Immunology and Cell Biology (2014), 1–6

www.nature.com/icb

SHORT COMMUNICATION

Human memory B cells isolated from blood and tonsils are functionally distinctive

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Human B-cell studies *in vitro* have routinely used B lymphocytes purified from spleen, blood or tonsils irrespective of potential differences in their immunological traits. In this study, we compared the functional responses of total ($CD19^+$) and memory B cells (B_{mem}; $CD19^+/CD27^+$) isolated from blood and tonsils to different stimuli. Peripheral B cells showed enhanced survival and proliferation compared with their tonsillar equivalents when stimulated for 10 days. Stimulated B cells from both tissues secreted significantly greater amounts of cytokines than unstimulated controls demonstrating their functional responsiveness. Analysis of CD27 expression over time indicated that the conditions that promoted survival and proliferation of peripheral B_{mem}, caused massive tonsillar B_{mem} death. Purified tonsillar B_{mem} failed to expand but rapidly differentiated in antibody secreting cells and subsequently underwent apoptosis. In contrast, circulating B_{mem} showed delayed activation and differentiation, but exhibited a longer lifespan and active proliferation. In addition, short-term stimulation of tonsillar B_{mem} resulted in the production of more immunoglobulin G (IgG) than their peripheral counterparts. At later time points, however, IgG production from the different B cells was reversed. Our findings imply that the tissue located and peripheral B_{mem} have distinct behaviors, indicating organ dependent functional responses that should not be generalizable to all B_{mem}. This work provides a greater understanding of how B_{mem} location is coupled to specialized roles of B lymphocytes.
Immunology and Cell Biology advance online publication, 22 July 2014; doi:10.1038/icb.2014.59

INTRODUCTION

Tonsils are lymphoepithelial structures that establish direct interactions with inhaled or ingested environmental antigens.[1] They are considered comparable to the nasopharynx-associated lymphoid tissue of rodents, a component of the mucosa-associated lymphoid tissue.[2] Human tonsils show similarities with lymph nodes and could participate as effector organs of local systemic type and mucosal humoral immunity.[3] In these specialized niches, B cells encounter antigen (Ag) and cognate T-cell help to drive proliferation and differentiation of Ag-specific naive B cells into memory B cells (B_{mem}) and plasma cells.[4] B_{mem} can then in turn be re-stimulated and subsequently expand to form effector cells. In humans, expression of CD27 distinguishes Ag-experienced B_{mem} ($CD27^+$) from Ag-inexperienced naïve B cells ($CD27^-$),[5–7] which reside in discrete regions of secondary lymphoid tissue or are detectable in the blood.[8–10]

T-cell memory is represented by central (T memory stem cells, T central memory cells) and effector (T effector memory) subsets.[11–13] These subsets identify T-cell functional specialization, migration and localization patterns within the population. In the B-cell field, immunologists have identified different subsets within the $CD27^+$ B_{mem} pool, which are generally based on surface isotype expression.[6,14] As yet, differences in the function, migration patterns and ontogenic relationship among these subsets remain largely undefined.

Our objective was to establish whether the functional responses of circulating B_{mem} population were equivalent to those of tonsil localized B_{mem} that are exposed to abundant Ags. We discovered that these B_{mem} populations exhibited different behaviors depending on their origin and location.

RESULTS AND DISCUSSION

To compare the proliferative capacity of B cells isolated from blood and tonsils we used a carboxyfluorescein succinimidyl ester (CFSE) dilution assay analyzed by flow cytometry. Cells were cultured for 10 days in medium supplemented with anti-immunoglobulins (αIgs) + CD40L + interleukin-2 (IL-2) + IL-4, mimicking B cells encountering their Ag and subsequently receiving T-cell help. The response of splenic human B cells following stimulation has been shown to be dominated by B_{mem} undergoing faster proliferation and more differentiation than naive B cells.[15] As proportion of B_{mem} within the $CD19^+$ B-cell population isolated from peripheral blood was less than in tonsil (40% ± 20% and 55% ± 15%, respectively), we also analyzed the expression of CD27 within the populations to determine whether putative differences in proliferation were related to changes in the frequency of $CD27^+$ and $CD27^-$ B cells on each tissue.

