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Beneficial effects of PCSK9 inhibition with alirocumab in familial hypercholesterolemia involve modulation of new immune players

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ABSTRACT

Familial hypercholesterolemia (FH) is associated with low-grade systemic inflammation, a key driver of premature atherosclerosis. We investigated the effects of inhibiting proprotein convertase subtilisin/kexin type 9 (PCSK9) function on inflammatory state, endothelial dysfunction and cardiovascular outcomes in patients with FH. Fourteen patients with FH were evaluated before and 8 weeks after administration of a PCSK9 blocking monoclonal antibody (alirocumab, 150 mg/subcutaneous/14 days). *In vivo* and *ex vivo* analysis revealed that alirocumab blunted the attachment of leukocytes to TNFα-stimulated human umbilical arterial endothelial cells (HUAEC) and suppressed the activation of platelets and most leukocyte subsets, which was accompanied by the diminished expression of CX_3CR1, CXCR6 and CCR2 on several leukocyte subpopulations. By contrast, T-regulatory cell activation was enhanced by alirocumab treatment, which also elevated anti-inflammatory IL-10 plasma levels and lowered circulating pro-inflammatory cytokines. Plasma levels of IFNγ positively correlated with levels of total and LDL-cholesterol, whereas circulating IL-10 levels negatively correlated with these key lipid parameters. *In vitro* analysis revealed that TNFα stimulation of HUAEC increased the expression of PCSK9, whereas endothelial *PCSK9* silencing reduced TNFα-induced mononuclear cell adhesion mediated by Nox5 up-regulation and p38-MAPK/NFκB activation, concomitant with reduced SREBP2 expression. *PCSK9* silencing also decreased endothelial CX_3CL1 and CXCL16 expression and chemokine generation. In conclusion, PCSK9 inhibition impairs systemic inflammation and endothelial dysfunction by constraining leukocyte-endothelium interactions. PCSK9 blockade may constitute a new therapeutic approach to control the inflammatory state associated with FH, preventing further cardiovascular events in this cardiometabolic disorder.

Abbreviations: apoB, apolipoprotein B; CAM, cell adhesion molecules; CVD, cardiovascular disease; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinases 1/2; FH, familial hypercholesterolemia; GROα, growth-regulated oncogene-α; HAEC, human aortic endothelial cells; HUAEC, human umbilical arterial endothelial cells; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular cell adhesion molecule-1; IFNγ, interferon-γ; Ig, immunoglobulin; IL, interleukin; LDL, low-density lipoprotein; LDL-R, low-density lipoprotein receptor; LPS, lipopolysaccharide; mAb, monoclonal antibody; MCP-1, monocyte chemoattractant protein-1; Mon1/2/3, type 1/2/3 monocytes; NF-κB, nuclear factor-kappa B; Nox5, NADPH oxidase 5; ox-LDL, oxidized low-density lipoprotein; p38-MAPK, p38 mitogen-activated protein kinase; PCSK9, proprotein convertase subtilisin/kexin type 9; PF-4, platelet factor-4; PH, primary hypercholesterolemia; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RANTES, regulated on activation normal T-cell expressed and secreted; ROS, reactive oxygen species; SEM, standard error of the mean; siRNA, small interfering RNA; SREBP1/2, sterol-responsive element binding protein 1/2; Th, T helper cells; TNFα, tumor necrosis factor-α; Treg, T-regulatory cells; VCAM-1, vascular cell adhesion molecule-1.

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1. Introduction

Heterozygous familial hypercholesterolemia (FH) is a genetic disorder characterized by elevated plasma levels of low-density lipoprotein (LDL)-cholesterol and has an incidence of 1 in 200–500 individuals worldwide (up to 34 million people); homozygous FH is a rarer and more severe condition, affecting 1 in 160,000–1,000,000 individuals worldwide) [1,2]. Primary hypercholesterolemia (PH), which includes both autosomal-dominant FH and the more frequent polygenic non-familial hypercholesterolemia, is associated with low-grade systemic inflammation and endothelial dysfunction, both key drivers of premature atherosclerosis [3–6]. Low-grade systemic inflammation is characterized by the enhanced production of inflammatory mediators including cytokines, chemokines and acute-phase proteins within the circulation, as well as by changes in the activation state of different immune cell populations [7]. Notably, individuals with FH have a 3–13-fold greater risk of premature atherosclerosis than peers with normal plasma levels of LDL-cholesterol [1,8]. Accordingly, early diagnosis and appropriate treatment (lipid-lowering therapy) is crucial to reduce cardiovascular risk. Generally, statins are the first-line treatment for FH; however, many patients treated with statins either do not achieve their target levels of LDL, are intolerant to statins, or statins are contraindicated [1,9]. Under these circumstances, an alternative lipid-lowering strategy or combination therapy is required.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease ubiquitously expressed in several tissues and cells (mainly hepatocytes) that controls the metabolism of LDL-cholesterol. PCSK9 circulates in the bloodstream and has affinity for the LDL-receptor (LDL-R), with which it forms a complex and promotes its degradation, reducing LDL uptake in the liver [10]. In addition to its role in LDL-R recycling, PCSK9 seems also to be involved in other physiological processes such as the immune response, hemostasis and glucose metabolism (reviewed in [11]).

Gain-of-function mutations in *PCSK9*, as well as loss-of-function mutations in LDL-R or apolipoprotein B (apoB), result in impaired LDL clearance by enhancing LDL-R degradation and/or impairing LDL-R/LDL binding [10,12]. Both conditions increase the plasma levels of LDL-cholesterol and, consequently, the atherogenic risk. Neutralizing monoclonal antibodies (mAbs) against PCSK9 have demonstrated good efficacy in lowering LDL-cholesterol in patients with FH [13]. According to the European Medicines Agency and the U.S. Food and Drug Administration, two mAbs against human PCSK9 are currently available for therapy–alirocumab and evolocumab–in addition to inclisiran, which is an inhibitor of hepatic PCSK9 translation [6,14]. Both mAbs are administrated subcutaneously [9]. Recent studies suggest that mAbs against PCSK9 may also have a favorable impact on FH and cardiovascular outcomes by not only reducing circulating LDL-cholesterol levels, but also by blunting the systemic inflammation associated with the disease and alleviating anxiety/depression [15,16]. However, the impact of their administration on the immune system has been scarcely studied.

We aimed to investigate the effect of PCSK9 inhibition on the immune system in patients with FH treated with alirocumab for 8 weeks by evaluating the activation state of platelets and leukocytes and examining changes to the chemokine profile. Concerning the latter, the fractalkine (CX_3CL1)/CX_3CR1 and CXCL16/CXCR6 chemokine axes are reported to be important for mononuclear cell attachment and endothelial transmigration of monocytes and lymphocytes, a critical step in the atherogenic process [17,18]. Likewise, CCL2 (monocyte chemoattractant protein-1, MCP-1), is known to regulate the migration and infiltration of monocytes through interaction with its receptor (CCR2) [19]. Given the evidence supporting the involvement of the CX_3CL1/CX_3CR1, CXCL16/CXCR6 and CCL2/CCR2 axes in the development of cardiovascular disease (CVD) in several cardiometabolic disorders [17,20–22], we also explored the role of these chemokine axes. Finally, as endothelial dysfunction is an early step in the atherogenic process, we evaluated the effect of PCSK9 gene silencing on endothelial dysfunction, focusing on the impact of endothelial PCSK9 inhibition on mononuclear cell adhesion and the underlying mechanisms involved in this response.

2. Materials and methods

2.1. Human study population

We included a total of 14 patients with FH in the present study with a case-crossover design. Patients were recruited at the outpatient Lipid Clinic of the Endocrinology and Nutrition Service of the University Clinic Hospital of Valencia (Valencia, Spain) by opportunistic sampling during a period of 6 months. Patients were treated with a PCSK9 inhibitor (alirocumab, Praluent®, 150 mg/subcutaneous/14 days, Sanofi, Paris, France) for 8 weeks. All procedures involving human samples complied with the principles outlined in the Declaration of Helsinki and were approved by the institutional ethics committee of the University Clinic Hospital, Valencia. All patients signed informed consent. Clinical and biological parameters of patients and age-matched controls are shown in Table 1. Further details can be found in the Supplementary file.

2.2. Cell culture

Human umbilical arterial endothelial cells (HUAEC) were isolated by collagenase treatment. Details are described in the Supplementary file.

2.3. Small interfering RNA-mediated gene silencing

HUAEC were transfected with either a control or a *PCSK9* siRNA using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA). At 48 h post-transfection, HUAEC were treated according to the experimental plan. Details are described in the Supplementary file.

2.4. Quantitative reverse transcription polymerase chain reaction

PCSK9 mRNA expression was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). HUAEC were stimulated or not with tumor necrosis factor-α (TNFα, 20 ng/mL, Sigma-Aldrich, Madrid, Spain) for 1, 4 or 24 h. Relative quantification of the different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using *GAPDH* as an endogenous control, normalized to vehicle. Further details are described in the Supplementary file. Primers are described in Table S1.

Table 1
Clinical features of patients before and after treatment.

