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Importance of NK Cells in Cellular and Humoral Responses Triggered by Pneumococcus Vaccination

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Keywords
Streptococcus pneumoniae · NK cells · Vaccination · T_H1 · IgG

Abstract

Introduction: Despite the success of vaccination in reducing overall rate of pneumococcal pneumonia, *Streptococcus* pneumoniae is still held responsible for high mortality and modality rates worldwide. Our study aimed to investigate the potential role played by NK cells in immune response generated by pneumococcal vaccination, which could contribute to the development of more effective vaccines. ***Methods:*** The study included mice with and without NK cell depletion which were immunized with pneumococcus polysaccharide-conjugated vaccine followed by pneumococcus polysaccharide vaccine (PPV). Serum samples and splenocytes were collected from mice sacrificed 4 weeks after the last PPV dose. Serum samples were used for antibody level quantification by ELISA assay, while splenocytes were treated with PPV in vitro before monitoring CD4+ T-cell subsets (T_H1, T_H2, and T_H17) and cytokine (IFN-γ, IL-4, and IL-17) secretion levels by flow cytometry and ELISA analysis, respectively. ***Results:*** Results demonstrated reduced pneumococcal IgG and T_H1 cell levels due to NK cell depletion. Nevertheless, in contrast to these observations, IFN-γ secretion levels after in vitro PPV-23 treatment of splenocytes did not exhibit any statistically significant difference between the two mice groups. ***Conclusions:*** The data indicate a positive contribution of NK cells to both T-cell and B-cell responses triggered against pneumococcal vaccination. Further studies are required to confirm our data and investigate the potential benefit of NK cell targeting in promoting vaccine efficacy, especially in the elderly population who continues to be affected significantly by pneumococcal pneumonia.

Introduction

Streptococcus pneumoniae continues to be major threat to public health; it is still the leading cause of morbidity and mortality from lower respiratory infections (more

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deaths than other agents combined) and is among the four common causes of deaths attributable to and associated with bacterial antimicrobial resistance globally [1, 2]. Age is a significant risk factor for pneumococcal infections as the highest *S. pneumoniae* incidence rates are reported for subjects from the two extreme age groups (i.e., children and elderly population) [3].

Currently, there are pneumococcus polysaccharide vaccine (PPV) and polysaccharide-conjugated vaccine (PCV) in use for protection against pneumococcal infections. In contrast to PPV that induces T-cell-independent immune responses, PCV elicits T-cell-dependent immunity characterized by long-term immunological memory, affinity maturation, and extensive antibody subclass switching but has a lower serotype coverage [4]. Therefore, under certain circumstances, PCV is recommended to be given prior to PPV which would augment the immune response against capsular PPVs included in PPV [5]. Today, while recommendations of pneumococcal vaccination differ from country to country, the US Advisory Committee on Immunization Practices (ACIP) still recommends sequential PCV/PPV vaccination for adults older than 64 years of age residing in nursing homes and settings who may not benefit from herd immunity provided by pediatric PCV in children [6].

Yet, despite the success of pneumococcal vaccination in reducing overall rate of pneumococcal infections [7], people of old age (>65 years) continue to be affected significantly and represent the age group with the highest rate of invasive pneumococcal diseases (IPDs) [8, 9]. While this can be due to the factors including elevated rates of antibiotic-resistant bacteria and increased disease burden of non-vaccine serotypes [7], persistence and increase of some vaccine-types (type 3 and 19A, respectively) imply ineffectiveness of pneumococcal vaccines to confer protection [10].

Therefore, identification of immune mechanisms induced by pneumococcal vaccination is of high importance for development of alternative strategies to improve vaccine immunogenicity. One such strategy involves targeting NK cells which were previously demonstrated as key regulators of adaptive immunity and suggested as a prominent therapeutic target for infection control [11, 12]. Our study aimed to evaluate the importance of NK cells in induction of protective immune responses triggered by pneumococcal vaccination by comparing PCV-13-specific IgG, CD4+ T-cell subset (T_H1, T_H2, and T_H17), and cytokine (interferon [IFN]-γ, interleukin [IL]-4, and IL-17) secretion levels between mice with and without NK cell depletion, after combined pneumococcal vaccination schedule.

