http:// ijp.mums.ac.ir
Original Article (Pages: 5327-5337)

Immunomodulation of TLR2 and TLR4 by G2013 (α-L-Guluronic acid) in CVID Patients

Laleh Sharifi[1], *Asghar Aghamohammadi [1,2], Monireh Mohsenzadegan[3], Nima Rezaei[1], Farzaneh Tofighi Zavareh[1], Mona Moshiri[4], Saied Bokaie[5], Anis Barati[6], Seyed Javad Sayedi[7], Gholamreza Azizi[8], *Abbas Mirshafiey[1,6]

[1]Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran. [2]Primary Immunodeficiency Diseases Network (PIDNet), Universal Scientific Education and Research Network (USERN), Tehran, Iran. [3]Department of Medical Laboratory Science, Faculty of Allied Medical Sciences, Iran University of Medical Sciences, Tehran, Iran. [4]Department of Microbiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [5]Department of Epidemiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. [6]Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [7]Department of Pediatrics, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. [8]Department of Laboratory Medicine, Imam Hassan Mojtaba Hospital, Alborz University of Medical Sciences, Karaj, Iran.

Abstract

Background: Common variable immunodeficiency (CVID) is a primary immune disorder associated with hypogammaglobulinemia, recurrent infections and autoimmune diseases. CVID patients are frequently in contact with infectious pathogens leading to the activation of innate immunity through Toll-like receptors (TLR) affecting adaptive immunity. The aim of the present study was to test the immunomedulatory effect of small molecule G2013, a novel designed non-steroidal anti-inflammatory agent in CVID.

Materials and Methods: After blood sampling from 16 CVID patients and 16 age- and sex-matched healthy controls, peripheral blood mononuclear cells (PBMCs) were isolated and treated with/without lipopolysaccharide (LPS), lipopolyteichoic acid (LTA), and G2013. Assessing the immunomodulatory effect of G2013, flowcytometry was done for quantify the protein expression of TLR2 and TLR4. Gene expressions of signaling molecules involved in the TLR2 and TLR4 pathways were assessed by real-time PCR. ELISA performed assessing the production of IL-1β and IL-6.

Results: G2013 significantly decreased the intensity of TLR2 expression in CVID PBMCs (p=0.001) also G2013 decreased significantly the NF-κB gene expression in PBMCs of CVID patients (p=0.006).

Conclusion: These results indicated that G2013 had immunomodulatory effect at least in part via TLR2 and NF-κB expression. G2013 by decreasing MFI of TLR2 expression and NFκB gene expression provide the possibility of designing new drugs for preventing or controlling autoimmunity in CVID patients.

Key Words: α-L-Guluronic acid, G2013, CVID, TLR2, TLR4, NF-κB, IL-6, IL-1β.

*Please cite this article as: Sharifi L, Aghamohammadi A, Mohsenzadegan M, Rezaei N, Tofighi Zavareh F, Moshiri M, et al. Immunomodulation of TLR2 and TLR4 by G2013 (α-L-Guluronic acid) in CVID Patients.Int J Pediatr 2017; 5(7): 5327-37. DOI: **10.22038/ijp.2017.23562.1985**

Corresponding Author:

Prof. Abbas Mirshafiey, Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. ***AND*** Prof. Asghar Aghamohammadi, Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran.
E-mail: mirshafiey@tums.ac.ir ***AND*** aghamohammadi@tums.ac.ir
Received date Apr.02, 2017; Accepted date: Apr.22, 2017

1- INTRODUCTION

Common variable immunodeficiency (CVID) is one of the primary immunodeficiency disorders characterized by decrease in at least two of three major immunoglobulin isotypes (IgG and IgA and/or IgM) and a poor/lack of response to pneumococcal polysaccharides following vaccination (1). The disorder is associated with impaired B-cell maturation, impaired Somatic hypermutation (or SHM), reduced numbers of circulating memory and isotype-switched memory B cells, and diminished differentiation of B cells to plasma cells (2, 3).

Toll-like receptors (TLRs), a family of endosomal and membrane proteins that recognize microbial products or endogenous molecules, play an important role in triggering and promoting inflammation (4). The mammalian TLR family consists of 11 members and each of them recognizes a different, ligand of different pathogens. For example, TLR4 is the receptor for the bacterial lipopolysaccharide (LPS), TLR2 recognizes lipoproteins of bacteria and members of the TLR9 subfamily (TLRs 7, 8 and 9) are receptors for microbial RNA and DNA (5, 6).

