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Interleukin-10 increases reverse cholesterol transport in macrophages through its bidirectional interaction with liver X receptor α

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ABSTRACT

Interleukin (IL)-10 is a prototypical anti-inflammatory cytokine that has been shown to attenuate atherosclerosis development. In addition to its anti-inflammatory properties, the anti-atherogenic effect of IL-10 has recently also been suggested to reflect a complex effect of IL-10 on lipid metabolism in macrophages. In the present study we examined the effects of IL-10 on cholesterol efflux mechanism in lipid-loaded THP-1 macrophages. Our main findings were: (i) IL-10 significantly enhanced cholesterol efflux induced by fetal-calf serum, high-density lipoprotein $(HDL)_2$ and apolipoprotein A-1. (ii) The IL-10-mediated effects on cholesterol efflux were accompanied by an increased IL-10-mediated expression of the ATP-binding cassette transporters ABCA1 and ABCG1, that was further enhanced when the cells were co-activated with the liver X receptor (LXR)α agonist (22R)-hydroxycholesterol. (iii) The effect of LXRα activation on the IL-10-mediated effects on the ATP-binding cassette transporters seems to include enhancing effects on the IL-10 receptor 1 (IL10R1) expression and interaction with STAT-3 signaling. (iv) These enhancing effects on ABCA1 and ABCG1 was not seen when the cells were stimulated with the IL-10 family members IL-22 and IL-24. This study suggests that the anti-atherogenic properties of IL-10 may include enhancing effects on cholesterol efflux mechanism that involves cross-talk with LXRα activation.

1. Introduction

Accumulation of modified low-density lipoprotein (LDL) in macrophages, leading to foam cell formation, is a key event in all stages of atherosclerosis from the formation of early fatty streaks to the induction of plaque rupture within an advanced lesion [8]. The interaction between modified lipoproteins and macrophages does not only lead to lipid accumulation within these cells, but also promotes the release of inflammatory mediators leading to enhanced vascular inflammation and plaque progression [11,12]. A crucial step in the formation of foam cell macrophages is the internalization of modified LDL through specific scavenger receptors, but the degree of lipid accumulation is also dependent on cholesterol efflux that involves high-density lipoprotein (HDL)-related mechanisms [18]. In this reversed cholesterol transport, the activity of the ATP-binding cassette transporters such as ABCA1 seems to be of particular importance [19].

Several studies have suggested atheroprotective effects of the anti-inflammatory cytokine interleukin (IL)-10 (reviewed by [21]). Elevated IL-10 serum levels are associated with a more favorable prognosis in patients with acute coronary syndromes [1]. Also, various studies in mice prone to develop atherosclerosis have shown attenuating effects of IL-10 on atherogenesis, at least partly involving effects on lipid accumulation [6]. We and others have previously shown that IL-10 promotes foam cell formation through anti-apoptotic mechanisms [4], and some recent studies have also suggested that IL-10 could influence cholesterol efflux [5]. However, the ability of IL-10 to modulate reversed cholesterol transport as well as its mechanism of action in this process is far from clear.

To further elucidate the role of IL-10 in foam cell formation in macrophages, we examined the ability of IL-10 to modulate

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cholesterol efflux mechanisms in more detail. As the ABCA-1 and other relevant ATP-binding cassette transporters are target genes for the liver X receptor (LXR)α, particular focus was directed against the interaction between IL-10 and LXRα.

