

Comparison of Antiallergic Effects of Pneumococcal Conjugate Vaccine and Pneumococcal Polysaccharide Vaccine in a Murine Model of Allergic Rhinitis

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Objectives/Hypothesis: Pneumococcal vaccines have been widely used, and *Streptococcus pneumoniae* has been suggested to be an effective therapeutic agent in allergic disease.

Objectives: The present study was performed to evaluate the effects of pneumococcal polysaccharide vaccine (PV) and pneumococcal protein conjugate vaccine (PCV), and to examine differences between the vaccines in a murine model of allergic rhinitis.

Study Design: In vivo study using an animal model.

Setting: Catholic Research Institutes of Medical Science.

Methods: Allergic rhinitis was induced in 40 BALB/c mice by intraperitoneal sensitization and intranasal challenge with *Dermatophagoides farinae* (Derf). The animals were divided into four groups: control, Derf, PV, and PCV. Interferon- γ , interleukin-13, and interleukin-10 levels in nasal lavage fluid and Derf-specific immunoglobulin E levels in serum were measured. The levels of T-bet, GATA-3, and Foxp3 mRNA expression in splenic mononuclear cells were determined. The number of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in splenic mononuclear cells was compared between groups by flow cytometry.

Results: Allergic symptom scores, T-bet and GATA-3 mRNA levels, serum Derf-specific immunoglobulin E levels, and tissue eosinophil counts were lower in the PV and PCV groups than the Derf group ($P < 0.05$). The regulatory T (Treg) cell indicators, Foxp3 mRNA, and percentages of CD4⁺CD25⁺Foxp3⁺ T cells were increased in the PV and PCV groups ($P < 0.05$).

Conclusion and Clinical Relevance: Both PV and PCV suppressed the allergen-specific T helper 2 response and induced regulatory T cells in a murine model of allergic rhinitis. However, PV and PCV may activate Treg cells via different mechanisms.

Key Words: Pneumococcal conjugate vaccine, pneumococcal polysaccharide vaccine, allergic rhinitis, a mouse model, regulatory T cells.

Level of Evidence: N/A.

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INTRODUCTION

Allergic rhinitis (AR) is a common illness throughout the world, and its prevalence has increased markedly over the last 180 years.¹ The management of AR encompasses education, pharmacotherapy, immunotherapy, and surgery. Despite recent advances in understanding of the mechanisms underlying allergic inflammation, current therapies only alleviate the symptoms of the disease.

Pneumococcal polysaccharide vaccine (PV) has been available since the mid-1980s. These vaccines contain purified capsular polysaccharides from pneumococcal

serotypes. Polysaccharides primarily induce a B cell-dependent immune response via release of immunoglobulin M.^{2,3} Such polysaccharide vaccines are not recommended for use in children <2 years of age, due to their immature immune systems. The pneumococcal protein conjugate vaccine (PCV) contains capsular polysaccharides from pneumococci most frequently involved in pediatric infections.⁴ Use of this PCV is particularly successful in the vaccination of young children.

In 1983, a vaccination for *Streptococcus pneumoniae* has been documented to reduce the incidence of inflammation. Schuller⁵ et al. reported that the pneumococcal vaccine appears efficacious for management of asthmatic children with recurrent otitis media. There was a mean yearly reduction in otitis media of 38% in the pneumococcal vaccine group and 90% in the group treated with pneumococcal vaccine. In 2005, the retrospective cohort study evaluated the effectiveness of a 23-valent pneumococcal polysaccharide, and they documented that a decrease in the risk of hospital admission for asthma, acute otitis media, chronic obstructive pulmonary disease and other respiratory infections was observed in vaccinated compared with nonvaccinated

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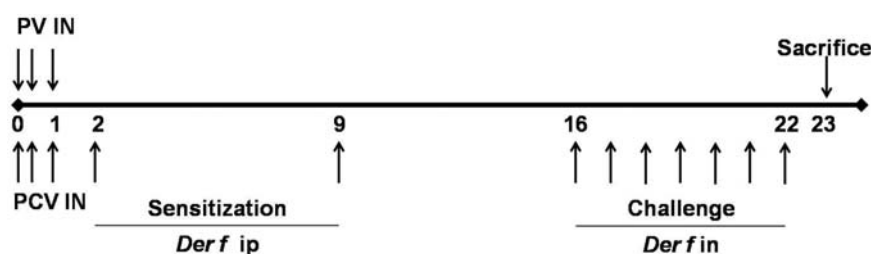


