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The Profile of Toll-like Receptor 2 (TLR2), TLR4 and Their Cytosolic Downstream Signaling Pathway in Common Variable Immunodeficiency (CVID) Patients

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ABSTRACT

Common variable immunodeficiency (CVID) is the most common clinical primary antibody deficiency, characterized by increased susceptibility to recurrent bacterial infections. Since Toll-like receptors (TLRs) play an important role in the maturation and differentiation of B-cells, TLRs' defect can be involved in the pathogenesis of CVID. Therefore, we evaluated the expression of TLR2 and TLR4 and their signaling pathway; also their association with autoimmunity, B-cell subtypes and response to pneumovax-23 were assessed in CVID patients.

Sixteen CVID patients were enrolled in the study. Flow cytometry was used for assessing the protein expression of TLR2 and TLR4, and real-time PCR was used for gene expression of myeloid differentiation primary response 88 (MyD88) and toll interacting protein (Tollip).

We found a higher protein expression of TLR2 in CVID patients which was associated with lower number of end stage B-cells and hyporesponse to pneumovax-23 vaccination. We showed a lower mRNA expression of MyD88 and an almost equal Tollip mRNA expression in CVID patients compared with controls. There was a profound association between MyD88 gene expression and autoimmunity in CVID patients.

According to the presence of the lower number of end stage B-cells and poor vaccine response in CVID patients and their correlation with the higher expression of TLR2, we hypothesized that there is a functional defect in this receptor and/or its downstream in the peripheral blood mononuclear cells (PBMCs) of CVID patients.

Keywords: Common variable immunodeficiency (CVID); Myeloid differentiation primary response 88 (MyD88); Toll-like receptor 2 (TLR2); Toll-like receptor 4 (TLR4); Toll interacting protein (Tollip)

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INTRODUCTION

Common Variable Immunodeficiency (CVID) is the most common clinical primary antibody deficiency characterized by hypogammaglobulinemia and poor production of specific antibodies. CVID is clinically associated with recurrent bacterial infections, inflammatory and autoimmune disorders, malignancies, gastrointestinal and allergic diseases.[1,2] In the recent years, multiple immunological defects in the innate and adaptive immune systems have been detected in CVID.[3-6] A small proportion of CVID patients, less than 15% of the cases, present several mutations in the genes involved in B-cell activation, differentiation and signaling.[7-9]

Since CVID is considered as hypogammaglobulinemia, most studies focused on the impairment of B cells; however, abnormalities in T-cells, dendritic Cells (DCs), monocytes, Natural killer cells (NK) and innate lymphoid cells (ILC) have also been reported.[10-16] In spite of the results obtained from the studies in the recent years, the basic molecular defect of this disease is yet unknown in most patients.

Toll-Like Receptors (TLRs) are evolutionary reserved receptors that sense a wide range of pathogen-associated molecular patterns (PAMPs)[17,18] which connect innate and adaptive immunity.[19] Since TLRs play an important role in proliferation and maturation of B-cells, a defect in TLRs could be involved in the pathogenesis of CVID.[20]

Cunningham-Rundles et al. reported an impairment of TLRs in CVID for the first time and showed a disability of TLR9 in activation of B-cells and cytokine production.[21] Later, further studies showed defects of some TLRs in peripheral blood mononuclear cells (PBMCs), B-cells, and dendritic cells of CVID patients.[22-26]

Activation of TLRs leads to inflammatory responses and extension of this condition develops to the autoimmunitycomplications.[27] Since TLR2 and TLR4 have an important role in the development of autoimmunity,[28] and some CVID groups are prone to progress to autoimmune disorders;[2,29] thus it is possible that the alterations of TLR2 and TLR4 predispose CVID patients to autoimmunity. Furthermore, TLR2 has an important role in the recognition of *Streptococcus pneumoniae* and CVID patients show higher susceptibility to *Streptococcus pneumoniae* infections;[13,30] hence abnormalities in TLR2 might be involved in the development of CVID. According to above-mentioned findings, we hypothesized that some alterations in TLR2 and TLR4 can be connected to CVID and the susceptibility to autoimmunity in these patients.

Until now, endosomal TLRs have been mainly studied in CVID and we had no comprehensive data about TLR2 and TLR4 in these patients. Therefore, we aimed to describe the profile of TLR2 and TLR4 via evaluating the surface protein expression of these receptors and mRNA expression of two important signaling molecules: myeloid differentiation primary response 88 (MyD88) as the most important adaptor molecule of TLR signaling pathway and toll interacting protein (Tollip) as a negative regulator of this pathway in the PBMCs of CVID patients with/without autoimmunity presentations. Furthermore, we investigated the association of our findings with subsets of B-cells and patients' response to Pneumovax-23.

PATIENTS AND METHODS

Study Population

Sixteen CVID patients and 16 healthy controls (age and sex-matched) who were referred to our clinic of immunodeficiency at the Children's Medical Center affiliated to Tehran University of Medical Sciences were enrolled in the study.

