

# A small CD11b<sup>+</sup> human B1 cell subpopulation stimulates T cells and is expanded in lupus

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**A primary function of B lymphocytes is immunoglobulin production; however, the therapeutic benefit of B cell depletion in autoimmune diseases previously thought to be T cell mediated suggests that some B cells fulfill other roles in autoimmunity. We examined the recently identified human B1 cell population for T cell stimulatory activity. We found two kinds of B1 cells that are distinguished by multiple surface markers and distinct transcriptomic profiles. In both umbilical cord and adult peripheral blood, a CD11b<sup>+</sup> subset constitutes ~1 out of every 8–10 B1 cells, whereas a CD11b<sup>−</sup> subset constitutes the remaining B1 cells. These B1 cell populations differ functionally. CD11b<sup>−</sup> B1 cells spontaneously secrete much more IgM than CD11b<sup>+</sup> B1 cells. In contrast, CD11b<sup>+</sup> B1 cells express more CD86, and more efficiently stimulate allogeneic CD4<sup>+</sup> T cell expansion, than CD11b<sup>−</sup> B1 cells. The frequency of these CD11b<sup>+</sup> B1 cells is markedly elevated in lupus patients. CD11b<sup>+</sup> B1 cells in lupus patients express more CD86 and have increased T cell–stimulating activity in disease. This work distinguishes a novel, T cell–interacting B1 cell population whose abundance and activity may be a reflection of, and a therapeutic target in, autoimmune disease.**

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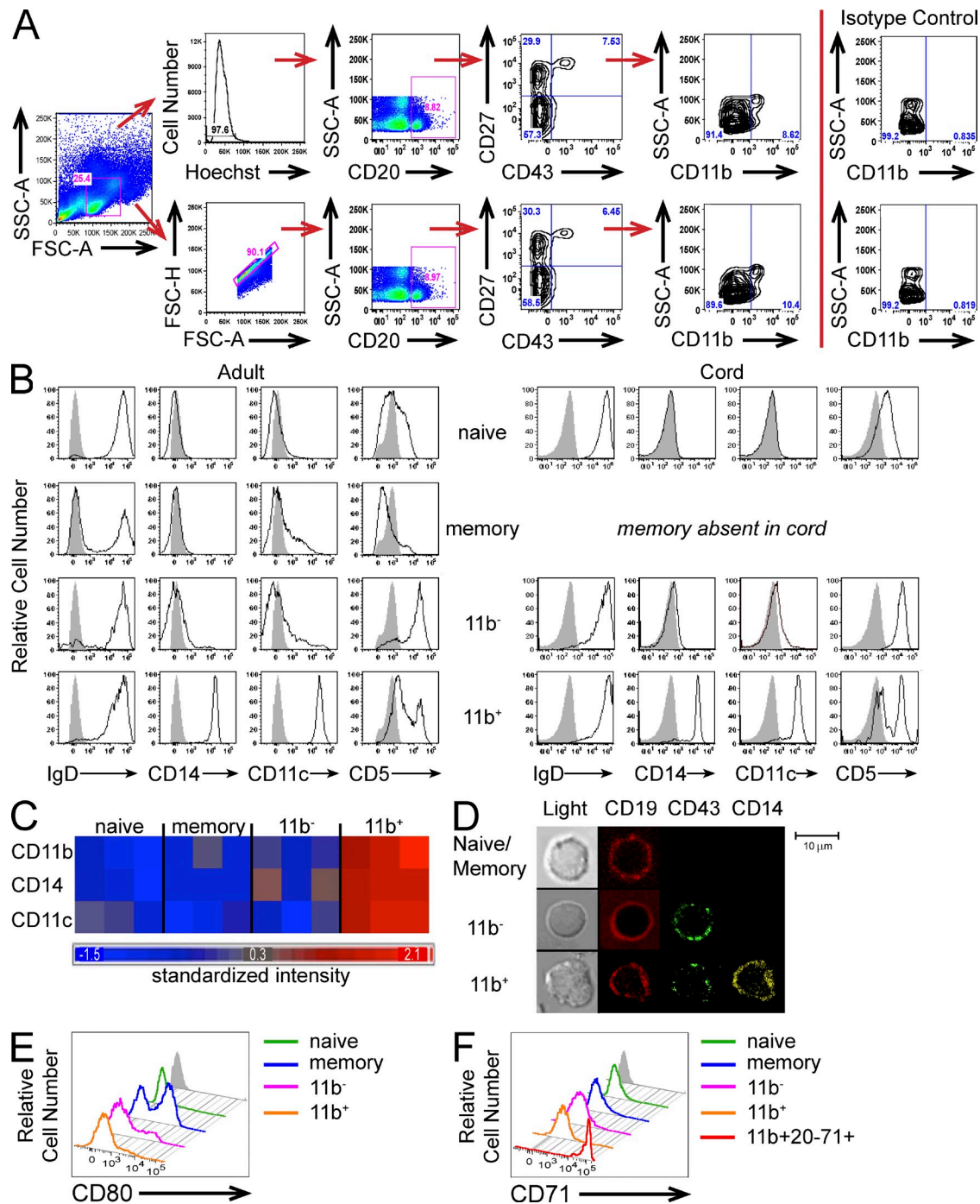
The immune response against foreign pathogens must arise promptly, develop effectively, and end appropriately, to counteract infection and avoid injury to normal tissue. In autoimmunity, however, the immune response is misdirected against self, resulting in tissue damage. Understanding of these processes has been advanced by recognition of individual cell types that carry out specific effector functions. Although the principal mission of B lymphocytes is considered to reside in immunoglobulin production, an effector role for these cells in regulating immune activity has been repeatedly noted (Zouali, 2008). With the recent success of B cell depletion therapy in autoimmune diseases, there is growing evidence that a population of cells contained within the B cell pool expresses immunostimulatory activity and is involved in clinical autoimmunity (Jacob and Stohl, 2010; Perosa et al., 2010; Sanz and Lee, 2010). Yet clear identification of the B cells that possess this function has remained a mystery.

