# A dynamic relationship between mucosal Th17 and Treg populations in nasopharynx evolves with age and associates with the clearance of pneumococcal carriage in humans

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# A dynamic relationship between mucosal Th17 and T regulatory cell populations that evolves with age and is a critical determinant for clearance of pneumococcal carriage in humans

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***Running title**: Mucosal Th17/Treg and pneumococcal carriage*

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**ABSTRACT**

*Nasopharyngeal carriage of Streptococcus pneumoniae* is a prerequisite of invasive pneumococcal disease. The carriage rate is high in young children and decreases with age. Host factors that determine the clearance or length of carriage which evolves with age remain unclear. We studied the relationship between mucosal IL17A-producing CD4+ T cells (Th17) and Foxp3+T regulatory cells (Treg) in human nasopharynx-associated lymphoid tissue (NALT) and their association with pneumococcal carriage in children and young adults. We show the frequencies of Th17 and Treg in NALT were inversely correlated ($p<0.01$) and both were considerably higher than in peripheral blood ($p<0.01$). Interestingly, we also show the Th17 frequency increases with age, whereas Treg frequency decreases with age. We further demonstrate the ratio of tonsillar Th17/Treg frequencies also increases with age ($p<0.01$) and the ratio was higher in pneumococcal carriage negative than in carriage positive individuals ($p<0.01$). Stimulation of tonsillar cells by a pneumococcal extract elicited a memory Th17 response that was more marked in the carriage– than in carriage+ children. Pneumococcal stimulation also induced Th17 differentiation from naïve tonsillar T cells, and the level of this Th17 induction in tonsillar T cells from young children was low but increased with age. By contrast, no such age-associated difference was seen in the induction of Treg following pneumococcal stimulation. Our results suggest a dynamic relationship between mucosal Th17 and Treg in human nasopharynx as children grow with age. The balance between Th17 and Treg in NALT evolves with age and appears to be a critical determinant for pneumococcal clearance from nasopharynx. Early priming of mucosal Th17 and/or through inhibiting Treg induction pathway in childhood may be a novel vaccination strategy against pneumococcal infection.

## INTRODUCTION

*Streptococcus pneumoniae* (pneumococcus) is a leading cause of community acquired bacterial meningitis and pneumonia worldwide [1, 2]. Nasopharyngeal carriage of *S. pneumoniae* is a prerequisite of invasive pneumococcal disease (IPD). Pneumococcal carriage is common in childhood, especially in young children that may be why they are prone to IPD such as pneumonia. Understanding local mucosal immunity in nasopharynx that mediates pneumococcal colonization/carriage and its clearance may inform novel vaccination strategy against pneumococcal diseases in humans.

*S. pneumonia* normally colonizes the mucosa of human nasopharynx. Within nasopharynx, the local mucosal immune system is likely to play an important role in mediating immunity against pneumococcal colonization and carriage. The adenoids and tonsils comprise nasopharynx-associated lymphoid tissue (NALT) in humans and are major part of the mucosal immune system in nasopharynx. It has been reported that adenoidectomy increased the risk of pneumococcal carriage in children and that appeared to be associated with impaired protein antigen-specific immunity[3, 4]. We previously demonstrated the presence of large numbers of naturally developed antigen-specific T and B cells to pneumococcal proteins in NALT of children [5, 6].

Pneumococcal carriage rate is high in young children and then decreases with age. The immunological factors responsible for the decrease in pneumococcal carriage or its clearance with age are not well understood [7]. Recent data in animal studies suggest a crucial role of CD4+ T cells [8-10] , and IL-17A-secreting $CD4^+$ T cells ($T_H17$) in particular, in mediating clearance of nasopharyngeal colonization of *S. pneumoniae*. [11, 12]. Whether this Th17 mediated mechanism operated in humans remain unclear. We recently reported the presence of memory Th17 in human NALT which was shown to increase following stimulation by domain 4 pneumolysin and shown a more marked Th17 response in carriage negative than positive children [13].

We previously also demonstrated the presence of highly suppressive $CD4^+CD25^{high}$Foxp3+T regulatory cells (Treg) in NALT tissue, and a higher number of Treg was shown to be associated with pneumococcal carriage in children [14]. This has been corroborated by others in that there is the presence of pneumococcal specific Treg in human nasopharynx [15]. In humans, unlike in mice, previous natural exposure(s) to *S. pneumoniae* in early childhood prime for memory Treg leading to accumulation of pneumococcal specific Treg in NALT [14]. Nevertheless, experimental pneumococcal colonization in mice induces Treg in nasopharynx through upregulating TGFβ pathway [16]. It is plausible that both Th17 and Treg contribute to mediating carriage in nasopharynx. However, it is not known how Th17 and Treg interact in nasopharynx and how that

interaction affects pneumococcal carriage and its clearance. It would be important to understand the relationship between Th17 and Treg in nasopharynx, and how they evolve with age and carriage. Recently, there is increasing interest on the possible reciprocal relationship between Th17 and Treg in mucosal immunity to pathogenic infections such as HIV [17-19]. It will be valuable to know whether and how the balance between Th17 and Treg in NALT affect pneumococcal carriage in human nasopharynx.