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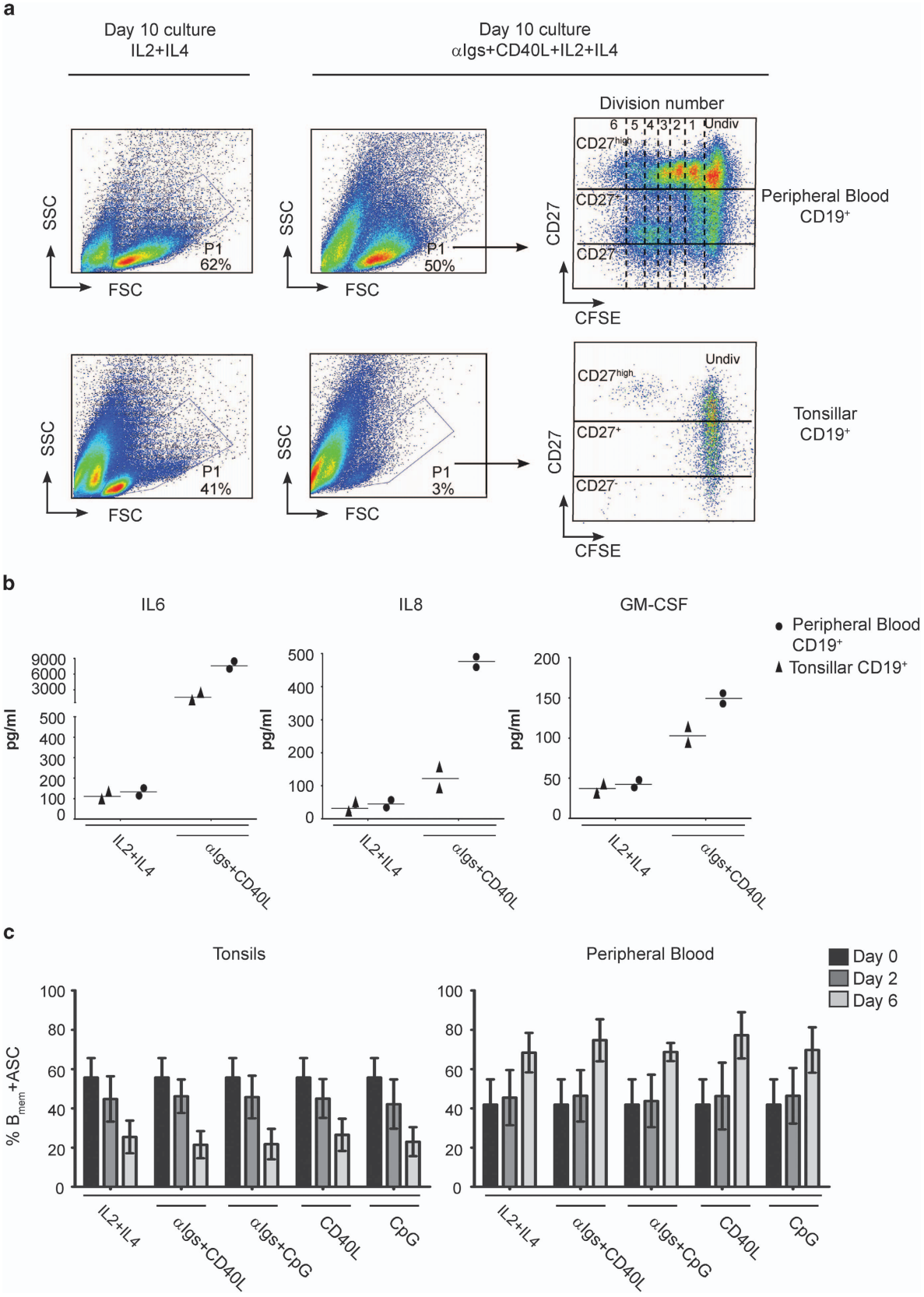
Received 6 March 2014; revised 17 June 2014; accepted 17 June 2014