B1 cells are a small innate B cell population that is responsible for constitutively producing protective natural immunoglobulin (Baumgarth, 2011). In the mouse system B1 cells have been

shown to arise early and to derive from a distinct progenitor, lending support to their status as a separate lineage in a layered immune system (Herzenberg and Tung, 2006; Montecino-Rodriguez et al., 2006). Current studies have revealed novel activities of B1 cells including, in particular, high level stimulation of T cell expansion (Zhong et al., 2007).

The recent identification of human B1 cells (Griffin et al., 2011) provides an opportunity to elucidate activities of this B cell population that may contribute to immune function and autoimmune disease. We found that human B1 cells are divisible into two readily separable, distinct populations and that T cell–stimulatory activity is a property of one population and not the other, which is instead characterized by heightened immunoglobulin secretion. The former, T cell–interacting population is markedly increased in patients with lupus.

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**Figure 1.** CD11b expression divides human B1 cells into two phenotypically distinct subsets. (A) Adult peripheral blood mononuclear cells were immunofluorescently stained for CD20, CD27, CD43, and CD11b, and were then evaluated by flow cytometric analysis. Two gating strategies are shown using nuclear staining with Hoechst 33342 (Hoechst) and FSC-H by FSC-A doublet gating to separate CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> B1 cells into CD11b<sup>+</sup> and CD11b<sup>-</sup> populations for a representative adult blood sample with isotype control displayed for CD11b. (B) Adult peripheral blood and umbilical cord blood mononuclear cells were stained for IgD, CD14, CD11c, and CD5 in addition to CD20, CD27, CD43, and CD11b, and then evaluated by flow cytometric analysis. Expression of IgD, CD14, CD11c, and CD5 by naive (CD20<sup>+</sup>CD27<sup>-</sup>CD43<sup>-</sup>) and memory (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>) B cells and by CD11b<sup>-</sup> and CD11b<sup>+</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>) is shown for representative adult and cord blood samples (one of three each) with isotype control in solid gray. (C) RNA was prepared from sort-purified populations of naive (CD20<sup>+</sup>CD27<sup>-</sup>CD43<sup>-</sup>) and memory (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>) B cells, and from CD11b<sup>-</sup> and CD11b<sup>+</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>), obtained from three normal adult individuals for each B cell population and analyzed for gene expression by microarray. Expression levels for CD11b, CD14, and CD11c transcripts are shown in the form of a heat map. (D) Adult peripheral blood mononuclear cells were stained with fluorescent antibodies that recognize CD19, CD43, and CD11b and sorted into populations containing naive/memory B cells, CD11b<sup>+</sup> B1 cells, and CD11b<sup>-</sup> B1 cells. Sort-purified B cells were then stained with a fluorescent antibody targeting CD14, fixed, adhered to poly-lysine-coated slides, and examined for immunofluorescence

## RESULTS AND DISCUSSION

Human B1 cells obtained from both umbilical cord and adult peripheral blood express index functional features not expressed by other mature B cells and phenotype as  $CD20^+CD27^+CD43^+CD70^-$  (Griffin et al., 2011). In further study we have now found that B1 cells can be divided into two distinct populations by phenotypic criteria. Immunofluorescent staining reveals that some  $CD20^+CD27^+CD43^+$  B1 cells express CD11b, whereas the bulk of B1 cells do not (Fig. 1, A and B). In view of evidence in the mouse system that B1 cells readily form aggregates (Ghosn et al., 2008), and the association of CD11b with the monocyte lineage, we verified that CD11b expression is an intrinsic property of some human B1 cells. We stained DNA with Hoechst 33342 and then analyzed only those cells that were singlets (Fig. 1 A). With this approach, we established that among cells defined on the basis of having only one nucleus, a specific fraction coexpressed CD20, CD27, CD43, and CD11b. Separately, we exerted strict FSC-H by FSC-A doublet gating (Fig. 1 A) but, regardless of the level of restriction,  $CD11b^+$  B1 cells were readily identified in similar proportions within the B1 population. Overall, we found that  $CD11b^+$  B1 cells represent  $\sim 1$  out of every 8–10 B1 cells for both adult peripheral blood ( $n = 67$ ) and umbilical cord blood ( $n = 6$ ) samples, with the remainder being CD11b negative.

Several phenotypic differences distinguish  $CD11b^+$  and  $CD11b^-$  B1 cells. Notably,  $CD11b^+$  B1 cells express CD14 and high levels of CD11c, whereas other B cell types ( $CD11b^-$  B1 cells, memory B cells, and naive B cells) do not (Fig. 1 B). The distinctive staining for CD11b, CD14, and CD11c is mirrored in the unique expression of CD11b, CD14, and CD11c transcripts (Fig. 1 C).

To further establish expression of CD14 by  $CD11b^+$  B1 cells, we visualized single sort-purified B1 cells by confocal microscopy. Sort-purified  $CD11b^+$  B1 cells coexpressed CD19, CD43, and CD14 (Fig. 1 D). In addition, and as expected, nominally  $CD11b^-$  B1 cells were positive for CD19 and CD43 but failed to express CD14 (Fig. 1 D). In contrast, naive and memory B cells, though positive for CD19, expressed neither CD43 nor CD14, as expected (Fig. 1 D).