The diagnosis of CVID in these patients was established according to the diagnostic criteria of PAGID (the Pan-American Group for Immunodeficiency) and ESID (the European Society for Immunodeficiencies), including: 1- increased susceptibility to infection, 2- reduction of IgG and a marked decrease of IgA with/without low IgM levels by 2 standard deviation (SD) from normal range, and 3- absent isohemagglutinins and/or a reduced specific response to vaccines. The exclusion criteria were 1- the age less than 4 years and 2- evidence of profound T-cell deficiency.[31] This study was approved by the Ethics Committee of Tehran University of Medical Sciences (No.93-03-154-27044-309056) and written informed consents were also obtained from all participants.

PBMC Isolation, Cell Culture and Treatments

Peripheral blood mononuclear cells (PBMCs) were

isolated from whole blood using Ficoll-Hypac (BioSera, UK) centrifugation gradient. PBMCs were seeded at a density of 1×10^6 cells per well in 24-well plates in RPMI 1640 improved by 10% of heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 2 mM of L-glutamine. PBMCs were treated within 24 hours with/without 10 µg/mL lipoteichoic acid (LTA) (Sigma-Aldrich, USA) and 1 µg/mL lipopolysaccharide (LPS) (Invivogen, USA) as agonists of TLR2 and TLR4, respectively.

Flow Cytometric Analysis of TLR2 and TLR4

The isolated PBMCs (5×10^5) were stained for cell membrane proteins, TLR2 and TLR4, with 5 µg fluorescein isothiocyanate (FITC) labeled anti-human CD282 (TLR2) and phycoerythrin (PE) anti-human CD284 (TLR4) monoclonal antibodies (Biolegend, USA) according to the company protocol. FITC Mouse IgG2a and PE Mouse IgG2a were used as isotype control antibodies. Flow cytometry was done using BD flow cytometer system (BD, USA) and the data were analyzed with flowjo software version 7.6.1.

RNA Extraction, cDNA Synthesis and Real-Time PCR

Total RNA was isolated manually from treated and non-treated PBMCs by Qiazole (Qiagen, USA). RNA was reverse transcribed to cDNA using reverse transcription kit (Takara, Japan) according to manufacturer's instructions. Quantitative Real-time PCR was done by SYBR Green PCR Master Mix (Takara, Japan). Expression of MyD88 and Tollip genes as well as β-actin gene was assessed using primer pairs as follows: β-actin: F; 5'-GTGGGGCGCCCCAGGCACCA-3', R; 3'-CTCCTTAATGTCACGCACGATTTC-5', MyD88: F; 5'-CGCCGCCTGTCTCTGTTC-3', R; 3'-GGTCCGCTTGTGTCTCCAGT-5', and Tollip: F; 5'-GCAAGGTGGAGGACAAGTG-3', R; 3'-GTAGGACATGACGAGGTTGATC-5'. The resulting transcripts were quantified using the ABI Step One Plus Real-time PCR system (ABI System, USA) according to the manufacturer's instructions. Amplification conditions were: 95°C for 15 s followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72° C for 30 s. β-actin housekeeping gene was used for normalization of amplification. The relative frequency of MyD88 and Tollip genes were calculated by $2^{-\Delta\Delta Ct}$ method.[32] Amplified products were analyzed by agarose gel electrophoresis for reassuring the size of PCR products.

Subtypes of B-Cells and Response to Pneumovax-23 Vaccination in CVID Patients

We retrieved our patients' flow cytometric data of numbers of B-cell subpopulations including naïve B-cells, transitional B-cells, marginal zone-like B-cells, total memory B-cells, switched memory B-cells, IgM-only memory B-cells, plasma blasts and a subset of $CD21^{low}$ expressing B-cells as well as information about antibody production before and after vaccination by Pneumovax-23 from our previous research project in the Research Center for Immunodeficiencies published in 2016.[33]

Statistical Analysis

The results were analyzed by SPSS software version 16 (IBM, USA). Kolmogorov–Smirnov and Shapiro-Wilk tests were used to determine whether our data were normally distributed. Independent samples t-test and paired samples t-test were used for detecting statistical differences in parametric analysis and Mann-Whitney and Wilcoxon tests were used for non-parametric analysis. Pearson Correlation test was considered for bivariate correlation of data. p values ≤0.05 were considered statistically significant. Prism software version 5 (Graph pad, USA) was used for drawing graphs.

RESULTS

Characteristics and Clinical Phenotypes of CVID Patients

Ten male and six female CVID patients with the age range of 18-40 years old were enrolled in this study. Eleven (68.75%) patients were born from a consanguineous marriage. The most common clinical presentations in the patients were enteropathy (12 patients, 75.00%), followed by sinusitis (11 patients, 56.25%), autoimmunity (9 patients, 56.25%), pneumonia (9 patients, 56.25%) and otitis media and bronchiectasis (3 patients, 37.5%). Demographic, clinical and immunologic profiles of the patients are presented in Table 1.

