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Increased Cellular Proliferation and Inflammatory Cytokines in Tonsils Derived From Children With Obstructive Sleep Apnea

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ABSTRACT: Adenotonsillar hypertrophy is the major pathophysiological mechanism underlying obstructive sleep apnea (OSA) and recurrent tonsillitis (RI) in children. The increased expression of various mediators of the inflammatory response in tonsils of patients with OSA prompted our hypothesis that the enhanced local and systemic inflammation in children with OSA would promote tonsillar proliferation. Mixed cell cultures from tonsils recovered during adenotonsillectomy in children with OSA and RI were established, and proliferative rates were assessed. Cells were also cultured to determine the levels of proinflammatory cytokines and antioxidant protein levels and mRNA expression. Global cell proliferative rates from OSA tonsils were significantly higher than RI ($p < 0.01$), with $CD3^+$, $CD4^+$, and $CD8^+$ cell proliferation being higher in OSA ($p < 0.05$). Moreover, proinflammatory cytokines, such as TNF-α, IL-6, and IL-1α, were highly expressed in OSA-derived tonsils. Furthermore, thioredoxin (TRX), an antioxidant protein, was also highly expressed in OSA tonsils at the mRNA and protein levels ($p < 0.01$). Thus, T cells are in a highly proliferative state in the tonsils of children with OSA and are associated with increased production of proinflammatory cytokines and TRX, when compared with children with RI. **(*Pediatr Res* 66: 423–428, 2009)**

Obstructive sleep apnea (OSA), a condition characterized by repetitive increases in upper airway resistance and collapse, is a common health problem in children affecting 1 to 3% of children during their first decade of life (1). In a series of previous studies from our and other laboratories, it has become apparent that children with OSA are at increased risk for a vast array of morbidities, principally affecting the CNS and cardiovascular systems (2–9). Adenotonsillar hypertrophy has been recognized as the major pathophysiological contributor of OSA in children (10), and it plays an important role as well in recurrent tonsillitis (RI) (11). Consequently, adenotonsillectomy (T&A) is currently the first line of treatment for children with these conditions (12,13). However, the exact mechanisms underlying follicular lymphoid proliferation and hyperplasia remain extremely poorly understood. In adults, there are several lines of evidence, suggesting that both local upper airway and systemic inflammation are implicated in the pathophysiology of this *a priori* mechanical dysfunction of the upper airway. For example, the number of immune cells is substantially increased in the upper airway mucosa and the muscle of adults with OSA (14). Similar increases in regional and systemic inflammatory markers have also been reported in children with OSA (15–17). In addition, increased expression of mediators of the inflammatory response, such as cysteinyl leukotrienes and glucocorticoid receptors, is consistently found in tonsillar tissues of children with OSA (18–20). Therefore, we hypothesized that cellular proliferation of tonsillar tissues in children with OSA would differ from that of children with RI, possibly reflecting different pathologic mechanisms and cell type involvement leading to the enlargement of upper airway lymphoid tissue in these two conditions.

METHODS

Subjects. The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained for each participant. Consecutive children with adenotonsillar hypertrophy who underwent tonsillectomy for either OSA or RI were identified. Overnight polysomnography was performed using standard methods (21–23). The diagnosis of OSA was defined as an obstructive apnea-hypopnea index (AHI) $\geq$5/h of total sleep time. Diagnosis of RI was based on a history of >5 tonsillar infections over a period of <6 mo, requiring administration of antibiotics, in the absence of any symptoms suggestive of OSA using a validated questionnaire (24). Overnight sleep studies were performed in the majority of children with RI (13 of 20), and showed AHI <1/h of total sleep time.

