10pro cell frequencies in control individuals and patients after stimulation with CD40L+CpG, with PIB added during the final 5 h of 48 h cultures. Linear mean fluorescence intensities (MFI) for $IL\text{-}10^+$ and $IL\text{-}10^-$ B

cells were determined using the gates indicated in (A) with the values shown representing a ratio of IL-10^{+} to IL-10^{-} MFIs. A linear regression line (± 95% prediction bands, dashed lines) is shown for reference.

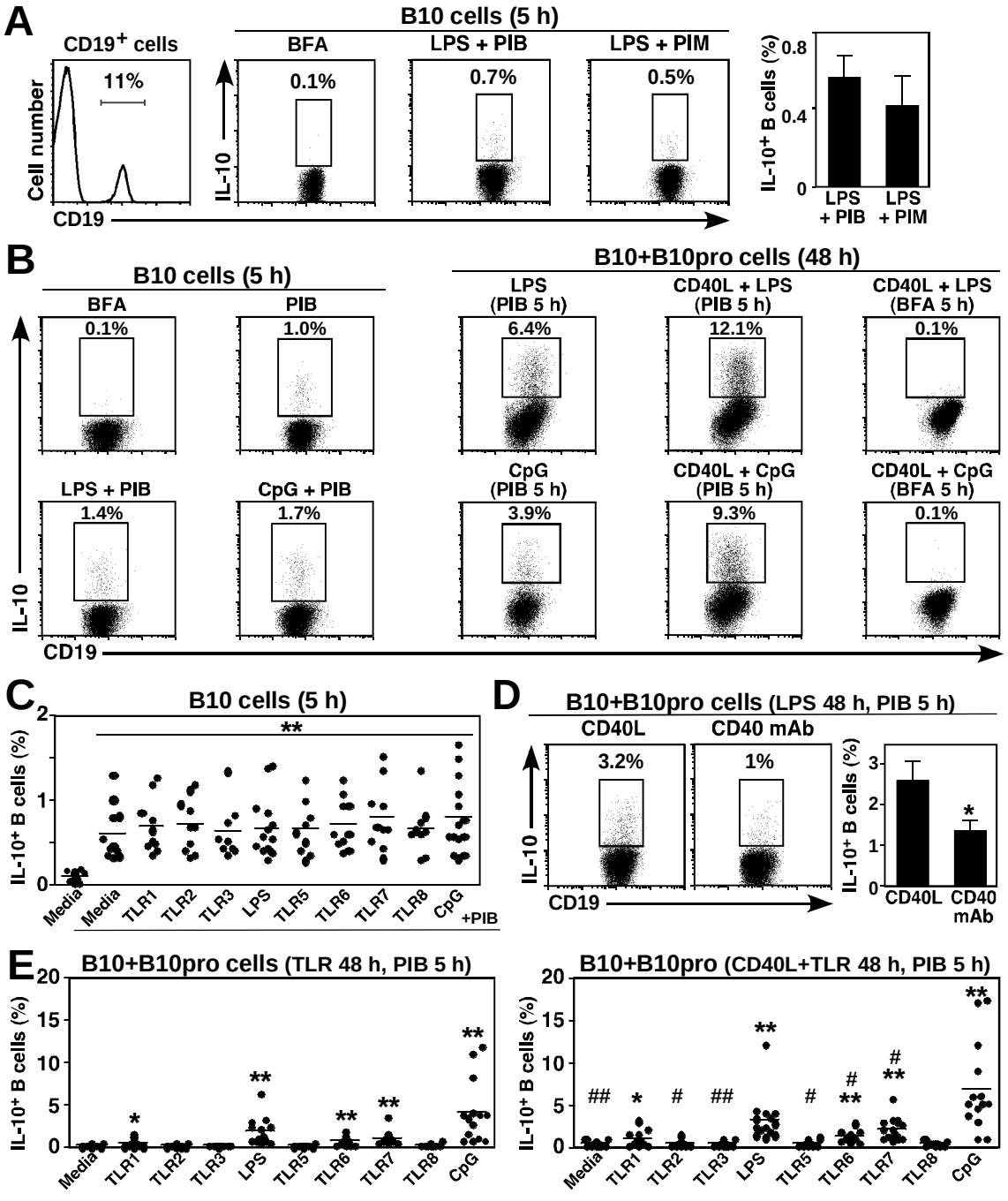

Figure 1
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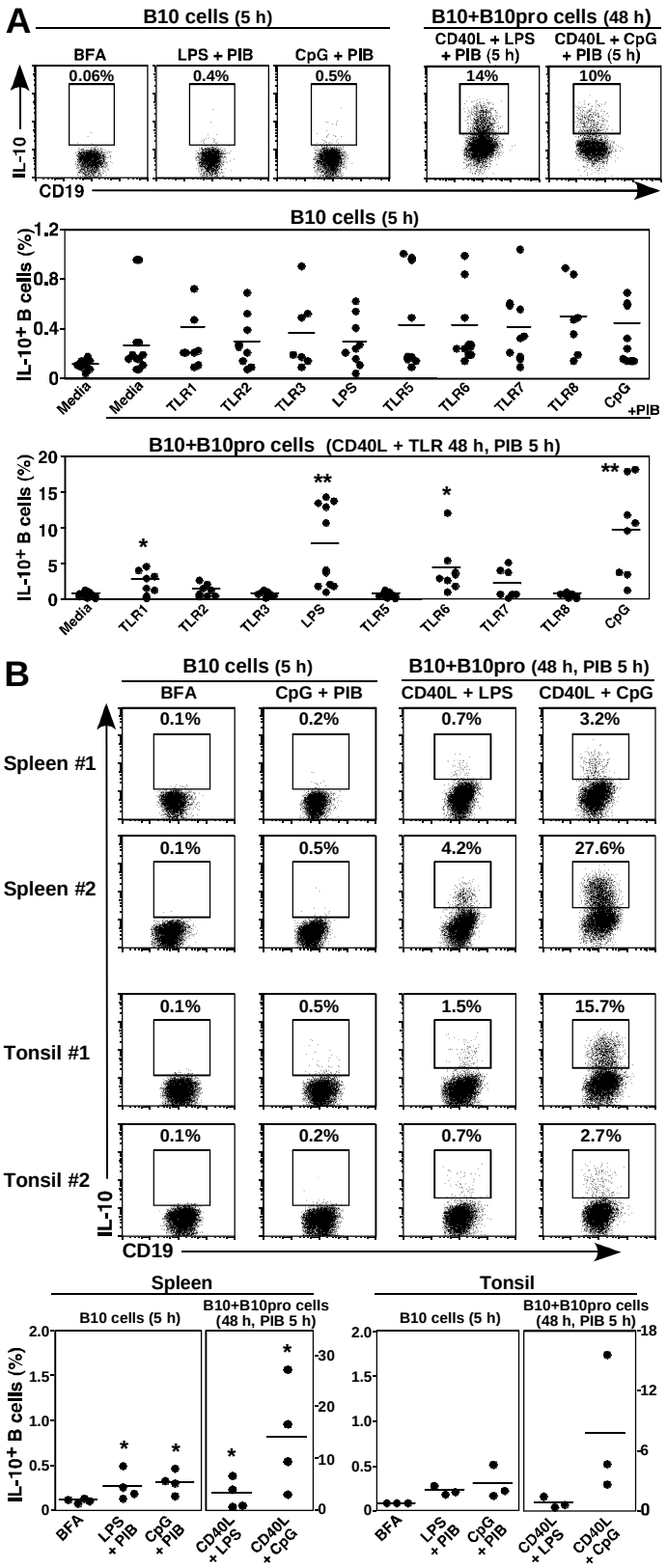

