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Expression of Homing Receptors in IgM$^+$IgD$^+$CD27$^+$ B Cells and Their Frequencies in Appendectomized and/or Tonsillectomized Individuals

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ABSTRACT

Background: In humans, blood circulating IgM$^+$IgD$^+$CD27$^+$ B cells are considered analogous to those described in the marginal zone of the spleen and are involved in important immunological processes. The homing receptors they express, and the organs involved in their development (for example, intestinal organs in rabbits) are only partially known. We recently reported that this population is heterogeneous and composed of at least two subsets: one expressing high levels of IgM – IgM^{hi} B cells – and another low levels – IgM^{lo} B cells.

Objectives: To evaluate the expression of homing receptors on IgD$^+$CD27$^+$ IgM^{hi} and IgM^{lo} B cells and quantify their frequencies in blood of control and appendectomized and/or tonsillectomized volunteers.

Materials and Methods: Using spectral flow cytometry, the simultaneous expression of 12 previously reported markers that differentiate IgM^{hi} B cells and IgM^{lo} B cells and of α4β7, CCR9, CD22 and CCR10 were evaluated in blood circulating B cells of control and appendectomized and/or tonsillectomized volunteers.

Results: The existence of phenotypically defined IgM^{lo} and IgM^{hi} B cell subsets was confirmed. They differentially expressed intestinal homing receptors, and the expression of α4β7 and CCR9 seems to determine new IgM subpopulations. IgM^{lo} and IgM^{hi} B cells were detected at lower frequencies in the appendectomized and/or tonsillectomized volunteers relative to controls.

Conclusions: Human blood circulating IgD$^+$CD27$^+$ IgM^{lo} and IgM^{hi} B cell subsets differentially express homing receptors, and it is necessary to investigate if mucosal organs are important in their development.

KEYWORDS
Appendectomy; homing receptors; marginal zone B cells; spectral flow cytometry; tonsillectomy

Introduction

Human marginal zone B cells (MZBC) respond to encapsulated blood-borne pathogens (Kruetzmann et al. 2003) and are altered in different pathologies, highlighting their importance in immunity (Jenks et al. 2018; Liechti et al. 2019; Tull et al. 2021; Woodruff et al. 2020). In children, MZBC have been reported to have a pre-diversified repertoire of

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immunoglobulin (Ig) genes in the absence of germinal centers (GC) or spleen (Reynaud et al. 2012; Weller et al. 2001, 2004), suggesting the presence of alternate places where the diversification of the repertoire takes place, as occurs in the intestine of rabbits (Weill et al. 2009; Yeramilli and Knight 2013). Although the spleen is the main organ where MZBC are located in humans (Lewis et al. 2019; Zhao et al. 2018), other potential zones equivalent to the marginal zone (MZ) have been identified in the inner wall of the subcapsular sinus of the lymph nodes (Cerutti et al. 2013) and subepithelial regions of the appendix (part of the GALT "*Gut Associated Lymphoid Tissue*") and tonsils (part of Waldeyer's ring and NALT *"Nasal Associated Lymphoid Tissue")* (Lettau et al. 2020; Zhao et al. 2018). How MZBC recirculate once they are stimulated in these organs is unknown, but clonal relationships exist between MZBC found in spleen and GALT, suggesting that recirculation between these organs is possible (Mandric et al. 2020; Meng et al. 2017; Zhao et al. 2018).

In humans, blood circulating $IgM^+IgD^+CD27^+$ B cells are considered analogous to those described in the MZ of the spleen (Berkowska et al. 2011; Weill and Reynaud 2020). One way to explore whether these cells have the potential to home to GALT and NALT is by evaluating their expression of homing receptors (Habtezion et al. 2016). Several of these homing receptors have been described: α4β7 is a heterodimeric integrin expressed by T and B cells that mediates homing to the small intestine and colon (Habtezion et al. 2016). CCR9 binds to CCL25 expressed in the small intestine but absent in the colon (Briskin et al. 1997; Habtezion et al. 2016), and CD22 has been described as a homing receptor binding to a 2–6-sialylated glycan expressed in GALT and tonsils (Kimura et al. 2007). Finally, CCR10 is a chemokine receptor expressed on plasma cells homing to the jejunum, ileum, stomach, colon and appendix (Kunkel et al. 2003). Although, tonsil specific homing receptors are unidentified, plasma cells expressing CCR9 and CCR10 have been identified in this organ (Kunkel et al. 2003). A previous report showed co-expression of β7 (used as surrogate of α4β7 expression) and CCR9 on approximately 12% of circulating MZBC (Magri et al. 2017), but the expressions of CD22 and CCR10 are unreported.

The activity of the appendix and tonsils are pronounced between the ages of 10 and 19, after which time the organs partially regress (Dasso et al. 2000; Gebbers and Laissue 2004; Lee et al. 2016). Appendectomy and tonsillectomy at an early age have been associated with an increased risk of developing diseases with an inflammatory or autoimmune component (Caetano and Ribeiro 2019; Janszky et al. 2011; Lee et al. 2018; Roshanisefat et al. 2011). Individuals tonsillectomized or appendectomized, but especially those lacking both organs, have lower serum secretory IgA (SIgA) levels, although the percentages of circulating cells in the blood seem to be unaffected (Andreu-Ballester et al. 2007). However, the latter study disregarded the age at which these organs were removed (a variable that may be important since these organs, as previously mentioned, regress after adolescence (Brandtzaeg 2015; Dasso et al. 2000; Gebbers and Laissue 2004)) and lacked evaluation of different circulating subtypes of B cells, including MZBC.

