Lipids, inflammation and atherosclerosis

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Chapter 3

INTERLEUKIN-9 INHIBITS β-VLDL INDUCED FOAM CELL FORMATION BY REDUCING RECEPTOR EXPRESSION IN MURINE MACROPHAGES

Miranda Stitzinger[1], Menno Hoekstra[1], Jacques van Snick[2], Theo J.C. van Berkel[1] and Johan Kuiper[1]
[1]Division of Biopharmaceutics, LACDR, Leiden University, The Netherlands
[2]Ludwig Institute for Cancer Research, Brussels, Belgium

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ABSTRACT

Previously, we found that treatment of LDLr deficient mice with IL-9 resulted in inhibition of atherosclerosis. To unravel the underlying mechanism of the atheroprotective effect of IL-9, we investigated the effect of IL-9 on murine macrophage RAW 264.7 cells loaded with β-VLDL. IL-9 inhibited β-VLDL induced foam cell formation of RAW cells with 50%. Quantitative real-time PCR-analysis showed that IL-9 caused a 1.28-fold reduction in the mRNA expression of the LDLr, a 2.13-fold reduction in LRP-1 and a 1.89-fold reduction in SR-BI, all tentative receptors for β-VLDL uptake. Experiments with [^{3}H]cholesterol loaded cells revealed that the reduction in foam cell formation was not due to a stimulatory effect of IL-9 on cholesterol efflux. In line with these results, IL-9 did not affect mRNA expression levels of the cholesterol efflux pumps ABCA1 and ABCG1. The mRNA expression of PPARα, a nuclear receptor whose activation leads to inhibition of foam cell formation, was increased 4.00-fold by IL-9. In addition, IL-9 caused a 1.75-fold and 2.08-fold reduction in the expression of SREBP-1 and SREBP-2, transcription factors involved in intracellular cholesterol homeostasis. The reduction in lipoprotein uptake receptors, the activation of the nuclear receptors PPARα and the reduction of the transcription factors SREBP-1 and SREBP-2 by IL-9 will all contribute to the protective effect of IL-9 in atherosclerosis in a coordinated way.

INTRODUCTION

Interleukin (IL)-9 is a pleiotropic cytokine that was first described as a T cell growth factor[1], but IL-9 also affects many other cells including mast cells, erythroid precursors, B lymphocytes, eosinophils, bronchial epithelial cells, neuronal precursors and macrophages[2,3]. IL-9 was primarily identified as a risk factor for asthma[4] and transgenic mice overexpressing IL-9 present features of asthma[5,6]. The treatment of mice with an IL-9 neutralizing antibody inhibits the development of allergic pulmonary inflammation and airway hyperresponsiveness[7,8]. In contrast, a T-helper (Th)2 response to allergen after sensitization is still present in IL-9 deficient mice as well as other features of asthma[9]. In addition, IL-9 is a susceptibility factor in *Leishmania major* infection[10] and leads to an early death in mice with chronic *Schistosoma mansoni* infection[11] by inducing a Th2 response.
On the other hand, several studies show that IL-9 may exhibit protective effects by inhibiting inflammatory responses. IL-9 shows these anti-inflammatory effects in the protection of mice from Gram-negative bacterial shock by suppression of tumor necrosis factor (TNF)-α, IL-12, and interferon (IFN)-γ, and induction of IL-10[12]. Other studies revealed a protective role of IL-9 in the host immunity to intestinal nematode infections[13,14]. IL-9 may directly deactivate lipopolysaccharide (LPS) stimulated blood mononuclear phagocytes and the mechanism of inhibition involves the increased transforming growth factor (TGF)β1 production by these cells[15]. In a recent study[16], we investigated the role of IL-9 in atherosclerosis, a disease characterized by chronic inflammation[17] and affected by pro- and anti-inflammatory mediators[18]. Treating low-density lipoprotein (LDL) receptor (LDLr) deficient mice with IL-9 led to a more than 60% reduction in atherosclerotic lesion development. Moreover, functional blocking of endogenous IL-9 resulted in an increase in atherosclerosis[16]. IL-9 not only affected the production of pro-inflammatory cytokines but also reduced the *in vitro* adhesion of monocytes to endothelial cells.
In the early stages of atherosclerosis, circulating monocytes infiltrate the vessel wall in response to the enhanced expression of adhesion molecules and chemoattractants in activated endothelial cells. The infiltrated monocytes differentiate into macrophages and, activated by inflammatory stimuli, synthesize and secrete various bioactive molecules, including cytokines, growth factors, and proteases. As a result of excessive accumulation of cholesteryl esters from modified lipoproteins, macrophages transform into foam cells, which have a prominent role in the initiation and progression of the atherosclerotic plaque[17-20].
Foam cells can be formed *in vitro* by receptor mediated uptake of either β-very low-density lipoprotein (β-VLDL)[21] or experimentally modified lipoproteins such as acetylated LDL (acLDL)[22] and oxidized LDL (oxLDL)[23]. In addition to the uptake of modified lipoproteins, cholesterol efflux is an important process in the development of foam cells, because foam cell formation is the result of an imbalance in cholesterol homeostasis[20]. ATP-binding cassette (ABC)G1[24-26] and ABCA1[27,28] are the key mediators of cholesterol efflux to high-density lipoprotein (HDL) and apolipoprotein (apo)A-I, respectively. Also scavenger receptor class B, type I (SR-BI) is able to facilitate cholesterol efflux to HDL[29].

