Changes in Tonsil B Cell Phenotypes and EBV Receptor Expression in Children Under 5-Years-Old

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Background: Palatine tonsils are principally B cell organs that are the initial line of defense against many oral pathogens, as well as the site of infection for others. While the size of palatine tonsils changes greatly in the first five years of life, the cellular changes during this period are not well studied. Epstein Barr virus (EBV) is a common orally transmitted virus that infects tonsillar B cells. Naive B cells are thought to be the target of primary infection with EBV in vivo, suggesting that they are targeted by the virus. EBV enters B cells through CD21, but studies of older children and adults have not shown differences in surface CD21 between naive B cells and other tonsil B cell populations.

Methods: In this study, we used an 11-color flow cytometry panel to detail the changes in B cell subpopulations in human tonsils over the first five years of life from 33 healthy US children.

Results: We provide reference ranges for tonsil B cell subpopulations over this age range. We show that the frequency of naive tonsil B cells decreases over the early years of life, and that naive B cells expressed higher surface levels of CD21 relative to other tonsil B cell populations.

Conclusions: We show that young children have a higher frequency of naive tonsil B cells, and importantly that these cells express increased surface EBV receptor, suggesting that young children have a larger pool of cells that can be infected by the virus. © 2017 International Clinical Cytometry Society

Key terms: tonsil; B cell; Epstein Barr virus; phenotyping; CD21

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Palatine tonsils are secondary lymphoid organs located in the oropharynx that are critical for defense against oral pathogens (1) [reviewed in (2)]. Palatine tonsils are comprised of an outer epithelial layer forming crypts which provide a high surface area, under which lie secondary lymphoid structures including follicles with...
germinal centers as well as extrafollicular T cell regions (3). Palatine tonsils change morphologically with age, the largest size being seen in children between 3- and 5-years-old, and subsequently declining in size through life (reviewed in (4)). As secondary lymphoid organs, palatine tonsils are also the sites of the final stages of B cell development, which involves trafficking of transitional B cells, antigen exposure, as well as affinity maturation and somatic hypermutation (2,5). B cells are the principal cellular component of palatine tonsils, comprising between 50 and 90% of subepithelial lymphocytes (3,6). B cells and their terminally differentiated form, plasma cells (PC), are the critical cell types of humoral immunity; with immunoglobulin G (IgG)-producing B cells responsible for binding blood-borne pathogens and IgA-producing B cells responsible for mucosal pathogens (7). Tonsils are known to be an important site for the development of antibody responses during exposure to oral antigens (8). The physical location of palatine tonsils in the oropharynx puts immune cells in close contact with pathogens in the oral cavity (3), and gives tonsils their important role in the immune defense against oral pathogens (8).

In addition to their role in the protection against oral pathogens, tonsils are also the sites of infection for some oral pathogens (9,10). One oral pathogen that is associated with significant morbidity and mortality is Epstein Barr virus (EBV) (11). The most common manifestation of EBV-associated disease is infectious mononucleosis, which is a self-limiting febrile illness of EBV-infected adolescents and IgA-producing B cells responsible for mucosal pathogens (7). Tonsils are known to be an important site for the development of antibody responses during exposure to oral antigens (8). The physical location of palatine tonsils in the oropharynx puts immune cells in close contact with pathogens in the oral cavity (3), and gives tonsils their important role in the immune defense against oral pathogens (8).

During primary infection, EBV infects naïve B cells in the tonsils through its receptor, CD21 (16,17). While EBV can infect all B cell types in vitro (18), naïve B cells more readily immortalize into proliferating lymphoblasts (19), and are the only cells to display the growth program classic for primary infection in vivo, suggesting that these cells are preferentially infected by the virus (16). While it is known that blocking CD21 severely inhibits the entry of EBV into B cells (20), studies in patients of undefined ages have not found differential CD21 surface expression on naïve tonsil B cells relative to other B cell types (18,19). Whether CD21 expression differs among tonsil B cells during early years of life is yet to be defined.

