

Human “Orchestrator” CD11b⁺ B1 Cells Spontaneously Secrete Interleukin-10 and Regulate T-Cell Activity

Daniel O Griffin^{1,2} and Thomas L Rothstein^{2,3}

¹Elmezzi Graduate School of Molecular Medicine and ²Center for Oncology and Cell Biology, The Feinstein Institute for Medical Research, Manhasset, New York, United States of America; and ³Departments of Medicine and of Molecular Medicine, Hofstra North Shore–Long Island Jewish (LIJ) School of Medicine, Manhasset, New York, United States of America

Immune regulation produced by B cells has been attributed to production and secretion of interleukin (IL)-10, which is a characteristic of mouse B1 cells. In view of the widespread clinical use of B-cell depletion therapies in autoimmune and malignant diseases, it is important to monitor the function and fate of regulatory B cells. However, there is no consensus regarding the phenotypic identity of human IL-10⁺ B cells. Here we show that human CD11b⁺ B1 cells, one of two recently described subpopulations of B1 cells, spontaneously produce IL-10 and suppress T-cell activation. In view of the capacity of these B cells to either stimulate T-cell proliferation or suppress T-cell activation, CD11b⁺ B1 cells are considered to be capable of orchestrating elements of immune responsiveness and thus are termed “orchestrator B1 cells,” or “B1orc,” whereas CD11b⁻ B1 cells that primarily secrete antibody are termed “secretor B1 cells,” or “B1sec.”

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INTRODUCTION

We identified the phenotype of human B1 cells as CD20⁺CD27⁺CD43⁺ (1). Human B1 cells are found both in umbilical cord blood and adult peripheral blood. Human B1 cells share with mouse B1 cells specificity for certain microbial and self-antigens. We recently described phenotypic and functional subdivision of human B1 cells: CD11b⁻ B1 cells secrete substantial amounts of antibody but stimulate T cells less efficiently than CD11b⁺ B1 cells; CD11b⁺ B1 cells express elevated levels of CD86 and stimulate T cells strongly but secrete less antibody than CD11b⁻ B1 cells (2). Most human B1 cells are CD11b⁻, whereas a smaller proportion are CD11b⁺, although this latter population is markedly increased in patients with lupus disease.

The ability of the immune system to reign in the inflammatory response and limit it to an appropriate level is critical to addressing potential immune-mediated damage. Failure to adequately regulate immune system activity is implicated in multiple clinical situations, including inflammatory bowel disease, respiratory asthma, food allergies, autoimmune diseases and transplant rejection. Elucidation of the cells responsible for immune regulation is expected to be invaluable in advancing the understanding of these and other diseases.

Recent work has led to the recognition that certain B cells can be regulatory in nature (Breg) and can suppress inflammatory and autoreactive responses (3). It is conceivable, then, that the current broad B-cell depletion therapies being

used therapeutically could erode an important B cell–derived modulatory influence. Thus, it is of great importance to understand how to recognize Breg cells so that their number can be monitored and their fate can be determined. Immune regulation by B cells has been attributed to production and secretion of IL-10, and it has long been known that IL-10 is produced by murine B-1 cells (4,5). More recently, IL-10⁺ mouse B cells were characterized as CD1d^{hi} in addition to CD5⁺ and may include B-1 cells, MZ B cells and T2-MZ precursors (6). Comparable studies with human B cells have yielded diverse results. Human B cells that produce IL-10 have been reported to be enriched within the CD24^{hi}CD38^{hi} gated population, or the CD24^{hi}CD27⁺ gated population, but also have been shown to be present among both CD24^{low} and CD38^{low/-} populations (5,7,8). It is notable that these reports involve the use of stimulatory regimens before assessment of IL-10 content, which could induce secretion in B cells that were not previously secreting spontaneously (5).

Moreover, the majority of the putative B-cell populations possessing B regulatory

Address correspondence to Thomas L Rothstein, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030. Phone: 516-562-3436; Fax: 617-562-2533; E-mail: tr@nshs.edu.

