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ORIGINAL ARTICLE

Anti-Pneumococcal Vaccine-Induced Cellular Immune Responses in Post-Traumatic Splenectomized Individuals

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Abstract
Purpose Splenectomy is associated with increased risk of overwhelming post-splenectomy infections despite proper anti-pneumococcal vaccination. As most studies concentrated on vaccination-induced humoral immunity, the cellular immune responses triggered in splenectomized patients are not yet well studied. The present study aims to investigate this area as it can contribute to the development of more effective vaccination strategies.
Methods Five healthy and 14 splenectomized patients were vaccinated with pneumococcal conjugate polysaccharide vaccine (PCV) followed by pneumococcal polysaccharide vaccine according to the guidelines established by Advisory Committee on Immunization Practices. PBMC samples collected 0, 8, and 12 weeks after PCV immunization were in vitro stimulated with PCV. Levels of lymphoproliferation, T_H cell differentiation, and cytokine release were assessed by carboxyfluorescein succinimidyl ester labeling, intracellular cytokine staining, and ELISA, respectively.
Results While T_H1-dominated immune response was detected in both groups, asplenic individuals generated significantly lower levels of T_H1 cells following in vitro stimulation. Similarly, levels of IFN-γ, IL-4, and IL-17 release and lymphoproliferation were significantly lower in asplenic patients.
Conclusions According to our data, splenectomy negatively influences the levels of PCV-induced lymphoproliferation, T_H1 differentiation, and cytokine release. Besides, PCV failed to induce T_H17-dominant immune response which is crucial for protection against extracellular pathogens.

Keywords Splenectomy · *Streptococcus pneumoniae* · vaccination · cellular immunity

Introduction

Splenectomy is associated with increased risk of overwhelming post-splenectomy infection (OPSI), which is mainly caused by *Streptococcus pneumoniae* (50–90% of cases) [1, 2]. Two types of pneumococcal vaccines are currently recomended for protection: the 23-valent pneumococcal polysaccharide vaccine (PPV-23) which induces T cell-independent immune responses and 13-valent pneumococcal conjugate vaccines (PCV-13) which, because of their conjugated nature to a carrier protein, can induce T cell-dependent immune responses characterized by immunological memory, affinity maturation, and extensive antibody subclass switching [3]. Among those, for more than two decades, PPV-23 has been recommended for both immunocompromised individuals and the elderly people [4]. However, PPV-23-driven hyporesponsiveness [5, 6] provided further support for the idea that new PCV-13 + PPV-23 combined schedules should be recommended for individuals at risk since 2012 (Advisory Committee on Immunization Practices-ACIP-2012) [7].

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As antibody-secreting B cells are thought to occupy a central role for the protection against encapsulated organisms, impairment of humoral response is considered the main cause for OPSI, and accordingly, most of the studies in literature concentrated on the vaccine-induced humoral immunity [8–15]. On the other hand, despite the fact that splenectomy was shown to influence unprimed T cells and/or their precursors by previous sudies [16, 17], vaccination-induced cellular immune responses in asplenic patients have not received enough attention.

Our aim in this study is to examine the effect of splenectomy on the levels of cell-mediated immune responses initiated against the anti-pneumococcal vaccination by measuring the levels of vaccine-specific PBMC proliferation; T_H1, T_H2, and T_H17 subpopulations generated; and IFN-γ, IL-4, and IL-17 secreted. Our results can contribute to the development of more effective vaccination strategies in order to protect splenectomized individuals from OPSI, as it can affect even those with appropriate immunization [18–20].

Methods

Subjects and Vaccination

Fourteen splenectomized patients who were splenectomized in Hitit University Corum Training and Research Hospital in Corum province of Turkey and five healthy patients were enrolled in this study. This study was approved by the ethics committee of Numune Training and Research Hospital in Ankara/Turkey (E-14-285). After getting all patients' informed consents, patients' database (clinical history, reason for splenectomy, vaccination status) was obtained from the hospital information system. All patient and control subjects were white and Caucasian.

Vaccination Schedule

All participants were vaccinated according to the updated Advisory Committee on Immunization Practices guidelines that recommend combined schedule of PCV-13 (Pfizer, Lot No. 305010) followed by PPV-23 (Merck, Lot No. K0 10839) vaccination 8 weeks later [7]. All individuals were vaccinated subcutaneously with one dose of the PCV-13 containing 2.2 μg of capsular PS 1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F, 6B, and 23F serotypes and 32 μg of CRM197 (a nontoxic, single-amino acid mutant of diphtheria toxin). After 8 weeks, the same patients were subcutaneously vaccinated with PPV-23 containing 25 μg of each 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F serotypes.