The Protein Expression of TLR2 and TLR4 at Baseline Levels and after Stimulation

Our findings showed that the PBMCs of CVID

Table 1. Demographic, immunologic, and clinical characteristics of patients with common variable immunodeficiency participated in a study of Profile of Toll-like Receptor (TLR) 2, TLR 4 (TLR2), and their cytosolic downstream signaling pathway

No	Sex	Age (year)	Age at onset (year)	Consanguinity	Clinical presentation	At the time of diagnosis							
						IgG[a]	IgA[a]	IgM[a]	CD3[b] (%)	CD4[b] (%)	CD8[b] (%)	CD19[b] (%)	WBC[c]
1	M	38	1	Unrelated	Sin, Ent, Lymp	520	75	180	83.0	31.0	58.0	9.0	11450
2	M	26	9	First cousin	Sin, Pne, Bron, Lymp	0	6	17	89.4	32.3	44.16	3.78	8900
3	M	35	17	First cousin	RA, Sin, Pne, Bron, Lymp, Spl	90	9	9	89.0	15.0	72.0	1.0	3770
4	M	21	4	First cousin	AE, Sin, Pne, Ent, Bron	360	0	44	74.0	18.0	55.0	6.0	11300
5	M	19	2	First cousin	ITP, AIHA, Sin, Otit, Ent, Hep, Spl	140	13	19	77.0	31.0	252	12.0	11000
6	F	37	28	Related	IBD, Ent	331	19	39	88.2	63.5	29.2	2.1	14880
7	M	26	1	Related	AIHA, Pne, Otit, Ent, Bron, Spl	480	36	36	63.0	21.0	18.0	2.16	6700
8	M	40	36	Unrelated	Sin, Pne, En	190	14	25	86.9	37.6	49.6	9.4	7400
9	F	40	6	First cousin	Sin, Otit, Ent, Alle	160	150	68	79.25	40.42	35.23	11.2	6800
10	M	18	12	Unrelated	Pne	290	11	20	79.6	29.4	46.7	15.8	11000
11	F	22	5	First cousin	AE, AIHA, Sin, Pne, Otit, Ent, Bron	295	5	69	83.89	21.89	32.15	7.13	11600
12	M	18	7	Unrelated	Sin, Ent	15	4	4	55.0	49.0	30.0	6.0	4600
13	F	18	4	First cousin	AIHA, Ent	126	13	0.7	77.6	54.0	18.0	15.2	7240
14	F	20	4	First cousin	ITP, AIHA, Ent	10.8	60	2.5	77.3	32.0	26.0	10.0	5220
15	M	22	11	Unrelated	JRA, Sin, Pne, Otit	45	0	11	76.6	23.4	40.3	26.74	13200
16	F	30	5	First cousin	Sin, Pne, Otit, Ent	0	0	0	87.0	27.0	66.0	8.0	7000

ITP, immune thrombocytopenic purpura; AE, adult autoimmune enteropathy; AIHA, autoimmune hemolytic anemia; IBD, inflammatory bowel disease; RA, rheumatoid arthritis; JRA, juvenile rheumatoid arthritis; Sin, sinusitis; Pneu, pneumonia; Otit, otitis media; Bron, bronchiectasis; Lymp, lymphadenopathy; Ent, enteropathy; Hep, hepatomegaly; Spl, splenomegaly; Alle, allergy.

a, Normal serum Ig ranges for adults: IgG , 650-1700 mg/dL, IgA 70-350 mg/dL, IgM 60-250 mg/dl; all levels before IVIG replacement.

b, Normal CD marker ranges for adults: CD3 (67.66 ±7.76), CD4 (39.22±6.7), CD8 (25.42±5.4) and CD19 (14.41±5.09) (34).

c, Normal WBC range for adults: 4.4-11×10^3 /µL.

patients are tended to a significantly higher expression of TLR2 at baseline level compared with healthy controls (17.93±9.70% *vs.* 12.76±5.20%, p=0.05).

In spite of the difference in TLR2 expression , no significant difference was observed comparing the baseline levels of TLR4 in the PBMCs of CVID patients with those of healthy controls (14.79±4.29% *vs*. 14.11±7.65%, p=0.34). Regarding the stimulated

cells, LTA-stimulated PBMCs of patients demonstrated a significantly higher expression of TLR2 compared to healthy individuals (15.53±6.24% *vs.*9.25±4.86%, *p*=0.013). Moreover, LPS-stimulated PBMCs of patients indicated a significantly increased expression of TLR4 compared with healthy controls (17.85±9.88% *vs.* 10.49±5.20%, *p*=0.027) (table 1).