In clinical medicine, lymphocytes and other white blood cells are commonly enumerated in peripheral blood as an indicator of the function of the immune system as a whole (21). It has been well documented that peripheral blood lymphocytes differ with age and sex (22,23), but whether these differences apply for lymphocytes in secondary lymphoid tissues of young children is not as well described. One early study using two-color flow cytometry described age-related changes in tonsil B cell markers with age (24), but B cell subpopulations could not be identified at that time. Since then, the proportions of different B cell subsets in human palatine tonsils have been documented for adults, adolescents, and older children (25), but to our knowledge no study has measured the changes in B cell subsets of human tonsils in early childhood (< 4 years of age). And despite interest in the cell types and function of tonsil B cells (26-28), studies have not focused on children under 5 years of age. This age range is critical for understanding the unique immune interface of the palatine tonsils when they are most highly developed (4) and during the ages when palatine tonsils play their most important physiological role in defense against oral pathogens.

Palatine tonsillectomy with or without adenoidectomy is a routine procedure performed in children and adults to remove hypertrophied tonsil tissue to improve breathing (29). Since palatine tonsils grow in size during the first several years of life, they can cause airway obstruction, leading some parents and children to elect to undergo tonsillectomy. Today almost 80% of tonsillectomies are performed for upper airway obstruction, in the setting of a baseline immune status at the time of removal, rather than infection (29). Tonsils are also a rich source of B cells and have been used for multiple immune phenotyping and microbiology studies (2,5,8,25,30,31). Unfortunately these studies frequently use different antibody combinations to identify different cell populations, resulting in an impaired ability to share findings among groups. Here we propose a standardized immune phenotyping strategy based largely on a commonly used B cell phenotyping panel for peripheral blood lymphocytes (32).

This study set out to answer several unanswered questions. First, what are the reference ranges of B cell subsets in tonsil tissue for young children between 1 and 5 years of age? Second, do B cell subsets differ with age, sex, and EBV infection in these children? Third, what is the tonsillar B cell expression pattern of CD21, the EBV receptor, during the early years of life? Here we define reference ranges of tonsil B cell subsets in young children based on a standardized immunophenotyping panel, and have uploaded our flow cytometry files to an open source repository for comparison with future study populations. We also report that mature naïve B cells are increased in tonsil mononuclear cells (TMC) early in life and, for the first time, that they express increased levels of CD21, suggesting a mechanism by which these cells may be preferentially infected by EBV in vivo.

**METHODS**

**Study Population**

Patients between 1 and 5 years of age undergoing routine tonsillectomy for tonsillar hypertrophy at a large
academic medical center were enrolled in the study. Consent was obtained from parents of study participants during a preoperative visit. A total of 37 children were enrolled in the study. Children were excluded for clinical evidence of acute infection, or if tonsils were not processed within 4 h from removal. The study was approved by the institutional review board at SUNY Upstate Medical University, Syracuse, New York, and was carried out according to the Declaration of Helsinki.

**Cell Preparation**

Tonsils were surgically removed and transferred into physiological saline for transport to gross pathology. Upon receiving a gross pathological diagnosis of benign hypertrophy, tonsils were placed in 25 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum, supplemented with penicillin/streptomycin and L-glutamine. Tonsils were transported to the laboratory for preparation within 4 h of surgery. Upon arrival tonsils were weighed, manually homogenized, and filtered twice over nylon to remove connective tissue and cell aggregates. Mononuclear cells were isolated over Ficoll-Hypaque and washed 3–4 times in complete RPMI 1640 medium. Mononuclear cells were frozen in complete RPMI 1640 with 20% FBS and 10% DMSO in liquid nitrogen prior to flow cytometric analysis.

**Flow Cytometry**

Cells were thawed and washed with complete RPMI 1640 medium. Two million viable cells per tonsil were prepared for flow cytometry. Cells were stained for viability using Fixable Aqua viability dye (Thermo Fisher Scientific, Waltham, MA) as per the manufacturer’s instructions. Cells were Fc blocked for 20 min with 20 μL Fc blocking reagent (eBioscience, San Diego, CA) in 30 μL flow buffer containing phosphate buffered saline, 1% BSA and 0.1% sodium azide. Cells were then stained with an antibody cocktail containing antibodies against CD3, CD19, CD10, CD27, IgD, IgM, CD24, CD38, CXCR4, and CD21 in a staining volume of 100 μL (Table 1). Cells were fixed in 2% paraformaldehyde in PBS and run on a BD LSR Fortessa flow cytometer equipped with FACS Diva software (BD Biosciences, San Jose, CA). Flow files were analyzed using FlowJo version 9.5.2 (Tree Star, Ashland, OR). Before analysis, all flow files were biexponentially transformed to better visualize separation of subsets.

