A human equivalent of mouse B-1 cells?

To the Editor:

In a recent issue of *The Journal of Experimental Medicine*, Thomas Rothstein and colleagues, a group with long-standing expertise in the field of mouse B1 cells, reported the description of a B1 B cell subset in human blood, a population that has thus far eluded identification (Griffin et al., 2011).

Mouse B1 cells are the main constituents of the B lymphocyte pool in pleural and peritoneal cavities, and are characterized as $CD5^+$ and/or $CD11b^+$ cells (Hardy, 2006; Baumgarth, 2011). However, CD5, which was initially identified on chronic lymphocytic leukemia tumors (Boumsell et al., 1978), is not a B1 marker in humans. Human CD5 marks immature/transitional B cells in bone marrow, blood, and spleen (Sims et al., 2005; Cuss et al., 2006), as well as in T cells. Mouse B1 cells are also described as $IgM^{high}IgD^{low}CD43^+$. The function of CD43 in B1 cells is unknown, and it is also expressed by T lymphocytes, B cell precursors, and plasma cells.

Griffin et al. (2011) described a $CD20^+CD27^+CD43^+CD70^-$ subset present in adult and human cord blood with functional characteristics that they describe as typical B1 cell attributes: spontaneous IgM secretion, constitutive BCR signaling, and ability to drive allogeneic T cell proliferation. It should be noted that this last feature has been shown to be displayed by switched memory B cells in humans, likely because of the high expression of CD80 and/or CD86 on these cells (Liu et al., 1995; Good et al., 2009).

A striking point of the observations of Griffin et al. (2011) was their quantitative aspect. Despite considerable variations between individual blood donors, the proportion of $CD43^+$ cells among $CD27^+$ B cells averaged 40–50% in adults, with a higher frequency in young individuals, and a lower frequency in the elderly. We find this puzzling, as these quantitative figures closely match the frequency of marginal zone–like (or IgM memory) B cells (Weill et al., 2009), with the difference being that marginal zone–like B cells are IgD^{int} whereas $CD43^+$ B cells are mainly IgD^{high} (Griffin et al., 2011). Therefore, we analyzed to what extent these two populations may superimpose.

$CD43^+$ B cells appear as large cells that require a wide lymphocyte gate to be detected. In so doing, the risk of inclusion of cell doublets in the analysis/isolation is high. Usually, specific selective criteria on the cell flow (FSC-W, SSC-W) are applied to remove doublets, unless the morphological characteristics of the cell population justify such an omission. In such cases, careful controls are obviously required to avoid the confusion between cell doublets and large cells.

We added anti-CD3 antibodies to the staining reaction, as T cells are the major lymphocyte subset in human blood compared with B cells (95:5). A large fraction of $CD20^+CD27^+CD43^+$ cells stained positive for CD3 (Fig. 1 A). We believe that these $CD20^+CD27^+CD43^+CD3^+$ cells are doublets involving T cells that account for the CD43 and CD27 labeling, and (mainly) naive B cells that account for the IgD^{high} phenotype (unpublished data). Pre-enrichment of peripheral blood B cells through $CD19^+$ selection strongly reduced the proportion of $CD20^+CD27^+CD43^+$ cells (Fig. 1 A).

Gating on $CD20^+$ cells excluded plasma cells, identified as $CD43^{++}CD27^{++}CD38^{++}$. The remaining $CD20^+CD3^-CD27^+CD43^+$ cells accounted for 2–3% of the total B cell pool (2.2% for adult samples, 2.8% for child samples; Fig. 1, B and C). These cells harbored either an IgD^+ or an IgD^- phenotype and a somewhat heterogeneous CD38 intensity (Fig. 1 B). Interestingly, the IgD^+ over IgD^- ratio among $CD43^+$ cells varied with age; IgD^+ cells dominated in children <5 yr, and IgD^- cells dominated in adults (Fig. 1 D). IgD^- cells include cells expressing IgG or IgA, as well as a minority of IgM-only cells (unpublished data). The presence of $CD20^+CD43^+$ cells displaying IgD, IgG, or IgA makes their possible B1 equivalence a more complex issue, even though B1 cells can give rise to IgA-producing plasma cells in the lamina propria (Suzuki et al., 2010).

