Association of Pneumococcal Carriage and Expression of Foxp3+ Regulatory T Cells and Th17 Cells in the Adenoids of Children

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Key Words
Foxp3+ regulatory T cells · Th17 cells · Pneumococcal carriage · Adenoid

Abstract
Background: Pneumococcal carriage in the nasopharynx is a primary means of transmission and a necessary prerequisite for pneumococcal disease. Objectives: We analyzed the relationship between expressions of Foxp3+ regulatory T (T\textsubscript{reg}) cells and Th17 cells, and pneumococcal carriage in the adenoids of children who were either positive or negative for pneumococci. Methods: We collected adenoidal tissue and nasopharyngeal swab samples from children undergoing an adenoidectomy. Adenoidal mononuclear cells were isolated, cultured and then stimulated with culture concentrated supernatant (CCS) obtained from a D39 bacterial strain. Results: Foxp3+ T\textsubscript{reg} cells were upregulated and Th17 cells were downregulated in populations of adenoidal mononuclear cells obtained from the pneumococcus-positive group. Following CCS stimulation, the increment in Foxp3+ T\textsubscript{reg} cells in the pneumococcus-positive group was significantly greater than that in the pneumococcus-negative group, while the increment in Th17 cells was less as compared to that in the pneumococcus-negative group. These results were consistent with variations in levels of Foxp3 mRNA and retinoic acid receptor-related orphan receptor-yt mRNA in adenoidal mononuclear cells. Levels of IL-17A and IL-6 in adenoid tissue were higher in the pneumococcus-negative group, and the levels of TGF-β in adenoid tissue were lower in the pneumococcus-negative group compared to the pneumococcus-positive group. Pneumococcal carriage in children was closely associated with the expressions of Foxp3+ T\textsubscript{reg} and Th17 cells in the adenoid. Conclusion: Upregulation of Foxp3+ T\textsubscript{reg} cells might downregulate the production of Th17 cells in the adenoid, resulting in decreased scavenging of \textit{Streptococcus pneumoniae} and chronic pneumococcal carriage.

Introduction

\textit{Streptococcus pneumoniae} (pneumococcus) is the most common cause of community-acquired pneumonia, which is the leading infectious cause of morbidity, mortality and severe pneumonia among children younger than 5 years in low and middle income countries [1]. It has been estimated that in 2010, there were 120 million episodes of pneumonia (14 million of which progressed to severe episodes) in children younger than 5 years, and...
in 2011, 1.3 million cases of pneumonia led to death [2]. Pneumococcus frequently colonizes the nasopharynx and may then spread directly via the airway to the lower respiratory tract to cause pneumonia, or to the sinuses or middle ears to cause various types of morbidity [1]. The adenoid, which is the primary area of pneumococcus colonization within the nasopharynx, is a mass of nasal lymphoid tissue. Pneumococcal carriage is both the primary means of transmission and a necessary prerequisite for invasive pneumococcal disease, and among young children, a high pneumococcal carriage rate is related to the occurrence of pneumococcal disease [3, 4]. Hence, promoting the elimination and shortening the carriage time are effective measures for preventing invasive pneumococcal disease [5].

CD4+ T cells can differentiate into corresponding types of effector CD4+ T cells (Th1, Th2 and Th17 subsets) which protect against different kinds of pathogens. By stimulating the production of distinct sets of cytokines and other soluble and cell-bound products, these cells may act as immune effectors which eliminate infected cells [6]. It has recently been shown that Th17 cells and IL-17 function during infections to eliminate pathogens such as Helicobacter pylori, Mycobacterium tuberculosis and Toxoplasma, etc. [7, 8]. The number and activation of CD4+ T cells, and especially Th17 cells, may adversely impact the cytokine network and a patient’s recovery from pneumococcal pneumonia, as well as their ability to eliminate streptococci [9, 10].