In immune cells, ligand binding to TLRs make recruitment of the adaptor proteins namely myeloid differentiation primary response 88 (MyD88), interleukin-1 receptor-associated kinase 1 (IRAK) and Tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) to cell membrane. Consequently, these proteins activate transcription factor including Interferon-regulatory factor 7 (IRF7) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) with phosphorylation of Inhibitor of κB (IκB). These transcription factors translocate to nucleus resulting in expression of IFN-α and inflammatory cytokines (7). In resting cells, Toll-interacting protein (Tollip) inhibits the NF-κB activation through complex formation with members of the IL-1 receptor-associated kinase (IRAK) family and blocking the phosphorylation of IRAK1 (8). Moreover, TLR4 interaction but not TLR9 activate all the three major mitogen-activated protein kinase (MAPK) pathways extracellular receptor kinase (ERK) 1/2, mitogen-activated protein kinase p38 (p38 MAPK) and c-Jun N-terminal kinase JNK cascades in the cells of the innate immune system, contributing significantly to the inflammatory response (9). It has been shown in CVID patients cluster of differentiation (CD)27+ and $CD27^{-}$ B cells stimulated with TLR7, TLR7/8, and TLR9 lack TLR-driven proliferation, Activation-induced cytidine deaminase (AID) expression, isotype switch, and immunoglobulin production. TLR7-, TLR7/8-, or TLR9-triggered plasmacytoid dendritic cells (pDCs) of CVID also produce IFN-α very little (10). These functional aberrations of TLR7 and TLR9 signaling in patients with CVID highlight the importance of TLR signaling between different cells in optimizing responses either innate or adaptive immunity (11).

Small molecule G2013, $C_6H_{10}O_7$ is a novel designed non-steroidal anti-inflammatory drug (NSAID) prepared in immunology section of Pathobiology Department of Tehran University of Medical Sciences. This new drug (G2013) is the epimer form of M2000 (β-D-Mannuronic acid) an Uronic acid with published anti-inflammatory properties (12, 13).

According to the position of TLRs for activation innate and adaptive immunity (11) and importance of G2013 as an immunomodularory and anti-aging agent (12, 14, 15), in the present study we investigated immunomodulatory effects of G2013 in the TLR2 and TLR4 protein expression and signaling downstream as well as cytokine production of PBMCs of CVID patients.

2- MATERIALS AND METHODS

2-1. Patients

Sixteen patients with definite diagnosis of CVID and regular use of Intravenous immunoglobulin (IVIG) who referred to clinic of immunodeficiency, Children Medical Center in Tehran, were satisfied to participate in this study. Definitive diagnosis of CVID was made based on criteria defined by International Union of Immunological Societies (16) and blood sampling were done just before IVIG obtaining.

The laboratory data of patients including hematological, serological and immunological evaluations which had been performed previously collected from the hospital lab. From blood donors who referred to Iran Blood Transfer Organization, sixteen age- and sex-matched healthy individuals were recruited study as control group. The participants filled printed informed consent. This study was approved by ethics committee of Tehran University of Medical Sciences (ID number: 154-27044).

2-2. Separation of PBMCs and Cell culture

In the department of intravenous immunoglobulin therapy (IVIG), 10 ml whole blood was obtained from patients just before IVIG by a nurse. The PBMCs were obtained from fresh blood samples by density gradient centrifugation method using Ficol-paque (Biosera, France) then isolated PBMCs were washed twice by Phosphate Buffered Saline to clean the residual of ficol. Staining with trypan blue, the viability of cells was recognized.

PBMCs were suspended in a culture medium containing RPMI 1640 enriched by 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM of L-glutamine.

2-3. G2013 and TLR ligands' treatments

After adding one million cells to each well of a 24-well cell culture plate, the PBMCs were incubated for an overnight by 5 µg/mL G2013 as low-dose and 25 µg/mL as high-dose drug, 10 µg/mL LTA (Sigma-Aldrich, USA) as specific ligand of TLR2, 1µg/ml LPS (Sigma-Aldrich, USA) as specific ligand of TLR4, and 25µg/m1 OxPAPC (Invivogen, USA) as an inhibitor of TLR4

2-4. Flowcytometry

Proteins of TLR2 and TLR4 were stained by 5µg monoclonal antibodies (Biolegend, USA) of FITC-labeled anti-human CD282 (TLR2) and PE labeled anti-human CD284 (TLR4). Staining procedure was carried out based on protocol of producing company. BD flow cytometer system (BD, USA) was used in this experiment. Monoclonal antibodies of FITC-labeled mouse IgG2a and PE-labeled Mouse IgG2a were used as isotype controls and the data was analyzed with FlowJo software version 7.6.1.