We therefore propose that the quantitative variations observed when counting $CD43^+$ B cells may be largely contributed by staining artifacts. The potential presence of doublet events in the $CD20^+CD43^+$ population analyzed by Griffin et al. (2011) obviously questions the in vitro functional characteristics described for this putative B1 subset. Whether $CD20^+CD43^+CD27^+IgD^+$ or IgD^- B cells are activated cells on their way to plasma cell differentiation or a new innatelike subset with B1 functional features remains to be seen.

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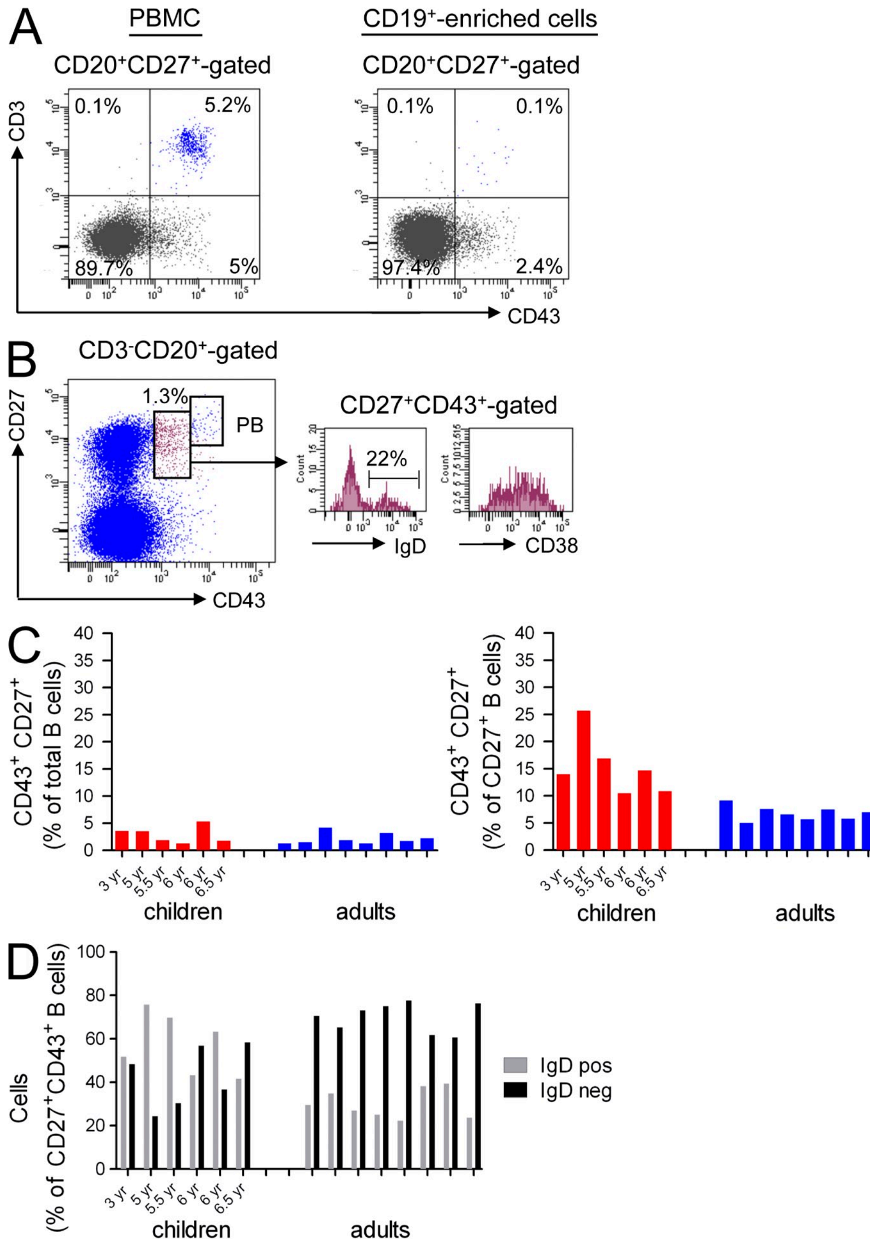

