The Impact of the PCSK-9 / VLDL-Receptor Axis on Inflammatory Cell Polarization

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Research Article

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Abstract

Background: Studies have shown that lipoproteins, including LDL, VLDL, and ApoE2 have an impact on macrophage polarization, important to atherosclerosis progression. PCSK9 is a key mediator regulating the expression of lipoprotein receptors.

The present study investigates the effect of the VLDL/VLDL-R axis on mononuclear cell polarization, as well as the role of PCSK9 and PCSK9 inhibitors (PCSK9i) within this network.

Methods: Human monocytic THP-1 cells and human monocyte-derived macrophages isolated from PBMC were treated with LPS/IFN-γ to induce a pro-inflammatory phenotype or with IL-4 /IL-13 to induce an anti-inflammatory phenotype. Cells were then subjected to further treatments including VLDL, LDL, PCSK9, PCSK9i, anti-LDL-R; PMA and TSP-1.

Results: LPS/IFN-γ treatment promoted a pro-inflammatory state with an increased expression of pro-inflammatory mediators e.g., TNF-α, CD80, and IL-1β. VLDL co-treatment induced a switch of this pro-inflammatory phenotype to an anti-inflammatory phenotype. In pro-inflammatory cells, VLDL significantly decreased the expression of the M1-markers e.g., TNF-α, CD80, and IL-1β. These effects were eliminated by PCSK9 and restored by co-incubation with a PCSK9i. Migration assays demonstrated that pro-inflammatory cells displayed a significantly higher invasive capacity compared to untreated cells or anti-inflammatory cells. Moreover, pro-inflammatory cell chemotaxis was significantly decreased by VLDL-mediated acquisition of the anti-inflammatory phenotype. PCSK9 significantly lessened this VLDL-mediated migration inhibition, which was reversed by the PCSK9i.

Conclusion: VLDL promotes macrophage differentiation towards the anti-inflammatory phenotype. PCSK9, via its capacity to inhibit VLDL-R expression, reverses the VLDL-mediated anti-inflammatory switch, thereby promoting a pro-inflammatory phenotype. Thus, anti-PCSK9 therapies may exert pro-inflammatory suppression within the vessel wall.

1. Introduction

Atherosclerosis is characterized by cell proliferation / migration and inflammation within the vessel wall, resulting from a thigh interaction of vascular smooth muscle, fibroblast and inflammatory cells [1]. Macrophages, dendritic cells, subsets of T and B lymphocytes, mast cells, and neutrophils are key players in the initiation and maintenance of the inflammatory process [2]. Mononuclear inflammatory cells display a high level of plasticity, allowing them to differentiate and activate depending on their local environment [3, 4]. Thus, based on their functional behavior in *in vitro* assays and their capacity to secrete different cytokines, subtypes have been characterized [4]. Traditionally, this includes the “classically” activated M1 subset, being the pro-inflammatory type [4, 5]. Another subset is the “alternatively” activated M2 macrophages, basically mediating anti-inflammatory effects and promoting tissue repair [4, 6]. Thus, M1 (pro-inflammatory) cells polarize upon treatment with lipopolysaccharide (LPS) and Th1 cytokines (i.e. IFN-γ and granulocyte colony-stimulating factor) and secrete mediators such as IL-1β, IL-6, IL-12 and

TNF-α, whereas M2-like cells are activated by Th2 cytokines (i.e. IL-4 and IL-13) and produce IL-10 and TGF-β, two key anti-inflammatory cytokines [7].

Lipoproteins are linked to inflammation via their capacity to stimulate pro-inflammatory/pro-atherogenic pathways, such as NF-κB, TNF-α receptor signaling, and reactive oxygen species (ROS)-induced inflammation [1]. Lipoprotein receptors including the LDL-Receptor (LDL-R), VLDL-Receptor (VLDL-R) and apoE-R2 are expressed on vascular cells including macrophages, vascular smooth muscle, and endothelial cells [8] [9]. High levels of serum LDL-Cholesterol (LDL-C) are a major cardiovascular risk factor and a treatment target [10]. Oxidized LDL-C diffuses the endothelial cell barrier, is taken up by intimal macrophages via scavenger receptors such as CD36, SR-A1 and LOX-1 and induces acetyl-CoA acetyltransferase-mediated lipid deposition, which leads to foam cell formation and sustained inflammation [11]. Very low-density lipoprotein (VLDL) is the precursor of low-density lipoprotein (LDL) [8, 9]. In contrast to LDL, normal triglyceride-rich lipoproteins, including normotriglyceridemic-VLDL (NTG-VLDL) show fewer pro-thrombotic effects, while hypertriglyceridemic VLDL (HTG-VLDL) are characterized by a higher and more diverse lipid content, including apoE and are thus more atherogenic [14, 15] [16]. Macrophages in atherosclerotic lesions express the VLDL-R and its activation promotes a phenotypic pro-inflammation to anti-inflammation switch *in vitro* [17, 18]. Accordingly, the deletion of VLDL-R elevated the expression of pro-inflammatory mediators, such as TNF-α and IL-1β, in the skin of pups nursed by $VLDLR^{-/-}$ mothers [20]. Comparable to VLDL, apoE a triglyceride-rich lipoprotein, which shares a high level of homology, has been demonstrated to inhibit the release of pro-inflammatory factors like IL-1β in vascular smooth muscle cells, thereby diminishing macrophages cytotoxicity [22].

Several of these lipoprotein receptors, including the LDL-R, VLDL-R and apoE-R are regulated via proprotein convertase subtilisin/kexin 9 (PCSK9) at the cell surface [19–21]. PCSK9 binds to the LDL-R in its EGF-A-domain, routing it to lysosomal degradation, thereby increases serum LDL-C [20, 21]. Clinical trials demonstrated that targeting PCSK9 either by monoclonal antibodies or by siRNA significantly reduces serum-LDL-C and thus major cardiovascular events (MACE) in high-risk patients [23–30]. In APOE3*Leiden-cholesteryl ester transfer protein (CETP) mice, targeting PCSK9 decreases atherosclerotic plaque burden and plaque inflammation [31]. However, the impact of this PCSK9-mediated VLDL-R degradation as well as of VLDL itself within this system is only incompletely understood.