Various mediators can affect foam cell formation. Nuclear receptors such as peroxisome proliferator-activated receptor (PPAR)α and PPARγ inhibit foam cell formation, but PPARδ does not influence foam cell formation[30]. In addition, many inflammatory mediators influence foam cell formation. Most studies suggest a foam cell inducing effect of pro-inflammatory mediators such as LPS and TNF-α[31,32] and a suppressive effect of anti-inflammatory mediators such as TGFβ1[33,34]. In contrast, the anti-inflammatory IL-10 enhances oxLDL induced foam cell formation of macrophages by anti-apoptotic mechanisms[35].
In the present study, we show that the anti-inflammatory cytokine IL-9 inhibited β-VLDL induced foam cell formation in RAW 264.7 murine macrophage cells. IL-9 reduced the expression of receptors for β-VLDL, whereas cholesterol efflux was not affected. Our data provide the first evidence for a direct effect of IL-9 on the gene expression of key players in foam cell formation, which may contribute to the anti-atherogenic effect of IL-9.

MATERIALS AND METHODS

Cell culture

RAW 264.7 murine macrophage cells were cultured in complete medium containing DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (P/S) and 4 mM L-glutamine (all from BioWitthaker™) at 37^0C in an humidified atmosphere with 5% CO_2.

Isolation of β-VLDL

β-VLDL was obtained from rats fed a RMH-B diet containing 2% cholesterol, 5% olive oil, and 0.5% cholic acid for 2 weeks (Abdiets). The rats were fasted overnight and anesthetized after which blood was collected by puncture of the abdominal aorta. Serum was centrifuged at 40,000 rpm in a discontinuous KBr gradient for 18 hours as reported earlier[36]. β-VLDL was collected and dialysed against phosphate buffered saline containing 1 mM EDTA. Isolated β-VLDL was characterized as described by Van Eck *et al*[37]. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the national laws. The Ethics Committee for Animal Experiments of Leiden University approved all experimental protocols.

Foam cell formation

RAW cells ($0.75x10^5$) were seeded in 24-well plates for 2 hours. Non-adherent cells were removed and adherent cells were cultured for 16 hours in absence or presence of 40 ng/ml recombinant murine IL-9 purified from baculovirus-infected Sf9 insect cell cultures[38]. Cells were incubated with β-VLDL (25-100 µg/ml) for 24 hours. The cells were stained for lipid accumulation with oil red O (Sigma) and photographed using a Leica DMRE microscope (1000x magnification) coupled to a video camera. Leica Qwin Imaging software (Leica Ltd.) was used to quantify foam cell formation by calculating the area of oil red O staining compared to the total cell area.

Cholesterol efflux

To study cholesterol efflux, RAW cells ($0.25x10^5$) were seeded in 24-well plates in complete medium. After 2 hours, non-adherent cells were removed and adherent cells were loaded with 0.5 µCi/ml ^{3}H-cholesterol (Amersham) in DMEM supplemented with 2% fatty acid free BSA (Sigma), P/S and L-glutamine (DMEM/2%BSA) for 24 hours at 37°C. Subsequently, cells were cultured in DMEM/2%BSA in presence or absence of IL-9 for 24 hours. Cholesterol efflux was studied by incubation of the cells with DMEM/2%BSA alone (negative control) or supplemented with either 5 µg/ml apoA-I (ABCA1-mediated) or 100 µg/ml HDL (ABCG1-mediated) for 24 hours. Radioactivity of media and lysed cells was determined with a Tri-Carb 2900TR liquid scintillation analyser (Packard) using Packard QuantaSmart software. Cholesterol efflux was defined as radioactivity in media compared to radioactivity in both media and cells.