Cell culture. Tonsils were placed in ice-cold PBS plus antibiotics, and processing began <30 min after surgical excision. Tonsils were manually dissected and gently grounded with a syringe plunger through a 70-μ mesh screen. Red blood cells were removed by lysis buffer. Cellular viability was determined by trypan blue exclusion. Specimens with a viability <70% were discarded. Cell cultures were established in standard medium RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics, which included streptomycin, fungisone, gentamicin, and penicillin. Mixed cell suspensions were transferred onto 96-round-bottom well plates at a concentration of 1×10^6 cells/well and cultured in a 5% CO_2 incubator at 37°C for 48 h. Cells were also cultured using 24-well plates to determine the proinflammatory cytokine levels, to conduct flow cytometric analysis, and to extract RNA for real time quantitative PCR assays or protein for western blot analyses.

Total cell proliferation assay. Cell proliferation was measured using a CellTiter 96 AQ nonradioactive cell proliferation assay (Promega, Madison, WI). Briefly, cells were plated in 96-well plates at a density of 1×10^6 cells/well in 200 μL of medium for 48 h; then MTS [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-phenozine methosulfate solution (30 μL/well) was added to the wells. After

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Abbreviations: AHI, Obstructive Apnea Hypopnea Index; **BrdU,** bromodeoxyuridine; **OSA,** obstructive sleep apnea; **RI,** recurrent infection; **TRX,** thioredoxin

incubation for 4 h, the absorbance was measured at 490 nm using a plate reader (Multiscan EX, Thermo Scientific, Waltham, MA). Data representing the average of four wells were considered as one experiment.

Bromodeoxyuridine (BrdU) cell proliferation assay with flow cytometry. To detect T-cell- and B-cell-specific proliferation, we used BrdU pulsed proliferation analysis using flow cytometry. All procedures were measured using the APC BrdU flow kit (BD Biosciences, San Diego, CA), as recommended by the manufacturer. In brief, at the end of 48 h of cell culture, cells were pulse-labeled with 1 mM BrdU for 4 h. BrdU-labeled cells were stained with a 3-color antibody combination consisting of mouse anti-human CD45/PerCP Cy7, CD3/PE, and CD19/APC-Cy7 antibodies (BD Biosciences, San Diego, CA) in 50 μL staining buffer for 15 min on ice. Cells were fixed and permeabilized with cytofix or cytoperm buffer, suspended with DNase (300 μg/mL) for 1 h at 37°C, and anti-BrdU APC antibody was added and incubated for 20 min at room temperature. Isotype controls relevant for each antibody were used to establish background fluorescence. Negative control was used as a sample, which was untreated with BrdU and was not stained with specific fluorescence antibodies. Data were acquired on a FACS Aria flow cytometer using the FACS Diva 5.5 software (BD Biosciences, San Diego, CA). After gating of lymphocytes based on $CD45^+$ cells, T-cell and B-cell numbers were calculated as $CD3^+/CD19^-$ and $CD3^-/CD19^+$ cell populations, respectively. Moreover, counting $CD3^+/BrdU^+$ and $CD19^+/BrdU^+$ cell populations identified proliferation of T cells and B cells. The results were displayed as two color dot-plots and analyzed by FlowJo software (Tree Star, San Carlos, CA). All data are expressed as the percentage of positive cell from the total cell population.

Quantitative real-time RT-PCR for cytokine expression and thioredoxin. Total RNA was isolated with Qiagen RNAeasy mini kit (Qiagen Inc, CA). Total RNA was quantified by spectrophotometer based on the absorbance A_{260}/A_{280} ratio. Quantitative real-time PCR were performed using TaqMan one-step RT-PCR reagents kits (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The genes examined included TNF-α (NM_000594), IL-6 (NM_000600), IL-1 α (NM_000575), thioredoxin (TRX) (×77584), CD4 (NM_000616.), and CD8a (NM_171827). In brief, all reactions were carried out in a final volume of 20 μL containing 11.5 μL 2× master mix, 0.5 μL RNase inhibitor mix, 1 μL primer (20 μM), 200 ng total RNA, and the volume of RNAse free water was adjusted with template. Thermocycling was run on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) as follows: 30 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. Samples were normalized using the housekeeping gene, ribosomal 18S rRNA. Individual gene expression was calculated using the comparative Ct method (25). Specific mRNA expression in OSA was expressed as the relative fold increase compared with RI samples.