Collection of Blood Samples and Isolation of Peripherial Blood Mononuclear Cells

Blood samples (20 ml) were collected before vaccination, 8 weeks after PCV-13 vaccination (PCV-13) and 4 weeks after PPV-23 vaccination (PCV-13 + PPV-23). PBMCs were isolated from peripherial venous blood by lymphocyte separation medium histopaque (Genaxxon, C4754.0100) and suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and streptomycin, and L-glutamine.

Lymphocyte Proliferation Assay

Previously described protocol was followed for lymphocyte proliferation assay [21]. Briefly, 2×10^6 cells were labeled with 10 μM carboxyfluorescein succinimidyl ester (CFSE) (Biolegend, 423801) in 1× PBS. Cells were incubated at 37 °C for 30 min. Labeling process was terminated by the addition of cell culture media, after which cells were centrifuged at 300×*g* for 5 min. Cells were then suspended in 500-μl cell culture media, and 5×10^5 cells in 0.125-ml media was transferred onto 96-well plates. For all patients, the following treatments were performed:

- Experimental group: anti-CD28 (2 μg/ml, Biolegend, 302933) + anti-CD49b (2 μg/ml, Biolegend, 359304) + PCV-13 (1:100).
- Positive control: anti-CD28 (2 μg/ml) + anti-CD49b (2 μg/ml) + anti-CD3 (2 μg/ml).
- Negative control: anti-CD28 (2 μg/ml) + anti-CD49b (2 μg/ml) but not with vaccine.

Cells were incubated with stimulants for 120 h, and CFSE positivity was analyzed with BD Accuri C6 flow cytometer.

Analysis of Vaccine-Specific T_H1, T_H2, and T_H17 Subpopulations

T_H1, T_H2, and T_H17 subpopulations were analyzed by following the protocol previously described [17, 22, 23]. PBMCs were diluted to 4×10^6 cells/ml, and 5×10^5 cells (in 0.125-ml media) were seeded into 96-well cell culture plate. For all patients, the following treatments were performed on PBMC samples collected:

- Experimental group: anti-CD28 (2 μg/ml) + anti-CD49b (2 μg/ml) + PCV-13 (1:100).
- Positive control: PMA (50 ng/ml) + ionomycin (1 μg/ml).
- Negative control: anti-CD28 (2 μg/ml) + anti-CD49b (2 μg/ml) but not with vaccine.

For helper T cell analysis, cells were treated with brefeldin A (Biolegend, 420601) at 5 μg/ml concentration at 16th hour of incubation and then incubated for 6 h. After 24 h of incubation, cells were collected, fixed, permeabilized, and stained against CD4, IFN-γ, IL-4, and IL-17 by using Human Th1/Th2/Th17 Phenotyping Kit (BD Biosciences, 560751) by following the manufacturer's guidelines. Cells were analyzed with BD Accuri C6 flow cytometer.

Analysis of Cytokine Secretion in Lymphocyte Culture Supernatants

For cytokine ELISA [24], cells were incubated with anti-CD28 (2 μg/ml), anti-CD49b (2 μg/ml), and PCV-13 (1:100) for 72 h. As positive control, PBMCs were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml), while cells incubated with antibodies but not with vaccine were used as negative control. Supernatant was collected, and IFN-γ, IL-4, and IL-17 concentrations were measured with IFN-γ ELISA kit (Biolegend, 430104), IL-4 ELISA kit (Biolegend, 430304), and IL-17 ELISA kit (Biolegend, 433914), respectively, by following guidelines provided by the manufacturer.

Statistical Analysis

Descriptive statistics were calculated and provided throughout the text. Mean ± standard error of mean. Within group and between group comparisons were performed using two-way repeated measures analysis of variance test. Between group comparisons were adjusted for errors by using Sidak's multiple comparisons test, while for within group comparisons, Tukey post hoc test was applied. Level of significance was accepted to be 0.05. All statistical analyses were performed, and graphs were drawn with GraphPad Prism (Version 7.00) software package.