Fig. 1. Schematic representation of the experimental allergic rhinitis model and treatment protocol. Derf = *Dermatophagoides farinae*; IN/in = intranasal administration; ip = intraperitoneal administration; PCV = pneumococcal protein conjugate vaccine; PV = pneumococcal polysaccharide vaccine. Units are days.

subjects.⁶ In 2007, killed *S.pneumonia* was administered, and the subsequent development of asthma was assessed. The results indicated the potential of *S. pneumonia* as an immunomodulatory therapy for asthma.⁷

However, limited data are available regarding the antiallergic effects of PV and PCV in AR. Thus, the present study was performed to evaluate the effects of both pneumococcal vaccines and to examine differences between PV and PCV in a murine model of AR.

MATERIALS AND METHODS

Experimental Animals

Six-week-old healthy female BALB/c mice (20–30 g) were used in this study. The experiment was performed with the approval of the Institutional Animal Care and Use Committee at the Catholic University of Korea.

Reagents

Dermatophagoides farinae (Derf) crude body extract (Arthropods of Medical Importance Resource Bank, College of Medicine, Yonsei University, Seoul, Korea) was used as the allergen. It was solubilized and stored at -70°C and dissolved prior to use. PV (Pneumovax 23; Merck Sharp & Dohme Corporation, West Point, PA) and PCV (Synflorac 10; GlaxoSmithKline Biologicals, Rixensart, Belgium) were prepared in sterile phosphate-buffered saline (PBS).

Production of the AR Model and Treatment Protocol

Forty mice were randomized into one of four groups: control ($n = 10$), Derf (AR, $n = 10$), PV (administered before sensitization, $n = 10$), and PCV (administered before sensitization, $n = 10$). Allergen sensitization and challenge for the development of the AR murine model are summarized in Figure 1. Briefly, on days 2 and 9, all mice except those in the control group were immunized by intraperitoneal injection of $100\text{ }\mu\text{g}$ Derf. Mice in the PV and PCV groups were treated intranasally with $33\text{ }\mu\text{g}$ PV and PCV on days 0, 0.5, and 1. One week after sensitization, all mice were challenged intranasally with $20\text{ }\mu\text{g}$ Derf for 7 consecutive days. The control group received PBS intranasally instead of Derf.⁸

Evaluation of Allergic Symptoms Induced After Allergen Challenge

The numbers of sneezing and nose-rubbing motions during the 15-minute period after the final allergen challenge were recorded and compared among the experimental groups by observers blinded to experimental groups.⁹

Nasal Lavage Fluid

Using ether anesthesia, thoracotomies were performed on the mice in each group 24 hours after the final allergen challenge. Nasal lavage fluid (NALF) was obtained after partial tracheal resection using a 22-gauge catheter. The catheter was inserted into the tracheal opening and advanced in the direction of the upper airway into the nasopharynx. The nasal passages were perfused with 1 mL PBS from the choana to the nostril, and NALF was collected from the nares.^{10,11}

Nasal Mucosal Tissue Evaluation

Mice were sacrificed and decapitated 24 hours after the final allergen challenge. The heads were fixed in 4% paraformaldehyde for 3 days at 4°C , washed in running water, decalcified for 3 days with Calci-Clear Rapid (National Diagnostics, Atlanta, GA) at room temperature, dehydrated by passage through a graded alcohol series, and embedded in paraffin blocks. The blocks were cut into $4\text{-}\mu\text{m}$ -thick sections and stained with hematoxylin and eosin to evaluate the general morphology and the number of eosinophils in the lamina propria. Eosinophils were counted under a light microscope. The average number of cells was counted in four areas around the nasal septa. The individual who counted the eosinophils was blinded to the animals' group assignments.

