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Research paper

Reduced IL-2 response from peripheral blood mononuclear cells exposed to bacteria at 6 months of age is associated with elevated total-IgE and allergic rhinitis during the first 7 years of life

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ABSTRACT

Background: Autoimmunity and allergy have been associated with decreased number and function of regulatory T-cells (Tregs) and low interleukin-2 (IL-2) levels. We aimed to investigate if the release of IL-2 from peripheral blood mononuclear cells (PBMCs) stimulated with pathogenic airway bacteria was associated with development of allergy-outcomes in early childhood.

Methods: PBMCs were isolated at age 6 months in 331 infants from the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) mother-child cohort, and subsequently stimulated with *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* in in vitro cultures. Levels of cytokines (IL-2, IL-10, IFN-γ, TNF-α, IL-5, IL-13 and IL-17A) were determined in the supernatant by electrochemiluminescence immunoassays. The immune profiles were analyzed for association with development of total-IgE, allergic sensitization and rhinitis during the first 7 years of life using regression models and principal component analysis (PCA).

Findings: An attenuated IL-2 response to stimulation with *H. influenzae* ($p = 0{\cdot}011$) and *M. catarrhalis* ($p = 0{\cdot}027$) was associated with elevated total-IgE at age 7, which was confirmed in a multivariate PCA model including all cytokine measurements (PC2, $p = 0{\cdot}032$). An immune profile with both reduced IL-2 and elevated IL-5 was associated with increased risk of allergic rhinitis (PC3, $p = 0{\cdot}038$). We found no associations with development of allergic sensitization.

Interpretation: A reduced IL-2 response from PBMCs exposed to common pathogenic airway bacteria at age 6 months was associated with elevated total-IgE and allergic rhinitis during the first 7 years of life. These findings suggest that suppressed Treg activity in early life may herald onset of allergy in early childhood, which could be a target for low-dose IL-2 trials in the future.

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1. Introduction

Reduced numbers and function of regulatory T-cells (Tregs) have been demonstrated in autoimmune and allergic diseases, suggesting that a dysregulated Treg system contributes to the pathogenesis of these conditions [1–4]. Interleukin 2 (IL-2) is critical for the development and survival of Tregs [5–7] and a possible role of the IL-2/Treg interplay in allergy has been suggested by a study showing that reduced IL-2 expression by cord blood CD4(+) T cells associated with decreased Tregs and increased risk of food allergy in childhood [8].

In the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) mother-child cohort we conducted repeated clinical assessments including total-IgE, specific-IgE, and allergic rhinitis through age 7 and biobanking of peripheral blood mononuclear cells (PBMCs) isolated from the infants at age 6 months. Recently, we investigated the PBMC response to in vitro stimulations with *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* measuring production of T cell-

Abbreviations: COPSAC, Copenhagen Prospective Studies on Asthma in Childhood; PBMC, Peripheral Blood Mononuclear Cell; PCA, Principal Component Analysis; Treg, regulatory T cell; SPT, Skin prick test.

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Research in context

Evidence before this study

It is well known that T regulatory cell (Tregs) play important roles in maintaining homeostasis of the immune system. Interleukin 2 (IL-2) is crucial for the development and survival of Tregs acting via its high affinity receptor CD25 and it has been demonstrated that low-dose IL-2 therapy results in significant expansion and activation of Tregs. It has been shown in randomized placebo-controlled trials that low dose IL-2 therapy is safe in humans and has the capacity to successfully control the manifestation of several diseases related to imbalanced immune responses, including hepatitis C virus-mediated vasculitis, graft-versus-host disease, alopecia areata and type I diabetes. Recently, it has been shown that a disturbed IL-2/Treg interplay may be of importance in childhood allergy development as reduced IL-2 expression by cord blood CD4(+) T cells was associated with decreased numbers of Tregs and increased risk of subsequent food allergy in the Barwon Infant study. Further, findings from a mouse model showed that low dose IL-2 treatment could dampen the allergic inflammatory process by inducing expansion and activation of Tregs. These findings suggest a potential application of low dose IL-2 therapy for prevention and treatment of childhood allergy, but further human studies are needed to disentangle the role of IL-2 expression in early life in relation to the development of allergic outcomes.

Added value of this study

In this study we investigated the functional immune properties of peripheral blood mononuclear cells (PBMCs) from 6-month-old healthy infants stimulated with pathogenic airway bacteria and show that immune responses characterized by reduced production of IL-2 are associated with development of elevated total-IgE levels and allergic rhinitis during the first 7 years of life. This suggests the existence of suppressed Treg activity in early life heralding the onset of allergy outcomes during early childhood, which may be reverted by low dose IL-2 therapy.

Implications of all the available evidence

These findings support the initiation of low dose IL-2 therapy trials for preventing and treating childhood allergic airway disorders.

related cytokines of the Treg (IL-2, IL-10), Th1 (interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α)), Th2 (IL-5, IL-13) and Th17 (IL-17A) cells [9,10].

The aim of the current study is to analyze the relationship between the PBMC response to bacterial stimulations and the development of allergy-related endpoints during the first 7 years of life, hypothesizing a protective role of IL-2.

2. Materials and methods

2.1. Study cohort

This study is part of the ongoing $COPSAC_{2000}$ prospective mother-child cohort of 411 children born to mothers with asthma [11]. The children were enrolled at age 1 month, excluded children born before gestational week 36 and children suffering from any respiratory disorder before enrollment. The children were followed prospectively at the COPSAC clinical research unit with regular follow-up visits every 6 months till age 7 and at episodes with respiratory symptoms. The clinical research unit was the primary health care facility for diagnosis and treatment of any respiratory symptoms, strictly adhering to validated algorithms [12].