2. Materials and methods

2.1. Experiments in THP-1 macrophages

The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD) was cultured in regular growth medium that consist of RPMI-1640 (Gibco, Life Technologies, Grand Island, NY) with 10% fetal calf serum (FCS; Sigma), 1 mM penicillin–streptomycin, 200 mM L-glutamine (Sigma, St. Louis, MI). Prior to all experiments the THP-1 cells were differentiated into macrophages by incubation for 48–72 h with 100 nM phorbol myristate acetate (PMA, Sigma) in 12-wells trays (Costar, Cambridge, MA; 1.0×10^6 -cells/ml). Fully differentiated THP-1 macrophages were then loaded with oxidized (ox)LDL (20 μg/ml) in regular growth medium for 24 h before additional culturing with and without recombinant human IL-10 (20 ng/ml; R&D System, Minneapolis, MN), the LXRα ligand (22R)-hydroxycholesterol (22R-OH(R); 2.5 μg/ml; Sigma) or a combination thereof. In a separate experiment, IL-22 and IL-24 (20 ng/ml; R&D System) was used instead of IL-10. In an additional experiment, an inhibitor peptide (PpYLKTK-mts) for signal transducer and activator of transcription 3 (STAT3) inhibitor (50 μM; Merck, Darmstadt, Germany) was added to cell culture 30 min prior to IL-10/22R-OH(R) activation. At different time points, cell pellets and supernatants were harvested and stored separately at −80 °C until further analyses.

2.2. Isolation of lipoproteins

2.2.1. LDL

For the cholesterol efflux studies the LDL was isolated from human endotoxin-free heparin plasma and oxidatively modified by Cu^{2+}-iones as previously described [3]. The oxidized LDL used throughout the rest of the studies was obtained from Kalen Biomedical, LLC (Montgomery Village, MD, USA).

2.2.2. Isolation of HDL_2 and HDL_3

Plasma from healthy individuals was stored in 0.6% sucrose at −80 °C. HDL_2 subfraction (density range 1.06–1.12 g/ml) and HDL_3 subfraction (density range 1.12–1.21 g/ml) were isolated by sequential ultracentrifugation at 8 °C in a Beckman Optima LE-80K, using rotor TI 80 as described by Henry and Pownall [7]. Density adjustments were made by the addition of solid NaBr (Sigma), and the 1.06–1.12 g/ml and 1.12–1.21 g/ml density range was used to isolate HDL_2 and HDL_3, respectively. The resulting HDL was dialyzed against phosphate-buffered saline (PBS, pH 7.4) before filter sterilization by 0.22 μm μStar LB (Costar).

2.3. Cholesterol efflux

PMA differentiated THP-1 macrophages were lipid loaded by incubation with ox LDL (20 μg/ml) in regular growth medium plus 0.5 μCi/l (18.5 mBq/l) H^3-Cholesterol (American Radiolabel Chemicals, Saint Louis, MO), dissolved in ethanol, was added. After 24 h, radiolabeled media were removed, and the foam cells were washed twice with 0.2% bovine serum albumin (BSA) (wt:v) in RPMI. Thereafter, the cells were incubated in RPMI 1640 with 10% FCS, HDL_2 (50 mg/l) or apoA-1 (15 μg/ml) as cholesterol acceptors, with and without IL-10 (20 ng/ml; R&D Systems). After 3–72 h , the cell medium was collected and the cells were harvested in 0.2 mol/L NaOH. The radioactivity was measured by liquid scintillation counting using TRI-CARB 2300 TR Scintillation Counter (Packard, Waltham, MA). Data are presented as fractional (%) cholesterol efflux calculated as dpm (media)/dpm (media + cell-associated)] × 100.

2.4. Real time quantitative PCR

Total RNA was isolated using Qiagen columns (Qiagen, Hilden, Germany), and stored −80 °C until further analysis. cDNA was synthesized using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time quantitative RT-PCR was performed using the ABI Prism 7700 (Applied Biosystems) and sequence-specific PCR primers were designed with the use of Primer Express software version 1.5 (Applied Biosystems). Primer sequences could be provided by request. SyBr Green assay was performed with the qPCR Master Mix for SYBR Green I (Eurogentec, Seraing, Belgium). Gene expression of the control gene β-actin was used for normalization.