During the last decade, regulatory T (Treg) cells expressing the transcription factor Foxp3, which play an important regulatory role during infections, have been identified as immune and inflammation suppressors and as being vitally important for the maintenance of immune homeostasis. However, excessive numbers of Foxp3+ Treg cells can lead to a persistent infection [6]. A further study showed that Foxp3+ Treg cells can manipulate inflammation mediated by Th17 cells [11]. An imbalance between Th17 cells and Foxp3+ Treg cells may play a central role in certain diseases. Defects in the Th17 cell differentiation axis may predispose a host organism to bacterial and fungal infections. Moreover, the differentiation mechanisms for Treg and Th17 cells are closely related, and TGF-β is a key factor in both processes [12].

An effective immune response by adenoid tissue promotes elimination of pneumococcus and can prevent the associated diseases. It has recently been reported that continuous pneumococcal carriage in the nasopharynx might be associated with the numbers and function of Foxp3+ Treg Cells in the adenoid [13]. Nevertheless, the evidence for a relationship between the numbers of Foxp3+ Treg and Th17 cells and pneumococcal carriage is poor, and the specific mechanism remains unclear. We hypothesized that upregulation of Foxp3+ Treg cells might downregulate the production of Th17 cells in the adenoid. This might suppress the scavenging activity of Th17 cells and lead to pneumococcal carriage. The present study was conducted to investigate the relationship between Foxp3+ Treg and Th17 cells in the adenoid tissues of children positive and negative for pneumococci, and explore the mechanism of pneumococcal carriage in the nasopharynx.

Materials and Methods

Specimen Collection

Nasopharyngeal swabs and samples of adenoid tissue were obtained from 47 children (age range, 3–8 years) on the day they underwent an adenoidectomy at the Children’s Hospital, Chongqing Medical University. Patients who received antibiotics or systemic steroids within 3 weeks of surgery, or who had any known immunodeficiency, chronic disease or respiratory tract infection at the time or 2 weeks prior to hospital admission were excluded from the study. No child in our study had been previously vaccinated against pneumococcus. The study protocol was approved by the Ethics and Human Research committees of the Children’s Hospital, Chongqing Medical University, and a written informed consent was obtained from the legal guardian of each patient prior to their enrollment in the study.

Identification of Streptococcus pneumoniae

Nasopharyngeal swabs were inoculated into culture plates containing Columbia blood agar and cultured overnight at 37 °C in an atmosphere of 5% CO₂. Afterwards, a grey, moist colony surrounded by a grass green hemolysis ring was inoculated into a second plate with Columbia blood agar that had an optochin test disk in its center. This plate was also incubated overnight at 37 °C in an atmosphere of 5% CO₂, after which the size of the hemolysis ring was measured. A hemolysis ring >14 mm in diameter indicated the colony was pneumococcus positive, while a ring <14 mm indicated it was pneumococcus negative.

Isolation and Culture of Adenoidal Mononuclear Cells

Samples of adenoid tissue were transported in minimum essential medium supplemented with glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) to our laboratory and processed within 1 h after the operation. The adenoid tissue samples were cut into pieces, filtered through a 300-mesh strainer and washed in phosphate-buffered saline. Adenoidal mononuclear cells were isolated by gradient centrifugation (2,000 rpm for 20 min, 20 °C) through a lymphocyte separation medium (TFD, Tianjing, China). The cell suspension was then divided into 4 layers. The adenoidal mononuclear cells contained in the second layer were washed twice with phosphate-buffered saline and then cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) containing penicillin, streptomycin, glutamine and 10% fetal bovine serum (Gibco, Grand Island, N.Y., USA). The viability of adenoi-
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Stimulation of Adenoidal Mononuclear Cells
The pneumococcal strains used in this study were the standard encapsulated type 2 (D39, NCTC7466). The concentrated culture supernatant (CCS) fraction from cultured D39 bacteria was used to stimulate adenoidal mononuclear cells. In brief, strain D39 bacteria were inoculated into a culture medium containing trypticase soy broth and grown to logarithmic phase (~10^8 cfu/ml) in 5% CO_2 at 37°C. The inoculum was then collected and centrifuged at 3,000 g for 30 min at 4°C. The culture supernatant was removed, passed through a 0.2-μm sterile filter and concentrated using a Vivaspin concentrator (Millipore, Boston, Mass., USA), and then adjusted to 1 μg/μl. Aliquots of CCS were then added to the adenoidal mononuclear cells. Some adenoidal mononuclear cells were cultured for 6 h, and others for 24 h in RPMI medium supplemented with 10% fetal bovine serum and antibiotics in 96-well plates in the presence or absence of CCS (1 μg protein/ml).