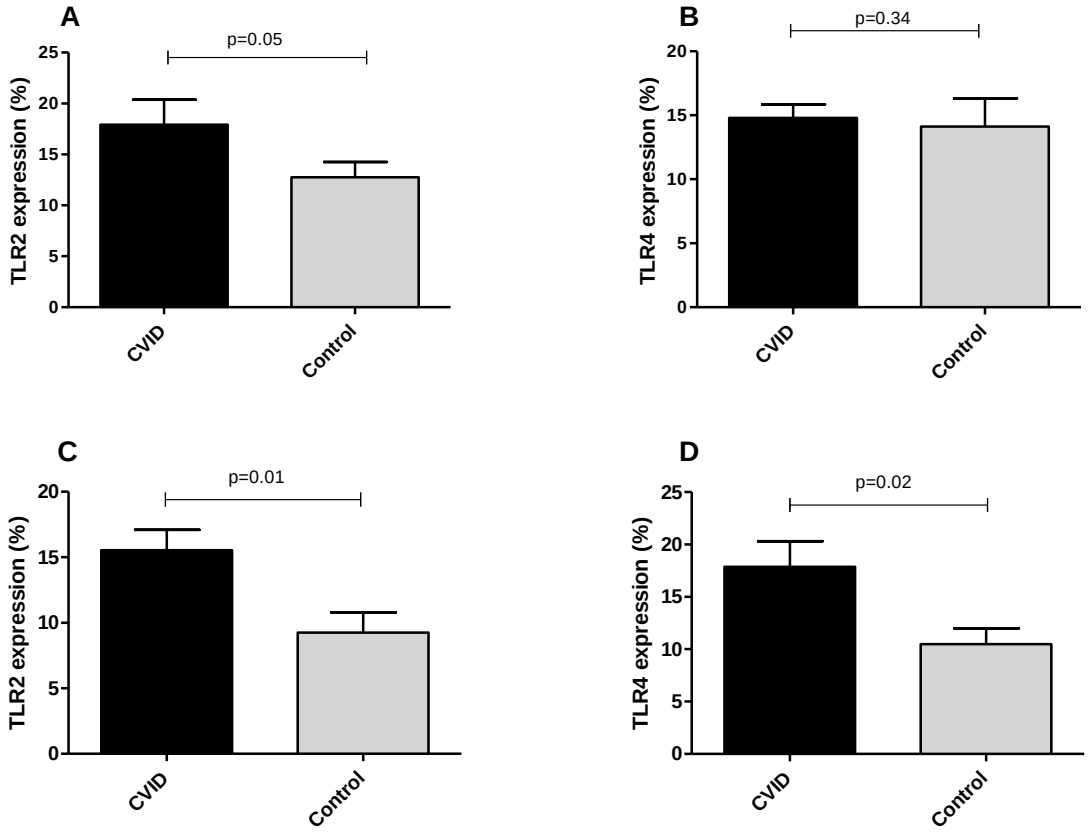

Figure 1. TLR2 and TLR4 expression in PBMCs of CVID patients and healthy controls. The expression of TLR2 and TLR4 were measured by flow cytometry in the PBMCs of CVID patients before and after stimulation with specific TLR ligands.
TLR2: toll-like receptor 2; PBMCs: peripheral blood mononuclear cells; CVID: common variable immunodeficiency; LPS: lipopolysaccharide; LTA: lipoteichoic acid
A-Columns representing TLR2 expression in PBMCs of CVID and controls at baseline level
B-Columns representing TLR4 expression in PBMCs of CVID and controls at baseline levelC-Columns representing TLR2 expression in PBMCs of CVID and controls after LTA treatment
D- Column bars representing TLR4 expression in PBMCs of CVID and controls after LPS treatment.

The Association of the Protein Expression of TLR2 and TLR4 in CVID Patients with Their Autoimmunity Presentation

Nine of our patients (56%) developed different kinds of autoimmunity which are presented in Table 1. To evaluate the effect of TLR2 and TLR4 expression in the development of autoimmunity in CVID patients, we categorized the patients according to autoimmunity into two groups: CVID patients with autoimmunity and CVID patients without autoimmunity.

Our results demonstrated that CVID patients with autoimmunity showed higher expressions of TLR2 (19.56±11.89% vs. 15.21±3.70%, *p*=0.404) and TLR4 (15.02±3.68% vs. 14.40±5.52%, *p*=0.787) at the baseline level in comparison to CVID patients without autoimmunity. Moreover, we tested the effect of ligand stimulation of these receptors in the two groups of CVID patients with and without autoimmunity. We

found that patients with autoimmunity showed a slightly higher expression of TLR2 after LTA stimulation (15.91±6.22% *vs.* 14.90±6.81%) and a higher expression of TLR4 after LPS stimulation compared to non-autoimmune CVID patients (18.97±11.71% *vs.* 15.99±6.29%); however, significant differences between TLR2 and TLR4 expressions of the stimulated PBMCs in autoimmune and non-autoimmune groups were not observed (p=0.766 and p=0.578, respectively) (Figure 2).

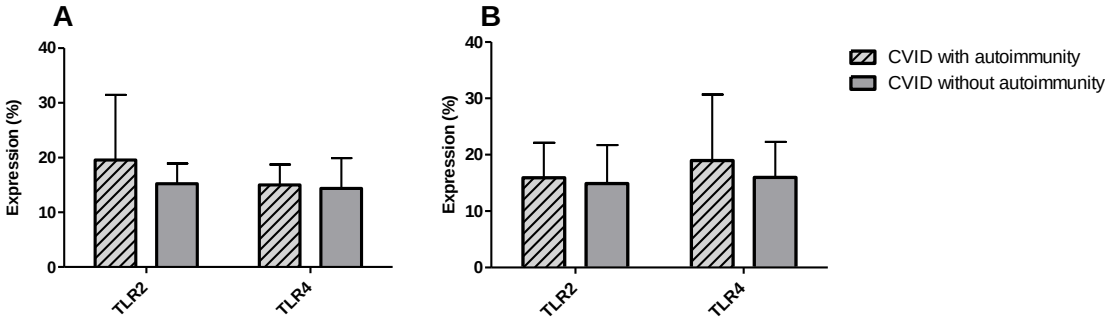

Figure2. The expression of TLR2 and TLR4 in PBMCs of CVID patients with/without autoimmunity. The expression of TLR2 and TLR4 were measured by flow cytometry in the PBMCs of autoimmune and non-autoimmune CVID patients.
TLR2: toll-like receptor 2; PBMCs: peripheral blood mononuclear cells; CVID; common variable immunodeficiency
A- Columns representing TLR2 expression in PBMCs of autoimmune and non-autoimmune CVID patients at the baseline level
B- Columns representing TLR4 expression in PBMCs of autoimmune and non-autoimmune CVID patients after TLR stimulation with specific ligands