We hypothesize that pneumococcal colonization in early childhood primes for both memory Th17 and Treg in NALT, and the balance between Th17 and Treg in NALT evolves as children grow older and that becomes a critical factor in the clearance of pneumococcal carriage from nasopharynx. In this study, we studied the frequencies of and relationship between Th17 and Treg in NALT over age. We demonstrate the presence of a prominent number of both Th17 and Treg in NALT, and there was an inverse relationship between the frequencies of Th17 and Treg. Interestingly, the frequency of Th17 was shown to increase with age, whereas the frequency of Treg appeared to decrease with age. We further demonstrate that the ratio of Th17/Treg also increased with age and was higher in individuals who were culture negative than those who were culture positive for *S. pneumoniae* in nasopharynx. Our results suggest there is a dynamic relationship between mucosal Th17 and T regulatory cell populations in NALT which is a critical determinant for clearance of pneumococcal carriage from nasopharynx in humans.

## MATERIALS AND METHODS

### *Patients and samples*

Adenotonsillar tissues and peripheral blood samples were obtained from patients (age 2-36 years) undergoing adenoidectomy and/or tonsillectomy due to upper airway obstruction. Patients with known immunodeficiency or who were prescribed antibiotics in the three weeks prior to surgery were excluded from the study. Nasopharyngeal swabs were taken on the day of operation for bacterial culture to determine pneumococcal carriage as described previously [20]. Each tissue sample was checked for any signs of gross inflammation and/or necrosis prior to processing and any samples that exhibited either of these features were excluded from the study. The Liverpool Paediatric Research Ethics Committee approved the study and written informed consent was obtained in all cases.

### *Cell separation and culture*

Mononuclear cells (MNC) from adenotonsillar tissues and peripheral blood (PBMC) were isolated by Ficoll density gradient centrifugation (GE Healthcare) using methods described previously [21,

22]. Cells were cultured in 96-well plate (Corning) in the RPMI medium supplemented with 2 mmol/L glutamine, 10μg/ml gentamycin and 10% fetal bovine serum (Sigma). Paired analysis of adenoidal and tonsillar MNC showed similar results in Treg and Th17 frequencies, so only results of tonsillar MNC are presented in this paper.

***Pneumococcal culture supernatant extract.*** *S. pneumoniae* was cultured and a concentrated pneumococcal culture supernatant (CCS) was prepared as described previously **[22]** from a wild type D39 (encapsulated type 2) *S. pneumonia* strain or a pneumolysin-deficient strain **[23]**. Briefly, the bacteria were grown to exponential phase (OD 0.4 at 620 nm, approx $10^8$ cfu/ml) in Todd-Hewitt broth supplemented with 5% yeast extract. After centrifugation (3000 g for 30 min), the culture supernatant was removed and passed through a 0.2-mm sterile filter and concentrated (tenfold) using a Vivaspin concentrator (Vivascience, Germany). The CCS contained secreted pneumococcal proteins including choline binding proteins and pneumolysin **[22]**. The protein concentrations of the CCS were determined using the Bio-Rad protein assay. The CCS were then used at a predetermined protein concentration of 1 ug/ml in cell stimulation experiments.

***Determination of Th17 and Treg frequencies by flowcytometry***

To determine the frequency of Th17 in NALT, freshly isolated tonsillar MNC were incubated in RPMI in the presence of Phorbol 12-myristate 13-acetate (PMA, 40pg/ml), ionomycin (0.5 μg/ml), and brefeldin A (eBioscience) for 5 hours, followed by analysis with intracellular staining for IL-17A-producing CD4+ T cells (Th17) [13]. Figure 1a describes the strategy for determination of frequency of Th17 (or Treg) by flow-cytometry. A memory Th17 response to pneumococcal stimulation was also analyzed following co-incubation of tonsillar MNC with a pneumococcal CCS for 24 hours, and by intracellular staining for Th17 [13] . The frequency of Treg in freshly isolated adenotonsillar MNC was analyzed by staining for intracellular Foxp3 and surface CD4, CD25 and CD127 expression. The use of CD4+CD25+ $CD127^{low}$ as a phenotype for Treg was shown to correlate well with CD4+Foxp3 expression. The frequency of Th17 or Treg is expressed as the percentage of IL-17A+ or Foxp3+ cells of all CD4+ T cells (Figure 1a).

Intracellular staining of IL-17A and Foxp3 was performed as described previously [14]. Briefly, following stimulation, tonsillar MNC were harvested and stained with fluorescence-labelled anti-human CD3 and CD4 (BD Bioscience), followed by fixation and permeabilization (eBioscience) and staining with fluorescence-labelled IL-17A, or Foxp3 (BD Bioscience). Intracellular expression of IL-17A or Foxp3 was subsequently analyzed by flow cytometry.

**Depletion of memory T cell and/or Treg cells from tonsillar MNC.**

To analyze the induction of Th17 cells from naïve T cells, CD45RO+ (memory and effector T) cells were depleted from tonsillar MNC using anti-human CD45RO antibody-labeled magnetic microbeads and magnetic cell sorting (MACS) according to manufacturer instructions (Miltenyi Biotec) [20]. CD45RO+ cell depletion removed Th17 cells from tonsillar MNC. For experiments on induction of Treg differentiation from naïve T cells, both CD45RO+ cells and CD25+ cells were depleted from tonsillar MNC using magnetic microbeads labeled with anti-CD45RO and -CD25 antibodies and MACS sorting. To ensure cell-depletion efficiency and cell purity, cell-depleted MNC were passed through a second column and cell purity was confirmed by CD3, CD4 and IL-17A or Foxp3 staining and flow-cytometry.