Methods

Vaccination Protocol and Collection of Biological Specimens

Prevnar 13 (PCV-13; Wyeth Pharmaceuticals Inc.) and Pneumovax (PPV-23; Merck and Co., Inc.) vaccines were commercially acquired and diluted in phosphate-buffered saline (PBS) solution to 1:10. Each animal received 100 μL of the final dilution by intraperitoneal administration. Seven-week-old control and NK-depleted C57 BL/6 mice were vaccinated with PCV-13 followed by PPV-23 4 weeks later. Mice were then sacrificed 4 weeks after receiving the last vaccine.

Splenocytes were obtained by meshing the spleen utilizing thumb piece of a plunger removed from a 1 mL syringe. The cells were then washed with cell culture media for 2 times and diluted in 10% DMSO-PBS before being stored in liquid nitrogen before use. Serum samples were isolated from blood specimens collected from sacrificed mice and stored as frozen at −80°C.

NK Cell Depletion

Mice received intraperitoneal injections of 25 μg anti-mouse NK1.1 antibody (PK136; Cytek Biosciences) six and 3 days prior to each PCV-13 and PPV-23 vaccination. Mock-treated animals received PBS on the same days. Following the confirmation of NK cell depletion by flow cytometry analysis, NK-depleted mice were vaccinated with either PCV-13 or PPV-23 on the next day, together with control mice.

In vitro Treatment of Splenocytes

Frozen splenocytes were first incubated in water bath at 37°C for 10 min. Cells were then diluted to 10 mL with RPMI-1640 media supplemented with 10% fetal bovine serum and centrifuged at 540 *g* for 10 min. After repeating the washing step one more time, cell counting was performed by using Agilent NovoCyte 3005 flow cytometer. Of those cells, 5×10^6 were removed and added into 15 mL falcon tubes for flow cytometry analysis of CD4+ T-cell subset levels after 24-h treatment with 5 μg/mL PPV-23 vaccine antigen and addition of 5 μg/mL brefeldin A (BioLegend, 420601) at 16th hour of incubation. The second group of cells were seeded onto 96-well microplates at concentrations of 2×10^5 cells/well and stimulated with 5 μg/mL PPV-23 vaccine antigen for 24 h before quantification of cytokine secretion by ELISA.

Flow Cytometry Analysis

NK cell depletion was confirmed by using blood samples collected from facial veins before the injection of the first anti-mouse NK1.1 antibody dose ($D = 0$) and 1 day prior to vaccination with PCV-13 ($D = 5$) and PPV-23 ($D = 33$). One hundred microliters of blood were incubated with anti-mouse NK1.1 PE (Cytek Biosciences) antibody for 20 min, in the dark, at room temperature. After the staining, red blood cells were lysed by incubating with Red Blood Cell (RBC) Lysis Buffer (Cytek Biosciences) for 15 min at room temperature. The samples were analyzed with flow cytometer, and percentage of NK cells was reported (Fig. 1).

At the end of the 24-h treatment with 5 μg/mL PPV-23 vaccine antigen, splenocytes were added 1 × PBS and centrifuged at 540 *g* for 5 min. Supernatants were then removed, and erythrocytes were lysed by incubation with RBC Lysis Buffer. This is followed by fixation and permeabilization of splenocytes with Fixation Buffer (Cytek Biosciences) and Perm Buffer (Cytek Biosciences), respectively. Splenocytes were then incubated with fluorochrome-conjugated monoclonal antibodies against CD3 (Cytek Biosciences; for total T-cells), CD4 (Cytek Biosciences; for T_H cells), IFN-γ

