

Infant 7-valent pneumococcal conjugate vaccine immunization alters young adulthood CD4⁺T cell subsets in allergic airway disease mouse model



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ABSTRACT

7-Valent pneumococcal conjugate vaccine (PCV7) immunization in adulthood can inhibit allergic asthma in mouse model. The aim of this study is to investigate the effects of infant PCV7 immunization on young adulthood CD4⁺T cell subsets in a murine allergic airway disease (AAD) model. Our study indicated that infant PCV7 immunization can inhibit young adulthood airway inflammation and airway hyperresponsiveness (AHR) by inducing the production of Foxp3⁺Treg, Th1 cells and their cytokines IL-10 and IFN- γ , inhibiting the production of Th2, Th17 cells and their cytokines IL-13 and IL-17A in BALB/c mice model. These results suggested that infant PCV7 immunization may serve as an effective measure to prevent young adulthood mice AAD.

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1. Introduction

Asthma is a common illness throughout the world which characterized with chronic airway inflammation, airway hyperresponsiveness (AHR) and airway remodeling. Despite advances in the understanding of the mechanisms of allergic asthma, current therapies only alleviate/control the symptoms of asthma. There is a need to look for other treatment approaches.

The recent world-wide changes in asthma prevalence imply significant environmental effects on asthma. Reduced exposure to bacteria or their products is associated with increased asthma, utilization of immunoregulatory treatments that based on bacterial components may have benefits for the suppression of asthma [1]. Studies demonstrated CpG-ODNs, BCG can inhibit allergic airway disease (AAD) in mouse models [2,3]. However, treatments with CpG-ODN may induce harmful side effects [2], while BCG has no

efficacy on allergic asthma in human trials [4]. Pneumococci is a common respiratory pathogen, causing pneumonia, otitis media, meningitis and septicemia. Pneumococcal vaccination is recommended to prevent invasive pneumococcal infection in high-risk groups including asthmatics [5]. Epidemiological studies demonstrated that 7-valent pneumococcal conjugate vaccine (PCV7) immunization reduce the incidence of asthma and associated hospitalizations in both children and the elderly [6,7]. Thorburn et al. [8] stated PCV7 immunization in adulthood mice inhibit the hallmark features of AAD through promotion of Tregs and suppression of Th2 cells production. Recent studies indicated Th17 cells play vital role in asthma pathogenesis [9–11]. Furthermore, PCV7 immunization is currently administered in infancy to prevent childhood pneumococci infections. Whether infant PCV7 immunization can alter young adulthood CD4⁺T cell subsets and inhibit AAD or not remains elusive. In this study we investigated the effects of infant PCV7 immunization on young adulthood AAD in mouse models. We demonstrated that infant PCV7 immunization attenuate young adulthood mice hallmark features of AAD by promoting Foxp3⁺Treg, Th1 cells and inhibiting Th2, Th17 cells production.

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2. Materials and methods

2.1. Mice, immunization, and treatment

BALB/c mice (6–8 weeks), free of specific pathogens, were maintained in individually ventilated cages, housed in autoclaved cages and fed on OVA-free diets, in an air-conditioned room on a 12 h light/dark cycle. Sterile special processing forage and water were provided adequately. Cages, bedding, food, and water were sterilized before use. Pregnant mice went into labor on 20 day of pregnancy and newborn mice were raised and maintained in the same conditions. Mice were divided into the following groups: (1) sensitizations and challenges with ovalbumin (OVA group); (2) treatment with PCV7 immunization in infant, sensitizations and challenges with OVA in adult (PCV7+OVA group); (3) the control group. On day 21, mice in the PCV7+OVA group were administered 7-valent pneumococcal conjugate vaccine (PCV7, Wyeth, USA) 33 μ l intranasally every 12 h for three doses [8]. The mice in the OVA and PCV7+OVA groups were sensitized intraperitoneally with 100 μ g ovalbumin (OVA, Sigma) diluted in 50% aluminum hydroxide (Pierce) to a total volume of 200 μ l on day 28 and day 42. From day 49 to 52, the mice were challenged with OVA aerosolized for 30 min every day lasting for 4 days. The control group mice were sensitized and challenged with sterile PBS at the same time. AAD was assessed 24 h after the final challenge. In our experiment, each experiment was repeated three times. Two to three mice were used in every experimental test described hereafter. This study was approved by the Institutional Animal Care and Research Advisory Committee at the Chongqing Medical University. All experimental animals were used in accordance with the guidelines issued by the Chinese Council on Animal Care.