Detection of TNF-α, IL-6, and IL-1α in cell culture supernatants. Proinflammatory cytokines levels, such as TNF-α, IL-6, and IL-1 α, were measured in cell-free supernatants with commercially available kits (R&D system, Minneapolis, MN) according to protocols provided by the manufacturer. Each of samples was assayed in duplicate. Interassay and intraassay coefficients of variability ranged from 3.4 to 5.3% and 3.4 to 4.5%, respectively.

Western blots. Cells were lysed using a nuclear extract kit (Active Motiff, Carlsbad, CA). Protein concentrations were measured using the DC protein assay (BioRad, Hercules, CA). TRX expression was assessed by immunoblotting with anti-human TRX MAb (1:1000, BD Biosciences, San Diego, CA) and mouse anti-rabbit IgGs that were conjugated to horseradish peroxidase in a Tris-buffered saline supplemented with 5% nonfat dry milk. Bands corresponding to TRX were visualized with ECL and quantified in a scanner with ImageQuant software (Molecular Dynamics, GE Health Science). Normalization of integrated densities was performed by reprobing membranes with β-actin antibody.

Immunohistochemistry. Coronal sections (40 μm) of tonsils from OSA and RI were initially incubated in 1× citrate buffer (Lab Vision Corporation, Fremont, CA) at 95°C for 45 min, washed several times in PBS, and blocked with a PBS or 0.4% Triton X-100/0.5% Tyramide Signal Amplification (TSA, Perkin Elmer Life Sciences, Boston, MA) blocking reagent or 10% normal horse serum for 1 h. Sections were then serially incubated with anti-CD4 antibody (1:300, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CD8 antibody (1:1000, BD Pharmagen, San Jose, CA) at 4°C for 24 h, and then washed in PBS six times for 5 min each wash. Sections were incubated for 1 h in horse anti-mouse biotinylated antibody (1:400, Vector Labs, Burlingame, CA) in a PBS or 0.4% TSA blocking reagent or 10% horse serum solution and then with streptavidin-horseradish peroxidase diluted 1:100 in PBS/0.5% TSA blocking reagent. Subsequently, the sections were incubated with TSA fluorescein reagents diluted 1:50 in amplification diluent (Perkin Elmer Life Sciences) for 2 min. Sections were then washed and mounted onto glass slides. Negative controls were prepared by either omitting the primary or the secondary antibody. Sections were prepared from five sets of tonsils from either OSA or RI groups and were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

Statistical analysis. All data were expressed by mean ± SD. Statistical analyses were performed using SPSS software (version 16.0; SPPS Inc., Chicago, IL). All p values reported are two-tailed with statistical significance set at <0.05.

RESULTS

Study population. The demographic, polysomnographic, and tonsillar cell distribution characteristics of the OSA and RI cohorts are shown in Table 1. The mean age of children with OSA and RI was 6.2 ± 2.8 y and 5.8 ± 2.2 y, respectively, and genders and ethnicities were similarly represented. All OSA subjects had AHI >5/hrTST, with 12 subjects having AHI <10/hrTST, 10 subjects with AHI ≥10 but <20 /hrTST, and 3 subjects with AHI >20/hrTST. The total percentage of lymphocytes was higher in children with OSA, and the percentage of $CD3^+$ T lymphocytes was also higher in children with OSA compared with RI (18.1 ± 4.1% *versus* 9.3 ± 3.9%, $p < 0.05$). In contrast, $CD19^+$ B lymphocytes tended to be less abundant in OSA (42.8 ± 3.8% *versus* 48.1 ± 3.5%; $p = 0.055$).