Clinical features	Before treatment	After treatment	P-value
Glucose (mg/dL)	99.50 ± 4.54	98.07 ± 4.36	0.66
Cholesterol (mg/dL)	244.93 ± 14.59	159.25 ± 44.64 * *	**< 0.01**
LDL (mg/dL)	164.07 ± 12.78	87.75 ± 31.57 * *	**< 0.01**
HDL (mg/dL)	61.43 ± 2.55	58.43 ± 3.07	0.17
Triglycerides (mg/dL)	134.57 ± 22.47	123.00 ± 18.37	0.32
ApoB (mg/dL)	114.00 ± 12.22	82.43 ± 8.00 *	**0.03**
Soluble PCSK9 (ng/mL)	575.90 ± 96.16	2859.00 ± 297.50 * *	**< 0.01**
GOT (U/L)	32.79 ± 4.47	31.14 ± 4.44	0.40
GPT (U/L)	33.57 ± 6.21	34.93 ± 8.82	0.86
Creatinine (mg/dL)	0.84 ± 0.05	0.84 ± 0.06	0.85
IgG (mg/dL)	939.64 ± 34.07	921.79 ± 36.92	0.27
IgM (mg/dL)	95.14 ± 16.35	95.43 ± 14.20	0.99
IgE (mg/dL)	42.00 ± 10.27	43.41 ± 10.49	0.74

Data are presented as mean ± SEM. Definition of abbreviations: ApoB = Apolipoprotein B; GOT = Glutamic Oxaloacetic Transaminase; GPT = Glutamyl Pyruvic Transaminase; HDL = High-Density Lipoprotein; Ig = Immunoglobulin; LDL = Low-Density Lipoprotein; PCSK9 = proprotein convertase subtilisin/kexin type 9. * $P < 0.05$ or * * $P < 0.01$ relative to values before treatment.

2.5. Flow cytometry

Full details are described in the Supplementary file. Gating strategies are described in Figs. S1–S5 and Tables S2–S3 (Supplementary file).

2.6. Measurement of soluble metabolic and inflammatory markers

Soluble metabolic and inflammatory markers were measured by enzyme-linked immunosorbent assay (ELISA) in plasma from heparinized whole blood. Relevant chemokines were similarly measured in the supernatant of transfected HUAEC stimulated or not with TNFα (20 ng/mL) for 48 h. Further details are described in the Supplementary file.

2.7. Leukocyte-endothelial cell interactions under flow conditions

To study leukocyte-endothelial cell interaction *ex vivo*, whole blood, treated or not with ethylenediaminetetraacetic acid (EDTA), was perfused across HUAEC monolayers unstimulated or stimulated beforehand with TNFα (20 ng/mL) for 48 h.

To test the functional role of endothelial PCSK9 in mononuclear cell adhesion to dysfunctional arterial endothelium, HUAEC were transfected with control or *PCSK9*-specific siRNAs and stimulated or not with TNFα (20 ng/mL) for 48 h. Mononuclear cells were then perfused across HUAEC monolayers. Details are described in the Supplementary file.

2.8. Western blotting

Western blotting was used to assess endothelial PCSK9 expression and to confirm the role of NADPH oxidase 5 (Nox5) in the endothelial expression of PCSK9. Full details are described in the Supplementary file.

2.9. Immunofluorescence

Immunofluorescence was used to assess PCSK9 expression and to confirm the flow cytometry analysis of endothelial intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), fractalkine/CX_3CL1 and CXCL16 expression. Full details are described in the Supplementary file.

2.10. Statistical analysis

All results were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Values are expressed as individual data points, percentages or mean ± standard error of the mean (SEM) where appropriate. For two-group comparisons, paired or unpaired Student's *t*-test was used in data that passed both normality (Kolmogorov-Smirnov) and equal variance (Levene) tests, as appropriate; otherwise, the non-parametric Mann-Whitney *U*-test was performed. For comparisons of multiple groups, one-way analysis of variance followed by *post hoc* Sidak analysis was used in data that passed both normality and equal variance tests; otherwise, the non-parametric Kruskal-Wallis test followed by Dunn's *post hoc* analysis was used. Statistical significance was set at $P < 0.05$. Some correlations between experimental findings and clinical features were calculated using the Pearson and Spearman correlation analysis procedures.

A sensitivity power analysis showed that with a sample size of 13 participants, and given $\alpha = 0.05$, effect sizes of $w = 0.9$ (based on previous data) could be detected in the model-based comparisons with a statistical power of $1 - \beta = 0.90$ [23].

3. Results

Fourteen patients with FH were included (see flow chart participation in Fig. S6, Supplementary file). Clinical and biological parameters of patients before and after alirocumab treatment are shown in Table 1. No significant changes were found in fasting levels of glucose, high-density lipoprotein-cholesterol, triglycerides, glutamic oxaloacetic transaminase, glutamyl pyruvic transaminase, creatinine, immunoglobulin (Ig)G, IgM or IgE after the 8-week regimen. By contrast, fasting levels of total cholesterol, LDL-cholesterol and apoB were all significantly lower, and levels of soluble PCSK9 were significantly higher in patients after the treatment than before. The increase in PCSK9 levels in patients treated with statins [24] or alirocumab [25] has been noted previously. However, PCSK9 likely has no functionality due to the neutralization of its activity by alirocumab.

3.1. Alirocumab treatment reduces leukocyte-platelet-endothelium, leukocyte-endothelium interactions and platelet activation in patients with FH

We determined the effects of alirocumab on platelet-leukocyte and leukocyte adhesion to dysfunctional arterial endothelium under dynamic flow conditions. Because FH is associated with low-grade systemic inflammation and elevated circulating levels of TNFα [26,27], we used this cytokine as an inflammatory stimulus on HUAEC to mimic endothelial dysfunctionality. As expected, TNFα stimulation (20 ng/mL, 48 h) increased leukocyte-platelet and leukocyte adhesiveness to HUAEC (Fig. 1A, B). Notably, alirocumab treatment reduced TNFα-induced leukocyte-platelet adhesion by 54.8% (Fig. 1A). Similarly, when platelets were dissociated from leukocytes with EDTA, alirocumab treatment significantly reduced leukocyte adhesion (54.5%) (Fig. 1B).

Regarding the contribution of platelets to leukocyte-endothelium interactions, while no significant changes were observed in the number of circulating platelets in patients before and after the treatment (Fig. 1C), the percentage of platelets expressing P-selectin (CD62P) and PAC-1 was significantly lower in patients after the treatment than before (Fig. 1D, E), indicating a reduction in their activation state. The increased percentage of activated (PAC-1^+) platelets positively correlated with levels of total and LDL-cholesterol (Fig. 1F, G). Elevated CX_3CR1 and CCR2 expression and levels of platelet activation-related soluble mediators have been reported in patients with FH [28,29]. Alirocumab treatment had no effect on platelet CX_3CR1 (Fig. 1H), CXCR6 (Fig. 1I) or CCR2 (Fig. 1J) expression, or on the plasma levels of soluble P-selectin (sP-selectin; Fig. 1K), platelet factor-4 (PF-4/CXCL4; Fig. 1L) or regulated on activation normal T-cell expressed and secreted (RANTES/CCL5; Fig. 1M).

3.2. Alirocumab treatment reduces neutrophil and eosinophil activation and lowers the circulating levels of IL-8/CXCL8 and eotaxin-2/CCL24 in patients with FH

We next measured several parameters related to the activation of different leukocyte subsets. No changes were detected in the percentage of circulating neutrophils, eosinophils (Fig. 2A, E) or in the percentage of neutrophil- and eosinophil-platelet aggregates after alirocumab treatment (Figs. S7A, B, Supplementary file). By contrast, neutrophil and eosinophil activation (CD11b expression) were significantly lower after alirocumab treatment than before (Fig. 2B, F). Of note, CD11b expression in eosinophils positively correlated with total and LDL-cholesterol levels (Fig. 2G, H). Several chemokines including interleukin (IL)− 8/CXCL8 and growth-regulated oncogene-α (GROα/CXCL1) or eotaxins (eotaxin-1/CCL11, −2/CCL24 and −3/CCL26) can induce human neutrophil or eosinophil activation/chemotaxis, respectively. Alirocumab treatment led to a significant decrease in the plasma levels of IL-8/CXCL8 and eotaxin-2/CCL24 (Fig. 2C, J), but did not affect GROα/CXCL1, eotaxin-1/CCL11 or eotaxin-3/CCL26 (Fig. 2D, I, K).