2-5. Real-time PCR

Total RNA was extracted manually PBMCs by Qiazole (Qiagen, USA) and reversed transcribed into CDNA by Takara kit (Takara, Japan); all procedures was done according to protocols of companies. Gene expressions of MyD88, Tollip, IκB, P38, and NFκB were defined by quantitative real-time polymerase chain reaction (PCR) using SYBR Green PCR Master Mix (Takara, Japan) on the ABI Step One plus Real-time PCR system (ABI System, USA); also β-actin was used as housekeeping and the relative abundance of each gene was computed using the $2^{-\Delta\Delta CT}$ method. Nucleotide sequences of specific primers and β-actin are presented in **Table.1**.

Table-1: Nucleotide sequences of primers used in this study

β-actin	F; 5'-GTGGGGCGCCCCAGGCACCA-3'- R; 3'-CTCCTTAATGTCACGCACGATTTC-5'
MyD88	F; 5'-CGCCGCCTGTCTCTGTTC-3'- R; 3'-GGTCCGCTTGTGTCTCCAGT-5'
Tollip	F; 5'-GCAAGGTGGAGGACAAGTG- R; 3'-GTAGGACATGACGAGGTTGATC-5'
IκBα	F; 5'-CTCCACTCCATCCTGAAGGCTA-3'- R; 3'-AGGTCCACTGCGAGGTGAAG-5'
P38	F; 5'-GAGGTGCCCGAGCGTTAC-3'- R; 3'-GGAGAGCTTCTTCACTGCCAC-5'
NFκB	F; 5'-GCTACACAGGACCAGGGACAGT- R; 3'-AGCTCAGCCTCATAGAAGCCATC-5'

2-6. Cytokine measurement

Detecting the concentration of cytokines, supernatants of cell cultures were gathered after incubation period and stored at -70°C. Quantities of IL-1β and IL-6 were detected by ELISA kit (eBioscience, USA) based on the protocol which was provided by the manufacturer.

2-7. Statistical analysis

Data were statistically analyzed by SPSS software version 16.0. Data are shown as mean ± standard deviation (SD) and statistical differences were statistically tested using the independent-samples t-test and paired-samples t differences were considered significant at p- value of equal or less than 0.05.

3- RESULT

Evaluating the immunomodulatory properties of G2013 in CVID patients, 16 CVID patients consist of 10 (62.5%) male and 6 (37.5%) female with age mean of 26.43±9.02 years old were entered the study. The surface protein expression of TLR2 and TLR4, signaling pathway and cytokine production were detected before and after treatment with low and high doses of G2013 as well as agonists and inhibitor of TLR2 and TLR4. Patients' characteristics are demonstrated in **Table.2**.

3-1. The effect of G2013 treatment on the protein expression of TLR2 and TLR4 in PBMCs of CVID patients

Mean Fluorescence Intensity (MFI) of TLR2 and TLR4 in patients and controls at baseline levels and after treatment of PBMCs with two doses of G2013 were investigated by flow cytometry. The baseline MFI of TLR2 was 76.62±8.47 in healthy controls and 85.95±12.09 in CVID group (P=0.56). In CVID patients, treatment of low-dose G2013 in culture of PBMCs decreased the MFI of TLR2 to 65.69±10.88 (P=0.42) and treatment of high-dose G2013 decreased significantly this amount to 20.65±6.39 (P=0.001). LTA-stimulation of CVID patients altered the MFI of TLR2 to 70.55±9.93 (P=0.39), moreover OxPAPC decreased significantly the MFI to 27.86± 3.16 (P=0.001).

We demonstrated that in healthy control individual the baseline MFI of TLR4 was 49.94±8.91 whereas this amount in CVID patients was 41.10±6.08 (P=0.17). The MFI of TLR4 after addition of low and high dosage of G2013 in controls group elevated to 53.45±6.80 (P=0.06) and 55.15±7.47 (P=0.06) in CVID patients, respectively. Treatment of CVID PBMCs with their specific ligand LPS increased the MFI of TLR4 to 46.77±7.34 (P=0.21). Using inhibitor in the culture of CVID PBMCs lead to significant reduction of intensity of TLR4 to 24.95± 3.68 (P=0.03).