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activity via secretion of IL-10, both CD24^{hi}CD38^{hi} cells and CD24^{hi}CD27⁺ cells, are negative for production of IL-10, as shown by intracellular staining. Only 10–15% of CD24^{hi}CD38^{hi} cells are producing IL-10 after several days of exposure to a stimulatory regimen (8), whereas only 3–4% of CD24^{hi}CD27⁺ cells are producing IL-10 after brief stimulation (7). Thus, the level of regulatory B-cell enrichment that can be obtained with these markers is severely limited. An additional issue is that neither of these gating strategies identifies a unique phenotype for IL-10-secreting cells, since the CD24^{hi}CD38^{hi} gate includes transitional cells as well as naive B cells, whereas the CD24^{hi}CD27⁺ gate includes true memory cells, circulating marginal zone (MZ) cells and B1 cells.

Because CD11b⁺ B1 cells are capable of driving T-cell proliferation through surface expression of CD86, we questioned whether members of this subpopulation might also be capable of modulating immune cell activity via production and secretion of IL-10, like mouse B1 cells. We found that human CD11b⁺ B1 cells do spontaneously produce and secrete IL-10 that is capable of downregulating T-cell responses.

MATERIALS AND METHODS

Donors and Samples

Adult peripheral blood samples were obtained by venipuncture of adult volunteers after obtaining informed consent in accordance with the Declaration of Helsinki. Additional samples in the form of leukopacks were obtained from the New York Blood Center on the day of donation. This study was approved by, and all samples were obtained in accordance with, the Institutional Review Board of the North Shore-LIJ Health System.

Processing

All blood samples were treated in a similar manner and processed promptly on receipt. Mononuclear cells were obtained by density gradient separation using lymphocyte separation medium

(Cellgro, Manassas, VA, USA). Except as otherwise noted, mononuclear cells were then washed and resuspended in RPMI 1640 (Cellgro) containing 10% fetal calf serum plus 2 mmol/L L-glutamine, 10 mmol/L HEPES (pH 7.25), 100 U/mL penicillin and 100 µg/mL streptomycin.

B-Cell Enrichment

For some experiments, B cells were enriched by CD19⁺ selection using the EasySep Human CD19⁺ B Cell magnetic bead selection kit (STEMCELL Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions.

Flow Cytometry Analysis and Cell Sorting

Enriched B cells and mononuclear cells were sort-purified on an Influx instrument (BD Biosciences, San Jose, CA, USA) after immunofluorescent staining, as described in Results. In all experiments displayed, CD20⁺ cells were studied. Flow cytometric analysis of immunofluorescently stained cells was carried out on an LSR-II instrument (BD Biosciences). When intracellular staining was performed, cells were fixed in 1.6% paraformaldehyde for 15 min at room temperature. Cells were permeabilized with 0.2% Tween for 15 min at 37°C.

Enzyme-Linked Immunosorbent Assay

B cells were cultured in RPMI medium for 5 d, after which supernatant fluid was assayed for IL-10 by enzyme-linked immunosorbent assay (ELISA; BD Biosciences) according to the manufacturer's directions.

T-Cell Activation Assays

Sort-purified CD19-enriched B cells were cocultured with negatively selected CD4⁺ T cells (STEMCELL Technologies Inc.), 1:1 in 100 µL RPMI medium (1,2) in the presence of plate-bound anti-CD3 monoclonal antibody (0.5 µg/mL) for 72 h. T-cell expression of tumor necrosis factor (TNF)-α was assessed by intracellular staining after treatment with phorbol myristate acetate (PMA) and ionomycin plus Golgi-stop for the last 6 h. IL-10 neu-

tralization experiments were performed by preincubating B cells with IL-10 neutralizing antibody for 30 min before introduction of B cells into the T-cell coculture.

Reagents

Anti-CD20-V450, anti-CD27-APC, anti-CD43-FITC, anti-CD11b-PE and anti-CD11b-PerCP, anti-CD3-PE, anti-CD19-PE-Cy7, anti-CD4-PerCP-Cy5.5, anti-TNF-α-APC and anti-CD24-PE were obtained from BD Biosciences, along with MsIgG₁κ isotype controls conjugated to V450, APC, FITC, PE and PerCP-Cy5.5, plus MsIgG_{2a}κ isotype controls conjugated to PE and PerCP. Anti-IL-10 eFluor 450 was obtained from eBiosciences. IL-10 neutralizing antibody was obtained from R&D Systems (Minneapolis, MN, USA).