Flow Cytometry
The expressions of Foxp3 and IL-17A in adenoidal mononuclear cells in each group were analyzed by flow cytometry. Briefly, adenoidal mononuclear cells were cocultured with phorbol myristate acetate + ionomycin, CCS or medium for 6 h, with Golgi blockers (BD Bioscience, San Jose, Calif., USA) being added during the last 4 h. The cells were then stained with fluorescence-labeled mouse anti-human antibodies to CD4 and CD25 (CD4-FITC and CD25-PE; 4A Biotech, Beijing, China), and the intracellular expression of IL-17A (IL-17A-PE; BD Bioscience) was analyzed by flow cytometry. The IL-17A-expressing cells were then fixed and permeabilized (BD Bioscience). Foxp3-PEcy5 antibody (eBioscience, San Diego, Calif., USA) was used for intracellular staining of Foxp3, and fixation/permeabilization solutions (eBioscience) and ×10 permeabilization solution (eBioscience) were used to prevent nonspecific binding of monoclonal antibodies. All samples were preincubated with purified rat IgG. The resultant data were analyzed on a FACSCalibur flow cytometer (BD Bioscience) equipped with FlowJo software.

RNA Analysis
The expressions of Foxp3 mRNA and retinoic acid receptor-related orphan receptor-γt (ROR-γt) mRNA in adenoidal mononuclear cells in each group were detected by real-time quantitative PCR analysis. Briefly, total RNA was extracted from different samples using Trizol reagent (Invitrogen; Carlsbad, Calif., USA) according to the manufacturer’s instructions. After quantification, the RNA was reverse transcribed using a PrimeScriptRT reagent kit (TaKaRa, Shiga, Japan). Real-time quantitative PCR was performed to detect the relative expression levels of Foxp3 and ROR-γt. The real-time PCR conditions were as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. The specific primer sequences for Foxp3 were: forward, 5′-ATCCCCAGAGTTCTCTCA-CAA-3′; reverse, 5′-ATTGAGTGTCCGTGCTTCT-3′. The sequences for ROR-γt were: forward, 5′-AGTGCTGCTGTTAGGATGTG-3′; reverse, 5′-AGGGATGGGAAAGTCAAAAG-3′. The relative expression levels of Foxp3 and ROR-γt were analyzed using the 2^(-ΔΔCq) method.

Enzyme-Linked Immunosorbent Assay Analysis
Adenoidal mononuclear cells were divided into a control group and CCS group. The mononuclear cells were cultured and stimulated for 24 h, after which ELISA was used to measure the levels of IL-17A, IL-6 and TGF-β in the supernatants of the cultured cells. The ELISA kits used for measurements of IL-17A were purchased from eBioscience, and ELISA kits used for detecting IL-6 and TGF-β were purchased from Neobioscience (Beijing, China).

Statistical Analysis
Data are shown as the mean value ± standard deviation. Statistical differences were analyzed by the χ^2 test and Student’s t test. All analyses were performed using PASW Statistics for Windows, version 18.0, SPSS Inc., Chicago, Ill., USA. p values <0.05 were considered statistically significant.

Results

Nasopharyngeal Pneumococcal Carriage Rate
A total of 47 nasopharyngeal swab samples were obtained from children aged 3–8 years, and the overall pneumococcus carriage rate was 32%. There was no significant difference in the carriage rates shown by males (30.8% of subjects) and females (33.3% of subjects). The carriage rate among children aged 3–5 years was 35.5%, and that of the children aged 5–8 years was 25% (p = 0.465).

