



## Original article

# A dynamic relationship between mucosal T helper type 17 and regulatory T-cell populations in nasopharynx evolves with age and associates with the clearance of pneumococcal carriage in humans<sup>☆</sup>

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## ABSTRACT

Pneumococcal carriage is common in young children, which may account for the high incidence of disease in this age group. Host factors determining the clearance of carriage in humans remain unclear. We aimed to study the relationships between T helper type 17 (Th17) and Foxp3<sup>+</sup> regulatory T (Treg) cells in nasopharynx-associated lymphoid tissue (NALT) and carriage in children and adults. Frequencies of Th17 and Treg cells in NALT were analysed by flow cytometry in association with age and pneumococcal carriage status. Cytokine responses following pneumococcal stimulation were analysed by cytometric beads array. The frequencies of Th17 and Treg cells in NALT were inversely correlated ( $R = -0.60$ ). Whereas Treg cell frequency decreased with age ( $R = -0.63$ ), both Th17 and the Th17: Treg ratio increased with age ( $R = 0.62$  and  $R = 0.64$ , respectively). Also, the Th17: Treg ratio was higher in carriage-negative than in carriage-positive children ( $p < 0.01$ ). Pneumococcal stimulation of tonsillar cells increased both Th17 and Treg cell numbers, but the Th17: Treg ratio and pattern of cytokine responses differed between carriage-negative and carriage-positive children. The former showed markedly higher Th17: Treg and interleukin-17A: interleukin-10 ratios than in the latter ( $p < 0.01$ ). Pneumococcal stimulation also induces Th17, although the capacity of this Th17 differentiation from naive T cells of young children was low, but increased with age. We demonstrated a dynamic relationship between Th17 and Treg cells in human nasopharynx that evolves with age. The balance between Th17 and Treg cells in NALT appears to be a major host factor closely associated with the clearance of *Streptococcus pneumoniae* from the nasopharynx. **A. Mubarak, CMI 2016;22:736.e1–736.e7**

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## Introduction

*Streptococcus pneumoniae* (pneumococcus) is a leading cause of pneumonia and meningitis [1,2]. Pneumococcal carriage is a

prerequisite of invasive pneumococcal disease [3]. Carriage is common in young children, which may account for the high incidence of disease in this age group. Pneumococcal carriage decreases with age, although host factors determining carriage clearance in humans remain unclear. Understanding local immunity in human nasopharynx that mediates pneumococcal clearance may inform novel vaccination strategies against pneumococcal diseases. Adenoids and tonsils comprise nasopharynx-associated lymphoid tissue (NALT), and in humans are major components of the mucosal immune system in nasopharynx. It has been reported that adenoidectomy increased the risk of pneumococcal carriage in

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children [4]. We previously demonstrated the presence of large numbers of pneumococcal-specific T and B cells in NALT [5–7].

Recent animal studies suggest a crucial role of interleukin-17A (IL-17A)-secreting CD4<sup>+</sup> T cells (T helper type 17; Th17) in mediating the clearance of pneumococcal colonization [8,9]. Whether this Th17-mediated mechanism operates in humans remains unclear. We recently reported the presence of memory Th17 in human NALT, which were shown to increase following stimulation by domain 4 pneumolysin [10]. We previously also demonstrated the presence of highly suppressive CD4<sup>+</sup> CD25<sup>high</sup> Foxp3<sup>+</sup> T regulatory (Treg) cells in NALT, and a higher Treg cell frequency was shown to be associated with pneumococcal carriage in children [11]. This has been corroborated in that pneumococcus-specific Treg cells are present in the nasopharynx [12]. In humans, unlike in mice, previous pneumococcal exposure primes for memory Treg cells, leading to accumulation of pneumococcal-specific Treg cells in NALT [11]. Nevertheless, experimental colonization in mice induced Treg cells in the nasopharynx through the transforming growth factor  $\beta$  (TGF- $\beta$ ) pathway [13].

There is increasing interest in the possible reciprocal relationship between Th17 and Treg cells in mucosal immunity to infections [14,15]. It is unknown how Th17 and Treg cells interact in human nasopharynx and how the balance between them affects pneumococcal carriage.

We studied the frequencies of and relationship between Th17 and Treg cells in NALT over age, and the ratio of Th17: Treg and pneumococcal carriage. We demonstrated a dynamic relationship between Th17 and Treg cell populations in NALT that evolves with age, and a higher ratio of Th17: Treg appears to be a critical determinant associated with the clearance of pneumococcal carriage.