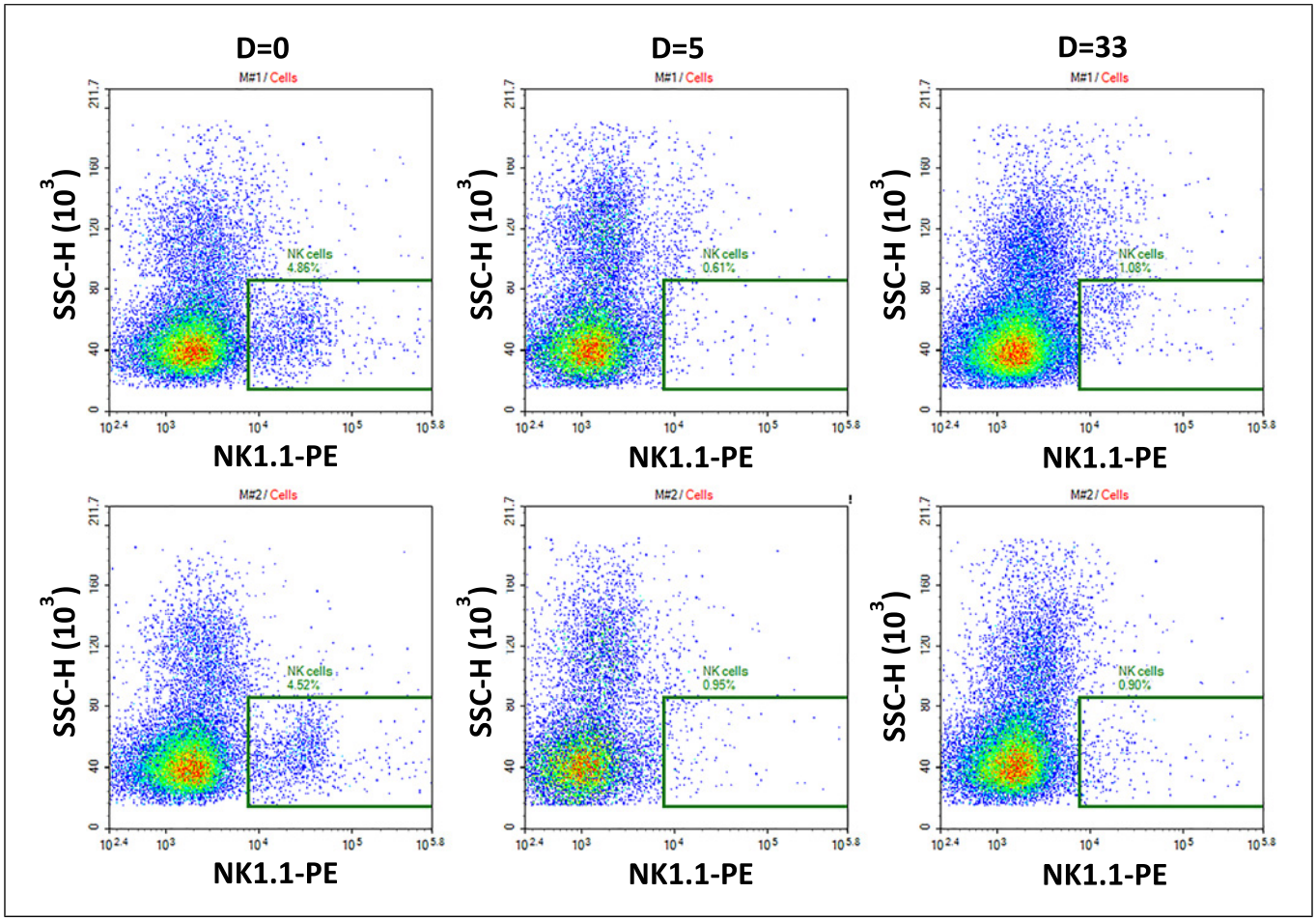

Fig. 1. Confirmation of NK cell depletion upon administration of anti-mouse NK 1.1 antibody. NK cell levels in the blood samples collected before the injection of the first anti-mouse NK1.1 antibody dose (D = 0) were compared with those obtained from the same mice one day prior to vaccination with PCV-13 (D = 5) and PPV-23 (D = 33) dose. Blood samples collected from facial veins were treated with RBC Lysis buffer and labeled with anti-mouse NK1.1-PE before flow cytometry analysis. The percentages of NK cells displayed a significant reduction 1 day prior to vaccination with PCV-23 (D = 5) and PPV-13 (D = 33) as a result of treatment with antibodies against mouse NK1.1.