Despite unique surface marker expression,  $CD11b^+$  B1 cells are similar to  $CD11b^-$  B1 cells (and naive B cells) in being predominantly  $IgD^+$ , whereas a majority of memory B cells are  $IgD^-$ . Surface immunoglobulin expression marks  $CD11b^+$  and  $CD11b^-$  B1 cells as B cells. It is highly unlikely that binding of anti- $IgD$ , or binding of other antibodies to defining determinants, is nonspecific inasmuch as additional

antibodies bound poorly (anti-CD80) to  $CD11b^+$  or  $CD11b^-$  B1 cells despite binding to memory B cells (Fig. 1 E), or did not bind at all (anti-CD71) to  $CD11b^+$  or  $CD11b^-$  B1 cells despite binding  $CD11b^+CD20^-CD71^+$  cells (Fig. 1 F).

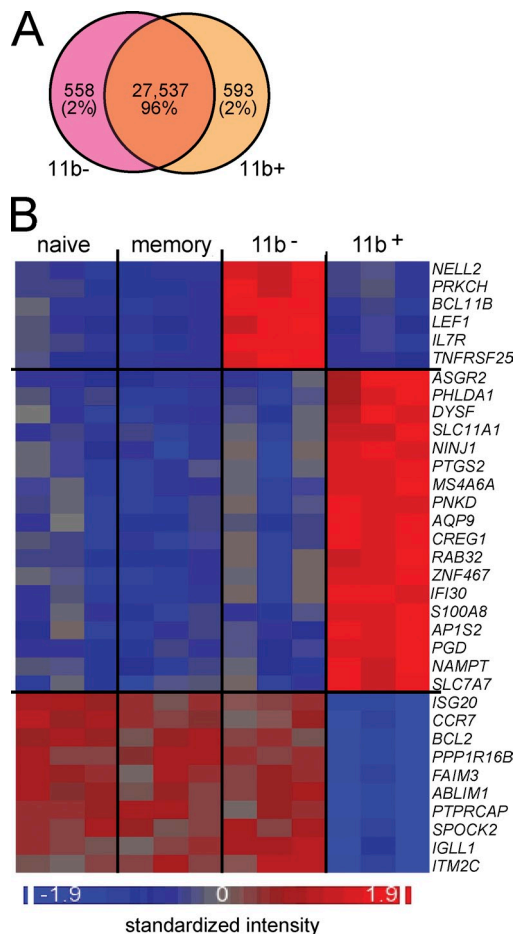
Unlike  $CD11b^-$  B1 cells that are mostly  $CD5^+$ , the majority of  $CD11b^+$  B1 cells either do not express CD5 or express very low levels of CD5 (Fig. 1 B). These results show that identification by CD5 expression not only omits  $\sim 1/4$  of all B1 cells (Griffin et al., 2011) but preferentially omits in particular one of two B1 cell subpopulations.

In sum, elevated expression of CD11b, CD11c, and CD14 constitutes a constellation of markers that defines a small subpopulation of B1 cells that is operationally  $CD11b^+$ . Further evidence that  $CD11b^+$  and  $CD11b^-$  B1 cells represent distinct groups is provided by the many genes differentially expressed between these two populations (Fig. 2 A), a subset of which is displayed in heat map format for each individual sample tested (Fig. 2 B).

We further characterized  $CD11b^+$  and  $CD11b^-$  B1 cells by examining isolated populations purified by cell sorting as described in Materials and methods. To avoid potential doublets,  $Ca^{2+}$  was depleted with 2 mM EDTA, dilute cell suspensions were agitated frequently, and strict doublet discrimination was used. We showed that sort-purified  $CD11b^+$  and  $CD11b^-$  B1 cells were single cells without evidence of doublets by post-sort analysis on the basis of nuclear staining with Hoechst 33342 and FSC-H by FSC-A discrimination (Fig. S1 A). In addition, we sorted Hoechst-stained cells in single droplets onto glass slides and showed the presence of only a single nucleus per event in  $>500$  events examined by direct visualization with fluorescence microscopy (Fig. S1 B). Furthermore, sort-purified B1 cells, both  $CD11b^+$  and  $CD11b^-$ , were  $>95\%$  viable by propidium iodide staining.

We examined  $CD11b^+$  and  $CD11b^-$  B1 cells by light microscopy after sort purification. These B1 cells display typical round lymphocyte morphology without dendritic projections or multilobulated nuclei, as seen in Wright-Giemsa-stained images, much like other B cells (Fig. S1 C). Notably, there was no change in expression of CD11b when sort-purified  $CD11b^+$  and  $CD11b^-$  B1 cells were separately cultured, with CD11b expression remaining high on  $CD11b^+$  B1 cells and remaining low on  $CD11b^-$  B1 cells, suggesting that these populations are stable and not in the process of transitioning one to another (Fig. S1 A). Moreover, stimulation of sorted  $CD11b^-$  B1 cells with PMA plus ionomycin, SAC plus IL-2, or anti-Ig plus anti-CD40 failed to induce surface expression of CD11b (or CD14) despite up-regulation of

sequentially at three wavelengths by confocal microscopy. Representative results from one of three comparable experiments are shown. (E) Adult peripheral blood mononuclear cells were stained for CD20, CD27, CD43, CD11b, and CD80 and were then evaluated by flow cytometric analysis. CD80 expression by naive ( $CD20^+CD27^-CD43^-$ ) and memory ( $CD20^+CD27^+CD43^-$ ) B cells, and by  $CD11b^+$  and  $CD11b^-$  B1 cells ( $CD20^+CD27^+CD43^+$ ), is shown as colored lines, along with isotype control (solid gray). Representative results from one of three comparable experiments are shown. (F) Adult peripheral blood mononuclear cells were stained for CD20, CD27, CD43, CD11b, and CD71 and were then evaluated by flow cytometric analysis. CD71 expression by naive ( $CD20^+CD27^-CD43^-$ ) and memory ( $CD20^+CD27^+CD43^-$ ) B cells, and by  $CD11b^+$  and  $CD11b^-$  B1 cells ( $CD20^+CD27^+CD43^+$ ) and  $CD11b^+CD20^-CD71^+$  cells, is shown as colored lines, along with isotype control (solid gray). Representative results from one of three comparable experiments are shown.



