Protective effect of the polarity of macrophages regulated by IL-37 on atherosclerosis

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ABSTRACT. As an anti-inflammatory cytokine, interleukin-37 (IL-37) provides certain protective effects against inflammatory and autoimmune diseases. Recent reports indicate that IL-37 is expressed in foam cells of atherosclerotic plaques in both the coronary and carotid arteries of humans, suggesting the possible involvement of IL-37 in the pathogenesis and progression of atherosclerosis. Current evidence supports the protective role that IL-37 plays against atherosclerosis via the regulation of different subtypes of macrophage. Atherosclerosis was induced in apolipoprotein E -/- mice through diet, and the mice were then given IL-37 to observe patterns in the aorta plaque. Furthermore, human peripheral blood-derived monocytes were cultured for seven days to induce the differentiation of macrophages. Specifically, we observed the effect of IL-37 on oxygenated low density lipoprotein (ox-LDL)-induced macrophage polarity, in addition to conducting an expressional assay of the M1 cell markers tumor necrosis factor (TNF)-α and CD86 and the M2 marker CD206. IL-37 effectively decreased the area ratio between the aorta plaque and vascular cavity. We also observed that M1 macrophages can be induced from peripheral monocytes by ox-LDL, with significant elevation of marker molecules

TNF-α and CD86. The co-stimulation of IL-37 and ox-LDL, however, inhibited the induction of M1 cells and facilitated the transformation of macrophages into M2 cells, as supported by the elevation of cell-specific marker CD206. These results indicate that IL-37 can prevent atherosclerosis by modulating macrophage polarity.

Key words: IL-37; Macrophage polarity; Atherosclerosis; Artery plaque

INTRODUCTION

Atherosclerosis is a current major health concern and demonstrates an increasing occurrence rate and decreasing age of primary onset. The lack of effective treatments contributes to its high mortality and disability rates. Inflammation is a major risk factor in the occurrence and development of atherosclerosis. Recent studies have shown the important role of the transformation between M1 and M2 sub-type macrophages in atherosclerosis (Wu et al., 2013). Different macrophage sub-types can be observed in atherosclerotic plaques. As previously reported, about 40% M1 cells and 20% M2 cells reside in the plaques of model mice (Adamson and Leitinger, 2011). Oxygenated low density lipoprotein (ox-LDL) can facilitate the transformation of macrophage or M2 cells into M1 cells and stimulate the secretion of pro-inflammatory cytokines including interleukin-6 (IL-6), IL-18, and monocyte chemotactic factor-1, thereby accelerating the progression of atherosclerosis (Duewell et al., 2010; Hirose et al., 2011; van Tits et al., 2011). Therefore, the modulation between different subtypes of macrophage may provide new drug targets for the treatment of atherosclerosis.

IL-37 is a novel anti-inflammatory cytokine in the IL-1 family and exerts a negative modulatory role in the inflammatory response of innate immunity. Mainly present in the brain, kidney, heart, and monocytes or dendritic cells in peripheral blood, IL-37 has been widely reported to have anti-inflammatory and immune suppressing functions (Boraschi et al., 2011; Dinarello and Bufler, 2013; Teng et al., 2014). Studies have also indicated the role IL-37 plays in inhibiting the activation of macrophages and discovered its expression in the foam cells of atherosclerotic plaques in the coronary artery and carotid (Ji et al., 2014). These studies indicated a potential protective role of IL-37 against atherosclerosis. Therefore, we hypothesized that IL-37 may be able to inhibit the occurrence and progression of atherosclerosis by modulating the transformation of different subtypes of macrophage. In this study, we investigated if IL-37 can regulate the polarity of macrophages and inhibit the formation of atherosclerotic plaques.

MATERIAL AND METHODS

Animal models

Sixteen male apolipoprotein E knockout mice (8 weeks old, body weight 22-25 g, provided by Weitong Lihua Corp., China) were randomly divided into the control group (N = 8) and IL-37 treatment group (N = 8). After 8 weeks of high fat and high cholesterol diet feeding, treatment animals were intraperitoneally injected with 2 mg recombinant IL-37 protein (R&D Corp., USA) daily for two weeks, while control mice were given 200 μL saline for two weeks.