## Materials and methods

### Patients and samples

Adenotonsillar tissue and peripheral blood samples were obtained (from May 2012 to January 2015) from immune-competent children and young adults who had elective adenoidectomy and/or tonsillectomy due to upper airway obstruction. All eligible patients were consecutively included. Patients with any immunodeficiency or who were prescribed antibiotics in the 3 weeks before surgery were excluded. Nasopharyngeal swabs were taken for bacterial culture using a standard method with blood agar plates to determine pneumococcal carriage as described previously [6]. Liverpool Paediatric Research Ethics Committee approved the study (Ref: 08/H1002/97) and written informed consent was obtained either from the guardians of the children or the patients.

### Cell culture

Mononuclear cells (MNC) from adenotonsillar tissues and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-gradient centrifugation as described previously [7]. Freshly isolated cells were cultured in 96-well plate in RPMI-1640 medium supplemented with 2 mmol/L glutamine, 10 mg/L gentamycin and 10% fetal bovine serum (Sigma, Dorset, England). Twelve paired adenoidal and tonsillar MNC were initially analysed for Treg and Th17 frequencies from 12 participants and showed similar results, so subsequently only tonsillar tissues were analysed and results presented.

### Pneumococcal culture supernatant extract

*Streptococcus pneumoniae* was cultured and concentrated pneumococcal culture supernatant (CCS) was prepared as

described [7] from wild-type (D39) strain or an isogenic pneumolysin-deficient strain [16]. Briefly, the bacteria were grown to exponential (log) phase (approx 10<sup>8</sup> CFU/mL) in Todd–Hewitt broth with 5% yeast extract. After centrifugation, culture supernatant was concentrated (ten-fold) using Vivaspin concentrators (Vivascience/Sigma, Dorset, England). The CCS contained secreted pneumococcal proteins including pneumolysin [7]. The CCS were used at a predetermined protein concentration of 1 mg/L in cell stimulation.

### Determination of Th17 and Treg frequencies

To determine Th17 frequency, tonsillar MNC were incubated in RPMI-1640 with PMA (40 pg/mL), ionomycin (0.5 mg/L), and brefeldin A (eBioscience, San Diego, CA, USA) for 5 h, followed by intracellular staining for IL-17A<sup>+</sup> CD4<sup>+</sup> T cells (Th17) [10]. Fig. 1(a) describes the strategy for determination of Th17 and Treg frequencies. The memory Th17 response to pneumococcal stimulation was analysed following co-incubation of tonsillar MNC with pneumococcal CCS for 24 h (optimal period for detection), followed by intracellular IL-17A staining [10]. Treg frequency in adenotonsillar MNC was analysed by staining for intracellular Foxp3 and surface CD4, CD25 and CD127 [11].

### Depletion of memory T cells and/or Treg cells

To analyse Th17 induction/differentiation from naive T cells, CD45RO<sup>+</sup> (memory/effector T) cells were depleted from tonsillar MNC using anti-human CD45RO microbeads and MACS sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) [6], which depleted Th17 from tonsillar MNC. For Treg cell induction/differentiation from naive T cells, both CD45RO<sup>+</sup> and CD25<sup>+</sup> cells were depleted from tonsillar MNC using anti-CD45RO and anti-CD25 MACS sorting. To ensure cell-depletion efficiency, cell-depleted MNC were passed through a second column and cell purity was confirmed by flow cytometry (purity >98%).

