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ORIGINAL ARTICLE

Interleukin-1β and interleukin-6 in Common Variable Immunodeficiency and their association with subtypes of B cells and response to the Pneumovax-23 vaccine

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ABSTRACT. *Introduction*: Common Variable Immunodeficiency (CVID) is the most common symptomatic form of primary immunodeficiencies. Current research data show altered B cells, TLRs, and cytokine profile in CVID patients. The aim of this study was to determine levels of IL-1β and IL-6 in CVID patients in response to TLRs stimulation and the association of these cytokines with subtypes of B cells and response to Pneumovax-23 vaccination. *Method*: Peripheral blood mononuclear cells of CIVD patients were stimulated with and without TLR2 and TLR4 agonist and specific inhibitors including lipopolysaccharide (LPS), lipoteichoic (LTA), and OxPAPC. The levels of IL-1β and IL-6 were assessed by ELISA in different treatment groups. Finally, association of cytokines levels was assessed among different subtypes of B cells and types of response to Pneumovax-23 vaccine. *Results*: Secretion of IL-6 and IL-1β was significantly diminished in CVID patients ($p = 0.015$ and $p = 0.019$), but ligand engagement of TLR2 and TLR4 leads to significant increase in IL-6 and IL-1β production. IL-6 was significantly lower in Pneumovax-23 hypo responder patients ($p = 0.05$) and significant correlations between the concentration of IL-6 and the number of switched memory and $CD21^{low}$ expressing B cells were found. *Conclusion*: Secretion of IL-6 and IL-1β is abolished in CVID patients. However, TLR2 and TLR4 are hyper responsive to stimulation with their cognate ligands resulting in the secretion of higher levels of proinflammatory cytokines. This characteristic of CVID TLRs leads to an improvement of cytokine secretion compared to baseline levels. Also, our novel findings about the association concentrations of serum IL-6 and the frequency of with switched memory and $CD21^{low}$ expressing B cells as well as the poor response to Pneumovax-23 should be substantiated by the use of a higher sample size in future studies.

Key words: CVID, IL-6, IL-1β, Pneumovax-23, $CD21^{low}$ expressing B cells, switched memory B cells

INTRODUCTION

Common Variable Immunodeficiency (CVID) is the most common symptomatic form of Primary Immunodeficiencies (PID). This disorder is the second common primary antibody deficiency (PAD) presenting hypogammaglobulinemia and poor production of specific antibodies against polysaccharide and protein antigens [1]. CVID patients are predisposed to recurrent sinopulmonary infections, autoimmune disorders, granuloma, and malignancy [2, 3].

Different alterations in cytokines pathways and their secretion level have been reported to contribute to the pathophysiology and manifestations of CVID. The results of these studies revealed a very dissimilar pattern of cytokines in CVID which can be due to the heterogeneous nature of this syndrome and partly because of restricted comparability of the findings [4, 5].

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Underlying genetic causes of CVID are unknown [6] and recently detected mutation is responsible only for 10% of the patients. [7-12]. To find the cause of the disease in the rest of the CVID patients, several options are proposed such as decreased cytokine levels [13], defects in T-cells [14], monocytes [15], and dendritic cells [16]. Since antibody deficiency is considered a hallmark of CVID and its consequent infections, B cells are one of the most important suspected causes of CVID [17]. Our previous research showed that there is a statically significant reduction in the percentage of end-stage B cells in CVID such as marginal zone-like, switched memory, IgM-only memory, total memory, and plasmablast B cells [18]. Even, scientists have tried to unravel clinical and immunological heterogeneity of CVID using classifications based on the variation of subsets of B cells [19-21].

The above-mentioned probable causes of CVID make the adaptive immune system the main area for research on identifying the cause of CVID. However, the role of recently found mutations of pattern recognition receptors (PRRs) in CVID suggests a potential role for these innate receptors in pathogenesis of CVID [22-24]. Toll-like receptors (TLRs) are well-known members of PPRs which are expressed by immune and nonimmune cells, which are able to identify pathogen-associated molecular pattern (PAMPs) or host-derived damage-associated molecular patterns (DAMPs) [25]. There is a set of data that identify defective signaling of TLRs in B cells in CVID, which leads to weaknesses in class-switch recombination, plasma cell differentiation, IgG or IgA production, response to polysaccharide antigens, cytokine production, and low memory B cell count [26].