The study was approved by the Copenhagen Ethics Committee (KF 01-289/96), the Danish Data Protection Agency (2002-41-2434), and followed the principles of the Declaration of Helsinki. Written and oral informed consent was obtained from the parents at enrolment.

2.2. Bacterial stimulation of PBMCs

The bacterial stimulations of PBMCs was previously described in details [9,10]. Briefly, PBMCs were isolated by density centrifugation from blood samples collected at 6 months and stored for up to 12 years at -140 °C. After thawing, the cells were stimulated with UV-inactivated *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* (50 μg/mL) or blank sterile culture media alone in U-bottomed 96-well plates at 5×10^5 cells/well (200 μL total volume/well) for 40 h at 37 °C and 5% CO_2 in a humidified incubator. Supernatants were harvested and stored at −80 °C until quantification of IL-2, IL-10, IFN-γ, TNF-α, IL-5, IL-13 and IL-17A levels by customized multiplex immunoassays from MesoScale Discovery read on a Sector Imager 6000 (MSD, Gaithersburg, MD, USA). All assays were highly sensitive with a detection limit below 1 pg/mL.

3. T cell immune phenotyping

The composition of the T cell compartment was analyzed on freshly thawed unstimulated PBMCs (5×10^5 cells) using flow cytometry. Staining and flow cytometry analysis were performed using the following antibody panel: CD3/eFlour450, CD8/FITC, TCRVa24-Ja18/PerCP-eFlour710, CD127/APC-eFlour780 (eBioscience, San Diego, CA), CD25/PC7, TCRγδ/PE (Beckman Coulter, Brea, CA) and CD4/V500 (BD Bioscience, San Jose, CA). T cell subsets were identified by a predefined gating strategy and analyzed in a blinded manner: Helper ($CD3^+CD4^+$), cytotoxic ($CD3^+CD8^+$), regulatory ($CD3^+CD4^+CD127^-CD25^+$), γδ ($CD3^{+-}TCR\gamma\delta^+$) and invariant NK ($CD3^+TCRV\alpha24\text{-}J\alpha18^+$) T cells. All population frequencies were calculated relative to the $CD3^+$ T cell compartment.

3.1. Allergy-related endpoints

Total-IgE level was determined at 6 years by ImmunoCAP (Pharmacia Diagnostics AB, Uppsala, Sweden) and analyzed as a continuous variable.

Allergic sensitization was assessed at 6 years by skin prick tests (SPT) and measurements of specific-IgE against house dust mites, cat, dog, horse, birch, grass, mugwort, and molds as previous detailed [13]. SPT was done using allergen extracts (ALK Abello, Soluprick® SQ, Copenhagen, Denmark) defining a positive test as any wheal ≥3 mm. Specific-IgE levels were measured with ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden), defining a positive test as any specific-IgE level ≥ 0·35kU_A/L. Sensitization was analyzed as a dichotomized variable, separately for SPT and specific-IgE as the overlap between test results is poor at young age [14].

Allergic rhinitis at age 7 was diagnosed by the COPSAC pediatricians based on a parental interview on the child's history of symptoms [15]. Significant sneezing or blocked or runny nose affecting the wellbeing of the child in the past 12 months in periods without cold or flu defined rhinitis. The diagnosis required sensitization and congruence between symptoms and allergen exposure.

3.2. Statistical analysis

Initially, supernatant levels of cytokines in response to bacterial stimulations were adjusted by subtracting the baseline levels of

cytokines secreted by PBMCs incubated with sterile media. Thereafter, the levels were square root-transformed. If an individual did not produce a measurable cytokine response in PBMCs after bacterial stimulation (non-responders), the cytokine level was set to zero.

First, we analyzed the association between the cytokines released upon bacterial stimulations at 6 months and total-IgE level (log-transformed), allergic sensitization by SPT (yes/no) and elevated specific-IgE (yes/no), and allergic rhinitis (yes/no) during the first 7 years of life using linear and logistic regression models. The associations with outcomes for cytokines from each bacterial stimulation were analyzed using forward stepwise selection while retaining IL-2. If the final model had multiple independent variables they were compared with a null model by ANOVA for goodness of fit. If only IL-2 was retained in the final model, the hypothesis test for its coefficient was used as assessment of goodness of fit. Only the models with significant overall *p*-values (≤0.05) are discussed in detail.

Thereafter, we conducted a data-driven principal component analysis (PCA) on the 21 independent variables (3 bacterial stimulation x 7 different cytokines) to capture immune patterns in the data, using principal component 1 to 4 (PC1–4) from the model for association analyses with the outcomes.

We also analyzed the association between T cell subsets and allergy-related outcomes. T cell composition data was transformed by isometric log ratio (ilr) using sequential binary partitioning and their association with outcomes was analyzed by multiple regression models [16].

All analyses were conducted with R version 3.4.2 [17]. The results are reported with 95% CI and *p*-values ≤.05 were considered statistically significant.

4. Results

4.1. Baseline characteristics

A total of 331 (81%) of the 411 infants in the cohort had PBMCs collected at age 6 months, which were subsequently exposed to *H. influenzae*, *M. catarrhalis,* and *S. pneumoniae* for assessment of the immune response by measuring supernatant cytokine levels. The T cell immune phenotyping and immune responses are described in Tables 1–2. Of the 331 infants, 259 (78%) had measurements of total-IgE (mean level, 117·6 IU/mL), 270 (82%) had SPT ($N = 251$) and/or sIgE ($N = 259$) with 100 (30%) being sensitized to one or more allergen either by SPT ($N = 40$) and/or sIgE ($N = 94$). Allergic rhinitis was assessed in 254 (77%) with a prevalence of 13% ($N = 34$) by age 7 years.