### Induction of Th17 and Treg cells by pneumococcal stimulation

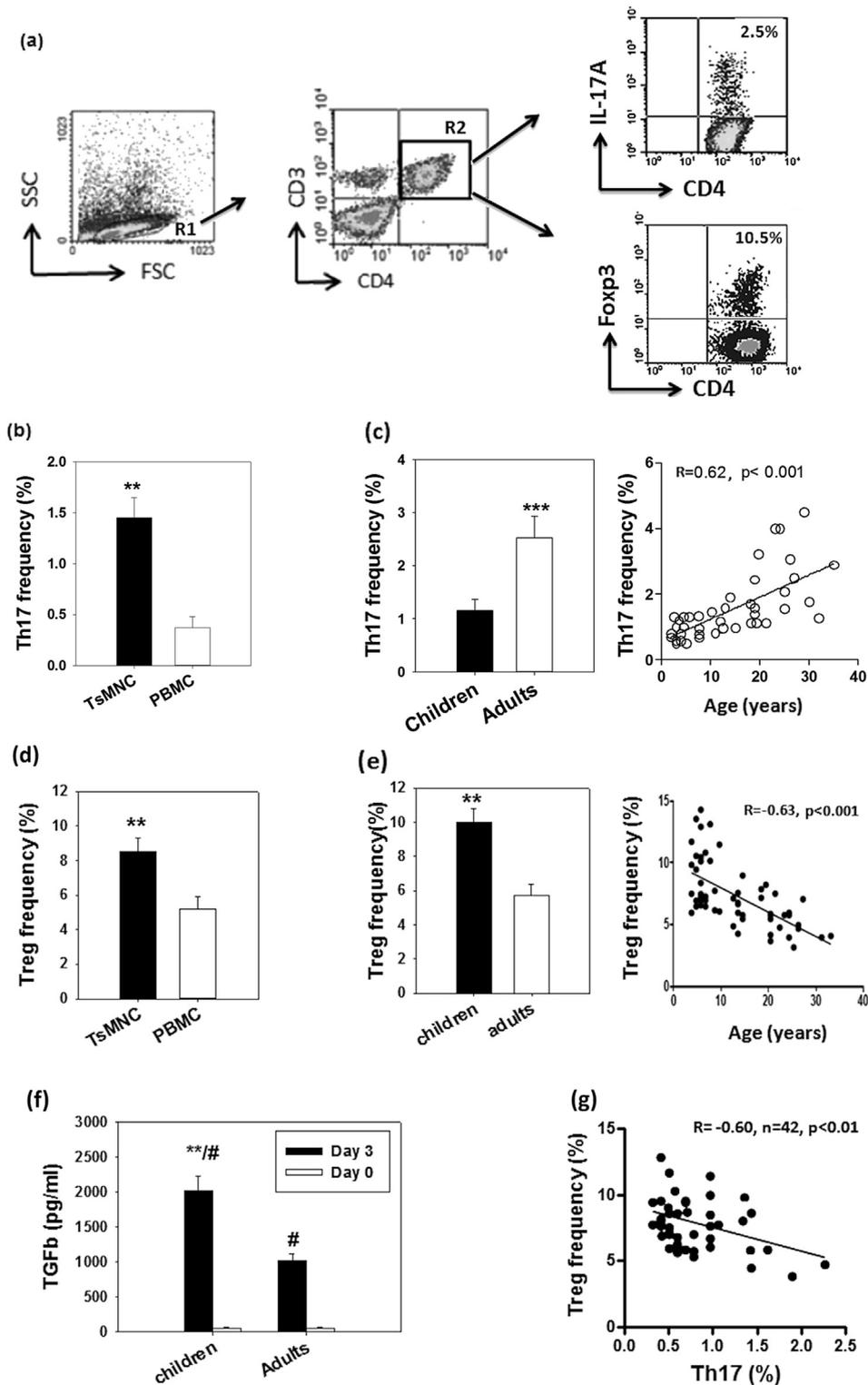
For Th17 induction, CD45RO<sup>+</sup> cell-depleted tonsillar MNC were co-cultured for 7 days with wild-type pneumococcal CCS and Th17 polarizing cytokines [17]. Interleukin-21 (50 ng/mL), IL-1 $\beta$  (50 ng/mL), and TGF- $\beta$ <sub>1</sub> (2.5 ng/mL) (R&D, Minneapolis, MN, USA) were used for optimal Th17 induction. On day 7, PMA/ionomycin and brefeldin A were added, and incubated for 5 h, followed by IL-17A staining [10]. For Treg cell induction, Treg-depleted tonsillar MNC were co-cultured for 7 days with pneumococcal CCS in the presence of TGF- $\beta$ <sub>1</sub> (2.5 ng/mL), followed by Foxp3 staining and flow cytometry [11].

### Measurement of cytokines

Concentrations of IL-2, IL-4, IL-6, IL-10, IL-17A, interferon- $\gamma$  and tumour necrosis factor- $\alpha$  in tonsillar MNC culture supernatants at day 3 following pneumococcal CCS stimulation were analysed by CBA array (BD) [11]. IL-17F, IL-22 and TGF- $\beta$ <sub>1</sub> were measured by a standard ELISA procedure following the manufacturer's instructions (eBioscience).

### Statistical analysis

Comparisons between groups were analysed using Student's *t*-test or analysis of variance. Correlation was analysed by Pearson's correlation. Where necessary, data transformation (to normality) by log<sub>10</sub> conversion was performed before parametric analysis.



**Fig. 1.** Frequencies of T helper type 17 (Th17) and regulatory T (Treg) cells in nasopharynx-associated lymphoid tissue (NALT) and correlation with age. Gating strategy for determination of Th17 and Treg frequencies (a). Lymphocytes (R1) gated based on typical FSC and SSC plots; CD4<sup>+</sup> T cells (R2) gated based on CD3<sup>+</sup>/CD4<sup>+</sup> staining and percentage of Th17 or Treg frequency (% of CD4<sup>+</sup> T cells) calculated. The Th17 frequency of 2.5% in the representative plot was from adult tonsillar mononuclear cells (MNC). Th17 frequencies in tonsillar MNC and peripheral blood mononuclear cells (PBMC) (b, \*\* $p < 0.01$ ,  $n = 15$ ). Comparison of tonsillar Th17 frequency between children ( $n = 35$ ) and adults ( $n = 22$ ) (c, \*\*\* $p < 0.001$ ), and correlation between Th17 frequency and age (c,  $R = 0.62$ ,  $p < 0.001$ ,  $n = 57$ ). Treg frequencies in tonsillar MNC compared with PBMC (d, \*\* $p < 0.01$ ,  $n = 23$ ), and in children compared with adults (e, \*\* $p < 0.01$ ), and the relationship with age (e,  $R = -0.63$ ,  $p < 0.001$ ,  $n = 59$ ). Transforming growth factor  $\beta_1$  (TGF- $\beta_1$ ) concentrations in cultured tonsillar MNC at day 3 compared with day 0, and in children ( $n = 30$ ) versus adults ( $n = 16$ ) (f, # $p < 0.001$  versus day 0, \*\* $p < 0.01$  versus adults). Relationship between tonsillar Treg and Th17 frequencies (g,  $R = -0.60$ ,  $p < 0.01$ ). Bar graphs represent means and standard errors.