Expressions of Foxp3+ Treg and Th17 Cells in Adenoid Tissues from Pneumococcus-Positive and -Negative Groups
Flow-cytometric analyses showed significantly higher numbers of Foxp3+ Treg cells among adenoidal mononuclear cells obtained from the pneumococcus-positive group compared to the pneumococcus-negative group (6.70 ± 0.53 vs. 4.49 ± 0.24%, p < 0.001). However, the numbers of Th17 cells among adenoidal mononuclear cells obtained from the pneumococcus-positive group were significantly lower compared to Th17 cell numbers in the pneumococcus-negative group (1.70 ± 0.14 vs. 3.84 ± 0.51%, p < 0.01). These results indicated that increased numbers of Foxp3+ Treg cells and decreased numbers of Th17 cells were associated with nasopharyngeal carriage of S. pneumoniae (fig. 1, 2).

Expressions of Foxp3 mRNA and ROR-γt mRNA in Adenoid Tissue from Pneumococcus-Positive and -Negative Groups
Foxp3 mRNA expression in adenoid mononuclear cells obtained from the pneumococcus-positive group was significantly higher than that in mononuclear cells from the pneumococcus-negative group (2.15 ± 0.35 vs. 1.01 ± 0.23,
Nevertheless, the expression of ROR-γt mRNA in adenoidal mononuclear cells from the pneumococcus-positive group was significantly lower than in mononuclear cells from the pneumococcus-negative group (0.19 ± 0.03 vs. 0.52 ± 0.09, p < 0.01). These expressions of Foxp3 mRNA and ROR-γt mRNA were consistent with the numbers of Foxp3+ Treg and Th17 cells, indicating that increases in Foxp3 mRNA and decreases in ROR-γt mRNA were associated with nasopharyngeal carriage of S. pneumoniae (fig. 3).

**Fig. 1.** Numbers of Foxp3+ Treg cells among CD4+ adenoidal mononuclear cells. The flow cytometry results from a pneumococcus-negative sample (a) and a pneumococcus-positive sample (b) are shown, respectively. c Comparative numbers of Foxp3+ Treg cells in populations of CD4+ adenoidal mononuclear cells from the pneumococcus-negative and pneumococcus-positive groups, (pneumococcus-negative group: n = 10; pneumococcus-positive group: n = 10).

**Fig. 2.** Numbers of Th17 cells among CD4+ adenoidal mononuclear cells. The flow cytometry results from a pneumococcus-negative sample (a) and a pneumococcus-positive sample (b) are shown, respectively. c Comparative results for numbers of Th17 cells among CD4+ adenoidal mononuclear cells from the pneumococcus-negative and pneumococcus-positive groups (pneumococcus-negative group: n = 10; pneumococcus-positive group: n = 10).

CCS Stimulation Induced Greater Production of Foxp3+ Treg Cells and Foxp3 mRNA in the Pneumococcus-Positive Group

CCS was used to stimulate CD4+ adenoidal mononuclear cells obtained from the pneumococcus-positive and -negative groups, and then the productions of Foxp3+ Treg cells and Foxp3 mRNA were analyzed. Our results showed that following CCS stimulation, the increments in Foxp3+ Treg cells and Foxp3 mRNA in the pneumococcus-positive group were significantly higher than in the pneumococcus-negative group (fig. 4).
The pneumococcus-positive group were significantly higher than those in the pneumococcus-negative group (3.37 ± 0.31 vs. 1.54 ± 0.50, p < 0.01, and 0.97 ± 0.26 vs. 0.14 ± 0.21, p < 0.05, respectively; fig. 4). These results indicated that CCS stimulation induced a greater production of Foxp3+ Treg cells and Foxp3 mRNA in the pneumococcus-positive group compared to the pneumococcus-negative group.