The aim of the present study was to characterize the impact of the VLDL/VLDL-R axis on macrophage polarization important for atherosclerosis progression, as well as the impact of PCSK9 and PCK9-inhibitors within this network.

2. Material And Methods

2.1. In vitro studies with human monocytic THP-1 cells

Human monocytic THP-1 cells were purchased from DSMZ (DSMZ No.: ACC16, Germany). Cells were grown routinely in growth media containing RPMI 1640 Medium (Gibco, Germany) with L-glutamine, 10%

Fetal Bovine Serum Advanced (Capricorn Scientific, Germany) and 1% Penicillin/Streptomycin (Biochrom, Germany) at 37°C and 5% CO_2 [39]. Exponentially growing cells were harvested by centrifugation (5 min. by 300 rpm) and resuspended in a fresh medium every 2 days. For pro-inflammatory priming, THP-1 cells were treated with LPS (10 ng/mL; L6529, Sigma, Germany) and Recombinant Human IFN-γ (10 ng/mL-;300-02-20, PeproTech, Germany) for 24 hr. For anti-inflammatory priming, THP-1 cells were treated with Recombinant Human IL-4 (20 ng/mL;200-04, PeproTech, Germany) and Recombinant Human IL-13 (10 ng/mL;200 – 13, PeproTech, Germany) for 24 hr. Untreated cells served as controls (M0 cells).

For experiments, LPS/IFN-γ or IL-4/IL-13 treated cells were either post treated with human VLDL (100 µg/mL; A50261H, Meridian Life Science, Germany) or human LDL (10 µg/mL;770200-4, Kalen Biomedical, Germany) for additioal 24 hr, as outlined in figure legends. In experiments cells were co-treated with Recombinant Human PCSK9 (5 µg/mL;3888-SE-010, R&D Systems, USA) with and without a specific monoclonal anti-PCSK9 antibody (10 µg/mL; 71207, BPS Bioscience, Germany) for 24 hr, or co-treated with a specific monoclonal anti-LDL-R antibody (2 µg/mL; BM5053, Acris, Germany) for 24 hr. For other experiments, pro-inflammatory polarized cells were treated with 0.15 µmol/L phorbol-12-myristate-13-acetate (PMA; P8139, Sigma, Germany) or 10 µg/mL Human Recombinant Thrombospondin-1 (Sigma, Germany), with or without VLDL for 24 hr. All experiments were performed in triplicate.

2.2. Isolation of human monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation from freshly collected blood of healthy young men and women (n = 7). Monocytes were isolated from PBMCs with human CD14 MicroBeads (Miltenyi Biotec, Germany) and then seeded into 6-well plates ($1x10^6$ cells/well) in RPMI-1640 medium with L-glutamine, 10% FCS, 1% penicillin/streptomycin, 4 ng/mL macrophage colony stimulating factor (M-CSF) (Peprotech, Germany) at 37°C; 95% humidity and 5% CO_2 for 3 days. For M1-like polarization, monocyte-derived macrophages were treated with LPS (10 ng/mL; L6529, Sigma, Germany) and Recombinant Human IFN-γ (10 ng/mL; 300-02-20, PeproTech, Germany) for 24 hr. Subsequently, cells were treated with 10 µg/mL human VLDL for 24 hr. Sample collection and the experimental protocol were approved by the institutional review board with the ethic vote EA4/154/16. All probands read and signed a consent form. All research was performed in accordance with the German guidelines.

2.3. RNA isolation and quantitative Real time (RT)-PCR

Total RNA was extracted with Trizol™ (Thermo Fisher, Germany). The RNA concentration was measured using the Biophotometer (Eppendorf, Germany) with UVette. Samples were stored at -80°C until use.

cDNA was synthesized using the SuperScript II (Invitrogen, Germany). Equal amounts of cDNA (20 ng) were used for RT-quantitative PCR (MX3000P, Stratagene, Germany) to detect the expression of M1 markers (TNF-α: forward primer: CTCTTCTGCCTGCTGCACTTTG, reverse primer: CTCTTCTGCCTGCTGCACTTTG; CD80: forward primer: CCAACCACAGCTTCATGTGTC, reverse primer: GGATGGGAGCAGGTTATCAGG; IL-1β: forward primer: AGCTGATGGCCC TAAACAGA, reverse primer:

GTCGGAGATTCGTAGCTGGA; IL-12A: forward primer: CACTCCCAAAACCTGCTGAG, reverse primer: TCTCTTCAGAAGTGCAAGGGTA) and of M2 markers (IL-10: forward primer: GATGCCTTCAGCAGAGTGAA, reverse primer: GCAACCCAGGTAACCCTTAAA; CD206: forward primer: CATATCGGGTTGAGCCACTT, reverse primer: GAGGGATCTCCTGTGTTCCA; CD209: forward primer: TTCACCTGGATGGGACTTTC, reverse primer: CTAAATTCCGCGCAGTCTTC; TGF-β: forward primer: CCCTGGACACCAACTATTGC, reverse primer: GTCCTTGCGGAAGTCAATGT) in THP1 cells. The amount of the gene of interest was normalized with 18S (forward primer: CGGCTACATCCAAGGAA, reverse primer: GCTGGAATTACCGCGGT). Duplicate tests on each sample were done.

2.4. Western blot analysis

The protein lysates were blotted as previously described [40] and subsequently immunostained with anti-VLDLR antibody (Biorbyt, #36044, Germany) or anti-LDL antibody (Abcam, ab30532, Cambridge UK) at 4°C overnight and afterwards with ECL™ Anti-Rabbit IgG, Horseradish Peroxidase-Linked Whole Antibody (GE Healthcare, NA934, Germany) for 2 hr. at room temperature and visualized with enhanced chemiluminescence (GE Healthcare, Freiburg, Germany). Actin (Sigma Aldrich, A2066, Germany) was used as reference protein.

2.5. ELISA

Supernatant of untreated, LPS/IFN-γ, and IL-4/IL-13 treated cells were collected and ELISA analysis for TNF-α (BioLegend, Germany) was performed. ELISA was done as recommended by the manufacture.

2.6. Flow cytometry

The purity of the CD14 isolated human monocytes was analyzed using following antibodies: human anti-CD45-VioBlue (Cat# 130-110-637) and human anti-CD14-APC-Vio770 (130-113-706) from Miltonyi Biotec. Flow cytometry experiments were performed with a MACSQuant® Analyzer 10 (Miltonyi Biotec, Germany). The MACSQUANTIFY™ software was used to analyze the data.