A comparison between children with vs. without immune response data has previously been published showing no differences [9,10].

4.2. Bacteria-induced immune response and allergy-related outcomes

4.2.1. Total-IgE

Multiple regression analyses showed inverse associations between IL-2 production in response to *H. influenzae* and *M. catarrhalis* at age 6 months and total-IgE level at age 6 years: IL-2-coefficient$_{hi}$ = −0·183 [95% CI; −0·324, −0·043], $p = 0·011$ and IL-2-coefficient$_{mc}$ = −0·150 [−0·282, −0·018], p = 0·027, respectively. The IL-2 association was only seen in response to the Gram-negative bacteria *H. influenzae* ($p_{Ftest} = 0·028$) and *M. catarrhalis* ($P_{Ftest} = 0·024$), whereas the model using cytokine data in response to the Gram-positive bacteria *S. pneumoniae* was not better than the null model ($P_{Ftest} = 0·139$): IL-2-coefficient$_{sp}$ = 0·151 [0·001, 0·301], $p = 0·0503$ (Fig. 1).

Besides IL-2, the production of IFN-γ and TNF-α in response to *H. influenzae* and *M. catarrhalis* were positively associated with total-IgE level: IFN-γ-coefficient$_{hi}$ = 0·019 [0·000, 0·0367], p = 0·046, and TNF-α-coefficient$_{mc}$ = 0·159 [0·027, 0·290], p = 0·019, respectively.

The result of a PCA analysis to identify individual cytokine profiles in response to the bacteria is depicted in Fig. 2 and Fig. e1. The PCA association analysis for total-IgE confirmed the findings from the regression models. The cytokine pattern in PC2 from *M. catarrhalis* was inversely associated with total-IgE level ($p = 0·032$) and the same trend was seen for PC2 from *H. influenzae* stimulation ($p = 0·051$); i.e. higher total-IgE was associated with a decreasing score dominated by IL-2 (Fig. 2).

4.2.2. Allergic sensitization

Higher IL-5 secretion in response to *S. pneumoniae* was significantly associated with sensitization diagnosed by sIgE at age 6: $OR_{sc} = 1·678$ [1·069, 2·633], p = 0·024, although the overall model with inclusion of all cytokines was not significantly better than the null model (overall *p*-value = 0·080). No associations were observed between the immune response to the other bacterial stimulations and development of allergic sensitization determined by neither sIgE nor SPT (Figs. 1–2).

4.2.3. Allergic rhinitis

IL-2 in response to *M. catarrhalis* and IL-13 in response to *H. influenzae* were inversely associated with allergic rhinitis by age 7: $OR_{mc} = 0·495$ [0·273, 0·898], $p = 0·021$, and $OR_{hi} = 0·429$ [0·197, 0·933], p = 0·033; i.e. higher IL-2 and IL-13 production reduced the risk of allergic rhinitis. We also found that elevated IL-5 in response to all the bacterial stimulations increased the risk of allergic rhinitis: $OR_{hi} = 1·954$ [1·037, 3·681], $p_{hi} = 0·038$, $OR_{mc} = 1·569$ [1·056, 2·330], $p_{mc} = 0·026$, and $OR_{sp} = 1·614$ [1·070, 2·434], $p_{sp} = 0·022$. The multiple regression models were all significantly better than the null models: $p_{chisq} = 0·048$ for *H. influenzae*, $p_{chisq} = 0·017$ for *M. catarrhalis*, and $p_{chisq} = 0·046$ for *S. pneumoniae* (Fig. 1).

The results from the PCA supported the main findings from the regression analyses regarding IL-5 and IL-2, but not for IL-13. We found an inverse association between development of allergic rhinitis and the immune pattern in PC3 upon *M. catarrhalis* stimulation

Table 2
Composition of T cell compartment at 6 age months ($n = 242$).

T cell profile	Median (IQR)
Helper T cells (%)	68·5(62·6–72·4)
Cytotoxic T cells (%)	24·1(20·6–29·8)
Regulatory T cells (%)	2·3(1·8–2·9)
γδ T cells (%)	3·0(2·2–3·9)
Invariant NK T cells (%)	0·1(0·1–0·1)

Values are percentage of all CD3+ T cells.
IQR = interquartile range.

Table 1
Supernatant cytokine levels in response to pathogenic airway bacteria at 6 months ($n = 331$).

Cytokine supernatant	Control, median (IQR)	*H. influenzae*, median (IQR)	*M. catarrhalis*, median (IQR)	*S. pneumoniae*, median (IQR)
IL-2 (pg/ml)	0·8 (0–4·2)	4·8 (1·2–14·6)	12·2 (3·2–28·5)	9·5 (1·4–41·8)
IL-10 (pg/ml)	0·0 (0–1)	15·0 (2·2–60·2)	22·5 (3·9–77·6)	1·8 (0–9·9)
IFN-γ (pg/ml)	0·4 (0–1)	4·5 (0·3–77·9)	19·9 (0·8–233·9)	1·4 (0·2–12·2)
TNF-α (pg/ml)	1·5 (0·5–4·1)	35·5 (8·2–169·2)	54·2 (17·7–201·7)	21·5 (5·2–101)
IL-5 (pg/ml)	0·0 (0·0–0·0)	0·0 (0·0–0·4)	0·4 (0·0–2·4)	0·0 (0·0–0·6)
IL-13 (pg/ml)	0·0 (0·0–0·0)	14·9 (0·0–75·5)	60·4 (5·7–203·3)	10·9 (0·0–71·6)
IL-17A (pg/ml)	0·0 (0·0–1·8)	1·9 (0·0–12·6)	9·4 (0·5–43·0)	2·2 (0·0–17·6)

IQR = interquartile range.