**CCS Stimulation Induced Less Production of Th17 Cells and ROR-γt mRNA in the Pneumococcus-Positive Group**

We analyzed the production of Th17 cells and ROR-γt mRNA in CD4+ adenoidal mononuclear cells obtained from the pneumococcus-positive and -negative groups after CCS stimulation. Our results showed that following CCS stimulation, the increments in Th17 cells and ROR-γt mRNA in the pneumococcus-positive group were significantly lower compared to those in the pneumococcus-negative group (0.3 ± 0.07 vs. 0.85 ± 0.14% and 0.03 ± 0.02 vs. 0.44 ± 0.16%, p < 0.05, respectively; fig. 5). These results indicated that CCS stimulation was less effective at inducing production of Th17 cells and ROR-γt mRNA in the pneumococcus-positive group compared to the pneumococcus-negative group.

**The Levels of IL-17A, IL-6 and TGF-β in Adenoids from the Pneumococcus-Negative and -Positive Groups before and after CCS Stimulation**

We next investigated the cytokines in the supernatant fractions of adenoidal mononuclear cells in each group. We found that prior to CCS stimulation, the levels of IL-17A and IL-6 in the pneumococcus-negative group were significantly higher than those in the pneumococcus-positive group (81.85 ± 17.20 vs. 28.72 ± 5.61 pg/ml, p < 0.05, and 149.67 ± 19.12 vs. 33.85 ± 6.11 pg/ml, p < 0.001, respectively), while TGF-β levels in the pneumococcus-negative group were lower than those in the pneumococcus-positive group (741.02 ± 48.53 vs. 971.77 ± 31.36 pg/
30
ml, respectively, p < 0.05). However, the pneumococcus-positive and -negative groups showed similar variations in the levels of IL-17A, IL-6 and TGF-β in the following CCS stimulation (fig. 6).
Foxp3+ T regulatory (T reg) cells can be activated and expanded against cells (fig. 6). The increment in Foxp3+ T reg cells in the pneumococcus-positive group was significantly greater than that in the pneumococcus-negative group, while the increment in Th17 cells in the positive group was lower compared to that in the negative group (fig. 4). These changes were consistent with the changes in levels of Foxp3 mRNA and ROR-γt mRNA (fig. 5). Finally, we demonstrated that IL-17A levels were higher in the pneumococcus-negative group, which might reflect the production of Th17 cells, and levels of IL-6 were higher in the pneumococcus-negative group, which might result from the high percentage of Th17 cells among CD4+ adenoidal mononuclear cells. TGF-β levels were lower in the pneumococcus-negative group, which, based on comments by Ishigame et al. [12], might result in the high production of Th17 cells from CD4+ adenoidal mononuclear cells (fig. 6).