Results

Characteristics of Study Groups

For all 14 splenectomized patients who were included in this study, the main reason for splenectomy was trauma. The study group consisted of 11 (79%) men and 3 (21%) women with a mean age of 37 ± 9.1 years (20–53 years). Mean weight and body mass index (BMI) were 74.8 ± 6.3 kg (64–80 kg) and 24.98 ± 2.02 kg/m^2 (22.1–27.5 kg/m^2), respectively. Mean time since splenectomy was 12.7 years, with a range of 3–36 years. All study individuals were healthy acccording to the physical examination and routine laboratory parameters. Control group included five individuals: two women and men men, with mean age 36 ± 9.7 years (25–48 years) who did not receive any pneumococcal vaccination before this study. Mean weight and BMI were 73.5 ± 7.12 kg (57–82 kg) and 23.81 ± 1.88 kg/m^2 (19.8–27.3 kg/m^2), respectively. There was no any acute illness or any other severe diseases detected in control groups.

Lymphocyte Proliferative Responses

In order to examine the effect of splenectomy on the anti-pneumococcal vaccination-induced cellular immune responses, at first, lymphoproliferative responses were compared. For this purpose, PBMC samples isolated from pre-vaccinated and post-vaccinated control and experiment groups were treated with PCV-13 in vitro. PCV-13 was chosen as the in vitro stimulant since PPV-23 is not able to induce cell-mediated immune responses [25].

In pre-vaccinated samples isolated from both groups, there was no any significant level of PCV-13-induced lymphocyte proliferation. This was reversed in the samples collected after PCV-13 vaccination and PPV-23 vaccination following the initial PCV-13 shot (PCV-13 + PPV-23). Removal of spleen seemed to have a negative impact on the lymphocyte proliferation as there is a significant reduction in the level observed in asplenic individuals (Fig. 1, Table 1).

On the other hand, time since the cellular immune response activation by the first PCV-13 vaccination shot did not seem to

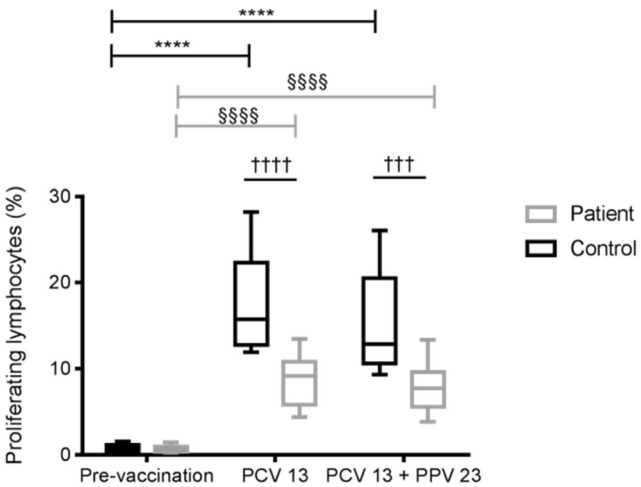


Fig. 1 Level of lymphoproliferation in response to PCV-13 treatment in vitro. PBMCs were collected 8 weeks following the initial PCV-13 vaccination (PCV-13) and 4 weeks after the second PPV-23 vaccination (PCV-13 + PPV-23) for both groups. Isolated PBMC samples were labeled with 10 μM CFSE and treated with anti-CD28, anti-CD49b, and PCV-13. For positive control, cells were stimulated with anti-CD3, anti-CD28, and anti-CD49b (data not shown). For negative control, cells were incubated anti-CD28 and anti-CD49b only (data not shown). The *asterisks*, and *section signs* signify significant differences against pre-vaccinated control and pre-vaccinated patient values (within group comparison), respectively. The *dagger* signifies significant difference between control and patient group values in pre-vaccinated, post-PCV-13-vaccinated and post-PCV-13 + PPV-23-vaccinated samples (between group comparison). *, §, † represent $p < 0.05$; **, §§, †† represent $p < 0.01$; ***, §§§, ††† represent $p < 0.001$; and ****, §§§§, †††† represent $p < 0.0001$

Table 1 Mean percentage (±SEM) of proliferating lymphocytes

Proliferating lymphocytes (%)			
	Pre-vaccination	Post-PCV-13 vaccination	Post-PCV-13 + PPV-23 vaccination
Control	0.77 ± 0.23	17.17 ± 2.88****	15.00 ± 2.91****
Patient	0.69 ± 0.10	8.76 ± 0.75$^{§§§§, ††††}$	7.96 ± 0.78$^{§§§§, †††}$