**FACS Isolation of B Cell Subsets**

Tonsil cells were thawed and washed in complete RPMI medium. Cells were stained with Zombie Aqua viability dye (BioLegend, San Diego, CA) as per the manufacturer’s instructions then suspended in blocking buffer (PBS, 5% bovine serum albumin, and purified anti-human Fc block) for 30 min prior to staining with antibodies. 5 × 10⁷ cells were stained with antibodies against CD19, CD24, CD38, CD27, IgD, and CXCR4 for 30 min. Fluorescent-activated cell sorting (FACS) was used to isolate purified naïve B cells, total memory B cells (non-class-switched, classic, and atypical), centroblast, and centrocytes, gating on populations as shown in Fig. 1. Postsort analyses were performed to determine purity of each isolated B cell population. Sorts were performed on a FACS Aria II flow cytometer (BD Biosciences).

**EBV Detection by Quantitative PCR from Tonsil Cells**

DNA was extracted from 1 × 10⁷ TMC or isolated B cell subsets using a Qiagen DNeasy kit by the blood and tissue protocol as per the manufacturer’s instructions (Qiagen, Valencia, CA). DNA was eluted in 200 μL H₂O and the concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific). A total of 120 ng of template DNA, corresponding to approximately 20,040 human cells (167 diploid human cells/ng) were run per PCR reaction. The qPCR protocol was as follows: 10 min at 95°C, 45 cycles of 15 s at 95°C and 1 min at 60°C, followed by a melt curve with a 0.5°C step down. The PCR was performed on an iCycler qPCR thermocycler equipped with an optical module (BioRad Laboratories, Hercules, CA). iQ supermix was used for all reactions (BioRad Laboratories, Hercules, CA). Previously designed primers were used to detect a 70 base pair region of EBV BALF-5 (33).
FIG. 1. Tonsil B cell flow cytometry gating strategy. After biexponential transformation, lymphocytes were gated by FSC-A versus SSC-A. Of lymphocytes, doublets were excluded using FSC-W/H and SSC-W/H sequentially. Of singlets, live cells were selected as viability dye negative. Viable cells were then plotted by CD19 versus CD3. B cells were gated as CD19+, CD3−, and T cells were gated as CD3+, CD19−. Next, B cells were plotted by CD38 versus CD24 differentiation markers. Transitional B cells were identified as CD38hi, CD24hi; mature B cells as CD24+, CD38−; germinal center B cells as CD38−, CD24+, and PC as CD38−, CD24+, and CD27+. Of mature B cells, IgD versus CD27 allowed discrimination between mature naive (IgD+, CD27−), non-switched memory (IgD+, CD27+), classical memory (IgD−, CD27+), and atypical memory (IgD−, CD27−). Of germinal center B cells, centroblasts were identified as CxCR4hi and centrocytes were gated as CxCR4+. 
RESULTS

Tonsil Characteristics

A total of 33 tonsils were analyzed in this study. There were 23 male and 10 female study participants. The mean age of study participants was 37 months, with a range of 17–59 months. The mean weight of tonsils obtained was 4.34 grams (g), with a standard deviation of 2.01 g. The mean number of mononuclear cells per gram of tissue obtained from male versus female tonsils (P = 0.36). There was also no relationship between the number of mononuclear cells/g and patient age (P = 0.31, R² = 0.034).