Cytokines in NALF and Derf-specific Immunoglobulin E in Serum

To evaluate the allergic reaction using NALF (the supernatant from each group), interferon (IFN)- γ for T helper 1 (Th1) immune reaction and interleukin (IL)-13 for T helper 2 (Th2) immune reaction were measured by enzyme-linked immunosorbent assay (ELISA; Multiplex; Millipore, Billerica, MA).¹² Serum Derf-specific immunoglobulin E (IgE) levels were measured with an ELISA kit (Indoor Biotechnologies, Manchester, UK). IL-10 levels were also measured with a Multiplex ELISA kit.

Real-Time Polymerase Chain Reaction

The spleen was removed from each animal aseptically 24 hours after the last challenge. Total RNA was extracted from splenic mononuclear cells using the TRIzol reagent (Invitrogen, Carlsbad, CA), and the first strand was reverse-transcribed using random primers (TaKaRa, Otsu, Japan). The oligonucleotide primer sequences were as follows: T-bet forward primer, 5'-GCCAGGGAACCGCTTATA-3' and T-bet reverse primer, 5'-CCTTGTTGTTGGTGAGCTTTA-3'; GATA-3 forward primer, 5'-CTG GATGGCGGCAAAGC-3' and GATA-3 reverse primer, 5'-GTGG GCGGGAAGGTGAA-3'; Foxp3 forward primer, 5'-GAAAGCGG ATACCAAATGA-3' and Foxp3 reverse primer, 5'-CTGTGAGGA CTACCGAGCC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer, 5'-GCACAGTCAAGGCCGAGAAT-3'.

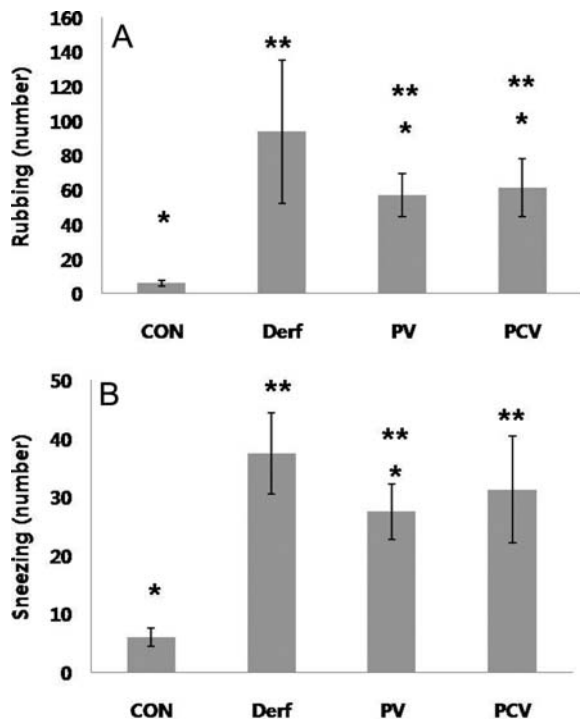


Fig. 2. Nasal symptom score. Nose rubbing motions (A) and sneezing motions (B) were measured within a 15-minute period. Error bars represent standard deviations. * $P < 0.05$ vs. *Dermatophagoides farinae* group; ** $P < 0.05$ vs. control group.

and GAPDH reverse primer, 5'-GCCTTCTCCATGGTGGTGAA-3'. The levels of T-bet, GATA-3, Foxp3, and GAPDH mRNA expression were determined by real-time polymerase chain reaction (PCR) using the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR master mix (TaKaRa).^{13,14} The levels of expression of these mRNAs were analyzed using the ABI 7300 Sequence Detection System (Applied Biosystems). The results were normalized relative to GAPDH expression and are shown as the fold increase over the expression of the control group.

Flow Cytometry

For cell-surface staining, aliquots of 10^6 splenic mononuclear cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (GK1.5) antibody (eBioscience, San Diego, CA). For intracellular staining, cells stained with CD4 were incubated with fixation/permeabilization working solution, and Fc receptors were blocked with excess mouse Fc block. Cells were then stained with phycoerythrin (PE)-Cy5-conjugated anti-mouse Foxp3 (FJK-16s) and allophycocyanin (APC)-CD25 antibody (eBioscience). CD4⁺CD25⁺Foxp3⁺ T cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA).^{15,16}