a
Day 10 culture
IL2+IL4
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αIgs+CD40L+IL2+IL4
Division number
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CD27high
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SSC
FSC
P1
62%
P1
50%
P1
41%
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3%
CFSE
Peripheral Blood
CD19+
Tonsillar
CD19+
b
IL6
IL8
GM-CSF
pg/ml
Peripheral Blood
CD19+
Tonsillar CD19+
IL2+IL4
αIgs+CD40L
c
Tonsils
Peripheral Blood
Day 0
Day 2
Day 6
% Bmem+ASC
IL2+IL4
αIgs+CD40L
αIgs+CpG
CD40L
CpG

Apoptotic and viable cells were distinguished by differences in forward and side scatter. Such scatter-based assay has good correspondence with results obtained by fluorescein isothiocyanate (FITC)-annexin staining for distinguishing the viability of untransformed human B cells.[16,17] We consistently observed that circulating B cells exhibited significantly higher viability than tonsillar B cells following αIgs + CD40L + IL-2 + IL-4 stimulation (Figure 1a, Supplementary Figure 1a). Unstimulated control cultures (IL-2 + IL-4 treatment) displayed comparable levels of viability for circulating and tonsillar B cells (Figure 1a; Supplementary Figure 1a), suggesting elevated levels of cell death were clearly linked to stimulation of tonsillar cultures.

Next, we analyzed CD27 expression in dividing B cells. Stimulated cultures from blood and tonsil presented a division linked, proliferating, $CD27^{high}$ population, typical of antibody secreting cells (ASC) generated *in vitro*[18–20] (Figure 1a, far right panels). Cultures from each tissue exhibited only $CD27^{+}$ or $CD27^{high}$ populations and few live $CD27^{-}$ cells, evidencing their response to stimuli. Dilution of CFSE in cultures of circulating B cells indicated high rate of proliferation (Figure 1a). In contrast, in stimulated tonsillar cultures, profound cell death made accurate analysis of cell division unfeasible. Most living B cells remained undivided but the few cells that had undergone proliferation were $CD27^{high}$.

As an alternative read-out for B-cell activation, cytokine production was analyzed using a multiplex biomarker immunoassay (Luminex Technology, Austin, TX, USA). We detected higher concentrations of IL-6, IL-8 and granulocyte macrophage colony-stimulating factor (GM-CSF) in stimulated B-cell cultures from blood and tonsils when compared with control cultures exposed to IL-2 + IL-4 only (Figure 1b). The overall concentration of cytokines was higher for circulating B cells as would be expected due to the high cell death in the tonsillar B-cell cultures. Circulating B cells secreted approximately fourfold more IL-6, and approximately fivefold more IL-8 than tonsillar B cells following stimulation. However, GM-CSF levels were only slightly higher in peripheral blood B-cell cultures (Figure 1b). Notably, although tonsillar B cells showed strongly reduced viability, the remaining B cells in the culture produced good levels of cytokine suggesting that on a per cell basis, tonsillar B cells potentially have a better capacity to produce cytokines. This may reflect the higher activation states for naïve[21] and B_{mem}[22,23] residing in tonsils.