Gene expression

RAW cells ($1.5x10^5$) were seeded in 24-well plates for 2 hours. Non-adherent cells were removed and adherent cells were cultured for 16 hours in absence or presence of 40 ng/ml IL-9. Quantitative gene expression analysis on these cells was performed as described[39]. In short, total RNA was isolated using TriZol® reagent (Invitrogen Life technologies) according to the manufacturer's instructions and reverse transcribed using RevertAid™ reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green technology (Eurogentec) with the primers displayed in **Table 1**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), acidic ribosomal phosphoprotein P0 (36B4) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as standard housekeeping genes. Relative gene expression numbers were calculated by subtracting the threshold cycle number (Ct) of the target gene from the average Ct of housekeeping genes and raising 2 to the power of this difference. The average Ct of three housekeeping genes was used to exclude that changes in the relative expression were caused by variations in the separate housekeeping gene expressions. The average Ct values for housekeeping genes (mean±SEM; n=6) were 17.1±0.2 for control cells and 17.0±0.2 for IL-9 treated cells.

Table 1: Primers for quantitative real-time PCR analysis

Gene	GenBank Accession	Forward primer	Reverse Primer
36B4	X15267	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
ABCA1	NM013454	GGTTTGGAGATGGTTATACAATAGTTGT	TTCCCGGAAACGCAAGTC
ABCG1	NM009593	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG
GADPH	NM008084	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
LDLr	Z19521	CTGTGGGCTCCATAGGCTATCT	GCGGTCCAGGGTCATCTTC
LRP-1	NM008512	TGGGTCTCCCGAAATCTGTT	ACCACCGCATTCTTGAAGGA
PPARα	NM011144	TGAACAAAGACGGGATG	TCAAACTTGGGTTCCATGAT
PPARγ	NM011146	CATGCTTGTGAAGGATGCAAG	TTCTGAAACCGACAGTACTGACAT
PPARδ	NM011145	GAGGGGTGCAAGGGCTTCTT	CACTTGTTGCGGTTCTTCTTCTG
SR-BI	NM016741	GGCTGCTGTTTGCTGCG	GCTGCTTGATGAGGGAGGG
SREBP-1	AB017337	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC
SREBP-2	AF374267	TGAAGCTGGCCAATCAGAAAA	ACATCACTGTCCACCAGACTGC
TGFβ1	NM011577	AGGGCTACCATGCCAACTTCT	GCAAGGACCTTGCTGTACTGTGT
VLDLr	NM013703	GGCTCTGGCGAGTGCATT	GCCGTCCTTGCAGTCAGG

Immunoblotting

RAW cells ($3.0x10^5$) were seeded in a 6 well-plate, after which they were treated with IL-9 and β-VLDL as described above. Subsequently, cells were washed with ice-cold PBS and lysed in 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1% SDS containing 0.02 µg/ml leupeptin, 0.02 µg/ml aprotinin, and 0.02 µg/ml trypsin inhibitor. Cell debris was removed by centrifugation at 10,000 rpm for 10 min. Equal amounts of protein (25 µg) were separated on 7.5% SDS-PAGE gels and electrophoretically transferred to Protran nitrocellulose membrane (Schleicher & Schnell). Immunolabeling was performed using rabbit polyclonal antibodies against SR-BI (amino acids 496-509, Abcam), SREBP-1 (amino acids 41-200, Santa Cruz Biotechnology) and SREBP-2 (amino acids 812-975, Santa Cruz Biotechnologies) as primary antibodies. Goat-anti-rabbit IgG (Jackson ImmunoResearch) was used as secondary antibody. Finally, immunolabeling was detected by enhanced chemiluminescence (ECL plus, Amersham Biosciences), scanned by a Typhoon 9400 variable mode imager (Amersham Pharmacia Biotech) and analysed using ImageQuant™ software (Amersham Pharmacia Biotech).