We recently reported that blood circulating $IgM^+IgD^+CD27^+$ B cells in humans are heterogeneous, composed of at least two different subsets, one expressing high levels of IgM and low levels of IgD – IgM^{hi}— and another expressing low levels of IgM and high levels of IgD – IgM^{lo}— (Bautista et al. 2020). Expression of twelve phenotypical markers, evaluated separately, differed between these subsets. The phenotype, function, transcriptome and Ig gene repertoire of these populations indicated that while IgM^{hi} B cells may be analogous to spleen MZBC, IgM^{lo}

B cells are less differentiated (possibly being precursors of MZBC) and that further heterogeneity may exist within these subsets (Bautista et al. 2020). Furthermore, we reported that circulating rotavirus-specific B cells, a pathogen that replicates in the small intestine, are enriched mainly in IgM^{hi} B cells, but also in IgM^{lo} and IgM-only B cells (Herrera et al. 2014; Narvaez et al. 2012). Recently, others have studied IgM^{hi} and IgM^{lo} B cells in the context of autoimmune diseases. One study showed that a decrease in the expression of CD32b on IgM^{hi} blood cells may be associated with the immunopathogenesis of multiple sclerosis in women (Trend et al. 2021). The role of IgM^{hi} and IgM^{lo} B cells in this and other pathologies remains to be determined.

In the present work, we further extended the characterization of circulating IgM^{hi} and IgM^{lo} B cells in healthy adults by simultaneously studying the expression of 12 previously reported markers and additionally assessing the expression of the intestinal homing receptors α4β7, CCR9, CD22 and CCR10. Furthermore, we compared the absolute numbers and percentages of these populations in individuals with appendectomy and/or tonsillectomy.

Materials and methods

Donor selection, purification of PBMCs and plasma storage

The donors selected for the study lacked symptoms of recent infections or underlying diseases at the time of sampling. The donors were divided into 2 groups matched by sex and similar age: with appendix and tonsils (controls: C. $n = 11$) or appendectomized and/or tonsillectomized (AP/T/APT $n = 14$). For some comparisons, tonsillectomized: T, $n = 5$, appendectomized: AP, $n = 7$ and tonsillectomized and appendectomized: APT, $n = 2$ were analyzed separately. Volunteers with tonsillectomy and/or appendectomy were recruited only if their surgery was performed before they were 19 years of age. Age, sex and absolute number of cells (coherent with those reported in the literature (Giraldo-Ocampo et al. 2022)) from volunteers included in this study are shown in Supplementary Table S1. Peripheral blood mononuclear cells (PBMC) were isolated by a ficoll gradient and processed immediately; plasma samples were stored at −80°C before use.

Evaluation of intestinal homing receptors

One million total cells were stained with Zombie NIR (Biolegend, San Diego, CA. United States # 423105) at optimal titer, as a viability stain, and incubated at room temperature. Subsequently, cells were stained in presence of TruStain FcXTM (Biolegend, San Diego, CA. United States # 422301) and brilliant stain buffer plus (BD Biosciences, San José, CA, United States # 566385) at the concentrations recommended by the manufacturer. Cells were then incubated with anti-human monoclonal antibodies (shown in Supplementary Table S2) at optimal titer after carrying out all quality and sensitive assays for detection of these markers. Cells were fixed with 1% paraformaldehyde and analyzed on a three laser (violet-blue-red) Cytek Aurora spectral cytometer (Cytek Biosciences, Fremont, CA, United States). Absolute lymphocyte counts were determined through an automated blood counter using the DxH 800 coulter cellular Analysis System (Beckman coulter, Brea, CA. United States # 628134).

Determination of Ig in plasma

Concentrations of total IgA, IgG and IgM were determined by an automated method using the VITROS MicroTip IgA: Immunoglobulin A Reagent Test (Ortho Clinical Diagnostics, Raritan, NJ # 6801732), VITROS MicroTip IgG: Immunoglobulin G Reagent Test (Ortho Clinical Diagnostics, Raritan, NJ # 6801733) and VITROS MicroTip IgM: Immunoglobulin M Reagent Test (Ortho Clinical Diagnostics, Raritan, NJ #6801734). An in-house sandwich ELISA previously described (Salas-Cuestas et al. 2017) was used to detect total SIgA. The presence of antibodies to the RBD of SARS-CoV-2 were undetected in the serum of all volunteers using a previously described assay (Townsend et al. 2021)), suggesting that they had not been infected by the virus and that results were not modified by this viral infection (Woodruff et al. 2020).

Data analysis and statistics

Flow cytometry data were analyzed using SpectroFlo version 2.2 software (Cytek Biosciences, Fremont, CA, United States), OMIQ (https://www.omiq.ai/) and FCS Express (https://denovosoftware.com/). Percentages, absolute numbers, mean fluorescence intensity (MFI) and integrated mean fluorescence intensity (iMFI, percentage of cells positive for a marker x MFI) of B cells populations were identified. Supervised (UMAP) (Becht et al. 2019) and unsupervised (FlowSOM) (Van Gassen et al. 2015) dimensionality reduction analyses were performed in OMIQ using cells from healthy donors ($n = 11$). In both analyses, we included concatenated data from all manually-gated $IgM^+IgD^+CD27^+$ B cells (1914–5525 cells per individual), excluding class-switched B cells, and 15 fluorescent markers were considered (IgM, IgD, CD1c, BAFFR, CD27, CD45RB, IL21R, CD69, CD23, CD5, CD184, CD38, α4β7, CCR9 and CD22). The following parameters were used in UMAP analysis: Neighbors = 120, Minimum Distance = 0.1, Components = 2, Metric = Euclidean, Learning Rate = 1, Epochs = 300, Random Seed = 268, Embedding Initialization = random. The FlowSOM analysis was performed with the following parameters: 144 clusters with xdim = 12 and ydim = 12, rlen = 10, Distance Metric = Euclidean, consensus metaclustering with k = 15, Random Seed = 7336.