Cell proliferation in tonsil mixed cell culture. Cellular proliferative assays showed that the tonsils of children with OSA had significantly higher proliferative rates than those of RI (Fig. 1; OD units: 1.45 ± 0.06 *versus* 0.82 ± 0.02, $p < 0.01$).

T-cell and B-cell proliferation in tonsils. To assess T-cell and B-cell proliferation, we used the BrdU proliferation assay in combination with flow cytometry. Figure 2*A* illustrates the strategy for identification of and sorting of T cells and B cells. As shown in Figure 2*B*, the proportion of proliferating of $CD3^+$ T cells was significantly higher in children with OSA compared with children with RI (OSA *versus* RI: 5.49 ± 2.10% *versus* 2.74 ± 0.97%, $p < 0.05$). However, proliferation of $CD19^+$ B cells in children with OSA was reduced (OSA *versus* RI: 2.80 ± 1.04% *versus* 4.3 ± 1.41%, $p < 0.05$). In addition, mRNA expression studies in tonsil cultures

Table 1. *Demographic, polysomnographic, and tonsillar lymphocyte distribution characteristics in 25 children with obstructive sleep apnea and 20 children with recurrent tonsillitis*

	OSA (n = 25)	RI (n = 20)
Age (y)	6.2 ± 2.8	5.8 ± 2.2
Gender (F/M)	14/11	11/9
African American (n/%)	9/36	7/35
AHI (per hrTST)*	13.3 ± 3.5	0.3 ± 0.1
Nadir SaO_2 (%)*	80.3 ± 3.7	95.1 ± 0.4
Arousal Index (per hrTST)*	19.7 ± 4.3	6.9 ± 0.3
$CD45^+$ Lymphocytes (%)*†	82.6 ± 3.2	72.3 ± 3.6
$CD19^+$ B cells (%)	42.8 ± 3.8	48.1 ± 3.5
$CD3^+$ T cells (%)†*	18.1 ± 4.1	9.3 ± 3.9

All data are expressed as mean ± SD; for RI, polysomnographic data were acquired in 13 subjects only with seven remaining subjects having no evidence of increased risk for OSA based on validated questionnaire (24).

* $p < 0.05$.

† Data are expressed as percentage of positive cell of total cell population.

$CD19^+$ B cells and $CD3^+$ T cells were calculated from the flow cytometer as $CD19^+/CD3^-$ and $CD19^-/CD3^+$ cell populations, respectively.

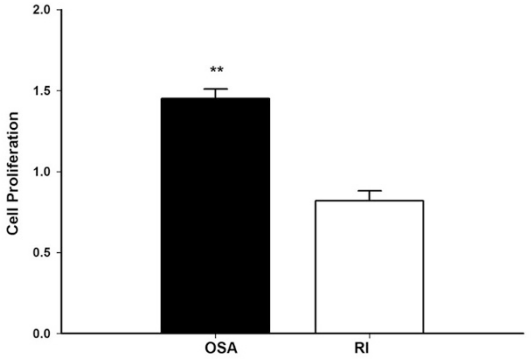

Figure 1. Tonsillar cellular proliferation in children with OSA ($n = 25$) and RI ($n = 20$) in a mixed cell culture system. Cell proliferation was assessed using a colorimetric method and is expressed in relative 490 nm absorbance units. $^{**}p < 0.01$.

from children with OSA and RI confirmed that both $CD4^+$ and $CD8^+$ mRNA were highly expressed in children with OSA compared with RI (Fig. 3), with $CD4^+$ being the predominant cell type (Fig. 3). Both these T cell types were primarily located in the tonsillar extrafollicular area.

Protein concentration and mRNA expression of TRX. Figure 4 shows mRNA and protein expression of TRX in cells derived from tonsils in children with OSA and RI. As seen in Figure 4*A*, there was a 2.6-fold increase in TRX mRNA expression in children with OSA ($p < 0.01$). In addition, western blots confirmed these findings, such that TRX protein expression was markedly increased in tonsils from children with OSA (OSA *versus* RI; relative intensity: 0.86 ± 0.19 *versus* 0.28 ± 0.16, $p < 0.01$).