RESULTS

Foam cell formation

Macrophage foam cell formation *in vitro* requires the uptake of modified lipoproteins such as oxLDL[23] or acLDL[22]. β-VLDL, a lipoprotein present in the plasma of cholesterol-rich fed animals[40], is also capable of inducing foam cell formation[21]. To induce foam cell formation, RAW 264.7 cells were incubated with β-VLDL (25-100 µg/ml) for 24 hours. The induction of foam cell formation by incubating RAW 264.7 cells with β-VLDL was clearly visible (**Fig. 1A**) and the lipid area of cells incubated with 25, 50 and 100 µg/ml β-VLDL was 2.3%, 4.4% and 9.5% of the total cell area respectively (**Fig. 1C**).

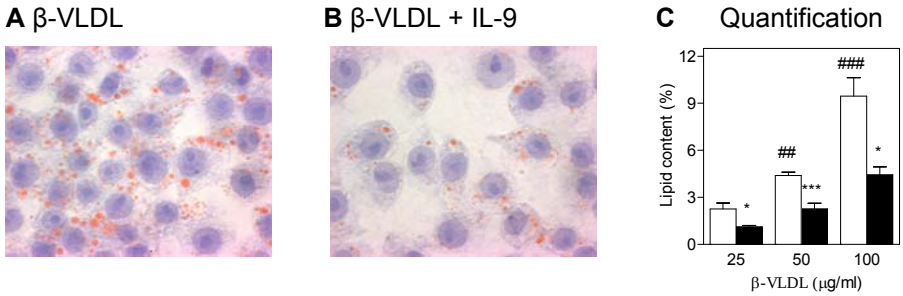

Fig. 1: Effect of IL-9 on β-VLDL induced foam cell formation. RAW cells were cultured for 16 hours in the absence (**A**) or presence (**B**) of IL-9 and subsequently incubated with 50 µg/ml β-VLDL for 24 hours. The cells were stained for lipid accumulation with oil red O and photographed. **C** RAW cells were cultured in the absence or presence of IL-9 for 16 hours, after which they were incubated with β-VLDL for 24 hours. Oil red O staining was quantified using Leica Qwin Imaging software. Values are expressed as mean±SEM of % lipid area compared to total cell area. Significant differences between control cells and cells pre-treated with IL-9: * $p<0.05$, *** $p<0.001$. Significant differences between control cells incubated with 25 µg/ml β-VLDL and control cells incubated with 50 or 100 µg/ml: ## $p<0.01$ and ### $p<0.001$

Since foam cell formation is affected by pro- and anti-inflammatory mediators, we studied the effect of IL-9, an anti-inflammatory cytokine, by incubating RAW cells with 40 ng/ml IL-9 for 16 hours prior to incubation with β-VLDL (**Fig. 1B**). After incubation with 25, 50 and 100 µg/ml β-VLDL for 24 hours, the lipid area of IL-9 pre-treated cells was 1.1%, 2.3% and 4.5% of the total cell area, respectively (**Fig. 1C**). Compared to the lipid area of control cells after incubation with β-VLDL between 25 and 100 µg/ml, a significant 2-fold reduction ($p<0.01$) in β-VLDL induced foam cell formation was observed after IL-9 treatment.

Receptors for β-VLDL

The major route for β-VLDL uptake by macrophages is the LDLr[41]. In addition, the VLDL receptor (VLDLr)[42], LDLr related protein-1 (LRP-1)[43] and SR-BI[44] are receptors involved in β-VLDL uptake. Therefore, we studied the effect of IL-9 on the expression of these receptors in RAW cells. RAW cells were cultured in the absence/presence of IL-9 for 16 hours, after which RNA was isolated for quantitative real-time PCR-analysis. LDLr, LRP-1 and SR-BI were expressed in untreated control RAW cells. IL-9 treatment showed a tendency to a 1.28-fold downregulation of LDLr gene expression ($p=0.06$). LRP-1 and SR-BI expression were significantly downregulated by IL-9, 2.13-fold and 1.89-fold respectively (**Fig. 2A-C**). In addition, Western Blot analysis confirmed that SR-BI protein expression was significantly 1.54-fold decreased by IL-9 treatment as determined by Western Blot analysis ($p<0.05$, **Fig. 2D**). The expression of the VLDLr was not detectable in both conditions (data not shown), suggesting a very low expression of VLDLr in macrophages.