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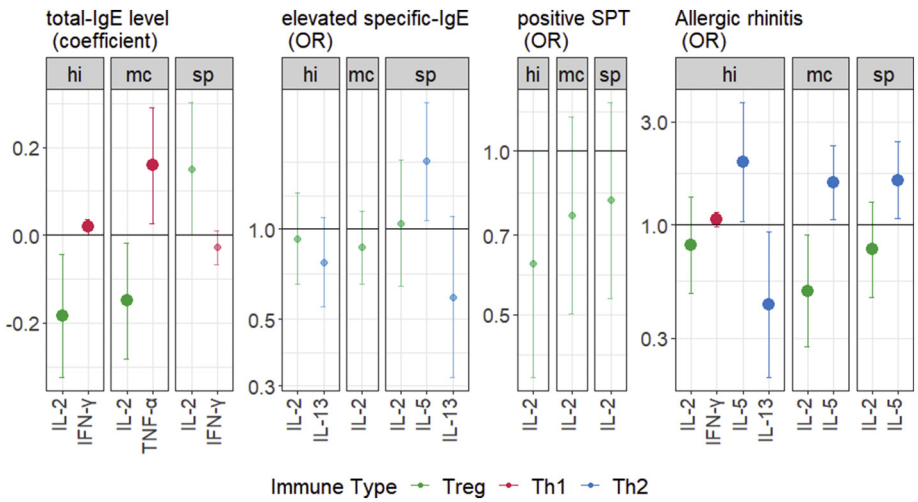

Fig. 1. Association between selected cytokines and allergic outcomes. Association between cytokines from 6 month (n = 331) upon stimulation with *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* and log transformed total IgE (mean = 1.505, SD = 1.115), allergic sensitization determined by skin prick test (case/control = 44/173) and specific-IgE level (case/control = 93/173) at 6 year, and allergic rhinitis (case/control = 38/185) at 7 year. The shown cytokines were from the best fitted models, which were defined by forward stepwise regression in relation to each clinical outcomes. Ordinary least squares regression for log transformed total-IgE level and logistic regression for the rest of allergy outcomes was each regressed on cytokines from each stimulation in a forward stepwise fashion by retaining IL-2 in the selection process. The overall fitness of selected models was accessed and relevant model statistics (Odds Ratio (OR) for binary outcomes, coefficients for continuous outcome and their associated 95% confidence intervals) for significant ones were shown in solid colors and bigger dot size, otherwise the colors were faint and the dots were smaller. Positive SPT = positive skin prick test, hi = H. influenzae, mc = M. catarrhalis, sp = *S. pneumoniae*.

($p = 0{\cdot}038$), i.e. lower IL-2 and higher IL-5 from PBMCs at 6 months increased the risk of allergic rhinitis at age 7. In addition, a decreasing PC4 score from the *H. influenzae* stimulation was significantly associated with risk of allergic rhinitis, i.e. lower IL-17A and higher IFN-γ release increased the risk of allergic rhinitis (p = 0·048) (Fig. 2).

4.2.4. Sensitivity analysis

As many of the children did not produce any IL-5 in PBMCs after bacterial stimulations (IL-5 non-responders), we conducted a sensitivity analysis excluding these (Table e1). This showed similar results for the univariate association between IL-5 and allergic rhinitis by age 7: $OR_{hi} = 2{\cdot}532$ [1·127, 5·690], $p_{hi} = 0{\cdot}025$, $OR_{mc} = 3{\cdot}231$ [1·274, 8·200], $p_{mc} = 0{\cdot}014$, and $OR_{sp} = 1{\cdot}729$ [1·084, 2·757], $p_{sp} = 0{\cdot}021$, and the multiple regression models were also significantly better than the null models: $p_{chisq} = 0{\cdot}040$ for *H. influenzae*, $p_{chisq} = 0{\cdot}001$ for *M. catarrhalis*, and $p_{chisq} = 0{\cdot}048$ for *S. pneumoniae* (Fig. e2). The PCA results were consistent showing an inverse association between allergic rhinitis and the immune pattern in PC3 upon stimulations from both *M. catarrhalis* ($p = 0{\cdot}036$) and *H. influenzae* ($p = 0{\cdot}038$) (Fig. e3).

4.3. T cell compartment composition

To study if the cytokine response profiles were related to an underlying difference in T cell subsets, we examined the T cell compartment composition in relation to the allergy-outcomes. The relative ratios of γδ T cells and iNKT cells amongst all T cells were associated with total-IgE level at age 6, but in opposite directions with an inverse association identified for iNKT cells (p = 0·030), and positive association for γδ T cells (p = 0·022) (Fig. 3, Fig. e4). No associations between the relative ratio of Tregs and development of allergy-related outcomes were observed (Fig. 3).

5. Discussion

5.1. Main findings

An attenuated IL-2 response to stimulation with the common Gram-negative pathogenic airway bacteria *H. influenzae* and *M. catarrhalis* in PBMCs collected at age 6 months was associated with elevated total-IgE at age 6 years. Furthermore, both reduced IL-2 and increased IL-5 production were associated with a higher risk of developing allergic rhinitis during the first 7 years of life. Altogether, these findings suggest a role of diminished IL-2 and increased IL-5 in development of allergy-related traits in early childhood.

