Immunology and Cell Biology (2015), 1–2

www.nature.com/icb

CORRESPONDENCE

Donors define whether CD19$^+$CD27$^+$ tonsillar B cells are mostly memory or germinal-center B cells

Immunology and Cell Biology advance online publication, 10 March 2015; doi:10.1038/icb.2015.19

CD27 has been used by numerous independent groups as a pan-memory B-cell marker not only for the identification of peripheral blood (PB) memory B cells (B_{mem}) but also for spleen B_{mem},[1,2] tonsillar B_{mem}[3] and bone marrow B_{mem}.[4] Through a Letter to the Editor, Kupperś group has criticized the experimental approach we used (that is, using only CD27 as a human-B_{mem} marker in tonsils) in the article in which we report a comparative analysis of human B_{mem} derived from PB and tonsils.[5] We would like, in turn, to respond and address the issue.

In their letter, Przekopowitz and colleagues provide data from a collection of 21 tonsillar samples. Using CD38 expression, they show that germinal-center (GC; CD20$^+$CD27$^+$ CD38$^+$) B cells in their pool of samples accounted on average for 49.3% of the CD20$^+$CD27$^+$ cells. Based on those records, they conclude that ~40–50% of the cells we used as tonsillar B_{mem} were in fact GC B cells. Notably, they suggest that levels of contaminating GC cells in the samples are age dependent, with higher levels at younger ages, as shown in Figure 1c. This is in agreement with our own experience, and actually it is logical considering that generally children are referred for tonsillectomy because of multiple and frequent episodes of bacterial tonsillitis. The high proportion of GC cells in the referred CD20$^+$CD27$^+$ population in children is compatible with the immune response against it.

Accordingly, people under 21 were excluded from our study as implicitly stated in the paper (see Methods section in Perez *et al.*: ´*cells were isolated from tonsils obtained from healthy adults…*´).

In general, adults are referred for tonsillectomy because of tonsillar hyperplasia indicated by obstructive sleep apnea (no history of recurrent tonsillitis); at least this is the situation in the Otolaryngology Services at Clinical Hospital. Adults with allergies or with a history of recurrent tonsillitis were also excluded from our study. Such selection of donors was not only to minimize the presence of GC B cells but also to match PB donors (in age and physiological condition).

Over three-quarters of Przekopowitz samples were people aged 21 or under 21. Hence, their population is different from ours and their results should not be extrapolated to our data.

Still, scientists from Kupperś group raise a fair point: that is, the need of clearly demonstrating that GC B cells do not constitute an important fraction of the CD19$^+$CD27$^+$ population when sorting that population as B_{mem} from secondary lymphoid tissues as tonsils or spleen. We proceeded with the questioned sorting strategy only upon confirmation through previous staining that GC B cells did not represent a significant proportion of CD19$^+$CD27$^+$ cells. We apologize for any inconvenience caused by omitting this methodological information in our manuscript, as we were restricted for being in a Short Communication format.

In our collection of samples, which ranged from 22 to 40 years old, the average fraction of tonsillar CD19$^+$CD27$^+$CD38low (B_{mem}) among all CD19$^+$CD27$^+$ cells was 80% (range 70–86%). The fraction of plasma cells was negligible and thus GCs accounted on average for 20% of CD19$^+$CD27$^+$ cells (Figure 1).

Even if contaminating levels were not as high as those alleged in the Letter to the Editor, we acknowledged that the sorting strategy used left around a 20% putative GC B cells in our B_{mem} sample that we disregarded. We did it on the basis of previous findings[6] that showed that 75% of such GC B cells are dead within 16 h of culture, which means that it would be unlikely that they would have any influence on the culture behavior observed at days 2, 5 and 6 (reported in Figure 2 in Perez *et al.*[5]). Generation of antibody-secreting cells (ASC), cell proliferation and Ig production at those time points are likely to result from the dominating fraction of tonsillar B_{mem} in culture, being indeed usual functional responses of such a population of cells.

To be precise, Przekopowitz cited in the current Letter to the Editor the same Nature paper from MacLennan and coworkers[6] indicating that according to this work ´*…the addition of crosslinked anti-immunoglobulin and soluble CD40 or other stimuli may retain viability of the GC B cells…*´. This is inaccurate. In the article cited, the authors emphasized that apoptosis of GC B cells is precluded only when conjugating stimulating anti-immunoglobulins to sheep red blood cells. This is in line with the hypothesis that GC B cells are rescued only by binding antigen displayed on the surface of follicular dendritic cells, and thus GC B-cell recognition of antigen has to be on a particulated surface, which we did not provide. Although observing that addition of CD40L by itself could rescue GC B cells from death for a maximum of 2 days, Liu and colleagues did not report any kind of functional response from the GC B-cell population in the cited manuscript under such a condition. Importantly, a number of papers subsequently reported substantial functionality of human GC B cells *in vitro* through the extra addition of other stimulants such as IL10,[7] IL21,[8] IL15[9] and BAFF,[10] which we did not use.

