Tonsillar immunity over time, from immune resistance to immune regulation

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Research Article

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Abstract

Background. The tonsils operate as a protection ring of mucosa at the gates of the upper aero-digestive tract. They show similarities with lymph nodes and participate as inductive organs of systemic and mucosal immunity. Based on the reduction of their size since puberty, they are thought to experience involution in adulthood. In this context, we have used tonsillar mononuclear cells isolated from patients at different stages of life, to study the effect of ageing and the concomitant persistent inflammation on these immune cells.

Results. We found an age-dependent reduction in the proportion of germinal center B cell population (B_{GC}) and its T cell counterpart (T follicular helper germinal center cells, Tfh_{GC})**. Also, we demonstrated an increment in the percentage of local memory B cells and mantle zone** T follicular helper cells (mTfh)**.** Furthermore**,** younger tonsils rendered higher proportion of proliferative immune cells than those from older ones. Finally, we demonstrated the growth of a B cell subset metabolically adapted to catabolize adenosine triphosphate ($CD20^+CD39^+CD73^+$ cells), as patients get older.

Conclusions. This paper shed light on the changing aspects of the immune cellular landscape, over the course of time and constant exposure, at the entrance of the respiratory and digestive systems. Our findings support the notion that there is a re-modelling of the immune functionality of the tonsils over time. They are indicative of a shift from an effector type of immune response early in life, to a regulatory role at later stages, when limiting the tissue damage gets critical. Understanding these features will not only contribute to comprehend the differences in susceptibility to pathogens among children and adults but would also impact on vaccine developments intended to target these relevant mucosal sites.

Background

In humans, the local mucosal immunity in upper airways is secured mostly by the Waldeyer ring (paired palatine tonsils, adenoids and lingual tonsils). Based on the reduction of their size, it has long been assumed that palatine tonsils undergo some kind of involution from puberty onwards [1]. Still, the mechanisms of such partial involution and their impact on immunity are yet to be determined.

Recurrent tonsillitis (RT) and tonsillar hypertrophy (TH) are the two main causes for tonsillectomy in children and teenagers [2]. In fact, TH is the major motive for tonsillectomy in young children [3], [4]. Around puberty, a shift occurs towards RT as the primary reason for the indication of surgery [3], [2]. RT is defined as a number of repetitive infections of tonsils per year, characterized by systemic and local symptoms including fever, sore throat and tonsillar exudate. On the other hand, TH has been long considered of non-infectious etiologyby ear, nose and throat (ENT) specialists.

The sequential incidence of TH and RT as the main cause of surgery at a population level, led us to speculate that these two conditions share a common pathogenesis. Hence, we have recently shown that TH is of infectious nature by evidencing bacterial penetration through the epithelial layer to the lymphoid compartment in TH samples [4]. Thus, the characteristic follicular hyperplasia that defines the TH

condition can be attributable to the persistent, sub-clinical infection detected. Our current hypothesis is that the age-related changes of tonsillar immunity combined with the regulatory mechanisms triggered by the local continual inflammation to avoid immunopathology, reflect on the number of tonsillectomies due to RT after puberty.

In this regard, we have previously demonstrated that HT tonsils rendered significantly lower percentages of IL10-producing B cells (Bregs) than tonsils excised due to RT [2]. There are also regulatory mechanisms exerted by B cells which are independent of IL10. For instance, adenosine (ADO)-producing $CD39^{+}CD73^{+}$B cells [5]. CD39 and CD73 are ectonucleotidases that can hydrolyze extracellular adenosine triphosphate (ATP) yielding adenosine 5'-monophosphate (5'-AMP) and ADO. Interestingly, human tonsil cryosections have long been used to study extracellular nucleotide catabolism [6], [7].

In the present work we demonstrate that the proportion of tonsillar germinal center B cell population (B_{GC}) and its T cell counterpart (T follicular helper germinal center cells, Tfh_{GC}), both decrease with increasing age. Moreover, we show the progressive loss of capacity of the GC in terms of proliferative aptitude. Finally, we extend our previous observations by evidencing the accumulation of B cells with regulatory function in samples from older children. These results imply that the appearance of recurrent tonsillar infections as the main cause of tonsillectomy in teenagers correlates with an escalation of local immune regulation and concomitant deterioration of the effector mechanisms. The paper provides a better comprehension of the dynamics forces of the immune response in the course of time at the gateway of the upper airways.

Results

3.1 Germinal Center population steadily decreases with increasing age

To explore age-related functional changes in the tonsils of our cohort of patients, tonsillar mononuclear cells (TMC) were analyzed by flow cytometry and B_{GC} cells were scored at different ages, as a read out of the effector immunological activity of these organs.

First, single lymphocytes were gated based on forward and side scatter properties, and tonsillar mature B lymphocytes were identified as $CD3^{-}CD20^{+}CD10^{+/-}$ cells within the singlets and lymphocyte gate ([8]). B_{GC} cells were defined by bright CD10 and low CD44 expression (Fig. 1A). We found that the proportion of B_{GC} cells within $CD20^{+}$ cell population steadily declined with increasing age. B_{GC} cells represented slightly over one third of all the B cells from tonsils within the (2–4) year old range (32.6% ± SD 14.7%). Tonsils from children between 5 and 8 years old rendered 30.8% ± SD 11.9% B_{GC} cells and in the pre-teens (9–11 years old) group we obtained 27.3% ± SD 11.2% B_{GC}. We found a statistically significant lower proportion of B_{GC} (17% ± SD 10.3%) in tonsils from teenagers (12–18 years old) when compared to the youngest group (Fig. 1B). Moreover, the frequency of memory B cells was significantly lower in the tonsils from the

toddlers (2–4 years old) than that of the oldest patients (Fig. 2A and B). Being the GC a crucial reserve of the effector B cell population, we concluded that from a cellular perspective, local effector immune responses appear reduced as patients aged. On the other hand, memory B cells accumulated over time.