2.5. Western immunoblotting

THP-1 macrophages were suspended in lysis buffer (Buffer A: 150 mM NaCl, 50 mM Tris–HCl, pH 7.6, 2.5 mM $CaCl_2$, 1% Triton X-100, and protease inhibitor cocktail [Roche, Mannheim, Germany]). The samples were agitated for 15 min at 4 °C followed by centrifugation for 15 min at 10,000g at 4 °C. The supernatants were stored at −70 °C until further analysis. Equal amounts of protein (30 μg) were subjected to SDS/10% PAGE (Bio-Rad, Hercules, CA), and electrophoretically transferred to PVDF filters (NEN™, Life Science, Boston, MA). Filters were blocked for 1 h at room temperature with 10% low-fat milk in Tris-buffered saline containing 0.02% Tween-20 (v/v), followed by incubation at room temperature for 1 h with anti-human P-STAT3 (Cell Signaling, Beverly, MA). Proteins were detected by enhanced chemiluminescence using horseradish peroxidase-labelled IgG (Cell Signalling). The enhanced-chemiluminescence-exposed films (Pierce, Rockford, IL) were scanned by densitometry Kodak 440 CF imaging station (Kodak, Boston, MA) and the software Total Laboratory v.1.10 (Phoretix, Newcastle, UK) was used for quantification. The filter was reprobed with anti-human-ß-tubulin (Sigma) to ensure equal loading.

2.6. Statistical analyses

The Student *t* test was used. *p* values (2-sided) were considered significant at levels <0.05.

3. Results

3.1. IL-10 enhances cholesterol efflux in lipid loaded THP-1 macrophages

We have previously reported that IL-10 increases foam cell formation in oxLDL stimulated THP-1 macrophages at least partly involving anti-apoptotic mechanisms [4]. To elucidate whether the net cholesterol homeostasis also could be more directly affected by IL-10, we studied the effect of this cytokine on cholesterol efflux in these cells. THP-1 macrophages was exposed to ^{14}C-cholesterol for 24 h before cholesterol efflux was induced by either 10% FCS, HDL_2 (20 μg/ml) or apoA-1 (15 μg/ml) as cholesterol acceptors. As depicted in Fig. 1, IL-10 (20 ng/ml) significantly enhanced cholesterol efflux induced by all these lipid acceptors after 24 h of IL-10 exposure, and for FCS- and HDL_2-induced cholesterol efflux, this increase in reverse cholesterol transport persisted for 72 h (Fig. 1A and B).