Statistical analyses were performed using GRAPHPAD PRISM.  $P < 0.05$  was considered significant.

## Results

### Patients' demographic data

A total of 94 patients (age 2–36 years) were recruited, and their demographic data are shown in Table 1. There was an age-associated decrease in pneumococcal carriage rate. No difference was found in carriage rates between males and females (data not shown). Out of 66 children, 46 (69.7%) received pneumococcal conjugate vaccination. There was no difference in the vaccination rate between the carriage-positive and carriage-negative children, although the serotypes (i.e. vaccine or non-vaccine types) of the carriage isolates were not characterized.

### Th17 frequency increases with age, whereas Treg frequency decreases with age

Th17 frequency in tonsillar MNC was analysed by flow cytometry following co-incubation with PMA/ionomycin, and was shown to be higher than in PBMC (Fig. 1b). Also, Th17 frequency in tonsillar MNC in adults was higher than in children ( $p < 0.01$ ), and there was an age-associated increase in the Th17 frequency (Fig. 1c;  $p < 0.01$ ). In general, younger children (<10 years old) had lower Th17 frequency than older children and adults.

Treg frequency in tonsillar MNC was also higher than in PBMC (Fig. 1d;  $p < 0.01$ ). But in contrast to Th17, the Treg frequency in tonsillar MNC in children was higher than in adults ( $p < 0.01$ ), and there was an age-associated decrease in Treg cells (Fig. 1e;  $p < 0.001$ ).

As TGF- $\beta$  is known to be important in Treg differentiation, we examined whether TGF- $\beta_1$  is produced in tonsillar cells. Fig. 1(f) shows that after 3 days of culture, there was a marked increase in TGF- $\beta_1$  in tonsillar MNC culture ( $p < 0.01$ ), and the concentration was higher in children than in adults ( $p < 0.01$ ).

### Th17 frequency is inversely correlated with Treg cells

To study whether there is any relationship between tonsillar Th17 and Treg cells, the Th17 frequency (detected following PMA/ionomycin stimulation) and the Foxp3<sup>+</sup> Treg frequency in freshly isolated tonsillar MNC were analysed in the same tissue samples from the same individuals. There was an inverse correlation between the Th17 and Foxp3<sup>+</sup> Treg frequencies in tonsillar MNC (Fig. 1g;  $R = -0.60$ ).

**Table 1**  
Demographic data of the study participants and nasopharyngeal carriage of *Streptococcus pneumoniae*

	Age group	Total number	Culture positive	% of colonized
Children	2–4 years	32	17	53.1
	5–9 years	20	8	40.0
	10–16 years	14	3	21.4
Adults	17–36 years	28	2	8.5
No. (%) of children who received conjugate vaccination		46 (69.7%)	20 (71.4%)	

### Th17: Treg ratio increases with age and a higher ratio is associated with a lower pneumococcal carriage rate

As the higher Th17 frequency in adults correlated with a low pneumococcal carriage rate, we further analysed the relationship between Th17 frequency and carriage status in children. There was a trend to show a higher Th17 frequency in carriage-negative than in carriage-positive children, although the difference was not significant (Fig. 2a). However, Treg frequency was higher in carriage-positive than in carriage-negative children (Fig. 2b;  $p < 0.05$ ).

We then examined whether the Th17: Treg ratio correlated with the carriage rate. The Th17: Treg ratio in adults was markedly higher than in children (Fig. 2c;  $p < 0.001$ ), which correlated with the low carriage rate in adults relative to children, and the ratio was shown to increase with age (Fig. 2d;  $p < 0.001$ ). Further analysis was performed to determine whether both the Th17: Treg ratio and age were independently associated with the carriage status in children. Th17: Treg ratio was shown to be higher in carriage-negative than in carriage-positive children (Fig. 2e;  $p < 0.01$ ), whereas no significant age difference was shown between carriage-negative and -positive children (Fig. 2f;  $p > 0.05$ ). The box-plots in Fig. 2(g) showed the distributions of Th17: Treg ratios and ages of children.