Statistics

Statistical analysis was performed using Prism Software, version 5.0d, for Mac (Graphpad Software Inc., La Jolla, CA, USA). Data are displayed when appropriate as mean plus or minus the standard error of the mean (SEM). Data were compared for statistically relevant differences by using Student's *t* test with two-tailed analysis. *P* values are shown in relevant figures.

RESULTS

We evaluated spontaneous B-cell IL-10 production by combined surface and intracellular immunofluorescent staining of fixed and permeabilized, unstimulated human peripheral blood B cells. We studied CD20⁺ B cells to avoid CD19⁺CD20⁻ plasmablasts (12). Circulating B cells were divided into four populations as previously described: naive (CD3⁻CD20⁺CD27⁻CD43⁻), memory (CD3⁻CD20⁺CD27⁺CD43⁻), CD11b⁻ B1 cells (CD3⁻CD20⁺CD27⁺CD43⁺CD11b⁻) and CD11b⁺ B1 cells (CD3⁻CD20⁺CD27⁺CD43⁺CD11b⁺) (Figure 1A). We assessed the fraction of each B-cell population that expressed intracellular IL-10. We found that naive, memory and CD11b⁻ B1 cells were essentially negative for IL-10, whereas the majority of CD11b⁺ B1 cells were positive for IL-10 (Figures 1B, C). We confirmed these results by sort-purifying

