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Adenoidal follicular T helper cells provide stronger B cell help than those from tonsils

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Abstract

Objectives—The tonsils and adenoids are secondary lymphoid organs, where antigen processing and immune cell development occurs to control bacterial colonization and infection in the upper respiratory tract. Both organs are abundant in follicular T helper cells (T_{FH}), a subset of T cells specialized for promoting B cell development. There are no prior studies on differences between the immune cells of the tonsils and adenoids and whether the cells function differently.

Study design—*In vitro* assays to assess cell phenotype of tonsils and adenoids from young children (median age 40 months).

Methods—Mononuclear cells from tonsils and adenoids were cultured with or without 1 µg/mL *Staphylococcus enterotoxin B* (SEB) for 4 days. Cell phenotype and function were assessed by flow cytometry and multiplex ELISA.

Results—We found that in resting adenoids, T_{FH} expressed higher CXCR5 and ICOS but lower PD-1 than those from the tonsils, and that adenoidal B cells expressed higher CD27. Upon polyclonal stimulation with SEB, both T_{FH} and B cells from the adenoids proliferated to a greater extent, and culture supernatants contained higher levels of IL-21.

Conclusion—We conclude that the cells of the adenoid are disposed toward the provision of more robust B cell help than the tonsils.

Keywords

Nasopharynx; mucosal immunity

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Introduction

Respiratory pathogens first infect the upper airways, including the nose, mouth, and pharynx¹. These airways are protected by an anatomical structure known as Waldeyer's tonsillar ring, which comprises the nasal-associated lymphoid tissue of the tonsils and adenoids and fills an immune sentinel role to prevent harmful pathogens from establishing invasive disease^{2,3}. This sentinel function depends on the generation of diverse B cells and the secretion of mucosal antibody. B cells undergo affinity maturation and proliferation in the germinal centers of secondary lymphoid organs^{2,4}, depending heavily upon help provided by specialized CD4 T cells known as follicular T helper cells (T_{FH}). The tonsils and adenoids contain abundant T_{FH}; in fact, the initial discovery of T_{FH} in 2000 was made in tonsils^{4,5}.

T_{FH} are defined as a subset of CD4+ T cells expressing a handful of characteristic markers. Their differentiation is governed by the master regulator B-cell lymphoma 6 (Bcl-6), which promotes expression of the T_{FH} markers CXCR5 and programmed death 1 (PD-1)⁶. CXCR5 is a chemotactic receptor driving T_{FH} localization to the B cell zones of lymphoid follicles, where its ligand, CXCL13, is highly expressed⁴. The other canonical T_{FH} markers, PD-1 and inducible costimulator (ICOS), are costimulatory T cell receptors. PD-1 has been generally described as a suppressor of immune function in general and T cell activity in particular^{7,8}, but appears to be crucial for the provision of proper help to B cells^{9,10}. ICOS has been shown to promote the expression of Bcl-6¹¹ and induces IL-21¹². T_{FH} provide help to B cells by means of both cell-cell interactions and the secretion of cytokines, most prominently IL-21^{13–15}, and a population of circulating T_{FH}-like cells has been proposed to represent a memory fraction of this population¹⁶.

Little is known about any differences between the adenoids and tonsils. Histologically, it is noteworthy that the gross structure of the tonsils is marked by a series of crypts¹⁷, which the adenoids do not possess. Further, the palatine tonsils but not the adenoids express the antimicrobial compound calprotectin². The tonsils may also be more permissive to the development of biofilms^{18,19}. Associations have been uncovered between adenoiditis and otitis media with effusion, which is also more frequent in children with allergies²⁰.

Removal of the tonsils and/or adenoids is a common operation in children²¹, but the immunological consequences have not been extensively studied, although a postoperative decrease in serum IgG has been described²². While tonsillectomy has been found significantly to improve remission rates of IgA vasculitis²³, this may be a secondary consequence of the removal of infected tissue and an ensuing drop in IgA secretion. Overall, little is known about immunity following adenotonsillectomy.

In light of these findings, we sought to compare T and B cell function in the tonsils and adenoids by analyzing their response to Staphylococcus enterotoxin B (SEB), a potent global stimulant of T cells, also known as a “superantigen” which activates T cell receptors independently of antigen-presenting cells²⁴. We describe for the first time multiple immune cell differences in adenoids and tonsils of young children, allowing the conclusion that T_{FH} in the adenoids provide stronger B cell help than those in the tonsils.

Materials and Methods

Samples

Tonsil and adenoid samples (n = 18 tonsils and 19 adenoids from children aged 19–76 months, median 40 months) were collected under protocol CIC 1257-B-10-1, approved by the Institutional Review Board of Rochester Regional Health Systems. Briefly, following surgery, tissue was minced in RPMI containing 10% fetal bovine serum (FBS) and strained through a 70 μ m filter to remove clumps. Cells were suspended in 5 mL ACK Lysing Buffer (Life Technologies) for 2 minutes, then washed twice in RPMI+10% FBS and resuspended in Recovery Cell Culture Medium (Life Technologies) for storage in liquid nitrogen.