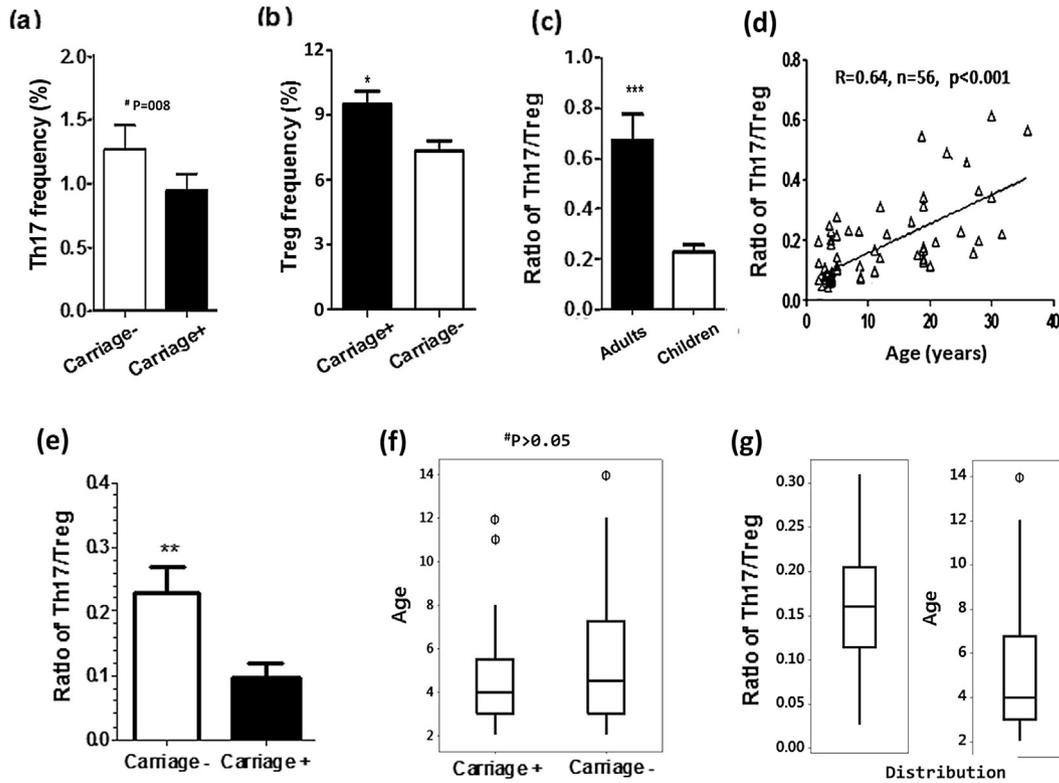
### Activation of memory Th17 cells by pneumococcal stimulation and correlation with carriage

As previous pneumococcal exposure/colonization in the nasopharynx in early childhood would have primed individuals with memory Th17 cells to *S. pneumoniae* in NALT, we examined memory Th17 response in tonsillar MNC following stimulation by a wild-type pneumococcal CCS. The stimulation induced a prominent increase in Th17 cells (Fig. 3a;  $p < 0.05$ ), which was higher in carriage-negative than in carriage-positive children (Fig. 3b;  $p < 0.05$ ). As pneumococcal stimulation also induced an increase in Treg cells (Fig. 3c;  $p < 0.05$ ), which may suppress the Th17 response, we analysed the Th17: Treg ratio following stimulation, and it was higher in carriage-negative than in carriage-positive children (Fig. 3d;  $p < 0.01$ ).