**Figure 2. CD11b expression divides human B1 cells into two transcriptionally distinct subsets.** Gene expression microarray analysis was performed on RNA that was isolated from sort-purified naive ( $CD20^+CD27^-CD43^-$ ) and memory ( $CD20^+CD27^+CD43^-$ ) B cells, and from  $CD11b^-$  and  $CD11b^+$  B1 cells ( $CD20^+CD27^+CD43^+$ ), obtained from three normal adult individuals for each B cell population. (A) The expression of coding transcripts with well established annotation (28,688) by  $CD11b^+$  and  $CD11b^-$  B1 cells is shown in the form of a Venn diagram. Transcripts differentially expressed twofold or greater by  $CD11b^+$  B1 cells are shown in light orange, transcripts differentially expressed twofold or greater by  $CD11b^-$  B1 cells are shown in pink, and transcripts expressed similarly by  $CD11b^-$  and  $CD11b^+$  B1 cells are represented by the dark orange overlap. (B) A representative set of genes demonstrating significant expression differences among B cell populations for each individual tested is displayed as a heat map along with gene designations.

CD69 (unpublished data), indicating that  $CD11b^+$  B1 cells are not simply an activated version of  $CD11b^-$  B1 cells.

We evaluated sort-purified  $CD11b^+$  and  $CD11b^-$  B1 cells for two key features by which human B1 cells were originally defined—spontaneous IgM secretion and efficient T cell stimulation—to assess functional distinctions that might differentiate these subsets. We first tested these two B1 cell populations for spontaneous secretion of immunoglobulin. We cultured  $CD11b^+$  and  $CD11b^-$  B1 cells, as well as naive ( $CD20^+CD27^-CD43^-$ ) B cells, from both adult and cord

blood, and memory ( $CD20^+CD27^+CD43^-$ ) B cells from adult blood without stimulation for 5 d and assayed supernatants for IgM by ELISA. We found that for both adult and cord blood samples,  $CD11b^-$  B cells secreted much more IgM than  $CD11b^+$  B1 cells (Fig. 3, A and B), whereas  $CD11b^+$  B1 cells still secreted more IgM than non-B1 B cells. Thus,  $CD11b^-$  B1 cells are specialized for spontaneous secretion of IgM.

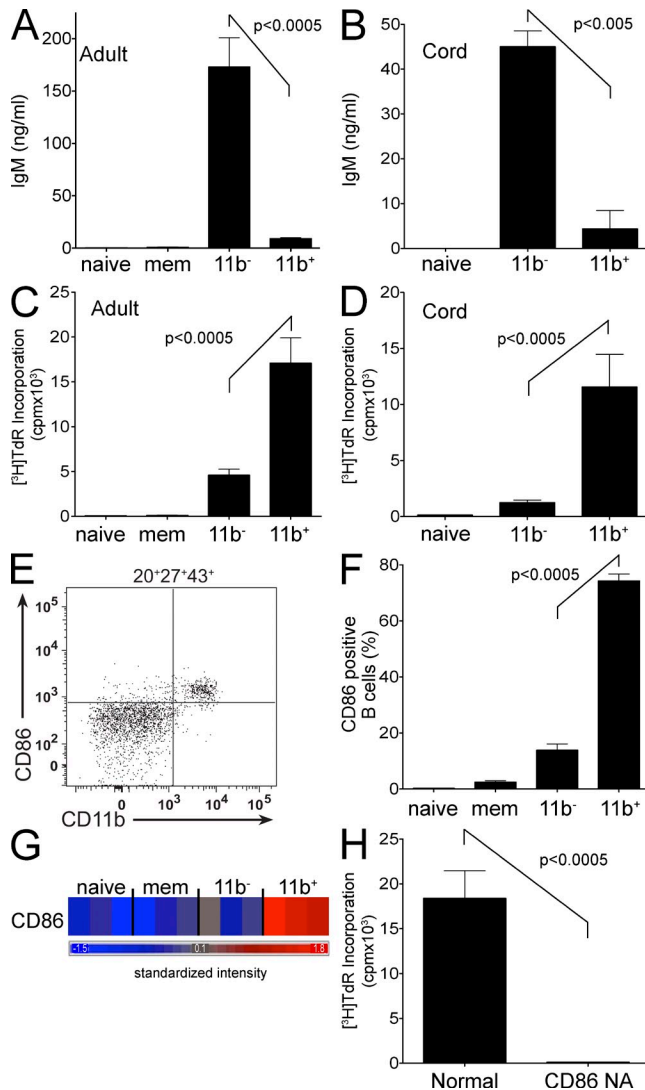
We then tested these two B1 cell populations for antigen presentation and T cell stimulation. We cultured irradiated, sort-purified  $CD11b^+$  and  $CD11b^-$  B1 cells, as well as irradiated naive B cells, from adult and cord blood, and irradiated memory B cells from adult blood, with allogeneically mismatched  $CD4^+$  T cells for 5 d and evaluated DNA replication by measuring tritiated thymidine incorporation. We found that for both adult and cord blood samples,  $CD11b^+$  B1 cells stimulated T cell proliferation to a much greater extent than  $CD11b^-$  B1 cells (Fig. 3, C and D), whereas  $CD11b^-$  B1 cells in turn stimulated T cell proliferation to a greater extent than non-B1 B cells.

