Interleukin-37 suppresses ICAM-1 expression in parallel with NF-κB down-regulation following TLR2 activation of human coronary artery endothelial cells

Yandan Xie, Yuguang Li, Xiangna Cai, Xin Wang, Jilin Li

Department of Cardiology, First Affiliated Hospital of Shantou University Medical College, Shantou City, Guangdong province, China

Department of Plastic Surgeon, First Affiliated Hospital of Shantou University Medical College, Shantou City, Guangdong province, China

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ABSTRACT

Introduction: The inflammatory receptor Toll-like receptors (TLRs) activation could induce endothelial inflammatory responses, which plays an important role in the development of many diseases including atherosclerosis. We already found that TLR2 activation of Peptidoglycan (PGN) stimulation could increase intercellular adhesion molecule-1 (ICAM-1) expression in HCAECs. Since anti-inflammatory cytokine interleukin (IL)-37 exhibits intra- and extracellular properties for suppressing innate inflammation, we want to investigate whether IL-37 suppresses ICAM-1 expression and this effect is in parallel with the inhibition of nuclear factor kappa B (NF-κB) activation upon PGN stimulation in HCAECs.

Methods: HCAECs were treated with IL-37-transfection plasmid or silent mRNA or nothing for 24 h, and we test IL-37 expression by immunoblotting. Same treatments prior to PGN stimulation (10 μg/ml), we analyzed the expression of ICAM-1 and NF-κB mRNA at 0, 30 min, 1 and 2 h by real-time PCR. ICAM-1 protein at 24 h and NF-κB activation at 0–2 h were measured by immunoblotting.

Results: IL-37 and silent IL-37 transfection change the expression of IL-37 protein. Stimulation of PGN increased both NF-κB activation and ICAM-1 expression at mRNA and protein level, but these inflammatory cytokines expression was significantly decreased in IL-37-transfection cells. Interestingly, both NF-κB activation and ICAM-1 expression were significantly increased when IL-37 was silent.

Conclusions: As an anti-inflammatory cytokine, IL-37 could decrease both NF-κB and ICAM-1 expression upon TLR2 activation in HCAECs. The suppressed effect of IL-37 on ICAM-1 may be due to its inhibition on NF-κB.

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1. Introduction

IL-37 is already considered as a fundamental inhibitor with intra- and extracellular properties for suppressing innate inflammation [1,2]. It is already proved to be involved in the systematic inflammatory diseases such as rheumatoid arthritis, atherosclerotic diseases and some others [3–6]. These diseases could be treated when inhibition of pro-inflammatory cytokines by IL-37 [3,7]. Evidence showed that IL-37 was expressed in the foam-like cells of atherosclerosis coronary and carotid artery plaques in IL-37-transgenic mice, suggesting that IL-37 is involved in the formation of atherosclerotic plaque [8]. It is still unclear how this factor is involved in the process and the role on the development of atherosclerosis.

As an inflammatory disease, atherosclerosis is characterized by extensive lipid deposition and atherosclerotic plaque formation in the intima, which is initiated and enhanced by inflammatory responses of endothelial cells. Endothelial dysfunction caused by inflammation is a key initiating event in atherosclerotic plaque formation. [9,10]. Some studies already found that IL-37 expression ameliorates inflammatory responses in epithelial cells, macrophages and dendritic cells and psoriasis disease [1,6,11,12]. It is not determined the role of IL-37 plays in the inflammation of HCAECs. And these studies also indicate that IL-37 possibly ameliorates the inflammation process on the development of atherosclerosis if it can reduce inflammatory response in HCAECs.
Toll-like receptors (TLRs) are pathogen pattern recognition receptors that recognize bacterial and viral products, and other pathogens. We already found that TLR2/4 stimulations cause inflammation in HCAECs including ICAM-1 and NF-κB [13]. It is already proved that IL-37 could inhibit innate immune responses upon the stimulation of TLR4 ligand Lipopolysaccharide (LPS) [14]. In this study, we want to investigate whether IL-37 could reduce ICAM-1 expression activated by PGN in HCAECs, and the possible mechanism of inhibition of IL-37 is in parallel with NF-κB, a key inflammatory factor on the TLR2 pathway.

