

# 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<sup>+</sup> and/or CD11b<sup>+</sup> 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<sup>high</sup>IgD<sup>low</sup>CD43<sup>+</sup>. 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD70<sup>-</sup> 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<sup>+</sup> cells among CD27<sup>+</sup> B cells averaged 40–50% in adults, with a higher frequency in young individuals, and a lower fre-

quency 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<sup>int</sup> whereas CD43<sup>+</sup> B cells are mainly IgD<sup>high</sup> (Griffin et al., 2011). Therefore, we analyzed to what extent these two populations may superimpose.

CD43<sup>+</sup> 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> cells stained positive for CD3 (Fig. 1 A). We believe that these CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD3<sup>+</sup> cells are doublets involving T cells that account for the CD43 and CD27 labeling, and (mainly) naive B cells that account for the IgD<sup>high</sup> phenotype (unpublished data). Pre-enrichment of peripheral blood B cells through CD19<sup>+</sup> selection strongly reduced the proportion of CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> cells (Fig. 1 A).

Gating on CD20<sup>+</sup> cells excluded plasma cells, identified as CD43<sup>++</sup>CD27<sup>++</sup>CD38<sup>++</sup>. The remaining CD20<sup>+</sup>CD3<sup>-</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup> or an IgD<sup>-</sup> phenotype and a somewhat heterogeneous CD38 intensity (Fig. 1 B).

Interestingly, the IgD<sup>+</sup> over IgD<sup>-</sup> ratio among CD43<sup>+</sup> cells varied with age; IgD<sup>+</sup> cells dominated in children <5 yr, and IgD<sup>-</sup> cells dominated in adults (Fig. 1 D). IgD<sup>-</sup> cells include cells expressing IgG or IgA, as well as a minority of IgM-only cells (unpublished data). The presence of CD20<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup> B cells may be largely contributed by staining artifacts. The potential presence of doublet events in the CD20<sup>+</sup>CD43<sup>+</sup> population analyzed by Griffin et al. (2011) obviously questions the in vitro functional characteristics described for this putative B1 subset. Whether CD20<sup>+</sup>CD43<sup>+</sup>CD27<sup>+</sup>IgD<sup>+</sup> or IgD<sup>-</sup> 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.