2.7. Measurement of total ROS

Cells were seeded into 6 well plates and incubated with 10 µmol/L H2DCFDA (Life Tech, Germany) for total ROS measurement for 30 min at 37°C as described in [41]. Briefly, cells were washed twice with PBS containing calcium-chloride (1 mmol/L) and lysed with Triton X-100 buffer. The fluorescence intensity was analyzed by excitation at 485 ± 10 nm and emission at 530 ± 10 nm using a ViktorX Multilable plate reader and subsequently normalized to protein level.

2.8. Migration assay

Transwell cell culture chamber with gelatin-coated (0.2%) polycarbonate membranes (8mm pores) (Becton Dickinson) was used. The number of cells per high power field (HPFs; magnification x 320) migrated to the lower surface of the filters after 4 hr. incubation was determined as described [42].

2.9. Phagocytosis assay

Phagocytotic effect of untreated, LPS/IFN-γ, and IL-4/IL-13 treated THP-1 cells was measured with the Vybrant™ Phagocytosis Assay Kit (V-6694, Molecular Probes, USA) according to the instructions of the manufacturer.

3.0. Statistical analysis

Data were evaluated using the Mann-Whitney test (nonparametric test), Kruskal-Wallis test (nonparametric test) or with two-way ANOVA. Statistical significance was defined as $p<0.05$. Statistical analysis was performed by using Prism 5 for Windows (GraphPad Software, 2003, San Diego, USA).

3. Results

3.1. VLDL has anti-inflammatory effects on pro-inflammatory polarized monocytic cells

First, the impact of VLDL on pro-inflammatory mediator mRNA levels (TNF-α, CD80 and IL-1β), as well as the ratio of pro-inflammatory IL-12 / anti-inflammatory IL-10 was investigated in untreated, LPS/INF-γ and IL-4/IL-13-primed cells. Results were compared to the effects for LDL. Pro-inflammatory and anti-inflammatory phenotype was validated by the expression of pro-inflammatory markers (i.e., TNF-α, CD80, IL-1β and IL-12) and anti-inflammatory markers (IL-10, CD206 and CD209) (Table 1). Whereas untreated and IL-4/IL-13 primed cells displayed generally low mRNA levels of TNF-α, CD80 and IL-1β, these inflammatory marker genes were greatly increased in pro-inflammatory cells ($p < 0.001$ vs untreated and IL-4/IL-13 treated cells; Fig. 1A-C). VLDL treatment significantly decreased the RNA expression of TNF-α, CD80, and IL-1β in pro-inflammatory cells ($p < 0.05$ vs untreated pro-inflammatory cells) but had no significant effect in untreated and IL-4/IL-13 treated cells (Fig. 1A-C). In contrast to VLDL, LDL had no effect on TNF-α, CD80 or IL-1β mRNA levels in either phenotypes ($p < 0.05$; Fig. 1D-F).

Table 1

Validation of the M1 and M2 phenotype of THP-1 cells. Relative mRNA expression analysis of TNF-α, CD80, IL-1β, IL-12, IL-10, CD206, and CD209 performed in undifferentiated, pro-inflammatory (LPS/IFN-γ, 24 hr), and anti-inflammatory (IL-4/IL-13, 24 hr) THP1 cells. Data are shown as the mean (Experiments: n = 5–6/group).

	untreated	pro-inflammatory	anti-inflammatory	p value
TNF-α	1.06	9.35	1.30	< 0.01
CD80	1.43	37.65	3.50	< 0.01
IL-1β	1.31	19.16	2.42	< 0.01
IL-12	1.02	4.52	1.30	< 0.01
IL-10	1.30	8.52	1.83	< 0.001
CD206	1.17	1.56	15.48	< 0.01
CD209	1.91	34.00	13.26	< 0.01

Analysis of the IL-12/IL-10 ratio revealed that VLDL, but not LDL, significantly increased the expression of IL-10 and decreased the expression of IL-12 (IL-12l^{ow}, IL-10^{high}) in pro-inflammatory polarized cells when compared to baseline ($p < 0.05$; Fig. 1G-H). Comparable to mRNA levels, TNF-α protein levels were significantly increased in these cells ($p < 0.05$ vs untreated and IL-4/IL-13 treated cells) and VLDL treatment significantly reduced TNF-α protein expression ($p < 0.05$ vs LPS/IFN-γ treated cells; Fig. 1I). In addition, pro-inflammatory polarized cells produced significantly more total ROS than untreated cells ($p < 0.001$; Fig. 1J), while VLDL treatment significantly reduced total ROS levels in these cells ($p < 0.001$ vs. untreated pro-inflammatory cells; Fig. 1J). Furthermore, VLDL treatment significantly increased the mRNA expression of the M2 marker CD206 when compared to untreated pro-inflammatory cells ($p < 0.01$; Fig. 2A). However, the mRNA levels of CD209 and TGF-β were reduced after VLDL treatment, when compared to pro-inflammatory cells ($p < 0.01$ and $p > 0.05$ respectively; Fig. 2B, C).

In addition, human PBMC-derived monocytes displayed high $CD45^+/CD14^+$ on FACS-analysis (Fig. 2D). Comparable to human monocytic THP-1 polarized pro-inflammatory cells, CD80 and IL-1β mRNA expression was significantly reduced after VLDL treatment in human pro-inflammatory monocyte-derived macrophages ($p < 05$ vs untreated LPS/IFN-γ treated cells; Fig. 2E, F).

3.2. LDL but not VLDL regulates the VLDL-R in pro-inflammatory polarized monocytic cells

Next, we compared VLDL-R and LDL-R gene and protein expression in undifferentiated and differentiated cells and investigated the impact of VLDL and LDL on these lipid receptors in pro-inflammatory cells. All three cell phenotypes expressed the VLDL-R and LDL-R, with pro-inflammatory cells displaying higher mRNA and protein levels of the LDL-R ($p < 0.05$ vs M0; Fig. 3A-C). When pro-inflammatory cells were

treated with VLDL, VLDL-R mRNA and protein levels were unaffected (p = non-significant vs untreated pro-inflammatory cells; Fig. 3A, D). In contrast, LDL-R mRNA and protein significantly decreased upon VLDL treatment ($p < 0.01$ vs untreated pro-inflammatory cells; Fig. 3B, D). When these cells were treated with LDL, VLDL-R mRNA significantly increased and LDL-R mRNA levels decreased (both $p < 0.01$ vs untreated LPS/IFN-γ treated cells; Fig. 3E, F).