In this study, we aimed to investigate the profile of two pro-inflammatory cytokines, IL-1β and IL-6, as the final products of TLRs signaling cascade in CVID patients and assess the influence of triggering TLR2 and TLR4 on cytokines production. In addition, we aimed to find the association between the cytokines levels with the frequency of B cell subtypes and patients' response to Pneumovax-23 vaccination in CVID patients.

MATERIAL AND METHODS

Study population

Sixteen CVID patients who referred to clinic of immunodeficiency at the Children's Medical Center (CMC), Tehran, Iran were entered into this study if they met inclusion criteria of: definitive diagnosis of CVID as defined by International Union of Immunological Societies [27], the age of $\geq$18 years old, and regular obtaining of intravenous immunoglobulin (IVIG). The exclusion criteria for the patients were having IVIG treatment for less than 3 weeks. For conducting this individual matching case-control study, 16 healthy controls with same age and sex were recruited to the patients. This study gained approval from the ethics committee of Tehran University of Medical Sciences and all the participants filled written informed consent forms before sampling.

Isolation of peripheral blood mononuclear cells (PBMCs)

The blood samples were obtained quickly before IVIG infusion using ethylene tetra acetic acid (EDTA) containing tubes. The density gradient centrifugation method was used for obtaining PBMCs from blood samples of patients and controls using Ficol-paque (Biosera, France). The cells were washed once by phosphate buffered saline (PBS) removing residual of ficol.

Cell culture and treatments

The viability of PBMCs was detected by trypan blue staining. Cells were suspended in complete culture media consisting of RPMI 1640 improved by10% of heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM of L-glutamine. For cell culture, 1×10^6 cells were divided into 24 wells of cell culture plate and stimulated for 18 hours with/without 1 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, USA) as an agonist of TLR4, 10 μg/mL lipoteichoic acid (LTA) (Sigma-Aldrich, USA) as an agonist of TLR2, and 25 μg/mL oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphoryl-choline (OxPAPC) (Invivogen, USA) as an inhibitor of both TLR2 and TLR4.

Cytokine assay

Cell culture supernatants were collected after 18 hours treatments and stored at -70 °C for the measurement of cytokines. The concentrations of IL-1β and IL-6 in cell cultures were measured by the ELISA kit (ebioscience, USA) according to the protocol provided by the manufacturer. The sensitivity of the ELISA kit was 2 *pg/ml* for both the cytokines.

Subtypes of B cells and response to Pneumovax-23 vaccination in CVID patients

Data of CVID patients' response to vaccination by Pneumovax-23 as well as the frequency of B cell subpopulations including naive B cell, transitional B cell, marginal zone-like B cell, total memory B cell, switched memory B cell, IgM-only memory B cells, plasmablasts, and subset of $CD21^{low}$ expressing B cell were retrieved from our previous research in Research Center for Immunodeficiencies [28].

Statistical analysis

Data were analyzed with SPSS software version 16. Data are shown as mean $\pm$ standard error (SD). Normal distribution was analyzed by the Shapiro-Wilk and Kolmogorov-Smirnov tests. The statistical differences were determined by using the Independent-Samples T Test and Paired-Samples T Test in the parametric analysis and the Mann-Whitney and Wilcoxon tests in the nonparametric analysis. Also the Pearson correlation analysis was used to find correlation between the number of B cell subtypes and

cytokines. Differences were considered statistically significant at p values of ≤ 0.05.

RESULTS

Sixteen adult CVID patients consisting of 10 (62.5%) male and 6 (37.5%) female with mean age of 26.43 $\pm$ 9.02 years old were entered into this study. The demographic and immunological data of the study population are demonstrated in *table 1*.

Cytokine production

The production of IL-6 by PBMCs of CVID patients and healthy controls

Cytokine assay for IL-6 indicates that PBMCs of CVID patients at the baseline level produced significantly lower amounts of IL-6 compared to healthy controls (2820.86 $\pm$ 825.88 ρg *vs.* 9760 $\pm$ 2389.47 ρg, p = 0.015) (*figure 1*).

The production of IL-6 by ligand-stimulated PBMCs of CVID patients

Interestingly, LTA stimulation of CVID PBMCs elevated the secretion of IL-6 to 14037.00 $\pm$ 3486.88 ρg compared to its baseline levels 2820.86 $\pm$ 825.88 ρg (p = 0.002) (*figure 1*).

Also, LPS stimulation of CVID PBMCs significantly increased the secretion of IL-6 to 16219.00 $\pm$ 3799.22 compared to baseline level of IL-6 production by CVID patients 2828.60 $\pm$ 825.88 (p = 0001) (*figure 1*).