3.3. Alirocumab treatment reduces monocyte activation and expression of CCR2 and CX_3CR1 in patients with FH

We analyzed monocyte subpopulations in peripheral blood by flow

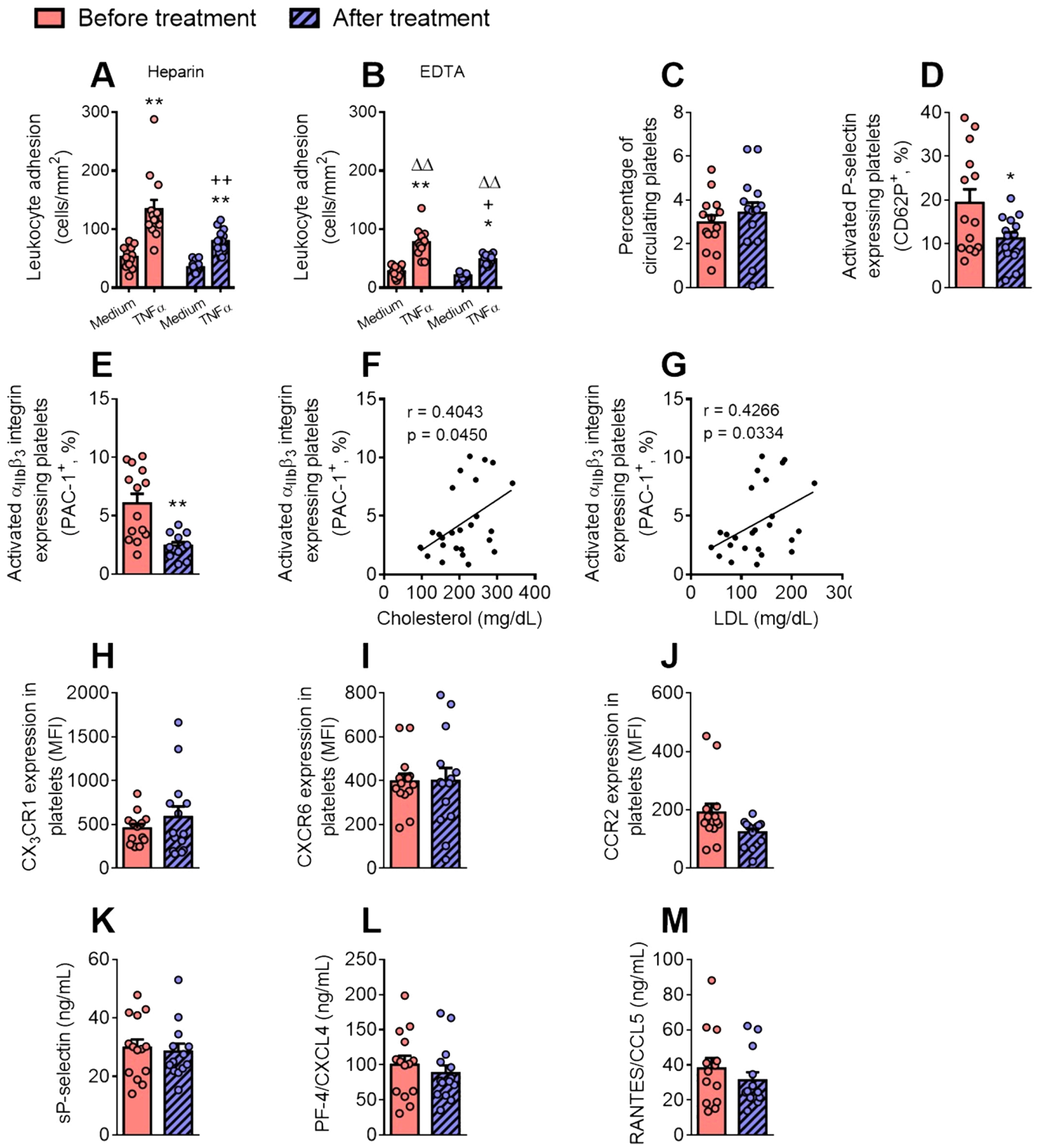

Fig. 1. Alirocumab treatment reduces leukocyte-platelet and leukocyte adhesion to TNFα-stimulated HUAEC and suppresses platelet activation in patients with FH. HUAEC were stimulated with TNFα (20 ng/mL) for 48 h. Subsequently, whole blood from patients (before and after treatment with alirocumab), incubated without (A) or with (B) EDTA, was perfused across endothelial monolayers for 7 min at 0.5 dyn/cm^2 and leukocyte adhesion was quantified. Results are presented as adhered leukocyte per mm^2 (cells/mm^2). Flow cytometry analysis of platelets labeled with fluorochrome-conjugated antibodies against CD41 (C), CD41 and P-selectin (D), CD41 and PAC-1 (E), CD41 and CX_3CR1 (H), CD41 and CXCR6 (I) and CD41 and CCR2 (J). Results are presented as the percentage of positive cells or mean fluorescence intensity (MFI). Soluble (s)P-selectin (K), platelet factor-4 (PF-4)/CXCL4 (L) and regulated on activation normal T cell expressed and secreted (RANTES)/CCL5 (M) plasma levels (ng/mL) were measured by ELISA. Values are expressed as mean ± SEM (n = 14 patients). * $P < 0.05$ or * * $P < 0.01$ relative to values in the respective control group (A and B) or relative to values detected before treatment (D and E); + $P < 0.05$ or + + $P < 0.01$ relative to values before treatment in TNFα-stimulated cells (A and B); ΔΔ$P < 0.01$ relative to the respective heparin group (A and B). Correlations between percentage of circulating platelets (PAC-1$^+$) and levels of total cholesterol (F) and LDL-cholesterol (G).

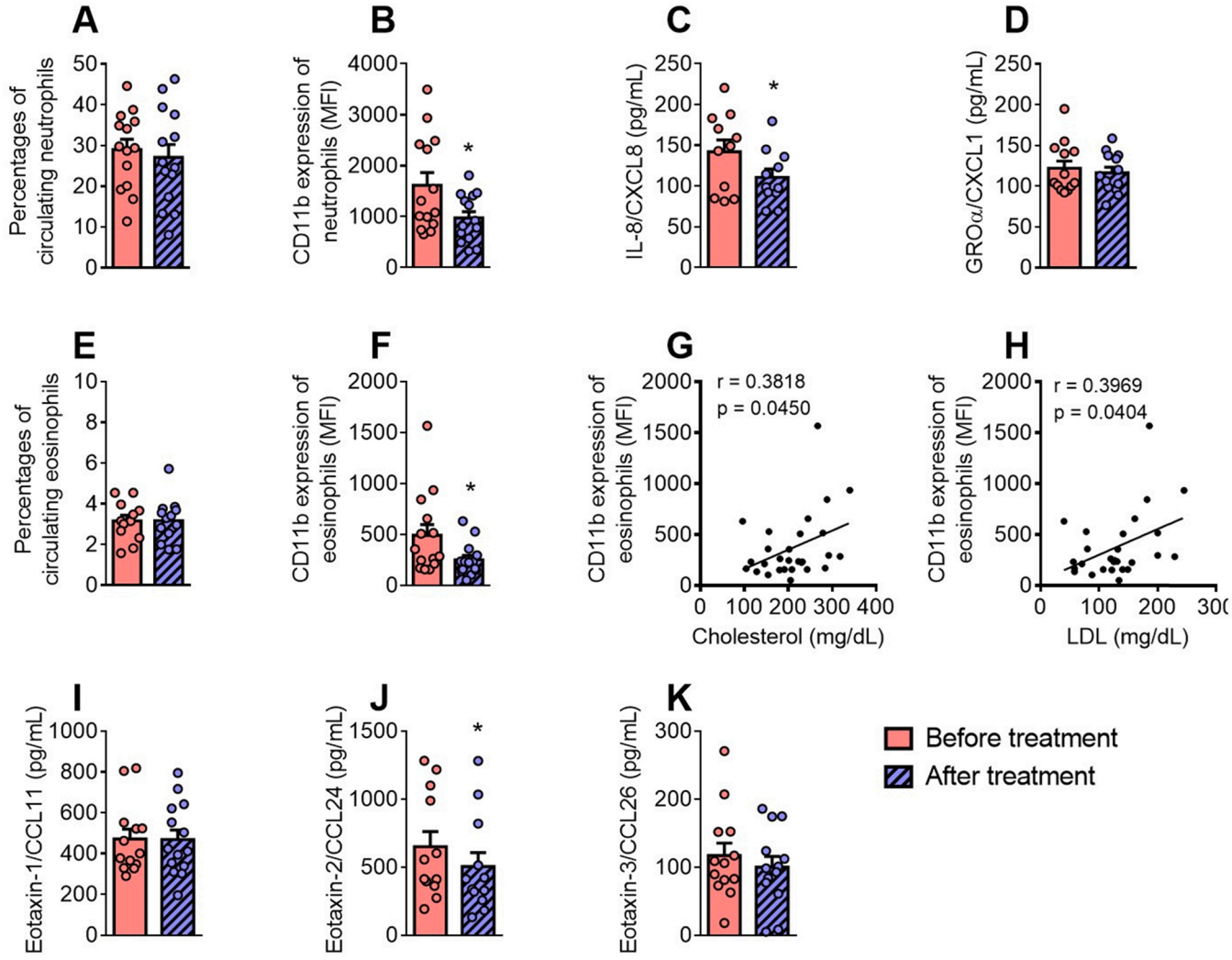

Fig. 2. Activation of neutrophils and eosinophils and circulating levels of IL-8/CXCL8 and eotaxin-2/CCL24 are reduced in patients with FH treated with alirocumab. Flow cytometry analysis of heparinized whole blood co-stained with fluorochrome-conjugated antibodies against specific markers for neutrophils (A) or eosinophils (E). Neutrophils and eosinophils were also stained with a CD11b fluorochrome-conjugated antibody (B and F). Results are presented as the percentage of positive cells or mean fluorescence intensity (MFI). Interleukin (IL)− 8/CXCL8 (C), growth-regulated oncogene-α (GROα)/CXCL1 (D), eotaxin-1/CCL11 (I), eotaxin-2/CCL24 (J) and eotaxin-3/CCL26 (K) plasma levels (pg/mL) were measured by ELISA. Values are expressed as mean ± SEM (n = 14 patients). * $P < 0.05$ relative to values detected before treatment. Correlation between CD11b expression of eosinophils and levels of total cholesterol (G) and LDL-cholesterol (H).