Figure 1. CD3, CD43, CD38 and IgD expression on human CD20⁺CD27⁺ PMBCs. (A) Human PMBCs were analyzed within a large lymphoid gate. The right dot plot represents cells after enrichment by CD19 microbeads (Miltenyi Biotec), whereas the left dot plot represents total PMBCs. The flow cytometric analysis shown was performed at a flow rate of <5,000 events per second. The data shown here for an adult are representative of eight adult blood samples analyzed in four separate experiments. (B) Representative analysis of an adult blood sample. PBMCs were analyzed within a large lymphoid gate, with doublets excluded by SSC-W criteria. Cells were further gated as $CD3^-CD20^+$ for analysis of CD27 and CD43 expression. The small top right gate in the dot plot indicates plasmablasts that are excluded from the cell estimates. The histograms show the expression of IgD and CD38 on the $CD43^+CD27^+$ population. (C) Blood samples from 6 children (3–6.5 yr old) and 8 adults were analyzed as in B. The proportion of $CD43^+CD27^+$ cells among $CD3^-CD20^+$ B cells (expressed either as percentage of total B cells or of $CD27^+$ B cells) is indicated. Each bar represents the results obtained for one individual. (D) The values obtained for the $CD27^+CD43^+$ population are expressed as percentage of total B cells, and IgD^+ and IgD^- values are shown in bar graphs, each bar representing the results obtained for one individual. The following antibodies were used: CD20 (APC-H7, clone 2H7), CD43 (FITC, clone 1G10), CD3 (PE, clone UCHT1), and CD27 (APC, clone M-T271) from BD; CD38 (PerCp-Cy5.5, clone HIT2) and IgD (PE-Cy7, clone IAG-2) from BioLegend. Flow cytometry analysis was performed on a FACSCanto II apparatus with FACSDiva software.

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The nature of circulating $CD27^{+}CD43^{+}$ B cells

To the Editor:

We recently read with great interest an article in *The Journal of Experimental Medicine* from the laboratory of Thomas Rothstein (Griffin et al., 2011). This paper described a new B cell population present in human cord blood and adult peripheral blood that displays functional characteristics shared with mouse B1 cells.

In contrast to B2 cells, B1 cells spontaneously produce IgM and are an important source of natural antibodies in the absence of antigen stimulation in mouse (Hayakawa et al., 1984; Sidman et al., 1986). Human B1 cells were defined based on coexpression of CD43 and CD27 (Griffin et al., 2011). In addition, human B1 cells were found to express CD20 and lack expression of CD69 and CD70. Notably, CD43 is also expressed on mouse B1 cells (Wells et al., 1994), as well as on human bone marrow B cell precursors and plasma cells (MacMichael et al., 1987; Wikén et al., 1988), whereas CD27 is generally regarded as a marker for memory B cells in humans (Agematsu et al., 1997; Tangye et al., 1998). Furthermore, CD27 and CD43 are coexpressed on the majority of blood T cells and plasma cells (MacMichael et al., 1987).

To gain further insight into the nature of $CD27^{+}CD43^{+}$ B1 cells in humans, we developed a flow cytometric strategy to discriminate between $CD27^{+}CD43^{+}$ B cells and $CD27^{+}$ memory B cells in human blood (Berkowska et al., 2011). Like Griffin et al. (2011), we systematically identified a $CD27^{+}CD43^{+}$ subset within the $CD19^{+}$ or $CD20^{+}$ B cell population in neonatal cord blood (n = 4 samples), in the peripheral blood of children and adults (n = 27 samples), and in childhood tonsils (n = 7 samples; Fig. 1, A–C). Their frequencies ranged from 1 to 25.5% of total $CD19^{+}$ or $CD20^{+}$ cells in blood (Fig. 1 D), and from 0.3 to 3.7% of $CD19^{+}$ cells in childhood tonsil (unpublished data).