The production of IL-1β by PBMCs of CVID patients and controls

With no treatments, PBMCs of CVID patients secreted significantly lower IL-1β compared with healthy individuals (163.21 $\pm$ 39.76 ρg IL-1β vs. 791.99 $\pm$ 234.26 ρg, p = 0.019) (*figure 2*).

The production of IL-1β by ligand-stimulated PBMCs of CVID patients

LTA stimulation of CVID patients led to a significant elevation of IL-1β compared to its secretion by unstimulated PBMCs of CVID patients (344.58 $\pm$80.47 ρg vs. 163.21 $\pm$ 39.76, ρg p = 0.008) (*figure 2*).

LPS triggering of PBMCs of CVID patients causes production of significantly higher amounts of IL-1β compared to baseline production of IL-1β by untreated PBMCs of CVID patients (1050.50 $\pm$161.10 ρg vs. 163.21 $\pm$ 39. 76, ρg p < 0.0001) (*figure 2*).

Table 1
Description of the CVID patients in the study.

Subjects		**Results**
Number of patients		16
Sex (male/female)		10/6
Age (mean $\pm$ SD)		26.43 $\pm$ 9.02
Age of onset		14.56 $\pm$ 9.24
Diagnosis delay		5.09 $\pm$ 3.83
Consanguinity	Non related	31.2%
	Related	18.8%
	First cousins	50.0%
Autoimmunity	ITP	12.5%
	AE	12.5%
	AIHA	31.2%
	IBD	6.2%
	RA	12.5%
Family history	Family history of recurrent infection	23.1%
	Family history of death with unknown cause	53.8%
	Family history of PID	44.4%
	Family history of cancer	15.4%
	Family history of asthma and allergy	8.3%
IgG (mg/dl)		234.13 $\pm$ 222.73
IgA (mg/dl)		25.35 $\pm$ 40.75
IgM (mg/dl)		36.11 $\pm$ 45.40
$CD3^+$ (%)		79.09 $\pm$ 9.75
$CD4^+$ (%)		32.97 $\pm$ 13.80
$CD8^+$ (%)		56.42 $\pm$ 56.42
$CD19^+$ (%)		9.03 $\pm$ 6.66

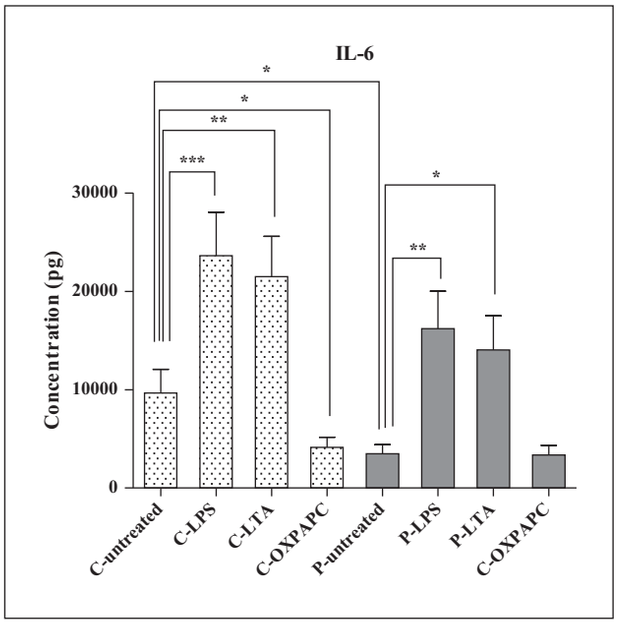

Figure 1
Secretion of IL-6 by PBMCs of CVID patients and healthy controls according to different treatments.
Results showed significant reduction in baseline production of IL-6 in CVID patients compared with healthy individuals. Although before and after analysis showed that ligand triggering of PBMCs led to significant elevated secretion of IL-6 compared with the baseline levels in both CVID and control groups, but we did not find any significant change in C-LPS group compared with P-LPS group as well as C-LTA and C-LPS groups.
Abbreviations: LPS: lipopolysaccharide; LTA: lipoteichoic acid. C-untreated indicates untreated PBMCs of controls; C-LPS: LPS-stimulated PBMCs of controls; C-LTA: LTA-stimulated PBMCs of controls; C-OX: OXPAPC-stimulated PBMCs of controls; P-untreated: untreated PBMCs of CVID patients; P-LPS: LPS-stimulated PBMCs of CVID patients; P-LTA: LTA-stimulated PBMCs of CVID patients; P-OX: OXPAPC-stimulated PBMCs of CVID patients.
*** P≤0.0001, ** p≤0.001, *p≤0.05