3.3. PCSK9 inhibits VLDL-mediated anti-inflammatory effects in pro-inflammatory polarized monocytic cells via VLDL-R regulation

VLDL-R and LDL-R regulation by PCSK9 was investigated in VLDL-treated pro-inflammatory polarized cells. Receptor gene and protein expression was compared in control (untreated) cells, cells co-incubated with PCSK9 and cells co-incubated with PCSK9 and concurrently treated with a specific monoclonal anti-PCSK9 antibody. Immunoblotting demonstrated, that PCSK9 significantly lessened LDL-R and VLDL-R protein levels (Fig. 4A). However, no effect was found on VLDL-R mRNA (Fig. 4B). Co-incubation and thus rescue of PCSK9 inhibitory effects with a specific monoclonal anti-PCSK9-antibody restored LDL-R and VLDL-R protein expression (Fig. 4A).

To investigate the impact of PCSK9 on VLDL/VLDL-R signaling cascade and thus VLDL-mediated anti-inflammatory effects in pro-inflammatory cells, mRNA expression of TNF-α and CD80 was investigated. VLDL-treatment significantly decreased both inflammatory markers ($p < 0.05$ vs pro-inflammatory cells; Fig. 5A, B). Upon co-incubation of VLDL-treated pro-inflammatory cells with PCSK9, mRNA levels of TNF-α and CD80 significantly increased ($p < 0.01$ vs. VLDL-treated pro-inflammatory cells; Fig. 5A, B). Inhibition of PCSK9 with a specific monoclonal anti-PCSK9-antibody reversed the effects of PCSK9 on CD80 mRNA levels ($p < 0.05$ vs. VLDL/PCSK9-treated pro-inflammatory cells; Fig. 5B). In contrast, a specific monoclonal LDL-R antibody had no impact on TNF-α or CD80 mRNA levels, neither in controls nor in VLDL-treated pro-inflammatory cells (Fig. 5C, D). To further elaborate VLDL-R dependent anti-inflammatory actions, we co-incubated pro-inflammatory differentiated cells with VLDL and either TSP-1 or the PKC-activator PMA as mediators of a sustained inflammatory response. In both cases, TSP-1 or PMA-mediated inflammation, evident by increases in TNF-α and CD80, were prevented by VLDL ($p < 0.05$ vs PMA or TSP-1 treated cells; Fig. 5E-H).

3.5. VLDL impacts on pro-inflammatory polarized monocytic cell phagocytosis and invasion

To assess the impact of VLDL on pro-inflammatory cell functionality, we investigated cell phagocytosis and invasion in untreated, LPS/IFN-γ and IL-4/IL-13 treated cells post treated with VLDL and cells post treated with VLDL and PCSK-9, co-incubated with or without a specific monoclonal anti-PCSK9 antibody.

Pro-inflammatory differentiated cells displayed a significantly higher phagocytotic capacity when compared to untreated or anti-inflammatory cells ($p < 0.05$ and $p < 0.01$, respectively; Fig. 6A). VLDL significantly reduced phagocytosis in pro-inflammatory cells when compared to untreated pro-inflammatory cells, down to the level found in anti-inflammatory cells ($p < 0.05$ vs pro-inflammatory cells; Fig. 6A). In Boyden-chamber invasion assays with gelatin-coated membranes, VLDL significantly inhibited the migratory capacity of pro-inflammatory cells towards MCP-1, when compared to untreated pro-inflammatory cells ($p < 0.001$; Fig. 6B). The number of migrated cells was comparable in pro-inflammatory cells post treated with VLDL to anti-inflammatory cells. PCSK9 eliminated this anti-migratory effect of VLDL on pro-inflammatory cells ($p < 0.001$ vs VLDL-treated pro-inflammatory cells; Fig. 6B). Accordingly, this was significantly lessened by co-treatment with a specific monoclonal anti-PCSK9 antibody ($p < 0.001$ vs VLDL/PCSK9-treated pro-inflammatory cells; Fig. 6B).

4. Discussion

The present study investigated the impact of VLDL on mononuclear inflammatory cell polarization. Here, we demonstrate that VLDL promotes an anti-inflammatory phenotypic change in inflammatory polarized cells, thereby significantly lessening cell invasion and phagocytosis. Furthermore, we show that PCSK9, via its capacity to regulate VLDL-R cell surface expression, is a master switch in VLDL/VLDL-R-mediated pro-inflammatory suppression.

Studies have shown, that apolipoprotein B-containing lipoproteins such as LDL and VLDL are crucial to atherosclerosis, although they vary in their apolipoprotein and triglyceride content [43]. Among the triglyceride-rich lipoproteins (TRLs), not all are atherogenic, as it was shown that large VLDL (in contrast to small VLDL remnants) do not cross the endothelial barrier [43]. It has been proposed that VLDL remnants are involved in macrophage foam cell formation via the TRL-lipoprotein lipase (LPL)-VLDL-R pathway; however, the effect of large VLDL on macrophages is still a matter of debate [48].

In our study, treatment of pro-inflammatory primed monocytic THP-1 cells or human monocyte-derived macrophages with VLDL, but not with LDL, significantly decreased the mRNA expression of M1 marker genes, including TNF-α, CD80, and IL-1β. This was accompanied by increases in CD206 used as an M2 marker. VLDL-treatment but not LDL-treatment increased the IL-10 mRNA expression in relation to IL-12 mRNA in pro-inflammatory primed cells, when IL-12 is the pro-inflammatory M1-cytokine, and IL-10 denotes the anti-inflammatory M2-cytokine in macrophage polarization [7]. Thus, a shift in the ratio of IL-12/IL-10 towards IL-10 ($IL\text{-}12^{low}/IL\text{-}10^{high}$) indicates an anti-inflammatory phenotype [49]. Accordantly, treatment of pro-inflammatory primed monocytic cells with the inflammatory stimulators PMA and TSP-1 resulted in a significant increase of TNF-α and CD80, which was reversed by VLDL co-treatment. TSP-1 is upregulated during inflammatory conditions and regulates the secretion of pro-inflammatory Th1 cytokines such as IL-6, IL-1β, and TNF-α via the NF-κB pathway [50]. PMA induces THP-1 macrophage differentiation/polarization and stimulates a high phagocytic capacity and expression of cytokines in response to TLR-ligands [51].