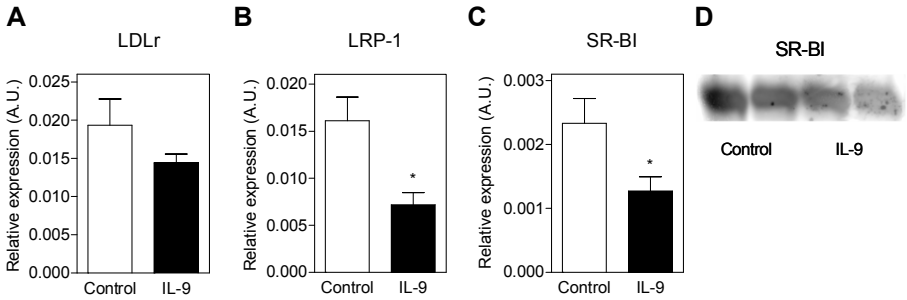

Fig. 2: Effect of IL-9 on mRNA expression of LDLr, LRP-1 and SR-BI. RAW cells were cultured for 16 hours in the absence or presence of IL-9, after which RNA or protein was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **A** LDLr, **B** LRP-1 and **C** SR-BI. Values are expressed as mean±SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units). Significant differences between control cells and cells treated with IL-9: * $p<0.05$. **D** Western blot of SR-BI

Cholesterol efflux

We investigated whether the effect of IL-9 on foam cell formation was due to a stimulatory effect on cholesterol efflux, because foam cell formation can be regarded as an imbalance in cholesterol homeostasis[20]. First, RAW cells were loaded with [^{3}H]cholesterol, after which they were incubated for 16 hours with medium alone or with medium containing IL-9. Cholesterol efflux was determined using BSA (negative control), apoA-I (ABCA1-

mediated) and HDL (ABCG1-mediated) as cholesterol acceptors. In both control and IL-9 treated cells, cholesterol efflux was 6-7% to BSA and increased significantly to 15-16% when the cells were incubated with apoA-I (**Fig. 3A**). After 24 hours of incubation with HDL, cholesterol efflux reached 39-41% in both groups of cells (**Fig. 3A**). The significantly higher efflux to HDL than to apoA-I is consistent with data obtained with peritoneal macrophages[45]. However, no differences were observed between the efflux of cholesterol from control cells and from cells treated with IL-9 at all conditions (**Fig. 3A**). Quantitative real-time PCR-analysis indicated that mRNA of ABCA1 and ABCG1 was expressed in RAW cells (**Fig. 3B/C**) probably mediating the efflux to respectively apoA-I and HDL. In agreement with the cholesterol efflux study, IL-9 had no significant effect on the mRNA expression of ABCA1 and ABCG1 (**Fig. 3B/C**).

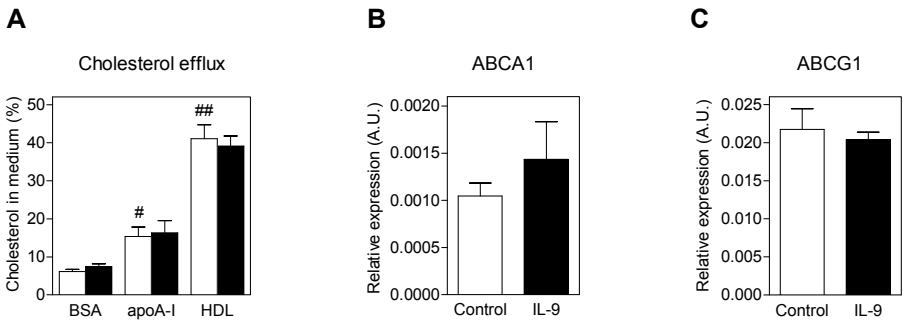

Fig. 3: Effect of IL-9 on cholesterol efflux. **A** RAW cells were loaded with ^{3}H-cholesterol for 24 hours at 37°C. Subsequently, cells were cultured in presence or absence of IL-9 for 24 hours. Cholesterol efflux was induced by incubating the cells for 24 hours with culture medium containing BSA or supplemented with apoA-I or HDL. Values are expressed as mean±SEM of % radioactivity in media compared to radioactivity in both media and cells. Significant differences between control cells and cells treated with IL-9: * $p<0.05$. Significant differences between cells incubated with 2% BSA and cells incubated with apoA-I or HDL: # $p<0.05$ and ## $p<0.01$. **B/C** RAW cells were cultured for 16 hours in the absence or presence of IL-9, after which RNA was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **B** ABCA1 and **C** ABCG1. Values are expressed as mean±SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units).

