Immunomodulatory Effect of G2013 (α-L-Guluronic Acid) on the TLR2 and TLR4 in Human Mononuclear Cells

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Abstract: Background: Inhibition of Toll-like receptors (TLRs) signaling has been established as a new method for the development of anti-inflammatory drugs instead of NSAIDs (non-steroid anti-inflammatory drugs). Since the immunomodulatory role of G2013 (α-L-Guluronic acid) was reported in some recent experiments, we decided to assess the effects of G2013 on the protein expression of TLR2 and TLR4, their downstream signaling cascade, and the secretion of pro-inflammatory cytokines in human peripheral blood mononuclear cells (PBMCs).

Methods: After blood sampling from 16 healthy donors, PBMCs were isolated and treated with/without lipopolysaccharide (LPS), lipopolysaccharide (LTA), and G2013. Flow cytometry was done for detecting the protein expression of TLR2 and TLR4, MyD88, IκB, Tollip, and NF-κB mRNA expression were assessed by real-time PCR. ELISA was performed for assessing the concentration of IL-1β and IL-6.
Results: G2013 at a concentration of 25 μg/mL (high dose) significantly down-regulated NF-κB, IκB and MyD88 mRNA expression and suppressed the secretion of IL-1β by PBMCs. The findings indicate that G2013 may exert its regulatory effect under normal condition via Tollip in a dose dependence pathway. Our results demonstrated that G2013 had no profound impact on the protein expression of TLR2 and TLR4.

Conclusion: In conclusion, our findings point to the immunomodulatory effect of G2013 on the TLR2 and TLR4 signaling cascade and cytokine production by PBMCs. These findings could lead to an establishment of new safe anti-inflammatory drugs in the future.

Keywords: Guluronic acid, G2013, TLR2, TLR4, MyD88, IκB, Tollip, NF-κB, IL-6, IL-1.

1. INTRODUCTION

Toll-like receptors (TLRs) are a family of immune receptors that play a crucial role in the innate and adaptive immunity [1]. These receptors identify highly conserved components derived from bacteria, viruses, fungi, and parasites called pathogen-associated molecular patterns (PAMPs) as well as endogenous molecules called endogenous damage-associated molecular patterns (DAMPs) and have the main role in triggering and enhancing inflammation [2, 3].

Fifteen kinds of TLRs have been recognized in mammalians and 13 of them have been identified in humans and mice together. Some of TLRs are expressed on the cell surface and others in endosomes. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface membrane; whereas TLR3, TLR7, TLR8, and TLR9 are expressed within endosomes [1]. TLRs recognize various components of microbial products. For example, TLR4 recognizes bacterial lipopolysaccharide (LPS), TLR2 recognizes lipoproteins and lipo-teichoic acid (LTA) of bacteria; TLR5 recognizes flagellin and members of the TLR9 subfamily (TLRs 7, 8 and 9) are receptors for microbial RNA and DNA [4]. TLR10 is an orphan receptor and strongly expressed in the human spleen [5] and B cells [6]. Ligand binding to TLRs leads to dimerization of the TLR proteins and initiates signaling pathways at the cell surface or endosomes. This is followed by recruitment of adaptor proteins such as myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP), TIR-domain-containing adaptor protein—including INF-β (Trif), and TRIF-related adaptor molecule (TRAM). Activation of adaptor proteins leads to phosphorylation of kinases eventually leading to activation and translocation of NF-κB and AP-1 transcription factors to nucleus, resulting in the expression of IFN-α, IFN-β, inflammatory cytokines and cost-