In contrast to most B lymphocytes, which are $CD43^{-}$, most T lymphocytes and plasma cells are $CD43^{+}$. To ensure that all identified $CD43^{+}CD27^{+}$ events constituted B lymphocytes, we further analyzed the $CD27^{+}CD43^{+}$ population. A substantial fraction (0.1–24%) of $CD19^{+}CD27^{+}CD43^{+}$ events was highly positive for CD3 and dimly positive for CD19. Analysis of CD19 versus CD3 expression suggested that these cells were T lymphocytes that were incorrectly included in the $CD19^{+}$ gate, and accounted for most $CD5^{+}$ events in the gate (Fig. 1, A–C). Of the remaining $CD3^{-}CD27^{+}CD43^{+}$ cells, the majority showed high expression of CD38 (0.1–4.6% of $CD19^{+}$). Coexpression of CD27 and high levels of CD38 is typically seen on plasma cells, as well as on germinal center cells and plasma cells in tonsil (Klein et al., 2003; van Zelm et al., 2007; Perez-Andres et al., 2010).

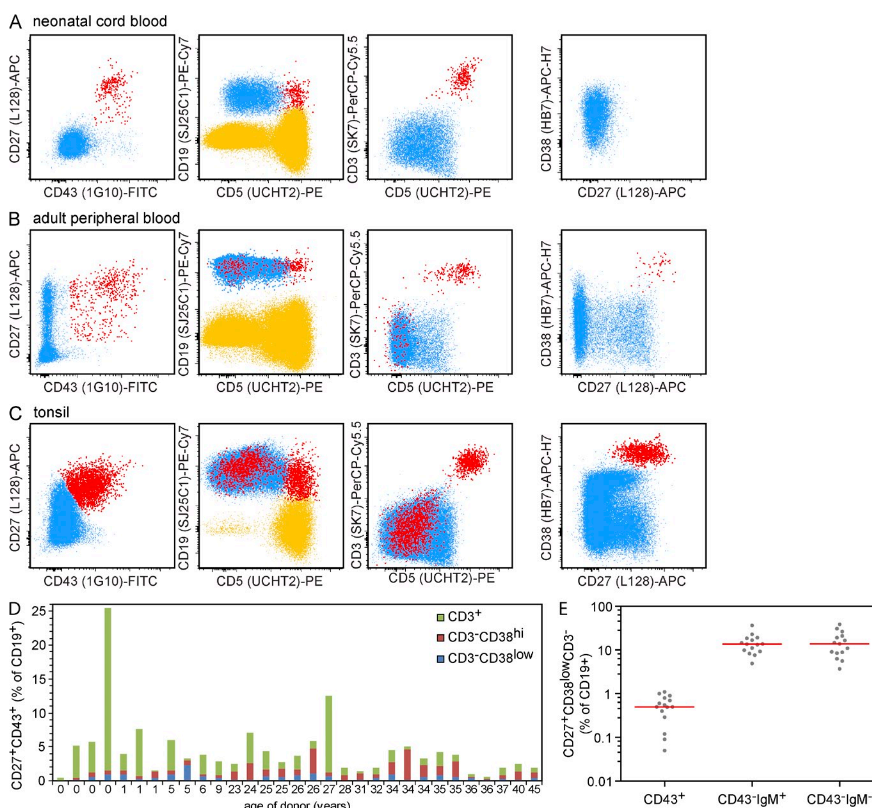

Figure 1. Flow cytometric analysis of $CD19^{+}CD27^{+}CD43^{+}$ cells in human neonatal cord blood, peripheral blood, and tonsil samples. (A–C) $CD27^{+}CD43^{+}$ cells (red) were defined within $CD19^{+}$ lymphocytes (blue; left plots). Where illustrative, the $CD19^{-}$ lymphocytes (T cells and NK cells) are shown in yellow. Samples were also stained with anti-CD3, anti-CD5, and anti-CD38 as indicated. The right plots only display $CD19^{+}CD3^{-}$ events. Data are representative of 4 cord blood, 27 blood, and 7 tonsil samples. (D) Frequencies of $CD27^{+}CD43^{+}$ cells among $CD19^{+}$ cells in neonatal cord blood and peripheral blood samples from individual children and adults of indicated ages. The relative contributions of $CD3^{+}$ T cells, $CD3^{-}CD38^{hi}$ plasma cells, and $CD3^{-}CD38^{low}$ B lymphocytes are indicated for each individual. (E) Frequencies of $CD43^{+}$, $CD43^{-}IgM^{+}$, and $CD43^{-}IgM^{-}$ cells among $CD19^{+}CD27^{+}CD3^{-}CD38^{low}$ cells within the blood of healthy adults. Individual frequencies are shown in gray and the median is indicated with a red bar. IgM expression was detected with a monoclonal IgM-HorizonV450 antibody (clone G20-127).

Only a minority of the CD19$^+$ CD27$^+$CD43$^+$ events appeared to be true B lymphocytes (0.3–2.3% of CD19$^+$CD3$^-$CD38^{dim} cells) and consisted of both IgM$^+$ and IgM$^-$ cells (unpublished data). Their total frequency was >10-fold lower than the frequencies of both CD27$^+$IgM$^+$ and CD27$^+$IgM$^-$ memory B cells in adult blood (Fig. 1 E). Therefore, we conclude that CD43$^+$ B lymphocytes constitute a small minority of total CD27$^+$ B lymphocytes.