5.2. Strengths and limitations

This is the first prospective cohort study to investigate infant's bacterial immune responses in PBMCs, which was done in a comprehensive manner by analyzing several cytokines of the Treg, Th1, Th2, and Th17 cells. The prospective nature of our study and the storage of PBMCs at age 6 months provide a unique possibility to investigate functional immunological properties in infancy before onset of allergy-related outcomes. As the PBMCs were collected in asymptomatic infants before onset of allergic disease, the bacterial immune response is unlikely to be affected by immune dysfunction caused by ongoing disease-driven inflammation.

It is an advantage of the study that the children participating in the $COPSAC_{2000}$ cohort solely used the COPSAC research pediatricians for diagnosis and treatment of any asthma and allergy-related disease. All diagnoses were done based on rigid standardized algorithms providing highly reliable homogeneous clinical outcomes.

It is a limitation that this was an in vitro study that may not reflect the complexity of the immune responses in vivo. Importantly, many of the infants stored PBMCs did not produce a measurable IL-5 response to any of the bacterial stimulations. However, analyses including the IL-5 non-responders as zeros as well as analyses excluding the non-responders showed similar associations with the clinical endpoints.

It is a limitation that the results are based on one concentration of antigen performed at one time point (40 h culture). Therefore, we are not able to determine how the results would look like at different time points and by stimulating with different concentrations, which is particularly important for IL-2, which is consumed in the cultures. However, this would have been of greater importance if we had not detected an association between IL-2 PBMC responses and allergy-outcomes.

Finally, it is a limitation that all the children participating in the cohort were born to mothers with a history of asthma, which may hamper the external validity of our findings.

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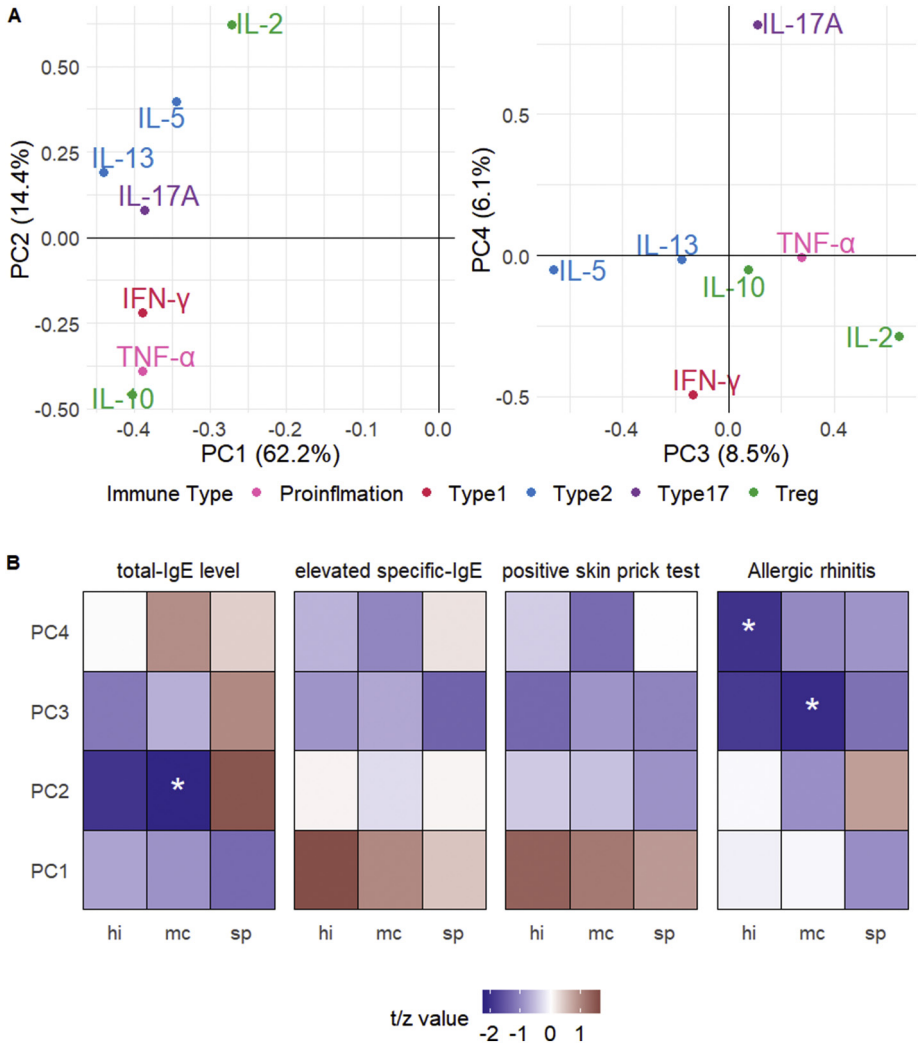

Fig. 2. Association between cytokine profiles from 6 month ($n = 331$) upon stimulation with *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* and log transformed total IgE, allergic sensitization determined by skin prick test and specific-IgE level at age 6 and allergic rhinitis at age 7. A. Cytokine loadings of PC1 to PC4 from principal components analysis (PCA). B. Ordinary least squares regression for log-transformed total-IgE level and simple logistic regression for the rest clinical outcomes was each regressed on PC1–4. The values of test statistic of regression coefficients are plotted in the heatmaps. Blue indicates negative association and red indicate positive association. Asterixis (*) indicates $p < .05$. hi = H. influenzae. mc = M. catarrhalis. sp = S. pneumoniae. Log transformed total IgE (mean = 1.505, sd = 1.115). Number of allergic rhinitis and control (38/185). Number of Kids with allergic sensitization assessed by the level of specific IgE and healthy control (93/173). Number of Kids with allergic sensitization assessed by skin prick test (SPT) and healthy control (44/173).