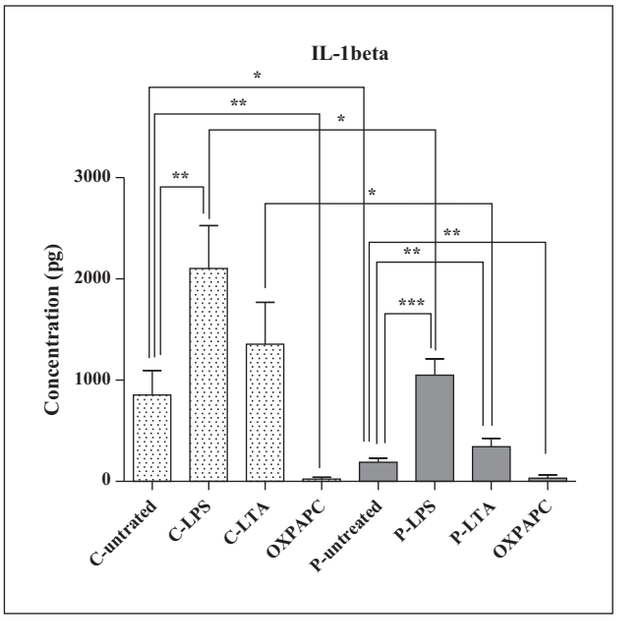

Figure 2
Secretion of IL-1β by PBMCs of CVID patients and healthy controls according to different treatments.
Results showed significant decrease in baseline production of IL-1β in CVID patients compared with healthy individuals similar to LPS- and LTA-stimulated PBMCs of CVID patients that showed significant reduction compared with healthy controls. The before and after analysis showed that ligand engagement of TLR2 and TLR4 in both controls and CVID PBMCs significantly promote secretion of IL-1β compared with untreated PBMCs. Abbreviations: LPS, lipopolysaccharide; LTA, lipoteichoic acid. C-untreated indicates untreated PBMCs of controls; C-LPS, LPS-stimulated PBMCs of controls; C-LTA, LTA-stimulated PBMCs of controls; C-OX, OXPAPC-stimulated PBMCs of controls; P-untreated, untreated PBMCs of CVID patients; P-LPS, LPS-stimulated PBMCs of CVID patients; P-LTA, LTA-stimulated PBMCs of CVID patients; P-OX, OXPAPC-stimulated PBMCs of CVID patients.
** p≤0.001, *p≤0.05

The correlation between the concentration of cytokines and B cell subtypes in CVID patients

Pearson correlation analysis showed a significant correlation between the number of switched memory B cells and the baseline IL-6 secretion by PBMCs of CVID patients (r = 0.52, p = 0.05) (*figure 3*).

Pearson correlation analysis showed a significant correlation between the number of $CD21^{low}$ expressing B cells and the concentration of IL-6 in both LPS- and LTA-stimulated PBMCs of CVID patients (r = 0.55, p = 0.042) and (r = 0.51, p = 0.06) respectively (*figure 4*).

The association between the response to Pneumovax-23 vaccination and the concentration of cytokines

We defined that hypo responder CVID patients secreted significantly reduced amounts of IL-6 compared with normal responders. The mean concentration of IL-6 in the normal vaccine responder group of CVID patients was 6862.7 ± 2881.2 ρg, while hypo responder patients secreted only 1776.7 ± 31.86.72 ρg (p = 0.05). Moreover, normal responder CVID patients produced 100.30 ± 3.63 ρg and hypo-responders secreted 212.98 ± 167.89 ρg of IL1beta (p > 0.05) (*figure 5*).

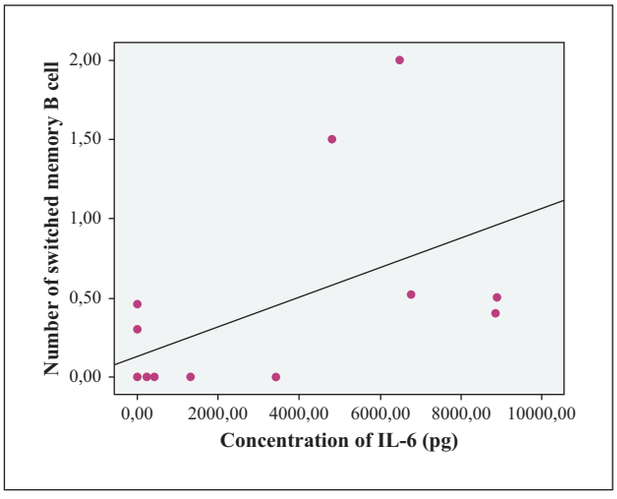

Figure 3
The correlation between the number of switched memory B-cells and concentration of IL-6 in nonstimulated PBMCs of CVID patients.
The graph shows a direct and significant correlation between the subset of switched memory B cells and IL-6 production by PBMCs of CVID patients at baseline level (r = 0.52, p = 0.05).