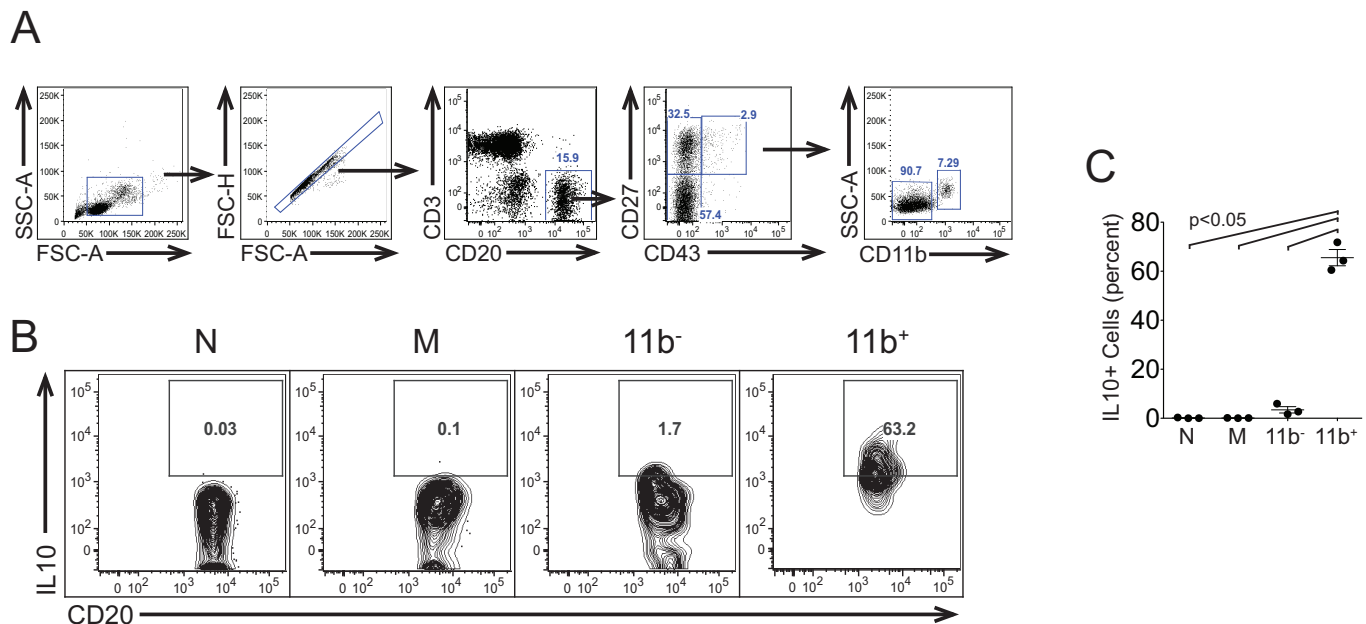


Figure 1. CD11b⁺ B1 cells express IL-10. (A) Adult peripheral blood mononuclear cells were immunofluorescently stained for surface CD3, CD20, CD27, CD43 and CD11b; and a representative sample with gating strategy used to identify naive (CD3⁺CD20⁺CD27⁻CD43⁻) and memory (CD3⁺CD20⁺CD27⁺CD43⁺) B cells and CD11b⁻ (CD3⁺CD20⁺CD27⁻CD43⁺CD11b⁻) and CD11b⁺ (CD3⁺CD20⁺CD27⁻CD43⁺CD11b⁺) B1 cells is shown. (B, C) Adult peripheral blood mononuclear cells were fixed, permeabilized and immunofluorescently stained for surface CD3, CD20, CD27, CD43 and CD11b, as well as intracellular IL-10. The fraction of naive (N) and memory (M) B cells and of CD11b⁻ (11b⁻) and CD11b⁺ (11b⁺) B1 cells is shown for a representative sample in (B) and for three independent samples in (C). Horizontal lines in (C) represent mean values and SEM. The cutoff for intracellular IL-10 positivity was determined on the basis of fluorescence-minus-one.

B-cell populations, culturing them in medium alone and then evaluating supernatants for spontaneously secreted IL-10 by ELISA. We found that IL-10 was produced by CD11b⁺ B1 cells in preference to other B1 or B2 cells (Figure 2).

We evaluated the regulatory function of CD11b⁺ B1 cell-generated IL-10 by coculturing sort-purified B cells at a 1:1 ratio with negatively selected CD4⁺ T cells stimulated by plate-bound anti-CD3. After 3 d, T-cell activation was assessed by intracellular staining for TNF- α (Figures 3A, B). We found that naive, memory and CD11b⁻ B1 cells failed to inhibit T-cell activation. In contrast, we found that CD11b⁺ B1 cells strongly inhibited expression of TNF- α by stimulated T cells. To directly assess the role of IL-10, CD11b⁺ B1 cells were cocultured with anti-CD3-stimulated CD4⁺ T cells in the presence of neutralizing anti-IL-10 antibody (Figures 4A, B). We found that CD11b⁺ B1 cell-mediated immunosuppression was largely reversed in the presence of anti-IL-10. Thus, among

B cells, only CD11b⁺ B1 cells spontaneously produce and secrete IL-10 that suppresses T-cell activation *in vitro*.

We evaluated the expression of surface antigens reported here (CD19, CD20, CD27, CD43, CD11b) and elsewhere (CD19, CD20, CD24, CD27, CD38) to identify IL-10-producing B cells (Figure 5). Naive, memory, CD11b⁻ and CD11b⁺ B1 cells expressed CD20, CD27 and CD43 as expected inasmuch as these represent selection criteria for these populations, along with CD19. We found that CD24 expression, although relatively high on CD11b⁺ B1 cells, did not distinguish CD11b⁺ B1 cells from CD11b⁻ B1 or memory B cells. Moreover, expression encompassed a broad range of CD24 levels, with some CD11b⁺ B1 cells being CD24^{lo} or CD24^{hi}. Along the same lines, we found that CD38 expression, although generally higher on CD11b⁺ B1 cells than on naive or memory B cells, did not distinguish CD11b⁺ B1 cells from CD11b⁻ B1 cells. Moreover, expression encompassed a broad range of CD38

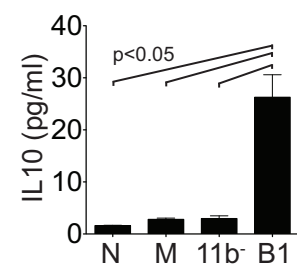


Figure 2. CD11b⁺ B1 cells spontaneously secrete IL-10. Adult peripheral blood mononuclear cells were immunofluorescently stained and sort-purified into populations of naive (CD20⁺CD27⁻CD43⁻) (N) and memory (CD20⁺CD27⁺CD43⁺) (M) B cells, total B1 (CD20⁺CD27⁻CD43⁺) cells and CD11b⁻ (CD20⁺CD27⁻CD43⁺CD11b⁻) (11b⁻) B1 cells that lack CD11b⁺ B1 cells. These populations were separately cultured for 5 d, after which supernatants were harvested and assayed for IL-10 by ELISA. Mean values (n = 3) are shown along with lines indicating SEM.