**Induction of Th17 and Treg cells by pneumococcal stimulation.** For Th17 induction experiment, CD45RO+ cell depleted tonsillar MNC were co-cultured cultured for 7 days with Th17 polarizing cytokines and wild type pneumococcal CCS or antigens. A number of cytokines were tested, and IL21 (50ng/ml), IL1-β (50ng/ml), and TGF-β (2.5ng/ml) (R&D systems) were used for optimal Th17 induction. On day 7, PMA/Ionomycin and brefieldin A (BFA) were added, and the cells were incubated for a further 5 hours. The cells were then harvested for intracellular staining of IL-17A **[13]**. For Treg induction experiment, tonsillar MNC depleted of CD45RO+ and CD25+ cells were co-cultured for 7 days with pneumococcal CCS or antigens in the presence of TGFβ (R&D systems). On day 7, the cells were harvested and stained for intracellular Foxp3 in addition to surface CD4/CD3 followed by flowcytometry on a FACSCalibur (BD Bioscience) [14].

**Measurement IL-17A, IL-17F, IL-22 and $TGF_{\beta}$ by ELISA**

Concentrations of IL-17A, IL-17F and IL-22 in cell culture supernatants were measured by ELISA (eBioscience) following manufacturer's instructions. Briefly, ELISA plate was coated with 100µl/well of anti-cytokine capture antibody overnight at 4°C. The plate was washed and blocked with blocking buffer for one hour. Cell culture supernatants diluted in blocking buffer were added and incubated for 2 hours. Detection antibody was subsequently added and incubated for 1 hour. Avidin-horse reddish protein (HRP) was added and incubated, followed by the addition of substrate solution. Optical density (OD) at 450nm was read and cytokine concentrations were calculated by the use of a microplate analysis Deltasoft software (Biometallics, Inc.).

Human $TGF_{\beta}$ ELISA kit (eBioscience) was used to detect $TGF_{\beta}$ in tonsillar MNC culture supernatants following stimulation by pneumococcal CCS. This assay was conducted to detect the active TGFβ following manufacturer's instructions. The samples (but not the $TGF_{\beta}$ standard) were treated with acid (1N hydrochloric acid, HCL) in order to activate latent TGFβ. Briefly, the samples

were diluted to 1:10 and then treated with 20µl of 1N HCL per 100µl sample for 10mins at RT. 20µl of 1N NaOH was subsequently added to neutralize the acid. The remaining procedure was the same as described for IL-17A measurement.

***Statistical analysis***

Two-group comparisons were analyzed using student's t test and multiple comparisons using ANOVA. Statistical analysis was performed using GraphPad Prism. $P<0.05$ was considered statistically significant.

## RESULTS

***Patients' demographic data***

A total of 94 patients (age 2–36 years) were recruited into the study, and pneumococcal carriage was assessed by pneumococcal culture of nasopharyngeal swabs. Table 1 shows the demographic data of the study subjects. As shown in Table 1, there is an age associated decrease in pneumococcal carriage rate. No difference was found in carriage rates between males and females ($P>0.05$, data not shown). Out of 66 children, 46 (69.7%) received pneumococcal conjugate vaccination, and there was no difference in pneumococcal vaccination rate between carriage-positive (71.4%) and carriage-negative subjects (65.8%). No individuals in the adult group received pneumococcal conjugate vaccination.

Table 1. Demographic data of the study subjects and nasopharyngeal carriage of *S. pneumoniae*

| Age group | Total number | Culture+ | Culture- | % of colonized |
|---|---|---|---|---|
| 2-4 yrs | 32 | 17 | 15 | 53.1 |
| 5-9 yrs | 20 | 8 | 12 | 40.0 |
| 10-16 yrs | 14 | 3 | 11 | 21.4 |
| 17-36 yrs | 28 | 2 | 26 | 8.5 |

***Th17 frequency in human NALT increases with age***

To determine the frequency of Th17 in NALT and peripheral blood, tonsillar MNC and PBMC were analysed by intracellular cytokine staining for IL-17A following stimulation with PMA/ionomycin for 5 hours. The frequency was expressed as the % of IL-17A-producing cells in CD4+ T cell population. The frequency of Th17 in tonsillar MNC was shown to be significantly

higher than in PBMC (Figure 1b, $p<0.001$). Also the frequency of Th17 in tonsillar MNC in adults was shown to be considerably higher than in children (Figure 1c, $p<0.001$). Further analysis revealed that there was an age-associated increase in the Th17 frequency of tonsillar MNC (Figure 1d, $p<0.01$). As can be seen in Figure 1d, in general, younger children less than 10 years had lower Th17 frequency than in older children and adults.

***Treg frequency in NALT decreases with age***

To determine the frequency of Treg in NALT, freshly isolated tonsillar MNC were stained with Foxp3, CD4, CD25 and CD127 followed by flowcytometry. The Treg frequencies detected by CD4+Foxp3+ staining and that by $CD4^{+}CD25^{high}CD127^{low}$ staining correlated well (data not shown), and therefore only frequency of Foxp3+ CD4+ Treg in tonsillar MNC is presented. As can be seen in Figure 2a, the frequency of Treg cells in tonsillar MNC was considerably higher than in PBMC ($p<0.01$). There was also a difference between children and adults in the frequency of Treg which was higher in tonsillar tissue of children than in adults (Figure 2b, $p<0.01\%$). Further analysis showed that there was an age-associated decrease in the Treg frequency in tonsillar tissues (Figure 2c, $r= -0.63$, $n=57$, $p<0.001$).

***Th17 frequency is inversely correlated with Treg frequency in NALT***

To study whether there is any relationship between tonsillar Th17 and Treg, the frequency of tonsillar Th17 (detected following PMA/ionomycin timulation) and the frequency of Treg detected in freshly isolated tonsillar MNC were anlyzed in the same tissue samples from the same individuals. As shown in Figure 2d, there was an inverse correlation between the frequencies of Th17 and Foxp3+ Treg in tonsillar MNC (Figure 2d, $r=-0.60$, $p<0.01$).