To extend our findings, we analyzed the temporal expression of CD27 over time (0, 2 and 6 days) in B-cell cultures stimulated through the toll-like receptor 9 agonist CpG in presence and absence of αIgs, as well as CD40L +/− αIgs. All cultures were also supplemented with IL-2 + IL-4 (Figure 1c). While the proportion of B_{mem} and ASC steadily declined over time in tonsillar cultures when exposed to differing stimuli, peripheral B cells exhibited the opposite trend. Bystander stimulations (CD40L + IL-2 + IL-4 or CpG + IL-2 + IL-4) target only B_{mem},[24,25] excluding influence of naïve B cell's functional response in culture behavior. Therefore, declination of tonsillar B_{mem} on those cultures could only be attributable to the inability of the initial B_{mem} population to proliferate and their tendency to cell death, as opposed to circulating B_{mem}. Analysis of cytokine secretion on supernatants from those cultures showed that circulating B cells secreted considerably higher levels of IL-6, IL-8 and GM-CSF than their tonsillar counterparts, but both B-cell subsets secreted much higher levels of cytokine than unstimulated control cultures (data not shown). Thus, different stimulating conditions that appear to support survival and proliferation of peripheral B_{mem} do not similarly support tonsillar B_{mem} and result in their death in culture.

Next we determined the kinetics of emergence of ASC in B-cell cultures from peripheral blood and tonsil (Figure 2a). Stimulation was restricted to the B_{mem} population by supplementing the medium with CD40L + IL-2 + IL-4 or CpG + IL-2 + IL-4, and examining the ASC proportion (R3) on day 2 and 6 of culture. On day 2, ~20% of the tonsillar B-cell population had become ASC when stimulated with CD40L + IL-2 + IL-4 as determined by acquisition of a $CD27^{high}$ phenotype. At the same time point, peripheral B-cell cultures had very few ASCs. On day 6, this situation was reversed with more ASCs evident in peripheral B-cell cultures. The pattern of ASC formation was similar for CpG stimulation. Thus, tonsillar B_{mem} were able to generate ASCs within 2 days of stimulation, while circulating B_{mem} took longer to differentiate but they exhibited greater capacity to proliferate and differentiate under *in vitro* conditions.

To specifically assess functional differences between nasopharynx-associated lymphoid tissue and peripheral B_{mem}, we sorted $CD19^{+}$ $CD27^{+}$ from tonsils and peripheral blood cells. Cell survival, blast formation and proliferation were monitored when challenged with αIgs + CD40L + IL-2 + IL-4 at different time points. The main