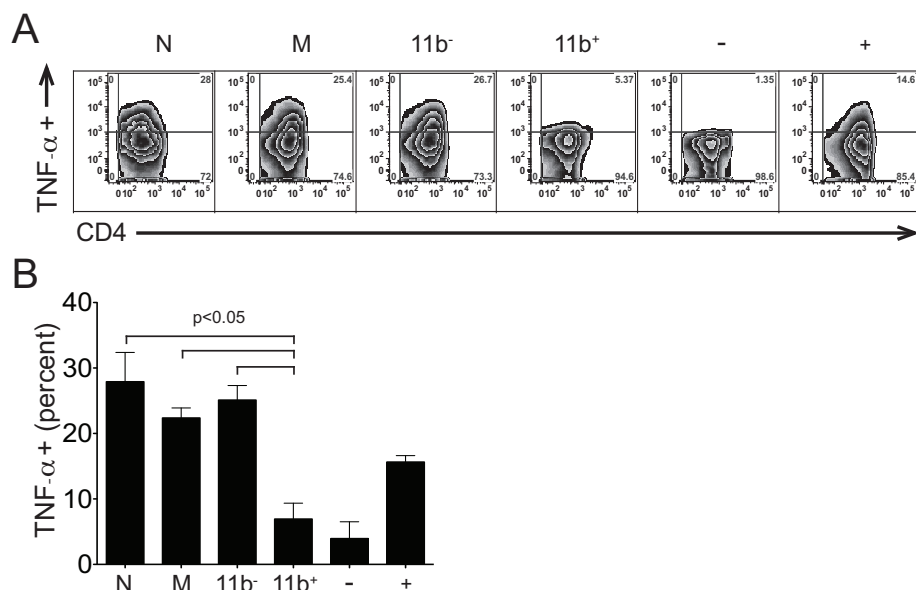


Figure 3. CD11b⁺ B1 cells suppress naive CD4⁺ T-cell activation. Sort-purified naive (CD20⁺CD27⁻CD43⁻) (N) and memory (CD20⁺CD27⁺CD43⁻) (M) B cells, and CD11b⁻ (CD20⁺CD27⁻CD43⁺CD11b⁻) (11b⁻) and CD11b⁺ (CD20⁺CD27⁺CD43⁺CD11b⁺) (11b⁺) B1 cells, were cocultured 1:1 with negatively selected naive CD4⁺ T cells plus plate-bound anti-CD3. Control cultures contained T cells with soluble anti-CD28 in addition to anti-CD3 but no B cells (+) and T cells with no antibody and no B cells (-). T-cell activation was assessed by staining for surface CD4 and intracellular TNF-α. (A, B) The fraction of CD4⁺ T cells expressing TNF-α after 3 d culture is shown for a representative experiment in (A) and for three independent experiments in (B). Vertical bars in (B) represent mean values, and lines indicate SEM. The cutoff for intracellular TNF-α positivity was determined on the basis of fluorescence-minus-one.

levels, with some CD11b⁺ B1 cells being negative for CD38. In terms of CD27, which marks B1 cells and memory B cells, we found that the levels expressed by CD11b⁺ B1 cells did not distinguish them from CD11b⁻ B1 or memory B cells and again encompassed a broad range. Thus, CD11b⁺ B1 cells, which produce and secrete IL-10, encompass both positive and negative expression of CD24 and CD38 and cannot be completely separated from other B cells by any combination of CD24, CD27 and CD38.

Our work identified B cells that spontaneously produce IL-10, in contrast to other reports, in which B cells were subjected to brief and/or prolonged stimulation before IL-10 assay. Despite this difference, we evaluated the extent to which IL-10-secreting CD11b⁺ B1 cells might coincide with phenotypes shown elsewhere to characterize B cells that produce IL-10 after various stimulatory regimens.

Mauri and colleagues (3) examined IL-10⁺ B cells after stimulation for 3 d with CD40L-expressing CHO cells followed by brief treatment with PMA and ionomycin plus brefeldin (8). They reported that such B cells are defined by CD24^{hi}CD38^{hi}. We plotted CD3⁻CD20⁺ B cells onto CD24 versus CD38 according to their gating scheme and generated a similar division of adult peripheral blood B cells into CD24^{hi}CD38^{hi}, CD24^{hi}CD38⁻ and CD24^{int}CD38^{int} populations (Figure 6A). When we displayed CD11b⁺ B1 cells on the same plot, we found a wide distribution that encompassed all three gating structures, with only a minority being captured within the CD24^{hi}CD38^{hi} gate (Figure 6B) and more than two-thirds outside this gate. Moreover, within the population of CD24^{hi}CD38^{hi} B cells, only about 10–15% were CD11b⁺ B1 cells. Whereas the CD24^{hi}CD38^{hi} gating parameters suggested by Blair *et al.* (8) produce