2. Materials and methods

2.1. Materials

HCAECs were purchased from ScienCell Research Laboratories (San Diego, California, USA). IL-37b plasmid vector with a GFP expression sequence and silent IL-37 mRNA were produced in Genepharma (Shanghai, China). Staphylococcus aureus PGN was from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were used for Western-blot analysis: mouse anti-human interleukin 37 (Abcam, Cambridge, MA, USA), mouse anti-human intercellular adhesion molecule (ICAM)-1 (Abcam, Cambridge, MA, USA), rabbit anti-human NF-κB p65 (Abcam, Cambridge, MA, USA), rabbit anti-human phospho-NF-κB p65 (Cell Signaling Technology, Boston, MA, USA), and rabbit anti-human GAPDH (Xianzi Biological Tech, Hangzhou, China). RNAeasy micro kit was purchased from PrimeScript RT Master Mix (TAKARA Bio, Dalian, China).

2.2. Culture of human HCAECs

Cells were grown in endothelial cell growth medium (ECM from ScienCell, San Diego, California, USA). For the experiments, cells were seeded in 1 ml complete medium in 12-well plates. After growing to confluence, medium was changed completely. PGN was diluted in complete cell culture medium and added to the cells. The final concentration of PGN was 10 μg/ml. For IL-37b transfection or silence, HCAECs were incubated with IL-37b or silent IL-37 plasmid (final concentrations were 1.0 μg/ml or 40 pmol/ml, respectively) using the Lipofectamine® 3000 (Invitrogen Life Technology, Shanghai, China), followed by a 24 hour recovery.

2.3. Immunoblotting

Immunoblotting was used to detect IL-37, ICAM-1, phosphorylated NF-κB p65, and GAPDH. After treatment, HCAECs were washed three times with cold PBS, and then lysed with lysis buffer (protease inhibitor cocktail and Mammalian Protein Extraction Reagent, Thermo Scientific, Waltham, MA, USA). Samples were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinyl membranes. Membranes were blocked for 1 h at room temperature with 10% dry milk in TBST (TBS containing 0.1% Tween 20) and then incubated with the appropriate primary antibodies (IL-37 antibody was diluted 1:500, ICAM-1 1:1000, Phosphor-NF-κB p65 1:1000, GAPDH 1:1000) overnight at 4 °C. After washing with TBST, membranes were incubated with Alkaline phosphatase-linked secondary antibodies (1:4000 dilution with TBST) at room temperature for 1 h. Bands were developed using ECL and exposed on X-ray films. Band density was analyzed using NIH Image software.

2.4. RNA isolation and real-time PCR

Confluent HCAECs were pre-treated with IL-37b or silent IL-37 plasmid for 24 h, and then treated with or without PGN. Thereafter cells were harvested with TRIzol reagent (Life Technologies, Shanghai, China) according to the manufacturer’s instructions. cDNA was prepared by reverse transcription. Real-time PCR was performed using CFX Connect Real-Time System (Applied BIO-RAD, Hercules, California, USA) and SYBR Premix Ex Taq (Tli RNaseH Plus) (TAKARA Bio, Dalian, China). Each reaction was carried out for a total volume of 25 μl (12.5 μl SYBR Premix Ex Taq (Tli RNaseH Plus) (2×), 1 μl PCR Forward Primer, 1 μl PCR Reverse Primer, 2.0 μl of synthesized cDNA, and 8.5 μl dH2O). Reaction mixtures were heated to 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing extension at 60 °C for 30 s. The following sets of primers were used to amplify specific cDNA fragments: GAPDH (house-keeping gene), forward: 5′-CTG GGA GGA GAA GAT GC; reverse: 5′-ACC TTT GTT CCA CGA CCC ATA GC-3′; ICAM-1, forward: 5′-AGC TTC TCC TTC CTG CCT GCA AC; reverse: 5′-GTC TGC TGG GAA TTT TCT GCA GC; NF-κB p65-1, forward: 5′-GCG AGA GCA GCA ACA GAT ACC; reverse: AGG GGT GTG TGT TGG TGG TGG GGC.

2.5. Statistics

Data are expressed as mean ± standard error of mean (SEM). Analysis of variance (ANOVA) was performed, and differences were considered significant when P < 0.05, as verified by Fisher post-hoc test.