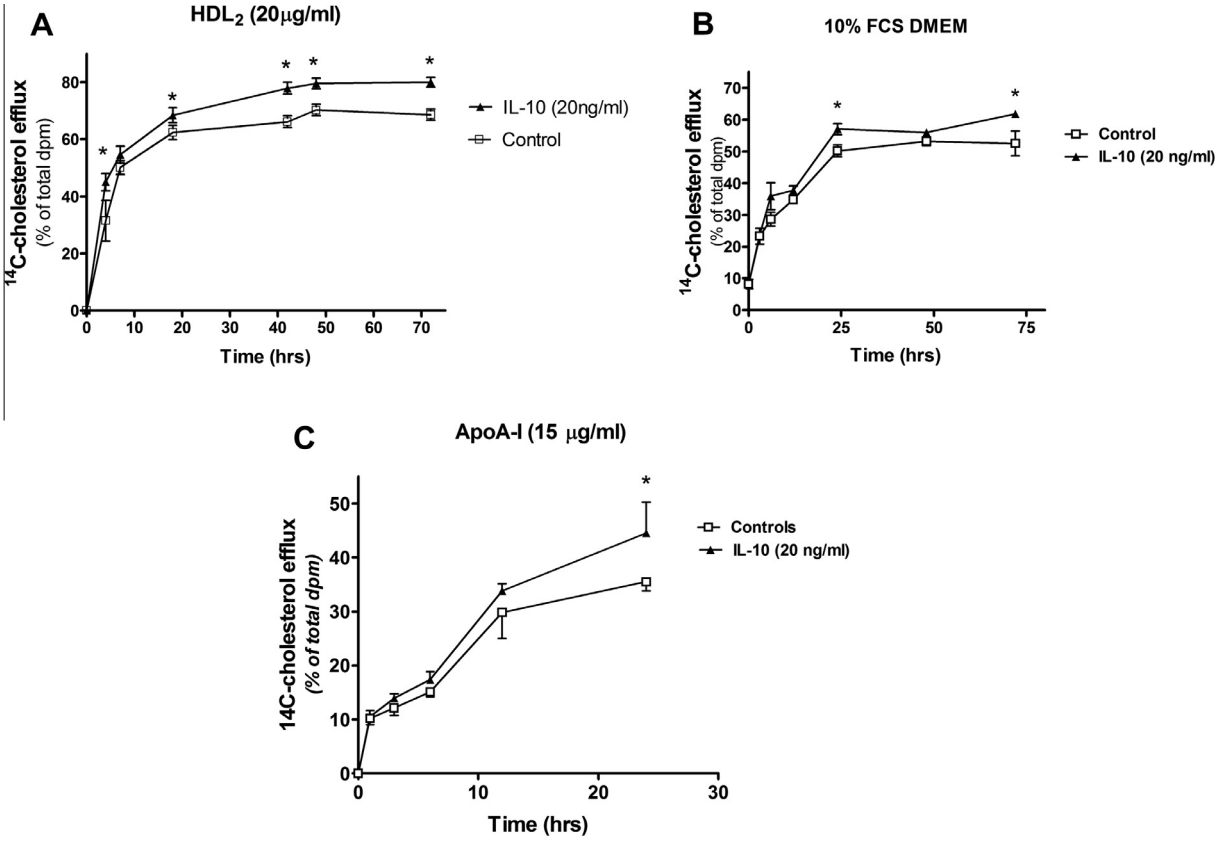

Fig. 1. Effects of IL-10 on efflux of ^{14}C-Cholesterol from lipid loaded THP-1 macrophages. PMA (100 nM)-differentiated macrophages were loaded for 24 h with 20 μg/ml oxLDL and 1 μCi/ml ^{14}C-Cholesterol in regular growth medium. The efflux was initiated by (A) 20 μg/ml HDL2, (B) 10% FCS or (C) 15 μg/ml ApoA-1. After 3–72 h the cells and conditioned media were harvested and the level of ^{14}C-Cholesterol was counted in scintillation counter. The % was calculated as outlined in Section 2. Data are presented as mean ± SEM ($n = 4$). $^{*}p < 0.05$ vs controls.

3.2. IL-10 increases the expression of mediators involved in reverse cholesterol transport in macrophages

To elucidate the mechanism for the enhanced removal of radiolabeled cholesterol from oxLDL-loaded THP-1 macrophages after IL-10 stimulation, we analyzed the impact of short term stimulation of IL-10 on the expression of ATP-binding cassette transporters ABCA-1 and ABCG-1, both involved in the reverse cholesterol transport, in lipid loaded THP-1 cells that were exposed to IL-10 (20 ng/ml). As shown in Online Fig. I, IL-10 significantly increased the expression of the ABCA-1 and ABCG-1 at the mRNA (real-time quantitative RT-PCR). Collectively, these data suggest that metabolic machinery that is involved in the reversed cholesterol transport from macrophages is enhanced by IL-10.

3.3. IL-10 potentates the effect of LXRα agonists on the ATP-binding cassette transporters in THP-1 macrophages

In macrophages the ATP-binding cassette transporters like ABCA-1 and ABCG-1 are known to be LXRα target genes [17]. In order to further examine the mechanism of the IL-10-induced increase in cholesterol efflux, we analyzed the gene expression of ABCA-1 and ABCG-1 in lipid loaded THP-1 macrophages after 3 h of stimulation with IL-10 (20 ng/ml), the LXRα ligand 22R-OH (1 μM) or a combination thereof. Similar to IL-10, the LXRα agonist increased the expression of ABCA-1 and ABCG-1, and notably, it also markedly enhanced the IL-10-mediated increase in the mRNA levels of these ATP-binding cassette transporters (Fig. 2A and B). One possible mechanism for this enhancing interaction could be that IL-10 increased the expression of LXRα in these cells. However, while 22R-OH increased mRNA levels of LXRα, IL-10 has no effect LXRα expression either alone or in combination with 22R-OH (Fig. 2C).