Comparisons between groups with more than three individuals were made with the Kruskal-Wallis, Wilcoxon and Mann-Whitney one-tailed nonparametric tests. Differences between groups in the multiparametric analysis were made with the edgeR algorithm, considering an FDR<0.05.

Results

IgM^{lo} and IgM^{hi} B cells differed in the expression of α4β7 and CCR9, and these homing receptors determined subpopulations within the $IgM^+IgD^+CD27^+$ B cells subsets

To further characterize previously described IgD^+CD27^+ IgM^{lo} and IgM^{hi} B cells (Bautista et al. 2020), we developed a flow cytometry panel with the 12 markers that are differentially expressed in IgM^{lo} and IgM^{hi} B cells (IgM, IgD, CD1c, BAFFR, CD27, CD45RB, IL21R, CD69, CD23, CD5, CD184, CD38) and the homing receptors α4β7, CCR9, CCR10 and

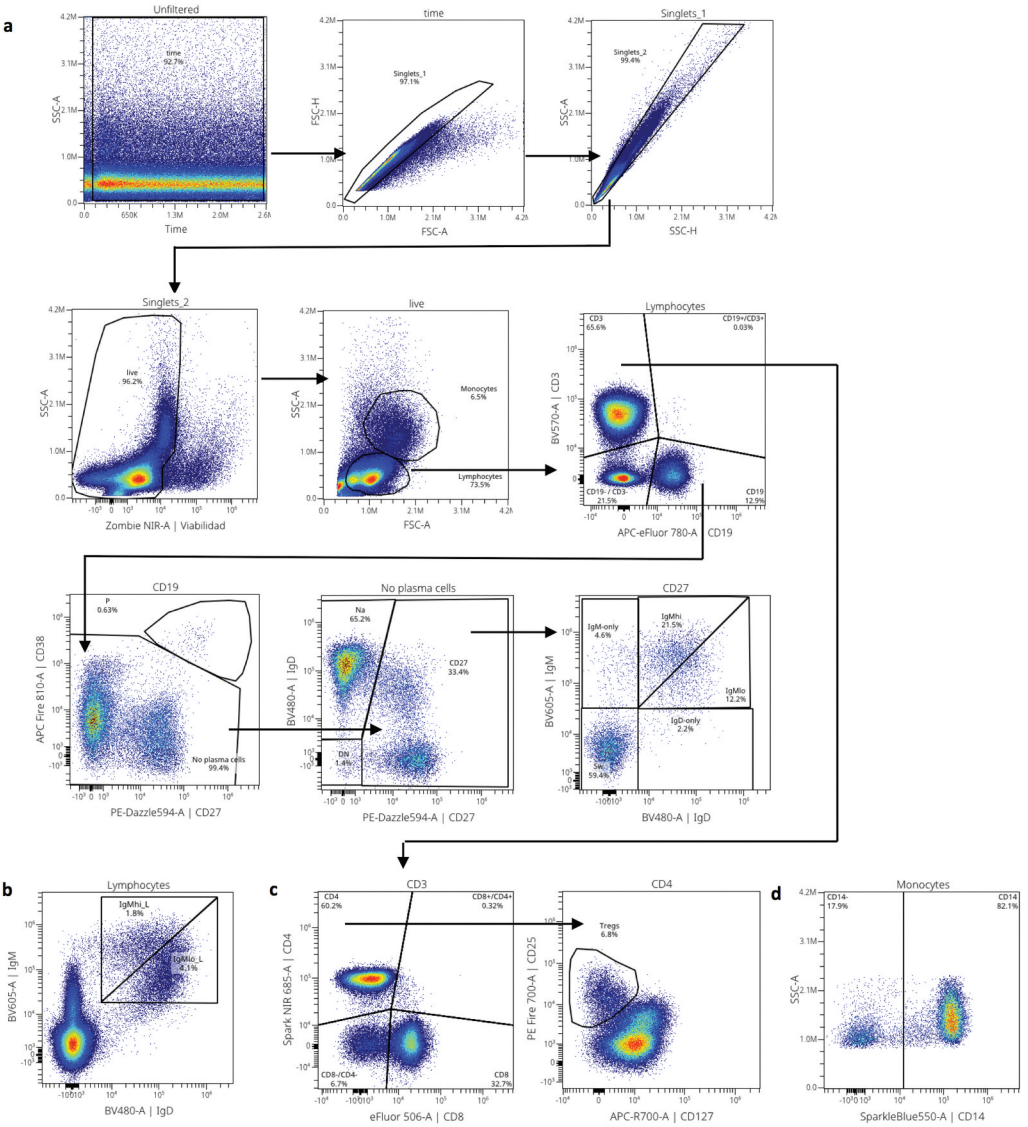

Figure 1. Flow cytometry analysis strategy. (a) After removing fluctuations in time, doublets and dead cells, lymphocytes and monocytes were gated based on FSC-A/SSC-A. B cells were defined based on the expression of CD19. After exclusion of plasma cells ($CD27^{hi}$ $CD38^{hi}$), B cells ($CD27^{lo/int}$ $CD38^{lo/int}$) were identified and then IgM-only ($IgM^{+}IgD^{-}CD27^{+}$), IgD-only ($IgM^{-}IgD^{+}CD27^{+}$), IgM^{lo} ($IgM^{lo}IgD^{hi}CD27^{+}$) and IgM^{hi} ($IgM^{hi}IgD^{lo}CD27^{+}$) B cells were analyzed. (b) the diagonal that separates IgM^{hi} and IgM^{lo} B cells was drawn taking into account the diagonal that forms on the lymphocyte gate. (c) T cells were also identified based on the expression of CD3 and of these CD4 ($CD4^{+}CD8^{-}$), CD8 ($CD4^{-}CD8^{+}$), $CD8^{-}CD4^{-}$ and Tregs ($CD127^{lo/-}CD25^{hi}$) were considered. (d) Monocytes cells were identified based on the expression of CD14.

CD22 (shown in Figure 1 and data not shown). For comparison, the expressions of α4β7, CCR9, CCR10 and CD22 were also evaluated in IgM-only and IgD-only B cells.

Percentages, absolute numbers, MFI and iMFI of cells expressing α4β7, CCR9 and CD22 in circulating B cell subsets from healthy individuals were evaluated (shown in

Supplementary Table S3 and Supplementary Figure S1). CCR10 was almost exclusively expressed in plasma cells (data not shown). While significant differences in percentages of IgM^{hi} and IgM^{lo} B cells expressing α4β7, CCR9 and CD22 were observed, the MFI only differed statistically for CCR9 and CD22 (shown in Supplementary Table S3). IgM^{hi} B cells had higher iMFI for CCR9 median 470,450 (range 121,303 to 1,040,279) *vs*. 223,344 (range 64,930 to 525,616) in IgM^{lo} B cells, and IgM^{lo} B cells had higher iMFI for CD22 median 8,082,015 (range 5,885,975 to 10,693,949) *vs*. median 4,671,274 (range 3,915,286 to 6,680,718) in IgM^{hi} B cells. The percentage of double positive $\alpha4\beta7^{+}CCR9^{+}$ IgM^{hi} cells was higher, median 26% (range 5% to 32%), than that of IgM^{lo} B cells, median 12% (range 5% to 23%) (shown in Supplementary Figure S1b and Supplementary Table S3), suggesting that they have a greater potential to home to the small intestine. In agreement with a previous report (Magri et al. 2017), percentages of $\alpha4\beta7^{+}CCR9^{+}$ cells were highest in IgM-only B cells, median 35% (range 13% to 41%), and the IgD-only population had the lowest, median 11% (range 4% to 22%) (shown in Supplementary Table S3). In conclusion, α4β7, CCR9 and CD22 homing receptors are differentially expressed in circulating $IgD^{+}CD27^{+}$ IgM^{lo} and IgM^{hi} B cells.