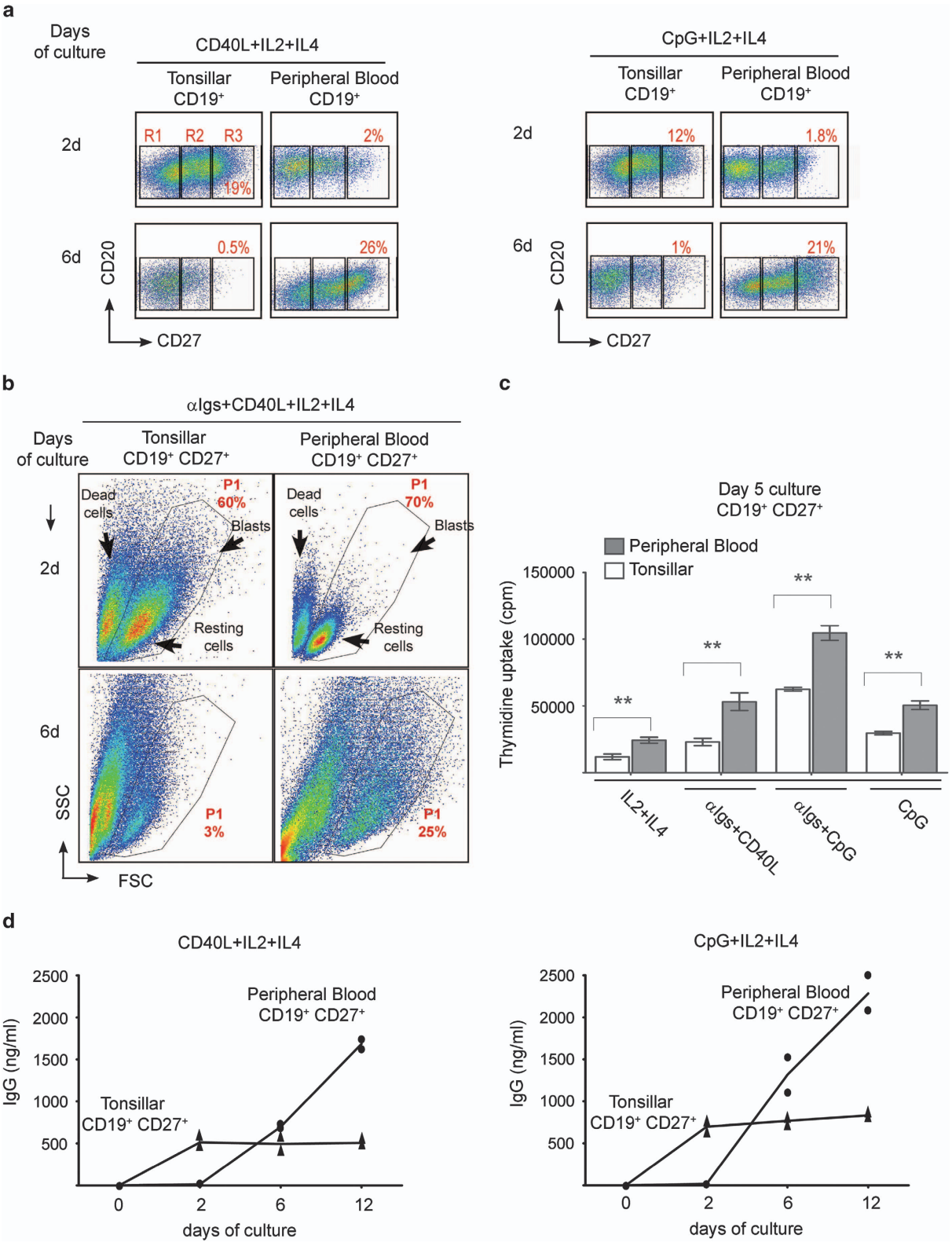
Table 1 Effect of T-cell-dependent and T-cell-independent activation on proliferation. Viable cell counts at different time points of B_{mem} culture

Culture	*B_{mem} + CD40L + IL2 + IL4*			*B_{mem} + CpG + IL2 + IL4*		
Day	*Tonsillar*		*Peripheral*	*Tonsillar*		*Peripheral*
Cell count (× 10^5)						
0	4.00	ns	4.00	4.00	ns	4.00
2	2.29 ± 0.70	***	2.54 ± 0.65	2.40 ± 0.55	**	3.11 ± 0.67
4	2.2 ± 0.64	***	4.27 ± 0.90	2.50 ± 0.82	***	4.41 ± 0.74
6	1.29 ± 0.85	***	8.87 ± 0.75	1.34 ± 0.85	***	9.34 ± 0.92
8	0.66 ± 0.50		9.84 ± 1.0	0.48 ± 0.35		10.54 ± 1.20

Abbreviations: Bmem, memory B cells; ns, not significant; PB, peripheral blood cells.
Tonsillar and PB B_{mem} (4×10^5 cells) were cultured for 8 days with CD40L or CpG in the presence of IL2 + IL4. At the indicated time points (2, 4, 6, and 8 days), the absolute number of viable cells were determined as in Methods. Data were collected from three independent experiments performed with samples from two patients each (six donors per sample). Values show the mean ± s.e.m. The statistical analysis of the results was performed by unpaired Student's *t*-test. $^{**}P<0.005$, $^{***}P<0.001$.