Effect on TGFβ1, SREBP-1 and SREBP-2

IL-9 is able to stimulate TGFβ1-production in LPS stimulated monocytes[15] and TGFβ1, in turn, can inhibit foam cell formation[33,34]. Therefore, we determined whether the effect of IL-9 on foam cell formation is mediated by induction of TGFβ1 expression in RAW cells. However, IL-9 did not affect the TGFβ1 expression in RAW cells (**Fig. 4A**) and the inhibitory effect of IL-9 on foam cell formation can not be explained by a stimulatory effect on TGFβ1.

Transcription factors sterol regulatory element binding protein (SREBP)-1 and SREBP-2 are involved in intracellular cholesterol homeostasis. We determined the expression of these transcription factors upon IL-9 treatment. The expression of SREBP-1 and SREBP-2 were significantly decreased by IL-9 treatment, 1.75-fold and 2.08-fold respectively (**Fig. 4B/C**). The downregulation of SREBP-1 and SREBP-2 was confirmed at protein level by Western Blotting ($p<0.05$, **Fig. 4D/E**)

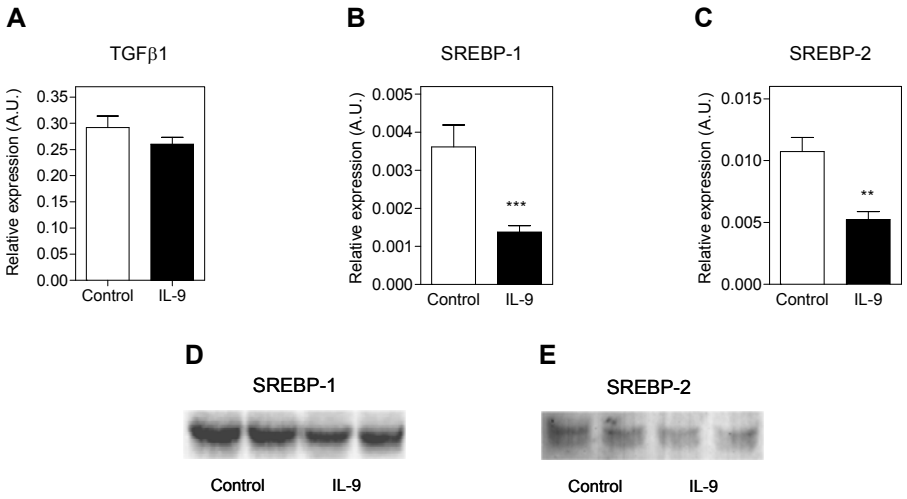

Fig. 4: Effect of IL-9 on TGFβ1, SREBP-1 and SREBP-2. RAW cells were cultured for 16 hours in the absence or presence of IL-9, after which RNA or protein was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **A** TGFβ1, **B** SREBP-1 and **C** SREBP-2. Values are expressed as mean±SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units). Significant differences between control cells and cells treated with IL-9: ** $p<0.01$ and *** $p<0.001$. **D** Western blot of SREBP-1. **E** Western blot of SREBP-2

Effect on PPARα, PPARγ and PPARδ

Nuclear receptors such as PPARs are also able to influence genes which are important for foam cell formation[30]. Therefore, we determined whether IL-9 had an effect on the expression of PPARα, PPARγ and PPARδ in RAW cells. PPARγ expression was not detectable in our system, suggesting that PPARγ expression was very low in RAW cells (data not shown). The expression of PPARδ was not affected by IL-9 (**Fig. 5A**), whereas IL-9 was able to significantly increase PPARα expression 4-fold (**Fig. 5B**).