In conclusion, our results confirm a decline with age of the overall population of CD27$^+$CD43$^+$ circulating human B cells, but indicate that caution should be taken in the definition of human B1 cells inside this heterogeneous cell population. Detailed flow cytometric analysis of CD19$^+$CD27$^+$CD43$^+$ cells reveals that the majority may be CD38^{hi} plasma cells or contaminating CD3$^+$ T cells. The large proportion of T cells in our analysis might explain the high frequency of CD5$^+$ cells reported by Griffin et al. (2011), as all T cells express CD5. Finally, we conclude that clearly defined CD3$^-$CD38^{dim}CD27$^+$CD43$^+$ B lymphocytes may be only a minor population of CD27$^+$ memory B cells in adult blood. Understanding the functional similarities between these cells and mouse B1 cells will be important topics for additional studies in regard to infectious diseases and lymphoid malignancies. However, these studies will require the exclusion of T cells and circulating plasma cells from such analysis.

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Human B1 cells are CD3$^-$: A reply to "A human equivalent of mouse B-1 cells?" and "The nature of circulating CD27$^+$CD43$^+$ B cells"

Griffin et al. respond:

In the January 2011 issue of the *JEM*, we identified a population of B cells in human umbilical cord and adult peripheral blood that manifests three key functional features of mouse B1 cells (spontaneous immunoglobulin secretion, tonic intracellular signaling, and efficient T cell stimulation). This population is

marked by the phenotype CD20$^+$ CD27$^+$CD43$^+$CD70$^-$ (Griffin et al., 2011). We appreciate the interest shown in this work by Perez-Andres et al. and Descatoire et al., who have written letters to report CD3$^+$ events detected within CD19$^+$CD27$^+$CD43$^+$ and CD20$^+$CD27$^+$CD43$^+$ gated populations, respectively, during their flow cytometry.

The problem of CD3 positivity among "a major fraction" (Perez-Andres et al., 2011) and "a large fraction" (Descatoire et al. 2011) of CD27$^+$CD43$^+$ B cells is not something we have experienced. In our hands, CD3$^+$ events within CD20$^+$CD27$^+$CD43$^+$ sort-purified populations are rare. In early work, we tested the function of B1 cells that were sort-purified from PBMCs without prior enrichment (Griffin et al., 2011); these CD20$^+$CD27$^+$CD43$^+$ populations contain <3% CD3$^+$ events (2.9 ± 0.66%; n = 3). In later work, we studied the function of B1 cells that were sort-purified from PBMCs after enrichment with anti-CD19 beads; these CD20$^+$CD27$^+$CD43$^+$ populations contain <1% CD3$^+$ events (0.57 ± 0.37%; n = 3; Fig. 1 a). The existence of CD27$^+$CD43$^+$ coexpressing B cells seems irrefutable; single cells expressing CD19, CD27, and CD43, and single cells expressing CD20, CD27, and CD43, can be readily visualized as confocal microscopic images (Fig. 1 b).