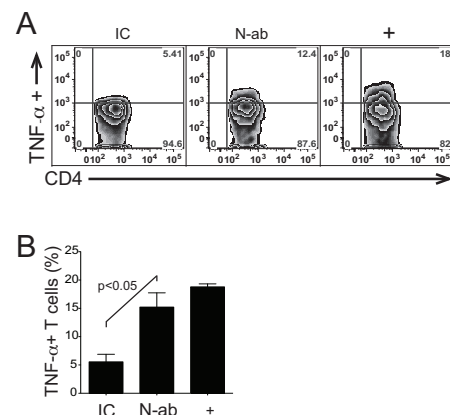


Figure 4. CD11b⁺ B1 cell suppression of naive CD4⁺ T-cell activation is dependent on IL-10. (A, B) Sort-purified CD11b⁺ B1 cells were cocultured 1:1 with negatively selected naive CD4⁺ T cells plus plate-bound anti-CD3 in the presence of neutralizing anti-IL-10 antibody (N-ab) or isotype-control antibody (IC). Control cultures contained T cells with anti-CD28 in addition to anti-CD3 but no B cells (+). The fraction of CD4⁺ T cells expressing TNF-α after 3 d culture is shown for a representative experiment in (A) and for three independent experiments in (B). Vertical bars in (B) represent mean values and lines indicate SEM.

a minor enrichment of CD11b⁺ B1 cells, the majority of CD11b⁺ B1 cells phenotype otherwise. Thus, it is clear that the CD24^{hi}CD38^{hi} phenotype is not particularly efficient in identifying spontaneously IL-10-secreting CD11b⁺ B1 cells.

Tedder and colleagues examined IL-10⁺ B cells after brief stimulation with PMA and ionomycin plus brefeldin (7). They reported that such B cells are defined by CD24^{hi}CD27⁺. With these criteria, they obtained an approximately fourfold enrichment of IL-10⁺ B cells from 0.8% of all B cells to 3.4% of CD24^{hi}CD27⁺ B cells. We plotted CD3⁻CD20⁺ B cells according to their gating scheme and generated a similar division of adult peripheral blood B cells into CD24^{hi}CD27⁺ and CD24^{lo}CD27⁻ populations; however, we also identified smaller numbers of CD24⁻ B cells (Figure 6C). When we displayed CD11b⁺ B1 cells on the same plot, we found a little more than half phenotyped as CD24^{hi}CD27⁺; however, the level of en-