2.2. Airway hyperresponsiveness

AHR was assessed *in vivo* by measuring changes in transpulmonary resistance using a mouse plethysmograph and methods similar to those previously described [12]. Briefly, 24 h after the final challenge, AHR was assessed in conscious, unrestrained mice by means of whole-body plethysmography (Emca instrument; Allmedicus, France). Each mouse was placed into a plastic chamber and exposed to aerosolized PBS followed by increasing concentrations of an aerosolized methacholine (Sigma-Aldrich, St. Louis, Mo. USA) solution (3.125, 6.25, 12.5, 25, and 50 mg/ml; Sigma) in PBS for 3 min exposures and then the mice had a rest for 2 min, after which a computer program was used to calculate Penh from the continuously recorded pressure and flow data for 5 min. Then take the average of the 5 min records as the value of Penh of this concentration. Penh is a dimensionless value and correlates with pulmonary airflow resistance. It represents a function of the ratio of peak expiratory flow to peak inspiratory flow and a function of the timing of expiration.

2.3. Lung histopathology

After formalin fixation, the left lung was dissected and embedded in paraffin. Sections of 4 μ m thickness were cut and stained with hematoxylin and eosin (H&E; Sigma). Tissues were subsequently mounted and coverslipped by use of Dako mounting medium (Dakocytomation, Denmark, CA). The degree of airway inflammatory cell infiltration was scored in a double-blind fashion by two independent investigators. Lung lesions were scored semi-quantitatively as described by other researchers [13]. The severity of inflammation was evaluated by assigning a value of 0 point for normal; 1 point for few cells; 2 points for a ring of inflammatory cells 1 cell layer deep; 3 points for a ring of inflammatory cells 2

to 4 cells deep; 4 points for a ring of inflammatory cells of >4 cells deep.

2.4. Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was obtained by instilling and collecting two aliquots of 1 ml each of PBS through an adapter cannula inserted through rings of the exposed trachea of euthanized mice 24 h after final challenge with OVA. BALF was pooled to obtain one sample for each mouse. Erythrocytes were lysed, and the remaining cells were cytocentrifuged 2500 rpm for 5 min. Total cell numbers in the BALF were determined using a standard hemocytometer. Differential cell counts were performed based on standard morphological and staining characteristics of at least 250 cells per sample. Supernatant was stored at -80°C . All slides were characterized by a single blinded examiner to eliminate bias.

2.5. BALF cytokine measurements

Cytokine concentrations in BALF were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. ELISA kits used for the measurement of IFN- γ , IL-5, and IL-10 were purchased from Sizhengbai (Beijing, China), ELISA kits for detection of IL-4 and TGF- β was purchased from Xinhosheng (Beijing, China), and the IL-17A and IL-13 detection ELISA kits were purchased from Bender.

2.6. Flow cytometric analysis of MLN CD4⁺T cell

The mediastinal lymph nodes (MLN) were removed and forced through a 70 μ m cell filter (BD, Bedford, MA, USA) to obtain single cell suspensions. Single cell suspensions in MLN were stained for surface-associated CD4(anti-CD4-FITC, BD Pharmingen, USA), CD3(anti-CD3-CyTM7, BD Pharmingen, USA), CD25(anti-CD25-PE, e Bioscience, USA), then fixed, permeabilized and stained for intracellular IFN- γ (anti-IFN- γ -PerCP-CyTM5.5, BD Pharmingen, USA), IL-17A (anti-IL-17A-PE, BD Pharmingen, USA), IL-4(anti-IL-4-APC, BD Pharmingen, USA) and Foxp3 (anti-Foxp3-PE-Cy5, e Bioscience, USA) and analyzed by flow cytometry (FACS Canto, BD Biosciences, USA).