Statistical Analysis

All measured parameters are expressed as means $\pm$ standard deviations. Differences among groups were analyzed using the Kruskal-Wallis test. In cases of statistical significance, the ranked parameters were compared by one-way analysis of variance and Bonferroni's multiple comparison tests (PASW Statistics 18; SPSS Inc., Chicago, IL, USA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

Comparison of Allergic Symptoms

Allergic symptoms were determined by the numbers of nasal rubbing motions and sneezes during the 15-minute period after the final challenge. The numbers of nasal rubbing motions were 6.1 ± 1.6 in the control group, 93.7 ± 41.4 in the Derf group, 57.2 ± 12.4 in the PV group, and 61.4 ± 16.8 in the PCV group. The numbers of sneezes were 6.0 ± 1.6 in the control group, 37.5 ± 6.9 in the Derf group, 27.5 ± 4.8 in the PV group, and 31.3 ± 9.1 in the PCV group. The number of nasal rubbing motions was significantly higher in the Derf group than in all other groups (all $P = 0.000$; Fig. 2A). The number of sneezes was significantly higher in the Derf group than in the control and PV groups (control, $P = 0.000$; PV, $P = 0.006$; Fig. 2B). The numbers of nasal rubbing motions and sneezes were significantly lower in the control group than in all other groups (Fig. 2).

Serum Derf-Specific IgE

Serum Derf-specific IgE levels were significantly higher in the Derf group (9 ± 1.84 ng/mL) than in the control (0.22 ± 0.08 ng/mL), PV (3.32 ± 1.04 ng/mL), and PCV (4.02 ± 4.12 ng/mL) groups (all $P < 0.05$). The control group had a significantly lower serum Derf-specific IgE level than did all other groups (all $P = 0.000$; Fig. 3).

Eosinophil Counts in the Lamina Propria

Figure 4 shows eosinophil infiltration in the lamina propria. Eosinophil numbers were 28.2 ± 11.9 in the control group, 110 ± 11.9 in the Derf group, 52.4 ± 6.54 in the PV group, and 52 ± 15.7 in the PCV group. The eosinophil count was significantly higher in the Derf group than in the control ($P = 0.000$), PC ($P = 0.005$), and PCV ($P = 0.005$) groups. The eosinophil count was significantly lower in the control group than in all other groups (Derf, $P = 0.000$; PV, $P = 0.019$; PCV, $P = 0.019$; Fig. 5).

Cytokine Analysis of NALF

IFN- γ levels in NALF were 2.44 ± 0.86 pg/mL in the control group, 1.71 ± 0.76 pg/mL in the Derf group,

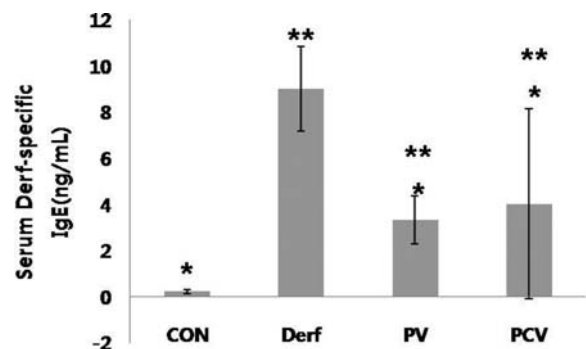


Fig. 3. Serum *Dermatophagoides farinae*-specific immunoglobulin E. Error bars represent standard deviations. * $P < 0.05$ vs. *Dermatophagoides farinae* group; ** $P < 0.05$ vs. control group.