(Biolegend; for T_H1 cells), IL-4 (Biolegend; for T_H2 cells), and IL-17 (Biolegend; for T_H17 cells) for 20 min in the dark at room temperature. Cells were then washed with 1 × PBS and suspended in 500 µL ×1 PBS, before being analyzed by flow cytometry. In flow cytometry analysis, T_H cell subpopulations were determined by following the gating strategy represented in Figure 2.

Evaluation of Cytokine and Antibody Levels by ELISA

Following in vitro treatment of splenocytes, cell culture supernatants were collected for determination of cytokine expression levels. Secreted levels of IFN-γ, IL-4, and IL-17 cytokines were determined by cytokine-specific ELISA, following the instructions provided by the manufacturer in the kits.

Peripheral blood samples collected from sacrificed animals were used for detection and comparison of serum antibody levels. Flat-bottom microtiter plates were incubated overnight at 4°C with 100 µL of 5 µg/mL PBS-diluted PCV-13. Plates were then washed 2 times with washing solution (0.05% Brij-35 Surfact-Amps [Thermo, Fisher Scientific]-PBS) and blocked with 5% bovine serum albumin-PBS for 1 h at room temperature. Serum samples were 1:20 diluted in PBS and applied to the plates for incubation of 2 h at room temperature. The plates were then washed 3 times with the washing solution. Mouse IgG levels were detected by using goat anti-mouse IgG antibody (Sigma A5153) conjugated with alkaline phosphatase (AP) and pNPP substrate tablets (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA). Absorbance was read at 415 nm.