The Association of the Protein Expression of TLR2 and TLR4 in CVID Patients with Their Response to the Vaccine

The CVID patients were classified into the normal- and hypo- responder groups based on their before and after responses to Pneumovax-23 vaccination. We found that at the baseline level, hypo-responder patients express significantly higher levels of TLR2 on their PBMCs compared with normal-responders patients (17.69±5.99% *vs*.13.35±0.35%, *p*=0.048), whereas this increase was not significant in TLR4 expression (14.84±4.69% vs.12.45±2.05%, *p*=0.507) (Figure 3). Also, there was no significant association between the protein expression of ligand-stimulated TLR2 and TLR4 and the patients' response to the vaccine (data are not shown).

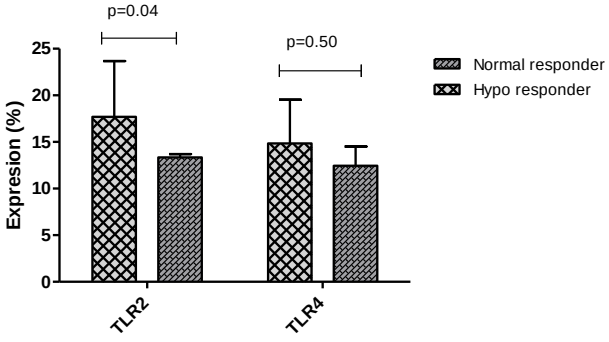

Figure 3. The expression of TLR2 and TLR4 in the PBMCs of CVID patients according to their response to Pneumovax-23.
TLR2: toll-like receptor 2; PBMCs: peripheral blood mononuclear cells; CVID; common variable immunodeficiency
The expression of TLR2 and TLR4 was measured by flow cytometry in the PBMCs of normal- and hypo- vaccine responders among CVID patients. Measured by flow cytometry in the CVID PBMCs of normal and hypo vaccine responder.

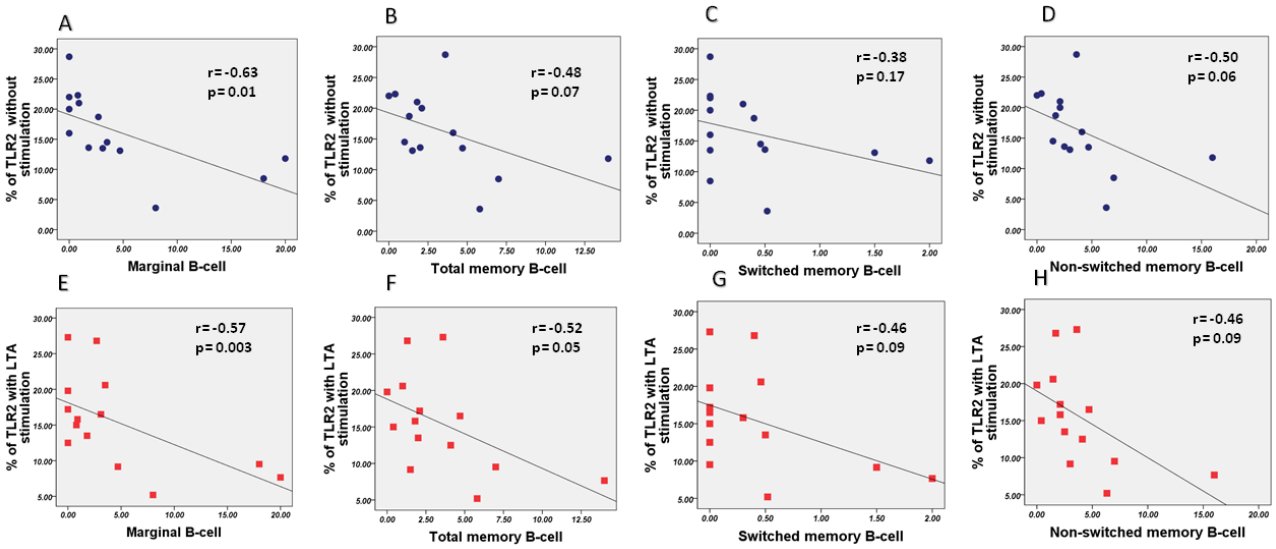

Figure 4. The correlation of B-cell subtypes with the expression of TLR2 in CVID patients before and after ligand stimulation.

TLR2: toll-like receptor 2; PBMCs: peripheral blood mononuclear cells; CVID: common variable immunodeficiency; LTA: lipoteichoic acid

The expression of TLR2 in the PBMCs of CVID patients was determined by flow cytometry before and after TLR2 specific ligand (LTA) stimulation.