Then, we assumed that under the condition of culture employed, GC B cells can hardly be responsible for generating blasts and viable ASC observed on day 2 as well as proliferating cells detected on day 5 (Figures 2a–c in Perez *et al.*[5]). We would also like to

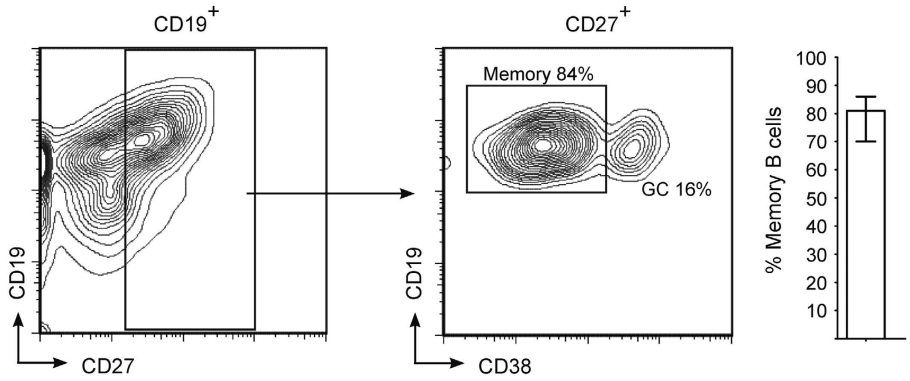

Figure 1 B_{mem} proportion in tonsillar samples used in *´Human memory B cells isolated from blood and tonsils are functionally distinctive´*. Left and Middle: flow cytometric analysis of a representative adult human tonsillar sample meeting the criteria to enter the study. CD19 MACS-sorted B cells were stained with CD19, CD27 and CD38. Left: gating on $CD19^+CD27^+$ B cells. Middle: gated population displaying CD19 and CD38 expression; $CD19^+CD27^+CD38^{low}$ identify B_{mem} and $CD19^+CD27^+CD38^+$ identify GC B cells. Right: proportion of B_{mem} among $CD19^+$ $CD27^+$ human tonsillar B cells according to gating in the left and middle panel. Data show the median percentage of $B_{mem} \pm$ range of samples ($N=35$) used for sorting B_{mem} through $CD19^+$ $CD27^+$ staining. Methods and reagents were described in the study by Perez *et al.*,[5] except for CD38 antibody (BD, San Jose, CA, USA, clone HIT2). The local ethical committee approved the studies. MACS, magnetic activated cell sorting.

point out that we showed replicates of our experiments without any CD40L but with CpG stimulation, which is only expected to activate B_{mem}.[11]

Of note, although we overlooked the difference between the actual starting number of cells capable of responding in the cultures of tonsil versus those of PB $CD19^+CD27^+$ cells, they both displayed comparable levels of cell death by day 2, as assessed by three different methods (Table 1, Figure 2b and Supplementary Figure 1b in Perez *et al.*[5]). Hence, at those critical early time points of culture, the contribution of the minor fraction of dying GC B cells to death rates in tonsillar $CD19^+CD27^+$ cultures seems to fall within the statistical variability in viability recorded from sorted $CD19^+CD27^+$ cells, irrespective of their tissue of origin. This is consistent with our assumption that their influence on the functional responses observed can be neglected.

By no means do we claim to have an ideal experimental approach; in fact, we doubt it exists, at least in the human system. However, in light of the above, we definitely defend that it was suitable to support the conclusions we have drawn in the article, as the sorted population from tonsils was indeed mostly composed of B_{mem} cells. We sincerely acknowledge the opportunity given by the editors to clarify these matters.

M Eugenia Pérez[1] and Eloísa Arana[1,2]

[1]Institute of Immunology, Immunogenetics Department, Genetics and Metabolism (INIGEM), Clinical Hospital, University of Buenos Aires, National Council for Scientific and Technological Research, Buenos Aires, Argentina and [2]Department of Immunology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina
E-mail: earana@hospitaldeclinicas.uba.ar

1 Tangye SG, Avery DT, Hodgkin PD. A division-linked mechanism for the rapid generation of Ig-secreting cells from human memory B cells. *J Immunol* 2003; **170**: 261–269.
2 Good KL, Avery DT, Tangye SG. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *J Immunol* 2009; **182**: 890–901.
3 Dorner M, Zucol F, Alessi D, Haerle SK, Bossart W, Weber M *et al.* Beta1 integrin expression increases susceptibility of memory B cells to Epstein–Barr virus infection. *J Virol* 2010; **84**: 6667–6677.
4 Giesecke C, Frölich D, Reiter K, Mei HE, Wirries I, Kuhly R *et al.* Tissue distribution and dependence of responsiveness of human antigen-specific memory B cells. *J Immunol* **192**: 3091–3100.
5 Perez ME, Billordo LA, Baz P, Fainboim L, Arana E. Human memory B cells isolated from blood and tonsils are functionally distinctive. *Immunol Cell Biol* **92**: 882–887.
6 Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan IC. Mechanism of antigen-driven selection in germinal centres. *Nature* 1989; **342**: 929–931.
7 Arpin C, Déchanet J, Van Kooten C, Merville P, Grouard G, Brière F *et al.* Generation of memory B cells and plasma cells *in vitro*. *Science* 1995; **268**: 720–722.
8 Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM *et al.* Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol* 2007; **179**: 8180–8190.
9 Park CH-S, Yoon S-O, Armitage RJ, Choi YS. Follicular dendritic cells produce IL-15 that enhances germinal center B cell proliferation in membrane-bound form. *J Immunol* 2004; **173**: 6676–6683.
10 Zhang X, Park CS, Yoon SO, Li L, Hsu YM, Ambrose C *et al.* BAFF supports human B cell differentiation in the lymphoid follicles through distinct receptors. *Int Immunol* 2005; **17**: 779–788.
11 Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* 2003; **101**: 4500–4504.