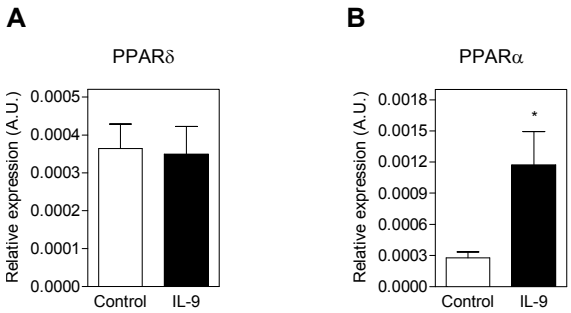

Fig. 5: Effect of IL-9 on PPARα and PPARδ. RAW cells were cultured for 16 hours in the absence or presence of IL-9, after which RNA was isolated. Quantitative real-time PCR analysis was used to determine the mRNA expression of **A** PPARδ and **B** PPARα. Values are expressed as mean±SEM of relative mRNA expression compared to housekeeping gene expression (arbitrary units). Significant differences between control cells and cells treated with IL-9: * $p<0.05$.

DISCUSSION

According to our previous studies in LDLr deficient mice, IL-9 is proposed to have a therapeutic potential in atherosclerosis due to its ability to inhibit atherogenesis[16]. During the development of atherosclerosis, macrophages play a central role in both inflammation and lipid deposition. It is already known that IL-9 is able to influence inflammation by inhibiting oxidative burst and cytokine release in both LPS stimulated monocytes[15] and alveolar macrophages[3]. On the other hand, the relation between inflammatory mediators such as IL-9 and foam cell formation in the macrophage remains poorly understood. We therefore investigated the effect of IL-9 on macrophage foam cell formation.

In addition to modified lipoproteins, β-VLDL, a lipoprotein that is naturally induced upon cholesterol-rich feeding in animals[40], is able to induce foam cell formation in mouse peritoneal macrophages *in vitro*[21]. Until now, only one study has been performed in which β-VLDL was used to induce cholesterol ester accumulation in murine macrophage RAW cells[31]. In our experiments, the induction of foam cells by incubating RAW cells with β-VLDL was clearly visible. β-VLDL in a concentration between 25 and 100 µg/ml caused a lipid area between 2.3% and 9.5% in RAW cells after 24 hours. Because of its pathological relevance in LDLr deficient mice, we used β-VLDL to study the effect of IL-9 on foam cell formation.

Pre-incubation of RAW cells with IL-9 resulted in a 2-fold reduction in foam cell formation induced by β-VLDL in a concentration between 25 and 100 µg/ml. The protective effect of the anti-inflammatory IL-9 is in line with our earlier results in peritoneal macrophages derived from LDLr deficient mice[16] and with the induction of foam cell formation by pro-inflammatory mediators. LPS[31] and TNF-α[32] are examples of pro-inflammatory mediators that stimulate foam cell formation either by downregulating mediators of efflux (LPS)[46] or by upregulating receptors for uptake of modified lipoproteins (TNF-α)[32,47]. Like IL-9, the anti-inflammatory TGFβ1 is able to inhibit foam cell formation. TGFβ1 mediated this effect through inhibition of macrophage LPL, inhibition of the expression and activity of the LDLr, SR-AI/II and CD36 and enhancement of macrophage cholesterol efflux[33]. Moreover, TGFβ1 is able to inhibit downregulation of ABCA1 expression and macrophage cholesterol efflux induced by IFN-γ[34].

Although IL-9 induces TGFβ1 in LPS stimulated human monocytes leading to inhibition of oxidative burst and TNF-α release[15], TGFβ1 expression in RAW cells was not affected by IL-9 in our study. Therefore, the inhibitory effect of IL-9 on foam cell formation is unlikely to be mediated by TGFβ1. IL-10, another anti-inflammatory cytokine, shares the ability with IL-9 to protect against atherogenesis[48,49]. However, unlike IL-9, IL-10 enhances the formation of foam cells induced by oxLDL[35]. It has to be taken into account that oxLDL, in contrast to β-VLDL, is able to induce apoptosis[50,51] and IL-10 exerted its foam cell promoting effect by inhibiting oxLDL induced apoptosis rather than by affecting macrophage lipid uptake or efflux[35].

We investigated the potential mechanism of the IL-9 induced inhibition of β-VLDL induced foam cell formation. Various macrophage receptors capable of binding and taking up β-VLDL are identified, including LDLr, LRP-1, VLDLr and SR-BI[41-44] of which LDLr is the major candidate. We studied the

effect of IL-9 on the expression of these receptors and indicated that IL-9 had a tendency to decrease the expression of the LDLr (1.28-fold, p=0.06) and clearly decreased the expression of LRP-1 and SR-BI in RAW cells, 2.13-fold and 1.89-fold respectively. Together the reduced expression of receptors for β-VLDL will lead to a lower uptake of β-VLDL, which can at least partly explain the inhibitory effect of IL-9 on foam cell formation. Downregulation of LRP-1 and SR-BI mRNA expression by IL-9 can explain why IL-9 was also able to inhibit β-VLDL induced foam cell formation of peritoneal macrophages deficient in LDLr[16].