3. Results

3.1. Expression of IL-37 reduce PGN-induced ICAM-1 production in endothelial cells

The expression of IL-37 protein was significantly increased in IL-37-transfection cells and significantly decreased when IL-37 was silent (Fig. 1). PGN increased NF-κB activation and ICAM-1 levels in HCAECs [13]. We examined the effect of IL-37 on ICAM-1 levels in cell lysate of HCAECs exposed to PGN by immunoblotting. Fig. 2 shows that PGN increased ICAM-1 protein production, and this effect was markedly reduced in IL-37 transfected HCAECs. Furthermore, silence of IL-37 could not reduce PGN-induced ICAM-1 levels. To confirm the effect of IL-37 on ICAM-1, we also analyzed ICAM-1 mRNA at 0, 30 min, 1 h and 2 h by real-time PCR (Fig. 3). The results show that ICAM-1 mRNA markedly decreased in IL-37 plasmid group compared to control group at 1 h and 2 h (P < 0.05). Interestingly, silence of IL-37 could increase ICAM-1 mRNA and protein production if compared to control when TLR2 activation (P < 0.05). These results indicate IL-37 could suppress PGN-mediated inflammatory responses in endothelial cells.

Fig. 1. HCAECs were treated with IL-37-transfection plasmid or silent mRNA for 24 h. IL-37 protein levels were analyzed by immunoblotting. IL-37 protein increased in IL-37 transfected cells while decreased in silent group compared to control.
3.2. Expression of IL-37 abrogate PGN-induced NF-κB activation in endothelial cells

PGN increased ICAM-1 levels dependent on NF-κB activation in HCAECs [13]. To test whether the suppression effect of IL-37 on ICAM-1 level is associated with change of NF-κB activation in HCAECs, we analyzed NF-κB activation at 10 min, 30 min, 1 h and 2 h by immunoblotting (Fig. 5), and NF-κB mRNA by real-time PCR (Fig. 4). It is shown that PGN-mediated NF-κB mRNA was markedly reduced at 30 min, 1 h and 2 h time point when exposed to IL-37 (P < 0.05), while it was markedly increased when exposed to silent IL-37 (P < 0.05). And NF-κB protein level was markedly decreased, while it was also markedly increased when IL-37 silent (Fig. 5). These results indicated that suppression of IL-37 on ICAM-1 production is in parallel with the reduction of NF-κB activation.

Fig. 2. Expression of IL-37 reduces ICAM-1 protein in HCAECs following TLR2 stimulation. 24 h prior to the treatment, HCAECs were treated with IL-37-transfection plasmid or silent mRNA or nothing, then were stimulated with PGN (10 μg/ml) for 24 h, and ICAM-1 protein levels were analyzed by immunoblotting. IL-37-transfection cells exhibited a greater decrease in ICAM-1 protein levels compared to control and silent group.

Fig. 3. Expression of IL-37 reduces ICAM-1 mRNA in HCAECs following TLR2 stimulation. 24 h prior to the treatment, HCAECs were treated with IL-37-transfection plasmid or silent mRNA or nothing, then were stimulated with PGN (10 μg/ml) for 24 h, and ICAM-1 mRNA levels were analyzed by real-time RT-PCR. IL-37-transfection cells expressed lower levels of ICAM-1 mRNA at 1 and 2 h (P < 0.05). The levels of ICAM-1 mRNA significantly increased when IL-37 was silent at 1 and 2 h (P < 0.05). Results are expressed as Mean ± SEM; n = 5; *P < 0.05 vs. cells treated with PGN.

Fig. 4. Expression of IL-37 reduces NF-κB mRNA in HCAECs following TLR2 stimulation. 24 h prior to the treatment, HCAECs were treated with IL-37-transfection plasmid or silent mRNA or nothing, then were stimulated with PGN (10 μg/ml) for 24 h, and NF-κB mRNA levels were analyzed by real-time RT-PCR. IL-37-transfection cells expressed lower levels of NF-κB mRNA at 30 min, 1 and 2 h (P < 0.05). But the levels of NF-κB mRNA significantly increased when IL-37 was silent at 1 and 2 h (P < 0.05). Results are expressed as Mean ± SEM; n = 5; *P < 0.05 vs. cells treated with PGN.
IL-37 is found to have an anti-inflammatory function in a variety of cell types in humans [14,15]. In this study, we firstly confirm that expression of IL-37 in HCAECs could be regulated when treated with IL-37-transfection plasmid or silent mRNA for 24 h. We found that IL-37, as an anti-inflammatory cytokine, could decrease ICAM-1 and NF-κB expression mediated by TLR2 activation in HCAECs. But ICAM-1 and NF-κB expression were increased when IL-37 was knocked out. The present study shows that IL-37 could effectively decrease inflammatory response of TLR2-NF-κB pathway.