Marc Descatoire, Jean-Claude Weill, Claude-Agnès Reynaud, and Sandra Weller

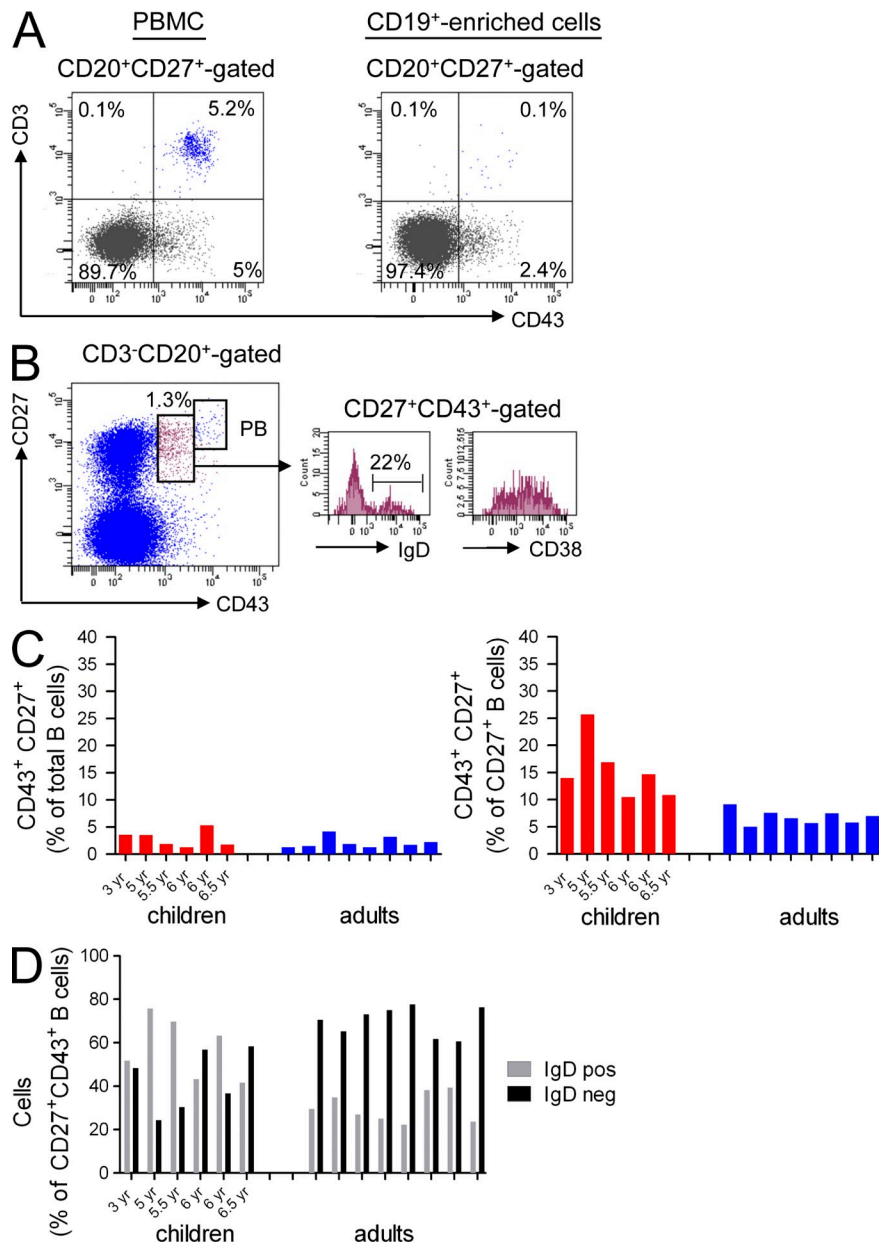
Institut National de la Santé et de la Recherche Médicale (INSERM) Unité 783 "Développement du système immunitaire", Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine, Site Necker-Enfants Malades, Paris, France

## CORRESPONDENCE

Sandra Weller: sandra.weller@inserm.fr

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**Figure 1. CD3, CD43, CD38 and IgD expression on human CD20<sup>+</sup>CD27<sup>+</sup> 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<sup>-</sup>CD20<sup>+</sup> 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<sup>+</sup>CD27<sup>+</sup> population. (C) Blood samples from 6 children (3–6.5 yr old) and 8 adults were analyzed as in B. The proportion of CD43<sup>+</sup>CD27<sup>+</sup> cells among CD3<sup>-</sup>CD20<sup>+</sup> B cells (expressed either as percentage of total B cells or of CD27<sup>+</sup> B cells) is indicated. Each bar represents the results obtained for one individual. (D) The relative distribution of IgD<sup>+</sup> and IgD<sup>-</sup> B cells among the CD27<sup>+</sup>CD43<sup>+</sup> B cell population is 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<sup>+</sup>CD43<sup>+</sup> B cells