cytometry. Monocytes can be divided into three subsets based on the differential expression of surface markers such as CD14, CD16 and CCR2 (Table S2, Supplementary file). No changes were detected in the percentage of circulating total monocytes, monocyte subsets (Fig. 3A, B) or in the percentage of monocyte-platelet aggregates after alirocumab treatment (Figs. S7C, D, Supplementary file). By contrast, alirocumab significantly reduced monocyte activation (CD11b expression) and, more specifically, the activation of type 1 and type 2 monocytes (Mon1 and Mon2) (Fig. 3C, F). CD11b expression in monocytes positively correlated with total and LDL-cholesterol levels (Fig. 3D, E). Alirocumab treatment also suppressed CCR2 expression on Mon1 and Mon2 subpopulations (Fig. 3G, H) and CX_3CR1 expression in Mon2 and Mon3 subsets (Fig. 3K, L). CCR2 expression in Mon1 and CX_3CR1 expression in Mon3 monocytes positively correlated with total and LDL-cholesterol levels (Fig. 3I, J, M, N). Despite these findings, no changes were detected in the plasma levels of MCP-1/CCL2 or fractalkine/CX_3CL1 after alirocumab treatment (Fig. 3O, P). Finally, no changes in monocyte CXCR6 expression were found after 8 weeks of alirocumab administration (Figs. S8A, B, Supplementary file).

3.4. Alirocumab treatment increases Treg cell activation and circulating IL-10 levels in patients with FH and reduces the levels of IFNγ and CXCL16

Mature T-cells express the general marker CD3, and either CD4 or CD8 depending on the T-cell type. Flow cytometry analysis showed that alirocumab treatment did not change the percentages of circulating $CD3^+$, $CD4^+$ or $CD8^+$ lymphocytes in patients (Fig. 4A). Closer inspection of the different $CD4^+$ T-lymphocyte subtypes also revealed no changes in the percentages of circulating T helper lymphocytes (Th)1, Th2, Th17 and T-regulatory (Treg) cells after the treatment (Fig. 4B). Likewise, no differences were detected in the percentage of T-lymphocyte-platelet aggregates (Figs. S7E-G, Supplementary file). In the context of T-lymphocyte activation ($CD69^+$), alirocumab treatment significantly increased the activation state of Treg cells (Fig. 4D) but had no effect on the activation of the other T-cell subsets (Fig. 4C and D).

Of note, whereas the circulating levels of interferon-γ (IFNγ), a cytokine mainly released by Th1 lymphocytes, were significantly lower in patients after alirocumab treatment than before (Fig. 4E), plasma levels of IL-10, a cytokine mostly produced by Treg cells, were significantly higher (Fig. 4L). Notably, the plasma levels of both cytokines

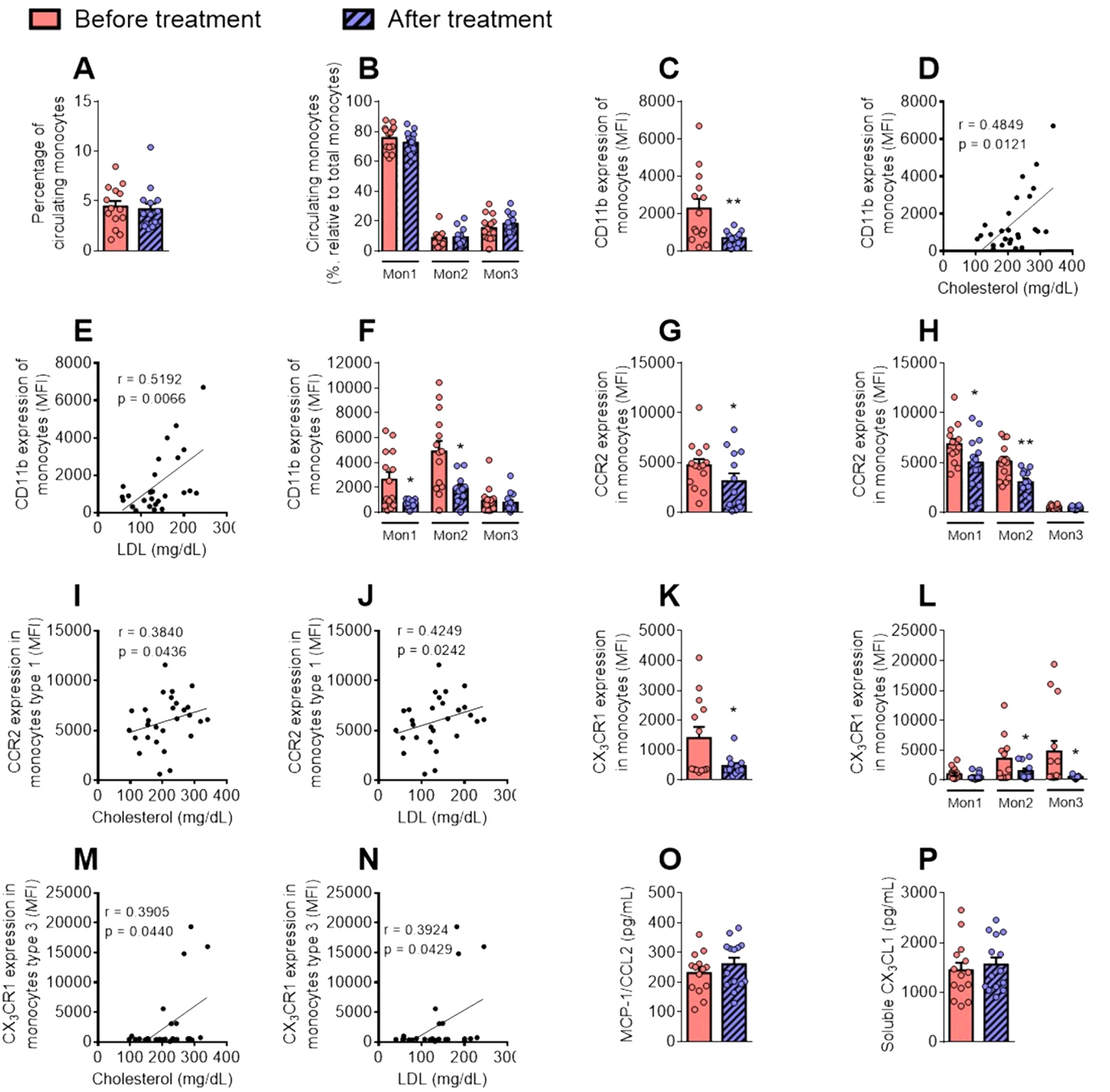

Fig. 3. Alirocumab treatment suppresses monocyte activation and CCR2 and CX_3CR1 expression in patients with FH. Flow cytometry analysis of heparinized whole blood co-stained with fluorochrome-conjugated antibodies against specific markers for monocytes and the different monocyte subsets (Mon1, Mon2 and Mon3; A and B). All monocyte subsets were also stained with fluorochrome-conjugated antibodies against CD11b (C and F), CCR2 (G and H) and CX_3CR1 (K and L). Results are presented as the percentage of positive cells or mean fluorescence intensity (MFI). Monocyte chemoattractant protein-1 (MCP-1)/CCL2 (O) and soluble CX_3CL1 (P) plasma levels (pg/mL) were measured by ELISA. Values are expressed as mean ± SEM (n = 14 patients). * $P < 0.05$ or * * $P < 0.01$ relative to values detected before treatment. Correlation between CD11b expression in monocytes and levels of total cholesterol (D) and LDL-cholesterol (E). Correlation between CCR2 expression of Mon1 and levels of total cholesterol (I) or LDL-cholesterol (J). Correlation between CX_3CR1 expression of monocytes type 3 and levels of total cholesterol (M) or LDL-cholesterol (N).

positively (IFNγ) or negatively (IL-10) correlated with the levels of total and LDL-cholesterol (Fig. 4F, G, M, N). By contrast, the plasma levels of other lymphocyte-related cytokines such as IL-4 (Fig. 4H), TNFα (Fig. 4I), IL-6 (Fig. 4J) and IL-12 (Fig. 4K) were unchanged after the treatment.

With regards to chemokine expression, CX_3CR1 expression in all T-lymphocyte subsets investigated was significantly lower in patients after alirocumab treatment than before (Fig. 4O, P), and CXCR6 expression decreased in all T-cell subsets with the exception of Treg cells (Fig. 4Q, T). Moreover, the expression of CXCR6 in $CD3^+$ $CD4^+$, $CD8^+$, Th1, Th2 and Th17 lymphocytes positively correlated with the levels of total and LDL-cholesterol (Fig. 4R, S and S9, Supplementary file). Finally, plasma levels of CXCL16 were also significantly reduced after the treatment (Fig. 4U).