2.7. Statistics

Results were analyzed using GraphPad Prism (version 5.0; GraphPad, La Jolla, CA) and expressed as mean \pm s.e.m. Results were interpreted using either one-way analysis of variance and Tukey's *post hoc* test, or two-way analysis of variance and Bonferroni's *post hoc* test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Infant PCV7 immunization suppressed young adulthood airway eosinophilic and neutrophilic inflammation in BALB/c mice asthma model

OVA sensitization and challenge induced the development of AAD: total inflammatory cells, eosinophils and neutrophils accumulation in BALF were significantly higher compared with controls ($14.58 \pm 2.50 \times 10^5$ cells/ml vs $2.34 \pm 0.36 \times 10^5$ cells/ml, $14.75 \pm 1.77 \times 10^4$ cells/ml vs $0.48 \pm 0.15 \times 10^4$ cells/ml, $63.39 \pm 9.28 \times 10^4$ cells/ml vs $9.30 \pm 1.10 \times 10^4$ cells/ml, respectively, $P < 0.001$). In contrast, the number of eosinophils and neutrophils was dramatically reduced in infant PCV7 immunized group mice compared with the OVA group ($2.15 \pm 0.29 \times 10^4$ cells/ml vs $14.75 \pm 1.77 \times 10^4$ cells/ml, $20.13 \pm 3.7 \times 10^4$ cells/ml

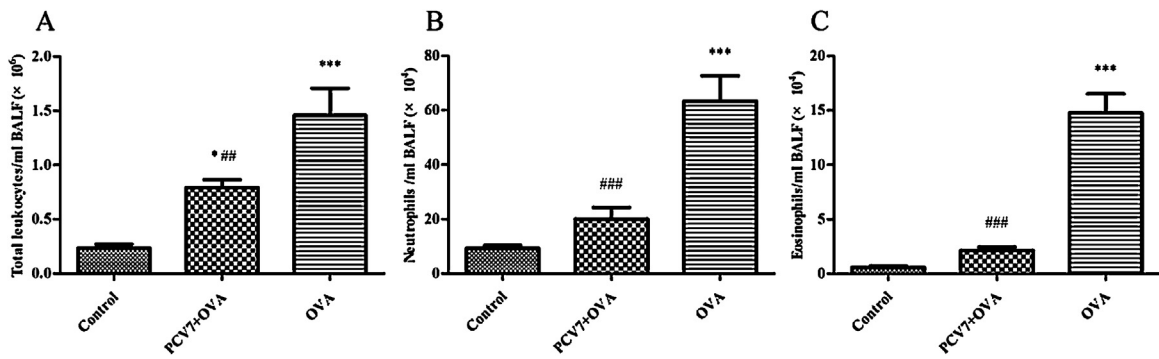


Fig. 1. Infant PCV7 immunization inhibited OVA-induced airway eosinophilic and neutrophilic inflammation in a BALB/c mouse AAD model. Mice in PCV7+OVA and OVA group were sensitized and challenged with OVA. 24 h after the final challenge, BALF samples were collected and total and inflammatory cells were counted. (A) Total inflammatory cells in BALF, (B) eosinophils in BALF, (C) neutrophils in BALF. Data were expressed as mean \pm s.e.m of the number of cells in each group of mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ as compared with OVA group ($n = 6-8$ /group).

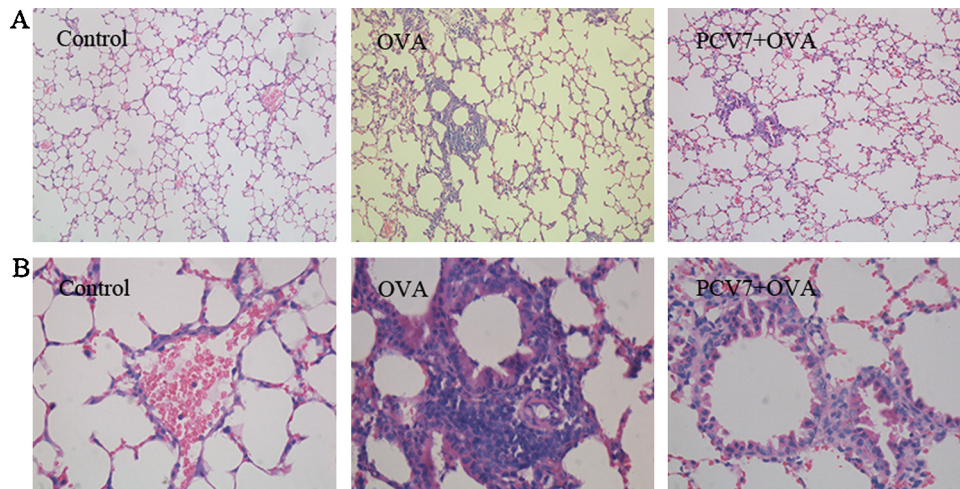


Fig. 2. Infant PCV7 immunization reduced OVA-induced tissue pathology of young adult BALB/c mice. Data of mouse lung tissue sections stained with hematoxylin and eosin (HE) 24 h after the final challenge are representative of each group mice. Magnification: A $\times 100$ and B $\times 400$.

vs $63.39 \pm 9.28 \times 10^4$ cells/ml, respectively, $P < 0.001$) (Fig. 1). These data demonstrated that infant PCV7 immunization can suppress OVA-induced airway eosinophilic and neutrophilic inflammation in young adulthood BALB/c mice asthma model.