The asterisks and section signs signify significant differences against pre-vaccinated control and pre-vaccinated patient values (within group comparison), respectively. The dagger signifies significant difference between control and patient group values in pre-vaccinated, post-PCV-13-vaccinated, and post-PCV-13 + PPV-23-vaccinated samples (between group comparison)

*, §, † represent $p < 0.05$; **, §§, †† represent $p < 0.01$; ***, §§§, ††† represent $p < 0.001$; and ****, §§§§, †††† represent $p < 0.0001$

influence the PCV-induced lymphoproliferation levels, since in both control and experiment groups, there was no any significant difference between the samples collected 8 weeks (PCV-13) and 12 weeks (PCV-13 + PPV-23) after the PCV-13 vaccination (Fig. 1, Table 1).

Vaccine-Specific T_H1, T_H2, and T_H17 Subpopulations

The effect of spleen on the cell-mediated immunity was further investigated by monitoring the levels of $CD4^+$ T cell subsets generated in response to PCV-13 stimulation in vitro. The subset levels were screened following the addition of brefeldin that is able to block the secretion of cytokines required for the detection of cell subsets by flow cytometry [22].

Pre-vaccinated samples from both groups did not generate any significant level of $CD4^+$ T cell subsets monitored, following the PCV stimulation. In contrast, PBMCs collected from PCV-13 and PCV-13 + PPV-23-vaccinated individuals (from both groups) displayed enhanced levels of T_H1 and T_H17 cells, while T_H1 cells were reported to be the dominant cell population (Fig. 2, Table 2). Spleen seems to influence the $CD4^+$ T cell differentiation since significantly lower level of T_H1 cell population was detected when PCV-13-vaccinated asplenic patient PBMCs were used for in vitro stimulation. However, this significant difference is lost in case of PCV-13 + PP23-vaccinated samples (Fig. 2a, Table 2 (A)).

On the other hand, in contrast to asplenic group, in which samples from both PCV-13 and PCV-13 + PPV-23 individuals showed significantly enhanced T_H2 levels, this change was significiant only in PCV-13 + PPV-23-vaccinated samples for the control group (Fig. 2b, Table 2 (B)).

Vaccine-Specific IFN-γ, IL-4, and IL-17 Levels

Cytokines play a major role in adaptive immune responses by both influencing naive T cell differentiation into various $CD4^+$ T_H cell subsets and mediating downstream immunological T cell effector functions. Because of this dual role in T cell-mediated immune responses, cytokines can be categorized into groups depending on which T_H cell-mediated immune response they are associated with. In our study, the effect of splenectomy on the PCV-13-mediated cellular immune response was further studied by monitoring the levels of IFN-γ (T_H1), IL-4 (T_H2), and IL-17 (T_H17) cytokine release in response to PBMC stimulation by PCV-13 in vitro.