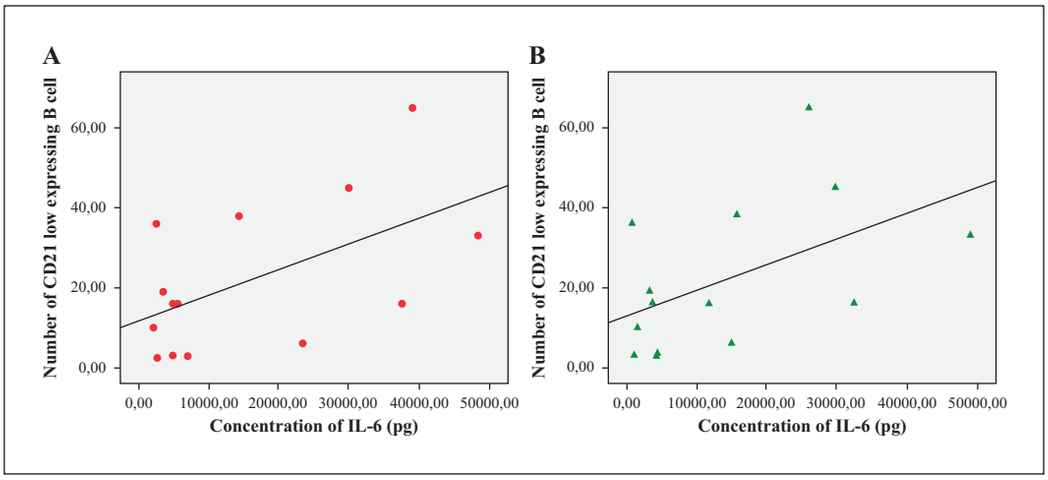

Figure 4

The correlation between the number of $CD21^{low}$ expressing B cells and concentration of IL-6 in ligand-stimulated PBMCs of CVID patients. A- The concentration of IL-6 secreted by LPS-stimulated PBMCs showed a direct and significant correlation with the number of $CD21^{low}$ presenting B cells ($r = 0.55$, $p = 0.042$). B- The concentration of IL-6 produced by LTA-triggered PBMCs exhibited a direct and significant correlation with the number of $CD21^{low}$ presenting subsets of B cells ($r = 0.51$, $p = 0.06$).

DISCUSSION

According to leading role of cytokines in the orchestration of antibody responses, they have been frequently studied in CVID. Until now, different defects of cytokine pathways have been proposed to be connected with CVID manifestations [4, 5, 29]. In this study, we showed a significant reduction in quantities of both IL-6 and IL-1β in the supernatant of PBMCs of CVID patients, but surprisingly we demonstrated that TLR2 or TLR4 of CVID patients respond properly to their cognate ligands and produce higher amounts of cytokines compared to their baseline levels.

Interleukin 1β is produced by macrophages and dendritic cells (DCs). This pro-inflammatory cytokine is a member of the IL-1 family and plays a crucial role in the inflammatory and immune regulation [41]. Formerly, a set of studies have assessed this cytokine in CVID patients and suggested heterogeneity in their results. Even though, there are divers reports about IL-1β level in CVID [4, 29] but similar to our findings Lollo et al., Trujillo et al., Heyden et al., and Junker et al. in different studies showed that LPS stimulation of CVID patients leads to the restoration of IL-1β secretion to normal or even increased levels [29-32].