Others have demonstrated that apoE induces macrophage polarization from a pro-inflammatory to an anti-inflammatory phenotype involving the apoER2 or the VLDL-R [22]. VLDL-R- or apoER2-expressing cells not only downregulated the expression of M1-markers, but also reduce M1-macrophage responses after treatment with ApoE in that study [53]. Likewise, ApoE-producing macrophages obtained from VLDL-R-deficient mice displayed a reduced secretion of M2 marker-cytokines, promoting a pro-inflammatory phenotype [22]. Interestingly, the VLDL-R and LDL-R display about 50% sequence homology, with the O-linked sugar (OLS)- and the intracellular domains as the regions with the most considerable divergence [52].

Several studies demonstrated that the cytokine repertoire of macrophages strongly depends on their local environment [54] [55] [56]. In our study, we show VLDL anti-inflammatory effects, since the pro-inflammatory differentiated THP-1 cells as well as human monocyte-derived macrophages produced less pro-inflammatory mediators and ROS formation following VLDL treatment. However, we could not show the generation of a “clear-cut” pro-inflammatory suppression following VLDL treatment, since M2 markers such as CD206 were not strongly regulated. However, others also found that a pro-inflammatory- or anti-inflammatory-specific stimulus e.g., with HDL or free fatty acids caused a different spectrum of macrophage activation than the initial M1 or M2 phenotype [57].

To further investigate VLDL-signal transduction, we assessed the expression of the VLDL-R and LDL-R, as well as their regulation by LDL and VLDL. We found that both receptors are expressed at mRNA and protein level in all cell phenotypes investigated. The VLDL-R was moderately lessened in pro-inflammatory derived cells, while the LDL-R was significantly increased, suggesting that a pro-inflammatory environment affects the expression of both receptors.

Other authors demonstrated that VLDL and LDL (among other lipoproteins) negatively regulated the LDL-R, while the VLDL-R is not regulated by lipoproteins in monocytic THP-1 cells [58, 59]. In accordance, LDL negatively regulated the expression of its receptor in this study. Treating pro-inflammatory polarized cells with VLDL resulted in a significant decreased of the LDL-R at mRNA and protein expression, while the levels of the VLDL-R did not change. Likewise, it has been shown that β-VLDL does not regulate LDL-R expression [58].

To further examine the importance of the VLDL-R, we investigated its regulation by PCSK9. Here, we demonstrate that PCSK9 is a key player in VLDL/VLDL-R mediated pro-inflammatory suppression in monocytic cells. Previous studies have shown that PCSK9 is related to a heightened expression of pro-inflammatory cytokines, chemokines, and adhesion molecules [60]. In endothelial cells, PCSK9 is induced by inflammatory mediators [61]. Furthermore, PCSK9 stimulates the secretion of pro-inflammatory markers in different cell-types including macrophages and is associated with the induction of pro-inflammatory pathways, including TLR4 and NF-κB, along with apoptosis and autophagy regulation [62]. Both, VLDL-R and apoER2 are degraded by PCSK9 [38]. For this reason, we treated pro-inflammatory differentiated monocytic cells with PCSK9, as well as with a specific monoclonal PCSK9-blocking antibody.

PCSK9 co-treatment resulted in a significant resurge of the pro-inflammatory markers TNF-α and CD80 in VLDL-treated inflammatory polarized monocytic cells. Concurrent inhibition of PCSK9 with its specific monoclonal antibody restored the VLDL-mediated anti-inflammatory phenotypic transition. Using immunoblotting and RT-PCR, we show that PCSK9 regulates VLDL-R expression post-translationally, comparable to the LDL-R [63]. To further clarify the importance of the VLDL-R, we compared VLDL-R regulation by PCSK9 to LDL-R regulation by PCSK9. Our study shows that both are downregulated at protein levels by PCSK9 in pro-inflammatory cells and that the inhibition of PCSK9 restores the protein expression of both receptors. This is in accordance with previous studies, showing that PCSK9 governs the degradation, not only of the LDL-R but also of the VLDL-R, as the latter is regulated in a LDL-R independent manner [64]. The VLDL-R comprises an EGF-A repeat, sharing about 60% sequence identity with the LDL-R to which PCSK9 can directly bind [64] [65]. Among the lipoprotein receptor-family, LDL-R and VLDL-R, but not apoER2, contend for PCSK9-mediated degradation, with VLDL-R being more prone to the effects of PCSK9 [38]. Taken together, PCSK9 is a key player in VLDL/VLDL-R anti-inflammatory signaling.

To further investigate PCSK9/VLDL/VLDL-R mediated inhibition of inflammation, we investigated its impact on cell functionality using pro-inflammatory polarized cells. Alternative activated macrophages undergo cell-functional changes, as they are less motile and cytotoxic [22]. Our results showed that treating these pro-inflammatory cells with VLDL significantly decreased the amount of phagocytosis, comparable to an anti-inflammatory phenotype. Treatment with VLDL also significantly decreased invasion of pro-inflammatory polarized cells. This was significantly inhibited by PCSK9, thus promoting invasion and abolished by PCSK9 inhibition.

5. Conclusions

In summary, our study demonstrates that VLDL via VLDL-R promotes the anti-inflammatory phenotype in mononuclear cells. Within this cascade, PCSK9 is a key regulator of VLDL-R protein expression, thereby inhibiting VLDL-mediated anti-inflammatory actions. PCSK9-targeting therapies thus potentially impact on inflammation important for the development and progression of atherosclerosis.

Abbreviations

ApoE2	Apolipoprotein E2
IL	Interleukin
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
PCSK9	Proprotein convertase subtilisin/kexin type 9

PMA	Phorbol 12-myristate 13-acetate
TSP-1	Thrombospondin 1
VLDL	Very low-density lipoprotein

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors approved the manuscript, and given consent of submission and subsequent publication of the manuscript.

Availability of data material

Materials and data that are reasonably requested by others will be made available.