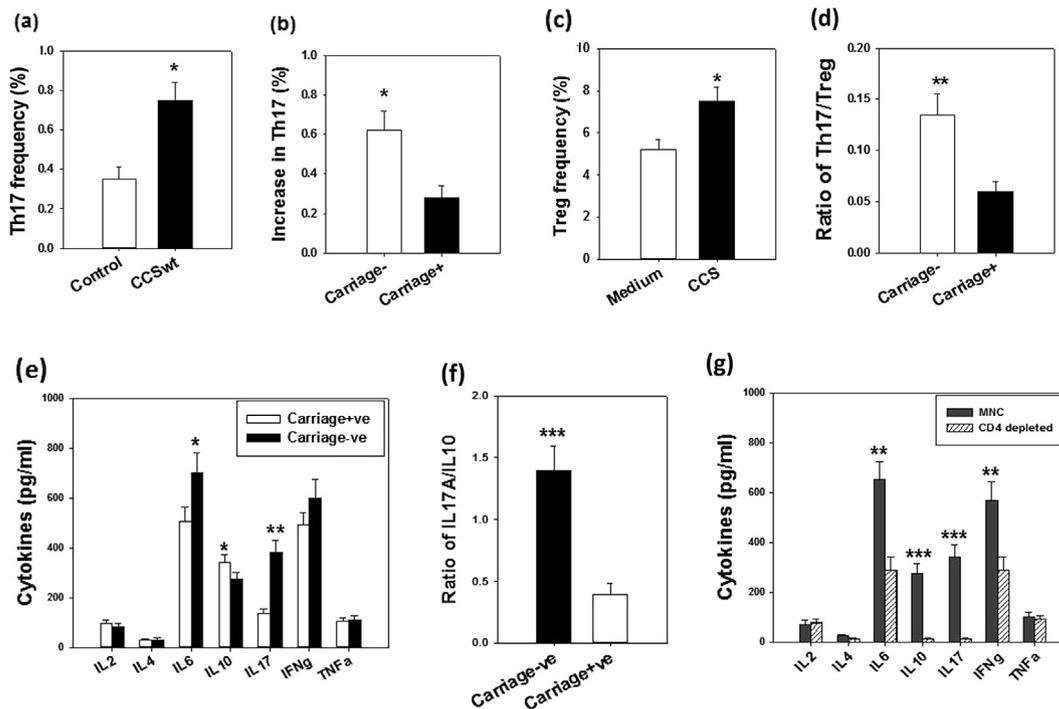
We next analysed the pattern of cytokine responses associated with a higher Th17: Treg ratio. Cytokines including IL-2, IL-4, IL-6, IL-10, IL-17A, interferon- $\gamma$ , tumour necrosis factor- $\alpha$  in tonsillar MNC culture were analysed by CBA array following the stimulation. As shown in Fig. 3(e), carriage-negative children (with higher Th17: Treg ratio) had higher IL-17A and IL-6 responses than carriage-positive children (\*\* $p < 0.01$ , \* $p < 0.05$ ), but the latter tended to have a higher IL-10 response. The former showed a markedly higher IL-17A: IL-10 ratio than the latter (Fig. 3f;  $p < 0.001$ ). CD4<sup>+</sup> T-cell depletion abrogated the IL-17A, IL-10 and reduced IL-6, interferon- $\gamma$  production ( $p < 0.01$ ), but there were no significant changes in the other cytokine levels (Fig. 3g).

### Induction of Th17 and Treg cells by pneumococcal stimulation

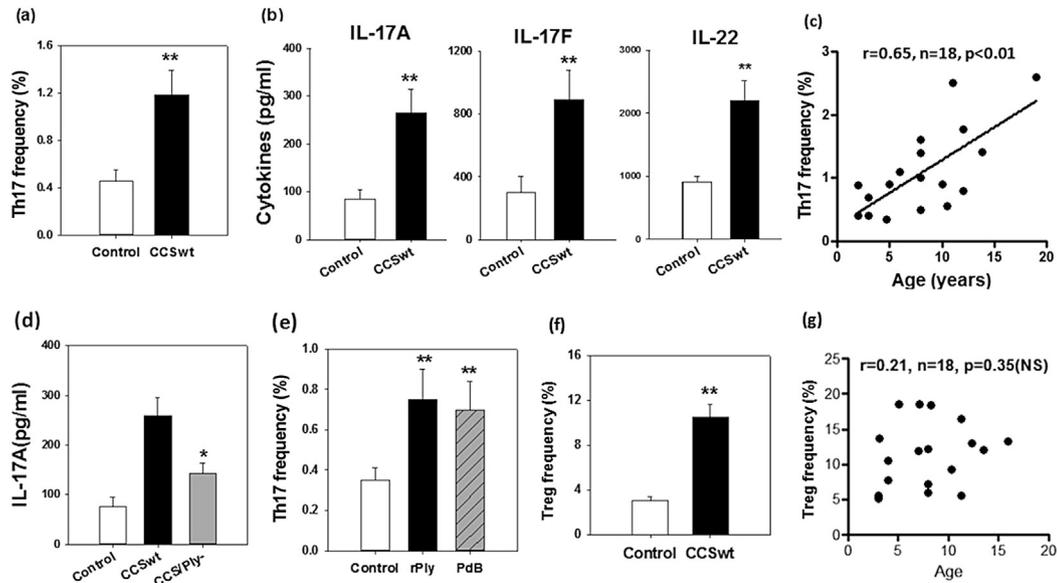
To examine whether pneumococcal stimulation induces Th17 differentiation, CD45RO<sup>+</sup> cell-depleted MNC that retained naive T cells but removed existing Th17 cells were stimulated in the presence of Th17-polarizing cytokines for 7 days. Stimulation with wild-type pneumococcal CCS induced Th17 differentiation (Fig. 4a;  $p < 0.01$ ), along with IL-17A, IL-17F and IL-22 production (Fig. 4b;  $p < 0.01$ ). The magnitude of Th17 differentiation from naive T cells of young children was low but increased with age (Fig. 4c;  $r = 0.65$ ). Compared with wild-type CCS, the CCS derived from an isogenic pneumolysin-deficient strain induced lower IL-17A (Fig. 4d;  $p < 0.05$ ). Stimulation with sublytic concentrations of recombinant