Foxp3+ T reg cells are generated in the thymus and are also induced from naive CD4+ T cells in peripheral sites. Foxp3+ T reg cells have been suggested as important immunoregulatory cells capable of suppressing the amplification and activation of effector CD4+ T cells [14, 15]. Foxp3+ T reg cells can be activated and expanded against a wide range of different pathogens in vivo. While such pathogen-specific Foxp3+ T reg cells may prevent infection-induced immunopathology, they may also prolong pathogen persistence by inhibiting protective immunity and thus favor the chronicity of infections. Th17 cells play an essential role in mucosal host defense by secreting signature cytokines (e.g., IL-17A, IL-17F and IL-22) to elicit neutrophil recruitment and production of antimicrobial peptides. A dysregulation of Th17 cells can result in immunologic derangement [16]. IL-17A released by Th17 cells was reported to have a host protective role during infections and chronic carriage of S. pneumoniae in animal models [17]. Delayed clearance and chronic pneumococcal carriage were first identified in IL-17 knockout mice [18, 19]. A recent study [20] demonstrated a protective role for Th17 cells against pneumococcal carriage. In our current study, we demonstrated that the numbers of Foxp3+ T reg cells in adenoidal tissues from pneumococcus-positive patients were significantly higher than those in adenoidal tissues from pneumococcus-negative patients, while Th17 cell numbers in pneumococcus-positive patients were significantly lower as compared to those in the pneumococcus-negative group. We also demonstrated that CCS stimulation in vitro could induce both Foxp3+ T reg and Th17 cell production. However, the increment in Foxp3+ T reg cells in pneumococcus-positive patients was significantly greater than that in pneumococcus-negative patients, while pneumococcus-negative patients showed a greater increase in Th17 cells. These results suggest that nasopharyngeal pneumococcal colonization may be associated with excess Foxp3+ T reg cell production in the adenoid. These excess Foxp3+ T reg cells may inhibit the effects of Th17 cells and contribute to the delayed clearance of pneumococci or the persistence of pneumococcal carriage in children. This mechanism would be consistent with the hypothesis that pneumococcal carriage in the nasopharynx might be associated with an abnormally high production of Foxp3+ T reg cells in the adenoid. The differentiation of Foxp3+ T reg and Th17 cells from naive CD4+ T cells is controlled by the local cytokine milieu and is critically dependent on TGF-β and IL-6. In the absence of IL-6, TGF-β mediates the generation of immunosuppressive T reg cells [21, 22]. The reciprocal balance between Foxp3+ T reg and Th17 cells is critical for immune homeostasis, and, if not properly regulated, Th17 cells can drive pathogenic inflammation [23]. High concentrations of IL-6 promote the differentiation of naive CD4+ T cells to form Th17 cells, and high concentrations of TGF-β promote the differentiation of naive CD4+ T cells to form Foxp3+ T reg cells through promoting expression of Foxp3 and suppressing expression of ROR-γt [12, 24]. It has been demonstrated that pneumococci can induce production of TGF-β1 by airway epithelial cells. This TGF-β1 then drives the generation of T reg cells in the nasopharynx. A blockade of TGF-β1 signaling can enhance neutrophil influx into the nasopharynx, leading to clearance of pneumococci [25]. In this study, we showed that levels of IL-6 and IL-17 in a group of pneumococcus-negative patients were significantly higher than those in pneumococcus-positive patients. Additionally, TGF-β levels in pneumococcus-negative patients were lower compared to those in pneumococcus-positive patients. These results suggest that high concentrations of TGF-β in adenoidal tissue may promote production of Foxp3+ T reg cells which suppress effector CD4+ Th17 cells.

In summary, we propose that pneumococcal carriage in children is closely associated with the levels of Foxp3+ T reg and Th17 cells in the adenoid, as well as secretion of cytokines such as TGF-β and IL-6. TGF-β is a key cytokine involved in generating T reg cell production in the nasopharynx. However, excessive numbers of Foxp3+ T reg cells may suppress effector CD4+ Th17 cells, leading to long-term pneumococcal carriage.
We have shown that upregulation of Foxp3+ T<sub>reg</sub> cell production may downregulate Th17 cells, resulting in less scavenging of <i>S. pneumoniae</i> and subsequent chronic pneumococcal carriage. However, our study has a limitation in that it involved only a small number of patients, and we did not classify the various strains of <i>S. pneumoniae</i> obtained from nasopharyngeal swabs. As our understanding of the complex relationships among Foxp3+ T<sub>reg</sub> cells, Th17 cells and adenoid tissue grows, there will undoubtedly be more opportunities for applying our knowledge of adaptive immunity to the management and ultimate prevention of streptococcal pneumonia.

Acknowledgments

The authors acknowledge financial support for this project from the National Nature Science Foundation of China (No. 81070015 and 81270086), the Chongqing Municipal Health Bureau (No. 2012-1-051) and the Chongqing Yuzhong District Science and Technology Commission (No. 20120213). We also wish to thank the Experimental Animal Centre at the Chongqing Medical University.

Financial Disclosure and Conflicts of Interest

The authors declare they have no conflicts of interest regarding the design or conduct of this study.

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