Phenotypic Characterization of Tonsil B Cell Subpopulations

To determine the B cell subpopulation frequencies in human tonsils, TMC were phenotyped by flow cytometry according to standardized panels for peripheral blood phenotyping (32,34), with some modification (Table 1). Figure 1 represents the gating strategy used to identify B cell subpopulations after exclusion of cellular debris by forward and side scatter area, clumps of cells by width and height of forward and side scatter, dead cells (viability dye positive), and T cells (CD3+, CD19+). As a control, a subset of tonsils were analyzed for B cell subsets before and after freezing, the result of which had no notable effects on any cell phenotype studied (data not shown). B cells were gated as CD19+, CD3–, and subpopulations were determined on the basis of CD38, CD24, IgD, IgM, CD27, CD10, and CXCR4. The relative fluorescence intensities for different markers are shown in Table 2. Transitional B cells (Tr) were identified as CD38hi, CD24hi, CD27+ and were also IgD–, IgM–, and CD10–. Mature B cells (M) were identified as CD38+, CD24+. This group was further subdivided based on expression of IgD and CD27 as mature naïve (MN: IgD–, CD27–) (37). As CM B cells are class-switched, the CM population was also gated to exclude IgM– cells. Other mature B cell subsets were further subdivided based on expression of IgM, with MN and NSM as largely IgM– and AM as largely IgM–. Germinal center B cells were identified as CD24–, CD38+, and were subdivided based on the expression of CXCR4 as centroblasts (CXCR4+) and centrocytes (CXCR4–). PC were identified on the basis of CD24–, CD38hi, and CD27hi, and were larger (increased FSC-A) than GC B cells. This is consistent with previous reports that GC B cells express low CD27, while PCs express high CD27 and are larger in size (38,39). Within the PC population, a group of IgM– PC was also enumerated.

Reference Ranges for Tonsil B Cell Subsets in Young Children

To provide reference ranges for future studies of tonsil B cells in young children, frequencies of B cell subpopulations were determined. The means with the 25th and 75th percentiles are shown in Table 3. Means for all samples tested are shown in Fig. 2. The mean frequency of T cells of total lymphocytes was 20.5% (95% CI 17.8–23.1). B cells made up 72.6% of all lymphocytes (95% CI 70.3–74.9). Transitional B cells made up 1.6% of all B cells (95% CI 1.2–2.0). Mature B cells comprised 52.3% of all B cells (95% CI 47.3–57.2). Germinal center B cells represented 40.3% of all B cells (95% CI 35.5–45.1). PC made up 1.5% of all B cells (95% CI 1.2–1.7). Mature B cells were further broken down on the basis of IgD and CD27 expression as described in Figure 1 and Table 3. Mature naïve B cells comprised 76.5% of all mature B cells (95% CI 73.8–79.2). Non-class-switched memory B cells were 3.9% of mature B cells.
B cells 72.6% (67.8–78.1)
Transition 1.6%
Mature 52.3% (43.1–63.2)
Mature Naive 76.5%
Non-switched Memory 3.9%
Classical Memory 8.1%
Atypical Memory 10.1%

| Cell type          | Frequency of parent and subsets |
|--------------------|---------------------------------|
| T cells            | 20.5% (14.1–26.4)               |
| B cells            | 72.6% (67.8–78.1)               |
| Transitional       | 1.6% (0.8–2.2)                  |
| Mature             | 52.3% (43.1–63.2)               |
| Mature Naive       | 76.5% (71.5–82.7)               |
| Non-switched Memory| 3.9% (2.8–5.1)                  |
| Classical Memory   | 8.1% (5.1–10.9)                 |
| Atypical Memory    | 10.1% (7.6–11.9)                |
| Germinal Center    | 40.3% (29.7–51.1)               |
| Centroblasts       | 65.3% (61.1–68.0)               |
| Centrocyes         | 26.2% (24.0–30.1)               |
| PC                 | 1.5% (1.0–1.7)                  |
| IgM+ PC            | 3.9%                            |
| IgM- PC            | 96.0% (95.2–98.9)               |

Data represent mean and 25–75th percentile values.

Reference Ranges for Palatine Tonsil Lymphocyte Populations of Young Children

(95% CI 3.4–4.5). The proportion of classical memory B cells (excluding IgM+ cells) of all mature B cells was 8.1% (95% CI 6.5–9.1). Atypical memory B cells were on average 10.1% of total mature B cells (95% CI 9.1–11.1).