**Fig. 2.** Correlation between the ratio of tonsillar T helper type 17 (Th17) to regulatory T (Treg) frequencies and pneumococcal carriage. Tonsillar Th17 (a) and Treg (b) frequencies in carriage-negative ( $n = 22$ ) and -positive ( $n = 14$ ) children (a,  $\#p = 0.08$ ; b,  $\#p < 0.05$ ). The tonsillar Th17: Treg ratio in adults ( $n = 21$ ) versus children ( $n = 36$ ) (c,  $***p < 0.001$ ), its relationship with age (d,  $p < 0.001$ ), and comparison between carriage-negative ( $n = 22$ ) and -positive ( $n = 14$ ) children (e,  $**p < 0.01$ ,  $t$ -test). No age difference was shown between carriage-negative and -positive children (f,  $\#p > 0.05$ , Mann–Whitney  $U$ -test). The distributions of Th17: Treg ratios (normal) and ages (non-normal) of children were shown in box-plots (g, medians, interquartile ranges and outliers ( $\Phi$ ) were shown).



**Fig. 3.** Activation of memory T helper type 17 (Th17) cells and cytokine response by pneumococcal stimulation and correlation with pneumococcal carriage. Stimulation of tonsillar mononuclear cells (MNC) with wild-type (D39) concentrated pneumococcal culture supernatant (CCS) activates the memory Th17 response (a,  $*p < 0.01$  versus control,  $n = 40$ ), and the response was higher in carriage-negative than -positive children (b,  $*p < 0.05$ ). Pneumococcal CCS also elicited increases in regulatory T (Treg) cells (c,  $*p < 0.05$ ,  $n = 30$ ). The Th17: Treg ratio was higher in carriage-negative ( $n = 22$ ) than in carriage-positive ( $n = 14$ ) children (d,  $**p < 0.01$ ). Cytokine responses (e) and ratio of interleukin-17A (IL-17A)/IL-10 (f) were analysed at day 3 following stimulation and compared between carriage-negative (with higher Th17: Treg ratio) and -positive children (with low Th17: Treg ratio) (e, f,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $n = 26$ ). Cytokine responses in CD4 $^{+}$  T-cell-depleted and unfractionated MNC following stimulation (g,  $**p < 0.01$ ,  $***p < 0.001$ ,  $n = 8$ ).



**Fig. 4.** Induction of T helper type 17 (Th17) and regulatory T (Treg) cells by pneumococcal stimulation. Induction of Th17 cells from naive tonsillar T cells (a,  $**p < 0.01$ ), and interleukin-17A (IL-17A), IL-17F and IL-22 production (b,  $**p < 0.01$ ,  $n = 18$ ) following concentrated pneumococcal culture supernatant (CCS) stimulation. There was an age-associated increase in Th17 cells induced in CD45RO<sup>-</sup> mononuclear cells (MNC) (c,  $r = 0.65$ ,  $p < 0.01$ ). IL-17A production induced by wild-type CCS compared with pneumolysin-deficient CCS (d,  $*p < 0.05$ ,  $n = 8$ ). Induction of Th17 cells by recombinant pneumolysin and toxoid compared with medium control (e,  $**p < 0.01$ ,  $n = 8$ ). Induction of Foxp3<sup>+</sup> Treg cells from Treg-depleted tonsillar MNC by wild-type CCS in the presence of transforming growth factor  $\beta_1$  (TGF- $\beta_1$ ) (f,  $**p < 0.01$ ,  $n = 18$ ), and there was no age-associated difference in the number of Foxp3<sup>+</sup> Treg cells induced (g,  $p$  NS).

pneumolysin (0.05 mg/L) or toxoid (PdB, 0.25 mg/L) also induced an increase in Th17 number (Fig. 4e;  $p < 0.01$ ).

To study Foxp3<sup>+</sup> Treg induction, tonsillar MNC depleted of CD45RO<sup>+</sup> and CD25<sup>+</sup> cells were stimulated by wild-type pneumococcal CCS in the presence of TGF- $\beta_1$  (2.5 ng/mL). The depletion removed existing Foxp3<sup>+</sup> Treg cells (>98%) and memory T cells (>99%) but retained CD45RO<sup>-</sup> naive T cells. The stimulation induced Foxp3<sup>+</sup> Treg differentiation from naive T cells (Fig. 4f;  $p < 0.01$ ), but in contrast to Th17 induction, there was no age-associated difference in the magnitude of Treg cell differentiation (Fig. 4g).