3.4. LXRα activation increases the IL-10-mediated effect on IL-10 and IL-10 receptor 1 (IL10R1) expression receptor

To further study the observed cross-talk between LXRα agonist and IL-10, we examined the effect of LXRα activation on IL-10 and its signaling pathway. First, we examined the ability of 22R-OH to enhance the expression of IL-10 and its receptor, consisting of two chain units IL-10R1 and IL-10R2 chain, in lipid loaded macrophages. As can be seen in Fig. 4A, both IL-10 on its own and 22R-OH increased the expression of IL-10, with a marked enhancing effect when these stimuli were combined. Moreover, while either IL-10 or 22R-OH had any significant effect on the high affinity IL-10R1 expression, the combination of these stimuli significantly increased mRNA levels of this IL-10R subunit (Fig. 3B). As for low affinity IL-10R2, both IL-10 and in particular 22R-OH increased its expression in lipid loaded macrophages, but with no further enhancement when these stimuli were combined (Fig. 3C). IL-22, a cytokine in the IL-10 family, binds to IL-10R2 in addition to its own high affinity receptor IL-22R. However, in contrast to IL-10, IL-22 had no effect on ABCA1 or ABCG1 expression in THP-1 macrophages either when given alone or when co-stimulated with 22R-OH (Supplemental Fig. II).

3.5. LXRα activation increases the IL-10-mediated effect on ATP-binding cassette transporters in macrophages through enhanced STAT3 signaling

STAT3 signaling is of major importance for IL-10-mediated effects in macrophages, and by using phosphor-specific antibody

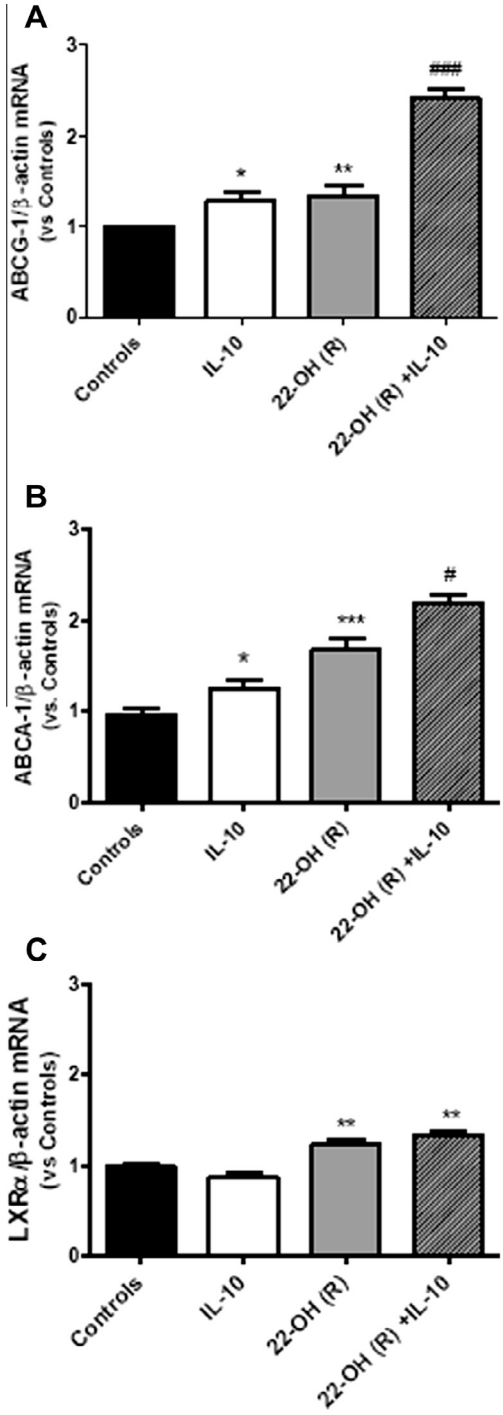

Fig. 2. The effect of IL-10 and LXRα activation on the gene expression of ABCG-1 (A), ABCA-1 (B) and LXRα (C) in THP-1 macrophages. PMA (100 nM)-differentiated macrophages were loaded for 24 h with 20 μg/ml oxLDL in regular growth medium. Thereafter, the cells were washed in serum-free medium, and stimulated with IL-10 (20 ng/ml), LXRα ligand 22-OH(R) cholesterol (2.5 μg/ml, or a combination thereof additional for 6 h before the cells were harvested for RNA extraction. Controls were given vehicle. The mRNA levels were determined by qRT-PCR using standard curve methods, and the gene expression was adjusted according to the level of the house keeper gene β-actin. Data are presented as mean ± SEM ($n = 3$). $^{*}p < 0.05$ vs controls and $^{**}p < 0.01$ vs controls; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ versus IL-10 or 22OH(R) alone.