***A higher ratio of Th17/Treg in NALT is associated with age and with an absence of pneumococcal carriage***

The fact that tonsillar Th17 frequency increases with age, and a higher Th17 frequency in adults that correlated with a lower carriage rate would suggest that Th17 may contribute to the age-associated decrease in carriage. To determine whether Th17 may be implicated in the reduction of pneumococcal carriage, we compared tonsillar Th17 frequency between children who were pneumococcal carriage- and those who were carriage+. There appeared a trend to show a higher Th17 frequency in the former, although there was no significant difference between the two (Figure 3a, $p=0.08$).

As both mucosal Th17 and Treg are suggested to mediate mucosal microbial colonization, we sought to determine whether the balance of tonsillar Th17/Treg is an important determinant in mediating pneumococcal carriage. The ratio of tonsillar Th17/Treg in adults was considerably higher than in children (Figure 3b, $p<0.001$) which correlates with the low carriage rate in adults and higher carriage rate in children (Table 1). Figure 3c shows the ratio of Th17/Treg increases with age ($p<0.001$). We further analyzed whether the ratio of Th17/Treg differs between carriage+ and carriage- children. As can be seen from Figure 3d, the ratio of Th17/Treg in tonsillar MNC was significantly higher in carriage- than in carriage+ children (Figure 3c, $p<0.01$). A general linear model of analysis of variance (ANOVA) was used to analyze the individual effect of age and carriage status on the Th17/Treg ratio, and both carriage status and age were shown to be independently correlated with the Th17/Treg ratios ($p<0.01$).

***Activation of memory Th17 by pneumococcal stimulation and correlation with pneumococcal carriage***

We hypothesize that previous pneumococcal exposure in nasopharynx in early childhood had primed individuals for memory Th17 to *S. pneumonia* in NALT, and pneumococcal stimulation of tonsillar cells from these individuals would induce a memory Th17 response. We therefore examined the memory Th17 response following stimulation of tonsillar MNC by a pneumococcal extract (pneumococcal CCS). We found that stimulation of tonsillar MNC by pneumococcal CCS induced a significant increase in Th17 number compared to medium controls (Figure 4a). Furthermore, the increase in Th17 number following the pneumococcal CCS stimulation was higher in children who were carriage- than in those carriage+ children (Figure 4b). In agreement with this, IL-17A concentration in tonsillar MNC culture supernatant increased following stimulation, and the IL-17A concentration was also shown to be higher in carriage - than in carriage + children (Figure 4c). As pneumococcal stimulation also elicited an increase in Treg number (mean increase 2.1%), the Th17 response detected was likely to be regulated by the concomitant Treg response in tonsillar MNC. When the ratio of tonsillar Th17/Treg numbers was analyzed following stimulation by pneumococcal CCS, it was also found to be higher in carriage- than in carriage+ children (Figure 4d). By contrast, concentration of TGFβ was also shown to increase following stimulation but was higher in carriage+ than in carriage- children (Figure 4e).

***Induction of Th17 by pneumococcal stimulation***

We then examined whether Th17 differentiation could be induced from naïve T cells by pneumococcal stimulation. Tonsillar MNC depleted of CD45RO+ (memory and effector T) cells were used to study Th17 induction, as we showed that the depletion retained CD45RO- naïve T

cells but removed all existing Th17 cells in the MNC (data not shown). The CD45RO- MNC were stimulated with pneumococcal CCS in the presence of Th17-polarizing cytokines (TGF-β:2.5ng/ml, IL1-β: 50ng/ml, and IL21: 50ng/ml) for 7 days. We show stimulation with the wild type CCS induced Th17 cells, along with the production of IL-17A and IL-17F in the cell culture supernatant (Figure 5a-c). Interestingly, the magnitude of Th17 induction from naïve tonsillar T cells was found to correlate and increase with age (Figure 5d, $r=0.62$, $p<0.01$). Compared with CCS from wild type *S.pneumoniae*, CCS derived from an isogenic pneumolysin-deficient strain induced a lower production of IL-17A (Figure 5e). Further experiment was performed to study whether purified recombinant pneumolysin or its toxoid could also induce Th17 differentiation. Under the same culture condition with Th17-polarizing cytokines, the addition of sublytic concentrations of pneumolysin (0.05µg/ml) or toxoid (PdB, 0.25µg/ml) to the CD45RO- MNC induced a marked increase in the number of Th17 cells (Figure 5f).

***Induction of Treg by pneumococcal stimulation***

To determine whether Foxp3+ Treg could be induced by pneumococcal stimulation, tonsillar MNC depleted of CD45RO+ and CD25+ cells were stimulated by pneumococcal CCS in the presence of $TGF_{\beta}$ (2.5ng/ml). The depletion procedure removed existing Foxp3+ Treg (>99%) and memory T cells (>99%) but retained CD45RO- naïve T cells in the MNC. As shown in figure 6a, stimulation by wild type pneumococcal CCS induced a marked increase in Foxp3+ Treg as compared to medium control. No difference was found between young and older children in the induction of Treg (Figure 6b).

## Discussion

There is increasing evidence to support an important role of Th17 in mediating mucosal immunity against microbial colonization. On the other hand, Treg are suggested to be important in mediating microbial colonization. In this study, we describe the relationship between mucosal Th17 and Treg in human NALT, the changes in the balance between the Th17 and Treg populations with age and their association with pneumococcal carriage.