To characterize the relationships and heterogeneity of B cell populations, data from IgM^{lo} and IgM^{hi} subsets from 11 healthy donors were concatenated and analyzed with the supervised dimensionality reduction UMAP algorithm. We additionally included in the analysis IgM-only and IgD-only populations, as controls. As expected, the analysis with the original markers clearly clustered the IgM^{lo} and IgM^{hi} populations separately (shown in Figure 2a, first panel and Figure 2b). The grouping of IgM^{lo} and IgM^{hi} B cells with CD22 (shown in Figure 2a, second panel and Figure 2c, third panel) was like that observed with the original markers. In contrast, α4β7 and CCR9 expression increased heterogeneity within the IgM^{lo}, IgM^{hi} and IgM-only B cells, generating $\alpha4\beta7^{+}$ and $\alpha4\beta7^{-}$ or $CCR9^{+}$ and $CCR9^{-}$ subsets (shown in Figure 2a, third and fourth panel and Figure 2c, first and middle panel). Added heterogeneity (determined by combinations of the two homing receptors) was observed by including all markers in the analysis (shown in Figure 2a, last panel).

To further assess subset heterogeneity, we used the FlowSOM algorithm to identify new B cells clusters in an unsupervised manner. The analysis revealed 15 clusters among IgM^{lo}, IgM^{hi}, IgM-only, and IgD-only B cells (shown in Figure 2d). The expression of IgM, IgD and homing receptors were analyzed in each cluster (shown in Figure 2e). As such, IgM^{lo} contained clusters with high and intermediate expression of α4β7, but most of them were $CCR9^{lo}$ and $CD22^{hi}$. Similarly, the IgM^{hi} subset contained cells with high, intermediate and low expression of α4β7 and CCR9, but most of them were $CD22^{hi}$. Cells in clusters 01, 03, 07, 09 and 11 were found amongst IgM^{lo} cells. Specifically, cells in clusters 01, 03 and 09 were mainly $\alpha4\beta7^{+}$, in cluster 07 $\alpha4\beta7^{+}CCR9^{+}$ and in cluster 11 $\alpha4\beta7^{-}CCR9^{-}$ (Shown in Figure 2e). Cells in clusters 02, 04, 05, 10, 13 and 14 were found amongst IgM^{hi} cells. In particular, cells in clusters 04 and 05 were mainly $\alpha4\beta7^{+}$, in clusters 02 and 13 $\alpha4\beta7^{+}CCR9^{+}$, in cluster 14 $CCR9^{+}$, and in cluster 10 $\alpha4\beta7^{-}CCR9^{-}$ (Shown in Figure 2e). Cells in clusters 06, 08 and 15 were IgM-only cells. Among them, cells in clusters 06 and 08 were $\alpha4\beta7^{+}$ while in cluster 15 $\alpha4\beta7^{+}CCR9^{+}$. Finally, cells in cluster 12 were equivalent to IgD-only and were $\alpha4\beta7^{-}CCR9^{-}$ (Shown in Figure 2e). Except for cells in cluster 15, all clusters presented a low to null expression of CCR10 (data not shown) and homogeneous