Figures 1 and 2 demonstrates the MFI of the expression of TLR2 and TLR4 at baseline level and after addition of low and high dosage of G2013 as well as their agonists LTA/LPS and their inhibitor OxPAPC.

3-2. Gene expression of TLRs signaling molecules in PBMCs of CVID patients

However our findings from real-time PCR showed no significant differences between NFκB gene expressions of healthy controls and CVID patient at baseline level (P=0.285), but treatment of CVID PBMCs by low-dose G2013 can significantly decrease the gene expression of NFκB (P=0.006), but high-dose of it, did not have similar effect (P=0.189) (**Figure.3**). Rather than some alterations, G2013 did not affect significantly other signaling molecules including Tollip, MyD88, IκB and P38 in CVID and controls.

3-3- Cytokines production in PBMCs of CVID patients

Both IL-1β and IL-6 had significant weaker production in CVID patients compared with healthy people. Before and after data comparison, showed that CVID PBMCs treated with G2013 either low or high doses show an increasing in IL-1β and IL-6 levels compared with baseline levels. However paired t test do not confirmed significant relations for these findings (P>0.05 for each comparison) (**Figures 4 and 5**).

Table-2: Demographic and clinical presentation of CVID patients

Subjects		Results
Number of patients		16
Gender (male/female)		10/6
Age (Mean ± SD)		26.43±9.02
Age of onset		14.56±9.24
Diagnosis delay		5.09±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%

SD: standard deviation; ITP: immune thrombocytopenic purpura; AE: adult autoimmune enteropathy; AIHA: autoimmune hemolytic anemia; IBD: inflammatory bowel disease; RA: rheumatoid arthritis; PID: primary immunodeficiency.

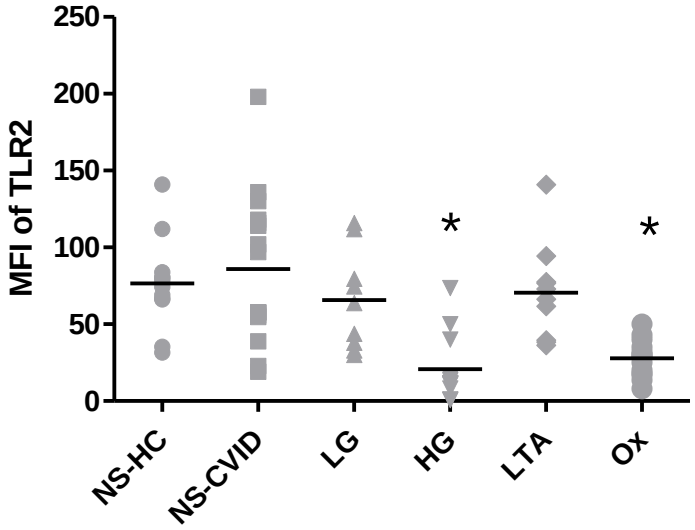

Fig.1: The effect of G2013 on the expression of TLR2 in PBMCs of CVID patients

Abbreviations: NS: non-stimulated; HC: healthy control; LG: low-dose G2013 (5µg/ml); HG: high-dose G2013 (25µg/ml), LTA: lipoteichoic acid; Ox: OxPAPC.

High dose of G2013 significantly reduced the baseline MFI of TLR4 in CVID PBMCs (p=0.001). *Indicates significant association with baseline expression of TLR2 in CVID patients.

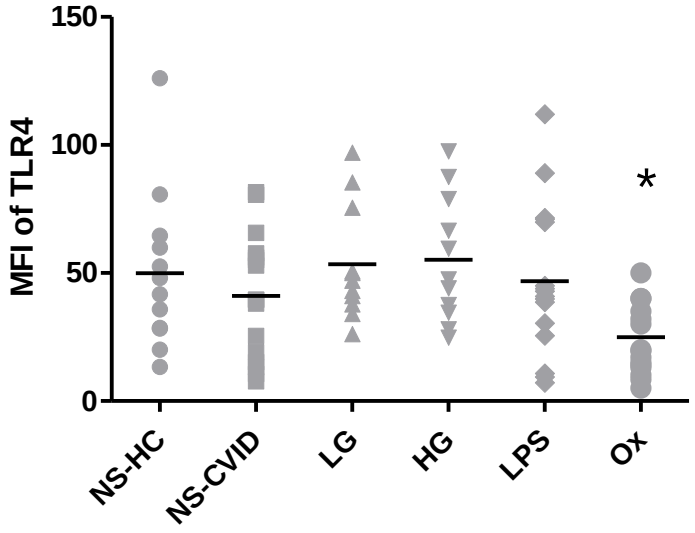

Fig.2: The effect of G2013 on the expression of TLR4 in PBMCs of CVID patients.