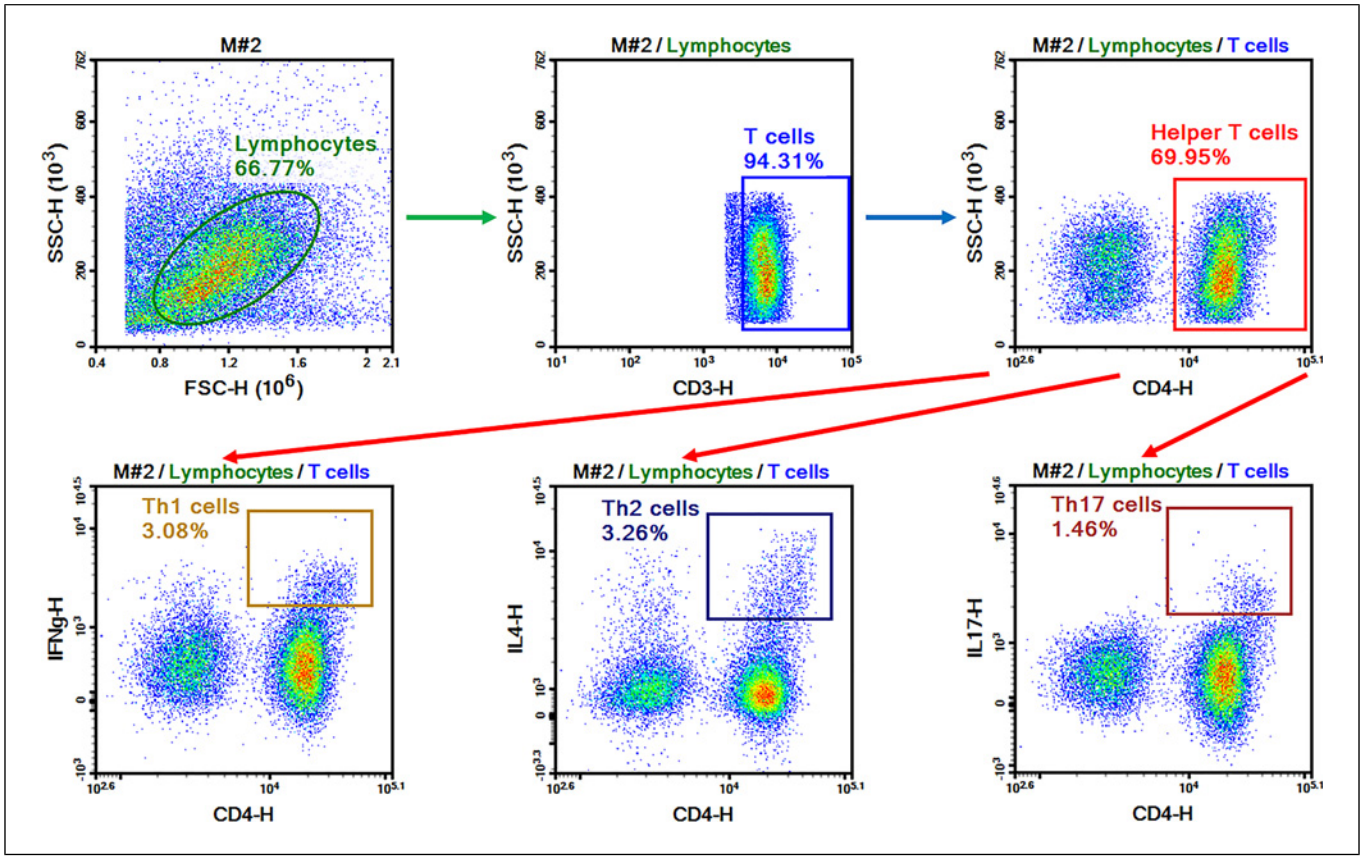

Fig. 2. Gating strategy to identify T_H populations of interest. Lymphocyte populations were gated based on FSC/SSC, excluding aggregates. T_H cells were then gated from CD3+CD4+ cells, and T_H1, T_H2, and T_H17 cell populations were identified based on IFN-γ, IL-4, and IL-17 positivity, respectively.

Statistical Analysis

Arithmetic mean ± standard deviation and median (minimum–maximum) values for quantitative variables were calculated for descriptive statistics. Since the data did not meet the parametric assumptions, nonparametric tests were applied. Mann-Whitney U test was performed to investigate the statistical significance of the group differences. The level of significance was accepted to be 0.05. GraphPad Prism (Version 9.5.0 macOS) was used for all calculations and graphs.

Results

PPV-23-Specific IgG Levels after Pneumococcal Vaccination

The importance of NK cells in pneumococcal vaccine-induced immune response was first investigated by monitoring capsular polysaccharide-specific IgG antibody levels, which is considered as a useful approach to evaluate the immune response triggered against vaccination [13]. For this purpose, serum samples collected after combined schedule of PCV-13 followed by PPV-23 vaccination 4 weeks later were used to compare PPV-23-specific IgG levels between control and NK cell-depleted mice. Analysis with ELISA revealed significantly lower serum pneumococcal IgG levels in NK-depleted mice than that of control animals without NK cell depletion (Fig. 3).

T_H1, T_H2, and T_H17 Cell Frequencies and Associated Cytokine Levels after Pneumococcal Vaccination

Reduced PPV-23-specific IgG levels in NK cell-depleted mice led us to question whether vaccination-induced cellular immune response levels also differ between control and NK cell-depleted animals. This was investigated by comparing T_H1, T_H2, and T_H17 cell frequencies after in vitro stimulation of splenocytes with PPV-23 vaccine antigen. Flow cytometry analysis reported significantly lower T_H1 but unaltered T_H2 and