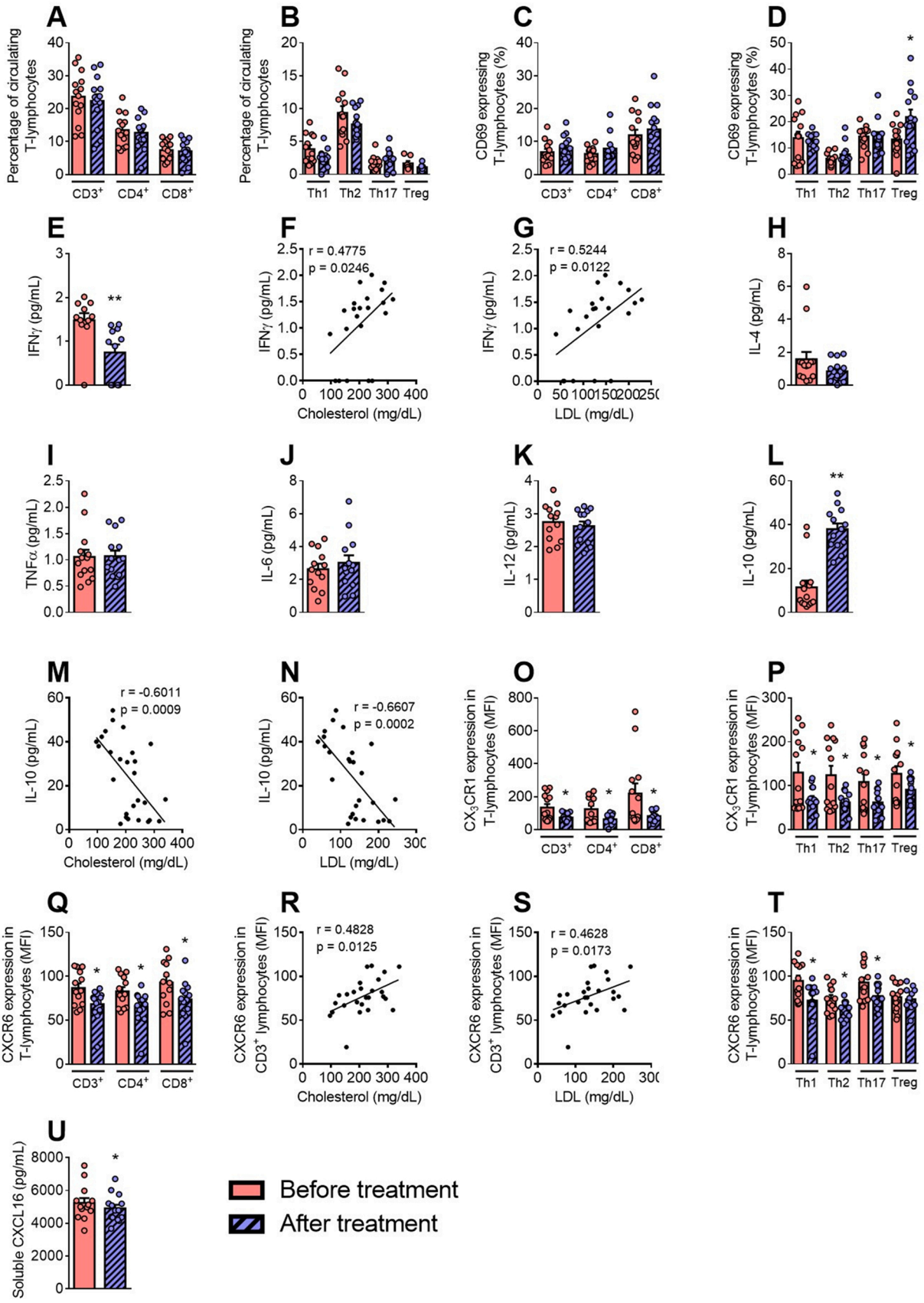

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Fig. 4. Alirocumab treatment increases the activation of Treg cells and IL-10 circulating levels in patients with FH, but decreases IFNγ and CXCL16 levels. Flow cytometry analysis of heparinized whole blood co-stained with fluorochrome-conjugated antibodies against specific markers for T-lymphocytes ($CD3^+$; A), T-lymphocyte subsets ($CD4^+$ and $CD8^+$; A), T-helper cells (Th1, Th2 and Th1 and Treg; B). All T-lymphocytes were also stained with fluorochrome-conjugated antibodies against CD69 (C and D), CX_3CR1 (O and P) and CXCR6 (Q and T). Results are presented as percentage of positive cells or mean fluorescence intensity (MFI). Interferon-γ (IFNγ; E), interleukin (IL)− 4 (H), tumour necrosis factor-α (TNFα; I), IL-6 (J), IL-12 (K), IL-10 (L) and soluble CXCL16 (U) plasma levels (pg/mL) were measured by ELISA. Values are expressed as mean ± SEM (n = 14 patients). * $P < 0.05$ or * * $P < 0.01$ relative to values detected before treatment. Correlation between plasma levels of IFNγ or IL-10 and levels of total cholesterol (F or M) or LDL-cholesterol (G or N). Correlation between CXCR6 expression in $CD3^+$ lymphocytes and levels of total cholesterol (R) or LDL-cholesterol (S).

3.5. TNFα induces PCSK9 expression in HUAEC

PCSK9 expression has been previously described in human endothelial cells stimulated with oxidized LDL (ox-LDL) or lipopolysaccharide (LPS) [30,31]. Given that circulating levels of TNFα are elevated in patients with FH [26,27], we questioned whether TNFα affected endothelial PCSK9 expression. TNFα stimulation (20 ng/mL) of HUAEC for 1 or 4 h failed to alter *PCSK9* mRNA expression (Fig. 5A, B),

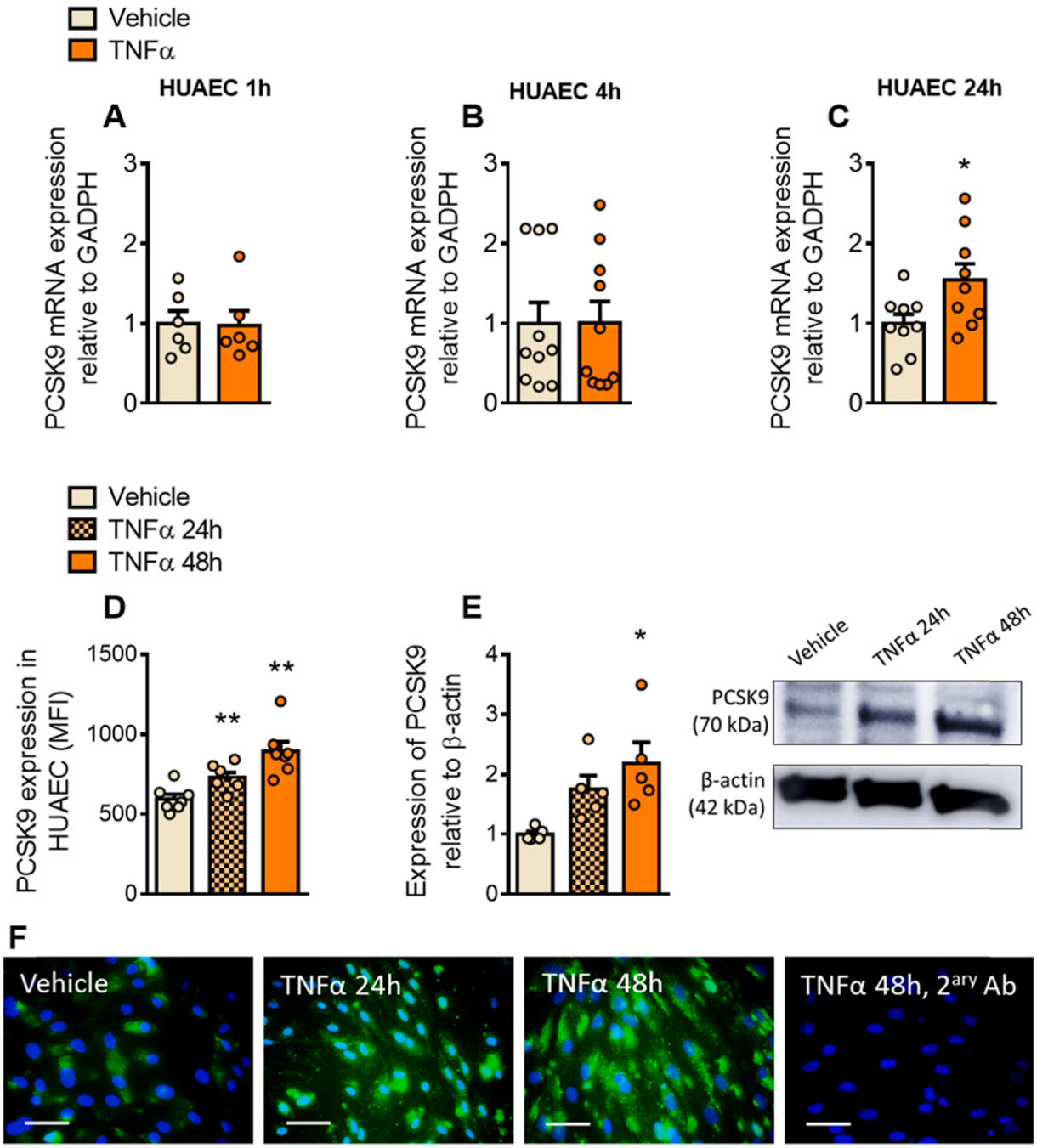

Fig. 5. TNFα induces PCSK9 expression in HUAEC. *PCSK9* mRNA expression in HUAEC after 1 (A), 4 (B) or 24 h (C) stimulation with TNFα (20 ng/mL). PCSK9 expression was assessed in HUAEC by flow cytometry after 24- and 48-h-stimulation with TNFα. (D), western blot (E) and immunofluorescence (F). Results are presented as relative mRNA expression (A–C), mean fluorescence intensity (MFI, D) or relative protein expression (E). Values are expressed as mean ± SEM (n = 4–9 independent experiments). *$P < 0.05$ or * *$P < 0.01$ relative to values in the vehicle group. Scale bar = 50 μm.

but a longer incubation (24 h) led to a significant increase in *PCSK9* mRNA expression (Fig. 5C). Flow cytometry analysis showed that PCSK9 protein expression was significantly enhanced 24 h after addition of TNFα, and robust expression was detected after 48 h (Fig. 5D). These results were confirmed by western blotting (Fig. 5E) and immunofluorescence (Fig. 5F). Accordingly, all remaining experiments were performed after 48 h stimulation with TNFα.