T cell stimulation by mouse B1a cells has been reported to be attributable to CD86 expression (Zhong et al., 2007). We found that among human adult peripheral blood B1 cells, expression of CD11b correlated with expression of CD86 (Fig. 3 E), and that a much higher percentage of  $CD11b^+$  B1 cells expressed CD86 above isotype control values than did  $CD11b^-$  B1 cells or naive or memory B cells (Fig. 3 F). This increased level of CD86 was mirrored by a much higher level of CD86 transcripts in  $CD11b^+$  B1 cells (Fig. 3 G). Importantly, T cell stimulation induced by  $CD11b^+$  B1 cells was markedly reduced by anti-CD86 neutralizing antibody (Fig. 3 H). Thus,  $CD11b^+$  B1 cells are specialized for efficient stimulation of T cells across an allogeneic barrier, at least in part as a result of elevated expression of CD86.

The present results indicate that segregation of B1 cells according to CD11b expression is accompanied by functional polarization reflected in two foundational features: one population, which expresses CD11b and is the smaller of the two, strongly stimulates T cells through CD86 but produces modest levels of secreted antibody; and, a second population, which is  $CD11b^-$  and is the larger of the two, secretes abundant amounts of IgM but is less effective in stimulating T cells. Collectively, these findings indicate a unique division of labor between two subpopulations of human B1 cells, one of which is specialized for secretion of IgM, the other of which is specialized for interaction with T cells.

Induction of T cell proliferation across an allogeneic barrier is a surrogate for antigen presentation, and in this sense the combination of elevated CD86 expression and efficient T cell stimulation on the part of  $CD11b^+$  B1 cells may speak to a role in the generation of T cell abnormalities in autoimmune disease. To address this possibility, we phenotyped circulating B cells in 15 patients with lupus and 67 normal controls (see Table S1 for patient characteristics). In lupus patients, as in normal controls,  $CD20^+CD27^+CD43^+$  B1 cells lacked CD70 expression and thus were readily recognizable as true B1 cells (Griffin et al., 2011).



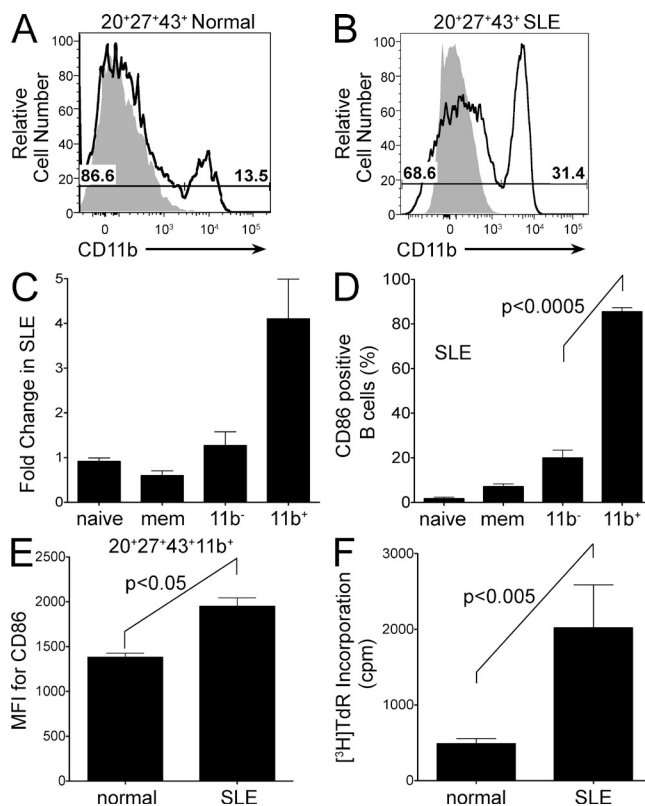


**Figure 3. CD11b expression divides human B1 cells into two functionally distinct subsets.** (A–D) Sort-purified populations of naive B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>), CD11b<sup>-</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>-</sup>), and CD11b<sup>+</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>+</sup>) were obtained from adult peripheral blood and umbilical cord blood samples, and sort-purified memory (mem) B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>) were obtained from adult blood. (A and B) B cells were cultured at 10<sup>6</sup> cells per ml for 5 d, after which supernatants were evaluated for secreted IgM by ELISA. Mean values are shown along with lines indicating SEM for six adult blood samples (A) and four cord blood samples (B) for each population. (C and D) B cells were irradiated and co-cultured 1:2 with negatively selected allogeneic CD4<sup>+</sup> T cells for 5 d, after which proliferation was measured by incorporation of tritiated thymidine during the last 8 h of triplicate cultures. Mean cpm values are shown, along with lines indicating SEM for four adult blood samples (C) and three cord blood samples (D) for each population. (E and F) Mononuclear cells from adult peripheral blood samples were immunofluorescently stained for CD20, CD27, CD43, CD11b, and CD86 and were then evaluated by flow cytometric analysis. (E) Levels of CD86 and CD11b expressed by gated CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> B1 cells in a representative adult peripheral blood sample are shown. Representative results from 1 of 15 comparable experiments are shown. (F) Mean values for the proportion of CD86<sup>+</sup> cells among naive B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>),