5.3. Interpretation

In this study, we aim to identify immune patterns in infancy in response to different bacteria in relation with development of allergic disorders in the first 7 years of life. The bacterial stimulations were chosen as we previously demonstrated that one-month-old infants colonized in the airways with *H. influenzae*, *M. catarrhalis*, and/or *S. pneumoniae* had an increased risk of asthma at age 5 years [18]. However, no direct link was shown between neonatal airway colonization with these bacteria and allergic outcomes, but studies indicate that environmental exposure to certain bacteria or bacterial components like lipopolysaccharide, which is contained in *H. influenzae* and *M. catarrhalis*, are associated with risk of allergic sensitization in childhood [19–21].

We speculate that our finding of a reduced IL-2 production in concert with increased IL-5 to stimulations with the Gram-negative bacteria *H. influenzae* and *M. catarrhalis* may be caused by imbalanced Treg and Th2 populations and thereby increase the risk of developing allergic diseases. This finding may be of clinical importance as low-dose IL-2 therapy is being investigated with the purpose of expanding the Treg population in several diseases. We did not observe similar findings when analyzing the cytokine responses from the Gram-positive *S. pneumonia* stimulation, which may be due to the fact that this bacteria does not contain the innate immune ligand lipopolysaccharide.

Recent clinical trials have studied the tolerability and efficacy of low-dose IL-2 therapy in the treatment of autoimmune disorders and graft-versus-host disease [22–25]. All trials reported the therapy to be safe and effective in mediating sustained expansion of systemic Tregs with clinically relevant improvement in patients with HCV-induced vasculitis [22], alopecia areata [25], and graft-versus-host disease [24]. We speculate that low-dose IL-2 therapy may have a beneficial role on allergy and propose initiation of clinical trials in children with allergy and subsequently trials investigating low-dose IL-2 as a possible therapeutic intervention or a prophylactic regimen in high-risk infants. Due to the proposed early window of immune programming for regulatory T cell development [26], it might be speculated to be even more important to propagate Treg development in early infancy, once an impaired IL-2 production has been detected. We here addressed the question of IL-2 production in PBMCs collected at 6 months of age, as bacteria-specific T cells will have been expanded at this age if the child has been exposed to the given bacteria in vivo. This is in contrast to studies performed on cord blood. We selected a relatively short stimulation protocol of 40 h

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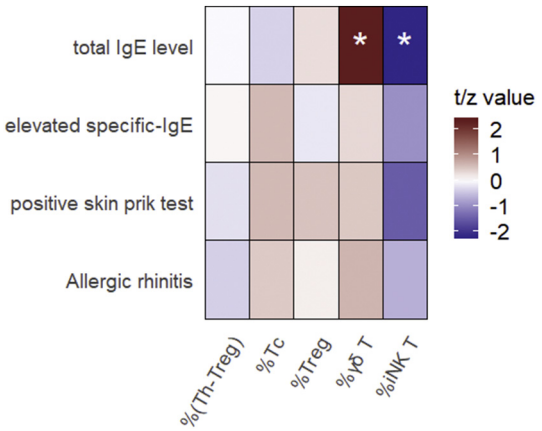

Fig. 3. The association between T cell compartment at 6 month and allergy outcomes at the age of 6 years. Multiple linear regression for log transformed total-IgE level and multiple logistic regression for the rest of allergy outcomes was regressed on isometric transformed T cell compartments. The values of test statistic of regression coefficients are plotted in the heatmaps. Blue indicates negative association and red indicate positive association. Asterixis (*) indicates $p < .05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with the aim of examining specifically the bacteria-specific memory and effector T cells present in the PBMC fraction that would be more readily activated for cytokine production than naïve T cells, which require a longer protocol. Although this study examines immune programming prior to disease development, it might also be valuable to use IL-2 therapy in already diseased individuals. Prior to trials in allergic diseases, it would valuable to investigate IL-2 PMBC responses in children with vs. without ongoing symptoms of allergic rhinitis.

Although IL-2 has pleiotropic effects and works on other types of Th effectors, the efficacy and specificity of low dosage IL-2 therapy may be supported by the selective responsiveness of Tregs against low does IL-2. It was reported that due to an enhanced expression of the IL-2 receptor (α and γ chain) and promoted activity of endogenous serine/threonine phosphatase protein phosphates 1 and/or 2A in Tregs about 10–100 fold lower levels of IL-2 was required to activate STAT5 in Tregs compared to the levels needed for memory T cells or activated T cells [27].

It is possible that reduced IL-2 production during the continuous immune activation induced by the host microbiome in infancy could affect the risk of developing allergic disease. It is recognized that the microbiome plays a key role in development of the immune system and the establishment of homeostatic tolerance by Tregs [28–30]. Thus, infants who respond to bacteria with lower IL-2 production could have an imbalanced immune system with blunted Treg function leading to proneness to develop higher circulating IgE levels, diminished control of tolerance to allergens, and subsequently develop symptomatic disease such as rhinitis.

We have previously within the same cohort reported abnormal bacterial immune response in PBMCs in infants developing asthma later in childhood [9]. The immune responses associated with asthma were dominated by increased Th2 cytokines (IL-5 and IL-13) in response to the same three pathogenic airway bacteria. However, in contrast to the current findings, no associations existed between IL-2 release and development of asthma, which underlines different immune patterns driving the heterogeneous phenotypes of allergy and asthma and suggests that low-dose IL-2 therapy might be targeted solely to the allergic phenotype. This fits well with a recent study from a food allergy mouse model showing that low dose IL-2 treatment could control the allergic inflammatory process by inducing expansion and activation of the Treg population [31]. In line with this, a very recent study with a murine respiratory allergy model also showed that application of IL-2-αIL-2 mAb complexes resulted in specifically induction of Treg population and protection from airway hyperresponsiveness thereafter [32]. Further experimental studies of i.e. allergen-specific stimulations of human PBMCs are needed to determine whether IL-2 therapy should solely be targeted to the allergic phenotype.