3.6. Endothelial PCSK9 silencing impairs mononuclear cell adhesion to dysfunctional arterial endothelium induced by TNFα through down-regulation of ICAM-1, VCAM-1, CX_3CL1 and CXCL16 expression and the generation of several chemokines

We investigated the functional role of endothelial PCSK9 in leukocyte arrest using an siRNA-based approach to knockdown *PCSK9* in HUAEC. siRNA silencing significantly suppressed PCSK9 protein expression 48 h after transfection (Fig. S10A, Supplementary file) and had no effect on cell viability (Fig. S10B, Supplementary file). TNFα stimulation of control siRNA-transfected HUAEC significantly increased mononuclear leukocyte-platelet adhesion, whereas silencing endothelial *PCSK9* expression before TNFα stimulation resulted in a significant decrease (55.8%) in mononuclear cell arrest to dysfunctional arterial endothelium (Fig. 6A). Likewise, when platelets were dissociated from leukocytes, the enhanced mononuclear cell adhesion induced by TNFα was significantly decreased when endothelial *PCSK9* was silenced (44.5% inhibition, Fig. 6B). Turning this approach on its head, we evaluated the impact of exogenous PCSK9 on leukocyte arrest. HUAEC were stimulated with PCSK9 (0.8 μg/mL, 24 h), which significantly increased mononuclear leukocyte-platelet adhesion (Fig. S11, Supplementary file), albeit to a lower extent than with TNFα. The dissociation of platelets from leukocytes resulted in a decrease in PCSK9-induced mononuclear cell adhesion (Fig. S11, Supplementary file).

As expected, TNFα stimulation of HUAEC increased ICAM-1, VCAM-1, CX_3CL1 and CXCL16 expression (Fig. 6C-F). Notably, the expression of TNFα-induced ICAM-1 (Fig. 6C), VCAM-1 (Fig. 6D) and the membrane-bound chemokines fractalkine/CX_3CL1 (Fig. 6E) and CXCL16 (Fig. 6F) was lower in *PCSK9*-silenced HUAEC than in control siRNA-transfected HUAEC. These results were confirmed by immunofluorescence (Fig. S12, Supplementary file). Moreover, TNFα stimulation of HUAEC led to the generation and release of GROα/CXCL1, IL-8/CXCL8, soluble CXCL16, MCP-1/CCL2, RANTES/CCL5 and soluble fractalkine/CX_3CL1 (Fig. 6G-L). Endothelial *PCSK9* knockdown significantly decreased the generation and release of TNFα-induced IL-8/CXCL8 by 42.9% (Fig. 6H), soluble CXCL16 by 41.2% (Fig. 6I), MCP-1/CCL2 by 16.8% (Fig. 6J) and soluble fractalkine/CX_3CL1 by 32.6% (Fig. 6L), but not that of GROα/CXCL1 and RANTES/CCL5 (Fig. 6G, K).

3.7. PCSK9 endothelial silencing blunts the enhanced TNFα-induced expression of Nox5, p38-MAPK and NF-κB/p65 activation, and SREBP2 up-regulation

The production of reactive oxygen species (ROS) and activation of redox-sensitive signaling pathways mediate many of the inflammatory responses of TNFα, including mononuclear cell recruitment [32]. Because TNFα induces mononuclear cell adhesion through Nox5 up-regulation in endothelial cells [33], we evaluated the potential effect of endothelial *PCSK9* silencing on Nox5 expression. Western blot analysis revealed that *PCSK9* endothelial silencing significantly suppressed the TNFα-induced expression of Nox5 (Fig. 7A). Overexpression of Nox5 in endothelial cells triggers a broad range of redox-sensitive pathways, including p38 mitogen-activated protein kinase (p38-MAPK) and extracellular signal-regulated kinases (ERK)1/2 phosphorylation [34]. In turn, the activation of these signaling pathways can provoke nuclear factor (NF)-κB transactivation [35], which is involved in PCSK9 expression [36–38]. TNFα induced p38-MAPK, ERK1/2 and NF-κB/p65 phosphorylation in control siRNA-control transfected HUAEC (Fig. 7B-D), but this was blunted in equivalent *PCSK9*-silenced cells (Fig. 7B and D) with the exception of ERK1/2 (Fig. 7C).

Sterol-responsive element binding proteins (SREBPs) are transcription factors that regulate cellular lipid metabolism [39]. The SREBP1 isoform preferentially promotes fatty acid synthesis, whereas SREBP2 regulates cellular cholesterol levels [39,40]. The regulation of PCSK9 synthesis is mediated by SREBP2 [41,42], and its expression is increased by TNFα [39]. As shown in Fig. 7E, SREBP2 expression was increased after 24- or 48-h-stimulation with TNFα in HUAEC (Fig. 7E). Notably, *PCSK9*-silenced HUAEC showed a decrease in TNFα-induced SREBP2 expression at these time points (Fig. 7E). Finally, both Nox (apocynin) and NF-κB (CAY10512) inhibition dramatically decreased the TNFα-induced expression of SREBP2 at 24 h (Fig. 7F).

4. Discussion

We demonstrate that an 8-week regimen of alirocumab in patients with FH suppresses leukocyte adhesion to dysfunctional arterial endothelium. This effect seems to be mainly due to the repressed activation of platelets and leukocyte subsets, improved endothelial dysfunction, the reduced levels of circulating inflammatory mediators, and enhanced anti-inflammatory Treg cell activation and IL-10 production. Based on these findings, PCSK9 neutralizing antibody treatment likely blocks the subendothelial infiltration of leukocytes, a critical step in the atherogenic process.

Platelet activation is associated with atherogenesis and cardiovascular morbidity [43]. Upon activation, platelets express specific cell adhesion molecules (CAMs) such as P-selectin, and release myriad inflammatory mediators. We previously showed that patients with PH present with a pro-thrombotic state characterized by increased platelet activation (P-selectin$^+$ and PAC-1$^+$ platelets) [5]. A recent study reported that PCSK9 increases platelet activation through a mechanism that relies on CD36, a scavenger receptor expressed in platelets and responsible for their hyperactivity in a dyslipidemic context [44]. We show that alirocumab inhibits platelet activation in patients with FH, which accords with recently published data in PH [45], and with observations in *Pcsk9*-knockout mice [46]. However, we found that alirocumab failed to alter the plasma levels of sP-selectin, PF-4/CXCL4 or RANTES/CCL5, contrasting with the results of Barale et al. [45] who reported a decrease in sP-selectin and PF-4/CXCL4 expression in patients using a similar treatment approach. We posit that a likely explanation for this discrepancy is the difference in the study length, as the aforementioned authors noted these reductions after 12 months of treatment but not at earlier time points. Overall, it is plausible that PCSK9 inhibitors ameliorate the prothrombotic state and associated cardiovascular consequences.

An increase in the activation of polymorphonuclear leukocytes *in vitro* has been reported in people at high cardiovascular risk (hyperlipidemia) [47], and similar effects have been reported in neutrophils of patients with PH [5]. In the present study, alirocumab treatment clearly impaired leukocyte activation (CD11b expression) and reduced the plasma levels of mediators involved in the activation/chemotaxis of neutrophils (IL-8/CXCL8) and eosinophils (eotaxin-2/CCL24), which would improve the pro-inflammatory status of patients.