Figure 1 Distinct functional response of human tonsillar and peripheral B cells upon stimulation. (**a**) Freshly isolated $CD19^{+}$ cells from normal adult volunteers (blood $n=2$, and tonsil $n=2$) were labeled with CFSE and cultured for 10 days either on αIgs + CD40L + IL-2 + IL-4 or IL-2 + IL-4 alone (control). P1 denotes percentage of living cells determined by FSC-SSC dot plot profiles by flow cytometry (FACS) under the stimulations described above on cultures of peripheral blood $CD19^{+}$ and of tonsillar $CD19^{+}$. Data show the expression of CD27 in each division of responding B cells. (**b**) Cytokine production by B cells cultured in **a**. Symbols (circles and triangles) show the mean amount of cytokine secreted by 0.5×10^6 cells for each individual from samples analyzed in duplicate. The mean of individuals analyzed in the experiment are indicated by a horizontal line. (**a**,**b**) Data show one representative experiment of three similar experiments ($n=2$ per experiment). Each sample was analyzed in duplicate. (**c**) CD19-purified human tonsillar (left) and peripheral blood (right) cells were stimulated in the presence of indicated stimuli. The proportion of B_{mem} and ASC ($CD27^{+/high}$) was determined by FACS on day 0, 2 and 6 of culture. Data show the mean percent $CD27^{+/high}$ ± s.d. pooled from eight independent experiments performed (blood: $n=2$ individuals and tonsil: $n=2$ individuals in each experiment).

findings are summarized in Figures 2b and c and Supplementary Figure 2. The forward/sideward scatter blots (Figure 2b) show cell survival (P1 gate) and blast formation on days 2 and 6 of culture. On day 2, tonsillar B_{mem} exhibited higher proportion of cells of bigger size, compatible with a blast phenotype, than circulating B_{mem} (Figure 2b, top panel), evidencing that the former seem to differentiate more rapidly. In light of the results shown in Figure 1c and CFSE profiles obtained at day 2 after stimulation (Supplementary Figure 2), we propose that tonsillar B_{mem} differentiation might occur with only marginal B-cell expansion. Conversely, on day 6, the



percentage of viable gated cells in circulating B_{mem} cultures was 10 times higher than that of tonsillar B_{mem} (Figure 2b; Supplementary Figure 1b). Moreover, these latter cells appeared small suggesting that they were senescent. In contrast, circulating B_{mem} reached their greatest size upon 6 days of culture (Figure 2b, lower panel).

For both subsets, DNA synthesis was measured by (^{3}H) thymidine incorporation after 5 days on medium supplemented with T-cell-derived stimuli. Uptake of (^{3}H) thymidine by circulating B_{mem} exceeded three times than that by tonsillar B_{mem} (Figure 2c). Analysis of CFSE profiles on day 5 further confirmed this finding (Supplementary Figure 2). Importantly, in two experiments we detected a minor population of tonsillar B_{mem} capable of proliferating (Supplementary Figure 2c) 5 days post stimulation. Detection of such minor population appeared to be linked to enhanced survival between 2 and 5 days post stimulation. In addition, we observed a significant proliferative advantage in circulating B_{mem} when cultures were stimulated with αIgs + CpG + IL-2 + IL-4, CpG + IL-2 + IL-4 or with IL2 + IL4 alone.

Our findings show that B_{mem} isolated from blood and tonsils display different kinetics of activation. A major proportion of tonsillar B_{mem} were found to differentiate rapidly but exhibited little expansion and higher cell death, which is compatible with their higher initial activation status. In contrast, circulating B_{mem} were slower to commence proliferation and differentiation but showed much higher survival rates. Consistent with these conclusions, the number of cells recovered from circulating B_{mem} cultures was higher than that of tonsillar B_{mem} irrespective of the culture conditions (Table 1).

Measurement of IgG secretion from sorted B_{mem} tonsils or peripheral blood at day 2, 6 and 12 showed that tonsillar B_{mem} reached their maximal IgG production at day 2 after CD40L + IL-2 + IL-4 and CpG + IL-2 + IL-4 stimulation, while IgG was undetectable on peripheral B_{mem} cultures at the same time point (Figure 2d). Nevertheless, peripheral blood B_{mem} gained the ability to produce IgG over the subsequent 4 days and reached high levels of production by day 12. Thus, tonsillar B_{mem} differentiated more rapidly into ASC but with a limited capacity of IgG secretion compared with circulating B_{mem}.