3.2. Effects of infant PCV7 immunization on lung histopathology during AAD

In control group mice, there was little tissue inflammation. Pulmonary alveolar, peribronchiolar and perivascular inflammatory

infiltrate of inflammatory cells in OVA group mice was denser than that in control group mice. In infant PCV7 immunized group mice, pulmonary alveolar, peribronchiolar and perivascular cellular infiltration significantly lower than that in OVA group (Fig. 2). As shown in Fig. 3, the inflammation scores of pulmonary alveolitis, pulmonary perivasculitis and pulmonary peribronchiolitis in infant PCV7 immunized mice were significantly lower than that in OVA group (3.00 ± 0.26 vs 1.17 ± 0.17 , $P < 0.001$, 3.67 ± 0.21 vs 2.17 ± 0.31 , $P < 0.001$, 3.33 ± 0.21 vs 1.83 ± 0.31 , respectively, $P < 0.01$). Thus, infant PCV7 immunization suppressed airway inflammation in young adulthood asthmatic mice.

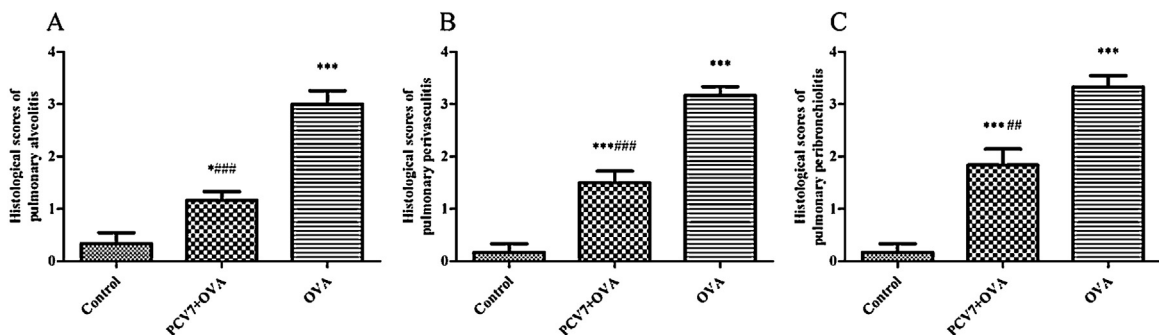


Fig. 3. After HE-staining, lung tissue section were scored for levels of alveolitis, perivasculitis and peribronchial inflammation. Infant PCV7 immunization decreased alveolitis, perivasculitis, and peribronchial inflammation. Data are shown as mean \pm s.e.m from three separated experiments. (A) Histological scores of pulmonary alveolitis, (B) histological scores of pulmonary perivasculitis, (C) histological scores of pulmonary peribronchiolitis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ as compared with OVA group ($n = 6-8$ /group).

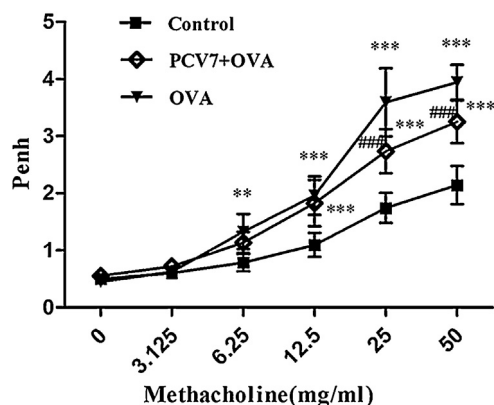


Fig. 4. Experimental mice were sensitized with OVA at 4 and 6 weeks of age, and they were challenged with allergen for 4 days at 7 weeks of age. AHR (Penh) in reaction to increased doses of nebulized methacholine was measured by a whole-body plethysmograph in the experimental groups and control group after the 24 h of the final challenge. Data are shown as the mean \pm s.e.m of Penh from experimental and control groups of mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ as compared with OVA group ($n = 6-8/\text{group}$).