The Correlation of the Protein Expression of TLR2 and TLR4 in CVID Patients with the Different Subtypes of B-Cells

We identified a significant correlation between the baseline expression of TLR2 and TLR4 with the number of the marginal zone-like B cells (r=-0.637, *p*=0.014 and r=-0.543, *p*=0.045, respectively). Also, ligand stimulation of TLR2 with LTA resulted in a strong correlation between TLR2 and marginal zone-like B-cells (r=-.579, *p*=0.003) as well as the total number of memory B-cells (r= -0.522, *p*=0.05); however, there was no significant correlation between the expression of and B-cell subsets (Figures 4 and 5). It should be noted that all B-cell subtypes showed a reveres correlation with TLR2 and TLR4 expression except $CD21^{low}$ expressing B-cells (data are not shown).

The mRNA Expression of MyD88 and Tollip in CVID Patients

To determine whether TLR2 and TLR4 downstream signaling are associated with CVID or not, we assessed the gene expression of MyD88, and found that relative quantification (RQ) of MyD88 mRNA in CVID patients was lower than in healthy controls (0.05±0.17 vs. 5.41±4.80, *p*=0.032). Even though we showed that LPS and LTA stimulation of TLR2 and TLR4 increased the gene expression of MyD88, this augmentation was less than healthy controls (8.32±2.17 vs. 11.04±4.84, *p*=0.823 and 0.17±0.34 vs. 1.47±1.58, *p*=0.418, respectively).We found a low difference in the gene expression of Tollip between patients and controls at baseline levels (5.65±3.13 vs. 5.69±4.80, *p*=0.994), but stimulation of TLR2 and TLR4 with LPS and LTA raised the expression of Tollip mRNA to higher levels compared with healthy controls (13.86±8.96 vs. 10.06±5.85, p=0.793 and 32.94±15.02 vs. 11.13±8.61, p=0.50, respectively) (Figure6).

The Association of mRNA Expression of MyD88 and Tollip in CVID Patients with Their Autoimmunity Presentation

Our results showed that in patients with autoimmunity, MyD88 mRNA was significantly elevated in comparison to patients without autoimmunity (0.08±0.006 *vs.* 0.01±0.006, *p*<001). Also, we showed that CVID patients with autoimmunity expressed higher levels of Tollip mRNA

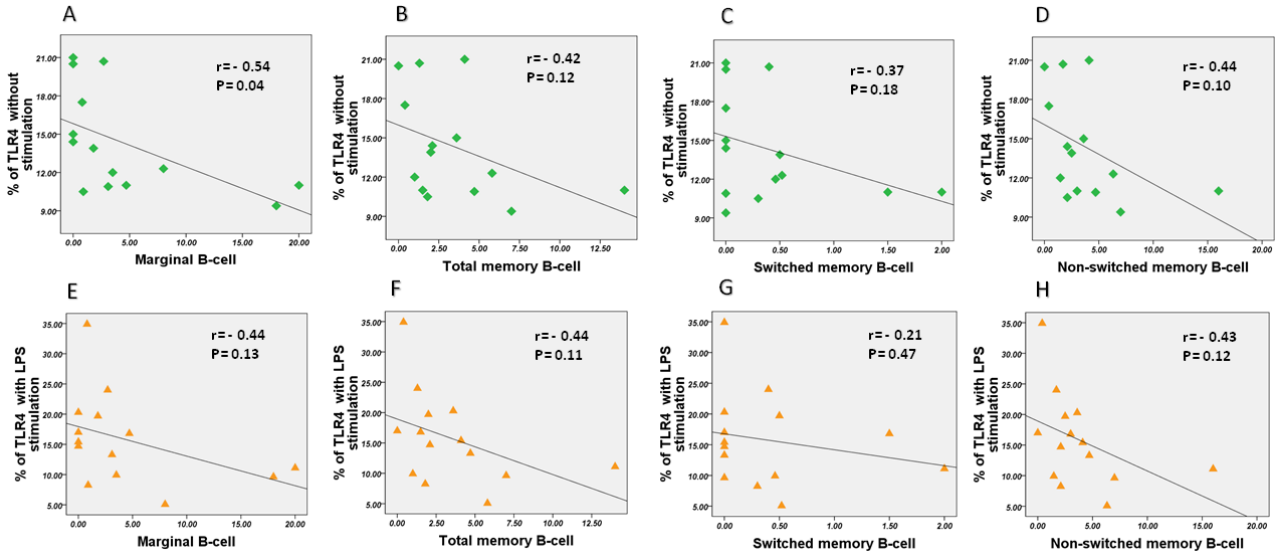

Figure 5. The correlation of B-cell subtypes with the expression of TLR4 in CVID patients before and after stimulation.