To discern the origin of the reported CD3$^+$ events, we attempted to recreate the observations of Perez-Andres et al. (2011) and Descatoire et al. (2011). We reproduced the high frequency of double staining events observed by these investigators only by changing our usual protocol for cell preparation. When we reconstituted mononuclear cells at a relatively high concentration, we observed CD3$^+$ events in the CD20$^+$CD27$^+$CD43$^+$ gate. FSC-H/FSC-A discrimination decreased, but did not completely eliminate, CD3$^+$ staining in that gate (Fig. 1 c). Diluting and vortexing the sample reduced the frequency of CD3$^+$ events in the CD20$^+$CD27$^+$CD43$^+$ gate, which was further reduced by applying FSC-H/FSC-A doublet dis-

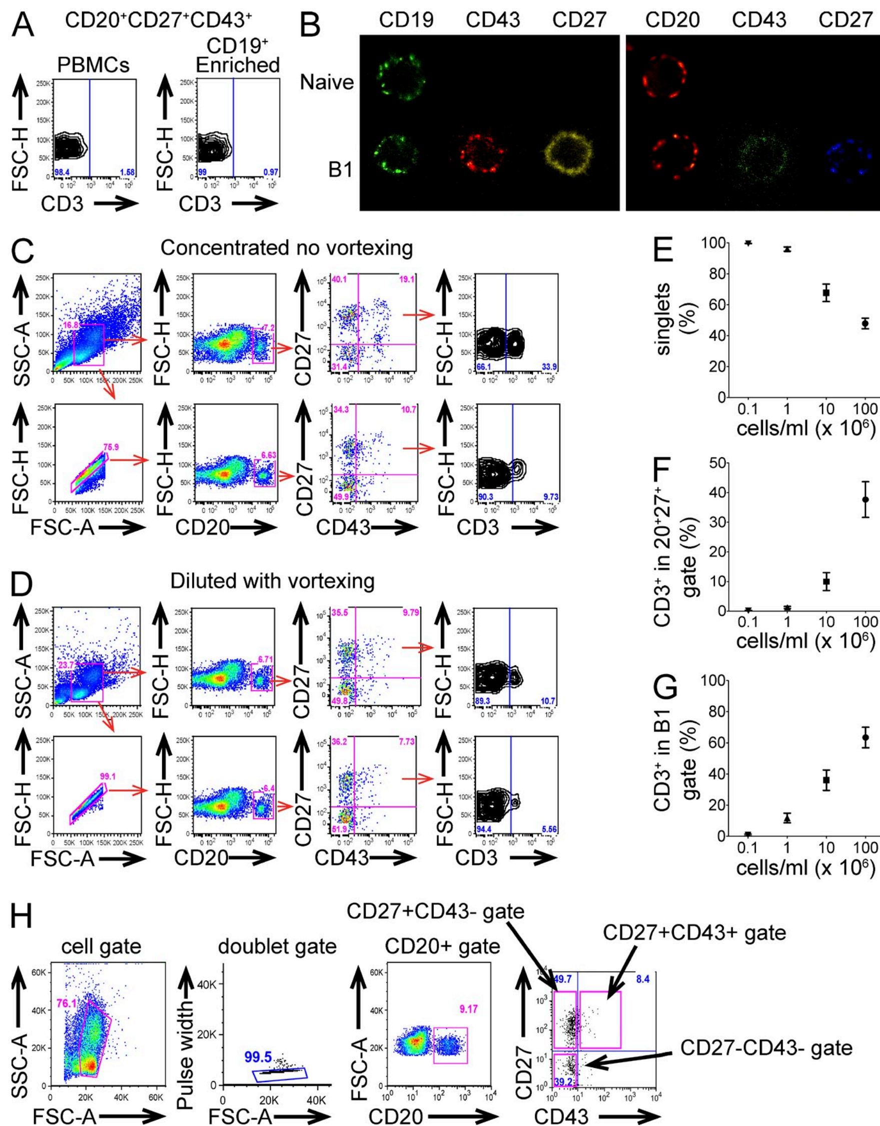

Figure 1. Human CD20$^+$CD27$^+$CD43$^+$ B1 cells do not express CD3. (A) Adult peripheral blood cells were immunofluorescently stained with specific antibodies. CD20$^+$CD27$^+$CD43$^+$ B1 cells were sort-purified from nonenriched, as well as CD19-enriched PBMCs and sorted B1 cells were analyzed for CD3 expression. Gating was based on an isotype control. Results for one of three comparable experiments are shown. (B) Adult peripheral blood cells were immunofluorescently stained, sort-purified for naive (CD19$^+$CD27$^-$CD43$^-$/CD20$^+$CD27$^-$CD43$^-$) and B1 (CD19$^+$CD27$^+$CD43$^+$/CD20$^+$CD27$^+$CD43$^+$) cells, and examined by confocal microscopy. (C) Immunofluorescently stained PBMCs were analyzed at high concentration without vortexing without (top row) or with (bottom row) FSC-H/FSC-A doublet discrimination. The last panel shows CD3$^+$ events among CD20$^+$CD27$^+$CD43$^+$ cells. (D) The same sample as in C was diluted and vortexed, and then analyzed without (top row) or with (bottom row) FSC-H/FSC-A doublet discrimination. Note that many more cells appear as singlets (99.1%) as compared with C (75.9%). The last panel shows CD3$^+$ events among CD20$^+$CD27$^+$CD43$^+$ cells. Results for one of three comparable experiments are shown. (E–G) Adult