3.3. Infant PCV7 immunization inhibited young adulthood mice AHR during AAD

AHR was evaluated by the calculation of Penh values (enhanced pauses) 24 h after the final challenge. OVA sensitization and challenge resulted in increased AHR. The Penh value for OVA group was significantly higher than that in the control group at methacholine concentrations 6.25 mg/ml ($P < 0.01$), 12.5 mg/ml ($P < 0.001$), 25 mg/ml ($P < 0.001$), and 50.0 mg/ml ($P < 0.001$). However, PCV7+OVA group mice had significantly lower Penh values compared to values obtained from mice in the OVA group from 25 to 50.0 mg/ml ($P < 0.001$, respectively) (Fig. 4).

3.4. Infant PCV7 immunization inhibited IL-13, IL-17A while increased IFN- γ , IL-10 cytokines production during AAD

To investigate the effects of infant PCV7 immunization on CD4⁺T cell subsets production during AAD, CD4⁺T cell cytokines in BALF were analyzed. As expected, OVA sensitized and challenged mice exhibited dramatically increased IL-13, IL-17A production (87.14 ± 7.12 pg/ml vs 40.62 ± 3.59 pg/ml, $P < 0.001$, 247.70 ± 35.81 pg/ml vs 158.90 ± 16.40 pg/ml, $P < 0.05$) and significantly decreased IFN- γ , IL-10 production (18.07 ± 1.13 pg/ml vs 33.16 ± 1.87 pg/ml, $P < 0.001$, 122.30 ± 18.53 pg/ml vs 223.10 ± 35.92 pg/ml, $P < 0.05$) compared with the control group mice. However, the production of IL-13, IL-17A in the infant PCV7 immunized group mice were significantly lower than that in the OVA group mice (31.93 ± 4.36 pg/ml vs 87.14 ± 7.12 pg/ml, $P < 0.001$, 120.90 ± 9.56 pg/ml vs 247.70 ± 35.81 pg/ml, $P < 0.01$). IFN- γ , IL-10 was significantly higher in the infant PCV7 immunized group mice than that in the OVA group mice. (27.89 ± 1.83 pg/ml vs 18.07 ± 1.13 pg/ml, $P < 0.001$, 228.50 ± 27.47 pg/ml vs 122.30 ± 18.53 pg/ml, $P < 0.05$). However, there was no significant difference of IL-4, IL-5 and TGF- β production between control, PCV7 + OVA and OVA group mice (Fig. 5).

3.5. Infant PCV7 immunization promoted Foxp3⁺Treg, Th1 while inhibited Th2 and Th17 cells production during AAD

To investigate the effects of infant PCV7 immunization on CD4⁺T cell subsets production during AAD, CD4⁺T cell subsets in MLN were analyzed. As expected, OVA sensitized and challenged mice exhibited dramatically decreased Foxp3⁺Treg, Th1 cells production ($8.66 \pm 0.37\%$ vs $10.49 \pm 0.57\%$, $P < 0.05$, $2.08 \pm 0.37\%$ vs $4.87 \pm 0.14\%$, respectively, $P < 0.001$) and significantly increased Th2, Th17 cells production ($0.75 \pm 0.07\%$ vs $0.35 \pm 0.04\%$, $P < 0.001$, $2.17 \pm 0.23\%$ vs $0.93 \pm 0.10\%$, $P < 0.001$) compared with the control group mice. However, the production of Foxp3⁺Treg and Th1 cells in the infant PCV7 immunized group mice was significantly higher than that in the OVA group mice ($12.53 \pm 0.28\%$ vs $8.66 \pm 0.37\%$, $P < 0.001$, $3.64 \pm 0.20\%$ vs $2.08 \pm 0.37\%$, $P < 0.001$), Th2 and Th17 cells were