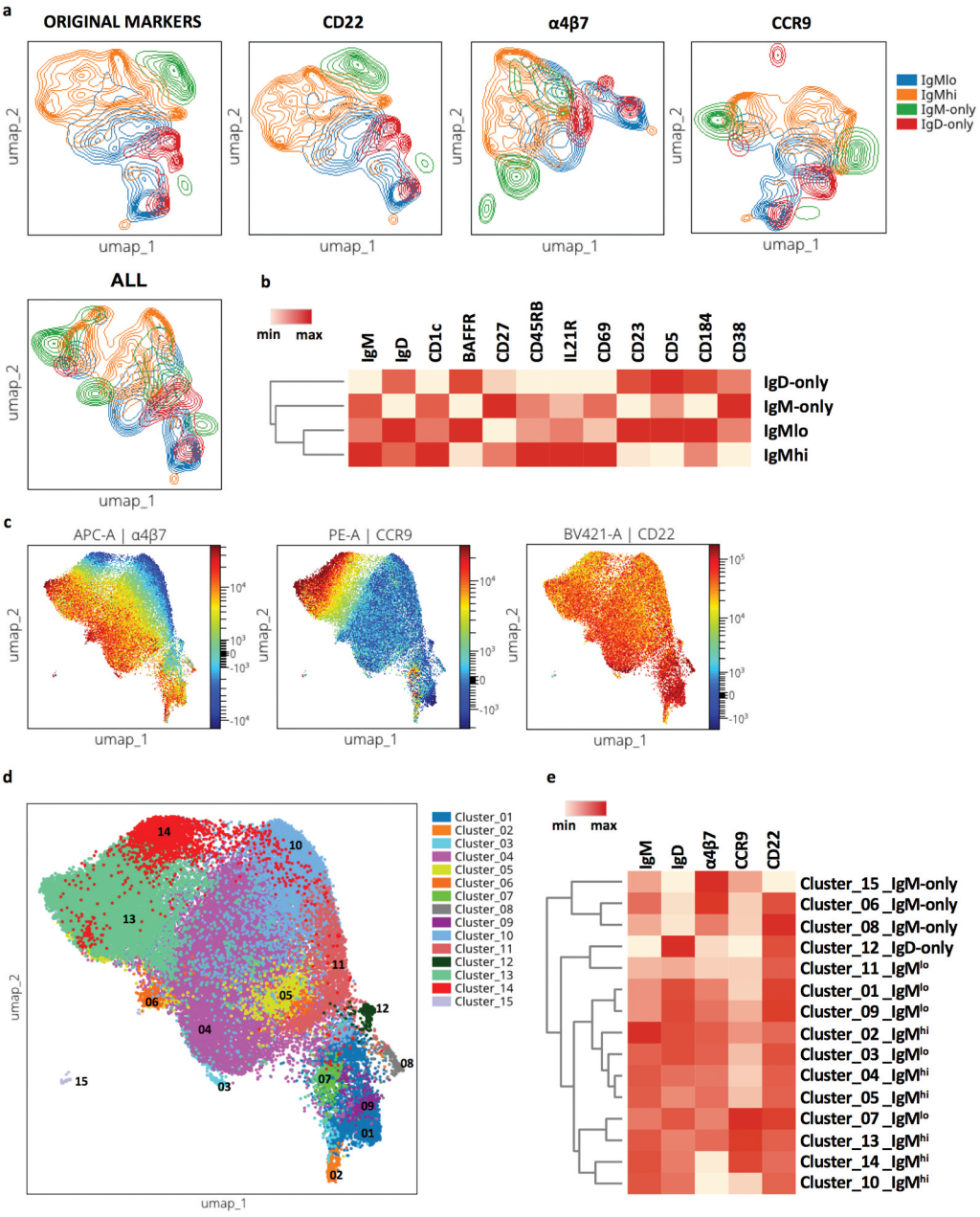

Figure 2. UMAP (supervised) and FlowSOM (unsupervised) dimensionality reduction analysis of IgM$^+$IgD$^+$CD27$^+$ B cells. Concatenated data from 11 control volunteers were used to show the localization of IgM^{lo} (represented in blue), IgM^{hi} (represented in orange), IgM-only (represented in green), and IgD-only B cells (represented in red), projected on two UMAP dimensions using the 12 basic markers of the panel (IgM, IgD, CD1c, BAFFR, CD27, CD45RB, IL21R, CD69, CD23, CD5, CD184, and CD38) (a-first panel); basic markers plus CD22 (a-second panel), basic markers plus α4β7 (a-third panel), basic markers plus CCR9 (a-four panel) and 15 markers (basic markers plus CD22, α4β7 and CCR9) (a-last panel) on a gate of IgM$^+$IgD$^+$CD27$^+$ B cells. (b) heat map of expression levels of the 12 markers described in a-first panel. Scale bars show color coding of median fluorescence intensity (dark red color indicates a greater expression). (c) Scatterplot of the expression of intestinal homing receptors α4β7 (first panel),

expression of CD22 (show in Figure 2c,e). Thus, supervised and unsupervised dimensionality reduction analyses revealed the existence of distinct IgM^{hi} and IgM^{lo} subpopulations, with a mixture of cells expressing α4β7 and/or CCR9, while others lacking these homing receptors.