We found that CD11b<sup>+</sup> B1 cells underwent changes in lupus. We found overall a large, approximately fourfold increase in CD11b<sup>+</sup> B1 cells (and only minimal changes in naive, memory, and CD11b<sup>-</sup> B1 cells; Fig. 4, A–C). Both lupus patients and control subjects were mostly female and predominantly of either European or Asian descent, and mean ages were 36 and 51 yr, respectively. Moreover, differences in B cell composition, particularly the increased frequency of CD11b<sup>+</sup> B1 cells, were similar when lupus samples were compared with samples from 15 of the 67 controls that were intentionally age- and gender-matched (whose B cells consisted of 66.4 ± 2.6% naive versus 60.2 ± 5.1%, 21.1 ± 2.2% memory versus 11.7 ± 2.1%, 9.1 ± 1.5% CD11b<sup>-</sup> B1 versus 16.1 ± 3.9%, and 0.7 ± 0.1% CD11b<sup>+</sup> B1 cells versus 5.3 ± 1.1%). Not only is the proportion of CD11b<sup>+</sup> B1 cells increased in lupus, but CD11b<sup>+</sup> B1 cell expression of CD86 is increased as well. In lupus patients, as in normal controls, the frequency of CD86 expression by CD11b<sup>+</sup> B1 cells far outweighed that of other B cell populations (Fig. 4 D), and at the same time the amount of CD86 on CD86<sup>+</sup>CD11b<sup>+</sup> B1 cells, quantified by mean fluorescence intensity, was greater in samples from lupus patients as compared with normal controls (Fig. 4 E). Furthermore, B1 cell function is altered in lupus. We found that lupus CD11b<sup>+</sup> B1 cells were more effective than normal CD11b<sup>+</sup> B1 cells in stimulating T cells (Fig. 4 F). Thus, in combination with the marked increase in CD11b<sup>+</sup> B1 cells associated with lupus, the increased efficiency of T cell stimulation, identified *in vitro*, would have the effect of markedly enhancing T cell activation and effector function *in vivo*. Because many of the lupus samples were obtained from patients with inactive disease, increases in CD11b<sup>+</sup> B1 cell numbers, CD86 expression, and T cell stimulation could not be correlated with clinical flares; however, the consistency of lupus-associated B1 cell abnormalities may suggest a fundamental disorder that drives disease regardless of its periodic inflammatory intensity which may, in turn, relate to other factors.

Recognition of CD11b<sup>+</sup> B1 cells as a new B cell type with distinct characteristics provides a potential explanation for prior observations regarding B cells in lupus. The increase in CD11b<sup>+</sup> B1 cells, which express much higher levels of

memory (mem) B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>), CD11b<sup>-</sup> (11b<sup>-</sup>) B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>-</sup>), and CD11b<sup>+</sup> (11b<sup>+</sup>) B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>+</sup>) are shown with lines indicating SEM for 15 adult blood samples. (G) RNA was prepared from sort-purified populations of naive B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>), memory (mem) B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>), and CD11b<sup>-</sup> and CD11b<sup>+</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>), and analyzed for gene expression by microarray. Expression of CD86 transcripts is shown for each population in the form of a heat map. (H) Sort-purified and irradiated CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>+</sup> B1 cells were co-cultured with allogeneic CD4<sup>+</sup> T cells, as in C, with (CD86 NA) or without (normal) anti-CD86 neutralizing antibody. For anti-CD86-treated cultures, B1 cells were exposed to antibody for 1 h before addition of T cells. Mean cpm values are shown along with lines indicating SEM for three adult peripheral blood samples analyzed in sextuplicate.



**Figure 4. CD11b<sup>+</sup> human B1 cells are increased and functionally altered in patients with systemic lupus erythematosus.**

(A and B) Adult peripheral blood mononuclear cells from normal individuals and from patients with SLE were immunofluorescently stained for CD20, CD27, CD43, and CD11b, and were then evaluated by flow cytometric analysis. Expression of CD11b is shown (black lines) on gated CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> B1 cells from a representative normal individual (A) and a representative SLE patient (B) with isotype control in solid gray. (C) Adult peripheral blood mononuclear cells from patients with SLE and from normal individuals were immunofluorescently stained for CD20, CD27, CD43, and CD11b, and were then evaluated by flow cytometric analysis. The percentages of naive B cells (CD20<sup>+</sup>CD27<sup>-</sup>CD43<sup>-</sup>), memory (mem) B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>), CD11b<sup>-</sup> (11b<sup>-</sup>) B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>-</sup>), and CD11b<sup>+</sup> (11b<sup>+</sup>) B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>+</sup>), among all B cells in SLE patients ( $n = 15$ ), were compared with the percentages of these populations among all B cells in normal controls ( $n = 67$ ). Ratios between these values are expressed as fold change for B cell populations in SLE patients versus normal controls along with lines indicating SEM. (D and E) Adult peripheral blood mononuclear cells from 15 lupus patients were immunofluorescently stained for CD20, CD27, CD43, CD11b, and CD86 and were then evaluated by flow cytometric analysis. (D) Mean values for the proportion of CD86<sup>+</sup> cells among naive B cells (CD20<sup>+</sup>CD27<sup>-</sup>CD43<sup>-</sup>), memory (mem) B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>), CD11b<sup>-</sup> (11b<sup>-</sup>) B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>-</sup>), and CD11b<sup>+</sup> (11b<sup>+</sup>) B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>+</sup>) are shown with lines indicating SEM ( $n = 15$ ). (E) CD86 mean fluorescence intensity (MFI) was evaluated for CD11b<sup>+</sup> B1 cells from 15 randomly selected samples from among the 67 normal control (normal) and 15 lupus patient (SLE) adult peripheral blood samples. Mean values are displayed along with lines indicating SEM. (F) Sort-purified and irradiated CD11b<sup>+</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>+</sup>) from three randomly selected samples from among the 67 normal control (normal) and 3 lupus patient (SLE) peripheral blood samples were cultured 1:2

CD86 than other B cell populations, is consistent with the previous finding that CD86<sup>+</sup> B cells are increased in lupus (Bijl et al., 2001). Furthermore, the increase in CD11b<sup>+</sup> B1 cells, which like memory B cells express CD27, could well be responsible for the reported lupus-associated increase in CD27<sup>+</sup> B cells (Jacobi et al., 2008).