## To the Editor:

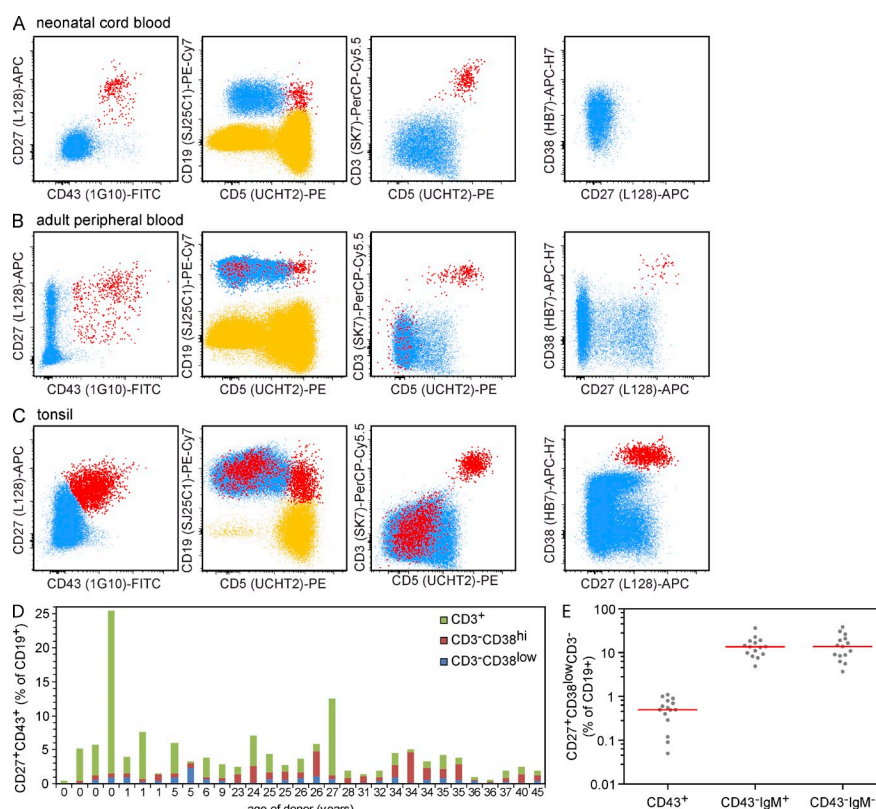
We recently read with great interest an article in *The Journal of Experimental Medicine* from the laboratory of Thomas Rothstein (Griffin et al., 2011). This paper described a new B cell population present in human cord blood and adult peripheral blood that displays functional characteristics shared 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<sup>+</sup>CD43<sup>+</sup> B1 cells in humans, we developed a flow cytometric strategy to discriminate between CD27<sup>+</sup>CD43<sup>+</sup> B cells and CD27<sup>+</sup> memory B cells in human blood (Berkowska et al., 2011). Like Griffin et al. (2011), we systematically identified a CD27<sup>+</sup>CD43<sup>+</sup> subset within the CD19<sup>+</sup> or CD20<sup>+</sup> 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<sup>+</sup> or CD20<sup>+</sup> cells in blood (Fig. 1 D), and from 0.3 to 3.7% of CD19<sup>+</sup> cells in childhood tonsil (unpublished data).

In contrast to most B lymphocytes, which are CD43<sup>−</sup>, most T lymphocytes and plasma cells are CD43<sup>+</sup>. To ensure that all identified CD43<sup>+</sup>CD27<sup>+</sup> events constituted B lymphocytes, we further analyzed the CD27<sup>+</sup>CD43<sup>+</sup> population. A substantial fraction (0.1–24%) of CD19<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup> gate, and accounted for most CD5<sup>+</sup> events in the gate (Fig. 1, A–C). Of the remaining CD3<sup>−</sup>CD27<sup>+</sup>CD43<sup>+</sup> cells, the majority showed high expression of CD38 (0.1–4.6% of CD19<sup>+</sup>). 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> cells in human neonatal cord blood, peripheral blood, and tonsil samples.** (A–C) CD27<sup>+</sup>CD43<sup>+</sup> cells (red) were defined within CD19<sup>+</sup> lymphocytes (blue; left plots). Where illustrative, the CD19<sup>−</sup> 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<sup>+</sup>CD3<sup>−</sup> events. Data are representative of 4 cord blood, 27 blood, and 7 tonsil samples. (D) Frequencies of CD27<sup>+</sup>CD43<sup>+</sup> cells among CD19<sup>+</sup> cells in neonatal cord blood and peripheral blood samples from individual children and adults of indicated ages. The relative contributions of CD3<sup>+</sup> T cells, CD3<sup>−</sup>CD38<sup>hi</sup> plasma cells, and CD3<sup>−</sup>CD38<sup>low</sup> B lymphocytes are indicated for each individual. (E) Frequencies of CD43<sup>+</sup>, CD43<sup>−</sup>IgM<sup>+</sup>, and CD43<sup>−</sup>IgM<sup>−</sup> cells among CD19<sup>+</sup>CD27<sup>+</sup>CD3<sup>−</sup>CD38<sup>low</sup> 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> events appeared to be true B lymphocytes (0.3–2.3% of CD19<sup>+</sup>CD3<sup>−</sup>CD38<sup>dim</sup> cells) and consisted of both IgM<sup>+</sup> and IgM<sup>−</sup> cells (unpublished data). Their total frequency was >10-fold lower than the frequencies of both CD27<sup>+</sup>IgM<sup>+</sup> and CD27<sup>+</sup>IgM<sup>−</sup> memory B cells in adult blood (Fig. 1 E). Therefore, we conclude that CD43<sup>+</sup> B lymphocytes constitute a small minority of total CD27<sup>+</sup> B lymphocytes.