Thaunat *et al.*,[26] elegantly demonstrated using high-resolution imaging analysis and transgenic murine models that B cells retaining larger stores of Ag exhibited a prolonged capacity to interact with T-follicular helper cells. This rendered them more prone to quickly transition from a germinal center gene expression program to that of an ASC which is more terminally differentiated and susceptible to cell death (decreasing potential hypothesis[27]). Along these lines, human tonsils present Ag-retaining crypts, which gather both airborne and alimentary Ags.[28] Our findings suggest that oversupply of Ag indeed shaped tonsillar B_{mem}, which presented a rapid ASC differentiation and increasing susceptibility to apoptosis, when compared with peripheral B_{mem} patrolling normally sterile tissues. Notably, it has been previously shown that human tonsillar B_{mem} express higher levels of β1 integrin[22,23] than circulating B_{mem}, which the authors related to a higher state of activation.[29]

In conclusion, our results demonstrate differences between peripheral and tonsillar B_{mem} subsets in relation to the quality and durability of their functional responses *in vitro*. We suggest that nasopharynx-associated lymphoid tissue B_{mem} are adapted to a strong and immediate response on site that is not sustained in time, but the issue requires further investigation about molecular mechanisms implied.

METHODS

Isolation of cells

Primary human mononuclear cells were isolated from tonsils obtained from healthy adults ($n = 50$) undergoing routine tonsillectomy or from buffy coats taken also from healthy adult donors. Tonsillar mononuclear cells were prepared as follows. Briefly, tonsils were collected in phosphate-buffered saline buffer containing 50 μg ml^{-1} amphotericin B (Richet, Buenos Aires, Argentina). Tissues were chopped with a scalpel in complete medium and passed through a 70 μm-pore-size cell strainer (BD Falcon, San José, CA, USA). For the isolation of B-cell subsets, tonsillar mononuclear cells were purified by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Little Chalfont, UK). Peripheral blood mononuclear cells were purified by Ficoll-Hypaque density gradient centrifugation according to the instructions of the Ficoll-Hypaque manufacturer. The viability of primary cells, as determined by trypan blue exclusion was >99% in all preparations. Informed consent was obtained from subjects before the study. The institutional ethics committee (Clinical Hospital, School of Medicine, Buenos Aires) approved the collection and the use of clinical material conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). B cells were isolated using the B-cell isolation kit II according to the instructions of the manufacturer (Miltenyi, San Diego, CA, USA). Further separation into naive and B_{mem} was performed using either Memory B-Cell Isolation Kit (Miltenyi) or by cell sorting with a FACSAria II flow cytometer (Becton Dickinson (BD, San José, CA, USA)) after CD19/CD27 staining. The purity of isolated B-cell subsets was determined by flow cytometry using antibodies to human CD19 (BD, clone SJ25C1) or CD20 (BD, clone L27) and CD27 (BD, clone M-T271 or Miltenyi clone LG.3A10) for discrimination between naive and B_{mem}. Purified cell populations used for experiments were >95%. All experiments were performed with freshly isolated cells. The number of samples (donors) assayed per experiment was restricted by the low frequency of B cells in peripheral blood mononucleated cells. This required up to 500 ml of blood processed as buffy coats per donor to achieve the number of B_{mem} required for the experiments described.

Cell culture

Primary human B cells were cultured in IMDM medium (Life Technologies, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES), 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA). Human IL2 (20 ng ml^{-1}; R&D Systems, Minneapolis, MN, USA) and