Figure 2
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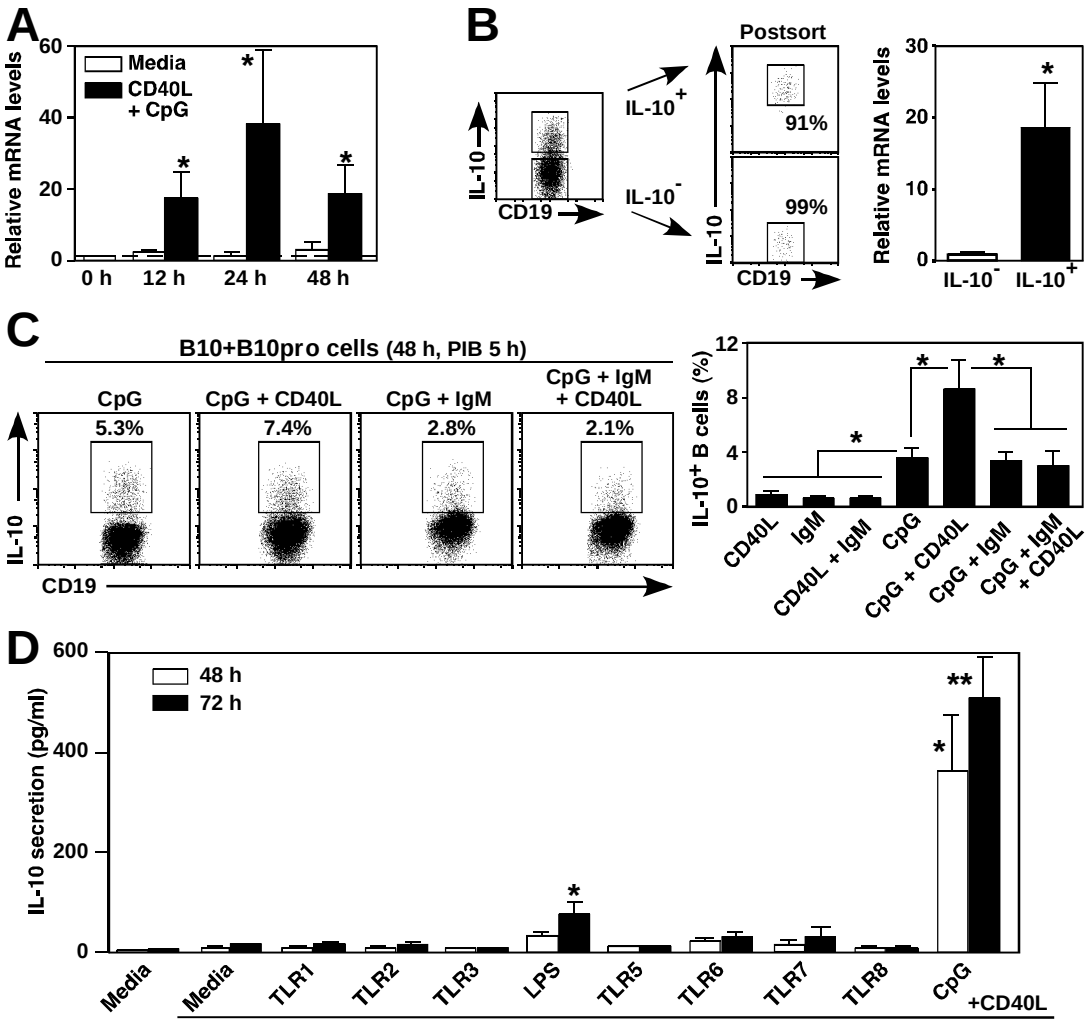

Figure 3
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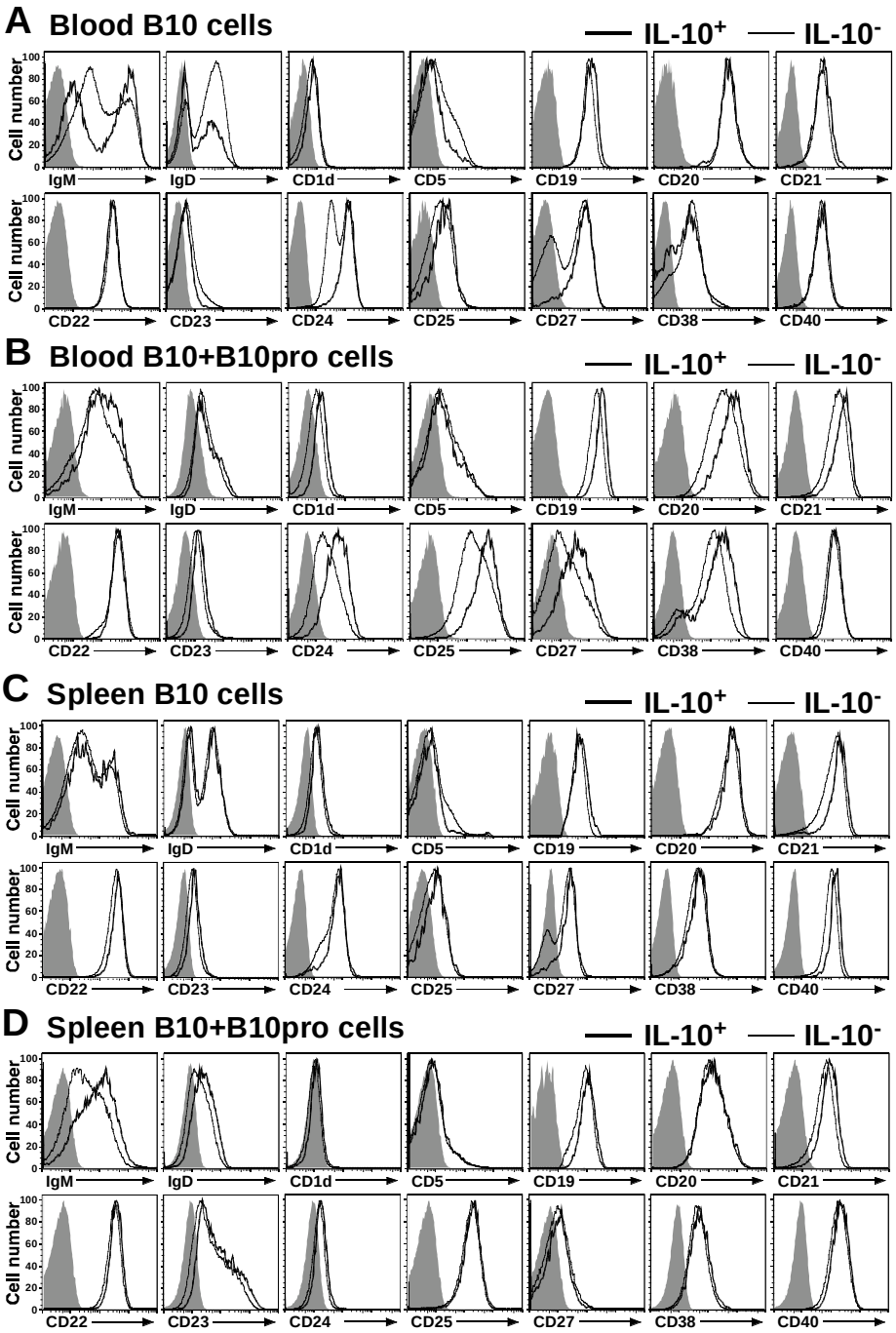