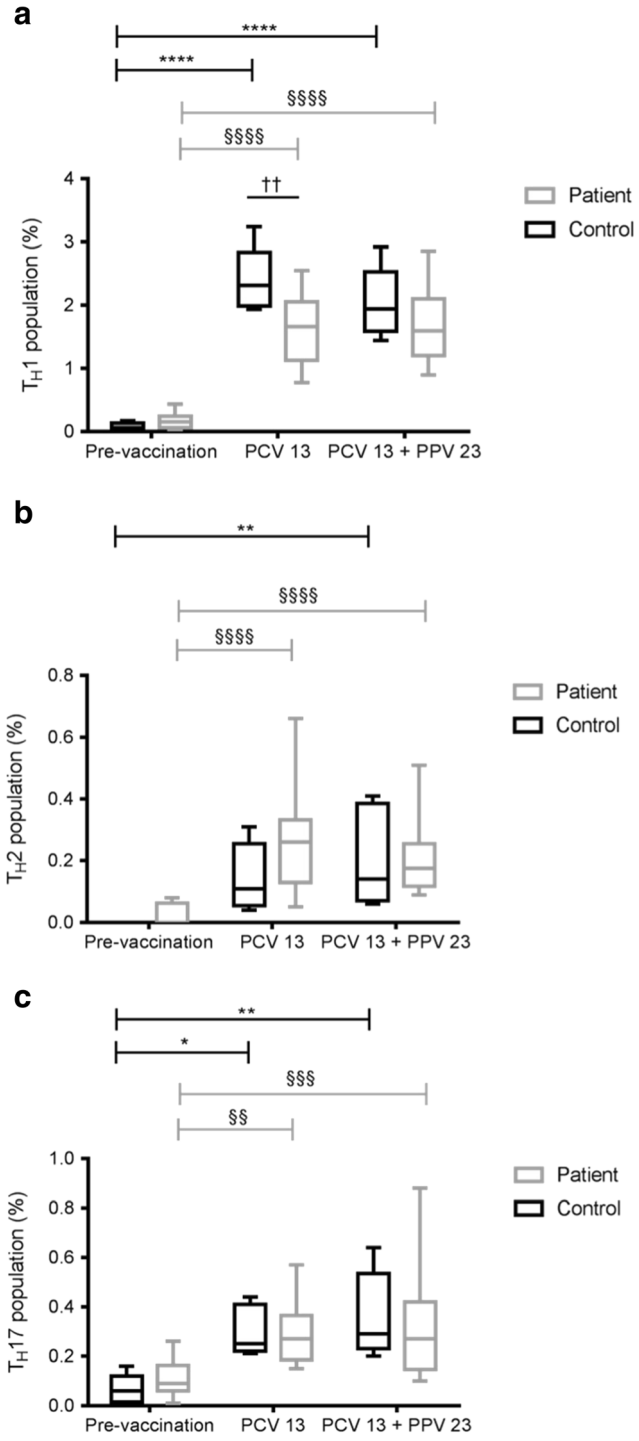
As with vaccine-specific lymphoproliferation and T_H cell subset results above, there was no any significant level of cytokine release in response to PCV-13 stimulation of unvaccinated patient and control group PBMCs. PBMCs collected from asplenic individuals after PCV-13 and PCV-13 + PPV-23 vaccination displayed significantly reduced levels of IFN-γ, IL-4, and IL-17 cytokines in comparison to that from the control group (Fig. 3, Table 3). In accord with the vaccine-specific T_H cell subset results above, IFN-γ (T_H1) seemed to be the predominant cytokine induced by anti-pneumococcal vaccination, since the corrosponding levels were higher than those for IL-4 and IL-17 in the same serum samples used (Fig. 3, Table 3).

Furthermore, as observed in lymphoproliferative results, there was no any significant difference between the post-PCV-13 and post-PCV-13 + PPV-23 control/asplenic patient PBMCs in the cytokine levels detected (Fig. 3, Table 3).

Discussion

Splenectomy is associated with lifelong risk of pneumococcal infections which are the most common cause of OPSI syndrome [1, 2]. Today, ACIP recommends initial PCV-13 vaccination followed by immunization with PPV-23 8 weeks later [7]. The PCV vaccination is able to trigger T cell-mediated immune response characterized by immunological memory, affinity maturation, and extensive antibody subclass switching [3, 25, 26], while PPV-23 vaccination triggers exclusively humoral immunity [17, 27].

Individuals who have undergone post-traumatic splenectomy were previously reported to have negative effect on humoral and cellular branches of immunity [8, 9, 16, 28].



◀ **Fig. 2** The levels of $CD4^+$ T_H cell subsets—T_H1 (**a**), T_H2 (**b**), and T_H17 (**c**)—induced by PCV-13 treatment in vitro. PBMCs were isolated from splenic and asplenic patients 8 weeks following the initial PCV-13 vaccination (PCV-13) and 4 weeks after the second PPV-23 vaccination (PCV-13 + PPV-23). Isolated PBMC samples were stimulated with anti-CD28, anti-CD49b, and PCV-13. For positive control, PBMCs were stimulated with PMA and ionomycin (data not shown), while cells incubated with antibodies but not with vaccine were used as negative control (data not shown). The *asterisks* and *section signs* signify significant differences against pre-vaccinated control and pre-vaccinated patient values (within group comparison), respectively. The *dagger* signifies significant difference between control and patient group values in pre-vaccinated, post-PCV-13-vaccinated, and post-PCV-13 + PPV-23-vaccinated samples (between group comparison). *, §, † represent $p < 0.05$; **, §§, †† represent $p < 0.01$; ***, §§§, ††† represent $p < 0.001$; and ****, §§§§, †††† represent $p < 0.0001$

However, the effects on anti-pneumococcal vaccine-induced cell-mediated responses are not yet investigated. The current study aims to investigate the effect of splenectomy on the persistence and effectiveness of cellular immune responses triggered by the anti-pneumococcal vaccination, which fail to give optimum level of protection after splenectomy [18–20].