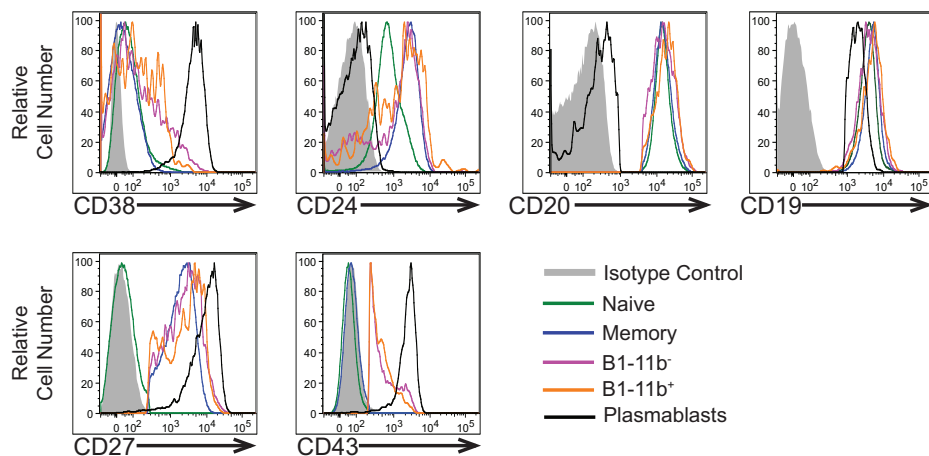


Figure 5. CD11b⁺ B1 cells express a unique phenotype. Adult peripheral blood mononuclear cells were immunofluorescently stained for surface CD3, CD19, CD20, CD27, CD43, CD11b, CD24 and CD38. Expression of CD38, CD24, CD20, CD19, CD27 and CD43 is shown for naive (CD3⁻CD20⁺CD27⁻CD43⁻) and memory (CD3⁻CD20⁺CD27⁺CD43⁻) B cells, and for CD11b⁻ (CD3⁻CD20⁺CD27⁺CD43⁺CD11b⁻) (B1-11b⁻) and CD11b⁺ (CD3⁻CD20⁺CD27⁺CD43⁺CD11b⁺) (B1-11b⁺) B1 cells, and for plasmablasts (CD3⁻CD19⁺CD20⁺), as indicated. Representative results from one of three comparable experiments are shown.

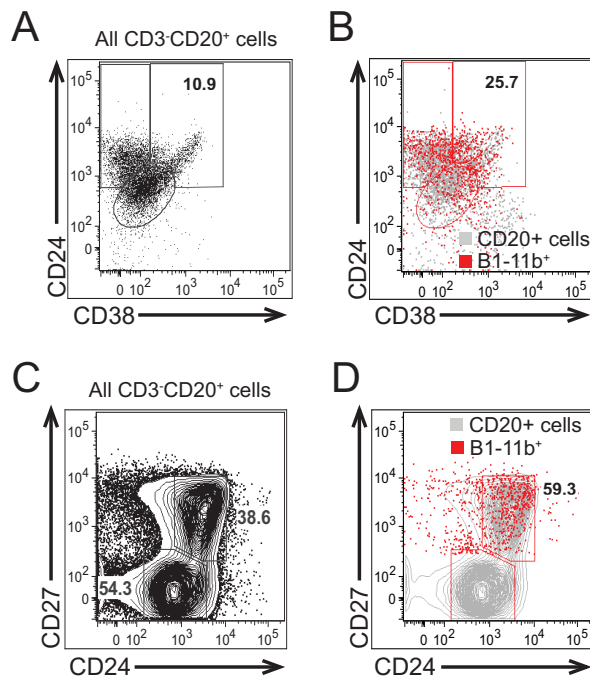


Figure 6. CD11b⁺ B1 cells are not the same as CD24^{hi}CD38^{hi} or CD24^{hi}CD27⁺ cells. Adult peripheral blood mononuclear cells were immunofluorescently stained for surface CD3, CD20, CD27, CD43, CD11b, CD24 and CD38. (A) CD3⁻CD20⁺ cells are displayed on a CD24 by CD38 dot plot. (B) CD3⁻CD20⁺ cells are displayed on a CD24 by CD38 dot plot in gray with CD11b⁺ B1 cells (CD3⁻CD20⁺CD27⁺CD43⁺CD11b⁺) (B1-11b⁺) overlaid in red. (C) CD3⁻CD20⁺ cells are displayed on a CD27 by CD24 contour plot. (D) CD3⁻CD20⁺ cells are displayed on a CD27 by CD24 contour plot in gray with CD11b⁺ B1 cells (CD3⁻CD20⁺CD27⁺CD43⁺CD11b⁺) (B1-11b⁺) overlaid in red.

richment is limited by the large number of true memory and other B cells contained within that gate, amounting to about one-third of all B cells (Figure 6D). Thus, the CD24^{hi}CD27⁺ gating parameters offered by Iwata *et al.* (7), although inclusive of many CD11b⁺ B1 cells, still omit a substantial fraction and at the same time are not particularly specific for CD11b⁺ B1 cells. For these reasons, gating for CD24^{hi}CD27⁺ B cells provides only modest enrichment of CD11b⁺ B1 cells and is not a practical guide to identifying or enriching these cells.