We show the frequencies of Th17 and Treg in NALT were inversely correlated and both are higher than in peripheral blood. The higher frequency of both cell subsets in NALT suggests there are locally induced Th17 and Treg and are important in mediating local mucosal immunity. The inverse relationship between the two cell populations suggests that they may perform opposite functions and the induction pathways for Th17 and Treg in NALT are differentially regulated and may inhibit each other. We also show a significant difference between young adults and children in the

frequencies of Th17 and Treg in tonsillar tissue. Whilst Th17 frequency in young adults was considerably higher than in children, in general, Treg frequency in young adults was lower than in children. Further analysis revealed that tonsillar Th17 frequency increases with age, whereas the Treg frequency decreases with age.

This is the first study to describe a dynamic relationship between mucosal Th17 and Treg in NALT that evolves with age, and its association with pneumococcal carriage. There is little data available on relationship between frequencies of Th17 or Treg and age from childhood to adulthood. PBMC from Swedish children produced lower levels of IL-17A in response to stimulation by a pneumococcal whole cell antigen compared to adults, although no such difference was noted from the samples from Bangladesh [24]. It has been reported that the elderly had a lower memory Th17 frequency than young adults (**). A moderate increase in Treg freqency in peripheral blood from young adults to elderly (aged 21-93 years) was reported [25], although others reported no difference between the young and elderly (*). Induction of pathogen-specific Treg has been shown during infections with HIV, TB and leishmania in lymphoid tissue [26-29]. We show here children around 2 years of age have already developed a prominent number/frequency of Treg in NALT. This suggests the induction of Treg in NALT is fairly efficient in early childhood (eg. less than 2 years). It is possible that there is a rapid increase in Treg number in NALT during early childhood due to the intensive exposure/colonization with microorganisms (eg. *S. pneumoniae)* in the nasopharynx. The efficient induction of antigen-specific Treg cells during this period leading to the enrichment of Treg in NALT that may contribute to the persistence of certain microorganisms (ie. carriage) such as *S pneumoniae* [14].

The frequency of Th17 in NALT of younger children (<10 years) was shown to be low in general, which suggests the induction of Th17 during this period is inefficient. It has been reported that Th17 induction could be negatively regulated by Treg [17-19]. We show here that there was a negative correlation between Treg and Th17 frequencies in tonsillar tissue. This, coupled with the fact that younger children (<10 years) had higher Treg but lower Th17 frequency, and vice versa for adults, would be consistent with the hypothesis that a strong Treg activity during young childhood suppresses the induction of Th17 in NALT. It is possible that the mucosal environment in nasopharynx in early childhood is highly favorable for Treg induction, but less so for Th17 induction. This possibility and the underlying mechanisms are the subject of a further study in our laboratory.

There is growing evidence supporting that Th17 play an important role in mediating mucosal clearance of extracellular pathogens including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida albicans* [30-33]. *S. pneumoniae* is a frequent colonizer in nasopharynx during early childhood. This frequent colonization may prime for pneumococcal specific memory Th17 in NALT and contribute to immunity against pneumococcal colonization later in life. It has been shown that experimental pneumococcal colonization in human volunteer adults elicits a marked increase in memory Th17 cells [34]. It is known pneumococcal carriage rate decreases with age [35, 36], as is also shown in this study (Table 1). Our finding that Th17 frequency in NALT increases with age and pneumococcal carriage decreases with age would be consistent with the hypothesis that Th17 contributes to clearance of pneumococcal carriage from nasopharynx [11, 12], and contributes to the age-associated decrease in pneumococcal carriage.

When we compared the tonsillar Th17 frequency (after PMA/ionomycin stimulation) between the culture- and culture+ children, there was a trend to show a higher Th17 frequency in culture-negative than in culture-positive children, although the difference was not statistically significant. We then examined the relationship between the ratio of Th17/Treg in NALT and age and pneumococcal carriage status. We show the ratio of Th17/Treg increases with age and is considerably higher in adults than in children which correlated with a low carriage rate in adults and a high carriage rate in children. We further show the ratio of Th17/Treg was significantly higher in carriage- than in carriage+ children. A general model of ANOVA revealed that both age and carriage status are independent factors correlated to the ratio of Th17/Treg. These findings suggest that there is a dynamic relationship between mucosal Th17 and Treg cell populations in NALT that evolves with age in humans, and the balance between the two cell populations is a critical factor determining the clearance of carriage of *S. pneumoniae* from nasopharynx.

We previously showed that the Th17 cells detected in NALT tissues were primarily memory Th17 cells, as depletion of CD45RO+ T cells abrogate Th17 in tonsillar MNC [37]. In this study, we used a pneumococcal extract (pneumococcal CCS) to stimulate tonsillar MNC overnight to examine pneumococcal specific memory Th17 response. We show this stimulation elicited a marked increase in Th17 number in tonsillar MNC. Again, prior depletion of CD45RO+ (memory) T cell abrogated this increase in Th17 number (data not shown). This supports the presence of pneumococcal-specific memory Th17 in NALT. Further analysis revealed that this memory Th17 response in tonsillar MNC following pneumococcal stimulation was stronger in carriage- children than in carriage+ children. Unlike the total Th17 frequency determined following PMA/ionomycin stimulation, this memory Th17 response is likely to be influenced and regulated by the concomitant

increase in Treg number, as pneumococcal stimulation also activates memory Treg [14]. The ratio of Th17/Treg following pneumococcal stimulation was also shown to be higher in carriage- than in carriage+ children. These findings provide further support for an important role of Th17 in mediating clearance of pneumococcal carriage in human nasopharynx. In agreement with the Th17 response, IL-17A concentration in tonsillar MNC culture supernatant was shown to increase following stimulation, and the concentration of IL-17A in the cell culture was also shown higher in pneumococcal carriage- than in carriage+ children.