Our previous work identifying CD27/CD43 coexpressing B cells as B1 cells emphasizes the peril in designating particular phenotypic determinants as negative criteria; had CD43-expressing cells been discarded, B1 cells would have been omitted from consideration along with myeloid and T cells. Our current work indicating important divisions within the human B1 cell population on the basis of CD11b expression, leading to the identification of CD11b<sup>+</sup> B1 cells, is again propelled by expression of surface determinants not classically associated with B cell status. This experience suggests the value of focusing on positive identification without preconceived exclusionary criteria.

Notably, CD11b<sup>+</sup> B1 cells are not the same as tissue-based FcRH4<sup>+</sup> memory B cells because those B cells are predominantly negative for IgD and CD27 (Ehrhardt et al., 2005), whereas circulating CD11b<sup>+</sup> B1 cells are positive for IgD and CD27. CD11b<sup>+</sup> B1 cells are not the same as circulating CD21<sup>lo</sup> B cells identified in CVID patients because those B cells express low levels of CD21 and CD27, and no CD23 (Rakhmanov et al., 2009), whereas circulating CD11b<sup>+</sup> B1 cells are strongly CD21 and CD27 positive and express CD23 (unpublished data). CD11b<sup>+</sup> B1 cells are not the same as circulating MZ B cells because those B cells express low levels of IgD and do not express CD23 (Weller et al., 2004), whereas circulating CD11b<sup>+</sup> B1 cells express substantial levels of both. Thus, CD11b<sup>+</sup> B1 cells constitute a distinct and unique human B cell population without known antecedent or parallel among human B cells.

Lupus autoimmune disease appears to result from a complex state of immune system dysfunction to which B cells contribute through their influence on T cells, among other mechanisms (Jacob and Stohl, 2010; Perosa et al., 2010; Sanz and Lee, 2010). In addition to earlier work in mouse models suggesting the importance of B cells in the pathogenesis of lupus (Reininger et al., 1996), the recent success of anti-CD20 therapy has further emphasized the role B cells play in initiating and/or perpetuating autoimmune dyscrasias (Lund and Randall, 2010). The impact of B cell depletion on T cell abnormalities has demonstrated that there is a relevant effect of B cells on T cell stimulation in the context of autoimmune disease (Stasi et al., 2007; Eming et al., 2008; Lioussis and Sfakakis, 2008). T cell-stimulating function is just the activity that we have shown to be concentrated within the very small CD11b<sup>+</sup> B1 cell population, which expands greatly in lupus, up-regulates CD86 expression, and becomes more immunostimulatory.

in triplicate with negatively selected allogeneic CD4<sup>+</sup> B cells for 5 d, after which proliferation was measured by incorporation of tritiated thymidine. Mean cpm values are shown along with lines indicating SEM.

These characteristics mirror previous findings in several mouse models of lupus, in which it has been shown that B1 cell numbers are increased and antigen presentation is enhanced (Mohan et al., 1998; Xu et al., 2004; Duan and Morel, 2006), suggesting a pathogenic mechanism in which B1 cells present self-antigens to autoreactive T cells. Although it might be thought that the low frequency of CD11b<sup>+</sup> B1 cells would be insufficient to substantially contribute to or alter immune activity, large effects from small populations are well known in the immune system, such as the generation of natural IgM from mouse splenic B1 cells, the influence of human T reg cells on lupus disease, and the protection from pathogens afforded by myeloid dendritic cells (Barreto et al., 2009; Holodick et al., 2010; Kassianos et al., 2010; Hambleton et al., 2011). Moreover, the full size of the human B1 cell population could well be larger than suggested by its circulating component if one or more tissue reservoirs exist, as is the case with the preponderance of mouse B1 cells within the peritoneal cavity.

Thus, our results suggest that subdivision of human B1 cells into CD11b<sup>+</sup> and CD11b<sup>-</sup> populations is relevant not only for localizing activities that shape T cell function but for autoimmune disease pathogenesis and pathopersistence as well. Identification of CD11b<sup>+</sup> B1 cells as a new B cell type may provide an important foundation for understanding how B cells influence the immune response in both health and illness, and suggests a new target for selective therapy in autoimmune disease that will leave the bulk of B cell responses intact.

## 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. These healthy controls were of mean age 51 yr, 78% female, 85% European, 13% Asian, and 2% Hispanic. Anonymous umbilical cord blood samples were obtained from the Tissue Donation Program at The Feinstein Institute for Medical Research. 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 upon receipt. Mononuclear cells were obtained by density gradient separation using lymphocyte separation medium (Cellgro). Except as otherwise noted, mononuclear cells were then washed and resuspended in RPMI 1640 (Cellgro) containing 10% fetal calf serum plus 2 mM L-glutamine, 10 mM Hepes, pH 7.25, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**B cell enrichment.** For some experiments (Fig. 1 C; Fig. 2, A and B; Fig. 3 G; and Fig. 4 F), B cells were enriched by CD19 positive selection using the EasySep Human CD19<sup>+</sup> B cell magnetic bead selection kit (STEMCELL Technologies) according to the manufacturer's instructions. For some experiments (Fig. 1 D), B cells were enriched by CD20 positive selection using the EasySep Human CD20<sup>+</sup> B cell magnetic bead selection kit (STEMCELL Technologies).