Abbreviations: NS: non-stimulated; HC: healthy control; LG: low-dose G2013 (5µg/ml); HG: high-dose G2013 (25µg/ml), LPS: lipopolysaccharide; Ox: OxPAPC.

The low and high doses of G2013 up-regulate the expression of TLR4 in PBMC of CVID patients (p=0.06 for both). *Indicates significant association with baseline expression of TLR4 in CVID patients.

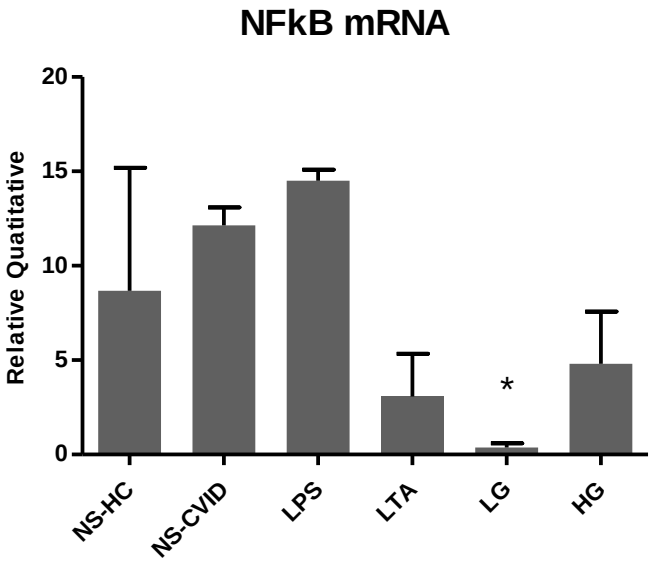

Fig.3: The effect of G2013 on the gene expression of NFκB in CVID patients.

Abbreviations: NS: non-stimulated; LPS: lipopolysaccharide; LTA: lipoteichoiec acid; LG: low-dose G2013; HG: high-dose G2013. Independent t test analysis of our data revealed that there is no significant differences between baseline levels of NFκB gene expression in controls and patients (p=0.285). Low-dose G2013-treated PBMCs in CVID patients significantly decrease the gene expression of NFκB (p=0.006) but high-dose of it do not have similar trend (p=0.189). *Indicates significant association with baseline gene expression of CVID patients.

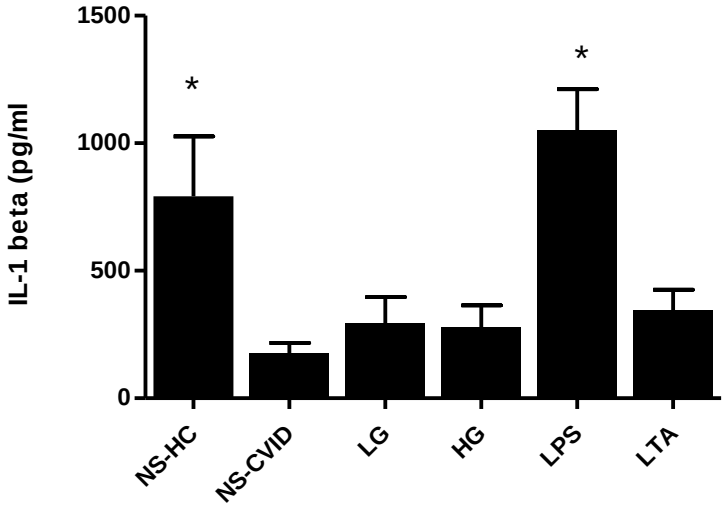

Fig.4: Effect of G2013 on IL-1β production by PBMCs of CVID patients.

Abbreviations: NS: non-stimulated; HC: healthy control; LG: low G2013; HG: high G2013, LPS: lipopolysaccharide; LTA: Lipoteichoic acid.