Competing interest

PS has received consultancy and lecture honoraria from Amgen, Novartis, Sanofi-Aventis, Bristol-Myers Squibb/Pfizer, Daiichi-Sankyo, Bayer, Boehringer Ingelheim, BerlinChemie, B. Braun, AstraZeneca, editor honoraria from Springer Nature and was an investigator in PCSK9-inhibitor outcome trails. The remaining authors declare that they have no conflict of interest. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all the authors.

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Authors' contributions

MB conceived the project, analyzed the data, prepared the figures, and draft the manuscript. ME draft the manuscript and analyzed the data. LM carried out the experiments in human monocytes. AB carried out the flow cytometry analyses. NH carried out the ROS measurements. PS conceived the project, analyzed the data, prepared the figures, and drafted the manuscript.

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Figures

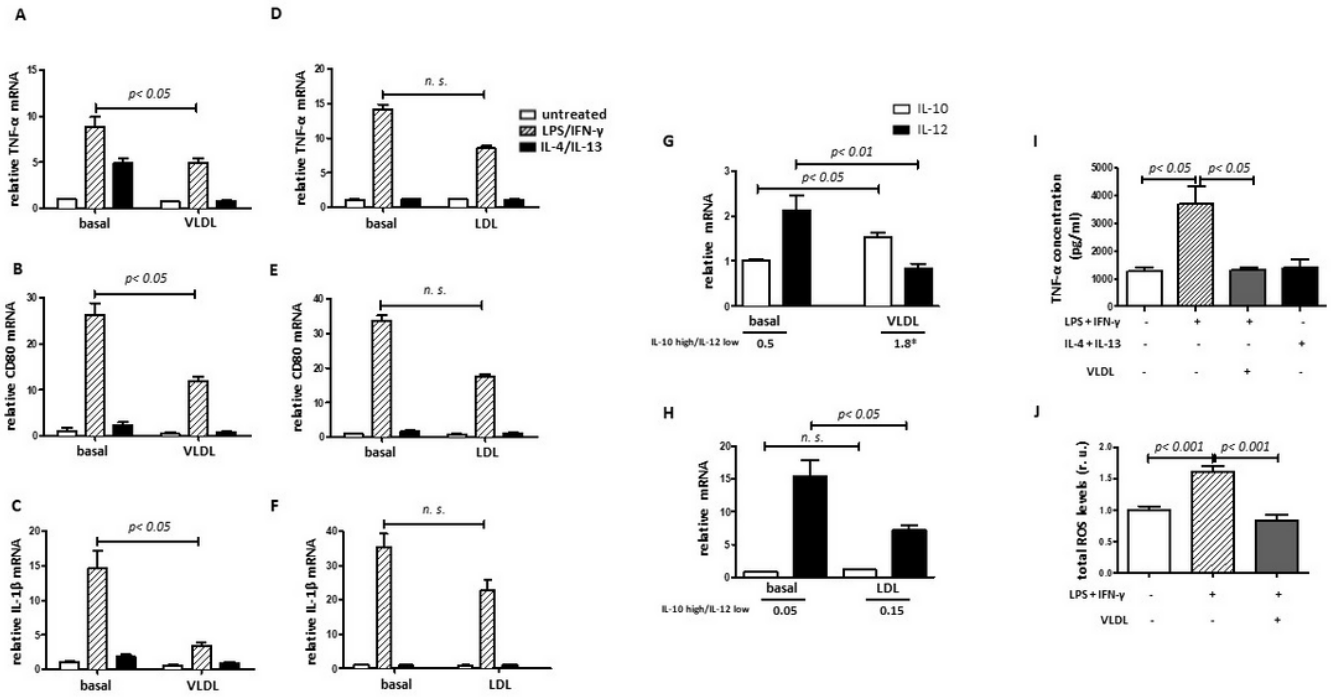

Figure 1

Figure 1

VLDL has anti-inflammatory effects on pro-inflammatory monocytic cells. Relative mRNA expression analysis of (A-F) TNF-α, CD80, and IL-1β performed in undifferentiated (M0), pro-inflammatory, and anti-inflammatory THP1 cells treated with VLDL (100 µg/mL) or with LDL (10 µg/mL) for 24h. (G-H) IL-12/IL-10 ratio (IL-10high/IL-12low) of pro-inflammatory (M1) monocytic cells treated with VLDL (100 µg/mL) or with LDL (10 µg/mL) for 24hr. (I) Elisa of TNF-α in cell culture media of pro-inflammatory (LPS + IFN-γ; 10 ng/ml) and anti-inflammatory (IL-4 + IL-13; 20 ng/mL and 10 ng/mL, respectively) THP-1 cells treated

with VLDL (100 µg/mL) for 24hr. (J) Total ROS levels of undifferentiated, and pro-inflammatory cells, treated with VLDL (100 µg/mL) for 24h. Data are shown as the mean ± SEM (Experiments: n= 5-6/group and technical duplicates).