TLR4: toll-like receptor 4; PBMCs: peripheral blood mononuclear cells; CVID: common variable immunodeficiency; LPS: lipopolysaccharide

The expression of TLR4 in the PBMCs of CVID patients was determined by flow cytometry before and after TLR4 specific ligand (LPS) stimulation

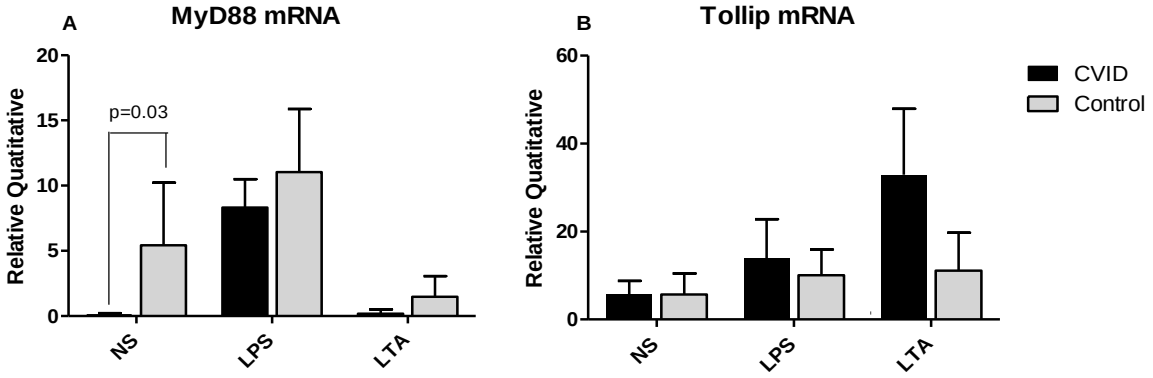

Figure 6. The gene expression of MyD88 and Tollip in the PBMCs of CVID patients and healthy controls based on different treatments.

MyD88: myeloid differentiation primary response 88; Tollip: toll interacting protein; PBMCs: peripheral blood mononuclear cells; CVID: common variable immunodeficiency; NS: non-stimulated; LPS: lipopolysaccharide; LTA: lipoteichoic acid

Real-time PCR was used to detect the gene expression of MyD88 and Tollip before and after specific ligands stimulation. A- Relative quantitative of MyD88 mRNA in the PBMCs of CVID patients and healthy controls before and after ligand stimulation. B- Relative quantitative of Tollip mRNA in the PBMCs of CVID patients and healthy controls before and after ligand stimulation.

in comparison to healthy controls; however, this increase was not statistically confirmed (7.93±5.17 *vs*.0.76±0.61, *p*=0.531) (Figure 7). Furthermore, LPS and LTA stimulation did not cause any significant association between MyD88 and Tollip mRNA and autoimmunity (data are not shown).

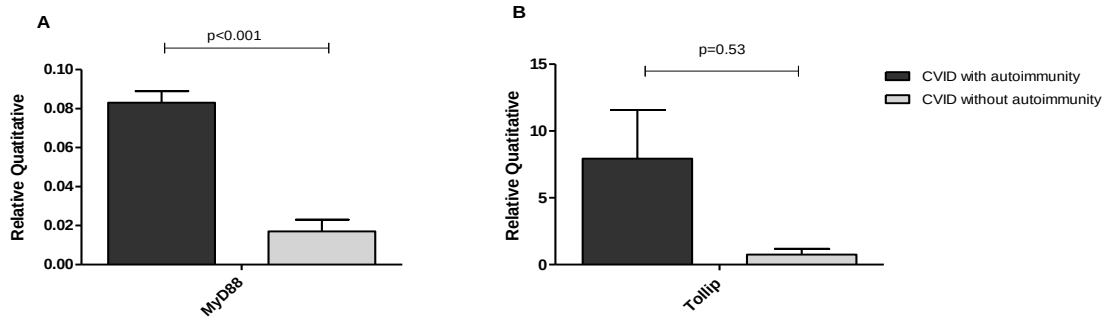

Figure7. The mRNA expression of MyD88 and Tollipin autoimmune and non-autoimmune CVID patients.
MyD88: myeloid differentiation primary response 88; Tollip: toll interacting protein; CVID: common variable immunodeficiency
Real-time PCR was used to detect the relative quantitative level of Tollip and MyD88 in autoimmune and non-autoimmune CVID patients.

DISCUSSION

In the present study, we evaluated whether an abnormality in the expression of TLR2 and TLR4 and their related downstream are associated with the pathogenesis of CVID. We found a significantly higher protein expression of TLR2 in the PBMCs of CVID and a slight increase in TLR4 expression as well as the higher responsiveness of these receptors to their ligands. On the other hand, we showed the in CVID patients compared with controls. Similarly, LPS and LTA ligation of TLR2 and TLR4 resulted in lower mRNA expression of MyD88. Decreased MyD88 expression recommends a defect in TLR2 and TLR4 signaling downstream since this molecule acts as an adaptor molecule in the TLRs' signaling cascade

It seems that increased TLR2 and TLR4 expression could be due to defective TLR signaling and the presence of compensatory mechanisms try to cover the functional defects of TLR2 and TLR4 by overexpression and hyper-responsiveness of these TLRs. Apart from MyD88, recently described adaptor molecule, Tolliplinks to TIR (TOLL/IL1 receptor) domain in cytoplasmic tails of TLRs and acts as a negative regulator for TLR2 and TLR4 by suppressing the IRAK activation.[35] Here, we showed that stimulation of TLR2 and TLR4 via their cognate ligands led to higher mRNA expression of Tollip in patients compared with healthy controls. Higher expression of this negative controller in response to ligand engagement could be due to the functional defects of TLRs. On the other hand, decreased Myd88 could be result of augmentation of this negative controller in response to ligand engagement in the patients; however, further studies are needed to confirm this association. Although MyD88 immunodeficiency has been recently described as a primary immunodeficiency (PID) due to innate immunity system.[36] Myd88 deficient patients suffer from invasive bacterial infections caused mainly by *S. pneumonia* and localized bacterial diseases caused mostly by *Staphylococcus aureus.*[37]