peripheral blood cells were immunofluorescently stained, and then presented for flow cytometric analysis at various cell concentrations, as indicated (n = 12). The proportion of single cells determined by FSC-H/FSC-A gating is shown as a function of cell concentration in E. The proportion of CD3$^+$ events among gated CD20$^+$CD27$^+$ cells is shown as a function of cell concentration in F. The proportion of CD3$^+$ events among gated CD20$^+$CD27$^+$CD43$^+$ cells is shown as a function of cell concentration in G. (H) Adult peripheral blood cells were immunofluorescently stained with specific antibodies, brought into a single cell suspension, and sort-purified on an Influx (BD) instrument. The gating strategy used for experiments reported in Griffin et al. (2011) is shown.

crimination (Fig. 1 d). Over a range of cell concentrations, we documented an inverse correlation between cell concentration and the proportion of singlets as determined by FSC-H/FSC-A, and a direct correlation between cell concentration and $CD3^+$ events in the $CD20^+CD27^+$ gate and in the $CD20^+CD27^+CD43^+$ B1 cell gate (Fig. 1, e and f). These results suggest that $CD3^+$ events within $CD20^+$ B cell populations result from the formation of B:T doublets.

To evaluate the possible existence of $CD19^+$ T cells, as suggested by Perez-Andres et al. (2011), we examined $CD27^+CD43^+$ B cells by confocal microscopy and failed to identify single cells that express both CD3 and CD19 (unpublished data). We examined B cells by integrated flow cytometry/image microscopy and again failed to identify single cells marked by either CD19 or CD20 in combination with CD3. Although the possibility of rare B cells that express CD3 (Wang et al., 2009) or rare T cells that express CD20 (Yokose et al., 2001) cannot be entirely ruled out, these results indicate that such cells must be exceedingly rare. We conclude that $CD19^+CD3^+$ cells observed by Perez-Andres et al. (2011), like the $CD20^+CD3^+$ cells observed by Descatoire et al. (2011), represent B:T doublets.