Monocytes are key leukocytes in the initial development of atherosclerotic lesions. Human monocytes are a heterogeneous cell population that are commonly classified into three subtypes: classical (Mon1), intermediate (Mon2), and nonclassical (Mon3) [48]. A previous report indicated that adults with FH display a pro-inflammatory imbalance in circulating monocyte subpopulations (Mon1) [49], and we found in an earlier study that the percentage of the Mon3 subtype was the unique subtype enhanced in patients with PH [5]. Although we found here that treatment of FH with alirocumab had no impact on the percentage of any circulating monocyte subtype, as described previously [29], the activation state of CCR2-expressing Mon1 and Mon2 monocytes (CD11b up-regulation) was clearly reduced and positively correlated with total

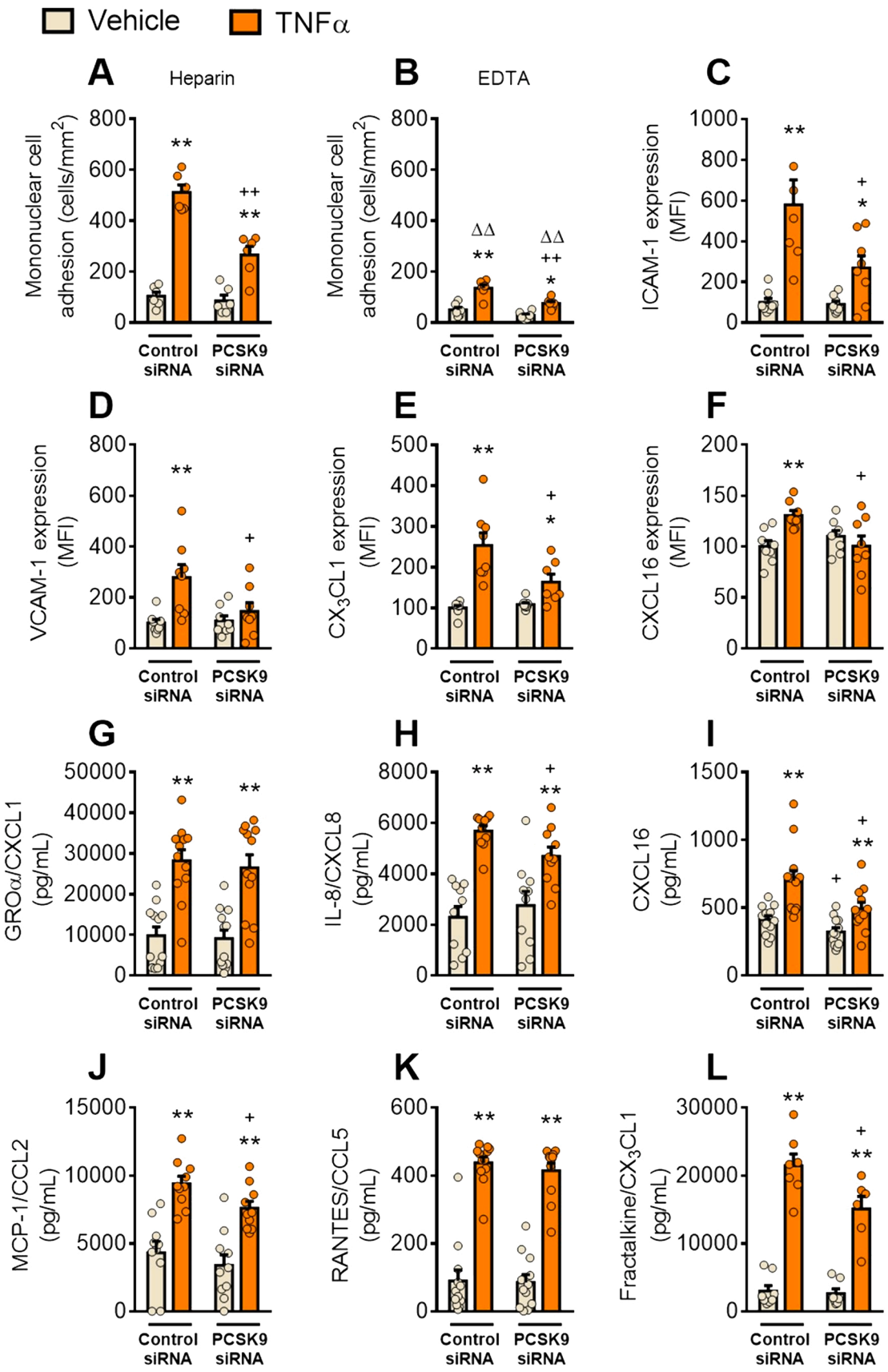

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Fig. 6. Endothelial *PCSK9* silencing impairs mononuclear cell adhesion to dysfunctional arterial endothelium induced by TNFα through down-regulation of ICAM-1, VCAM-1, CX_3CL1 and CXCL16 expression and the generation of several chemokines. The effect of endothelial *PCSK9* silencing on mononuclear cell adhesion to dysfunctional arterial endothelium, induced by TNFα (20 ng/mL, 48 h), was assessed using the parallel-plate flow chamber assay (A and B). The expression of endothelial ICAM-1, VCAM-1, CX_3CL1 and CXCL16 was determined by flow cytometry (C–F). Levels of GROα/CXCL1 (G), IL-8/CXCL8 (H), soluble CXCL16 (I), MCP-1/CCL2 (J), RANTES/CCL5 (K) and soluble fractalkine/CX_3CL1 (L) were determined in culture supernatants by ELISA. Results are expressed as number of adhered cells per mm^2 (A and B), mean fluorescence intensity (MFI, C–F) or as pg/mL (G–L). Values are expressed as mean ± SEM (n = 6–12 independent experiments). * * $P < 0.01$ relative to values in the respective vehicle group; $+P < 0.05$ relative to values in the respective control siRNA group stimulated with TNFα; $\Delta\Delta P < 0.01$ relative to values in the respective heparin group.

and LDL-cholesterol levels. The difference between our findings and those of others [29] is likely due to the different approaches to select monocyte subpopulations. Given that classical monocytes (Mon1) are the most abundant subpopulation and the primary cells interacting with the dysfunctional endothelium, PCSK9 inhibition in an environment of FH might affect their pro-adhesive properties by limiting their infiltration into the arterial wall.

Analysis of the different T-lymphocyte populations in the context of PCSK9 inhibition revealed unexpected and potentially relevant results. Although no effect was found in the percentage of circulating T-lymphocyte subsets or in the activation state of most of their subpopulations, we detected a clear increase in the abundance of activated Treg cells (CD69 expression). Treg cells are specialized T-cells that suppress the immune response [50]. Indeed, we observed a significant increase in the plasma levels of the anti-inflammatory cytokine IL-10, produced chiefly by these cells [50], and a decrease in circulating IFNγ levels. Moreover, circulating IL-10 and IFNγ levels negatively and positively correlated, respectively, with total and LDL-cholesterol serum concentrations. In a cross-sectional study testing the effects of airborne particulate matter in people with obesity, IFNγ plasma levels were found to positively correlate with circulating PCSK9 levels [51]. These findings add further support to the anti-inflammatory actions of PCSK9 inhibition in patients with FH to trigger an anti-atherogenic environment. Consistent with our observations, animal studies with a PCSK9 vaccine [52] found that while splenic production of IFNγ was decreased, IL-10 was increased in an atherosclerotic milieu.

As previously outlined, there is evidence to support a role for the CX_3CL1/CX_3CR1, CXCL16/CXCR6 and CCL2/CCR2 axes in