In conclusion, our results confirm a decline with age of the overall population of CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> cells reveals that the majority may be CD38<sup>hi</sup> plasma cells or contaminating CD3<sup>+</sup> T cells. The large proportion of T cells in our analysis might explain the high frequency of CD5<sup>+</sup> cells reported by Griffin et al. (2011), as all T cells express CD5. Finally, we conclude that clearly defined CD3<sup>−</sup>CD38<sup>dim</sup>CD27<sup>+</sup>CD43<sup>+</sup> B lymphocytes may be only a minor population of CD27<sup>+</sup> 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.

Martin Perez-Andres,<sup>1,2</sup> Christina Grosserichter-Wagener,<sup>3</sup> Cristina Teodosio,<sup>1,2</sup> Jacques J.M. van Dongen,<sup>3</sup> Alberto Orfao,<sup>1,2</sup> and Menno C. van Zelm<sup>3</sup>

<sup>1</sup>Centro de Investigación del Cáncer, University of Salamanca-CSIC, 37007 Salamanca, Spain

<sup>2</sup>Service of Cytometry, Department of Medicine, University of Salamanca, 37007 Salamanca, Spain  
<sup>3</sup>Department of Immunology, Erasmus MC, University Medical Center, 3015 GE Rotterdam, Netherlands

#### CORRESPONDENCE

Menno C. van Zelm: m.vanzelm@erasmusmc.nl

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## Human B1 cells are CD3<sup>−</sup>: A reply to “A human equivalent of mouse B-1 cells?” and “The nature of circulating CD27<sup>+</sup>CD43<sup>+</sup> B cells”