Other pro-inflammatory cytokine, IL-6, is produced by different cell types such as DCs, Th2 cells, and B cells [71]. It has been mentioned that IL-6 in immunodeficiency cases acts as a plasma cell growth factor [74]. Among studies have explored IL-6 in CVID, results are different from increased production [55, 79] and normal range [46] to decreased production after cells stimulation [80]. In accordance with our findings Junker et al., Heyden et al., Pandolfi et al., Pons et al.,

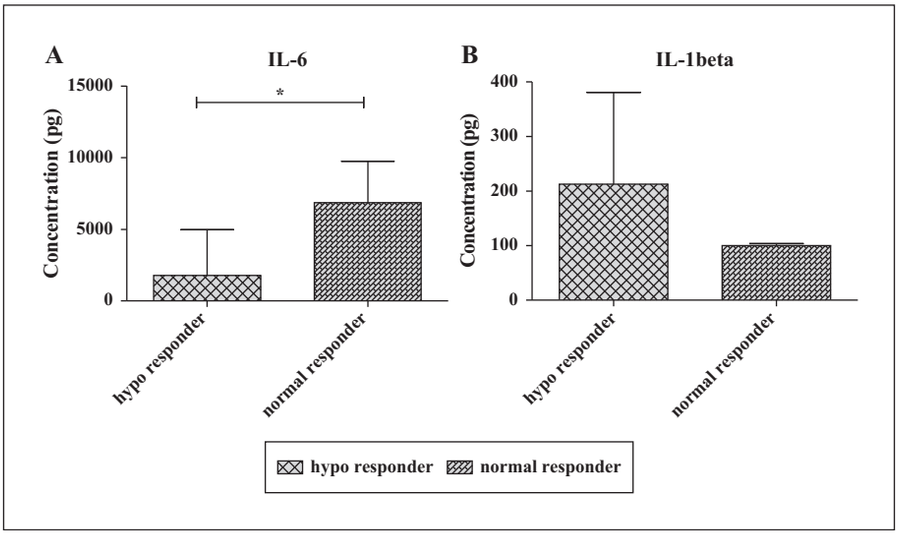

Figure 5

The association between response to Pneumovax-23 vaccination and the concentration of cytokines in CVID patients.

A. The PBMCs of hypo responder patients secreted significantly lower quantities of IL-6 compared with healthy controls ($p = 0.05$).

B. The PBMCs of hypo responder patients secreted higher amounts of IL-1β compared with healthy controls but this alteration was not significant ($p > 0.05$)

Trujillo et al., and Lollo et al. showed that CVID peripheral blood cells can produce elevated levels of IL-6 after stimulation [29–32][29-32].

However, it should be noted that the heterogeneous nature of CVID, using different laboratory methods, presence or absence of ligand stimulation, type of stimulations, and different cell types make the conclusion difficult about the cytokines situation in CVID [47].

Owing to the impaired response of CVID patients to the Pneumovax-23 vaccine and the role of TLR2 and IL-6 in protection against pneumococcal disease, we compared the levels of IL-6 in different groups of vaccine response among CVID patients. Our findings indicate a significant association between diminished IL-6 secretion and poor response to Pneumovax-23 vaccine in CVID patients which may present the role of IL-6 deficiency in poor response to Pneumovax-23 vaccination. Our results are in accordance with Hong et al. They test IL-6 quantities after TLR2 stimulation with Pneumovax-23; their results showed a significant reduction of IL-6 in patients with CVID as compared with their controls. They concluded that deficient IL-6 may be involved in amplified susceptibility to *Streptococcus pneumoniae* in CVID patients [33].

We found a significant correlation between the concentration of IL-6 and the number of switched memory B cells in CVID patients. It was confirmed that secretion of IL-6 by T helper cells leads to the proliferation and differentiation of the B cells into antibody-secreting plasma cells [34].

Also, our results revealed a significant correlation between the concentration of IL-6 and the number of switched memory and $CD21^{low}$ expressing B cells in ligand-stimulated PBMCs of CVID patients. It is well defined that $CD21^{low}$ B cells are increased significantly in the CVID, but they are a controversial population that defined differently as an autoreactive unresponsive clone [35] or human innate-like B cell [36], or tissue-like memory B cells [37].

For a more clarification of cytokine imbalance in CVID and its association with B cells and response to Pneumovax-23, we suggest considering immunological phenotypes and clinical manifestations of patients for future studies which enable researchers to dissect the intricacy of various immunological abnormalities in CVID.

CONCLUSION

However, we found lower secretion amounts of IL-1β and IL-6 in CVID patients but a concomitant responsiveness of TLR2 and TLR4 to stimulation with their cognate ligands to secret higher amounts of cytokines. This characteristic of CVID TLRs leads to the improvement of the cytokine secretion compared to its baseline level. These findings showing the association between IL-6 levels and the frequency of switched memory and $CD21^{low}$ expressing B cells as well as poor response to Pneumovax-23 should be evaluated precisely by higher sample size in future works.

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