Absolute numbers of $IgD^{+}CD27^{+}$ IgM^{lo} and IgM^{hi} B cells were lower in appendectomized and/or tonsillectomized donors

Next, the absolute numbers of CD14, CD4, CD8, Tregs, $CD8^{-}CD4^{-}$ cells, plasma cells, IgM^{lo}, IgM^{hi} and IgM-only B cells in blood circulation of control and appendectomized and/or tonsillectomized volunteers were compared. A statistically significant lower absolute numbers of circulating IgM^{lo} and IgM^{hi} B cells were detected in appendectomized and/or tonsillectomized volunteers (IgM^{lo} median = 6.5/μl range 3–19, IgM^{hi} median = 25 range 7–45) *vs.* controls (IgM^{lo} median = 12.5 range 5–22, IgM^{hi} median = 34 range 17–66) (shown in Figure 3a). Differences in the absolute numbers of CD14, CD4, CD8, Tregs and $CD8^{-}CD4^{-}$ cells and plasma cells between controls and appendectomized and/or tonsillectomized volunteers were undetected (shown in Figure 3b, and data not shown). When appendectomized or tonsillectomized individuals were considered separately, we found significant lower absolute numbers of IgM^{lo} B cells in appendectomized volunteers and lower numbers of IgM^{hi} B cells in tonsillectomized volunteers (shown in Supplementary Figure S2a). Changes in the absolute numbers of CD14, CD4, CD8, Tregs and $CD8^{-}CD4^{-}$ cells between controls and appendectomized or tonsillectomized volunteers were undetected (shown in Supplementary Figure S2b). However, we observed lower absolute numbers of CD19 cells in tonsillectomized volunteers (shown in Supplementary Figure S2b).

Furthermore, we determined if the lower frequencies of IgM^{lo} and IgM^{hi} B cells detected depended on their expression of homing receptors. To this end, we compared, for each subset, the absolute numbers of circulating $\alpha4\beta7^{+}$ and $\alpha4\beta7^{-}$ (shown in Supplementary Figure S3a), $CCR9^{+}$ and $CCR9^{-}$ (shown in Supplementary Figure S3b) and $CD22^{+}$ and $CD22^{-}$ (shown in Supplementary Figure S3c) B cells between controls and appendectomized and/or tonsillectomized donors. Statistically significant lower absolute numbers of nine of the twelve comparisons were observed (shown in Supplementary Figures S3a-c). For the three remaining subsets: IgM^{hi} $\alpha4\beta7^{+}$, IgM^{hi} $CCR9^{-}$ and IgM^{lo} $CD22^{-}$ B cells, trends for lower numbers of cells were present, but the differences were not statistically significant (shown in Supplementary Figures S3a-c). Thus, the lower frequencies of $IgD^{+}CD27^{+}$ IgM^{lo}

CCR9 (second panel) CD22 (last panel). Scale bars at the right of each UMAP plot show color coding of fluorescence intensity. (d) Unsupervised dimensionality reduction analysis of $IgD^{+}IgM^{+}CD27^{+}$ B cells, excluding class-switched B cells from concatenated data of 11 control donors displaying FlowSOM clusters projected on two UMAP dimensions including 15 markers (IgM, IgD, CD1c, BAFFR, CD27, CD45RB, IL21R, CD69, CD23, CD5, CD184, CD38, α4β7, CCR9 and CD22). (e) heat map of the expression level of IgM, IgD, α4β7, CCR9 and CD22 on each cluster. For each cluster the corresponding B cell subset composition is indicated. Scale bars show color coding of median fluorescence intensity (dark red color indicates a greater expression). Parameters used for the UMAP and FlowSOM analysis are described in materials and methods.