Tfh cells are the primary drivers of T cell–dependent GC responses. To extend our findings, we also assessed tonsillar T $CD4^+$ cell populations. The Tfh cells locating within the GC (Tfh_{GC}) express high levels of CXCR5 and PD-1 ($CXCR5^{high}$ $PD\text{-}1^{high}$). There are also Tfh in secondary lymphoid organs which express intermediate levels ($CXCR5^{int}$ $PD\text{-}1^{int}$) of those markers and localize in the mantle zone of the follicle (mTfh) (Fig. 3A). The proportion of the latter steadily increased with increasing age (Fig. 3C). Conversely, the frequencies of Tfh GC cells were significantly lower in children over 9 years old when compared with younger children, consistent with the decay of the proportion of GC B cell´s population (Fig. 3B).

Since we mostly work with a paediatric otolaryngology service, we usually receive samples up to 18 years old. We have previously shown that within such a cohort, the frequency of tonsillectomies peaked at 5–6 years old [9]. We further confirmed the data in the present manuscript. In Fig. 1B we present data from 76 samples, 42% of those fall in a single interval of age, the one including tonsils from children ranging from 5 to 8 years old. Children younger than 5 years old account for another 26% of the samples. Therefore, puberty specimens represent only a third of our samples. Moreover, samples from children over 12, represented 10% of the samples analyzed.

It has long been established that the cause of tonsillar disease is associated with the age of the patients. Notably, while tonsillar hyperplasia is the most frequent cause of tonsillectomy in children younger than 10, abscesses and recurrent acute infections are responsible for most tonsillectomies in teenagers [3] [10]. Our observations provide a mechanistic foundation to the temporal pattern reported for the causes of tonsillectomy, suggesting the decrease in immunological functionality holds critical consequences for some individuals around or after puberty.

3.2 Decreased CG reactivity after puberty

It is known that B_{GC} cells and plasmablasts exhibit the highest levels of Ki-67 expression of all tonsillar lymphocytes [11] (Fig. 4). Ki-67 antigen expression is strictly associated with cell proliferation as it is expressed in the nucleus of dividing cells but not during the G0 phase. In order to confirm the progressive loss of competence of the GC with the age of the patients, Ki-67 was used to identify and relatively quantify the proliferating cellpopulations within fresh TMC. We were able to incorporate adult samples for the assay (samples from people in their second and third decades of life). We detected proliferating cells in the B and T cell compartments which evidenced immune activity in all samples tested. As expected, the B cell compartment exhibited the highest proportion of proliferating cells driven by B_{GC} cells (Fig. 4A and B). There was a higher percentage of $CD20^+Ki\text{-}67^+$ cells in children under 10 years old than that of teenagers and adults (Fig. 4B, left panel), albeit the difference did not result statistically relevant. However, when scoring the proliferative capacity of T cell populations, we found that the youngest

children presented statistically significant higher frequencies of $CD4^+Ki\text{-}67^+$ (9% ± SD 1.6%) than teenagers (4.7% ± SD 2.5%) and adults (4.7% ± SD 1.6%) (Fig. 4B, middle panel). The differences of statistical strength between the proliferative aptitude of B and T cell populations suggest that the well characterized involution of the thymus precedes tonsillar decline. Therefore, the impairment in naïve T cell output has a clear effect on tonsillar GC responses, which adds to intrinsic aged B cell defects. Moreover, we found that teenagers and adults exhibited very similar parameters, confirming the notion that tonsillar functional senescense initiates around 10 years old [12].

We next determined the percentage of $CD8^+Ki\text{-}67^+$ cells as we did for the other lymphocyte populations. Once again, the percentage of proliferating cells from adult samples were significantly lower than those from the youngest children (Fig. 4B, right panel).

Therefore, these findings further validate the decline of tonsillar effector immune responses over time, which become dramatic around the the early onset of puberty. Taken all together, we decided to work with the samples divided in two groups, taking 10 years old as the dividing time point.

3.3 Co-expression of CD39 and CD73 membrane proteins in freshly isolated tonsillar B cells increases with age

As the patients approach youth, a number of biological factors could be related to the deterioration of their tonsillar immune responses. We have previously shown that hyperplastic tonsils from children endure bacterial growth able to breach the epithelial barrier [2]. Such damage to epithelial tissues might trigger tolerance programs intended to restrain immunopathology, assisting the transition of tissues from pro-inflammatory to anti-inflammatory states [13], [14]. To investigate a potential metabolic adaptation of B cells promoting a suppressive behavior upon years of hyperplasia and chronic inflammation, we compared co-expression of CD73 and CD39 on $CD20^+$ cells (Fig. 5A) from TMC from children younger and older than 10 years old. We found that samples from patients over 10 years old presented a statistically significant higher double positive $CD73^+CD39^+CD20^+$ cell population than those from younger children (35.3% ± SD 8.9% vs 26.1% ± SD 10.9% respectively, $n = 69$, $p < 0.005$). Thus, we confirmed that the impairment of GC reaction with ageing and chronic inflammation, correlated with and increment in the proportion of a B cell phenotype associated with immunosuppressive activity. These findings further confirmed our previous observations in relation to the higher proportion of IL10 secreting B cells (Bregs, [15]) and GC declining when comparing samples from recurrent tonsillitis and hypertrophy [2]. Collectively, these results indicate a strong correlation between the appearance of recurrent tonsillar infections in teenagers and the boost of immune regulation of GC reactions with increasing age.