Memory Th17 cells have been shown to confer heterologous and antibody-independent mucosal immune protection against several serotypes of *K. pneumonia* [38]. Our results lend further support to a protective role for mucosal Th17 against extracellular bacteria [39, 40]. IL-17A produced from Th17, is involved in the recruitment and activation of neutrophils that is associated with phagocytic killing [41]. It has also been suggested that the clearance of pneumococcal colonisation by activated monocyte/macrophages could be mediated via IL-17A [42]. Recombinant IL-17A exposure to human neutrophils or alveolar macrophages significantly increased the killing of opsonised *S. pneumoniae* by these phagocytes [43, 44]. It is possible that IL-17A produced by Th17 promotes pneumococcal clearance through recruitment of neutrophils and monocyte/macrophages. Studies in mice showed that the response of IL-17A following immunisation is associated with decreased carriage supports a critical role of IL17A in pneumococcal clearance [41].

We further studied whether pneumococcal stimulation induces differentiation of Th17 and Treg from naïve T cells in NALT. We show stimulation (for 7 days) by pneumococcal CCS induced Th17 and Treg differentiation from naïve T cells in tonsillar MNC under respective polarizing cytokine conditions. Interestingly, we show the magnitude of Th17 induction from naïve T cells in younger children was low and increase with age (Figure 5d). However, no such age associated difference was shown in the level of Treg induction which was similar in younger children to older children (Figure 6b). These findings provide support to the hypothesis that there is a favorable environment for Treg induction, but not for Th17 induction in NALT in young children.

Recent interests have focused on Th17-targeted vaccine strategy including that against pneumococcal infection[45-48], and efforts are made to identify candidate antigens that promote Th17 cells [47, 48]. Our finding that an isogenic pneumolysin-deficient mutant strain-derived CCS induced less Th17 than the wild type, and recombinant Ply and toxoid were shown to induce Th17 would support that pneumolysin contributes to activation of memory Th17 and able to induce differentiation of Th17 cells.

TGF-β promotes the development of Foxp3+Treg cells and suppresses IL-23 expression thus inhibit the differentiation of Th17 [49]. In this study, we show that there was a marked increase in TGF-β1 concentration in tonsillar MNC following pneumococcal stimulation. A number of different cell types including antigen-presenting cells, epithelia cells and Treg of mucosal tissues were shown to produce TGF-β [50, 51]. It is possible that within tonsillar tissue, upon stimulation by bacterial colonization such as *S. pneumoniae*, a number of cells produce TGF-β leading to a relatively high level of TGF-β in nasopharynx [16], a favorable condition for Treg induction in NALT. It has been shown in mice that carriage of *S. pneumonia* induces Treg that were associated with high levels of TGF-β in nasopharynx [16]. We show in this study that the level of TGF-β was higher in tonsillar cells from carriage+ children compared to carriage-children. Together with the finding of a higher Treg frequency in carriage+ than in carriage- children, these results support the hypothesis that TGF-β signalling plays an important role in Treg induction in NALT in early childhood that suppresses Th17 induction and leads to nasopharyngeal carriage of *S. pneumoniae*.

Taken together, our data suggest that the balance between mucosal Th17 and Treg in NALT evolve with age from early childhood to adulthood, and become a critical determinant for pneumococcal clearance from nasopharynx. The ability of Th17 priming by *S. pneumonia* is generally low in young children that may be related to the rapid and efficient priming of Treg during early childhood (due to intensive microbial exposure) that inhibit Th17 induction. As Treg in NALT decreases with age, the level of Th17 induction increases. This leads to a shift in the balance between Th17 and Treg in NALT to an environment favourable for Th17-mediated pneumococcal clearance in nasopharynx. Efficient priming of Th17 in NALT in early childhood to interrupt Treg-mediated carriage may become an effective vaccination strategy against pneumococcal infection [52].

Figure legends

Figure. ***Frequency of Th17 in NALT increases with age***. Gating strategy for determination of frequencies of Th17 and Treg by flow-cytometry (a). Lymphocytes were gated (R1) based on typical forward and sideward scatter (FSC and SSC) plots; CD4+ T cells (R2) were then gated based on CD3+ and CD4+ staining and percentage of IL-17A+ CD4+ T cells or Foxp3+ CD4+ T cells was subsequently calculated as Th17 or Treg frequency. Comparison of frequencies of Th17 in tonsillar MNC between children and adults ((b), ***p<0.001 compared to children); and comparison between Th17 in tonsillar MNC and PBMC ((c), **p<0.01 compared to PBMC). Correlation between frequency of tonsillar Th17 and age ((d), *p<0.05, **p<0.01, ***p<0.001 compared to 2-4 year group).

Figure 2. ***Frequency of Treg in NALT decreases with age.*** Frequency of Foxp3+ Treg in tonsillar MNC was analyzed and compared with PBMC ((a), **p<0.01) and compared between children and adults ((b), **p<0.01). Correlations between frequency of tonsillar Treg and age (c) and between Treg and Th17 frequencies in tonsillar MNC.

Figure 3. ***Correlation between the ratio of tonsillar Th17/Treg frequencies and pneumococcal carriage.*** Tonsillar Th17 frequencies were compared between carriage+ and carriage- children ((a), #p=0.08 compared to carriage+ children). The ratio of tonsillar Th17/Treg frequency was compared between adults and children ((b), ***p<0.001 ). The ratio of tonsillar Th17/Treg frequencies was analyzed and was found to increase with age ((c), p<0.001), and was higher in carriage- than in carriage+ children ((d), **p<0.01 compared to carriage+ children ).