Sample collection and preparation

All mice were perfused with saline and 4% paraformaldehyde and were then sacrificed for collection of the heart and aorta, which were then fixed in 4% paraformaldehyde after separating peripheral tissues. The heart was then dissected horizontally into two parts. The upper section was dehydrated, embedded in paraffin, and cut into consecutive slices 5-mm thick. Six slices were chosen every 40 mm for further hematoxylin-eosin and oil red O staining. The tissue morphology of atherosclerotic plaques was observed under a light field microscope. The Image Pro Plus software package (Version 6.0 for Windows) was used to analyze tissue morphology for parameters including vessel cavity area, plaque are and plaque/cavity area ratio. Averaged values were obtained for further statistical analysis.

Separation and induction of human macrophages

Venous blood (5 mL) was collected from healthy donors in EDTA-treated tubes. Monocytes were separated using the density gradient centrifugation method and were re-suspended in RPMI 1640 medium. Cells were cultured with 5% CO_2 for 60 min to collect adhesion cells. Giemsa staining confirmed the monocyte nature of such cells with purity >90%. Trypan blue staining revealed the cell viability was >95%. Collected monocytes were re-suspended in RPMI 1640 containing 20% human serum. Cells at 1 x 10^6 /mL were seeded on 24-well plates for further induction of macrophages in three groups: 1) control; 2) ox-LDL (50 mg/L), and 3) ox-LDL (50 mg/L) and IL-37 (50 mg/L). All cells were cultured for 7 days to induce the differentiation of macrophages.

Western blotting for macrophage markers

Cultured cells in all groups were lysed in RIPA lysing buffer (with 1% PMSF) on ice for 30 min, followed by centrifugation at 13,000 rpm for 30 min. Supernatants were quantified for protein concentration. Equal amounts (30 mg) were separated by SDS-PAGE and transferred to a PVDF membrane, which was then blocked in 5% defatted milk powder for 1 h. Rabbit antibody against tumor necrosis factor (TNF)-α (Cell Signaling, USA), CD68 (Cell Signaling, USA), CD206 (Abcam, USA), and β-actin (Cell Signaling, USA), all at 1:1000 dilutions, were added to incubate the membrane overnight. On the next day, the membrane was incubated with goat anti-rabbit IgG (Cell Signaling, USA), diluted 1:500, for 1 h. Colors were developed using ECL reagents. Staining images were captured under a light-field microscope, and the Image J software was used to quantify the protein expression level based on the optical density of blotting bands.

Statistical analysis

The SPSS 13.0 software package was used to process all collected data, which were first tested for normality. Measurement data are reported as means ± standard deviation. Between-group comparisons were performed by the Student *t*-test while the analysis among multiple groups was performed with a one-way analysis of variance (ANOVA) followed by the post-hoc SNK test. Results were considered statistically significance when $P < 0.05$.

RESULTS

IL-37 decreases the aorta plaque/vessel cavity area ratio of atherosclerotic mice

Histological observations showed that IL-37 intervention effectively decreased the ratio of aorta plaque/vessel cavity area in atherosclerotic mice, suggesting an inhibitory role of IL-37 in the formation of atherosclerotic plaques (Table 1).

Table 1. Plaque area and vessel cavity area in atherosclerotic mice.

Group	N	Cavity area (μm^2)	Plaque area (μm^2)	Plaque/cavity area ratio
Control	8	213.40 ± 48.60	168.20 ± 60.82	0.78 ± 0.42
IL-37	8	263.65 ± 48.68	30.13 ± 10.75	0.11 ± 0.04*

*P < 0.05 compared to control group.

IL-37 modulates ox-LDL-induced macrophage polarity

Western blot results (Figure 1) showed that ox-LDL induced the transformation of human peripheral blood-derived monocytes into primarily M1 type macrophages, with significant elevation in the protein expression of the major cell markers TNF-α and CD86. When co-applied with ox-LDL, IL-37 inhibited the transformation into M1 cells and facilitated the differentiation into M2 cells, as supported by an elevation in the major cell marker CD206.