Discussion

Human tonsils are considered comparable to the nasopharynx-associated lymphoid tissue (NALT) of rodents, they are part of the mucosa-associated lymphoid tissue (MALT). They also show similarities with lymph nodes and participate as effector organs of local systemic immunity as well, which make them

rather unique lymphoid structures. Here, we have used them as a model to study the impact of chronic inflammation and time on B cell mediated immunity, in particular.

We found that tonsils from young children (before puberty) yielded a significantly higher proportion of GC cell populations (B_{GC} cells and Tfh_{GC} cells) than samples from teenagers and adults. Moreover, younger GC cells presented higher proportion of proliferative immune cells than those from older ones. In contrast, the older children presented higher percentage of Ag-experienced B cells, like memory B cells. Such observations are in line with previous studies on B and T cells in a number of lymphoid compartments, as people age [16] [11].

The depreciation of the tonsillar immune effector mechanisms with advancing age observed *ex-vivo* correlates with a shift in the causes of surgery. Abscesses and chronic or recurrent tonsillitis are the main grounds for tonsillectomy after puberty, which evidence the failure of the local immune system to restrain infections and their systemic manifestations, in teenagers and young adults.

It is generally believed that the partial waning of immune capacity with time is the result of a number of factors. And one of these, in the tonsils we work with, is the fact that they are excised due to malfunction. The persistent sub-clinical infection underlying the HT of the samples from young children as well as the manifested recurrent infections present in older donors, both should have an impact on the tonsillar immune landscape, contributing to precipitate the effects of time, hormones, genetics, habits, etc.

Bacterial and viral infections are thought to be a major source of tonsillar disease pathogenesis. ADO is a purine nucleoside associated with various immune-pathological processes [17]. In this paper we also demonstrate that $CD73^{+}CD39^{+}CD20^{+}$ cell population expand post puberty. CD39 and CD73 are two ectonucleotidases that act sequentially to catabolize ATP to ADO. This biochemical pathway conducing to ADO generation is very active in inflammatory microenvironments. The immunological outcome of ADO production is an immunosuppressive microenvironment. This is not unexpected upon years of constant inflammation, as organisms have evolved mechanisms to regulate immunity to keep the physiological function of the tissue in the context of the persistent presence of an insult, as it is the case of the ENT patients.

We have previously reported on the higher proportion of Bregs as defined by IL10 secretion [15] and GC declining when comparing samples from recurrent tonsillitis and hypertrophy [2]. Therefore, we have extended those findings here, by monitoring an alternative Breg population and also by taking in account the effect of the age of the samples.

A point of particular interest to discuss is whether the variations in the lymphocyte subsets and the consequent shift from a pro-inflammatory microenvironment to a more suppressive one, can be extrapolated to people that do not present any disease that would justify tonsillectomy. In a prospective family-based cohort study, 16% of all the adults recruited reported an illness with sore throat and fever over a 1-year time frame. Such fraction scales up to 47% in teenagers from 12 to 18 years old, and to 40%, in children below 12 years old [18]. Hence, teenagers and children in general seem to be much more

vulnerable to tonsillitis caused by the most usual pathogens, than adults. The accumulation of B cell memory population with age that we observed in the tonsillar samples would account for the lower incidence in adulthood. On the other hand, while a predominant and consolidated memory B cell pool should be protective on re-exposure to the typical pathogens, it would leave adults more vulnerable to upper respiratory infections and tonsillitis from novel pathogens, than children. Interestingly, that was the case when COVID 19 emerged [19]. Of note, tonsils were identified as crucial sites of SARS-CoV-2 infection in children for an undetermined prolonged time, and surprisingly, with no symptoms [20].

Conclusions

Our data supports the notion of a link between the age-dependent causes of tonsillectomy and a reduction in the proportion of effector cells (B_{GC}), an increase in the proportion of memory B cells and an increase in the proportion of B cells with regulatory function as the patients age. With regards to T cells, Thf_{GC} decrease and mThf increase as age increases. Moreover, the proliferative status of local $CD4^+$ and $CD8^+$ T cells resulted even more affected than that of the B cell compartment.

The study contributes to the comprehension of the changes in the tonsillar immune response when advancing life stages and would help to predict the different results, in terms of immune responses, to local antigenic challenges in the life span of individuals.

Material and Methods

Isolation of cells. Primary human mononuclear cells were isolated from tonsils obtained from patients undergoing tonsillectomy. The particular number of samples per experiment were detailed in the corresponding Figure legends. Tonsillar mononuclear cells (TMC) were prepared as follows. Briefly, tonsils were collected in phosphate buffered saline (PBS) buffer containing 50 μg ml^{-1} amphotericin B (Richet, BA, Arg). Tissues were chopped with a scalpel and passed through a 70 μm-pore-size cell strainer (Falcon, Thermo Fisher, BA, Arg). TMC were purified by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). The viability of primary cells, as determined by trypan blue exclusion was greater than 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 use of clinical material, conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Informed consent was obtained from all participants and/or their legal guardian/s. FACS experiments were performed with freshly isolated cells and cultured cells.