Proinflammatory cytokine expression in mixed tonsil cell culture system. Figure 5*A* shows the concentrations in supernatants of proinflammatory cytokines. TNF-α and IL-6 concentrations were higher in children with OSA than in cultures from children with RI (OSA *versus* RI: TNF-α: 29.2 ± 6.2 pg/mL *versus* 10.5 ± 5.6 pg/mL, $p < 0.01$; IL-6: 49.6 ± 7.3 pg/mL *versus* 22.6 ± 9.7 pg/mL, $p < 0.01$). Similarly, IL-1α levels were also increased in OSA samples (OSA *versus* RI: 42.2 ± 9.81 pg/mL *versus* 25.5 ± 8.7 pg/mL, $p < 0.05$). As seen in Figure 5*B*, mRNA expression of TNF-α, IL-6, and IL-1α in tonsillar cells from children with OSA was significant higher than in children with RI ($p < 0.05$).

DISCUSSION

In this study, we show that proliferative rates are increased in tonsil mixed cell cultures harvested from children with OSA during routine T&A compared with children with RI, and this process seems to be mediated by T cells, whereas the reverse seems to occur in RI, with B cells assuming a more predominant role. Furthermore, both expression and release of proinflammatory cytokines to the supernatants, such as TNF-α, IL-6, and IL-1α, were increased in OSA, and in addition, evidence for up-regulation of the gene TRX and its transcript protein in OSA-derived cells. Taken together, the

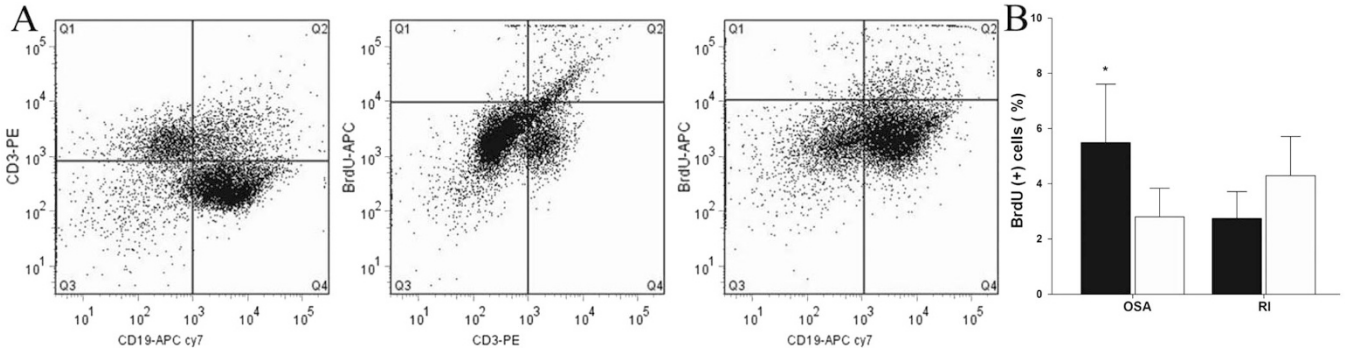

Figure 2. Proliferation of CD3+ and CD19+ cells in tonsils from children with OSA and RI in a mixed cell culture system. *A*. Illustrative example of the flow cytometry-based strategy for detection of CD3+ and CD19+ cell proliferation. *B*. CD3+ (■) and CD19+ (□) cell proliferation in tonsillar cultures in a mixed cell culture system in children with OSA ($n = 12$) and RI ($n = 11$). Data are expressed as the percentage of BrdU+ cells out of total CD3+ and CD19+cell populations. $^{*}p < 0.05$.