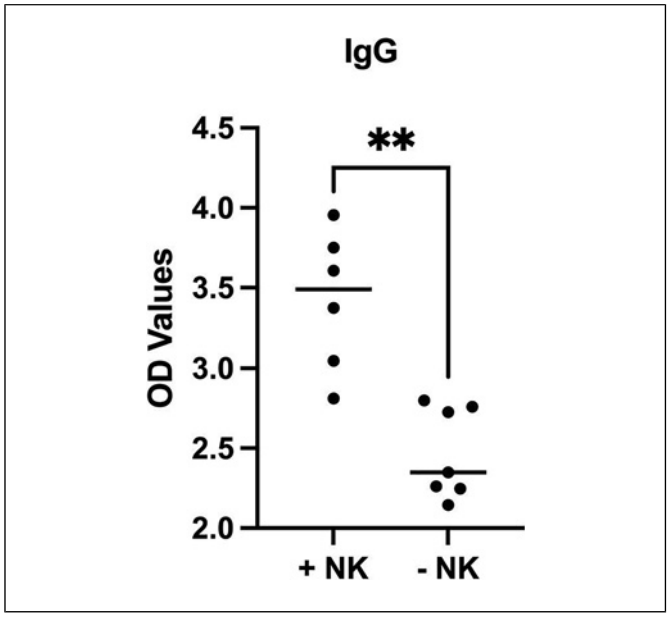

Fig. 3. Comparison of PPV-23-specific IgG antibody levels. Serum samples collected 4 weeks after PPV-23 vaccination were used in ELISA analysis for evaluation of pneumococcal IgG levels. Significantly lower OD values were obtained from mice with NK cell depletion (NK–), when compared with that from control animals without NK cell depletion (NK+). ** represents $p < 0.01$.

T_H17 cell frequencies in NK cell-depleted mice, when compared with those of the control mice group (Fig. 4). On the other hand, both animal groups were also compared in terms of secretion levels of cytokines associated with T_H1 (IFN-γ), T_H2 (IL-4), and T_H17 (IL-17) cells, in supernatants collected from PPV-23-induced splenocytes after 24-h treatment with PPV-23 antigen. Nevertheless, in contrast to the flow cytometry results, the ELISA analysis revealed lack of any statistically significant difference in IFN-γ, IL-4, and IL-17 levels between control and NK cell-depleted mice (Fig. 5).

Discussion

Albeit NK cells are specialized in targeting virus-infected and tumor cells, they were also reported to express cell surface toll-like receptors (TLRs) which may facilitate the induction of NK cell responses after direct interaction with microbial molecules [14]. Recently, NK cells were also shown to be the immune cell subset with the highest level of activation marker expression in whole blood samples after in vitro stimulation with different pneumococcal capsular polysaccharides [15]. That led us to investigate the potential role played by NK cells in T-cell and B-cell-mediated immune responses generated by pneumococcal vaccination. The results would contribute towards developing a more effective pneumococcal vaccination strategies which is urgently needed since *S. pneumoniae* serotypes found in the pneumococcus vaccines are still held responsible for significant numbers of IPD despite the vaccination programs [10].

In our study, NK cell depletion during both PCV and PPV administration resulted reduction in vaccination-induced pneumococcal IgG levels, as revealed by ELISA analysis of serum samples collected after the last PPV shot. Similarly, flow cytometry analysis of splenocytes showed lower T_H1 cell levels in response to in vitro stimulation with PPV-23 due to NK cell depletion. These results are in correlation with the literature highlighting NK cells as an important early source of IFN-γ cytokine which promotes T_H1 differentiation and IgG antibody class switching [12]. However, in contrast to these observations, ELISA analysis of supernatants collected from PPV-23-treated splenocytes revealed lack of difference in IFN-γ secretion levels between mice with and without NK cell depletion.

While the conflictive data can be due to low statistical power which is a major weakness of our analysis, it can also be explained by NK cells promoting T-cell responses and antibody production by mechanisms other than releasing IFN-γ cytokine. Accordingly, NK cells were previously demonstrated to induce antibody production by secreting T_H1 cytokines other than IFN-γ such as tumor necrosis factor (TNF)-α [16] as well as by cellular contact through CD40-CD40 ligand interaction [17]. Moreover, apart from through cytokine secretion, NK cells were suggested to influence T-cell-mediated responses indirectly by eliminating immature DCs (iDCs) which would reduce competition between iDCs and mature DCs, thereby enhance T-cell priming [11, 18]. Future studies using isolated cells in vitro are required to evaluate our suggestions and further enlighten the mechanism responsible.