Stimulation and CFSE labeling

Frozen cells were cultured at 2×10^6 cells/mL in 96-well U-bottom plates for 4 days in 250 μ L RPMI with 10% FBS with or without 1 μ g/mL SEB (Sigma Aldrich) at 37°C. Prior to stimulation cells were labeled with 0.05 μ M CFSE (Invitrogen) in PBS for 10 minutes at 37°C, followed by 3 rounds of washing in PBS+1% FBS.

Flow Cytometry

Following stimulation, cells were spun at 1300 RPM for 5 min at 4°C. Supernatant was removed for further analysis. Cells were then washed with PBS+1% FBS, then stained with fluorochrome-conjugated antibodies against CD3 (Pacific Blue), CD4 (PE/Dazzle 594), CXCR5 (APC), ICOS (PerCP-Cy5.5), PD-1 (PE-Cy7), CD19 (Q-dot 605), CD27 (AlexaFluor 700), and IgM (APC-Cy7). All antibodies were purchased from BioLegend. Following staining, cells were washed once with PBS+1% FBS and then acquired on a BD LSRII flow cytometer. Lymphocytes were selected on the basis of size, and then divided into T and B cells following doublet discrimination. Following established gating strategies for the identification of T_{FH}^{9,10,25,26}, we found the cells as a subset of CXCR5⁺ CD4⁺ T cells expressing both PD-1 and ICOS. B cells were identified as CD19⁺ cells and assessed for expression of CD27 and IgM (Fig. 1). Analysis was performed using FlowJo version 10.

ELISA

Secretion of IL-2, IL-4, IL-12p70, and IL-21 was assessed by a multiplex ELISA kit (Mitenyi). Samples were analyzed on a BioPlex plate reader (BioRad).

Statistics

Statistical analyses were performed using R version 3.1.1 (The R Foundation). The Bonferroni correction for multiple comparisons was applied to calculated p values. Dixon's Q test was used to exclude outliers. Statistical tests employed were the Mann-Whitney U test and Student's *t* test, as noted in figure legends.

Results

Heightened expression of co-stimulatory markers by resting adenoidal T_{FH}

We found that in unstimulated cultures, the adenoids and tonsils had similar frequencies of T_{FH} as a fraction of CD4+ T cells. However, adenoidal T_{FH} expressed significantly more CXCR5 and ICOS but less PD-1 than T_{FH} from tonsils (Fig. 2A). Baseline B cell frequencies did not differ between adenoids and tonsils, but we observed higher expression of the memory marker CD27 on B cells from the adenoids (Fig. 2B).

Increased responsiveness of adenoidal cells to polyclonal T cell stimulation

We next sought to determine whether the heightened expression of costimulatory markers on adenoidal T_{FH} corresponded with an increased response to stimulation. We challenged mononuclear cells from tonsils and adenoids with 1 µg/mL SEB for 4 days, then performed flow cytometry to assess the proliferation of T and B cells as determined by CFSE dilution. Upon stimulation with SEB, we observed that CFSE intensity was significantly reduced in both T_{FH} and B cells from adenoids but not tonsils (Fig. 3), indicating that adenoidal cells proliferated significantly in response to stimulation while tonsillar cells did not.

Various cytokines are important for the maintenance and functioning of T_{FH}. IL-12 is known to play a role in the differentiation of T_{FH}²⁷, and deficiency in the IL-12 receptor impairs T_{FH} responses in humans²⁸. IL-21 is one of the primary means by which T_{FH} provide help to B cells^{13–15}, and T_{FH} have also been found to secrete IL-4 T_{FH} cytokine²⁹. By examining these as well as IL-2, an established driver of T cell proliferation and survival, we sought to determine whether the differences in T_{FH} function apparent between the tonsils and adenoids was connected to differential secretion of cytokines upon stimulation. In culture supernatants from tonsil and adenoid cells stimulated for 4 days with 1 µg/mL SEB, we found that significantly more IL-21 was produced by cells from the adenoids (Fig. 4). It is important to note that not all cytokines had comparable concentrations; IL-2 was generally the most highly expressed, while production of IL-12 was negligible in all samples, and no cytokine was produced to a significantly different degree by resting cells.

Discussion

We performed *in vitro* assays aimed at assessing functional differences between the adenoids and tonsils of young children. We focused on T_{FH} and B cells since these are abundant in secondary lymphoid organs and important for the maintenance of mucosal immunity in the upper respiratory tract^{2,30}. In the course of our experiments, for the first time, we found evidence that adaptive immunity functions more robustly in the adenoids compared to the tonsils.