imulatory molecules [7]. The presence of endogenous inhibitors of TLRs is crucial to modulate the signaling pathways and maintain the balance of inflammatory responses. Negative regulatory proteins such a MyD88s, IRAK-M, SOCS1, A20, SyK and Tollip by blocking adaptor proteins or kinases can terminate inflammation. Hyper responsiveness of TLRs leads to inflammation and predisposing the autoimmunity disorders [8]. TLR2 and TLR4 are known to have a key role in the progress of autoimmune disorders such as Autoimmune Diabetes, Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Psoriasis, Multiple Sclerosis (MS), Systematic Sclerosis (SSc), and Sjogren’s Syndrome (SjS) [9-15]. Therefore, antagonists of TLR2 and TLR4 and their signal transduction inhibitors are proposed for the treatment of autoimmune complications. The non-steroidal anti-inflammatory drugs (NSAIDs) have been known as pain relievers and are used widely in clinical medicine [16]. NSAIDs play the main role in controlling inflammatory diseases such as spondyloarthritides [17], rheumatoid arthritis [18], scleroderma [19] and lupus [20]. They inhibit enzymes that synthesize prostaglandins, by reducing the level of prostaglandin E2 (PGE2). Although NSAIDs have therapeutic efficacy, they are also known to cause significant renal toxicities, gastrointestinal (GI), and cardiovascular, circumstances that limit their use. It has been shown that indomethacin (a NSAID) can injure the small intestine via a TLR4/MyD88-dependent pathway [21].Therefore, it is essential to develop anti-inflammatory drugs that modulate the immune system and could lead to new therapies for autoimmunity and inflammatory disorders with no side effects of NSAIDs.

The small molecule G2013 or α-L-Gluronate acid (C6H10O7) patented (DE-102016113017.6) is a
novel drug prepared as NSAID with immunomodulatory features prepared at the Immunology section of the Pathobiology Department of Tehran University of Medical Sciences (TUMS). It has less toxicity on the GI tract and kidney function compared with other NSAIDs [22]. This novel drug (G2013) is the epimer form of the M2000 drug. The molecular structure of M2000 is β-D-Manuronic acid, a uronic acid whose anti-inflammatory properties have been published previously [23]. Considering the importance of TLR activation to be an integral mechanism for activation of innate and adaptive immunity against microbial infections and the importance of G2013 as an immunomodulary agent [22, 24], in the present study we evaluated the immunomodulatory effects of G2013 on the protein expression of TLR2 and TLR4, and their signaling transduction molecules, including mRNA expression of MyD88, Tollip, IκB, and NF-κB, as well as IL-6 and IL-1β production of mononuclear cells from healthy subjects.

2. MATERIALS AND METHODS

2.1. The Participants

Sixteen blood donors who referred to Iranian Blood Transferring Organization in Tehran were enrolled in this study. The health status of participants was confirmed by a physician just before obtaining blood. All study participants filled the written informed consent; this study was approved by ethics committee of the Tehran University of Medical Sciences.

2.2. Isolation of PBMCs and Cell Culturing

The PBMCs were isolated from blood samples using density gradient centrifugation on Ficoll-paque (Biosera, France). Cells were washed in PBS (Phosphate Buffered Saline) to remove residual ficoll. The viability of PBMCs was verified by trypan blue staining. PBMCs were suspended in a culture medium containing RPMI 1640 supplemented with 10% of FBS (heat-inactivated fetal bovine serum), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM of L-glutamine.

2.3. Treatments

One million cells were added to each well of a 24-well cell culture plate. The PBMCs were treated overnight with 10 μg/mL LTA (Sigma-Aldrich, USA), or 1μg/mL LPS (Sigma-Aldrich, USA) as TLR2 and 4 stimulators, respectively; 25µg/mL OxPAPC (Invivogen, USA) was used as an inhibitor of both TLR2 and 4, as well as 5 µg/mL G2013 (low dose) and 25 µg/mL (high dose).

2.4. Flow Cytometry

Cell membrane proteins of TLR2 and TLR4 were stained with 5μg monoclonal antibodies (Bi-olegend, USA) of FITC-labeled anti-human CD282 (TLR2) and PE labeled anti-human CD284 (TLR4) using 5x10^5 cells. The staining procedure was performed according to the protocol of the manufacturing company. FITC-labeled mouse IgG2a and PE-labeled mouse IgG2a monoclonal antibodies were used as isotype control antibodies. A BD flow cytometer system (BD, USA) was used in this experiment. Flow cytometry data were analyzed with FlowJo software version 7.6.1.