Antibodies and fluorescence-activated cell sorting (FACS). Fluorochrome conjugated mAbs specific for human CD3 (Pacific Blue, clone SK7, BioLegend), human CD20 (FITC, clone L27 and APC H7 clone 2H7), human CD4 (PerCP, clone SK3, BioLegend), CD8 (APC Cy7 clone SK1, BioLegend), CD39 (APC, clone TU66, BD Pharmingen), CD73 (PE, clone AD2, BD Pharmingen), CD27 (FITC, clone M-T271, BD Pharmingen), CD38 (APC, clone HIT2, BD Pharmingen), CD44 (Bv510, clone IM7, BioLegend), CXCR5 (AF488 clone RF8B2, BD Pharmingen), PD1 (Bv711, clone EH12.2H7, BioLegend), CD10 (PE, clone ALB1,

Beckman Coulter), and respective isotype control mAbs were purchased from BD Biosciences (CA, USA) and Biolegend (CA, USA).

To detect Ki 67 transcription factor in the cells, the latter were incubated with Fixation/Permeabilization (eBioscience FOXP3/Transcription, Invitrogen) for 45 minutes and washed with Permeabilization Buffer (eBioscience FOXP3/Transcription, Invitrogen). Then, the cells were stained with anti-Ki67 mAb.

Cells were acquired using FACSAria II (BD Biosciences, CA, USA) and analyzed with FlowJo software (Treestar, OR, USA). Single stained controls were used to set compensation parameters. Fluorescence minus one and isotype-matched Ab controls were used to set analysis gates.

Immunohistochemistry. The 5 µm tissue sections mounted on silanized glass slides were deparaffinizated by two consecutive 5 min incubations xylene each, hydrated in decreasing concentrations of alcohol (100%, 96% and 70%) for 5 min each, followed by antigenic unmasking with Sodium Citrate Buffer (0,05 M, pH 6.0) in a thermostatic wáter bath at 95°C for 45 min.

The endogenous peroxidase was blocked by incubating tissue sections with 35% H_2O_2, 98.8% methanol PA (Synth) diluted in PBS pH 7.6 for 30 min, then washed with PBS-1X pH 7.6. The nonspecific binding sites were blocked with Bovine Serum Albumin (BSA) 5%. Ki67+ cells were detected by incubating with the primary antibody. The antibody dilution was 1/100 Ki-67 (Rabbit monoclonal clon SP6, TecnoLab, 275R-15). Slides were incubated one hour at 4°C and washed three times with PBS 1X, pH 7.6.

For the immunohistochemical staining system and counterstaining with hematoxylin, a fully automatic staining device, the Ventana BenchMark XT (Ventana Medical Systems, Roche Diagnostics Division) was used. The device uses a biotin-free, HRP multimer-based hydrogen peroxide substrate and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) chromogen (UltraVIEW Universal DAB Detection, Catalog number 760-500, Ventana Medical Systems, Tucson, USA)

The slides were examined using the Leica DM500 optical microscope (Leica Camera, Wetzlar, Alemania) at magnification of 20x and 40x.

Declarations

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Ethical Approval and Consent to participate. The institutional ethics committee (Clinical Hospital, School of Medicine, Buenos Aires) approved the collection and use of clinical material, conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Informed consent was obtained from all participants and/or their legal guardian/s.

Consent for publication. All authors of this manuscript concur with its submission.

Availability of supporting data. The authors confirm that the data supporting the findings of this study are available within the article. In case of need of further details, Eloísa Arana would provide them upon reasonable request.

Competing interests. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions. R.P and J.P processed most of the samples used in this study, performed most of the experiments and analyzed the data. PdlG and LSV performed a number of experiments. G.B, N.S and APL performed cryosections and H&E stainings. SC advised on design of FACS experiments. M.E A, B.P, AB and IAS provided samples. R.P, J.P, L.S.V, BP and FCh reviewed the manuscript. E.A supervised and designed research, analyzed the data and wrote the manuscript

References

1. Brandtzaeg P: **Potential of nasopharynx-associated lymphoid tissue for vaccine responses in the airways.** *Am J Respir Crit Care Med* 2011, **183:**1595-1604.
2. Sarmiento Varon L, De Rosa J, Machicote A, Billordo LA, Baz P, Fernández PM, Kaimen Maciel I, Blanco A, Arana EI: **Characterization of tonsillar IL10 secreting B cells and their role in the pathophysiology of tonsillar hypertrophy.** *Sci Rep* 2017, **7:**11077.
3. Mattila PS, Tahkokallio O, Tarkkanen J, Pitkäniemi J, Karvonen M, Tuomilehto J: **Causes of tonsillar disease and frequency of tonsillectomy operations.** *Arch Otolaryngol Head Neck Surg* 2001, **127:**37-44.
4. Sarmiento Varón L, De Rosa J, Rodriguez R, Fernández PM, Billordo LA, Baz P, Beccaglia G, Spada N, Mendoza FT, Barberis CM, et al: **Role of Tonsillar Chronic Inflammation and Commensal Bacteria in the Pathogenesis of Pediatric OSA.** *Frontiers in Immunology* 2021, **12**.
5. Saze Z, Schuler PJ, Hong CS, Cheng D, Jackson EK, Whiteside TL: **Adenosine production by human B cells and B cell-mediated suppression of activated T cells.** *Blood* 2013, **122:**9-18.