Figure 4
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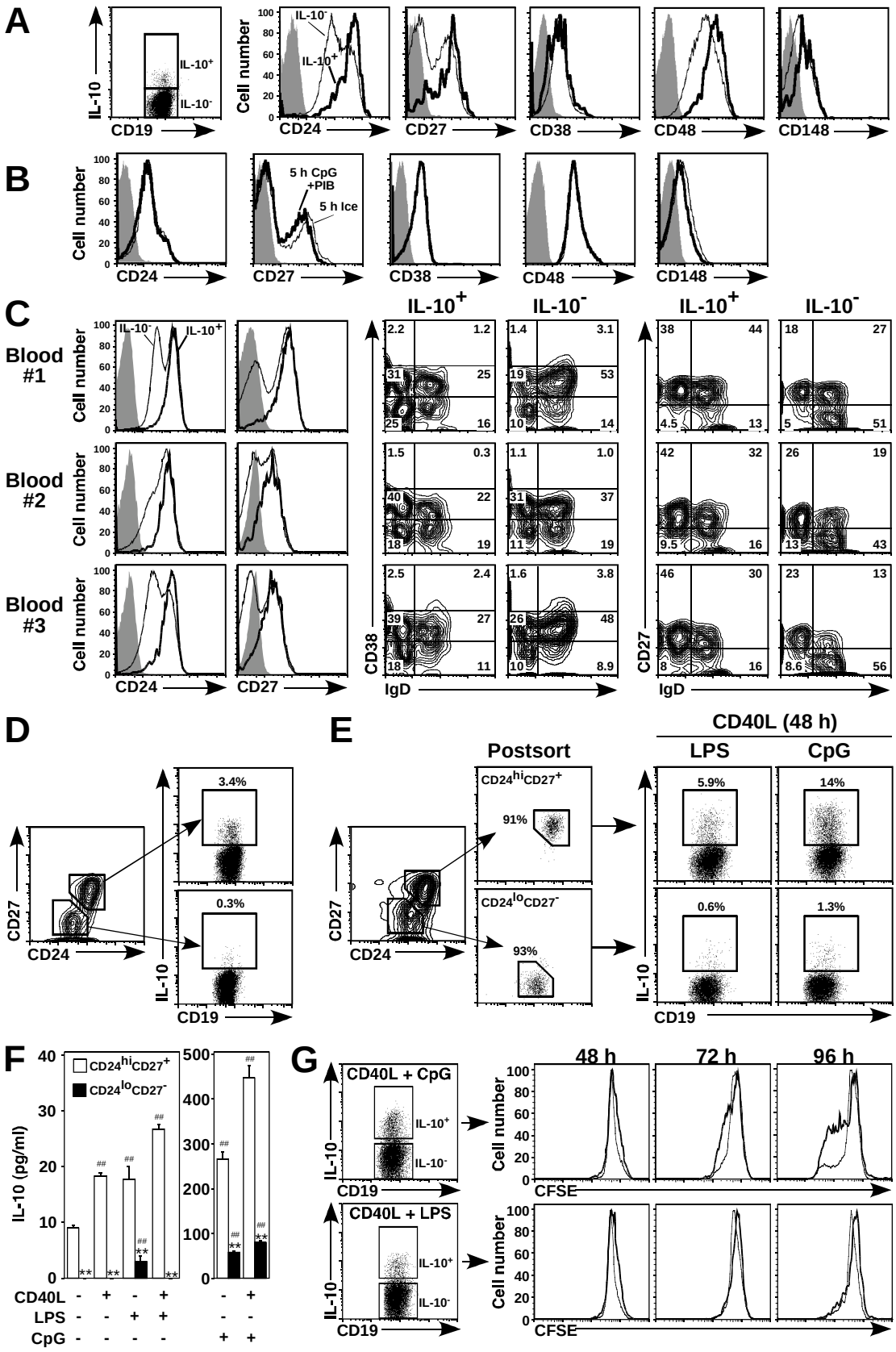

Figure 5
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