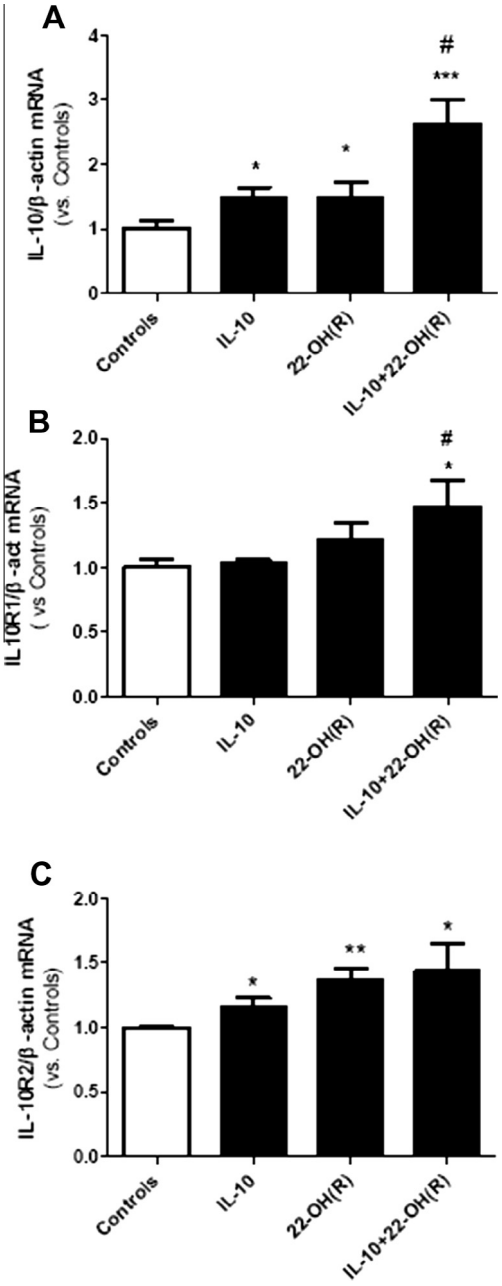

Fig. 3. The effect of IL-10 and LXRα activation on the gene expression of IL-10 (A), IL-10R1 (B) and IL-10R2 (C) in THP-1 macrophages. PMA (100 ng/ml)-differentiated macrophages were loaded for 24 h with 20 μg/ml oxLDL in regular growth medium. Thereafter, the cells were washed in serum free medium and stimulated with IL-10 (20 ng/ml), LXRα ligand 22-OH(R) cholesterol (2.5 μg/ml) or a combination thereof for additional 6 h before the cells were harvested for RNA extraction. Controls were given vehicle. The mRNA levels were determined by qRT-PCR using standard curve methods, and the gene expression was adjusted according to the level of the house keeper gene β-actin. Data are presented as mean ± SEM ($n = 4$). $^{*}p \leqslant 0.05$ and $^{***}p < 0.001$ vs control; $^{\#}p < 0.05$ vs 22-OH(R) or IL-10 alone.

in Western blotting, we found that IL-10 (20 ng/ml) markedly induced phosphorylation of STAT3 after 30 min of stimulation as compared to control (Fig. 4A). Moreover, while the LXRα agonist 22R-OH had no effects of its own, it enhanced the phosphorylation of STAT3 as compared to IL-10 alone (Fig. 4A). Also, the STAT3 inhibitor peptide (PpYLKTK-mts), significantly attenuated the combined effect of 22R-OH and IL-10 as well as the effect of IL-10 on its own on ABCA-1 and ABCG-1 expression (Fig. 4B and C). Interestingly, IL-24 another member of the IL-10 cytokine family that also induces STAT3 phosphorylation, but does bind to IL-10R1 or IL-10R2, had no effect on ABCA1 or ABCG1 expression in THP-1 macrophages either when given alone or when co-stimulated with 22R-OH (Supplemental Fig. II).