6. Barankiewicz J, Dosch HM, Cohen A: **Extracellular nucleotide catabolism in human B and T lymphocytes. The source of adenosine production.** *J Biol Chem* 1988, **263:**7094-7098.
7. Losenkova K, Paul M, Irjala H, Jalkanen S, Yegutkin GG: **Histochemical Approach for Simultaneous Detection of Ectonucleotidase and Alkaline Phosphatase Activities in Tissues.** *Methods Mol Biol* 2020, **2041:**107-116.
8. Pascual M, Alignani D, Vilas-Zornoza A, Delgado JA, Vázquez I, Malumbres R, Rodriguez I, Barriuso R, Calasanz MJ, Paiva B, et al: **Use of human pharyngeal and palatine tonsils as a reservoir for the analysis of B-cell ontogeny in 10 paired samples.** *Clin Otolaryngol* 2016, **41:**606-611.
9. Sarmiento Varón L, De Rosa J, Rodriguez R, Fernández PM, Billordo LA, Baz P, Beccaglia G, Spada N, Mendoza FT, Barberis CM, et al: **Role of Tonsillar Chronic Inflammation and Commensal Bacteria in the Pathogenesis of Pediatric OSA.** *Front Immunol* 2021, **12:**648064.
10. Rosenmann E, Rabinowitz R, Schlesinger M: **Lymphocyte subsets in human tonsils: the effect of age and infection.** *Pediatr Allergy Immunol* 1998, **9:**161-167.
11. Lee J, Chang DY, Kim SW, Choi YS, Jeon SY, Racanelli V, Kim DW, Shin EC: **Age-related differences in human palatine tonsillar B cell subsets and immunoglobulin isotypes.** *Clin Exp Med* 2016, **16:**81-87.
12. Gülay A: **Surgical Anatomy of the Tonsils.** In *Oral and Maxillofacial Surgery.* Edited by Gokul S. Rijeka: IntechOpen; 2020: Ch. 2
13. Medzhitov R, Schneider DS, Soares MP: **Disease tolerance as a defense strategy.** *Science* 2012, **335:**936-941.
14. McCarville JL, Ayres JS: **Disease tolerance: concept and mechanisms.** *Current opinion in immunology* 2018, **50:**88-93.
15. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK: **Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation.** *Immunity* 2002, **16:**219-230.
16. Farber DL, Yudanin NA, Restifo NP: **Human memory T cells: generation, compartmentalization and homeostasis.** *Nat Rev Immunol* 2014, **14:**24-35.
17. Antonioli L, Blandizzi C, Pacher P, Haskó G: **Immunity, inflammation and cancer: a leading role for adenosine.** *Nat Rev Cancer* 2013, **13:**842-857.
18. Danchin MH, Rogers S, Kelpie L, Selvaraj G, Curtis N, Carlin JB, Nolan TM, Carapetis JR: **Burden of acute sore throat and group A streptococcal pharyngitis in school-aged children and their families in Australia.** *Pediatrics* 2007, **120:**950-957.
19. Ludvigsson JF: **Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults.** *Acta Paediatr* 2020, **109:**1088-1095.
20. Lima TM, Martins RB, Miura CS, Souza MVO, Cassiano MHA, Rodrigues TS, Veras FP, Sousa JF, Gomes R, Almeida GM, et al. **Tonsils are major sites of prolonged sars-cov-2 infection in children.** *medRxiv* 2023**:**2023.2001.2021.23284592.

21. Dan JM, Havenar-Daughton C, Kendric K, Al-Kolla R, Kaushik K, Rosales SL, Anderson EL, LaRock CN, Vijayanand P, Seumois G, et al: **Recurrent group A Streptococcus tonsillitis is an immunosusceptibility disease involving antibody deficiency and aberrant T(FH) cells.***Sci Transl Med* 2019, **11**.