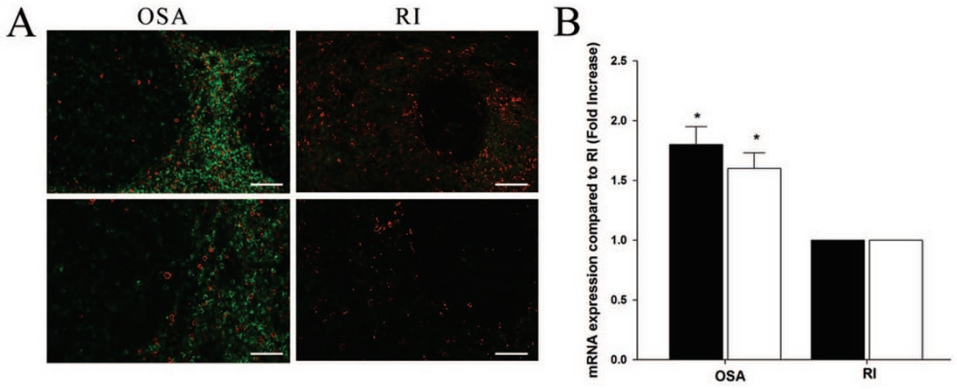

Figure 3. CD4+ and CD8+ expression in intact tonsillar tissues and in a mixed cell culture system in children with OSA and RI. *A*. Representative immunohistochemical assessment for CD4+ and CD8+ in tonsils from children with OSA and RI. A higher abundance of CD4+ (Green) and CD8+ (Red) is apparent in the children with OSA. Scaling bars indicate 200 μm for upper panels and 50 μm for lower panels. *B*. CD4 (■) and CD8 (□) mRNA expression in a mixed tonsillar cell culture system in children with OSA ($n = 12$) and RI ($n = 11$). Data are expressed as relative fold increases relative to RI. $^{*}p < 0.05$.

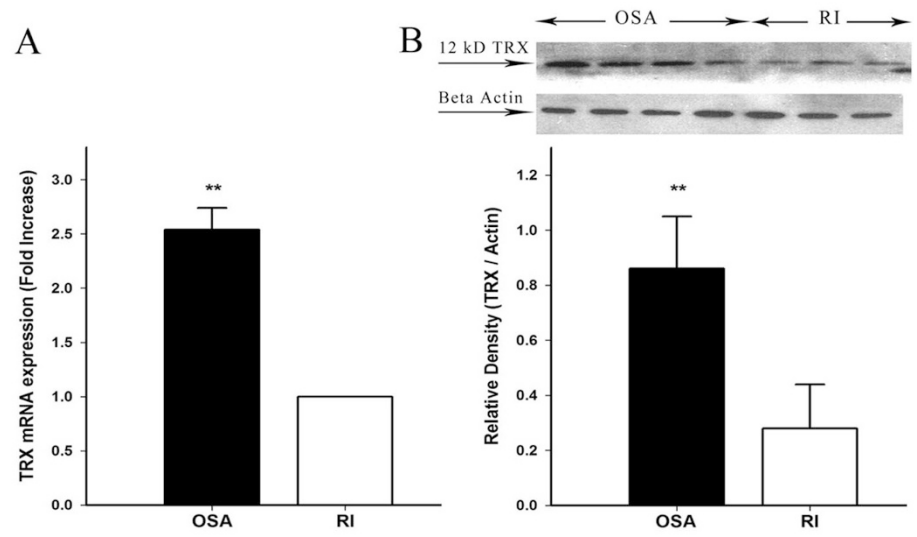

Figure 4. TRX expression at both protein and mRNA levels in children with OSA ($n = 12$) and RI ($n = 11$) in a mixed tonsillar cell culture system. *A*. TRX mRNA expression in children with OSA compared with children with RI, Data are expressed as fold increase relative to RI. *B*. Representative immunoblots are shown of TRX (detected at 12 kD) and β-actin. Data are summarized graphically in relative intensity units. $^{**}p < 0.01$.