Our key findings were: (1) While the overall frequency of T_{FH} was similar between the tonsils and adenoids, T_{FH} in the adenoids expressed higher CXCR5 and ICOS but lower PD-1 than those in the tonsils. (2) B cells in resting adenoids expressed higher CD27. (3) When challenged with the superantigen SEB, increased proliferation was measured by adenoidal T_{FH} and B cells compared to those from tonsils. (4) Cells from the adenoids expressed significantly more IL-21 than tonsillar cells upon SEB stimulation. This

corresponds with our observation of increased ICOS expression by adenoidal T_{FH}, since ICOS is known to promote the expression of IL-21¹². While it is possible that other cell types besides T_{FH} may have contributed to the observed cytokine production, T_{FH} constituted 30–40% of total CD4+ T cells in the samples, so we are confident in concluding that the T_{FH} in the adenoids produce significantly more IL-21 upon stimulation. IL-12 was only minimally induced by SEB, but this can likely be ascribed to the mechanism of SEB stimulation, which does not involve the antigen-presenting cells²⁴ that are the main producers of IL-12³¹.

This may point to the provision of less potent B cell help by T_{FH} in the tonsils. Indeed, other reports have found abundant tolerogenic Treg cells in the tonsils³², and that dendritic cells isolated from the tonsils are poor activators of T cells³³. The precise role played by PD-1 in T/B cell interactions is contentious. The presence of a circulating PD-1+ T_{FH} population was found to correlate with a protective response to influenza vaccination³⁴, as well as the production of neutralizing antibodies against HIV⁹. An examination of T_{FH} resident in lymph node germinal centers in rhesus macaques found that high PD-1 expression correlated with B cell maturation and antibody production¹⁰. In contrast, PD-1 was shown to inhibit B cell function during *Streptococcus pneumoniae* infection³⁵. The higher expression of PD-1 we found on resting tonsillar T_{FH} may therefore be consistent with a less-inflammatory environment, but the role of PD-1 requires further investigation.

These differences may be explicable by the different environments to which the tonsils and adenoids are exposed. While the adenoids are located at the rear of the nasal passages, the palatine tonsils are near the entrances to the trachea and the esophagus, where they are exposed to both inhaled and alimentary antigens. It may be that the cells of the tonsils are broadly more disposed towards tolerogenic functions so as to avoid the development of allergic or autoimmune reactions against ingested antigens. This could help to explain the permissibility of the tonsils to colonization by biofilms^{18,19}.

This research has significant implications for pediatric otolaryngology clinical practice. When recommending young children for adenotonsillectomy, it will be important to take into account the increased sensitivity shown by T_{FH} and B cells of adenoids. For example, it may be beneficial to delay removal of the adenoids, so that the child's immune system can make use of the greater activity of adenoidal B and T cells. This corroborates previous findings that adenotonsillectomy reduces secretion of antigen-specific IgA against poliovirus as well as total serum and salivary IgA for years after surgery³. While van den Akker et al. found that the decrease in circulating antibody following adenotonsillectomy does not result in increased upper respiratory infections during the year following surgery³⁶, longer-term effects were not examined, and specifically it will be important in the future to consider the tonsils and adenoids independently. Further study of the differences in function between adenoidal and tonsillar lymphocytes is likely to substantially alter the understanding of immune ontogeny and treatment of upper respiratory disorders in early life.

Conclusion

To our knowledge, this is the first report directly comparing tonsillar and adenoidal lymphocytes. Our finding of more potent B cell help provided by T_{FH} in the adenoids opens

intriguing possibilities for the development of mucosal vaccines and treatments of upper respiratory infections, including otitis media ²⁰. The mechanisms underpinning this differential provision of B cell help remain to be investigated.

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Figure 1. Gating Strategy

Identification of T_H and B cells; a representative tonsil sample is depicted, but the same gating strategy was used for both tonsil and adenoid samples (FSC-H, forward scatter height; FSC-A, forward scatter area, used to identify doublets).

Figure 2. Differences Between T_{FH} and B cells from Unstimulated Adenoids and Tonsils Cultured 4 Days A)

Intensity of CXCR5, PD-1, and ICOS expression on T_{FH} was assessed by flow cytometry.

B) Intensity of CD27 and IgM expression by B cells was assessed by flow cytometry. (Data are represented as individual points with median and interquartile range. A = adenoid, T = tonsil; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, Mann-Whitney U test)

Figure 3. Proliferation of T_{FH} and B cells from Tonsil and Adenoid Cells Stimulated 4 Days with 1 µg/mL SEB

Flow cytometry was used to measure CFSE dilution according to the change in MFI. **A)** Representative flow cytometry histograms from adenoids and tonsils (white = unstimulated, grey = SEB). **B)** Statistical comparison of CFSE dilution in response to SEB stimulation in T_{FH} and B cells from tonsils and adenoids (***) $p < 0.0005$, paired Mann-Whitney U test).

Figure 4. Cytokine Production by Tonsil and Adenoid Cells Stimulated 4 days with 1 µg/mL SEB
 Charts depict mean $\pm$ SEM of cytokines in supernatants measured by multiplex ELISA (grey = adenoids, black = tonsils, * $p < 0.05$, Student's t test).