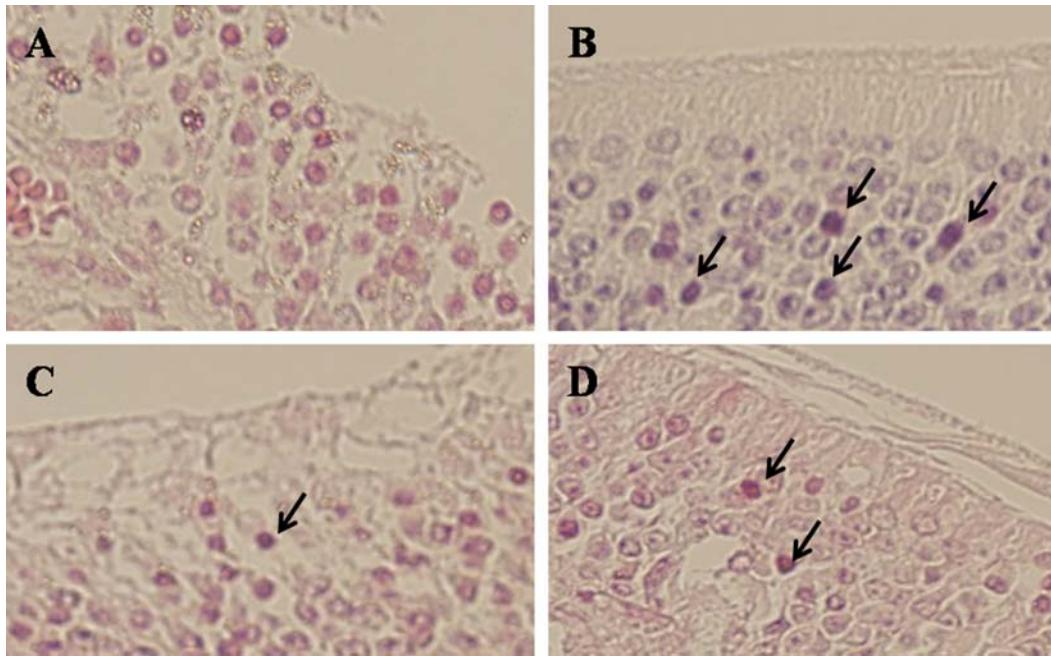


Fig. 4. Infiltration of eosinophils (arrows) in the nasal mucosa of BALB/c mice: (A) control group, (B) *Dermatophagoides farinae* group, (C) pneumococcal polysaccharide vaccine group, and (D) pneumococcal protein conjugate vaccine group. (Hematoxylin and eosin staining; original magnification, $\times 400$).

2.28 ± 0.9 pg/mL in the PV group, and 1.27 ± 0.57 pg/mL in the PCV group. IFN- γ levels were lower in the PCV group than in the PV ($P = 0.047$) (Fig. 6A).

IL-13 levels in NALF were 23.3 ± 6.08 pg/mL in the control group, 131.36 ± 54.33 pg/mL in the Derf group, 61.41 ± 32.45 pg/mL in the PV group, and 92.92 ± 37.94 pg/mL in the PCV group. The IL-13 level was significantly higher in the Derf group than in the PV ($P = 0.039$) and control ($P = 0.000$) groups (Fig. 6B).

IL-10 levels in NALF were 35.7 ± 34.3 pg/mL in the control group, 7.9 ± 6.5 pg/mL in the Derf group, 52.14 ± 44.52 pg/mL in the PV group, and 12.2 ± 6.03 pg/mL in the PCV group. The IL-10 level was significantly higher in the PV group than in the Derf group ($P = 0.037$; Fig. 6C).

Real-Time PCR Analysis

T-bet, GATA-3, and Foxp3 mRNA expression in splenic mononuclear cells was evaluated by real-time PCR. T-bet mRNA expression was decreased in the PV and PCV groups compared with the control (both $P = 0.000$) and Derf (PV, $P = 0.000$; PCV, $P = 0.004$) groups. However, no significant difference was observed between the PV and PCV groups (Fig. 7A).

GATA-3 mRNA expression was significantly higher in the Derf group than in all other groups (all $P = 0.000$). The control group showed significantly lower GATA-3 mRNA expression than all other groups. No significant difference was observed between the PV and PCV groups (Fig. 7B).

Foxp3 mRNA expression levels were significantly higher in the PV and PCV groups than in the control and Derf groups (both $P = 0.000$; Fig. 7C).

Flow Cytometric Analysis of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells from Splenic Mononuclear Cells

Cells were sorted based on Foxp3 and CD25 expression levels and whether they expressed CD4 (Fig. 8A). $CD4^+$ $CD25^+$ $Foxp3^+$ T cells accounted for $3.06\% \pm 1.11\%$ of all splenic mononuclear cells in the control group, $2.0\% \pm 0.91\%$ in the Derf group, $13.05\% \pm 4.04\%$ in the PV group, and $7.53\% \pm 3.45\%$ in the PCV group. The PV and PCV groups had significantly higher percentages of these cells than the control and Derf groups (all $P = 0.000$). The PV group had a significantly higher percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells than the PCV group ($P = 0.045$). These results indicated that PV and PCV induced $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Fig. 8B).