With no stimulation CVID PBMCs produce significantly lower amount of IL-1β than healthy controls (p=0.019). In spite of increasing the IL-1β in CVID patients after treatment of low and high doses of G2013 but according to paired t test analysis this increasing is not significant.(p=0.376 and p=0.380 respectively). *Indicates significant association with baseline cytokine production of CVID patients.

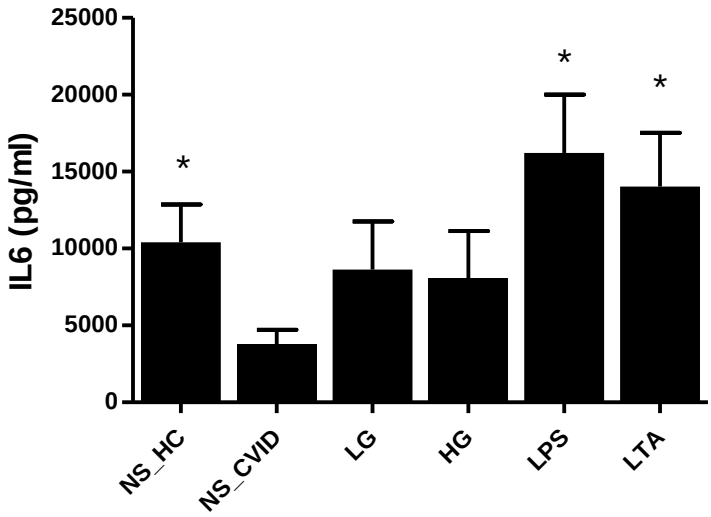

Fig.5: Effect of G2013 on IL-6 production by PBMCs of CVID patients.

Abbreviations: NS: non-stimulated; HC: healthy control; LG: low G2013; HG: high G2013, LPS: lipopolysaccharide; LTA: Lipoteichoic acid.

At baseline level CVID patients produce significantly lower IL-6 compared with healthy controls (p=0.015). In spite of increasing the IL-6 in CVID patients after treatment of low and high doses of G2013 but according to paired t test analysis this increasing is not significant.(p=0.139 and p=0.691 respectively). *Indicates significant association with baseline cytokine production of CVID patients.

4- DISCUSSION

Although defects observed in B cells, T cells, and dendritic cells in part by TLR7/TLR9 signaling impairment have been reported in CVID, blood mononucleolar cell functions of CVID via TLR2 and TLR4 engagement, has been less studied (17). de Lollo et al. (2016) showed that the TLR4 signaling was dysfunctional in CVID patients, because in response to TLR4 stimulation was observed decrease of CCL2 and CXCL8 production and increase of TNF-α secretion as compared with healthy control individuals (18). The most common clinical presentation in patients with CVID is recurrent infections, and pneumonia is one of the most common infectious organisms (19). Among TLRs, TLR2 play an important role in the recognition of Streptococcus pneumoniae and in the activation of the inflammatory and antimicrobial innate immune responses (20). In the present study, statistical analysis showed that there was no difference in TLR-2 and TLR-4 intensity of expression on PBMCs of CVID patients as compared with healthy control. However, G2013 significantly decreased the intensity of TLR-2 protein expression in CVID patients in a dose dependent pathway, whereas was not observed any significance of G2013 effect in healthy control individuals. In addition to microbial products, endogenous components such as released intracellular proteins, heat shock protein and oxidation-modified lipids can activate TLR2 and TLR4 and result in autoimmune diseases (21). Moreover, it has been reported that some of CVID patients develop autoimmune diseases including; rheumatoid arthritis, vitiligo, hemolytic anemia, thrombocytopenia, neutropenia associated with pyoderma gangrenosum, and gastrointestinal diseases (22). G2013 via decreasing intensity of TLR2

expression on mononuclear cells at least in part may attenuate autoimmune outbreak in CVID patients. However, additional studies are required to evaluate G2013 as an immunomodulatory factor for CVID patients. Anti-inflammatory properties of G2013 was demonstrated in experimental autoimmune encephalomyelitis (EAE) and shown that G2013 modulates EAE by decreasing NO production which was confirmed by histopathological and clinical findings (14).

Furthermore, our previous results showed that after treatment by G2013 in a dose dependent, IRAK1 expression reduced between 5 to 8-fold in HEK-293 TLR4 cells (embryonic kidney cells) (15). At present, we are evaluating effect of G2013 in clinical trial I on ankylosing spondylitis (IRCT2016091813739N4) and rheumatoid arthritis (IRCT: 2016092813739N5) patients. The current study demonstrated that TLR4 activation with LPS result in significant decrease of NF-κB gene expression in CVID in comparison with healthy control that may be sign of some deficiencies in signaling pathway via NF-κB activation and function.