Figure 4. ***Pneumococcal stimulation activates memory Th17 response that is correlated with pneumococcal carriage***. Stimulation of tonsillar MNC for 24 hours with wild type pneumococcal CCS activates a memory Th17 response in tonsillar MNC (a, **p<0.01 compared with pneumococcal culture medium only control). Stimulation with pneumococcal CCS elicits increases in Th17 number ((b), p<0.05), IL-17A concentration ((c), p<0.05) and in the ratio of Th17/Treg in tonsillar MNC which was higher in carriage- than in carriage+ children ((d), **p<0.01); but it elicits a higher level of TGFβ production in tonsillar MNC in carriage+ than in carriage- children ((e), *p<0.05).

Figure 5. ***Induction of Th17 by pneumococcal stimulation.*** Memory/effector T cell-depleted tonsillar MNC were stimulated by pneumococcal CCS in the presence of IL1β/TGFβ/IL21 for 7 days followed by co-incubation with PMA/Ionomycin for 5 hours. Induction of Th17 cell differentiation from naïve tonsillar T cells (a), and IL-17A (b) and IL-17F (c) production following stimulation with wild type CCS. **p< 0.01 compared to medium control. The level of Th17 indcution was shown to correlate with age (d. p<0.01). Comparison between IL-17A induced by wild type CCS and by pneumolysin-deficient CCS (e, *p<0.05). Induction of Th17 cells was shown by stimulation with recombinant pneumolysin and toxoid (f, p<0.01).

Figure 6. ***Induction of Treg by pneumococcal stimulation.*** Induction of FOxp3+ Treg from Treg-depleted tonsillar MNC (by CD25+/CD45RO+ cell-depletion) was shown following stimulation with wild-type pneumococcal CCS (a, **p<0.01 compared to medium control), and no difference was shown in the level of induction between different aged children (b).

1. Siber GR. Pneumococcal disease: prospects for a new generation of vaccines. Science **1994**; 265:1385-1387.
2. Peltola H. Burden of meningitis and other severe bacterial infections of children in africa: implications for prevention. Clinical Infectious Diseases **2001**; 32:64-75.
3. Mattila PS, Hammarén-Malmi S, Saxen H, Kaijalainen T, Käyhty H, Tarkkanen J. Adenoidectomy and nasopharyngeal carriage of Streptococcus pneumoniae in young children. Archives of Disease in Childhood **2010**; 95:696-702.
4. Mattila PS, Hammarén-Malmi S, Saxen H, Kaijalainen T, Käyhty H, Tarkkanen J. Adenoidectomy in young children and serum IgG antibodies to pneumococcal surface protein A and choline binding protein A. International Journal of Pediatric Otorhinolaryngology **2012**; 76:1569-1574.
5. Zhang Q, Choo S, Finn A. Immune responses to novel pneumococcal proteins (Pneumolysin, PspA, PsaA and CbpA) in adenoidal B cells from children. Infection & Immunity **2002**; 70:5363-5369.
6. Zhang Q, Bagrade L, Clarke E, et al. Bacterial lipoproteins differentially regulate human primary and memory CD4+ T and B cell responses to pneumococcal protein antigens through Toll-like receptor 2. Journal of Infectious Diseases **2010**; 201:1753-1763.
7. Lipsitch M, Whitney CG, Zell E, Kaijalainen T, Dagan R, Malley R. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? PLoSMed **2005**; 2:e15.
8. Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, Andrew PW. Host cellular immune response to pneumococcal lung infection in mice. Infection & Immunity **2000**; 68:492-501.
9. Kadioglu A, Coward W, Colston MJ, Hewitt CR, Andrew PW. CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. Infection & Immunity **2004**; 72:2689-2697.
10. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4 T cells mediate antibody-independent acquired immunity to pneumococcal colonization Proceedings of the National Academy of Sciences of the United States of America **2005**; 102:4848-4853.
11. Lu YJ, Gross J, Bogaert D, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS Pathogens **2008**; 4:e1000159.
12. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. The Journal of Clinical Investigation **2009**; 119:1899-1909.
13. Gray C, Ahmed MS, Mubarak A, et al. Activation of memory Th17 cells by domain 4 pneumolysin in human nasopharynx-associated lymphoid tissue and its association with pneumococcal carriage. Mucosal Immunol **2014**; 7:705-717.
14. Zhang Q, Leong SC, McNamara PS, Mubarak A, Malley R, Finn A. Characterisation of regulatory T cells in nasal associated lymphoid tissue in children: relationships with pneumococcal colonization. PLoS pathogens **2011**; 7:e1002175.
15. Pido-Lopez J, Kwok WW, Mitchell TJ, Heyderman RS, Williams NA (2011) Acquisition of Pneumococci Specific Effector and Regulatory Cd4+ T Cells Localising within Human Upper Respiratory-Tract Mucosal Lymphoid Tissue. PLoS Pathog 7(12): e1002396. .
16. Neill DR, Coward WR, Gritzfeld JF, et al. Density and duration of pneumococcal carriage is maintained by transforming growth factor beta1 and T regulatory cells. Am J Respir Crit Care Med **2014**; 189:1250-9.
17. Zhou L, Lopes JE, Chong MMW, et al. TGF-[bgr]-induced Foxp3 inhibits TH17 cell differentiation by antagonizing ROR[ggr]t function. Nature **2008**; 453:236-240.
18. Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and Regulatory T Cell Differentiation Mediated by Retinoic Acid. Science **2007**; 317:256-260.