Figure 2 Tonsillar B_{mem} exhibit a faster but less efficient response to activation than peripheral B_{mem}. (**a**) CD19-purified human tonsillar and peripheral blood cells were stimulated as indicated. Flow cytometry (FACS) analysis of CD20 and CD27 expression on cells gated on P1, after 2 and 6 days of culture. R1 designates CD20^{low}/CD27$^-$ cells, R2 indicates CD20^{low}/CD27$^+$ cells and R3 specifies ASC population (CD20^{low}/CD27^{high}). Data show one experiment representative of eight independent experiments ($n = 2$ individuals in each experiment). (**b**) CD19$^+$ and CD27$^+$ sorted human tonsillar (left) and peripheral blood (right) cells were cultured in the presence of αIgs + CD40L + IL-2 + IL-4. Data show analysis on days 2 (top panels) and 6 (lower panel). Dead cells, resting cells and B-cell blasts in the FSC-SSC analysis are marked by arrows in the top panels and lower panels. Data show one experiment representative of five independent experiments ($n = 2$ individuals in each experiment). (**c**) Proliferation was measured by [^{3}H] thymidine incorporation after 5 days of the indicated stimuli. Bars show the mean CPM ± s.e.m. of three independent experiments ($n = 6$ individuals, 2 individuals per experiment). $^{**}P < 0.01$ (**d**) CD19$^+$ and CD27$^+$ sorted human tonsillar and peripheral blood cells were cultured with the indicated stimuli. Supernatants from 0, 2, 6 and 12 day cultures were analyzed by ELISA to dose IgG level. Symbols (circles and triangles) show averaged duplicates of IgG levels secreted by cultures of each individual. Data shown are one of the two independent experiments performed with two individuals per experiment with similar results.

IL4 (20 ng ml^{-1}; R&D Systems) were added immediately before experiments also as supplements. When indicated, human recombinant hCD40L (250 ng ml^{-1}; R&D Systems) or 25 μM CpG-ODN 2006 (InvivoGen, San Diego, CA, USA) with or without 5 μg ml^{-1} goat anti-human IgM + IgG (H + L) F(ab′)$_2$ (Jackson Immunoresearch, West Grove, PA, USA) was used. Cells were cultured at 1×10^6 cells per ml either in 24-well culture plates (1 ml) or 48-well culture plates (0.5 ml).

Cytokine and proliferation analysis

The concentrations of cytokine in cell-free supernatants were quantified using the Bio-PlexTM 200 system (Bio-Rad, Hercules, CA, USA). Proliferation was measured either by CFSE (Molecular probes, Carlsbad, CA, USA) labeling according manufacturer's instructions or using DNA synthesis by [^{3}H] thymydine uptake (Perkin Elmer Life, Waltham, MA, USA). Cells were analyzed using a FACSAria II and analyzed with Flow Jo software (Treestar, Ashland, OR, USA).

Detection of IgG

Ninety-six-well plates were coated overnight at 4 °C with 1 μg ml^{-1} of goat anti-human IgM + IgG (H + L) F(ab′)$_2$ (Jackson Immunoresearch). Following washing with phosphate-buffered saline-Tween 0.05% and 1 h blocking with phosphate-buffered saline-bovine serum albumin 10%, plates were incubated for 1 h at 37 °C serial dilutions of supernatants from stimulated B cells. After washing, plates were incubated for 1 h at 37 °C with detection antibody: Biotin anti-human IgG, FCγ fragment specific (Jackson Immunoresearch). Binding was detected using Extra Avidin Alkaline Phosphatase (Sigma, St Louis, MO, USA) and 4-nitrophenyl phosphate (Fluka, St Louis, MO, USA). Absorbance was measured at 405 nm.

Statistical analyses

Results were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc, La Jolla, CA, USA). The statistical analysis of the results was performed by Mann–Whitney test, which is non-parametric and does not assume Gaussian distributions. A P-value < 0.05 was considered significant, unless indicated otherwise.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was funded by grants from governmental Argentinean agencies: ANPCyT (PICT PRH 00078, granted to scientists returning to the country), CONICET (PIP 0101, granted to young investigators) and UBA (UBACyT 20020110200026, granted to newly formed groups) to EA. MEP was the recipient of a CONICET postgraduate scholarship. We are very grateful to Otolaryngology and Hemotherapy Services at Clinical Hospital for providing samples.

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The Supplementary Information that accompanies this paper is available on the Immunology and Cell Biology website (http://www.nature.com/icb)