Interestingly, in comparison with un-stimulated cells after stimulating PBMCs with LPS in CVID no increase of NF-κB gene expression was observed. Consistence with these data, Keller et al. (2016), also demonstrated classical NF-κB signaling mediated by BCR (B cell receptor) was impaired in mature naïve B cells of CVID patients (especially patients with CD21 low B cells) (23). G2013 did not affect the MyD88, IκB, P38 and Tollip gene expression, while in CVID group G2013 at low dose significantly decrease the NF-κB gene expression in CVID PBMCs indicating immunomodulatory effect of this drug. This finding can be beneficial for designing anti-autoimmunity drugs based on G2013 molecule in CVID patients; of course, further detailed studies is needed for proving this hypothesis. Polysaccharide capsule of Streptococcus pneumoniae induces secretion of cytokines such as IL-6 and TNF-α from monocytes but not by B cells or T cells. Hong et al. (2010) showed that secretion of these cytokines induced by Pneumovax-23 (component of polysaccharide capsule of S. pneumoniae) is impaired in CVID (24); also a leading research groups in this field has been reported reduced IL-6 in CVID patients (25).

Consistent with these data, in the present study, CVID mononuclear cells in both un-stimulated and stimulation condition with LPS showed decrease in IL-1β and IL-6 level as compared with healthy controls indicating that in CVID PBMCs under inflammatory condition pro-inflammatory cytokines production are decreased, However, our findings were in conflict with other study in terms of inflammatory cytokine production in CVID patients.

They showed that there was normal secretion of TNF-α, IL-6, and IL-12 from PBMCs stimulated through TLR1, TLR2 TLR3, TLR4, TLR5, TLR7, or TLR9 in CVID patients (10). However IL-1β and IL-6 production are weaker in non-treated and G2013-treated CVID PBMCs compared with healthy controls but treating the CVID PBMCs with both doses of G2013 raise up the cytokines from the baseline levels.

5- CONCLUSION

In conclusion, we observed that despite of healthy individuals, G2013 significantly decrease the intensity of TLR2 protein expression and NF-κB gene expression in CVID PBMCs as well as increasing cytokines indicating immunomodulatory effect of this drug. G2013 by decreasing MFI of TLR2 expression on PBMCs and NFκB gene expression may control autoimmune presentation in CVID patients. However, additional studies are required to evaluate G2013 as an

immunomodulatory factor for CVID patients. This finding can be used for designing anti-autoimmunity drugs for prevention or treatment of this condition in CVID patients. Of course, due to the heterogeneity and diversity of inflammatory conditions and clinical signs in CVID, future studies should be investigated according to immune phenotype classes of patients to understand better the immunomodulatory phenomena of G2013 as well as its autoimmune preventing capacity for CVID patients.

6- CONFLICT OF INTEREST

The authors declare that they had no conflict of interest.

7- ACKNOWLEDGMENT

This research project was supported by a grant from Research Center for Immunodeficiencies, Tehran University of Medical Sciences.

8- REFERENCES

1. Shabashova NV, Filippova LV, Uchevatkina AE, Frolova EV. Common variable immunodeficiency in adults. Ter Arkh. 2016;88(11):94-8.

2. Goldacker S, and Warnatz K. Tackling the heterogeneity of CVID. Curr Opin Allergy Clin Immunol 2005;5(6):504-9.

3. Salzer U, Chapel HM, Webster AD, Pan-Hammarstrom Q, Schmitt-Graeff A, Schlesier M, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. Nat Genet. 2005;37(8):820-8.

4. Gatti G, Quintar AA, Andreani V, Nicola JP, Maldonado CA, Masini-Repiso AM, Rivero VE, and Maccioni M. Expression of Toll-like receptor 4 in the prostate gland and its association with the severity of prostate cancer. Prostate. 2009;69(13):1387-97.

5. Roshan MH, Tambo A, Pace NP. The Role of TLR2, TLR4, and TLR9 in the Pathogenesis of Atherosclerosis. Int J Inflam. 2016;2016:1532832.

6. Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, Harris KW, and Selander KS. Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro. Prostate. 2007;67(7):774-81.