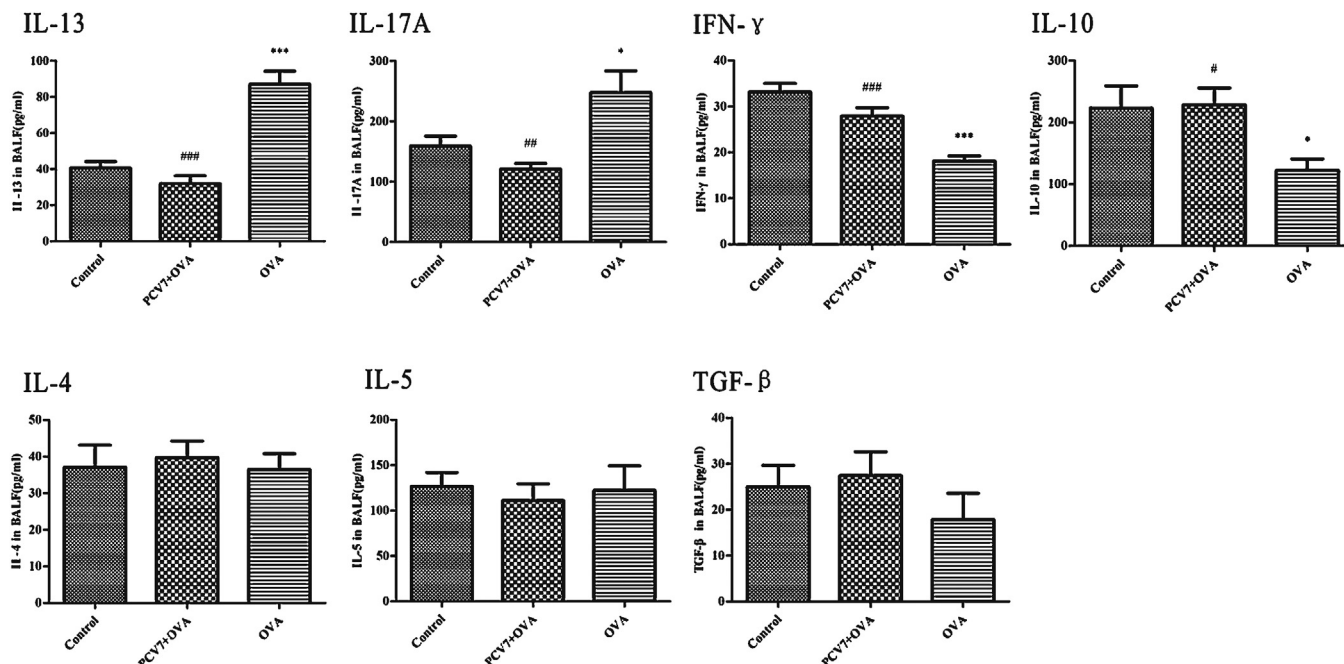


Fig. 5. Infant PCV7 immunization altered CD4⁺T cell cytokines in a BALB/c AAD model. Cytokines of CD4⁺T cells in BALF were measured by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ as compared with OVA group ($n = 6-8/\text{group}$).