Figures

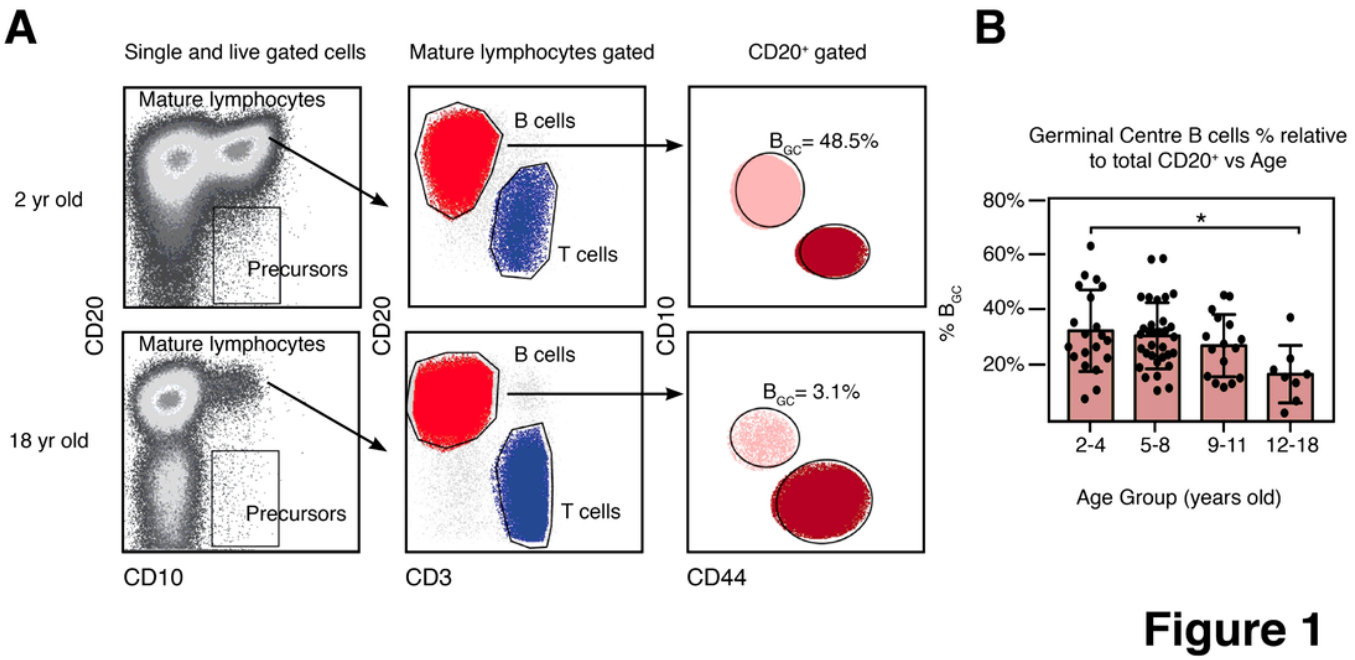

Figure 1

Figure 1

Age dependent distribution of tonsillar B-cell subpopulations. A) Fresh TMC were stained for surface CD20, CD10, CD3, CD44, CD38, CD27 and a dead/live stain as in [8]. Samples were subsequently analyzed by FACS. The gating strategy to identify the different B-cell populations is partially illustrated. Singlets were gated by plotting FSC-H vs FSC-A for each sample (not shown). Within the singlets population, dead cells were determined by a viability dye (not shown). Within the viable gate, lymphoid gate was determined through SSC-A vs FSC-A (not shown). Dot plot depicting the representative percentage of the tonsillar B cells subsets for individuals from the youngest (top) and oldest (bottom) groups of patients analyzed. Percentages and colors designate frequencies of the populations indicated. B) Histograms presenting the mean percentage ± SD of the B_{GC} cell population frequencies determined as in A), from 76 individuals, distributed according their age. *p* value was calculated through unpaired *t* test, *p<0.05.

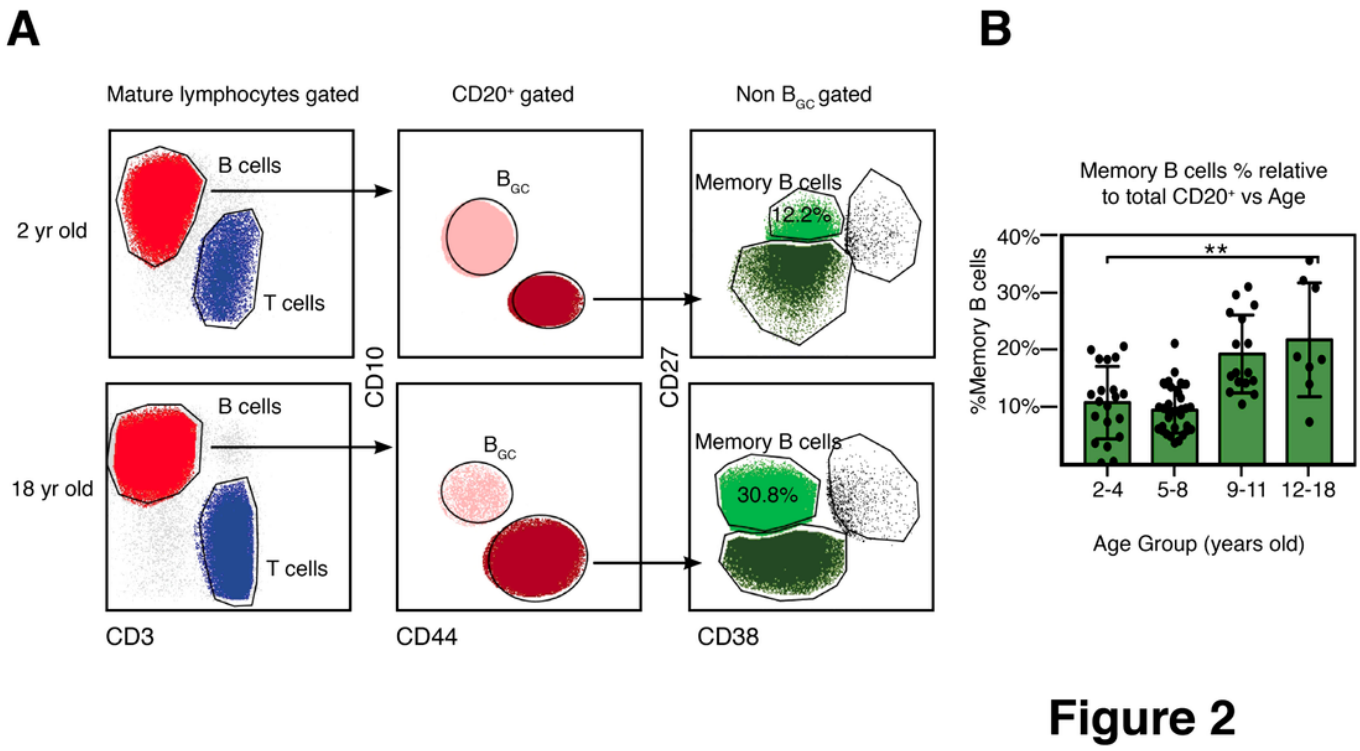

Figure 2

Figure 2

Memory B-cells displace B_{GC} with advancing age. A) Fresh TMC were stained for surface CD20, CD10, CD3, CD44, CD38, CD27 and a dead/live stain as in [8]. Samples were subsequently analyzed by FACS. The gating strategy to identify the different B-cell populations is partially illustrated as in Fig. 1. B) Histograms presenting the mean percentage ± SD of the memory B cell population frequencies determined as in A), from 76 individuals, distributed according their age. *p* value was calculated through unpaired *t* test, **p<0.01.