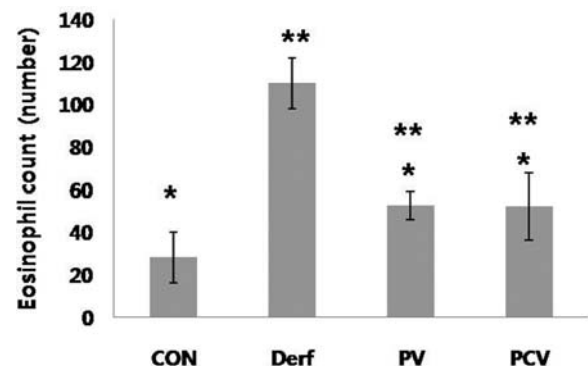


Fig. 5. Eosinophil counts in the nasal mucosa of each study group under a high power field. Error bars represent standard deviations. * $P < 0.05$ vs. *Dermatophagoides farinae* group; ** $P < 0.05$ vs. control group.

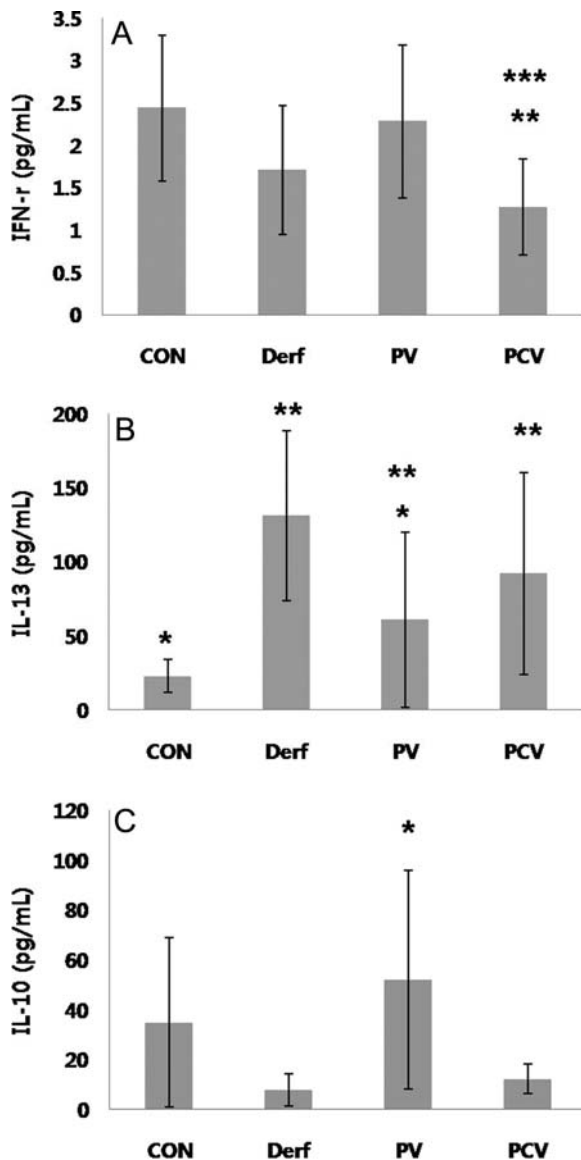


Fig. 6. Cytokine levels in the nasal lavage fluid of each study group: (A) interferon- γ , (B) interleukin-13, and (C) interleukin-10. Error bars represent standard deviations. * P < 0.05 vs. *Dermatophagoides farinae* group; ** P < 0.05 vs. control group; *** P < 0.05 vs. pneumococcal polysaccharide vaccine group.