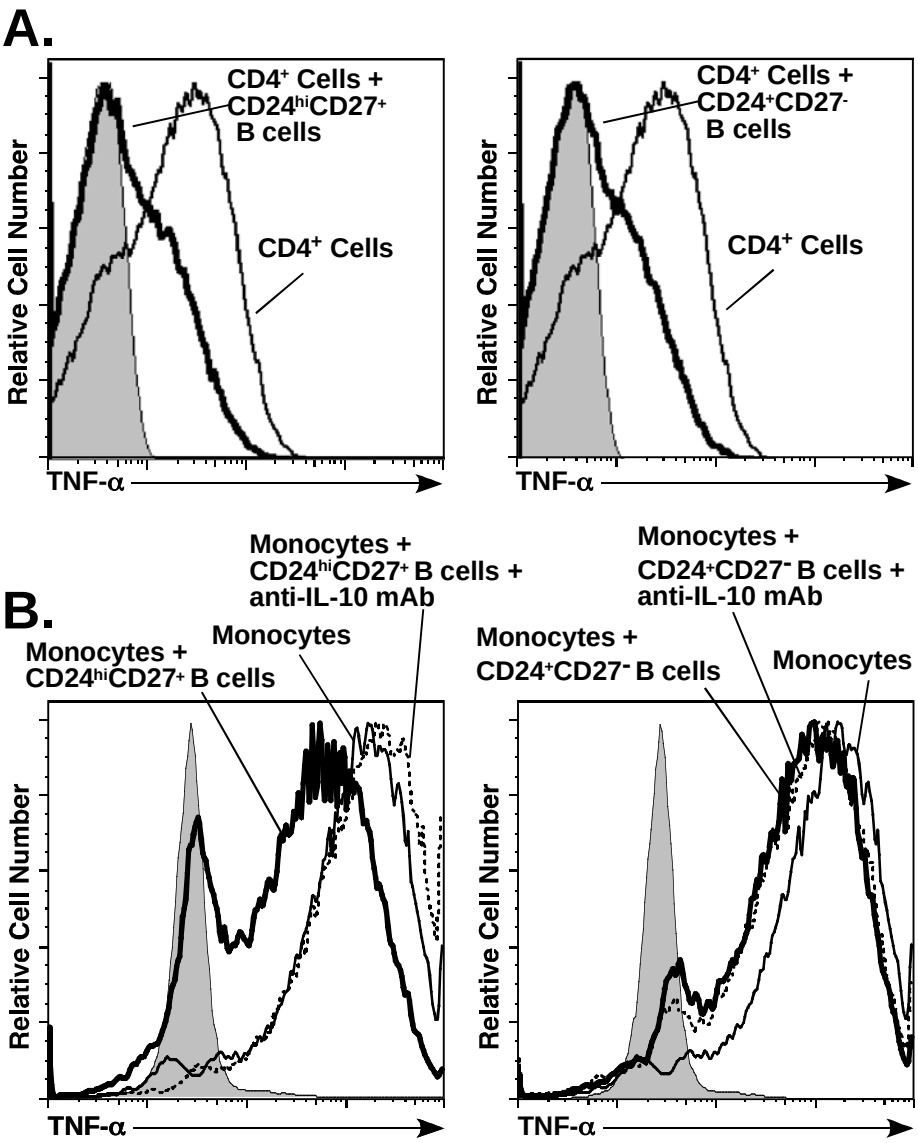

Figure 6
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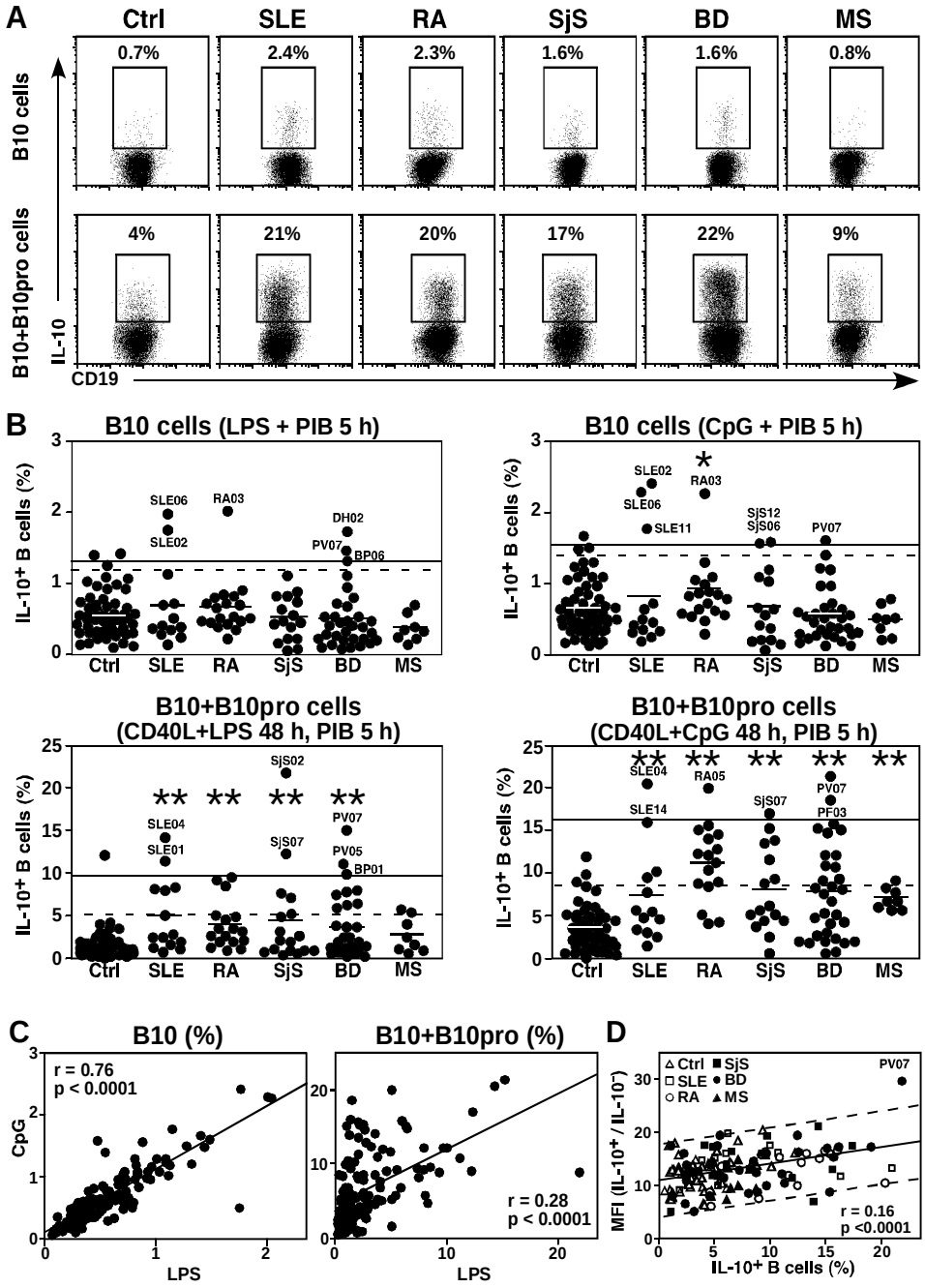

Figure 7
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