DISCUSSION

We have shown here that human CD11b⁺ B1 cells spontaneously produce and secrete IL-10 and suppress T-cell activation. Inasmuch as a high percentage of CD11b⁺ B1 cells stain positive for IL-10, and CD11b⁺ B1 cells represent a very small proportion of all B cells, focusing on CD11b⁺ B1 cells leads to very high enrichment for IL-10 positivity, which far exceeds that reported from a focus on CD24^{hi}CD38^{hi} or CD24^{hi}CD27⁺ B cells, as suggested elsewhere (7,8). It is important to note that, in these latter studies, B cells were stimulated with PMA plus ionomycin for 5–6 h before assay, and in some cases, with additional regimens for longer periods of time. Inasmuch as B cells would not be exposed to PMA or ionomycin *in vivo*, examination of B cells thus stimulated may be difficult to translate into what is actually occurring *in vivo*. Our approach differs in that B cells were unstimulated and IL-10 expression was, as far as can be determined, truly spontaneous. This work furthers the understanding of how effector functions of the immune system can be regulated by B cells in general and by B1 cells in particular.

Our results indicate that IL-10 is associated with a distinct identified B-cell subset in the human system that expresses CD20, CD27, CD43 and CD11b (CD11b⁺ B1 cells). A majority of these cells produce IL-10, in contrast to the B cells identified in the CD24^{hi}CD38^{hi} or CD24^{hi}CD27⁺ B-cell gating schemes reported by others, which miss many IL-10⁺ B cells

and which include heterogeneous groupings of cells, of which only a small proportion express IL-10 (7,8). An interesting and important question is whether IL-10 production and B-cell regulatory function is limited to CD11b⁺ B1 cells. In our work, removal of CD11b⁺ B1 cells eliminated B cells that express IL-10 (Figure 2) as well as B cells that possess regulatory function (Figure 3). However, in the well-studied murine system, although B-1 cells are the main source of B cell-derived IL-10, they are not the sole source (4,6). It is notable that some key characteristics of murine B-1 cells are recapitulated by stimulated B-2 cells. In that sense, our results speak only to homeostatic production of IL-10 in the absence of exogenous manipulation or stimulation, but do not rule out the possibility that other B-cell populations secrete IL-10 and suppress immune responses after activation *in vivo*.

IL-10 is spontaneously produced by unstimulated CD11b⁺ B1 cells, and immunosuppression by CD11b⁺ B1 cells is dependent on IL-10. This does not mean that the former represents the sum and substance of the latter. We have observed that various stimulatory regimens can increase the amount of IL-10 secreted by CD11b⁺ B1 cells and thus cannot dismiss the idea that individual CD11b⁺ B1 cells may increase their secretion of IL-10 above the level they produce spontaneously and that this may increase regulatory function. Conversely, although IL-10 may be the dominant means of B-cell regulatory function, not all B-cell regulatory function is mediated through secretion of IL-10 (10–13).

A number of clinical conditions are linked to dysregulation of IL-10. Patients suffering from asthma have lower levels of serum IL-10 as well as lower production of IL-10 from immune cells (14). Further, lower levels of IL-10 and IL-10 gene polymorphisms appear to correlate with disease severity in asthma and α_1 -antitrypsin deficiency as well as other allergic conditions (15,16). Moreover, inflammatory conditions affecting the gut, such as Crohn's disease and inflammatory bowel disease, are associated with defects in immune regulation and IL-10 in partic-

ular (17,18). From the therapeutic standpoint, it is critically important to discern the identity and role of regulatory B cells in patients with autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis and Sjögren's Syndrome who undergo treatment with B-cell depletion therapy, lest beneficial modulatory B cells be removed along with pathogenic B cells. Thus, understanding the origin of immunoregulatory IL-10 and determining the role of regulatory B cells are key issues for clinical medicine.

CONCLUSION

Previous work showed that human B1 cells are divided on the basis of surface CD11b expression and demonstrated that CD11b⁺ B1 cells drive T-cell proliferation due, at least in part, to elevated expression of the costimulatory molecule CD86. Here we show that this same CD11b⁺ B1 cell population secretes IL-10 and suppresses CD3-mediated T-cell activation. In view of the capacity of CD11b⁺ B1 cells to stimulate T-cell proliferation and to modulate T-cell activation, these B1 cells appear capable of orchestrating aspects of immune responsiveness. We propose that CD11b⁺ B1 cells that directly affect T-cell activation and proliferation be termed "orchestrator B1 cells," or "B1orc," and that CD11b⁻ B1 cells, which spontaneously secrete antibody but have little effect on T cells, be termed "secretor B1 cells" or "B1sec."

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DO Griffin and TL Rothstein designed the study. DO Griffin performed the experiments. DO Griffin and TL Rothstein analyzed the data and contributed to the final manuscript.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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