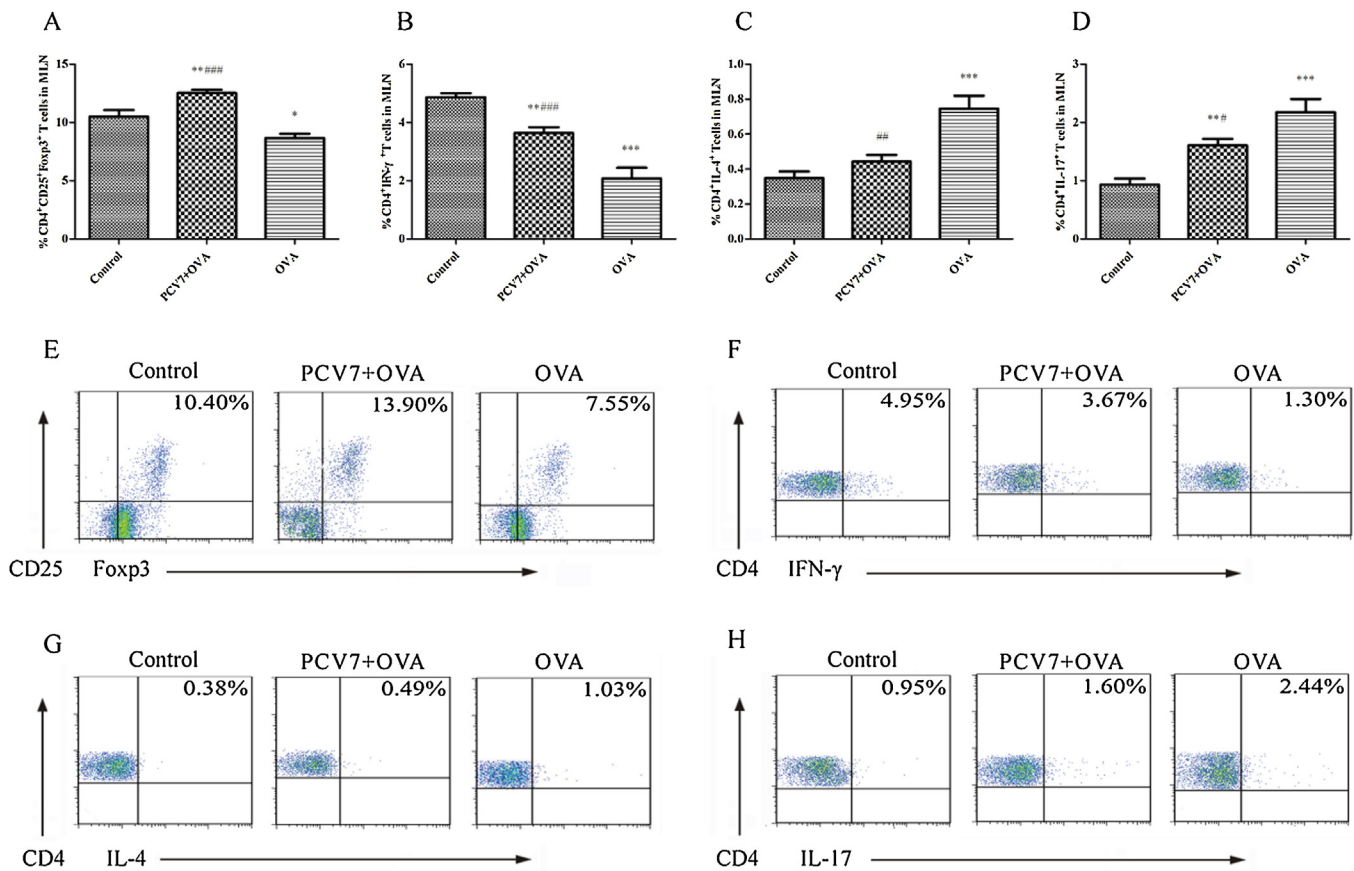


Fig. 6. Infant PCV7 immunization altered CD4⁺T cell immune response in a BALB/c AAD model. Subsets of CD4⁺T cells in MNL were measured by flow cytometric. (A) CD4⁺CD25⁺Foxp3⁺Treg; (B) CD4⁺IFN- γ ⁺ T(Th1); (C) CD4⁺IL-4⁺ T(Th2); (D) CD4⁺IL-17⁺ T(Th17). The data represent mean \pm s.e.m. The data (E–H), respectively, represent percentages of positively stained cells of Foxp3⁺Treg, Th1, Th2 and Th17 within the lymphocyte gate of MLN in BALB/c mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ as compared with OVA group ($n = 6-8$ /group).

significantly lower in the infant PCV7 immunized group mice than that in the OVA group mice ($0.44 \pm 0.04\%$ vs $0.75 \pm 0.10\%$, $P < 0.01$, $1.63 \pm 0.10\%$ vs $2.17 \pm 0.23\%$, $P < 0.05$) (Fig. 6A–H). These data indicated that infant PCV7 immunization promoted Foxp3⁺Treg, Th1 while suppressed Th2, Th17 cells production in young adulthood mice during AAD.

4. Discussion

Epidemiological studies in humans and experimental work in animals suggest that PCV7 can suppress allergic airway inflammation [7,8]. Previous studies suggested PCV7 immunization in adult mice inhibited hallmark features of AAD through the induction of Tregs and suppression of Th2 cells [8]. In this investigation we have demonstrated infant PCV7 immunization suppress young adulthood hallmark features of AAD in mouse models. Our study indicated that infant PCV7 immunization not only promote Foxp3⁺Treg and Th1 cells, but also inhibit Th2 and Th17 cells production, which resulted in the increased secretion of IL-10, IFN- γ and decreased production of IL-13, IL-17A during AAD mouse model. Infant PCV7 immunization can alter adaptive immune response in young adulthood life and suppress the development of young adulthood mice allergic asthma, which suggested its potential role as an immunoregulatory treatment to prevent young adulthood asthma.