Higher IL-13 production upon bacterial stimulation of the PBMCs was observed to reduce the risk of allergic rhinitis, which was however not significant in the multivariate PCA approach. This is an unexpected finding as co-expression of IL-4, IL-13 and IL-5 are normally observed in patients with ongoing symptoms of rhinitis. A non-coordinate expression of IL-4, IL-13 and IL-5 can occur when distinct Th2 clones produce each cytokine individually. Thus, the discrepancy in expression of IL-5 and IL-13 may suggest a transient expression pattern associated with the initial stage of Th2 priming as we investigated the response from stimulated PBMCs in 6-month-old asymptomatic infants long before onset of any symptoms of allergic rhinitis.

Our T cell compartment data showed an association between the composition of γδ T cells and total-IgE level suggesting a possible function of γδ T cells in the development of allergic diseases. Such association has previously been reported in children with atopic dermatitis aged 1 to 10 years, whereas no association was observed for total-IgE level within these children with atopic dermatitis [33]. Finally, we also identified an inverse association between the composition of iNKT cells and total-IgE, which is consistent with previous findings in adult asthmatics [34].

6. Conclusion

Reduced IL-2 production and increased IL-5 from PBMCs in response to bacterial stimulations in early life were associated with development of elevated total-IgE and allergic rhinitis during the first 7 years of life. This finding is of possible clinical relevance as experimental studies have shown that low-dose IL-2 therapy can restore immune tolerance by the expansion of Tregs.

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Conflict of interests

The authors declare no conflicts of interest.

Author contributions

The guarantor of the study is HB who is responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data and writing of the manuscript. HB had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. BC and NW were responsible for data analysis and wrote the first draft of the manuscript. AMS, JML, AT, SBP, MAR, JS and KB contributed to design of the study, interpretation of data and writing of the manuscript. JML, AT and SBP were responsible for generating the PMBC immune response data. All co-authors have contributed substantially to the analyses and/or interpretation of the data and have provided important intellectual input and approval of the final version of the manuscript. No honorarium, grant, or other forms of payment was given to anyone to produce the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.04.047.

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Table e1. Supernatant IL-5 levels in response to pathogenic airway bacteria at 6 months (n=239).

IQR=Interquartile range.

	Control, median (IQR)	***H influenzae, median (IQR)***	***M catarrhalis, median (IQR)***	***S pneumoniae, median (IQR)***
Cytokine supernatant				
IL-5 (pg/ml)	0·0 (0-0·04)	0·12 (0-0·77)	1·29 (0·24-4·11)	0·14 (0-1·25)

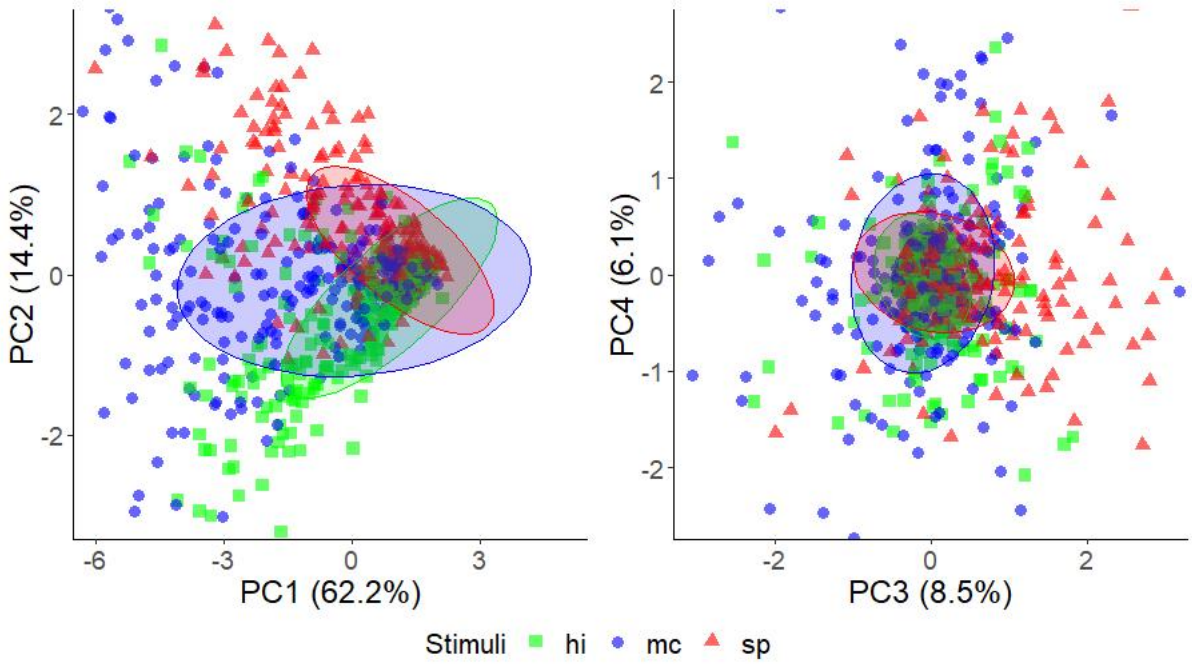

Figure e1. The score plots of principle components 1-4 (PC1-4) from principle components analysis (PCA) based on cytokine profiles from 6 month (n=331) upon stimulation with *H. influenzae, M. catarrhalis and S. pneumoniae*. The 90% confidence ellipse are added.