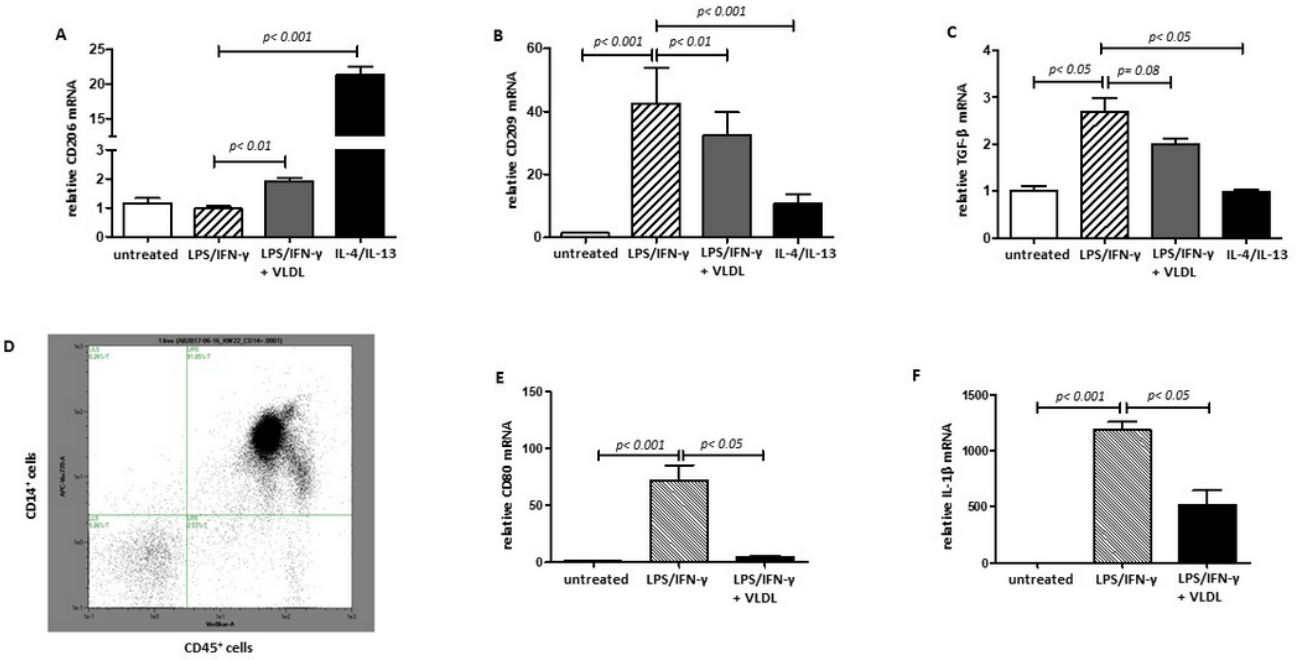

Figure 2

Figure 2

VLDL modulates the expression of M2 markers in pro-inflammatory monocytic cells and has anti-inflammatory effects on pro-inflammatory human monocyte-derived macrophages. Relative mRNA expression analysis of (A) CD206, (B) CD209 and (C) TGF-β in undifferentiated, pro-inflammatory THP-1 cells treated with and without VLDL (100 µg/mL) for 24 hr. and anti-inflammatory cells. Data are shown as the mean ± SEM (n= 5-6/group). (D) Flow cytometry of CD14 and CD45 in human CD14+-monocytes isolated from PBMCs. Relative mRNA expression analysis of (E) CD80, and (F) IL-1β performed in undifferentiated and pro-inflammatory human monocyte-derived macrophages treated with VLDL (100 µg/mL) for 24h. Data are shown as the mean ± SEM (Experiments: n= 7/group and technical duplicats).

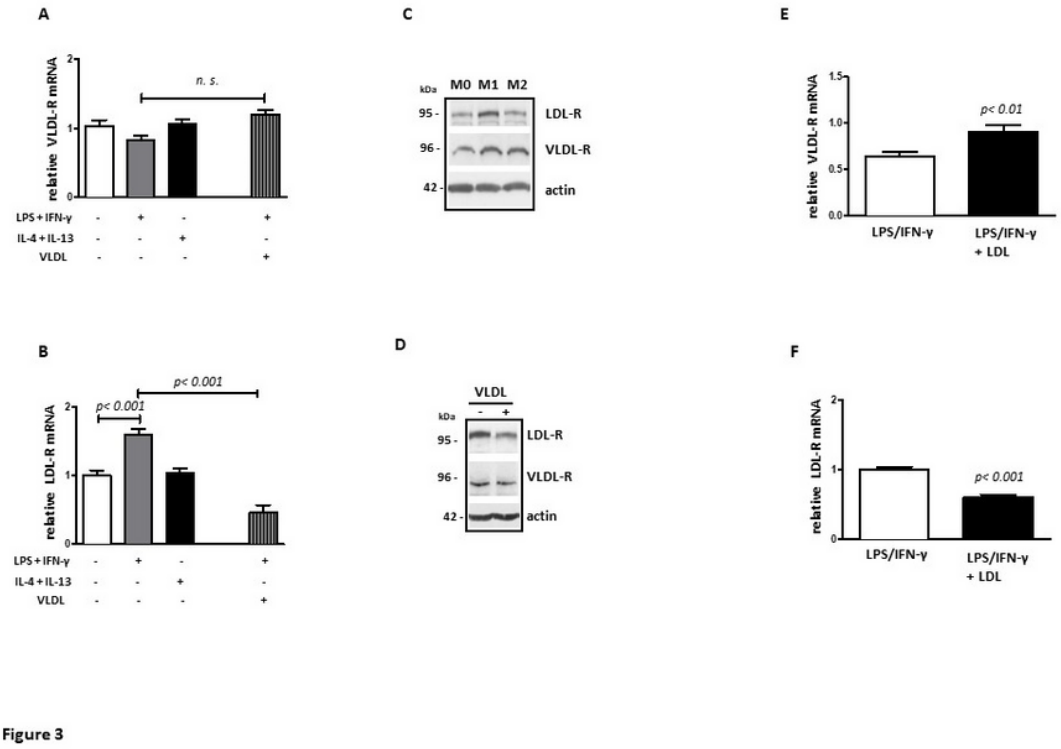

Figure 3

Figure 3

LDL, but not VLDL, regulates the VLDL-R in pro-inflammatory polarized monocytic cells. (A-B) Relative mRNA expression of the VLDL-R and LDL-R in undifferentiated, pro-inflammatory, and anti-inflammatory THP-1 cells treated with VLDL (100 µg/mL) for 24hr. (C) Protein expression of the VLDL-R and LDL-R in undifferentiated, pro-inflammatory, and anti-inflammatory cells. (D) VLDL-R and LDL-R protein expression in pro-inflammatory THP-1 cells treated with VLDL (100 µg/mL) for 24hr. (E-F) relative mRNA expression of the VLDL-R and LDL-R in pro-inflammatory THP-1 cells treated with LDL (10 µg/mL) for 24hr.mRNA data are shown as the mean ± SEM (Experiments: n= 5-6/group and technical duplicates). Representative images of western blot analysis, the lanes were run on the same gel.