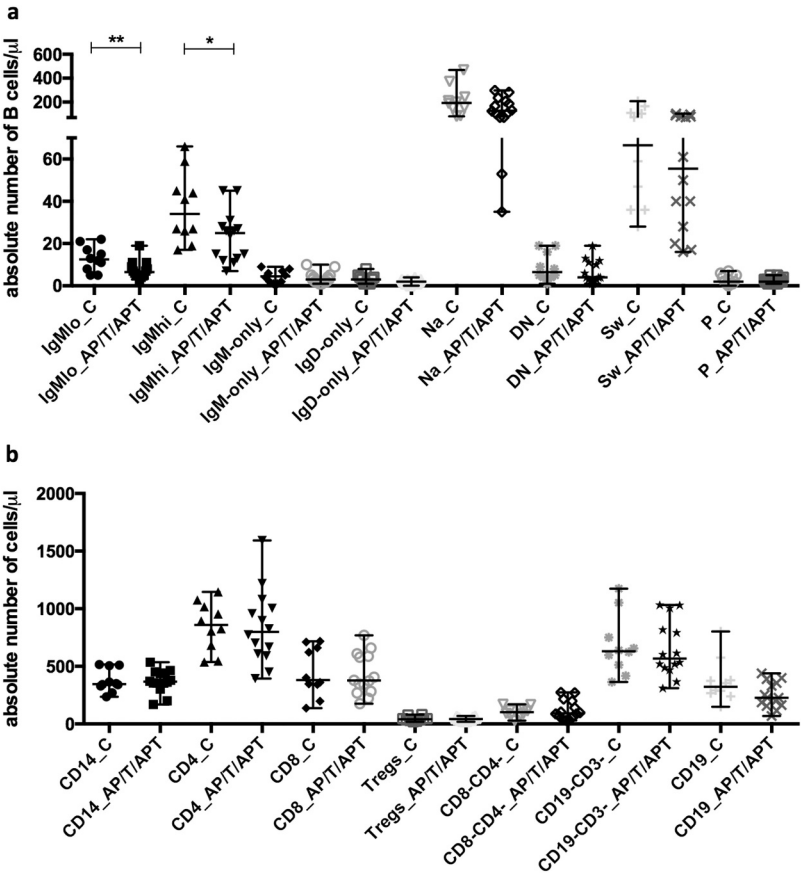

Figure 3. Comparison of absolute numbers of IgM^{lo}, IgM^{hi}, IgM-only and IgD-only B cells and other subsets of cells in appendectomized and/or tonsillectomized individuals. (a) Absolute numbers of IgM^{hi} and IgM^{lo} B cells and naive (Na), double-Negative (DN), class-switched (Sw) and plasma (P) cells in Control (C), $n = 10–11$ and appendectomized and/or tonsillectomized individuals (AP/T/APT), $n = 14$. (b) Absolute numbers of CD14, CD4, CD8, Tregs, CD8$^-$CD4$^-$, CD19$^-$CD3$^-$ and CD19 cells in Control (C), $n = 10–11$ and appendectomized and/or tonsillectomized individuals (AP/T/APT), $n = 14$. Differences between groups were analyzed by Kruskal-Wallis and Mann-Whitney U tests, $p < 0.05$ =*, $p < 0.01$=**.

and IgM^{hi} B cells in appendectomized and/or tonsillectomized donors seem to be independent of their expression of homing receptors.

Finally, the Ig concentration in plasmas of volunteers was determined. Control and appendectomized and/or tonsillectomized donors had equivalent concentrations of IgG (shown in Supplementary Figure S4a), IgM (shown in Supplementary Figure S4b), and total IgA and SIgA (shown in Supplementary Figures S4c and S4d) in plasmas.

Discussion

This work confirms that IgD$^+$CD27$^+$ IgM^{hi} and IgM^{lo} B cells are two distinct phenotypically defined populations (shown in Figure 2), shows that these cells differentially express the homing receptors α4β7, CCR9 and CD22 (shown in Supplementary Table

S3 and Supplementary Figure S1) and suggests that α4β7 and CCR9 expression determine new IgM B cell subpopulations (shown in Figure 2 and Supplementary Figure S3). Moreover, IgM^{hi} and IgM^{lo} B cells were detected in lower absolute numbers in individuals with appendectomy and/or tonsillectomy (shown in Figure 3a and Supplementary Figure S2a). Further studies are needed to establish if the appendix and tonsils may be involved in the development of human $IgD^{+}CD27^{+}$ IgM^{hi} and IgM^{lo} B cells.

Several recent publications have identified different subpopulations of human $IgM^{+}IgD^{+}CD27^{+}$ B cells (Grimsholm et al. 2020; Martin et al. 2015; Siu et al. 2022; Stewart et al. 2021). The first group characterized two populations called – MZBC-1 and MZBC-2— in mesenteric lymphoid nodules, spleen and blood (Siu et al. 2022). A second group found evidence for the existence of $CD27^{+}$ IgM^{hi} and IgM^{lo} populations (Martin et al. 2015), and more recently described two blood subsets (referred to as M-mem-1 and M-mem-2) of circulating IgM $CD27^{+}$cells using single-cell transcriptomics (Stewart et al. 2021). Finally, a third group identified two subsets of IgM^{+} $CD27^{+}$ B cells differing in the expression of CD27: $CD27^{bright}$ "remodeled IgM memory B cells" and $CD27^{dull}$ "innate memory" B cells (Grimsholm et al. 2020). These authors propose that $CD27^{bright}$ cells coincide with IgM^{hi} B cells and $CD27^{dull}$ cells overlap with IgM^{lo} B cells (Carsetti et al. 2021). Like previously reported (Bautista et al. 2020), we observed a higher expression of CD27 on IgM^{hi} compared with IgM^{lo} cells in volunteers from the present study (data not shown), suggesting that $CD27^{bright}$ and $CD27^{dull}$ cells could be related to IgM^{hi} and IgM^{lo} B cells, respectively. Similarly, M-mem-1 and MZB-1 B cells could be related to IgM^{hi}, while the M-mem-2 and MZB-2 could resemble IgM^{lo} B cells: Like IgM^{hi}, MZB-1 and M-Mem-1 have higher expression of CD1c, and higher percentage of mutations in their Ig gene repertoire, compared with IgM^{lo}, MZB-2 and M-Mem-2 cells (Bautista et al. 2020; Siu et al. 2022; Stewart et al. 2021). In addition, IgM^{hi} cells share with M-mem-1 cells the expression of activation genes (Stewart et al. 2021) and with MZB-1 cells the expression of β7 (Siu et al. 2022). Further studies are necessary to determine the relationship between MZBC-1/MZBC-2, M-mem-1/M-mem-2, $CD27^{bright}$/$CD27^{dull}$ and IgM^{hi}/IgM^{lo} subpopulations.