For the first time in literature, our results showed that splenectomy has a negative effect on the levels of lymphoproliferation, T_H1 cell differentiation, and cytokine (IFN-γ, IL-4, and IL-17) release stimulated by PCV treatment in vitro. This is in correlation with a previous study by Balsalobre and Carbonell-Tatay that showed defective cutanous delayed hypersensitivity reaction in splenectomized patients [29].

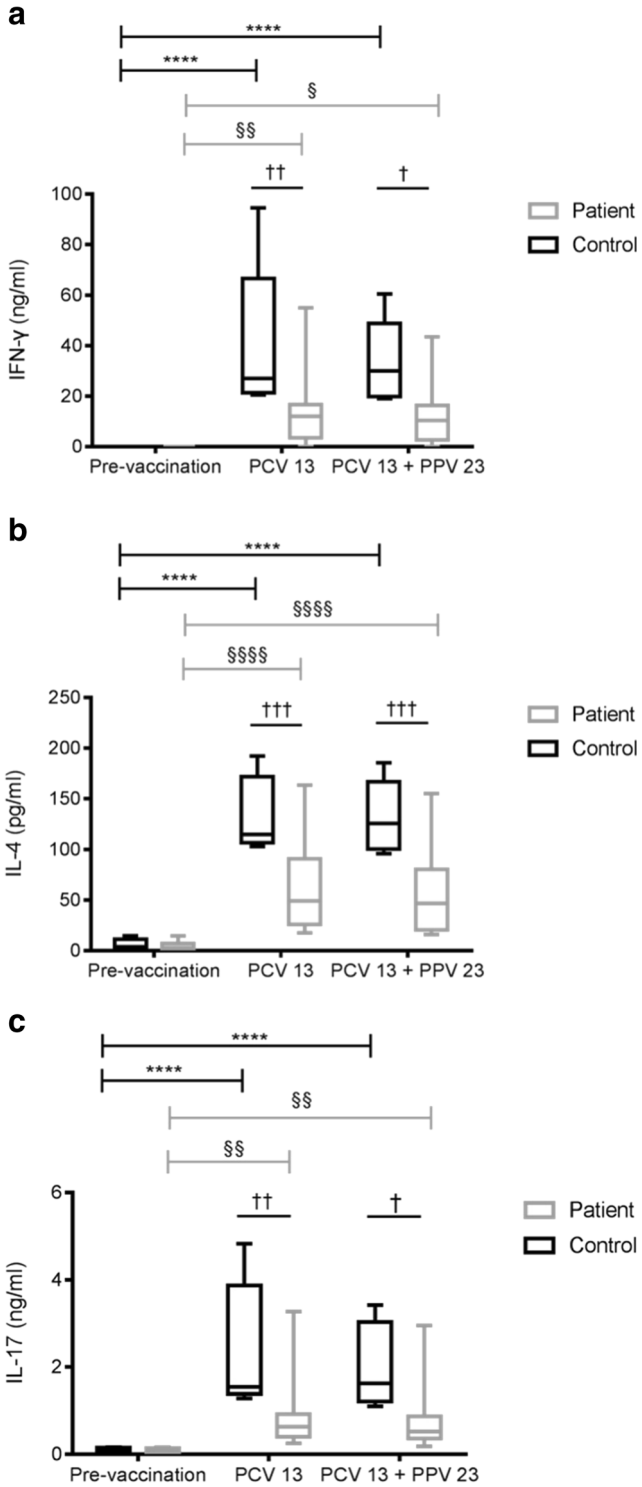
Among the cytokines and $CD4^+$ T_H cell subsets investigated, IFN-γ and T_H1 cells are known to provide protection especially against intracellular bacteria by eliciting macrophage activation, while IL-4 and T_H2 cells are protective against extracellular parasites via humoral immune responses [30]. Together with ELISA results, flow cytometry analysis revealed that the PCV-13 vaccination induces T_H1-dominant immune response which was negatively influenced by