Germinal center B cells were also broken down further on the basis of CXCR4 expression, representing centroblasts (CXCR4+) and centrocyes (CXCR4+) (40). Centroblasts made up 65.3% of GC B cells (95% CI 62.5–68.0), while centrocyes made up 26.2% of GC B cells (95% CI 23.8–28.6).

PC were also subdivided based on the expression of IgM, which are phenotypically and functionally distinct from IgG PC and may be important for mucosal immunity (41). Immunoglobulin M positive PC were 3.9% of all PC (95% CI 2.6–5.2).

Changes in Tonsil B Cell Populations with Age and Sex

Next we sought to identify changes in B cell subpopulations with age over the first five years of life. Tonsil lymphocyte populations were plotted against age of study participants (Fig. 3). We identified no age-related changes in the proportions of T or B cells of all live mononuclear cells in human tonsils ($R^2 = 0.003$, $P = 0.78$; $R^2 = 0.017$, $P = 0.47$, respectively). Of mature B cell subsets, mature naive B cells decreased with age ($R^2 = 0.159$, $P = 0.02$) and atypical memory B cells increased with age ($R^2 = 0.199$, $P = 0.01$). No other age-related changes were observed for B cell populations including Transitional, Mature, Germinal Center, or PC ($R^2 < 0.001$, $P = 0.96$; M—$R^2 = 0.008$, $P = 0.62$; GC—$R^2 = 0.007$, $P = 0.64$; PC—$R^2 = 0.002$, $P = 0.83$). Non-class-switched memory B cells and classical memory B cells did not change in frequency with age ($R^2 = 0.022$, $P = 0.42$; $R^2 = 0.074$, $P = 0.13$, respectively). The frequency of centroblasts and centrocyes of all GC B cells did not change significantly with age ($R^2 = 0.032$, $P = 0.33$; $R^2 = 0.035$, $P = 0.31$, respectively).

The frequencies of lymphocyte subsets were also compared by sex (data not shown). Neither T cells nor B cells differed by sex ($P = 0.99$, $P = 0.98$, respectively). The frequencies of major B cell subsets including transitional, mature, germinal center, and PC did not differ by sex ($P = 0.77$, $P = 0.13$, $P = 0.11$, $P = 0.95$, respectively). Of mature B cells, no differences were observed for MN, CM, or AM by sex ($P = 0.31$, $P = 0.54$, $P = 0.58$, respectively). However, NSM B cells were found at higher frequency in boys than girls ($t = 2.08$, $P = 0.05$). Germinal center B cell subsets CB and CC did not differ in frequency by sex ($P = 0.70$, $P = 0.72$, respectively). The frequency of IgM+ PC of all PC also did not differ significantly by sex ($P = 0.45$).

EBV Infection and Tonsil B Cell Phenotypes

To determine whether EBV infection has an effect on tonsil B cell phenotypes, we separated the EBV-positive tonsils from EBV-negative tonsils and compared B cell subpopulation frequencies. EBV was detected in 19 (57.6%) of the 33 tonsils studied. There was no difference in frequency EBV positive tonsils by sex ($P = 0.85$). Over the age range studied, there was no association between EBV positivity and age ($R^2 = 0.004$, $P = 0.75$). Frequencies of lymphocyte subsets were compared between EBV- and EBV+ tonsils. The mean CD3+ T cell frequency observed in EBV+ tonsils was higher than in EBV- tonsils (23.0% and 17.0%, respectively, $P = 0.02$. However, the proportion of B cells between
EBV+ and EBV- tonsils was not significantly affected ($P = 0.16$). Major B cell populations including Tr, M, GC, and PC were not significantly different between EBV+ and EBV- tonsils ($P = 0.55$, $P = 0.60$, $P = 0.65$, $P = 0.48$, respectively). Mature B cell subpopulations including MN, NSM, CM, and AM did not differ between EBV+ and EBV- tonsils ($P = 0.95$, $P = 0.27$, $P = 0.88$, $P = 0.37$, respectively). Frequencies of IgM$^+$ cells within mature B cell subpopulations did not differ by EBV status (MN—$P = 0.11$; NSM—$P = 0.34$; CM—$P = 0.90$; AM—$P = 0.72$).