2.5. Real-Time PCR

Total RNA was extracted manually from cells using Qiazole (Qiagen, USA), and reversed transcribed into cDNA using a Takara kit (Takara, Japan); all procedures were carried out according to the protocols of the companies. Gene expressions of MyD88, Tollip, IκB, and NF-κB were assessed by quantitative real-time PCR using SYBR Green PCR Master Mix (Takara, Japan) on the ABI Step One plus Real-time PCR system (ABI System,

Table 1. Sequences of primers used in the study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
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<tr>
<td>NF-κB</td>
<td>F: 5'-GCTACACAGGACCAAGGGACAGT-3' and R; 3'-AGCTCAAGGCTCATAGAAGCCTAC-5'</td>
</tr>
<tr>
<td>MyD88</td>
<td>F: 5'-CGCCGCCTGTCTGTTC-3' and R; 3'-GGTCCGCCTTCTGCTCCAGT-5'</td>
</tr>
<tr>
<td>Tollip</td>
<td>F: 5'-GCAAGTGAGGAGGACACTG-3' and R; 3'-GTAGGACATGAGGTTGATC-5'</td>
</tr>
<tr>
<td>IκB</td>
<td>F: 5'-CTCCACTCCATCTGAGGCTA-3' and R; 3'-AGGGTCCACTGCGAGTGAAG-5'</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5'-GTGGGGCGCCCAAGGACCACA-3' and R; 3'-CTCCCTAAATGTCAAGCGACATTTG-5'</td>
</tr>
</tbody>
</table>
USA). The encoding gene for β-actin was used as housekeeping gene for normalization of amplification; nucleotide sequences of specific primers and β-actin are shown in Table 1. Finally, the relative abundance of each gene was computed using the 2^(-ΔΔCt) method.

2.6. Cytokine Assay

To measure the cytokines the supernatants of cell cultures were collected and stored at -70°C. Concentrations of IL-1β and IL-6 were assessed using ELISA kits (ebioscience, USA) based on the protocol provided by the manufacturer.

2.7. Statistical Analysis

SPSS software version 16 was used for statistical analysis of data. Data are shown as means ± standard deviation (SD) and statistical differences were detected using the Paired-Samples T Test in parametric analysis and Wilcoxon in the non-parametric analysis. Differences were presumed significant at p values of ≤0.05.

3. RESULTS

Ten male and 6 female healthy donors with the age range of 19-41(26.88±7.33) years old were enrolled in the study. To find the immunomodulatory effect of G2013, the surface expression of TLR2 and TLR4, signaling pathway and cytokine production were assessed before and after treatment with low and high doses of G2013.

3.1. The Protein Expression of TLR2 and TLR4 After G2013 Treatment

The baseline protein expression of TLR2 in the PBMCs was 12.77±5.20% and its expression in low-dose (5μg/ml) G2013 treated cells was similar; 12.52±9.15% (p=0.28). Its expression in the high-dose (25μg/ml) G2013 treated cells was decreased although not statistically significant 10.17±3.01%; (p=0.07). The expression of TLR4 (14.12±7.65%) was not influenced by treatment with low or high G2013 doses 11.97±2.92% (p=0.35) and 13.60±10.49 (p=0.89), respectively (Fig. 1).

3.2. Gene Expression of Signaling Molecules of TLR2 and TLR4

3.2.1. The Effect of G2013 on the Gene Expression of NF-κB in the PBMCs

At baseline level, the mRNA expression of NF-κB was 8.22±1.07 and its expression in the low-dose G2013 group were not different 8.03±1.10 (p=0.83) while its expression in high dose G2013 group significantly decreased to 0.31±0.3 (p=0.009) (Fig. 2).
In the PBMCs, the baseline mRNA expression of Tollip was 5.21±1.07. After low-dose G2013 treatment, the mRNA expression significantly increased to 11.03±1.00 (p=0.03) (Fig. 3). In contrast, the mRNA expression in the high dose G2013 group significantly decreased to 0.48±0.45 (p=0.03) (Fig. 3).

The baseline expression of IκB mRNA in the PBMCs was 35.06±5.01. After low-dose G2013 treatment, its expression decreased significantly to 6.29±1.57 (P=0.007). After high dose of G2013 treatment, its expression decreased to 0.39±0.52 (p=0.008) (Fig. 4).