7. Joosten LA, Abdollahi-Roodsaz S, Dinarello CA, O'Neill L, and Netea MG. Toll-like receptors and chronic inflammation in rheumatic diseases: new developments. Nat Rev Rheumatol. 2016;12(6):344-57.

8. Akira S, and Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499-511.

9. Menon MB, and Gaestel M. TPL2 meets p38MAPK: emergence of a novel positive feedback loop in inflammation. Biochem J. 2016;473(19):2995-99.

10. Yu JE, Knight AK, Radigan L, Marron TU, Zhang L, Sanchez-Ramon S, et al. Toll-like receptor 7 and 9 defects in common variable immunodeficiency. J Allergy Clin Immunol. 2009;124(2):349-56, 56 e1-3.

11. Sharifi L, Mirshafiey A, Rezaei N, Azizi G, Magaji Hamid K, Amirzargar AA, et al. The role of toll-like receptors in B-cell development and immunopathogenesis of common variable immunodeficiency. Expert Rev Clin Immunol. 2016;12(2):195-207.

12. Mirshafiey A, Hosseini S, Afraei S, Rastkari N, Zavareh FT, Azizi G. Anti-aging property of G2013 molecule as a novel immunosuppressive agent on enzymatic and non-enzymatic oxidative stress determinants in Rat model. Current drug discovery technologies. 2016;13(1):25-33.

13. Jahromi SS, Jamshidi MM, Farazmand A, Aghazadeh Z, Yousefi M, Mirshafiey A. Pharmacological effects of beta-d-mannuronic acid (M2000) on miR-146a, IRAK1, TRAF6 and NF-kappaB gene expression, as target molecules in inflammatory reactions. Pharmacol Rep. 2017;69(3):479-84.

14. Afraei S, Azizi G, Zargar SJ, Sedaghat R, and Mirshafiey A. New therapeutic approach by G2013 in experimental model of multiple sclerosis. Acta neurologica Belgica. 2015;115(3):259-66.

15. Hajivalili M, Pourgholi F, Majidi J, Aghebati-Maleki L, Movassaghpour AA,

Samadi Kafil H, et al. G2013 modulates TLR4 signaling pathway in IRAK-1 and TARF-6 dependent and miR-146a independent manner. Cellular and molecular biology. 2016;62(4):1-5.

16. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol. 1999;93(3):190-7.

17. Vincent FB, Saulep-Easton D, Figgett WA, Fairfax KA, Mackay F. The BAFF/APRIL system: emerging functions beyond B cell biology and autoimmunity. Cytokine Growth Factor Rev. 2013;24(3):203-15.

18. de Lollo C, de Moraes Vasconcelos D, da Silva Oliveira LM, de Oliveira Titz T, Carneiro-Sampaio M, Jacob CM, et al. Impaired CD8(+) T cell responses upon Toll-like receptor activation in common variable immunodeficiency. J Transl Med. 2016;14(1):138.

19. Park MA, Li JT, Hagan JB, Maddox DE, Abraham RS. Common variable immunodeficiency: a new look at an old disease. Lancet. 2008;372(9637):489-502.

20. Kerr AR, Irvine JJ, Search JJ, Gingles NA, Kadioglu A, Andrew PW, et al. Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection. Infect Immun. 2002;70(3):1547-57.

21. Lollo C, de Moraes Vasconcelos D, Oliveira LM, Domingues R, Carvalho GC, Duarte AJ, et al. Chemokine, cytokine and type I interferon production induced by Toll-like receptor activation in common variable immune deficiency. Clin Immunol. 2016;169(121-7.

22. Bergler-Czop B, Brzezinska-Wcislo L. Pyoderma gangrenosum in a patient with common variable primary immunodeficiency. Postepy Dermatol Alergol. 2013;30(3):188-91.

23. Keller B, Cseresnyes Z, Stumpf I, Wehr C, Fliegauf M, Bulashevska A, et al. Disturbed canonical nuclear factor of kappa light chain signaling in B cells of patients with common variable immunodeficiency. J Allergy Clin Immunol. 2016.

24. Hong R, Agrawal S, Gollapudi S, Gupta S. Impaired pneumovax-23-induced monocyte-derived cytokine production in patients with common variable immunodeficiency. J Clin Immunol. 2010;30(3):435-41.

25. Cunningham-Rundles C, Radigan L, Knight AK, Zhang L, Bauer L, Nakazawa A. TLR9 activation is defective in common variable immune deficiency. J Immunol. 2006;176(3):1978-87.