In our results, $CD20^+CD27^+CD43^+$ B1 cells are individual single cells by post-sort analysis. The gating strategy that we have used, and continue to use, is displayed in Fig. 1 h. Virtually all (99.9%) events fell within a tight FSC-H/FSC-A discrimination gate, and virtually all (99.9%) events corresponded to single nuclei after DNA staining with Hoechst33342 when analyzed post-sort (unpublished data). In addition, we showed by direct visualization of Hoechst33342-stained nuclei that single cell–sorted $CD20^+CD27^+CD43^+$ B1 cells deposited onto glass slides are individual single cells, with not a single doublet observed in over 1,000 cells examined (unpublished data). Thus, the B1 cell functions delineated in our previous work correspond to the characteristics of single $CD20^+CD27^+CD43^+$ B cells.

Descatoire et al. (2011) notes that in the human system allogeneic T cell stimulation has been ascribed to memory B cells (Good et al., 2009). However, $CD20^+CD27^+$ B cells, historically considered memory B cells, fail to allogeneically stimulate T cells after removal of CD43-expressing constituents (Griffin et al., 2011). Furthermore, doublets formed by naive B cells and T cells would not be expected to manifest T cell stimulatory capacity nor would their removal be expected to adversely affect any memory B cell function. This emphasizes in another way, through negative depletion, the existence of functionally distinct, CD43-expressing $CD27^+$ B cells.

Descatoire et al. (2011) suggests that their $CD3^-CD20^+CD27^+CD43^+$ B cells were either IgD^+ or IgD^-; in our hands, $CD20^+CD27^+CD43^+$ B1 cells predominantly expressed IgD, and gave rise to amplified and rearranged IgM sequences as shown in our earlier study (Griffin et al., 2011), although we identified $IgD^-CD20^+CD27^+CD43^+$ B1 cells that may be responsible for IgA produced by B1 cells, as in the mouse system (Baumgarth, 2011). Perez-Andres et al. (2011) noted that their $CD3^-CD19^+CD27^+CD43^+$ lymphocytes expressed elevated levels of CD38 and were most likely plasma cells. CD19 does not exclude plasmablasts and plasma cells, as does CD20 (Jego et al., 2001), which we used to identify B cells in our study. Descatoire et al. (2011) raised the possibility that $CD20^+CD27^+CD43^+$ B cells might be activated cells on their way to plasma cell differentiation. We addressed this in our earlier work by showing that B1 cells do not express elevated levels of activation antigens, such as CD69 and CD70, and that culture of B1 cells with IL-6 for 5 d did not lead to loss of CD20 or acquisition of CD138 (Griffin et al., 2011).

Both Perez-Andres et al. (2011) and Descatoire et al. (2011) state that after excluding $CD3^+$ events, they indeed identified the phenotypically unique $CD27^+CD43^+$ B cell population that we first reported in January (Griffin et al., 2011), amounting to 2–3% of all B cells in Weller's hands. Furthermore, Perez-Andres et al. (2011) stated that their results confirm a decline with age among $CD27^+CD43^+$ B cells, recapitulating our published results (Griffin et al., 2011). It appears that the major issue raised by these two letters relates simply to different numbers for the frequency of $CD27^+CD43^+$ B1 cells. There are numerous reasons why different laboratories might arrive at different frequencies, including variations in sample size, donor age, cell preparation, antibody/fluorophore selection, and instrument and run characteristics, not to mention the efficacy of doublet discrimination. With respect to the first two reasons, in our January 2011 article (Griffin et al., 2011), we averaged noncord peripheral blood samples to obtain a mean $CD27^+CD43^+$ B cell frequency across 25 highly variable samples; however, many of these samples were obtained from children, whose B1 cell frequencies are elevated with respect to middle aged adults, and we included values that would now be considered outliers. In our current work with samples from individuals in the third through seventh decades of life, we obtain values over a wide range (1–9%) of $CD20^+CD27^+CD43^+$ B1 cells among all circulating B cells, a range recapitulated by other groups, including the letter writers. Our recent identification of human B1 cells provides the means to explore the regulation and role of this unique population in human health and disease. Functional B1 cells in the human system phenotype as $CD20^+$ $CD27^+CD43^+CD70^-$ and do not express CD3.

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