Sensitization and challenge with OVA induces strong polarized Th2 immune response. Th2 cells have important role in the pathogenesis of asthma [14,15]. Th2 cells recruited into the airway cause

mucus hypersecretion, airway remodeling, and AHR. Th2 cells associated cytokines can initiate and accelerate allergic inflammation [14,16]. IL-13 may play a vital role in asthma pathogenesis. IL-13 can induce airway inflammation, AHR, mucus secretion, and tissue remodeling [16–18]. IL-13 can facilitate the production of antigen specific antibodies [19] and mucous cells in the bronchial epithelium [20]. In this study, we demonstrated that infant PCV7 immunization could alter young adulthood mice adaptive immune response to OVA antigen, and that resulted in the suppression of Th2 cells, IL-13 production and eosinophilia recruitment. There was no difference of IL-4 and IL-5 production between control and OVA group mice, which may be associated with the increased Th17 cells inhibiting the production IL-4 and IL-5 [21,22].

Th17 is a pro-inflammatory CD4⁺T effector cell population that is different from Th1 and Th2 [23,24]. Th17 cells and related cytokines play pivotal role in the pathogenesis of allergic asthma [25,26]. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling [27]. Antigen specific Th17 cells can promote Th2-cell-mediated eosinophil recruit into the airways [9]. Allergen driven Th17 cells resulted in asthma exacerbations or accelerated tissue damage. Studies indicated that enhanced IL-17A levels correlate with increased AHR in asthmatics and allergic asthma mice [28,29]. IL-17A can also induce human bronchial epithelial cells to produce mucus proteins acting in concert with IL-6 [30]. IL-17A can induce lung structural cells to secrete pro-inflammatory cytokines and neutrophil chemotactic proteins, thereby inducing neutrophil infiltration [29,31,32]. Furthermore, IL-17A can mediate allergic

reactions by enhancing IgE class-switch recombination in B cells. [26,33] Here we demonstrated that infant PCV7 immunization may correct the imbalance of Th17 cells, inhibit harmful effect of Th17 and IL-17A, thus inhibit AAD in mouse model.

Foxp3⁺Treg cell is a distinct subset of CD4⁺T cells which can suppress effector CD4⁺T cells responses [34,35]. Studies showed that Foxp3⁺Treg cells play a crucial role in allergic diseases including asthma [36–39]. Foxp3⁺Treg cells can suppress Th2 and Th17 cells mediated inflammation and prevent airway inflammation, AHR both in asthmatic patients and in animal experiments [39,40]. The functions of Foxp3⁺Treg cells are impaired in asthma [41,42]. We showed here that infant PCV7 immunization can promote the production of Foxp3⁺Treg cells and inhibit Th2, Th17 cells and their cytokines IL-13, IL-17A, which resulted in relieving the manifestations of AAD.

A recent study showed respiratory streptococcus pneumoniae infection suppresses hallmark features of AAD and has potential benefits for asthma. Streptococcus pneumoniae infection suppresses allergic airways disease by inducing regulatory T-cells [43]. In this study, we demonstrated infant PCV7 immunization suppress young adulthood hallmark features of AAD in mouse models. Whether there are any key immunoregulatory components in streptococcus pneumoniae which can inhibit hallmark features of AAD needs further investigation.

But there were some limitations in this study. First, as previous study [8] have demonstrated intramuscular administration of PCV7 had limited effects on AAD, we did not design a study to investigate the effect of infant PCV7 immunization intramuscularly on young adulthood CD4⁺T cell subsets. Second, we did not investigate the mechanism of infant PCV7 immunization increased Foxp3⁺Treg cells in AAD mouse model. Literatures showed immature DC can promote the production of Foxp3⁺Treg cells [44–46], whether infant PCV7 immunization can alter the maturation of DC or not remains unclear, which is the work we will do hereafter.

In conclusion, infant PCV7 immunization may be an effective measure to prevent young adulthood asthma through promoting Foxp3⁺Treg and Th1 cells, and inhibiting Th2 and Th17 cells.

Authorship contributions

Conception and design: Hui Gao, Zhengxiu Luo; conducted experiments: Liqun Zhang, Ting Yang, Baohui Yang, Xiaoli Jiang, Lijia Wang, Qinghong Wang; data analysis and interpretation: Liqun Zhang, Hui Gao, Ting Yang, Baohui Yang, Xiaoli Jiang; writing of the manuscript: Liqun Zhang, Zhengxiu Luo.

Conflict of interest statement

We declare that there is no conflict of interest.

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