## Discussion

We demonstrated a dynamic relationship between mucosal Th17 and Foxp3<sup>+</sup> Treg cells in human nasopharynx. Whereas Treg cells decreased with age, Th17 and the ratio of Th17: Treg in NALT increased with age, which correlated with the reduction in pneumococcal carriage. Also, the Th17: Treg ratio was significantly higher in carriage-negative than in carriage-positive children. Further, pneumococcal stimulation elicited a stronger memory Th17/IL-17A response associated with higher Th17: Treg and IL-17A: IL-10 ratios in carriage-negative than in carriage-positive children. Collectively, these results suggest that the balance between Th17 and Treg cells in NALT is a critical determinant associated with the clearance of pneumococcal carriage from nasopharynx.

It is thought that Th17 cells promote bacterial clearance through IL-17-mediated recruitment and activation of neutrophils/macrophages [8,9]. Treg cells may promote pneumococcal carriage through its suppression on Th17 induction and activation [14,15]. The inverse correlation between Treg and Th17 frequencies, and that young children had lower Th17 but higher Treg frequencies, and vice versa for adults are consistent with the hypothesis. A recent study also reported a higher Treg frequency and activity in early life than in adults [18].

The increase in Th17 with age may be attributed to the accumulation of memory Th17 cells over time with microbial exposures. *Streptococcus pneumoniae* is a common nasopharyngeal colonizer during early childhood [19,20], which would prime for memory Th17 cells. A higher IL-17A response to *S. pneumoniae* stimulation was shown in PBMC from children and adults in Bangladesh (higher carriage rate) than those in Sweden (lower carriage), supporting the accumulation of pneumococcal memory Th17 cells due to exposure [21]. This is also supported by the finding that experimental pneumococcal colonization in adults elicited a marked increase in memory Th17 cells [22]. Previous pneumococcal exposures are likely to be the reason for increased antigen-specific Th17 responses and a potential reason for those children being culture-negative.

To understand how memory Th17 and Treg cells in the nasopharynx may respond upon exposure to *S. pneumoniae*, we analysed the memory Th17 response, Th17: Treg ratio and cytokine responses following stimulation by pneumococcal CCS. The stimulation of tonsillar cells elicited an increase in both Th17 and Treg cells, but the Th17: Treg ratio and pattern of cytokine responses differed between carriage-negative and carriage-positive children. The former showed a higher Th17: Treg ratio with higher IL-17A response, whereas the latter had a higher IL-10 response. Further, the former showed a markedly higher IL-17A: IL-10 ratio than the latter. These support the theory that a higher Th17: Treg ratio with a stronger IL-17A response promotes clearance of *S. pneumoniae*, whereas a low Th17: Treg ratio with a low IL-17A but higher IL-10 response favoured pneumococcal persistence/carriage. The correlated increase in IL-6 with IL-17A is consistent with the notion that IL-6 is important in Th17 induction [23].

The fact that CD4<sup>+</sup> T-cell depletion abrogated IL-17A production confirms that the IL-17A-producing cells in tonsillar tissue are primarily CD4<sup>+</sup> Th17 cells. We previously showed that Treg depletion reduced IL-10 and enhanced IL-17 production [11], which indicates that Treg cells and associated IL-10 production suppress the Th17/IL-17A response.

We further demonstrated that pneumococcal stimulation induced both Th17 and Treg cells from naive tonsillar T cells. The lower capacity of Th17 induction from naive T cells of younger children is consistent with the lower Th17 frequency in young children. TGF- $\beta$  is known to promote the development of Treg cells [14]. We showed that tonsillar MNC produced TGF- $\beta$ 1 following culture and that was higher in children than in adults, which suggests a favourable environment for Treg cell induction in NALT of young children. Carriage of *S. pneumoniae* in mice was shown to induce Treg cells associated with the TGF- $\beta$  pathway [13]. A higher level of TGF- $\beta$  in nasopharynx in childhood is likely to promote Treg cell induction and facilitate carriage.

Recent interest has focused on Th17-targeted vaccine strategies, including that against pneumococcal infection [24–28], and efforts have been made to identify candidate antigens that promote Th17 cells [27,28]. Our findings that an isogenic pneumolysin-deficient mutant strain-derived CCS induced less Th17 than the wild-type, and recombinant Ply and toxoid-induced Th17 may support the potential of pneumolysin derivatives as candidate vaccine components through promoting Th17.

Our results provide new insights into the relationship between mucosal Th17 and Treg cells in human nasopharynx and pneumococcal carriage. The balance between memory Th17 and Treg in NALT may be a critical host determinant associated with the clearance of carriage. Our results support that early priming of mucosal Th17 in the nasopharynx in childhood may be a novel vaccination strategy [29], and that understanding age-associated changes in paediatric immune responses will be valuable for developing novel formulations to induce early protective Th17 response in young childhood [30].

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## Transparency declaration

The authors have no conflicts of interest to disclose.

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