We also examined EBV status against sex and EBV load to determine if these measures affect B cell phenotypes and EBV infection. EBV positive samples did not differ significantly by sex (Supporting Information Fig. S1A). Of EBV positive tonsils, the mean EBV load per $10^6$ TMC was $3.2 \times 10^6$ copies (SEM $\pm 1.8 \times 10^6$) and did not differ by sex ($P = 0.41$). The frequencies of lymphocyte populations were next plotted against EBV load for EBV-positive samples. There were no significant differences in any lymphocyte subset frequencies by EBV status.
Collectively, a high frequency of tonsils from young children were positive for EBV with a variably high EBV copy number, EBV infection was associated with a reduced T cell frequency, but minimal changes in the tonsillar B cell compartment, and EBV viral load was also not associated with significant perturbations in tonsillar B cells.

EBV Receptor Expression on Tonsil B Cell Subsets

To determine whether different tonsil B cell subsets in young children express different amounts of surface EBV receptor CD21, we compared CD21 mean fluorescence intensity (MFI) among CD19⁺ B cell subpopulations. A summary of tonsil B cell CD21 expression is shown in Figure 4. The frequency of CD21 positive B cells was determined for the major B cell subsets analyzed. CD21⁺ B cells represented at least 97% of all B cell populations studied. As a control, the frequency of CD21 positive T cells was < 3% of all CD3⁺ T cells. Although the vast majority of B cells studied expressed surface CD21, we asked whether B cell subsets expressed different amounts of surface CD21, which could explain why in vivo, EBV preferentially infects naïve B cells (16). The MFI of CD21 was quantified for the B cell subpopulations studied. To control for donor-to-donor differences in CD21 expression, CD21 MFI of B cell subsets was converted to the fold change (FC) from overall B cell CD21 MFI for that donor, which was expressed as 1. Major B cell subpopulations including Tr, M, GC, and PC exhibited variations in the expression of surface CD21, with Tr and PC expressing a mean CD21 similar to that of overall B cells (1.024 and 0.977 FC, respectively). Mature B cells expressed increased surface CD21 (1.18 FC), while GC B cells expressed reduced surface CD21 (0.86 FC). Of GC B cells, CB expressed lower CD21 than CC (0.80 vs. 0.91 FC). There were also large differences in mean CD21 expression within these populations. Within M B cells, the highest surface CD21 expression was found in MN (1.27 FC) and NSM (1.49 FC) B cells, and relatively low expression was found in AM (0.81 FC). Classic memory B cells expressed low normal surface CD21 (0.90 FC). Within all four mature B cell subpopulations, expression of CD21 was significantly higher in IgM⁺ cells than IgM⁻ cells (MN: 1.31 vs. 1.08 FC; NSM: 1.62 vs. 0.92 FC; CM (CD27⁺, IgD⁺ B Cells): 1.74 vs. 0.90 FC; AM: 1.19 vs. 0.72 FC, respectively). Similarly, within the PC subset, IgM⁺ PCs expressed significantly higher CD21 than IgM⁻ PCs (1.21 vs. 0.98 FC).

We also determined the difference in mean CD21 surface expression between EBV⁺ and EBV⁻ samples to confirm that variations in CD21 were not due to EBV infection (Supporting Information Fig. S2). When cell subsets were broken down by CD21 surface expression for EBV⁺ and EBV⁻ tonsil samples, no major differences were found with populations including T, M, and GC B cells, but PC surface CD21 was significantly reduced in the EBV⁺ samples (1.08 vs. 0.90 FC). Of GC B cells, there were no differences in CD21 expression between CB and CC by EBV status. Of the PC subset, IgM⁺ PCs from EBV⁻ tonsils had significantly higher surface CD21 than from EBV⁺ tonsils (1.59 vs. 0.94 FC), whereas there was no difference in surface CD21 expression in IgM⁻ PCs. Of mature B cells, no B cell subset had different mean CD21 expression in EBV⁺ versus EBV⁻ samples.