**3.2.2. The Effect of G2013 on the Gene Expression of Tollip in the PBMCs**

At baseline level, the mRNA expression of Tollip was 5.21±1.07 and its expression in low-dose G2013 were significantly increased to 11.03±1.00 (p=0.03) while its expression in the high dose G2013 group significantly decreased to 0.48±0.45 (p=0.03) (Fig. 3).

**3.2.3. The Effect of G2013 on the Gene Expression of IκB in the PBMCs**

The baseline expression of IκB mRNA in the PBMCs was 35.06±5.01 and its expression decreased significantly after low-dose G2013 treatment 6.29±1.57 (P=0.007), and its expression after high dose of G2013 decreased to 0.39±0.52 (p=0.008) (Fig. 4).
3.2.4. The Effect of G2013 on the Gene Expression of MyD88 in the PBMCs

The baseline expression of MyD88 mRNA in the PBMCs was 5.14±1.02 and its expression increased after low-dose G2013 treatment 8.03±1.00 (P=0.13) but its expression after a high dose of G2013 decreased significantly to 0.33±0.52 (p=0.007) (Fig. 5).

3.3. Cytokines Production

3.3.1. The Effect of G2013 on IL-1β Secretion by the PBMCs

With no stimulation, PBMCs produced 0.63.3±0.75 ng/mL and the treatment of PBMCs with low-dose of G2013 reduced this amount to 0.43±0.52 ng/mL (p=0.21) and high-dose G2013

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**Fig. (4). The effect of G2013 on the gene expression of IκB in human PBMCs.** Abbreviations: NS: non-stimulated; G: G2013; LPS: lipopolysaccharide. Data are shown as mean±SD. * Indicates significant statistical difference with control group. # Indicates significant difference with LPS group.

**Fig. (5). The effect of G2013 on the gene expression of MyD88 in human PBMCs.** Abbreviations: NS: non-stimulated; G: G2013; LPS: lipopolysaccharide. Data are shown as mean±SD. * Indicates significant statistical difference with control group. # Indicates significant difference with LPS group.
decreased the production of IL-1β to 0.17±0.24ng/mL when compared with healthy controls (p=0.05). Furthermore, LPS dramatically increased the IL-1β production (p=0.01); also we found significant relations between LPS group and low-dose G2013 group (p=0.01), as well as high-dose group (p=0.04). We found no significant increase in LTA group compared with NS group (p=0.18) (Fig. 6).

Fig. (6). The effect of G2013 on IL-1β production by human PBMCs. Abbreviations: NS: non-stimulated; G: G2013; LPS: lipopolysaccharide; LTA: lipoteichoic acid; OX: OxPAPC. Data are shown as mean±SD. * Indicates significant statistical difference with control group. # Indicates significant difference with LPS group and the sign $ indicates significant difference with LTA group.

3.3.2. The Effect of G2013 on IL-6 Secretion by the PBMCs

At the baseline level, the PBMCs produced 10.4±8.8 ng/mL IL-6 and this amount increased in low-dose G2013 group to 14.5±13.5 ng/mL but this increase was not statistically significant (p=0.13). Also, high-dose G2013 treatment could not significantly increase the IL-6 amount 12.2±9.8 ng/mL (p=0.66) (Fig. 7).

Fig. (7). The effect of G2013 on IL-6 production by human PBMCs. Abbreviations: NS: non-stimulated; G: G2013; LPS: lipopolysaccharide; LTA: lipoteichoic acid; OX: OxPAPC. Data are shown as mean±SD. * Indicates significant statistical difference with control group. # Indicates significant difference with LPS group, $ indicates significant difference with LTA group.