We showed that the lower expression of MyD88 in CVID patients may be linked to TLRs functional defects and the susceptibility of CVID patients to *S. pneumonia*. Our data about poor vaccine response and its association with the higher expression of TLR2 support the hypothesis of possible functional defects in TLR2. Previously, it was demonstrated that TLR2 has an important effect against *S. pneumonia* infections and the lack of TLR2 is associated with the severe clinical presentation of the disease in mice;[38] therefore, the absence or functional impairment of TLR2 could be expected in CVID as a disorder in which the patients are susceptible to *S. pneumonia*. Our findings of significantly higher TLR2 expression in poor vaccine responder patients reveal the ineffectiveness of TLR2 expressed in CVID patients. In agreement with our findings, Hong et al, showed that Pneumovax-23, which acts via TLR2, up-regulates TLR2 on the cell membranes of monocytes in CVID patients.[13]

TLRs in combination with B-cell receptor (BCR) signaling play important roles in B-cell differentiation and activation. Transitional B cells can differentiate into mature B cells, plasma cells and IgM memory B cells upon TLR activation. The proliferative role of

TLR2 accompanied by BCR stimulus have been confirmed in previous studies;[39-42] also it has been demonstrated that TLR4 have poor expression and function in B-cells.[40]

Interestingly, our results revealed that TLR2 and TLR4 expression in the PBMCs of patients are associated with the number of end-stage B-cell populations; this association was more profound in the case of TLR2. It is demonstrated by this study and other studies that CVID patients show a decrease interminal B-cell subsets,[33,43,44] suggesting that the over expression of impaired TLR2, which is associated with a lesser count of end stage B-cells, may be due to the abolished proliferation ability of TLR2.

The inflammation caused by endogenous ligands derived from damage associated molecular pattern (DAMPs), may result in sustained TLR activation and this condition could be involved in the progression of CVID to autoimmune disorders. It has been recently demonstrated that the higher expression of TLR2 and TLR4 or the higher responsiveness to their ligands are established in the development of autoimmune diseases such as rheumatoid arteritis (RA), systemic lupus erythematosus (SLE), Systematic Sclerosis (SSc), Sjogren's Syndrome (SjS), psoriasis, multiple sclerosis (MS) and autoimmune diabetes.[45-51]According to the well-known role of TLR2 and TLR4 in the development of autoimmunity[28] and the increased susceptibility of CVID patients to autoimmune disorders,[2] we hypothesized that these receptors may be involved in the predisposition of CVID patients to autoimmunity. We found higher expressions of TLR2 and TLR4 in CVID patients with autoimmune disorders but this association was proven insignificant, therefore we decided to test the association between downstream molecules of these receptors and autoimmunity. Interestingly, we found that the CVID patients who presented autoimmunity were associated with a significantly higher expression of MyD88.In agreement with our results, Silver et al showed MyD88 deficiency entirely stopped the ANA and anti-ds DNA antibodies, hyperglobulinemia and the development of glomerulonephritis in a murine model of SLE.[52] Furthermore, results of a recent research by Pacheo et al demonstrated that MyD88 expression in patients with SLE was significantly higher than in controls.[53]

The association of MyD88 and autoimmunity underlines the capacity of establishment of therapeutic regimens considering TLR2 and TLR4 as new drug targets for treatment of autoimmune disorders. Also, the correlation between TLR2 and TLR4 expression and the number of marginal-zone like B cells make these receptors proper targets for B-cell modulation. However, more studies are needed to identify efficient TLR-targeted therapies.

Due to the use of different laboratory methods, cell types, kinds of TLRs and their ligands in different researches, it is difficult to reach a specific conclusion about defects of TLRs in CVID. Above-mentioned reasons underscore our inadequate data and the necessity of more profound insight into the role of TLRs in the pathogenesis of CVID. Our limitations in this research were the lack of immunophenotyping of the patients and the use of PBMCs rather than specific cell types like DCs, B-cells and T-cells. For future studies, we highly recommend considering the immunological phenotypes of the patients as well as exploring other specific cell types.

According to the correlation of the low number of end stage B-cells and poor vaccine response with the higher expression of TLR2, we hypothesized that there is a functional defect in this receptor and/or it's downstream in the PBMCs of CVID patients which leads to the susceptibility of CVID patients to *S. pneumonia.* Furthermore, we showed that the higher expression of MyD88 was associated with the autoimmune presentations in CVID patients. Our unpublished data about lower IL-6 and IL1beta in CVID patients confirm the abolished TLR2 and TLR4 in CVID patients. However future studies are needed to confirm the association between defects in TLR2 and TLR4 signaling and the pathogenesis of CVID.

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