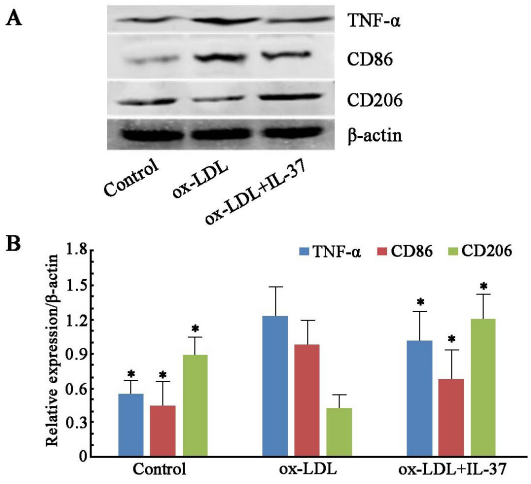

Figure 1. Effects of IL-37 on ox-LDL-induced macrophage polarity. **A.** Representative bands from western blotting. **B.** Quantified results showing relative protein expression levels. *P < 0.05 compared to ox-LDL group.

DISCUSSION

As an important component of the body's immune system, macrophages are believed to have pleiotropic roles, such as in immune induction and lipid phagocytes. They thereby play an important role in the pathogenesis and progression of atherosclerosis by facilitating inflammation and lipid deposition (Trpkovic et al., 2015; Lundqvist et al., 2015). Macrophages

can differentiate into the M1 and M2 types, which can be inter-transformed depending on the body's microenvironment (Medbury et al., 2014; Chen et al., 2015). In atherosclerotic plaques, both M1 and M2 macrophages exist, although they are responsible for distinct functions (Fadini et al., 2014). These macrophages can be distinguished by the differential expression of cytokines, surface molecules, and arginine metabolic pathways (Peled and Fisher, 2014). Under the stimulation of lipoprotein, macrophages can differentiate into M1 cells, which induce the secretion of large amounts of pro-inflammatory factors including IL-12, IL-23, IL-6, and TNF-α, all of which can participate in the inflammatory response and facilitate reactive oxygen release, thereby facilitating the progression of atherosclerosis (Singla et al., 2014). M2 cells, on the other hand, can be induced from macrophages under the direction of IL-4 and IL-13. M2 cells can inhibit the inflammatory response and facilitate the recovery of tissue injuries via the release of IL-10, arginase, and the scavenger receptor (Spivia et al., 2014). It is interesting to note that the major component of atherosclerotic plaques, oxygenated cholesterol, primarily facilitates the differentiation of M1 cells through activation of the MAP signaling pathway (Fang et al., 2010). All of these studies support a critical role of M1/M2 cell differentiation in the pathogenesis and progression of atherosclerosis.

As a newly identified member of the IL-1 family, IL-37 has been shown to have anti-inflammatory and immune suppressing functions (Boraschi et al., 2011), and it is known to be involved in the progression of various inflammatory diseases (Nold et al., 2010). Current studies have demonstrated that such functions of IL-37 are mainly related to its inhibition of the release of inflammatory cytokines, including IL-1β, IL-18, and TNF-α (Li et al., 2015; Nold-Petry et al., 2015). For example, IL-37 can potentiate the inhibitory function of IL-18BP on IL-18 via specific binding (Bufler et al., 2004). It is worth noting that recent studies have identified IL-37 expression in foam cells of atherosclerotic plaques in the human coronary artery and carotid (Wu et al., 2013; Ji et al., 2014), suggesting the potential role of IL-37 in the pathology of atherosclerosis. The nature of this function, i.e, whether a protective or aggravating role, has not been illustrated so far. Due to its anti-inflammatory nature, IL-37 was hypothesized in this study to play a protective role against atherosclerosis. Our *in vitro* studies showed that ox-LDL induced peripheral blood-derived monocytes to differentiate into M1 cells, as supported by the significant up-regulation of the M1 cell markers TNF-α and CD86. The co-stimulation of ox-LDL and IL-37, however, induced the macrophages to instead differentiate into M2 cells, with a corresponding elevation in CD206 protein expression. These results suggest that IL-37 can modulate the response of macrophages to liposomes and facilitate the differentiation towards anti-inflammatory M2 cells. Such results were also confirmed in atherosclerotic mice, in which recombinant IL-37 protein injection effectively inhibited the formation of atherosclerosis.

In summary, this study has demonstrated the modulatory role of IL-37 on macrophage polarity, along with its protective function against atherosclerosis. The critical role of macrophages in atherosclerosis suggests that the transformation between different subtypes of macrophages may provide novel drug targets in treating atherosclerosis.

Conflicts of interest

The authors declare no conflict of interest.

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