On the other hand, in addition to their regulatory function on B- and T-cells, NK cells are also known to exhibit adaptive and memory-like phenotypes despite being regarded as an innate immune system component. These led NK cells to be considered as an important target to increase vaccination outcomes for infection control [11, 12]. Targeting NK cells can be especially important for elderly subjects (age group with the highest rate of IPDs despite the presence of pneumococcal vaccines [8, 9]) who display NK cell dysfunction characterized by elevated NK cell levels but with reduced NK cell activities [19].

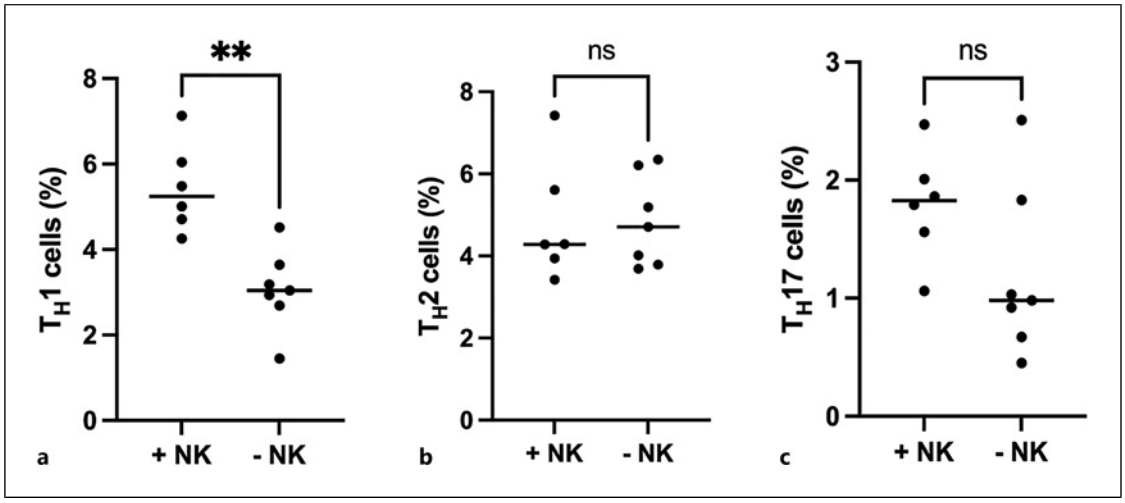

Fig. 4. Comparison of CD4+ T-cell subset levels. Splenocytes collected 4 weeks after the PPV-23 vaccination were stimulated by PPV-23 vaccine antigen for 24 h. Antibodies against IFN-γ, IL-4, and IL-17 were utilized to detect the frequencies of T_H1 (**a**), T_H2 (**b**), and T_H17 (**c**) cells among CD4+ T-cell population, respectively, by flow cytometry analysis. While T_H1 percentages were reduced, T_H2 and T_H17 cell ratios stayed unaltered in NK cell-depleted (NK−) mice in comparison to the control mice (NK+). ** represents $p < 0.01$.