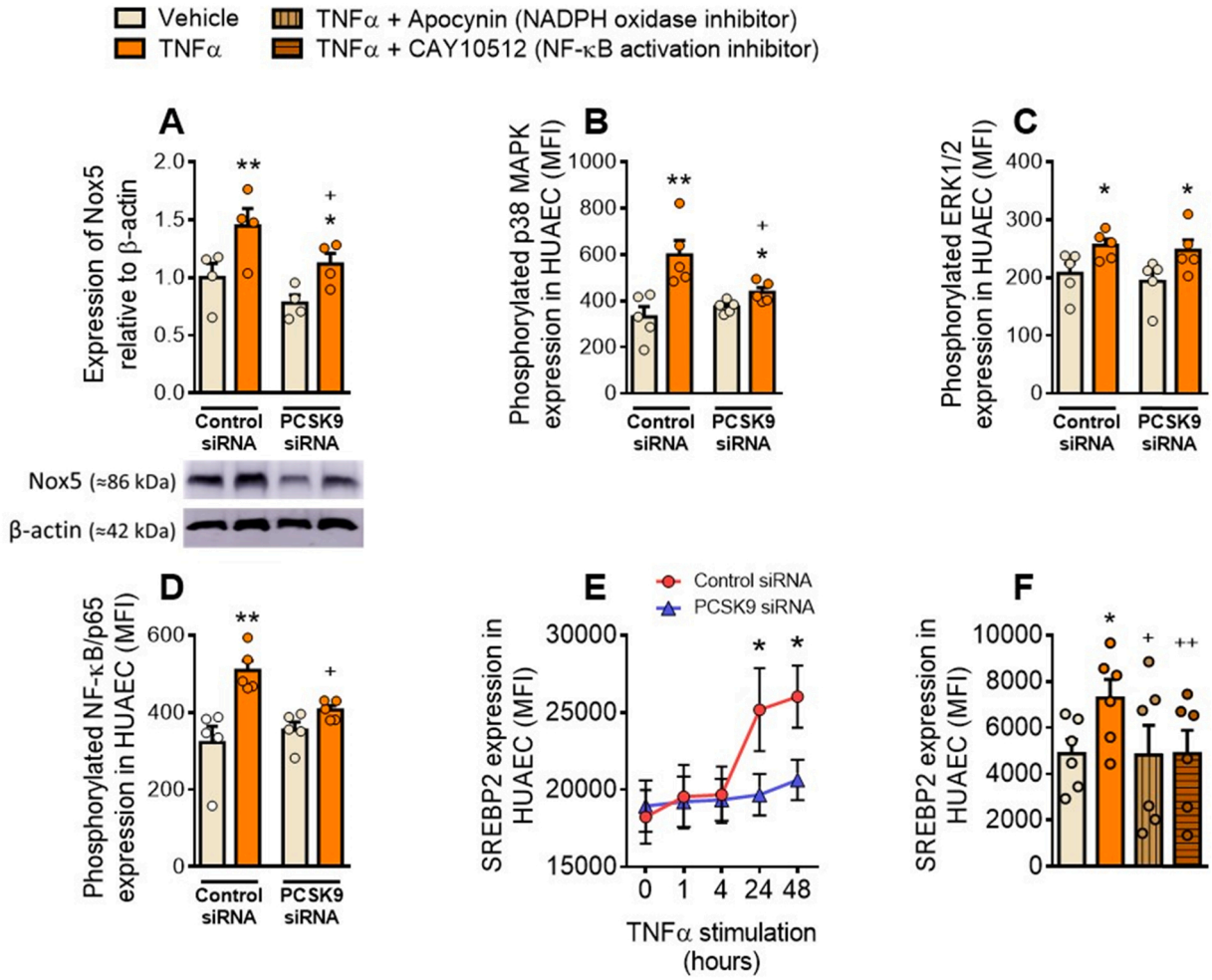

Fig. 7. *PCSK9* endothelial silencing tempers the enhanced TNFα-induced expression of Nox5, p38-MAPK and NF-κB/p65 activation, and SREBP2 expression. Protein expression of Nox5 (A) was assessed by western blot. Flow cytometry was employed to determine the phosphorylation of p38-MAPK (B), ERK1/2 (C) and NF-κB/p65 (D), or the expression of SREBP2 (E and F). HUAEC were stimulated or not with TNFα (20 ng/mL) for 1 h (A–E), 4 h (E), 24 h (E and F) or 48 h (E). Results are expressed as protein expression relative to β-actin (A) or mean fluorescence intensity (MFI, B–F). Values are expressed as mean ± SEM (n = 4–8 independent experiments). * $P < 0.05$ or * * $P < 0.01$ relative to values in the respective vehicle group; + $P < 0.05$ or + + $P < 0.01$ relative to values in the respective control siRNA group stimulated with TNFα; $\Delta P < 0.05$ relative to values in the PCSK9 siRNA group.

atherogenesis [20,53,54]. In fact, an increase in the mRNA expression of the fractalkine/CX_3CL1 receptor (CX_3CR1) was reported in monocytes of patients with FH [28], and an increase in the percentage of monocytes expressing CX_3CR1 was observed in patients with PH [5]. We show here that alirocumab treatment reduces the expression of CX_3CR1 in monocytes and T-lymphocytes. While the CXCL16/CXCR6 axis has not been investigated in the context of FH, as far as we know, we also detected reduced plasma levels of CXCL16, as well as lower CXCR6 expression in all T-lymphocyte subsets. As TNFα increases the expression of endothelial CX_3CR1 and CXCR6 counter-ligands (CX_3CL1 and CXCL16, respectively), the reduced expression of these receptors might partly explain the reduced adhesion of these leukocyte subpopulations to the dysfunctional endothelium in the context of PCSK9 inhibition. Finally and in agreement with a previous report [29], CCR2 expression was reduced in Mon1 and Mon2 monocytes (Mon3 do not express CCR2) in patients with FH after treatment with alirocumab for 8 weeks, which is also associated with reduced atherogenic risk.

The immunophenotypic changes upon PCSK9 neutralization seem to improve endothelial dysfunction in the context of leukocyte adhesiveness in a background of FH, but little is known with regards to endothelial PCSK9 loss-of-function. Here, we demonstrate *in vitro* that TNFα—a key cytokine in FH [26,27]—increases arterial endothelial PCSK9 expression analogous to that previously demonstrated for ox-LDL or LPS stimulation and low shear [30,31]. In this line, TNFα was also found to induce PCSK9 expression *via* SOCS3, albeit in a human liver cancer cell line (HepG2) [55]. Notably, endothelial *PCSK9* silencing resulted in a significant reduction in mononuclear cell adhesion to dysfunctional arterial endothelium (TNFα-stimulated). These effects were the consequence of the down-regulation of ICAM-1 and VCAM-1 and also membrane-bound chemokines (fractalkine/CX_3CL1 and CXCL16) induced by TNFα. In atheroslcerotic mice, knockdown of *Pcsk9* or alirocumab treatment leads to a marked decrease in macrophage numbers in aortic lesions [38] and impairs aortic plaque T-cell infiltration [56]. Additionally, down-regulation of *Pcsk9* decreases LPS-induced VCAM-1 expression, albeit in vascular smooth muscle cells [36], and decreases LDL-induced ICAM-1 expression in aortic endothelial cells from *Pcsk9*-knockout mice [57]. Here we found that MCP-1/CCL2, soluble fractalkine/CX_3CL1, soluble CXCL16 or IL-8/CXCL8 generation and release were dampened in *PCSK9*-silenced HUAEC upon stimulation with TNFα. Similarly, reduced MCP-1/CCL2 expression was detected in lentivirus-*Pcsk9* shRNA mice [38] and in aortic endothelial cells from *Pcsk9*-knockout mice [57], and vaccination against Pcsk9 in mice resulted in reduced circulating levels of other chemokines [58].

Having established the consequences of endothelial *PCSK9* silencing on the aforementioned components of the canonical leukocyte recruitment cascade, we sought to determine the effect on some intracellular signaling cascades triggered by TNFα. NADPH oxidases are important sources of ROS in endothelial cells and are induced by TNFα [32]. Among them, Nox5 seems to be the most important in the mononuclear leukocyte arrest on dysfunctional endothelium [33]. We show here that PCSK9 inhibition markedly impairs TNFα-induced Nox5 expression in arterial endothelial cells. TNFα-induced ROS production can boost the activation of different redox-sensitive pathways such as the MAPKs [35]. Indeed, PCSK9 deficiency was found to decrease p38-MAPK phosphorylation stimulated by TNFα but not ERK1/2 stimulated by ox-LDL in HUVEC [59]. p38-MAPK can transactivate downstream targets such as NF-κB, and the phosphorylation of NF-κB subunit p65 by TNFα was reduced in *PCSK9*-silenced cells, which has been previously reported in *in vitro* and *in vivo* models [36–38].

We found that the expression of SREBP2, a transcription factor related to cholesterol homeostasis that regulates the synthesis of PCSK9 [39–42], was induced by TNFα through Nox/NF-κB-dependent mechanisms in HUAEC and its expression was reduced after PCSK9 downregulation. The induction of PCSK9 by SREBP2 is well known [42,60], yet SREBP2 downregulation by PCSK9 silencing has not previously been addressed. Therefore, it is tempting to speculate that PCSK9 downregulation increases LDL-R functionality, which in turn attenuates SREBP2 expression. Moreover, as SREBP2 seems to bind and activate inflammatory target genes in TNFα-stimulated macrophages [39] and to play an important role in macrophage foam cell formation [61], our results suggest that SREBP2 overexpression could contribute to the pro-inflammatory milieu in endothelial cells induced by TNFα.

Our study has some limitations. There was no placebo group or control group without dyslipidaemia to confirm the effect of alirocumab.

In conclusion, our data add further support and extend the findings of the landmark FOURIER trial [62]. We provide evidence that PCSK9 inhibition reduces the activation of platelets and most leukocyte subsets in patients with FH, as well as CX_3CR1, CXCR6 and CCR2 expression on different immune players. By contrast, Treg lymphocytes are activated, promoting the release of the anti-inflammatory cytokine IL-10, which counteracts the inflammatory response elicited by TNFα and other cytokines, dampening leukocyte and leukocyte-platelet adhesiveness to dysfunctional arterial endothelium. Our results suggest that endothelial *PCSK9* silencing inhibits mononuclear cell arrest through inhibition of the TNFα-induced Nox5/p38-MAPK/NF-κB/SREBP2 pathway, limiting the subsequent expression and/or release of different CAMs and chemokines that are clearly implicated in mononuclear cell recruitment. Therefore, in addition to its lipid lowering effects, PCSK9 inhibition can trigger other favorable cardiovascular outcomes in FH by dampening the low-grade systemic inflammation and the endothelial dysfunction associated with this metabolic disorder.

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CRediT authorship contribution statement

Patrice Marques: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Elena Domingo:** Investigation, Data curation. **Arantxa Rubio:** Investigation, Data curation. **Sergio Martinez-Hervás:** Investigation, Data curation, Writing – review & Editing. **Juan F. Ascaso:** Methodology, Data curation, Writing – review & editing. **Laura Piqueras:** Methodology, Data curation, Writing – review & editing, Supervision. **José T. Real:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Funding acquisition. **Maria-Jesus Sanz:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Funding acquisition.

Authors' contributions

MJS and JTR contributed to the conception and design of the study; PM, ED, AR, SM-H, LP, JFA, JTR and MJS acquired, analysed and interpreted the data; PM, JTR and MJS wrote the manuscript and SM-H, LP and JFA revised it critically. All the authors have read and approved the submission of the manuscript.

Conflict of Interest Statement

No potential conflicts of interest relevant to this article were reported.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112460.

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