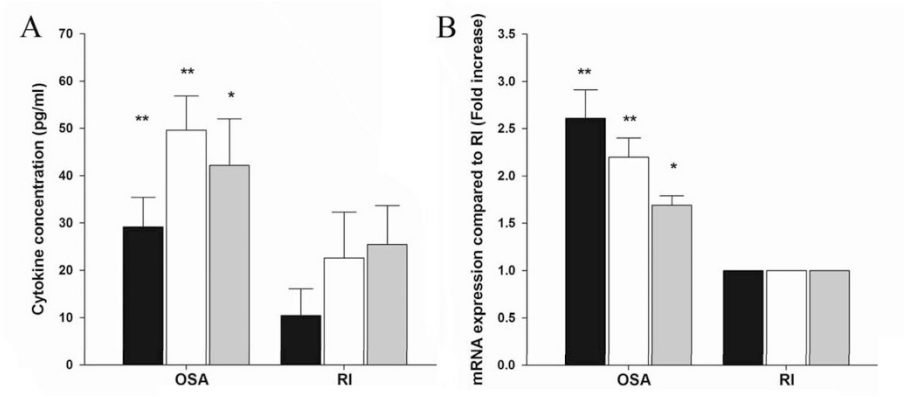

Figure 5. Pro-inflammatory cytokine expression at both protein and mRNA levels in children with OSA and RI in a mixed tonsillar cell culture system. *A*. TNF-α (■), IL-6 (□), and IL-1α (▨) pro-inflammatory cytokine concentrations in cell-free supernatants in children with OSA ($n = 23$) compared with children with RI ($n = 19$). *B*. TNF-α (■), IL-6 (□), and IL-1α (▨) mRNA expression in children with OSA ($n = 12$) compared with children with RI ($n = 11$). Data are expressed as fold increase. $^{*}p < 0.05$, $^{**}p < 0.01$.

data presented herein suggest that the mechanisms underlying upper airway lymphadenoid tissue proliferation in OSA and RI are distinct and may allow for future nonsurgical disease-specific therapeutic interventions that may ultimately obviate the need for T&A, while leading to involution of the hypertrophic adenoids and tonsils.

Several methodological issues deserve to be addressed. First, the remarkable similarity between the qualitative findings in the tonsillar immunohistochemistry regarding cell type distribution and the percentage of lymphocytes subtypes identified in cell culture using flow cytometry would suggest that processing of the tonsillar tissues harvested during surgery did not alter the constitutive cell populations of these tissues. Moreover, the standard procedures used herein would be expected to have a similar influence on tonsils collected from children with OSA and RI. Thus, the differences in proliferation, cytokine release, and TRX expression between the two cohorts seem to reflect the divergent intrinsic properties of these tissues, rather than reflect consequences of procedural methodologies. However, we should also remark that some differences in lymphocyte properties have been noted when different tissue processing procedures were used (26). Second, only palatine tonsils were included in this study, and therefore, differences in cytokine networks could emerge between nasopharyngeal lymphadenoid tissues and palatine tonsils, and future studies will have to examine this issue (27). Third, the two groups had similar age, gender, and ethnicity, and overnight sleep studies were conducted in the majority of children with RI, such as to more objectively confirm the two major diagnoses leading to T&A in children. Obviously, tonsil tissues from healthy children are unavailable for ethical reasons. Finally, we did not specifically assess the contributions of allergic sensitization and viral exposures on the etiology of tonsillar proliferation in either OSA or RI. Previous studies have reported a high prevalence of allergic sensitization in children with OSA (28,29), and early exposure of respiratory viruses could modify the proliferative properties of tonsils through up-regulation of nerve growth factor and neurokinin 1 receptor dependent pathways (22,30,31). Thus, further studies regarding the potential contributions of these factors are needed.