Table 2 Mean percentage (±SEM) of $CD4^+$ T_H cell subsets generated

	Pre-vaccination	Post-PCV-13 vaccination	Post-PCV-13 + PPV-23 vaccination
(A) $CD4^+$ T_H1 cells (%)			
Control	0.07 ± 0.03	2.39 ± 0.23****	2.03 ± 0.25****
Patient	0.17 ± 0.03	1.63 ± 0.14§§§§, ††	1.67 ± 0.16§§§§
(B) $CD4^+$ T_H2 cells (%)			
Control	0.00 ± 0.00	0.15 ± 0.05	0.21 ± 0.07**
Patient	0.02 ± 0.01	0.26 ± 0.04§§§§	0.20 ± 0.03§§§§
(C) $CD4^+$ T_H17 cells (%)			
Control	0.07 ± 0.03	0.30 ± 0.05*	0.36 ± 0.08**
Patient	0.11 ± 0.02	0.29 ± 0.04§§	0.31 ± 0.06 §§§

The asterisks and section signs signify significant differences against pre-vaccinated control and pre-vaccinated patient values (within group comparison), respectively. The dagger signifies significant difference between control and patient group values in pre-vaccinated, post-PCV-13-vaccinated, and post-PCV-13 + PPV-23-vaccinated samples (between group comparison)

*, §, † represent $p < 0.05$; **, §§, †† represent $p < 0.01$; ***, §§§, ††† represent $p < 0.001$; and ****, §§§§, †††† represent $p < 0.0001$



◀ **Fig. 3** The levels of IFN-γ (**a**), IL-4 (**b**), and IL-17 (**c**) cytokine release induced by PCV-13 treatment in vitro. PBMCs were collected from both groups, 8 weeks following the initial PCV-13 vaccination (PCV-13) and 4 weeks after the second PPV-23 vaccination (PCV-13 + PPV-23). Isolated PBMC samples were incubated with anti-CD28, anti-CD49b, and PCV-13. As positive control, PBMCs were stimulated with PMA and ionomycin (data not shown). For negative control, cells were incubated with antibodies but not with vaccine (data not shown). ELISA kits are used to measure IFN-γ (**a**), IL-4 (**b**), and IL-17 (**c**) concentrations in the supernatants collected. The *asterisks* and *section signs* signify significant differences against pre-vaccinated control and pre-vaccinated patient values (within group comparison), respectively. The *dagger* signifies significant difference between control and patient group values in pre-vaccinated, post-PCV-13-vaccinated, and post-PCV-13 + PPV-23-vaccinated samples (between group comparison). *, §, † represent $p < 0.05$; **, §§, †† represent $p < 0.01$; ***, §§§, ††† represent $p < 0.001$; and ****, §§§§, †††† represent $p < 0.0001$

splenectomy. Having reduced T_H1 cell and IFN-γ levels in aplenic patients is in correlation with another study by Kuranaga et al. that suggested spleen as an important organ for T_H1 immune responses against bacterial infections [31].

On the other hand, IL-17, which was the other cytokine investigated, is considered to be essential for protection against extracellular pathogens, including those that can cause OPSI [32]. Accordingly, protective immunity against pneumococcal colonization was previously shown to be mediated by IL-17A production by T_H17 cells [33, 34]. Furthermore, in contrast to IFN-γ and IL-4-deficient mice, animals lacking IL-17 receptor expression were not able to develop protection against pneumococci [30]. Therefore, we suggest that development of T_H17, rather than T_H1, dominant immune response-inducing vaccination strategies may provide a better protection for splenectomized patients.

One such strategy involves intranasal administration of polysaccharides with mucosal adjuvant which was previously shown to confer resistance against nasopharyngeal colonization by capsulated *S. pneumoniae* via $CD4^+$ T cells and IL-17 activity [35]. Furthermore, quite recently, new protein candidates that provide complete protection from pneumococcal colonization via a T_H17-mediated immune response has been

Table 3 Mean concentration (±SEM) of cytokines released

	Pre-vaccination	Post-PCV-13 vaccination	Post-PCV-13 + PPV-23 vaccination
(A) IFN-γ (ng/ml)			
Control	0.00 ± 0.00	40.50 ± 13.86****	33.40 ± 7.53****
Patient	0.00 ± 0.00	14.07 ± 4.06§§, ††	12.11 ± 3.33§, †
(B) IL-4 (pg/ml)			
Control	5.83 ± 2.47	134.23 ± 16.64****	131.91 ± 16.15****
Patient	4.74 ± 1.23	62.58 ± 12.11§§§§, †††	57.31 ± 11.34§§§§, †††
(C) IL-17 (ng/ml)			
Control	0.10 ± 0.02	2.41 ± 0.67****	2.02 ± 0.44****
Patient	0.09 ± 0.01	0.88 ± 0.22§§, ††	0.81 ± 0.20§§, †