IL-37 is found to have an anti-inflammatory function and has been demonstrated in a variety of cell types from different species, including in humans and rodents [14,15]. It is not well demonstrated that inhibited mechanism of IL-37 on inflammation both in extra- and intracellular. Previous studies show that intracellular IL-37 can translocate to the nucleus to down-regulate pro-inflammatory cytokine expression [13,16], and extracellular forms of IL-37 utilizes IL-1R8 to exert its effect of inflammation inhibition both in vitro and in vivo [2]. Furthermore, a mutation on the caspase-1 cleavage site of IL-37 makes it incapable of reducing LPS-induced immune responses in IL-37 transfected cells [1]. In the present study on HCAECs, we found that ICAM-1 and NF-κB expression significantly decreased by overexpression of recombinant IL-37b, while this effect is not exhibited even abrogated markedly when IL-37 mRNA is silent. We can consider that the effect and mechanism of IL-37 on ICAM-1 expression may be due to the reduction of NF-κB.

TLR activation is associated with an increase in intranuclear NF-κB phosphorylation. NF-κB is a key transcription factor that translocates to the nucleus to initiate the transcription of pro-inflammatory genes, so that mediates cellular inflammatory response including the expression of chemokines and cytokines [17]. The activation of NF-κB occurs through phosphorylation of IκBα by IKKβ and/or phosphorylation of p100 by IKKα, leading to the degradation of IκBα and/or the processing of p100 into smaller form (p52). This process allows two forms of activated NF-κB (p50–p65 and p52-ReB) to become free, resulting in the translocation of the activated NF-κB into the nucleus for binding to NF-κB-specific DNA-binding sites, which, in turn, regulates the transcription of target genes, including ICAM-1 [18]. In this study, we demonstrate the effect of IL-37 on ICAM-1 level by decrease of NF-κB phosphorylation. But we are not sure whether this effect of IL-37 could inhibit NF-κB phosphorylation directly or decrease upstream regulatory factors indirectly, such as IκBα, or IKKβ, or IKKα. This needs to be further explored in the future study.

Another possibility of inhibited effect of IL-37 on ICAM-1 may be independent on NF-κB phosphorylation, but may be due to the inhibition of IL-18. IL-18 could increase ICAM-1 expression [19,20], while IL-37 was reported to inhibit IL-18 by enhancing the effect of IL-18BP [15], so the decreasing effect of IL-37 on ICAM-1 may be through IL-18 pathway. So far, we can exclude this possibility, and have to make more studies to make it clear in the future study.

IL-37 is found to exhibit anti-inflammatory function in a variety of cell types in humans [14,15]. When this cytokine mRNA was silent, the present study in HCAECs shows that inflammation was significantly enhanced. This should be because anti-inflammatory function of IL-37 was also exhibited in control, but disappeared in silent group. This result further confirmed our hypothesis that IL-37 has anti-inflammatory function upon TLR2 activation in HCAECs.

Inflammatory activation of the endothelial cells is a critical step in the development of many diseases, including atherosclerotic plaque formation [21–23]. IL-37 was found to ameliorate the inflammatory process in psoriasis by suppressing proinflammatory cytokine production [11]. Since TLR2 activation can increase ICAM-1 expression [13], the inhibition of TLR2-NF-κB-ICAM-1 pathway in HCAECs can significantly decrease the development of many diseases. Present study indicates that the effect of IL-37 in HCAECs may improve endothelial dysfunction, so that control the development of many diseases. And increasing recent studies already confirm the effect and mechanism of this anti-inflammatory cytokine. So how to enhance the effect of IL-37 may be the next study point in the future.

5. Conclusion

In our study, the results of present study in HCAECs show that NF-κB-ICAM-1 expression upon TLR2 activation could be decreased by IL-37. The decreasing expression of IL-37 on TLR2-NF-κB-ICAM-1 pathway in HCAECs may help to control many diseases including atherosclerosis.

Authors’ contributions

XC and JL are involved in experimental design, acquisition and analysis of data, and drafted the manuscript. YX and YL participated in the experiment and the acquisition and analysis of data. YL and XW were involved in drafting the manuscript. All authors read and approved the final manuscript.
Conflicts of interest

The authors have no conflicts of interest to disclose.

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