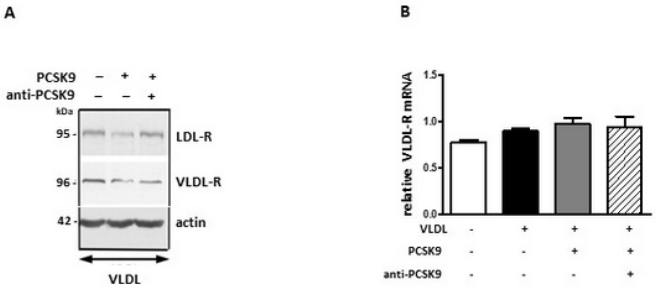

Figure 4

Figure 4

PCSK9 decreases VLDL-R and LDL-R on pro-inflammatory monocytic cells. (A) Relative protein expression of the VLDL-R and LDL-R on pro-inflammatory (M1) THP1 cells treated with VLDL (100 µg/mL) and PCSK9 (5 µg/mL); VLDL (100 µg/mL) and PCSK9 (5 µg/mL) and anti-PCSK9 (10 µg/mL) for 24hr. (B) Relative mRNA expression of VLDL-R on pro-inflammatory THP-1 cells treated with VLDL (100 µg/mL) alone, with VLDL (100 µg/mL) and PCSK9 (5 µg/mL) or with VLDL (100 µg/mL) and PCSK9 (5 µg/mL) and anti-PCSK9 (10 µg/mL) for 24 hr. mRNA data are shown as the mean ± SEM (Experiments: n= 5-6/group and technical duplicates). Representative images of western blot analysis, the lanes were run on the same gel.

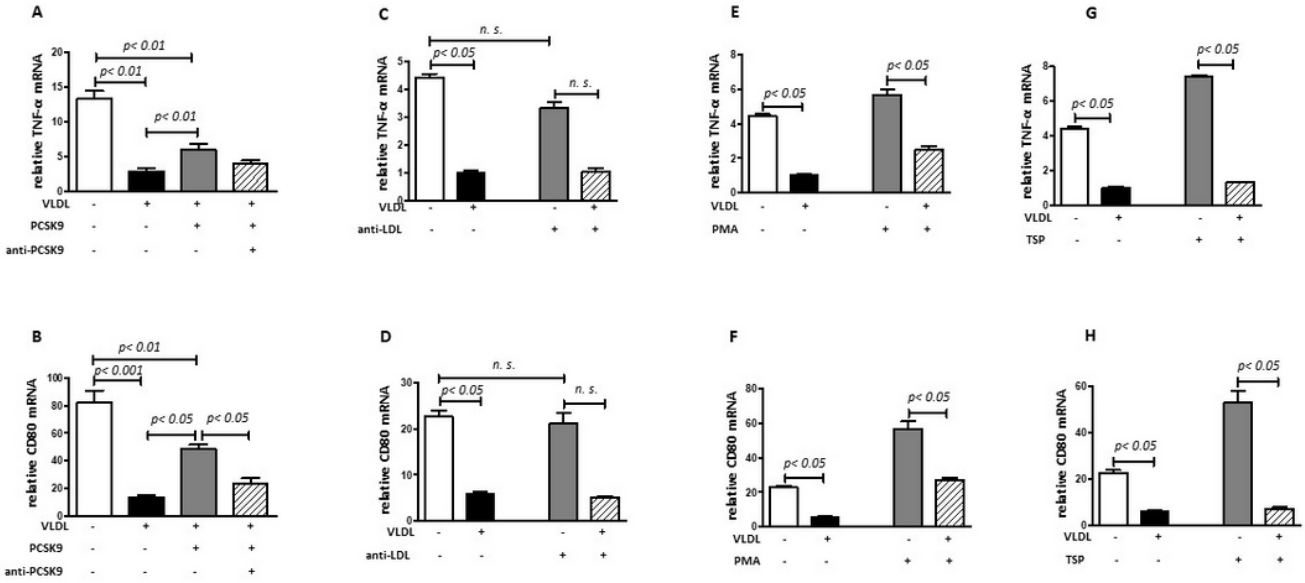

Figure 5

Figure 5

PCSK9 inhibits the anti-inflammatory effects of VLDL on pro-inflammatory polarized monocytic cells. Relative mRNA expression analysis of TNF-α and CD80 performed in pro-inflammatory THP-1 cells treated with (A-B) VLDL (100 µg/mL) alone or with VLDL (100 µg/mL) and PCSK9 (5 µg/mL); VLDL (100 µg/mL) and PCSK9 (5 µg/mL) and anti PCSK9 (10 µg/mL), (C-D) VLDL (100 µg/mL) alone or with anti-LDL (2 µg/mL), (E-F) VLDL (100 µg/mL) alone or with PMA (0.15 µg/mL) or (G-H) VLDL (100 µg/mL) alone or with TSP-1 (10 µg/mL) for 24hr. Data are shown as the mean ± SEM (Experiments: n= 5-6/group and technical duplicates).

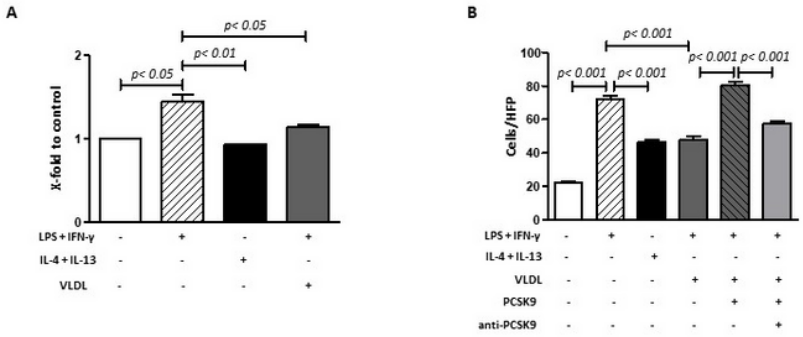

Figure 6

Figure 6

VLDL promotes an anti-inflammatory phenotype in pro-inflammatory polarized monocytic cells. (A) X-fold change of phagocytic undifferentiated, pro-inflammatory, and anti-inflammatory THP-1 cells treated with VLDL (100 µg/mL) for 24hr. (B) Amount of migrated cells per high power field (hpf) of undifferentiated, pro-inflammatory, and anti-inflammatory THP-1 cells treated with VLDL (100 µg/mL) alone or with VLDL (100 µg/mL) and PCSK9 (5 µg/mL) or VLDL (100 µg/mL) and PCSK9 (5 µg/mL) and anti PCSK9 (10 µg/mL) for 24hr. Data are shown as the mean ± SEM (Experiments: n= 5-6/group and technical duplicates).