**Cell sorting and flow cytometric analysis.** Enriched B cells and mononuclear cells were immunofluorescently stained and then sort purified on an Influx instrument (BD). Cells to be sorted were suspended in 2 mM EDTA in dye-free RPMI supplemented with 10% fetal calf serum at a concentration <50 million cells per milliliter. Flow cytometric analysis of immunofluorescently stained cells was performed on an LSR-II instrument (BD).

Post-sort analysis showed <1% CD3 positivity for sort-purified CD19-enriched B cells and <3% CD3 positivity for sort-purified mononuclear (not pre-enriched) B cells.

**ELISA.** Immunoglobulin secretion was determined by ELISA assay, as previously described (Griffin et al., 2011).

**Allogeneic stimulation.** Naive CD4<sup>+</sup> T cells were negatively selected from PBMC and co-cultured with sort-purified, irradiated B cells at a ratio of 2:1, as previously described (Griffin et al., 2011) For the allogeneic stimulation assays shown in Fig. 3 (C and D), 50,000 B cells were seeded per well. For the allogeneic stimulation assays shown in Fig. 4 F, comparing lupus patients to normal controls, 1,000 B cells were seeded per well to accommodate the smaller cell numbers that could be obtained from lupus patient samples. In some experiments B cells were exposed to 10 µg/ml of neutralizing anti-CD86 antibody for 1 h before, and then during, co-culture. Cultures were pulsed with 0.75 µCi [<sup>3</sup>H]thymidine for the last 8 h of 5-d cultures, and cpm was determined by scintillation counting.

**Microarray analysis.** RNA was isolated from sort-purified naive B cells (CD20<sup>+</sup>CD27<sup>-</sup>CD43<sup>-</sup>), memory B cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>-</sup>), CD11b<sup>+</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>+</sup>), and CD11b<sup>-</sup> B1 cells (CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>-</sup>) from three normal individuals per subset, using the RNeasy plus Mini kit according to the manufacturer's instructions (QIAGEN). RNA was analyzed for gene expression using the Illumina platform. The human HT-12 v4 Expression BeadChip was used that interrogates ≥47,000 target probes for genes obtained from the National Center for Biotechnology Information Reference Sequence RefSeq Release 38 and other sources. Initial data analysis and quality control checks were performed using GenomeStudio software (Illumina). The data for all 12 samples was quantile normalized without background subtraction and exported as a project for additional analysis using Partek software (Genomic Suite v6.5). An ANOVA analysis was performed for all samples and heat maps were generated for genes with differential expression between B1 cell subsets. Data are publically available with accession no. GSE29717 at the Gene Expression Omnibus.

**Photomicroscopy.** Sort-purified B cells were cytocentrifuged (Shandon), stained with Wright-Giemsa, and imaged at 40× (Carl Zeiss). CD11b<sup>+</sup> B1 cells stained with Hoechst 33342 were single-cell sorted onto an Ampligridd (Beckman Coulter), excited by UV illumination, and imaged at 40×. Sort-purified B cells for confocal imaging were fixed in paraformaldehyde, adhered to poly-lysine-coated slides, excited at 488, 568, and 647 nm, and imaged at 60× (FluoView 300; Olympus).

**B1 cell stimulation.** CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD11b<sup>-</sup> human B1 cells were sort purified from adult peripheral blood and were cultured with PMA at 100 ng/ml plus ionomycin at 400 ng/ml (P + I), SAC at 0.005% plus IL-2 at 5 ng/ml (SAC + IL2), and anti-IgM at 7 µg/ml plus anti-CD40 at 10 µg/ml (αIgM + αCD40) in RPMI medium in round bottom wells.

**Reagents.** Fluorescently labeled antibodies (anti-CD5-PE-Cy7, anti-CD11b-percp, anti-CD11b-PE, anti-CD11c-PE, anti-CD14-PE, anti-CD20-V450, anti-CD20-APC-Cy7, anti-CD21-PE, V500-streptavidin, anti-CD23-PE, anti-CD27-V450, anti-CD27-APC, anti-CD43-FITC, anti-CD70-PE, anti-CD86-PE, anti-CD86-biotin, anti-CD69-percp, anti-CD70-PE, anti-CD71-PE, anti-CD80-PE, anti-IgD-PE, and anti-IgM-PE) were obtained from BD. Anti-CD11b-percp was obtained from Abcam. Anti-CD14 Alexa Fluor 647 was obtained from BioLegend. Anti-CD19 Alexa Fluor 488 and Alexa Fluor 568 streptavidin were obtained from Invitrogen. Anti-CD43-biotin was obtained from Thermo Fisher Scientific. Anti-CD43-APC was obtained from eBioscience. Streptavidin was obtained from Biomed. Anti-CD86 neutralizing antibody was obtained from eBioscience. Wright-Giemsa stain was obtained from Ricca chemical.

**Statistics.** Statistical analysis was performed using Prism software (version 5.0d for Mac). For calculation of fold change in SLE samples, the mean value



from the analyzed normal samples was calculated, and the values for the SLE samples were calculated as fold change from normal mean for each sample. For age and gender matching of controls to lupus, normal individuals with ages and gender matching the lupus patients were chosen at random in a blinded manner relative to B cell analysis results from the normal individuals to create a control population with the same gender frequency and mean age of the lupus patients.

**Online supplemental material.** Fig. S1 shows that sort-purified CD11b<sup>+</sup> and CD11b<sup>−</sup> B1 cells are singlets whose phenotype does not change with in vitro culture; that by direct visualization, CD11b<sup>+</sup> B1 cells are singlets after sort purification; and that CD11b<sup>+</sup> B1 cells display typical lymphocyte morphology similar to other B cell populations. Table S1 shows lupus patient information. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20110978/DC1>.

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