### Griffin et al. respond:

In the January 2011 issue of the *JEM*, we identified a population of B cells in

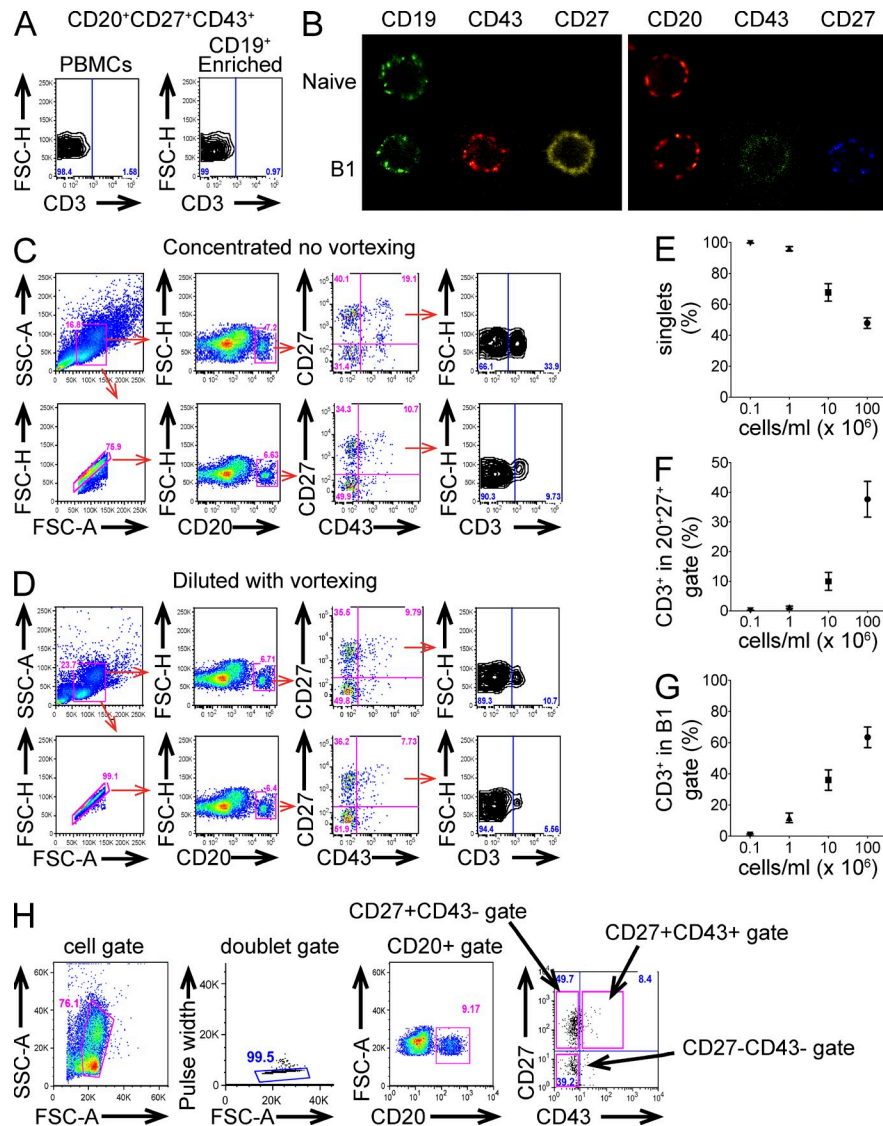
human umbilical cord and adult peripheral blood that manifests three key functional features of mouse B1 cells

(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 ( $2.9 \pm 0.66\%$ ;  $n = 3$ ). 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 ( $0.57 \pm 0.37\%$ ;  $n = 3$ ; 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 discrimination.



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<sup>+</sup> events in the CD20<sup>+</sup>CD27<sup>+</sup> gate and in the CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> B1 cell gate (Fig. 1, e and f). These results suggest that CD3<sup>+</sup> events within CD20<sup>+</sup> B cell populations result from the formation of B:T doublets.

To evaluate the possible existence of CD19<sup>+</sup> T cells, as suggested by Perez-Andres et al. (2011), we examined CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD3<sup>+</sup> cells observed by Perez-Andres et al. (2011), like the CD20<sup>+</sup>CD3<sup>+</sup> cells observed by Descatoire et al. (2011), represent B:T doublets.

In our results, CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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 characteris-

tics of single CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD27<sup>+</sup> 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<sup>+</sup> B cells.

Descatoire et al. (2011) suggests that their CD3<sup>-</sup>CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> B cells were either IgD<sup>+</sup> or IgD<sup>-</sup>; in our hands, CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>-</sup>CD20<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>-</sup>CD19<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> lymphocytes expressed elevated levels of CD38 and were most likely plasma cells. CD19 does not exclude plasma blasts 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup> events, they indeed identified the phenotypically

unique CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup> 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<sup>+</sup>CD27<sup>+</sup>CD43<sup>+</sup>CD70<sup>-</sup> and do not express CD3.

Daniel O. Griffin,<sup>1,2</sup> Nichol E. Holodick,<sup>2</sup> and Thomas L. Rothstein<sup>2,3,4</sup>

<sup>1</sup>Elmezzi Graduate School of Molecular Medicine and <sup>2</sup>Center and for Oncology and Cell Biology, the Feinstein Institute for Medical Research, Manhasset, NY 11030

<sup>3</sup>Department of Medicine and <sup>4</sup>Department of Molecular Medicine, Hofstra University School of Medicine, North Shore-LIJ School of Medicine, Manhasset, NY 11030

## CORRESPONDENCE

Thomas L. Rothstein: tr@nshs.edu

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