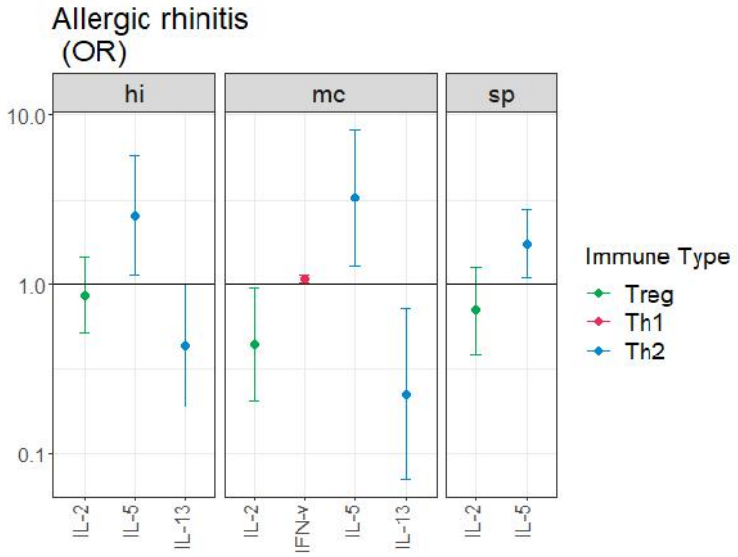

Figure e2. Sensitivity analyses of the association between selected cytokines and allergic rhinitis

Association between cytokines from age 6 month (n=239) after removal of the IL-5 non-responders upon stimulation with *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* and allergic rhinitis (case/control=27/120) at 7 year. The shown cytokines are derived from the best fitted models defined by forward stepwise regression in relation to allergic rhinitis.

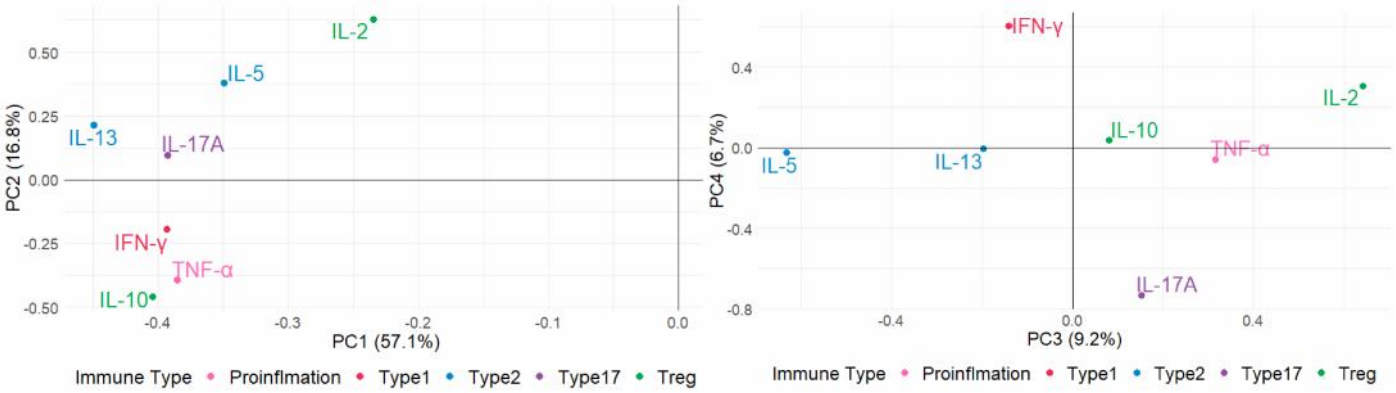

Figure e3. Cytokine loadings of PC1 to PC4 from principle component analysis (PCA)

Loading plots from PCA based on cytokines from 239 children, after removal of IL-5 non-responders.

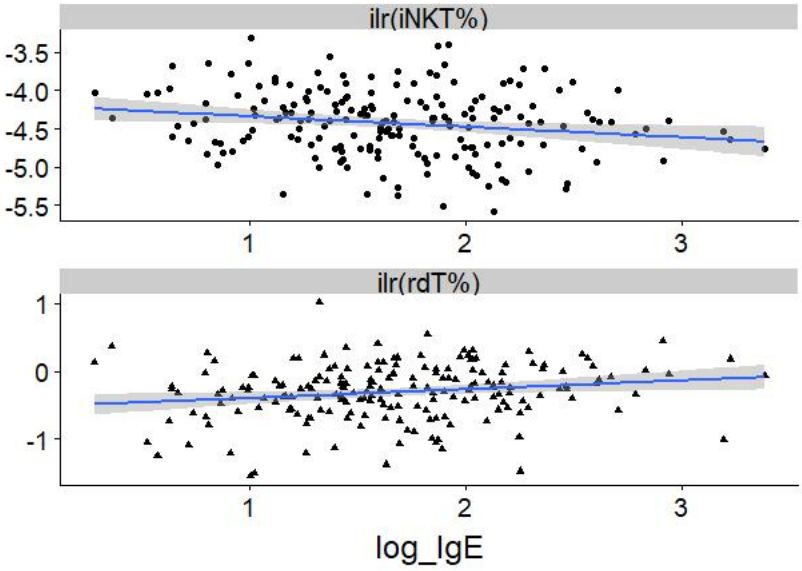

Figure e4. The scatter plot about association between T cell compartment and log transformed IgE level at age 6.