There is no doubt that adenotonsillar hypertrophy constitutes the primary contributor to OSA in children, and even if T&A does not always result in cure, significant improvements in the severity of sleep-disordered breathing are usually the rule (32–35). However, very little is known on the mechanisms that mediate proliferation of tonsils in children with either OSA or RI. We have previously shown that pathways of inflammation play a role, because both topical corticosteroids and leukotriene receptor modifiers were found to be highly

effective in reversing tonsillar hypertrophy, and their receptors display increased expression patterns in the upper airways of children with OSA (16,18–21,36,37). Moreover, nerve growth factor and substance P, a major controller of sensorineural development and neuroimmuno-inflammatory responses, are also overexpressed in the tonsils tissues from children with OSA (22). Notwithstanding such encouraging results, these studies did not quantitatively assess the proliferative characteristics of tonsillar tissues in OSA or RI and did not provide information as to the major cellular populations that are regulated in this process. Therefore, we used a mixed *in vitro* cell culture model to more quantitatively investigate potential relationships between inflammatory responses and cell proliferation.

Considering the increased inflammatory markers in the upper airway of pediatric patients with OSA (16–19,21,22), the increased proliferative activity in tonsils from children with OSA was not unanticipated. However, and of particular interest, were the findings on the differential patterns of proliferation among T cells and B-cells in the two patient cohorts, with a T cell predominant response in OSA being associated with higher expression and release of the proinflammatory cytokines TNF-α, IL-6, and IL-1α. It is now accepted that recurrent vibration in the upper airway may promote the development of local inflammatory responses, leading to mucosal swelling (38,39), lymphadenoid tissue proliferation, and upper airway obstruction. Indeed, $CD4^+$, $CD8^+$, and activated $CD25^+$ T cells were preferentially present in the mucosa and underlying muscle of the upper airway of adult patients with OSA (14). These findings have been recently confirmed (40). In children, Li *et al.* (17) showed marked increases in sputum neutrophil counts in OSA that correlated with the severity of the disease. The increases in serum high sensitivity C-reactive protein levels in children OSA would further attest to the presence of a systemic, inflammatory process that could also contribute to the increased proliferation of the upper airway lymphoid tissues (41–44). Based on aforementioned considerations, the favorable response of children with OSA to topical corticosteroids (37,45), and current findings, we propose that local and systemic activation of inflammatory pathways will promote preferential T-cell proliferation and upper airway lymphoid hyperplasia.

TRX has been characterized as a new oxidative stress inducible protein, and it plays an important role in intracellular signaling and resistance against oxidative stress (46). A multitude of stimuli may lead to increased TRX cellular expression, including hypoxia, lipopolysaccharide, hydrogen peroxide, and viral infections (47,48). TRX is actively secreted by a variety of normal and transformed cells, including fibroblasts, airway epithelial cells, and activated T cells (49). In a previous study, Park and Suzuki (50) proposed that TRX is mechanistically involved in intermittent hypoxia-mediated alterations in the susceptibility of the heart to oxidative stress. Recently, and in support for a potential role of TRX in OSA, adult patients with this condition had higher plasma TRX levels that were reduced after treatment with continuous positive airway pressure (51). In this study, we found that expression of TRX was markedly higher in tonsillar cells derived from children with OSA. Although we are still unclear as to the mechanisms responsible for the differential expression of TRX, we postulate that TRX may be involved in protection from oxidative stress and may also be modulating T-cell proliferative activity, and we cannot exclude a role in other inflammatory cell types, such as neutrophils. If the latter proves to be accurate, it may provide a viable target for development of interventional approaches for treatment or prevention of tonsillar hypertrophy in children with OSA.

In summary, we have established that T cells are in a highly proliferative state in the tonsils of children with OSA and are associated with increased production of proinflammatory cytokines and TRX, when compared with children with RI. These findings not only shed additional light on the differential regulatory mechanisms underlying tonsillar hypertrophy in two common pediatric conditions, namely RI and OSA, but also provide an *in vitro* model that should permit objective characterization and development of specific treatments for these disease processes.

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