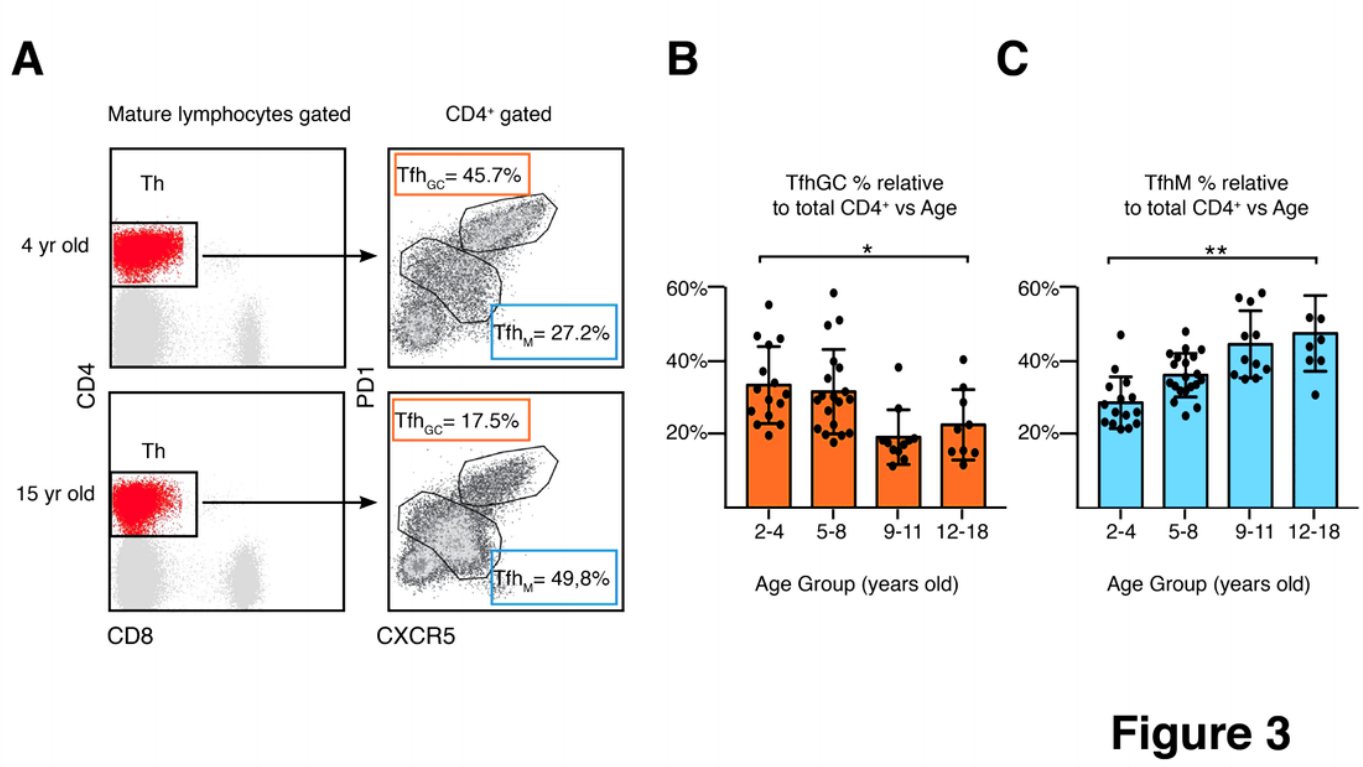

Figure 3

Age dependent distribution of tonsillar follicular T-cell subpopulations. A) Fresh TMC were stained for surface CD4, CD8, PD 1, CXCR5 and a dead/live stain. Samples were subsequently analyzed by FACS. The gating strategy to identify the different follicular T-cell populations is partially illustrated and was performed as in [21]. Dot plots depicting the percentage of the tonsillar Tfh subsets of selected individuals, from the youngest (top) and oldest (bottom) groups of patients analyzed. Percentages and colors designate frequencies of the populations indicated. B) Histograms presenting the mean percentage ± SD of the Tfh_{GC} population frequencies determined as in A), from 54 individuals, distributed according their age. *p* value was calculated through unpaired *t* test, *p<0.05, **p<0.01.