4. DISCUSSION

Considering the fundamental role of TLR2 and TLR4 in innate and adaptive immunity and their effect in developing defense mechanisms against invading pathogens, inflammatory and autoimmune disorders we performed this study to evaluate the immunomodulatory properties of the novel NSAID drug G2013 on these receptors. In the present study, we did not observe any considerable alteration in the protein expression of TLR2 and TLR4 on the PBMCs after incubation with G2013. However, along with our previous studies [24, 25], we showed that G2013 had a modulatory effect on gene expression of downstream molecules in the TLR signaling pathway. Profound decrease was observed in mRNA expression of MyD88 and NF-κB in cells treated with G2013 with high dose, in comparison with untreated control cells. In addition, the G2013 significantly down-regulated IkB gene expression after treatment with both low and high doses of G2013, presenting a dose dependent pattern which by increasing the concentration of G2013 the gene expression of IkB was progressively decreased. Tollip is an inhibitory adaptor protein inhibited NF-κB activation through complex formation with members of the IRAK family [26]. Our results showed that LPS did not affect the Tollip expression while G2013 had contradictory effects on Tollip expression, so that G2013 up-regulated significantly Tollip gene expression at low-dose and down-regulated its expression at
high-dose in comparison with un-stimulated cells. This opposing behavior of Tollip in response to G2013 suggests this molecule as a target for the immunomodulatory effect of G2013. However, it should be noted that the apparent contradictory effect on Tollip mRNA may be due to the particular time point chosen for the assay, so we recommend that validation of this time point should be considered in future works.

Consistency with these data, we have showed in our previous studies on HEK h TLR2 and TLR4 cell lines that G2013 or its epimer form M2000 (β-d-mannuronic acid) could significantly diminish interleukin-1 receptor associated kinase-1 (IRAK1) and (TNF) receptor associated factor 6 (TRAF6) and NF-κB without altering miR-146a (an anti-inflammatory factor) [25, 27]. The anti-inflammatory effects of the patented G2013 have been investigated on experimental autoimmune encephalomyelitis (EAE) and they showed that G2013 modulates EAE, at least in part, by suppressing nitric oxide (NO) production and this inhibition was in agreement with clinical and histopathological findings [24]. Another aspect of previous studies represents the safety effect of G2013 and β-D -mannuronic acid (analog of α-L-guluronic acid) in animal models of MS, rheumatoid arthritis, nephrotic syndrome, and acute glomerulonephritis [28].

In addition, high-doses G2013 suppressed the production of proinflammatory cytokines. We observed a significantly reduced IL-1β secretion by PBMCs after treatment with G2013 in comparison with un-stimulated PBMCs. Unlike our finding, it was observed that the unsaturated guluronate polymer induces IL-1α, IL-1β, IL-6 and TNFα secretion from a mouse macrophage cell line. It was suggested that the structural conformation and molecular size of the oligomer were important for stimulation of cytokine production.

Rather than 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 [29]. Although the main role of TLRs in autoimmune diseases remains to be established; it is clear that engagement of TLRs results in the activation of the NF-κB pathway, which enhances secretion of inflammatory cytokines such as IL-6, IL-1, TNF-α and type I IFNs and these may drive inflammation in autoimmune diseases such as ankylosing spondylitis and rheumatoid arthritis [30]. Until recently, targeted therapeutics towards inflammatory cytokines is a long way in the treatment of autoimmune diseases; therefore, studies have been focused on identifying safer and more effective types of NSAIDs during recent years. Developing anti-inflammatory new drugs can allow for selective modulating of TLR signaling pathways including inflammatory adaptor proteins and transcription factors. These results could be ground to employ the small molecule G2013 as a new NSAID drug with immunomodulatory properties in therapeutic options in the future in ankylosing spondylitis, rheumatoid arthritis, and other autoimmune diseases. Under such ground, at present, we are evaluating the effect of G2013 in the clinical trial I on ankylosing spondylitis (IRCT2016091813739N4) and rheumatoid arthritis (IRCT2016092813739N5) patients.

However, it is essential that future studies provide relevant kinetics data about G2013. Due to the variability of our data, we suggest that further works should include a larger group of individuals and to investigate the properties of G2013 on the inhibitory protein signaling pathway.

CONCLUSION

Overall, our results showed that the immunomodulatory effect of G2013 occurred under normal condition through down-regulating NF-κB, IκB and MyD88 expression and a decline in IL-1β production. These findings indicate that G2013 may exert its regulatory effect under normal conditions via Tollip in a dose dependence pathway. These findings can be beneficial for developing anti-autoimmunity drugs based on the G2013 molecule in patients with autoimmune disorders.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the ethics committee of Tehran University of Medical Sciences.

HUMAN AND ANIMAL RIGHTS

No animals were used for studies that are base of this research. All human procedures were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.
CONSENT FOR PUBLICATION

Human subjects used in the study provided informed consent to participate.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

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