The asterisks and section signs signify significant differences against pre-vaccinated control and pre-vaccinated patient values (within group comparison), respectively. The dagger signifies significant difference between control and patient group values in pre-vaccinated, post-PCV-13-vaccinated, and post-PCV-13 + PPV-23-vaccinated samples (between group comparison)

*, §, † represent $p < 0.05$; **, §§, †† represent $p < 0.01$; ***, §§§, ††† represent $p < 0.001$; and ****, §§§§, †††† represent $p < 0.0001$

identified [36]. As splenectomized patients can be considered as immunocompromised [37], and *S. pneumoniae* remain risk factors for invasive pneumococcal disease as long as they are colonized [38], reduction in bacterial colonization can have a vital role in conferring optimum level of protection against OPSI. Future studies correlating the bacterial colonization with the vaccination-induced IL-17 and T_H17 levels can further help to enlighten this area of research.

Our results also showed that lymphoproliferation is negatively influenced by splenectomy. This together with ELISA and flow cytometry results suggests that, respectively, lower level of antigen-specific memory cells may be generated in asplenic patients since memory T cells were shown to be the predominant T cell responding to recall antigens in vitro [39, 40]. Further studies on the levels of memory cells generated upon vaccination in asplenic patients are still ongoing in our laboratory.

On the other hand, our data is in contrast to Wolf et al. which did not detect a significant difference in the level of recall antigen-mediated lymphoproliferation between splenic and asplenic patients [17]. The conflicting data can be explained, at least partially, by the differences in the methodology utilized. For instance, while ^{3}H-thymidine incorporation assay was used to monitor lymphoproliferation by Wolf et al., in our work, CFSE staining was preferred. Among those, the former measures DNA synthesis during S phase, while the latter detects cells at the M phase of the cell cycle during which daughter cells are generated [41]. Since DNA synthesis can be triggered during processes independent of cell divison, such as gene duplication, repair, or apoptosis, ^{3}H-thymidine incorporation assay measures the level of DNA synthesis and not of cell division [42]. Accordingly, CFSE staining was recently reported to be more sensitive for lymphocyte proliferation assays than ^{3}H-thymidine incorporation [41].

Another important aspect investigated by our study is the effect of time since the initial PCV shot on the conjugated vaccine-induced cellular responses. For this purpose, PBMCs collected 4 weeks after PPV vaccination (i.e., 12 weeks after the PCV-13 immunization) were used, and detected responses were compared with that obtained from post-PCV-13 samples (i.e., 8 weeks after the PCV-13 immunization). PPV vaccination cannot boost any cellular immune response [17, 24], and therefore, cellular immune responses initiated by the PCV-13 vaccination are not affected by PPV vaccination in PCV-PPV samples.

Our results showed that there was no significant difference between splenic and asplenic patients in the levels of immune responses (i.e., lymphoproliferation, $CD4^+$ T_H subset differentiation, and cytokine release levels) detected 8 and 12 weeks after the initial PCV-13 vaccination. This was in correlation with a previous study showing increased level of lymphoproliferation and IFN-γ production 4 weeks following PCV vaccination that persists until week 24 [43].

The only exception was the T_H1 cell levels. Even though the levels did not seem to significantly differ between week 8 and week 12 post-PCV vaccination in both control and asplenic patients, the significant difference that was observed between control and asplenic patients in post-PCV-vaccinated samples is lost in post-PCV + PPV samples. This can be due to the low sample numbers used, which is a limitation of our study. This can also explain why the difference in T_H2 levels between unvaccinated and PCV-13-vaccinated samples did not reach the significant level, even though significantly high level of IL-4 was produced.

Conclusions

In conclusion, splenectomy negatively influences the anti-pneumococcal vaccine-induced immune responses including lymphocyte proliferation, $CD4^+$ T_H cell differentiation, and cytokine release. This may be as a result of reduced level of memory cells generated in asplenic patients following anti-pneumococcal vaccination, which is an area for further investigation. Our results also showed that PCV-13 vaccination induces T_H1, rather than T_H17, dominant immune response which is in general important for protection against rather intracellular microbes. Future vaccine development strategies to reverse splenectomy-associated effects and/or induce T_H17-dominant immune responses may help to minimize risk of OPSI in asplenic individuals.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the ethics committee of Numune Training and Research Hospital in Ankara/Turkey (E-14-285).

Informed Consent Informed consent was obtained from all individual participants included in the study.

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