DISCUSSION

Several reports have discussed the possibility of developing *S. pneumoniae*-based immunoregulatory treatments for allergic disease. A retrospective cohort study reported a decrease in the risk of hospital admission for asthma in PV-vaccinated compared with nonvaccinated subjects.⁶ Thorburn et al.¹⁷ compared the efficacy of PV and PCV in preventing allergic disease. They showed that treatment of ovalbumin-sensitized mice with PCV, but not PV, suppressed the development of hallmark features of allergic airway disease. Although few studies have compared these two vaccines in models of AR, the difference between PV and PCV may be due to their different compositions. PV includes purified capsular polysaccharide antigens. This vaccine has been

suggested to elicit T cell-independent antibody responses and to suppress allergic inflammation by inducing Th1 responses in allergic disease.¹⁸ PCV is comprised of pneumococcal polysaccharide antigens that are conjugated to immunogenic diphtheria toxoid.¹⁹ The co-administration of polysaccharides with an immunogenic protein has been suggested to induce regulatory T (Treg) cells that suppress allergic responses via their capacity to inhibit variable immune responses.^{17,20}

An important strength of this study was as follows. First, this study is the first to compare the efficacies of both vaccines in a murine model of AR. Second, we have used intraperitoneal sensitization and intranasal challenge with *D. farinae*, which is the most common aeroallergen to elicit AR in human patients.

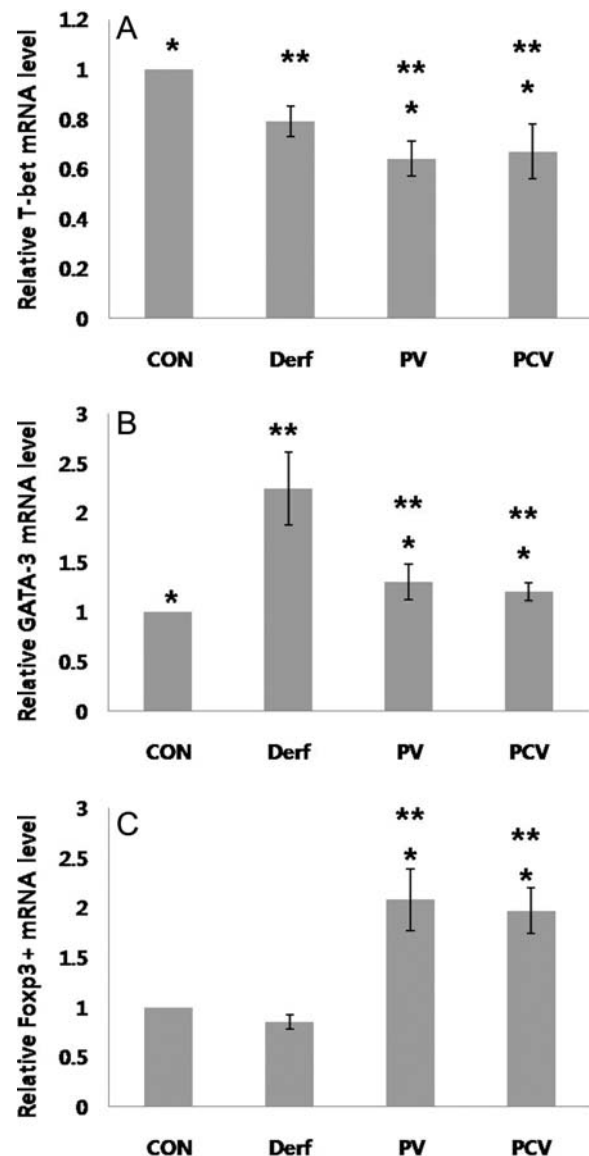


Fig. 7. Quantitative analysis of T-bet (A), GATA (B), and Foxp3 (C) mRNA expression by real-time PCR from splenic mononuclear cells. The results were normalized relative to GAPDH expression. Error bars represent standard deviations. * P < 0.05 vs. *Dermatophagoides farinae* group; ** P < 0.05 vs. control group.