Finally, we sorted tonsil B cell populations in an EBV positive tonsil sample that had the highest EBV load to determine the site of EBV carriage. We sorted B cells into four subsets (mature naïve, total memory, centroblast and centrocyte). Each subset was confirmed to have a purity of > 96% after sorting. Using quantitative PCR, we found that EBV was primarily detected in the memory B cell subset in these asymptomatic EBV-infected children.

DISCUSSION

This study sought to determine the following characteristics of TMC: reference values for B cell subsets in tonsils of children under 5-years-old, how these subsets
change with age, sex, or EBV status, and to determine the expression pattern of the EBV receptor CD21 on B cell subsets. We have provided the first detailed report of B cell subsets in children < 5 years of age, with the aim of benefiting future studies on other human populations. We also observed a high frequency of tonsils from...
young children were positive for EBV with a variably high EBV copy number. EBV infection was associated with a reduced T cell frequency but minimal changes in the tonsillar B cell compartment, and EBV viral load was also not associated with significant perturbations in tonsillar B cells.

Our results show that mature naïve B cells are the largest component of the tonsil B cell compartment in children under 5 years old, making up about 40% of all tonsil B cells in healthy children. Interestingly, we observed a proportion of B cells that were IgD+, IgM-, which has previously been described in germinal center and peripheral blood B cells (42). We hypothesize that these cells may represent a pre-GC phenotype of naïve B cells or an autoreactive population, consistent with previous reports of these populations (42,43). We also observed that the proportion of mature tonsil B cell subsets change with age over the first 5 years of life. Mature naïve B cells decreased in frequency with age, while atypical and classical memory B cells increased, consistent with age-dependent antigen exposure. These results add to a previous study of older children (> 4 years of age) and adults showing increased germinal center B cells, and reduced memory B cells in children compared to adolescents and adults (25). We did not observe major changes in B cell subsets by sex, and though NSM B were found more frequently in boys than in girls, this may be incidental, and the physiological importance of this finding is not known.

We observed a relatively high frequency of EBV infection during the first five years of life (~60%). The EBV viral load was much higher per cell (3.2 X 10^6 copies/10^6 TMC) than has been reported in peripheral blood mononuclear cells (44,45). This observation is not surprising considering that tonsils are the site of EBV entry and persistence in the lymphocyte compartment (46). The variable but high viral load we observed indicates that tonsils are a reservoir of viral persistence. Accordingly, in sorted B cell subsets, EBV was overwhelmingly found in the memory B cell compartment, consistent with the model that EBV infects naïve B cells during primary infection and is carried in memory B cells during latency through the life of the host (47,48). EBV infection was associated with an increase in the proportion of CD3+ T cells of TMC. This is consistent with the role of cytotoxic T cells in controlling primary EBV infection. The length of time between EBV infection and tonsillectomy was not possible to determine for this study, but the fact that children enrolled in this study were not acutely ill suggests that EBV infection may be associated with subacute to chronic alterations in tonsil T cell frequencies. Importantly, EBV infection was not associated with major changes in the B cell compartment, suggesting both that tonsils were not sampled during primary infection when B cell proliferation is high, and that EBV infection does not alter the B cell subset distribution during persistent infection.

We found large differences in the surface expression of CD21 on tonsil B cell subsets, with mature naïve B cells expressing significantly higher CD21 than classical and atypical memory B cell subsets in children under 5-years-old. These results support the well-documented observation that naïve B cells are more susceptible to EBV-induced transformation (19) and are more susceptible to primary EBV infection in vitro and in vivo (16,19). This contrasts with limited data from patients of undocumented ages showing that the expression pattern of CD21 was not different in tonsil B cell subpopulations (18,19). While we did not observe changes in CD21 surface expression in B cell subsets with age over the first five years of life (data not shown), it is possible that as children age, CD21 expression normalizes across different B cell subsets. It is also possible that previous studies that did not show differences in CD21 expression on B cell subsets (18,19) were underpowered to detect differences. Our data provide an important measure of susceptibility of B cell subsets to EBV infection during the first years of life. Studies on children of this age range are important for understanding primary EBV infection dynamics in general, as well as how EBV infection is modulated in the setting of sub-Saharan Africa, leading to increased risk of eBL. Furthermore, studies involving primary tonsil tissue provide much more physiologically relevant information for EBV infection than those involving peripheral blood.