SREBPs are known to regulate the expression of the LDLr in various cell types. In a human renal mesangial cell line, the pro-inflammatory cytokines TNF-α and IL-1β were able to induce LDLr expression by upregulation of SREBP-1[52], suggesting a link between inflammation, SREBPs and LDLr expression. In contrast to TNF-α, IL-9 reduced the expression of SREBP-1 in RAW cells correlating with the observed IL-9 induced downregulation of the LDLr. SREBP-2 is possibly involved in the regulation of the expression of LRP-1, because LDL induced LRP-1 upregulation is reduced by an inhibitor of SREBP-2 in human vascular smooth muscle cells[53]. In our experiments, IL-9 was able to reduce SREBP-2 expression, which coincided with the downregulation of LRP-1 in RAW cells. However, PPARγ has been shown to regulate LRP-1 gene expression and function in adipocytes[54]. Unfortunately, we were not able to detect PPARγ in our experimental set-up.

SR-BI promotes a bi-directional flux of cholesterol between cells and HDL[55] and although we observed a downregulation of its expression by IL-9 in RAW cells, we did not observe an effect of IL-9 on cholesterol efflux either to HDL or apoA-I, suggesting that SR-BI mediated efflux is not active under our conditions. SR-BI appears to play a dual role in the development of atherosclerosis: SR-BI induces the development of small fatty streak lesions, while it protects against the development of more advanced atherosclerotic lesions[56]. We determined the effect of IL-9 on the expression of SR-BI in RAW cells that were not loaded with cholesterol and therefore in a situation in which SR-BI probably functions as a mediator of cholesterol uptake rather than as a mediator of cholesterol efflux.

Recently, is was reported that liver X receptor (LXR)/retinoid X receptor (RXR) treatment of macrophages lacking ABCG1 failed to stimulate cholesterol efflux to HDL, whereas in wild type macrophage cholesterol efflux was increased by this treatment[25]. Together with other studies[24,26], this suggests that ABCG1 is a key gene involved in cholesterol efflux from the macrophage to HDL. We observed no effect of IL-9 on the expression of ABCG1 in RAW cells, explaining why cholesterol efflux to HDL was not affected by IL-9 in our study in spite of a reduced SR-BI expression.

Cholesterol efflux from macrophages to lipid-poor apoA-I is primarily regulated by ABCA1[57-59]. The expression of ABCA1 in vascular endothelial cells is downregulated by activation of SREBP-2[60]. Although IL-9 reduced the expression of SREBP-2 in RAW cells, the expression of ABCA1 in these cells was not affected by IL-9. In agreement with the unchanged expression of ABCA1, cholesterol efflux to apoA-I was not affected by IL-9 in our studies.

Several studies show the effects of PPARs on foam cell formation and the development of atherosclerosis. PPARα is described to protect against foam cell formation by inducing cholesterol efflux. However, Li et al[30] found that PPARα agonists reduced atherosclerosis and foam cell formation, but they did not observe an effect on the expression of ABCA1 nor on the expression of ABCG1. We found that treatment of RAW cells with IL-9 led to a 4-fold increase in PPARα expression. In agreement with Li et al, we did not find any changes in the expression of ABCA1 and ABCG1 upon IL-9 treatment. PPARγ and PPARδ can also effect cholesterol homeostasis[61,62], but changes in expression of these nuclear receptors by IL-9-treatment were not observed in RAW cells.
In conclusion, IL-9 is able to reduce the expression of receptors for β-VLDL uptake, to induce the nuclear receptor PPARα and to reduce the transcription factors SREBP-1 and SREBP-2, resulting in reduced foam cell formation. Although IL-9 is considered a risk factor for the development of asthma and some parasitic infections, it shows beneficial properties by protecting against bacterial and nematode infections and atherosclerosis. Our data provide the first evidence for a direct effect of IL-9 on the gene expression of key players in foam cell formation providing a possible mechanism for the anti-atherogenic effect of IL-9.

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