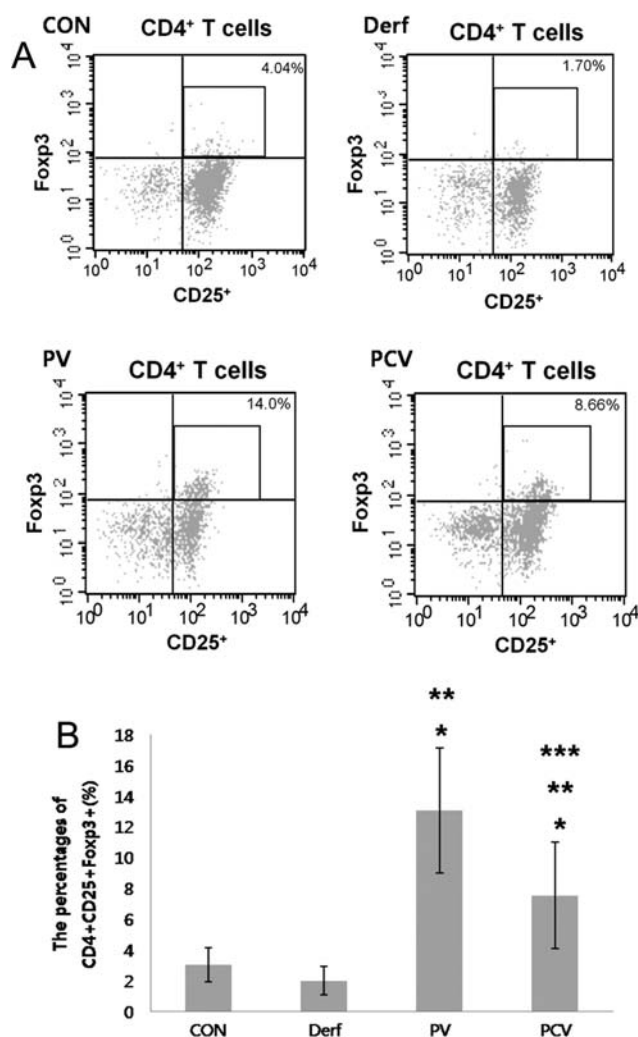


Fig. 8. (A) Flow cytometric analysis of CD4⁺CD25⁺Foxp3⁺ T cell subsets. Representative fluorescence-activated cell sorting analysis in each group. (B) The percentage of splenic mononuclear cells that were CD4⁺CD25⁺Foxp3⁺ T cells. RU = upper right quadrant, which represents CD4⁺CD25⁺Foxp3⁺ T cells. Error bars represent standard deviations. **P* < 0.05 vs. *Dermatophagoides farinae* group; ***P* < 0.05 vs. control group; ****P* < 0.05 vs. pneumococcal polysaccharide vaccine group.

In previous studies, PCV was delivered to mice at different times relative to sensitization in allergic airway disease. In these previous studies, treatment before, during, or after sensitization generally had the same effects and suppressed ovalbumin-induced Th2 cytokine release from lung-draining lymph node T cells.¹⁸

Previous studies, including our unpublished data, indicated that the antiallergic effects of PV were more potent when administered before than after sensitization. In the present study, mice in the PV and PCV groups were treated before sensitization.

In our study, tissue eosinophil counts, Derf-specific IgE levels, and GATA-3 mRNA levels were suppressed in both PV and PCV groups. Our observations suggested that both PV and PCV decrease the activity of the Th2 pathway, thus; both vaccines may be considered to relieve the allergic response.

Levels of Treg cell indicators, that is, Foxp3 mRNA expression and the percentages of CD4⁺CD25⁺Foxp3⁺ T cells, were increased in the PV and PCV groups.^{15,16} These findings did not correspond well with those of an earlier study suggesting that PV does not induce Treg cells.¹⁷ However, T cell-independent antigens such as PV do not prevent T cells from influencing responses, either directly or by cytokines produced by T cells.³

In addition, in the PV group increased IL-10 levels indicated that Treg cells affect inflammation, mainly via IL-10. In contrast, in the PCV group low IL-10 levels suggested that Treg cells could be acting through other cytokines, such as IL-2.²¹ Therefore, PV and the PCV may activate Treg cells via different mechanisms.

CONCLUSION

Both PV and PCV suppressed the allergen-specific Th2 response and induced Treg cells in a murine model of house dust mite AR, although the mechanisms by which they activate Treg cells appear to differ. Further studies are needed to determine the role of each vaccine and differences between vaccines.

ACKNOWLEDGEMENT

The experiment was performed with the approval of the Institutional Animal Care and Use Committee at the Catholic University of Korea.

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