Overall, our results provide reference ranges of B cell subsets that can be used in future studies of tonsillar tissue of different patient groups or other experimental conditions. We also show that mature naïve B cells decrease in frequency in human tonsils with age, and for the first time, that these cells express increased surface expression of the EBV receptor CD21, suggesting a mechanism for the observation that these cells are more susceptible to EBV infection in vivo (16). Our results also support the hypothesis that EBV infection early in life alters viral pathogenesis by increasing the pool of cells that can be infected by the virus. This enhanced infection, alongside other factors such as malaria that are known to profoundly affect EBV infection and the immune system as a whole, could help explain the extremely high burden of Burkitt lymphoma in the sub-Saharan African population.

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**LITERATURE CITED**

1. Ogra PL, Effect of tonsillectomy and adenoidectomy on nasopharyngeal antibody response to poliovirus. N Engl J Med 1971;284:59-64.
2. Van Kempen MJ, Rijkers GT, Van Cauwenberge PB, The immune response in adenoids and tonsils. Int Arch Allergy Immunol 2000;122:8-19.
3. Nave H, Gebert A, Pabst R. Morphology and immunology of the human palatine tonsil. Anat Embryol (Berl.) 2001;204:367-373.
10. Hug M, Dorner M, Frohlich FZ, Gysin C, Neuhaus D, Nadal D, Berger C. Pediatric Epstein-Barr virus carriers with or without tonsilar enlargement may substantially contribute to spreading of the virus. J Infect Dis 2010;202:1192–1199.

11. Okano M, Gross TG. Acute or chronic life-threatening diseases associated with Epstein-Barr virus infection. Am J Med Sci 2012;343:483–489.

12. Luzuriaga K, Sullivan JL. Infectious mononucleosis. N Engl J Med 2010;362:1993–2000.

13. Delecluse HJ, Lowdell MK, Lambert B, de Sanjosé S, Weiderpass E. Burkitt’s lymphoma in Africa, a review of the epidemiology and etiology. Afr. Health Sci 2007;7:166–175.

14. Pirisó E, Asito AS, Sumba PO, Fiore N, Middeldorp J, Moorman AM, Ploutz-Snyder R, Rochford R. Early age at time of primary Epstein-Barr virus infection results in poorly controlled viral infection in infants from Western Kenya: Clues to the etiology of endemic Burkitt lymphoma. J Infect Dis 2012;205:906–913.

15. Condon LM, Cederberg ER, Rabinovitch MD, Liebo RV, Go JC, Delaney AS, Schmeling DO, Thomas W, Balfour HH. Age-specific prevalence of Epstein-Barr virus infection among Minnesota children: Effects of race/ethnicity and family environment. Clin Infect Dis Off Publ Infect Dis Soc Am 2014;59:501–508.

16. Joseph AM, Babcock GJ, Thorley-Lawson DA. Cells expressing the EBV membrane antigen encoded by EBNA-1 are highly enriched in B cells isolated from blood and tonsils. B cells are functionally distinctive. Immunol Cell Biol 2015;93:175–182.

17. Heath E, Begue-Pastor N, Chaganti S, Croom-Carter D, Shannon-Lawson M. Cells expressing the EBV membrane antigen encoded by EBNA-1 are highly enriched in B cells isolated from blood and tonsils. B cells are functionally distinctive. Immunol Cell Biol 2015;93:175–182.

18. Halliley JL, Tipton CM, Liesveld J, Rosenberg AF, Darce J, Gregoretti C. Epstein-Barr virus growth program are present in and restricted to B cells isolated from blood and tonsils. B cells are functionally distinctive. Immunol Cell Biol 2015;93:175–182.

19. Milne ME, Kazi Cyclone B, Blackwell NL, Gallico GB, Krasnyanski A, Arrowsmith D, Morenas JJ, et al. Tonsil B cell phenotypes and EBV receptor in children. Cytometry Part B: Clinical Cytometry 2014;84(1):187–198.