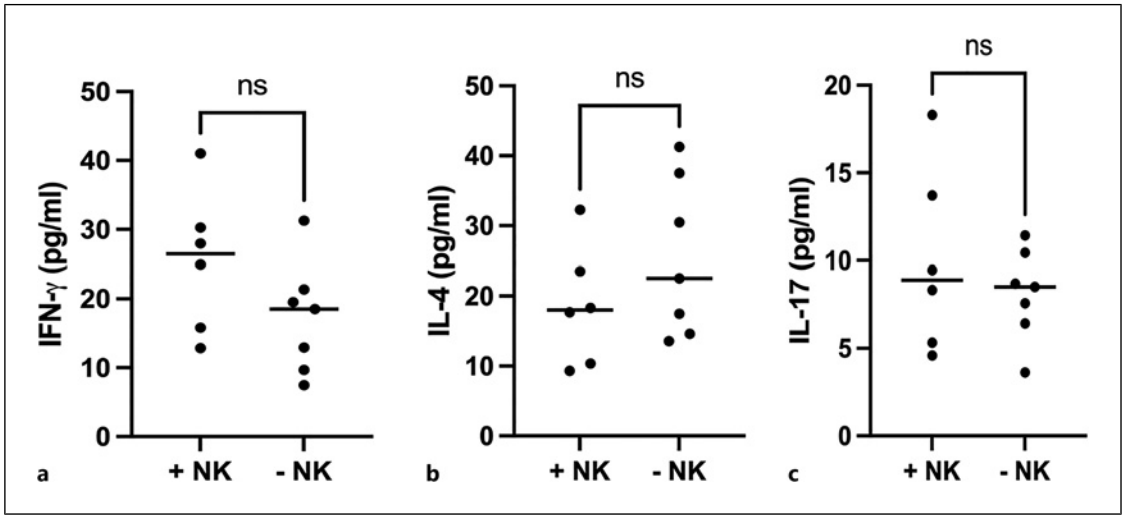

Fig. 5. Comparison of cytokine secretion levels. Splenocytes obtained 4 weeks after the last dose of combined pneumococcal vaccination were stimulated with PPV-23 vaccine antigen in vitro, and secretion levels of IFN-γ (**a**), IL-4 (**b**), and IL-17 (**c**) cytokines were quantified by ELISA assay of the supernatants collected. None of the cytokines displayed statistically significant difference in secretion levels between control (NK+) and NK cell-depleted (NK−) mice.

One approach to modulate NK cell function is through changing adjuvants which are important components of vaccines as they enhance and shape the immune response triggered. Among the adjuvants approved for human use, aluminum salts (alum) in conjugated pneumococcal vaccines induce T_H2-skewed immune responses, while oil (adjuvant system 03 [AS03], MF59), TLR adjuvants (e.g., CpG 1018), and combinational adjuvants (AS01, AS04) promote NK cell activities [12]. In support of changing adjuvants to provide better protection for the elderly through NK cell activation, a recent study by Li et al. [20] demonstrated improved influenza vaccine efficacy in older

adults upon using oil-based adjuvants. Additionally, NK cell function can further be modulated by the inclusion of pathogen-associated molecular patterns and cytokines (e.g., IL-2, IL-12, IL-15) involved in NK cell activation [11].

Our study has weaknesses that need to be acknowledged. First, our results need to be interpreted with caution due to low sample size. Moreover, even though NK1.1 is predominantly expressed on NK cells, it can also be detected on NKT cells which were also among blood cells displaying high level of activation (following NK cells) after in vitro stimulation with pneumococcal capsular polysaccharides [15]. Therefore, studies using alternative approaches such as those utilizing NK cell-deficient mice are of prime importance to confirm our data and conclusions. Additionally, it was not possible to evaluate the effect of NK cells in T-cell and B-cell responses triggered by each PPV and PCV shot due to lack of funding, which also needs to be addressed by future studies.

To conclude, our results demonstrated diminished T-cell and B-cell responses initiated by pneumococcal vaccination upon NK cell depletion. While further studies are required to confirm our data, it suggests targeting NK cells may be a useful strategy to increase efficacy of pneumococcus vaccination which is urgently needed.

Acknowledgments

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Statement of Ethics

This study was approved by the Local Ethics Committee of Kobay Laboratory Animals Center (approval number: 377, date: May 24, 2019).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

Study conception and design and supervision: U.G. and A.T.O.; data collection: U.G., M.K.D., D.K., and A.S.G.; interpretation of results: U.G. and O.T.; draft manuscript preparation: U.G. All authors revised and edited the manuscript and have read and agreed to the published version of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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