19. Kanwar B, Favre D, McCune JM. Th17 and regulatory T cells: implications for AIDS pathogenesis. Curr Opin HIV AIDS **2010**; 5(151-157. .
20. Zhang Q, Bernatoniene J, Bagrade L, et al. Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children. Journal of Infectious Diseases **2007**; 195:1194-1202.
21. Zhang Q, Choo S, Finn A. Immune responses to novel pneumococcal proteins pneumolysin, PspA, PsaA, and CbpA in adenoidal B cells from children. Infect Immun **2002**; 70:5363-9.
22. Zhang Q, Bernatoniene J, Bagrade L, et al. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. European Journal of Immunology **2006**; 36:46-57.
23. Berry AM, Ogunniyi AD, Miller DC, Paton JC. Comparative virulence of Streptococcus pneumoniae strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. Infection and immunity **1999**; 67:981-5.
24. Lundgren A, Bhuiyan TR, Novak D, et al. Characterization of Th17 responses to Streptococcus pneumoniae in humans: Comparisons between adults and children in a developed and a developing country. Vaccine **2012**; 30:3897-3907.
25. Gregg R, Smith CM, Clark FJ, et al. The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. Clinical & Experimental Immunology **2005**; 140:540-546.
26. Andersson J, Boasso A, Nilsson J, et al. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. J Immunol **2005**; 174:3143-7.
27. Nilsson J, Boasso A, Velilla PA, et al. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. Blood **2006**; 108:3808-3817.
28. Shafiani S, Tucker-Heard G, Kariyone A, Takatsu K, Urdahl KB. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. The Journal of experimental medicine **2010**; 207:1409-20.
29. Belkaid Y, Piccirillo CA, Mendez S, et al. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature **2002**; 420:502-507.
30. Wu Q, Martin RJ, Rino JG, Breed R, Torres RM, Chu HW. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory Mycoplasma pneumoniae infection. Microbes and infection / Institut Pasteur **2007**; 9:78-86.
31. Huang W, Na L, Fidel PL, Schwarzenberger P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. The Journal of infectious diseases **2004**; 190:624-31.
32. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nature immunology **2007**; 8:942-9.
33. Curtis MM, Way SS. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. Immunology **2009**; 126:177-85.
34. Wright AKA, Bangert M, Gritzfeld JF, et al. Experimental Human Pneumococcal Carriage Augments IL-17A-dependent T-cell Defence of the Lung. PLoS Pathog **2013**; 9:e1003274.
35. Granat SM, Ollgren J, Herva E, Mia Z, Auranen K, Makela PH. Epidemiological evidence for serotype-independent acquired immunity to pneumococcal carriage. The Journal of infectious diseases **2009**; 200:99-106.
36. Bogaert D, De Groot R, Hermans PW. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis **2004**; 4:144-54.
37. Gray C, Ahmed MS, Mubarak A, et al. Activation of memory Th17 cells by domain 4 pneumolysin in human nasopharynx-associated lymphoid tissue and its association with pneumococcal carriage. Mucosal immunology **2013**.

38. Chen K, McAleer Jeremy P, Lin Y, et al. Th17 Cells Mediate Clade-Specific, Serotype-Independent Mucosal Immunity. Immunity **2011**; 35:997-1009.
39. Happel KI, Dubin PJ, Zheng M, et al. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. The Journal of Experimental Medicine **2005**; 202:761-769.
40. Kolls JK, Lindén A. Interleukin-17 Family Members and Inflammation. Immunity **2004**; 21:467-476.
41. Lu YJ, Forte S, Thompson CM, Anderson PW, Malley R. Protection against Pneumococcal colonization and fatal pneumonia by a trivalent conjugate of a fusion protein with the cell wall polysaccharide. Infect Immun **2009**; 77:2076-83.
42. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. The Journal of clinical investigation **2009**; 119:1899-909.
43. Wright AK, Bangert M, Gritzfeld JF, et al. Experimental human pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. PLoS pathogens **2013**; 9:e1003274.
44. Lu YJ, Gross J, Bogaert D, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS pathogens **2008**; 4:e1000159.
45. Dubin PJaK, J. K. Th17 cytokines and mucosal immunity. Immunological Reviews **2008**; 226:160-171.
46. Belyakov IM, Ahlers JD. What Role Does the Route of Immunization Play in the Generation of Protective Immunity against Mucosal Pathogens? The Journal of Immunology **2009**; 183:6883-6892.
47. Moffitt KL, Gierahn TM, Lu Y-j, et al. TH17-Based Vaccine Design for Prevention of Streptococcus pneumoniae Colonization. Cell host & microbe **2011**; 9:158-165.
48. Moffitt KL, Malley R, Lu Y-J. Identification of Protective Pneumococcal T<sub>H</sub>17 Antigens from the Soluble Fraction of a Killed Whole Cell Vaccine. PLoS ONE **2012**; 7:e43445.
49. Zhou L, Lopes JE, Chong MM, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature **2008**; 453:236-40.
50. Pyzik M, Piccirillo CA. TGF-beta1 modulates Foxp3 expression and regulatory activity in distinct CD4+ T cell subsets. Journal of leukocyte biology **2007**; 82:335-46.
51. Chen W, Konkel JE. TGF-beta and 'adaptive' Foxp3(+) regulatory T cells. Journal of molecular cell biology **2010**; 2:30-6.
52. Malley R, Anderson PW. Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity. Proceedings of the National Academy of Sciences **2012**; 109:3623-3627.