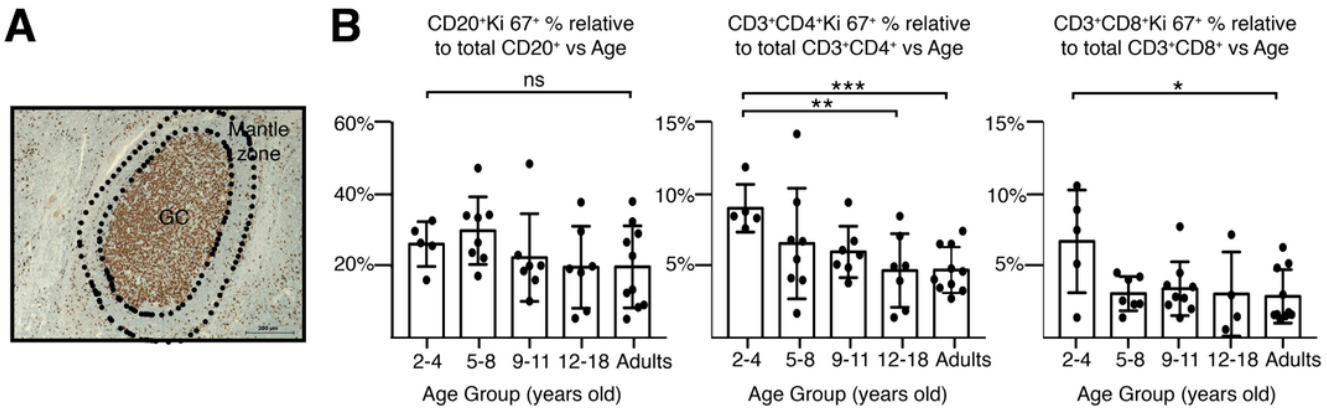

Figure 4

Figure 4

Teenagers and adults' tonsils have less proliferative cells than children. A) Representative Ki67-stained section showing single a germinal center (GC) with its mantle zone. Scale bar, 200 mm. B) Fresh TMC were stained for surface CD4, CD8, CD20 and CD3, also for intra-nuclear Ki 67 and a fixable viability dye. Samples were subsequently analyzed by FACS. Histograms presenting the mean percentage ± SD of the $CD20^{+}Ki\ 67^{+}$ cell population frequencies determined from 37 individuals distributed according their age (left panel), the mean percentage ± SD of the $CD3^{+}CD4^{+}Ki\ 67^{+}$ cell population frequencies determined from 37 individuals distributed according their age (middle panel), the mean percentage ± SD of the $CD3^{+}CD8^{+}Ki\ 67^{+}$ cell population frequencies determined from 35 individuals distributed according their age (right panel), *p* value was calculated through unpaired *t* test, *p<0.05, **p<0.01.

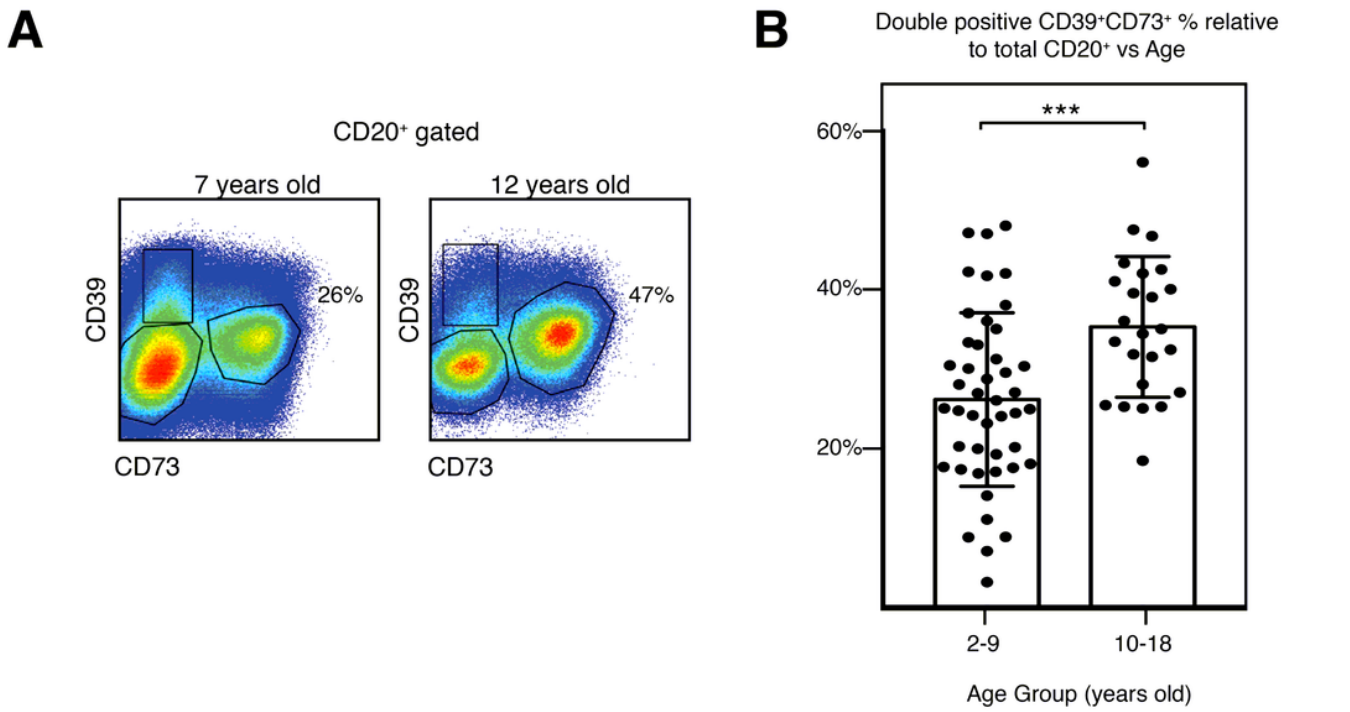

Figure 5

Figure 5

B-cells metabolically adapted to catabolize ATP increase with increasing age. A) Fresh TMC were stained for surface CD20, CD3, CD73, CD39 and a dead/live stain. Samples were subsequently analyzed by FACS. The gating strategy to identify the B-cell population analyzed, is partially illustrated. Singlets were gated by plotting FSC-H vs FSC-A for each sample (not shown). Within the singlets population, dead cells were determined by a viability dye (not shown). Within the viable gate, lymphoid gate was determined through SSC-A vs FSC-A as well as the CD20 gate (not shown). Dot plots depicting the percentage of the tonsillar B cells co-expressing CD73 and CD39 of selected individuals, from the children and teenagers group. B) Histograms presenting the mean percentage ± SD of the double positive population frequencies determined as in A), from 69 individuals, distributed according their age. *p* value was calculated through unpaired *t* test, ***p<0.005.