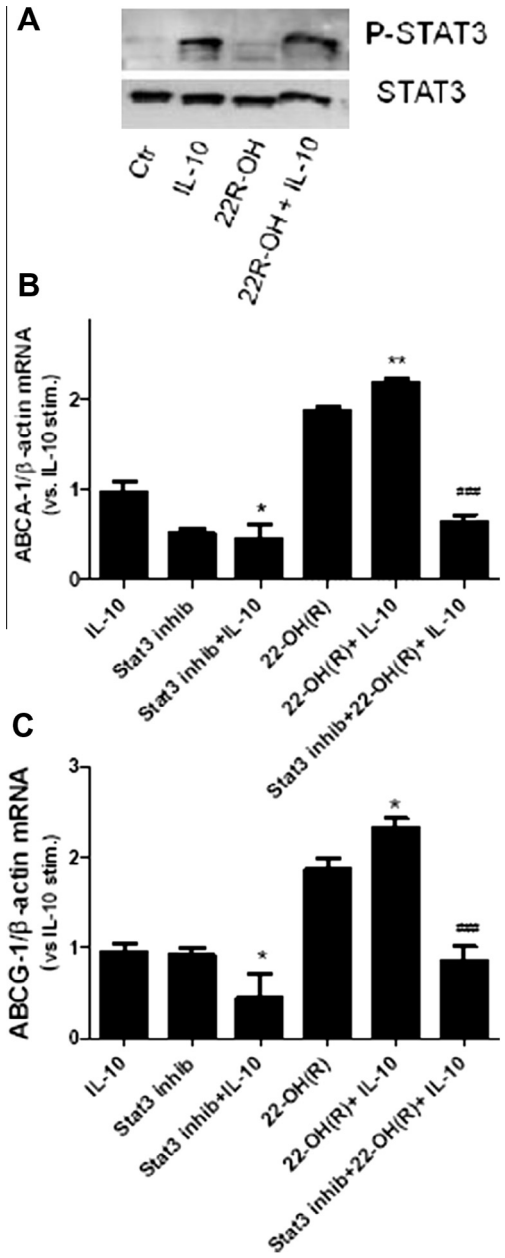

Fig. 4. IL-10 mediated STAT-3 signaling in THP-1 macrophages. PMA (100 ng/ml)-differentiated macrophages were loaded for 24 h with 20 μg/ml oxLDL in regular growth medium. The cells were then washed in serum-free medium and stimulated with IL-10 (20 ng/ml) for additional 15 min before the cells were harvested for Western blotting. The STAT-3 phosphorylation (A) was assessed by Western blotting using Phosho-STAT3 specific antibody as detailed in Section 2. Panel B and C, shows the gene expression of ABCA-1 (B) and ABCG-1 (C) after stimulation with IL-10 (20 ng/ml), STAT-3 inhibitor peptide (50 μM), LXRα ligand 22-OH(R) cholesterol (2.5 μg/ml) or combination thereof for additional 6 h before the cells were harvested for RNA extraction. Controls were given vehicle (solid bars). The STAT-3 inhibitor was added 15 min prior to the stimulation. mRNA levels were determined by qRT-PCR using standard curve methods, and the gene expression were adjusted according to the level of the house keeper gene β-actin. Data are presented as mean ± SEM ($n = 4$). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs control; $^{\#\#\#}p < 0.001$ vs similar conditions without the STAT-3 inhibitor.