Subpopulations of IgM^{lo}, IgM^{hi} and IgM-only B cells, and their corresponding clusters, differentially expressed α4β7 and CCR9, both in supervised (shown in Figure 2a) and unsupervised analyses (shown in Figure 2d,e). IgM^{hi} B cells seem to have a higher homing potential for the small intestine than IgM^{lo} cells due to their expression of both α4β7 and CCR9 (shown in Supplementary Figure S1a-b and Supplementary Table S3) and CCR9 (shown in Supplementary Fig S1d and Supplementary Table S3). In contrast, the expression of α4β7 (shown in Supplementary Table S3 and data not shown) and CD22 (shown in Supplementary Figure S1e and Supplementary Table S3 and data not shown) of IgM^{lo} B cells seem closer to that of naïve B cells (Spencer et al. 2019). $α4β7^{+}CCR9^{+}$ B cells probably originated in the small intestine, where there are Peyer's patches with particularly active large GC that project towards the intestinal lumen (Spencer et al. 2019). In contrast, $α4β7^{\pm}$ $CCR9^{-}$ B cells probably originated in other organs, such as the colon, where the GC are absent or small, less active and tend to locate below the muscularis mucosae (Spencer et al. 2019). The capacity of α4β7 and CCR9 to contribute to the heterogeneity of circulating B cells highlights the importance of mucosal surfaces (particularly the human small intestine) in the development of circulating B cells.

Several subsets of IgM^{hi} and IgM^{lo} B cells expressing or not α4β7, CCR9 and CD22 were found at lower frequencies in appendectomized and/or tonsillectomized volunteers, mirroring the lower frequencies of IgM^{hi} and IgM^{lo} populations in these same individuals (shown in Figure 3a and Supplementary Figure S2a), and suggesting that the lower levels of these cells are independent of their expression of the homing receptors. However, it is possible that the homing of B cells to tonsils and appendix depends on these receptors: MAdCAM-1 expression has been reported in tonsils at very low levels and in the appendix (Briskin et al. 1997; Salmi et al. 2001). To our knowledge, direct evidence of the expression of CCL25 (CCR9 ligand) in the appendix or tonsils is lacking, but plasma cells expressing CCR9 have been reported in these organs, although in low percentages (Kunkel et al. 2003). Besides, there is *in vitro* evidence that plasma cells of tonsils migrate towards CCL25 (Brandes et al. 2000). Moreover, in the cecal plate of mice, an organ equivalent to the human appendix, both $CCR9^{+}$ and $CCR10^{+}$ plasma cells are generated, the former with the capacity to migrate to the small intestine and the latter to the large intestine (Masahata et al. 2014). Further studies are required to understand why IgM^{lo} and IgM^{hi} B cells, both expressing and lacking the homing receptors, are detected at lower frequencies in appendectomized and/or tonsillectomized individuals. One hypothesis is that these organs may be necessary for the development of both mucosal IgM^{hi} and IgM^{lo} B cells and of precursors that develop into IgM^{hi} and IgM^{lo} B cells in systemic organs, like the spleen.

Controversies persist regarding the origin and function of human $IgM^{+}IgD^{+}CD27^{+}$ B cells. Some authors consider that these cells are generated from post-GC reactions and are T-dependent (Küppers 2021), while others propose that these cells can be stimulated by antigens from the microbiota and may function as innate cells (independent of GC and T cells) (Weill and Reynaud 2020; Zhao et al. 2018). It is probable that $IgM^{+}IgD^{+}CD27^{+}$ B cells exhibit elements of both innate and adaptive immunity, specially depending on the age of the individual, with gradual recruitment of MZB cells into GC responses (Kibler et al. 2021; Nemazee 2021; Tull et al. 2021). Future functional studies of these cells obtained from the spleen and mucosal organs (as suggested by our present study) may help in elucidating their role in immunity.

Finally, contrary to what was previously reported (Andreu-Ballester et al. 2007), similar concentrations of plasma SIgA were detected in control and appendectomized and/or tonsillectomized individuals, probably because of the low number of samples analyzed in this study (shown in Supplementary Figure S4).

Study limitations

The number of volunteers in our two main study groups is relatively low (C. $n = 11$ and AP/T/APT. $n = 14$), and these volunteers lacked antibodies against SARS-CoV-2. It is difficult to increase the n of the study groups with comparable volunteers from the post-pandemic period who have been, in many cases, infected/or vaccinated against SARS-CoV-2 and these process alter circulating B cells subpopulations (José-Cascón et al. 2022; Kato et al. 2022; Woodruff et al. 2020).

Conclusion

In conclusion, this study supports the existence of two subpopulations of human circulating $IgD^{+}CD27^{+}$ IgM^{hi} and IgM^{lo} B cells, it further identifies heterogeneity in these cells determined by their differential expression of intestinal homing receptors. Further studies are necessary to clarify the role of tonsils and appendix in their development.

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Author contributions

JA, MAF were involved with conceptualization, data curation, funding acquisition, project administration, resources, supervision, writing – original draft, writing – review & editing. DB was involved with conceptualization, data curation, performing the experiments, writing – original draft, writing – review & editing. MCR was involved in experiments to measure SIg, resources, writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethics statement

This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. This study protocol was reviewed and approved by the ethics committee of the School of Medicine of Pontificia Universidad Javeriana, approval number [0203–19]. Written informed consent approved by the ethics committee of the School of Medicine of Pontificia Universidad Javeriana was obtained from all participants. Data from study volunteers was anonymized. This process did not affect interpretation of the results.

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Data availability statement

All data needed to evaluate the conclusions made in this paper are included in the main body of the manuscript. Additional data will be made available by the corresponding author upon reasonable request

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