4. Discussion

IL-10 is a prototypical anti-inflammatory cytokine that has been shown to attenuate atherosclerosis development in experimental models. In addition to its anti-inflammatory properties, the anti-atherogenic effect of IL-10 has recently also been suggested to reflect a complex effect of IL-10 on lipid metabolism in macrophages. On the one hand, IL-10 has been shown to increase lipid accumulation in these cells at least partly through anti-apoptotic mechanisms [4]. On the other hand, some previous studies have suggested that IL-10 could up-regulate ABCA1 and ABCG1 in macrophages, involving LXRα-related mechanisms [15], potentially leading to increased cholesterol efflux, but these issues are far from clear. In the present study we extend these findings by showing that the IL-10-mediated up-regulation of the ATP-binding cassette transporters in macrophages is accompanied by increased cholesterol efflux, indicating that IL-10 enhances reverse cholesterol machinery at the functional levels. Also, our findings further suggest a boosting effect of LXRα activation on the enhancing effect of IL-10 on ABCA-1 and ABCG-1 expression, at least partly reflecting through enhanced STAT3 phosphorylation. Finally, while IL-10 had no effect on LXRα expression in lipid-exposed macrophages, we show that LXRα activation in combination with IL-10 enhanced expression of IL-10 as well as the high affinity IL-10 binding receptor subunit IL-10R1 in these cells. Our findings further support the ability of IL-10 to modulate not only inflammation, but also lipid metabolisms, representing the two major and interacting arms in atherogenesis.

Several experimental studies have shown anti-atherogenic effects of IL-10 [1,9,14,20], but its mechanism of action during atherogenesis is not fully understood. It has been suggested that its anti-atherogenic potential primarily rely on its anti-inflammatory effects including (i) the ability to induce a shift from pro-inflammatory Th1 cells to the anti-atherogenic Th2 cells [2], (ii) its role as a mediator of the anti-atherogenic effects of regulatory T cells[10], (iii) its ability to attenuate an inflammatory response in lipid-exposed endothelial cells, [13] and (iv) IL-10 has also been suggested to promote an anti-atherogenic response in B cells [12]. In addition, both *in vitro* [4] and *in vivo* studies [12,20] have shown that IL-10 inhibits macrophage apoptosis, leading to enhanced foam cell formation. While this may be beneficial in relation to plaque destabilization, it could be harmful in the early stage of atherosclerosis promoting lipid accumulation within the lesion. Previously, IL-10 has been reported to lower plasma levels of LDL and VLDL cholesterol, potentially involving direct and indirect effect on insulin production in pancreas and lipoprotein lipase activity [20]. Based on our findings in the present study, we suggest that the ability of IL-10 to enhance cholesterol efflux from macrophages could be of importance for its attenuating effect on plaque formation, and if operating *in vivo* within an atherosclerotic lesion, this mechanism will be beneficial also in the early stage of the atherosclerotic process.

We herein show that IL-10 increases the expression of the ATP-binding cassette transporters ABCA-1 and ABCG-1 in lipid-loaded macrophages, accompanied by a functional improvement in cholesterol efflux in these cells irrespectively of using FCS, HDL_2 or apoA-1 as cholesterol acceptors. As also reported by Rubic and Lorenz [15], we showed a positive interaction between IL-10 and LXRα activation in the regulation of ABCA-1 and ABCG1 that seems to involve enhanced phosphorylation of STAT3. In the present study we extend these previous findings by showing that this positive interaction involves up-regulation of IL-10 and its high-affinity receptor sub-unit IL-10R1. Thus, while we found no effect of IL-10 on LXRα expression in lipid loaded macrophages, the combination of IL-10 and LXRα activation markedly enhanced the expression of IL-10R1. Moreover, whereas IL-10 and LXRα activation modestly increased the expression of IL-10 when given alone, the combination of these stimuli markedly increased IL-10 expression in lipid-exposed macrophages. These findings suggest that the boosting effect of LXRα activation on the IL-10-mediated up-regulation of the ATP-binding cassette transporters in macrophages involve their synergistic effect on IL-10 and IL-10R1 expression. Interestingly,

two other members of the IL-10 cytokine family, IL-24, that induces STAT3 phosphorylation, but does not interact with IL-10R1 and IL-10R2, and IL-22, that binds to IL-10R2, but not to IL-10R1 [16], did not had any effects on ABCA-1 or ABCG-1 expression in lipid loaded macrophages either alone or in combination with LXRα activation, indicating the importance of IL-10R1 activation for the mediation of these effect.

This study suggests that the anti-atherogenic properties of IL-10 may include enhancing effects on cholesterol efflux mechanism that involves cross-talk with LXRα activation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.036.

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