European Heart Journal (2017) **00**, 1–10
doi:10.1093/eurheartj/ehx002

PCSK9 monoclonal antibodies reverse the pro-inflammatory profile of monocytes in familial hypercholesterolaemia

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Received 10 May 2016; revised 27 September 2016; editorial decision 30 December 2016; accepted 2 January 2017

Aims Migration of monocytes into the arterial wall contributes to arterial inflammation and atherosclerosis progression. Since elevated low-density lipoprotein cholesterol (LDL-C) levels have been associated with activation of plasma monocytes, intensive LDL-C lowering may reverse these pro-inflammatory changes. Using proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibodies (mAbs) which selectively reduce LDL-C, we studied the impact of LDL-C lowering on monocyte phenotype and function in patients with familial hypercholesterolaemia (FH) not using statins due to statin-associated muscle symptoms.

Methods and results We assessed monocyte phenotype and function using flow cytometry and a trans-endothelial migration assay in FH patients ($n = 22$: LDL 6.8 ± 1.9 mmol/L) and healthy controls ($n = 18$, LDL 2.9 ± 0.8 mmol/L). Monocyte chemokine receptor (CCR) 2 expression was approximaterly three-fold higher in FH patients compared with controls. C–C chemokine receptor type 2 (CCR2) expression correlated significantly with plasma LDL-C levels ($r = 0.709$) and was positively associated with intracellular lipid accumulation. Monocytes from FH patients also displayed enhanced migratory capacity *ex vivo*. After 24 weeks of PCSK9 mAb treatment ($n = 17$), plasma LDL-C was reduced by 49%, which coincided with reduced intracellular lipid accumulation and reduced CCR2 expression. Functional relevance was substantiated by the reversal of enhanced migratory capacity of monocytes following PCSK9 mAb therapy.

Conclusions Monocytes of FH patients have a pro-inflammatory phenotype, which is dampened by LDL-C lowering by PCSK9 mAb therapy. LDL-C lowering was paralleled by reduced intracellular lipid accumulation, suggesting that LDL-C lowering itself is associated with anti-inflammatory effects on circulating monocytes.

Keywords Hypercholesterolaemia • Inflammation • Atherosclerosis • PCSK9 • CCR2

Introduction

The cardiovascular (CV) benefit conveyed by statins is attributed to low-density lipoprotein cholesterol (LDL-C) lowering[1,2] as well as to anti-inflammatory effects,[3,4] evidenced by an independent linear relation between CV-benefit and both LDL-C as well as C-reactive protein (CRP) lowering in statin trials.[4] Mendelian randomization studies, however, have not substantiated a causal role of CRP in atherogenesis.[5] Subsequently, focus shifted towards immune cells which are

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considered causal players in atherogenesis and plaque progression.[6,7] Monocytes infiltrate atherosclerotic lesions,[8] where monocyte-derived macrophages contribute to a local pro-inflammatory milieu.[9] Distinct monocyte subsets, based on CD14 and CD16 expression, are distinguished, displaying differential surface expression of membrane receptors and concomitant variable migratory behavior.[7] The receptor for monocyte chemo-attractant protein 1 (MCP-1), C–C chemokine receptor type 2 (CCR2), is of particular importance in the recruitment of monocytes to the arterial wall[10] and its expression is increased in patients with hypercholesterolaemia.[11,12]

Since statins decrease both LDL-C and CRP,[13] it is difficult to disentangle the impact of lipid lowering from inflammatory changes. Several proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibodies (mAbs) are now available, which increase hepatic LDL-receptor (LDLR) expression following antibody-mediated scavenging of the free PCSK9 protein.[14] PCSK9 mAbs injected subcutaneously once every 2–4 weeks provide a consistent and potent LDL-C reduction of 60%[15–17] with only minimal changes in other lipoprotein fractions, such as high-density lipoprotein (HDL)-cholesterol and triglycerides (TAGs). In contrast to statin therapy, the marked reduction in LDL-C upon PCSK9 mAb administration is not accompanied by a CRP reduction,[18] implying the absence of an anti-inflammatory effect by this class of therapeutic agents. However, the impact of PCSK9 mAbs on other key inflammatory mediators, including monocytes, has not been reported.

Here, we assessed the impact of elevated LDL-C levels on monocyte phenotype and function in patients with familial hypercholesterolaemia. (FH) not using statins due to statin-associated muscle symptoms (SAMS) versus normolipidemic control subjects. Subsequently, we assessed the impact of PCSK9 mAbs in FH patients on monocytes, compared with a reference group of FH patients on stable statin dose for at least 6 months. Overall, these studies demonstrate that selective lowering of LDL-C with PCSK9 mAbs reduces the pro-inflammatory profile of circulating monocytes.

Methods

Patient selection

This single-center study comprised 22 patients with definite or probable FH[19] not receiving statin therapy due to SAMS and 18 age- and gender-matched healthy controls. In 17 patients with FH who started PCSK9 mAb treatment, we assessed the effect of LDL-C lowering following 24 weeks of PCSK9 mAb administration. Patients received either Alirocumab ($n = 10$; 150 mg every 4 weeks or 75 mg every 2; see Supplementary material online, methods) or Evolocumab ($n = 7$; 140 mg every 2 weeks). For comparison of treatment effects, we also included age and gender matched FH patients ($n = 14$) with stable statin treatment (>24 weeks). Exclusion criteria for both patients and controls included recent CV-events in the past 12 months, infection or diabetes. Subjects visited the hospital after an overnight fast for physical examination, medical history recording and blood withdrawal.

The study protocol was approved by the institutional review board of the Academic Medical Center in Amsterdam, the Netherlands, and written informed consent was obtained from each participant.

Flow cytometry

After removing red blood cells, white blood cells were stained with antibodies for various surface markers (see Supplementary material online, Table S1). Fluorescence was measured with BD Canto II and analysed with FlowJo software version 7.6.5 (FlowJo, LLC, Ashland, OR). Monocytes were gated based on CD14, CD16, and HLA-DR expression.[20] Monocyte area was gated by forward/side scatter, CD14+ and/or CD16+ cells were gated, and HLA-DR positive cells were considered monocytes, which were divided into classical (CD14++/CD16-), intermediate (CD14++/CD16+), or non-classical ($CD14^{dim}$/CD16+). The expression of surface markers was calculated as delta (Δ) median fluorescence intensity (MFI) (ΔMFI = MFI surface staining - MFI isotype control).

Monocyte characterization

Mononuclear cells were isolated through density centrifugation using Lymphoprep™ (Axis-Shield, Dundee, Scotland) and isolated using human CD14 magnetic beads and MACS® cell separation columns (Miltenyi, Bergisch Gladbach, Germany).

Migration assays

To functionally assess adhesive and migratory capacity, a trans-endothelial migration (TEM) assay was performed,[8] outlined in the Supplementary material online, methods. To investigate whether migration was mediated by MCP-1, we performed representative chemotaxis assays, detailed in the Supplementary material online, methods.

Lipid accumulation

Quantification of lipid droplets was performed on monocytes mounted on fibronectin coated glass (see Supplementary material online, methods) using a Leica TCS SP8 Confocal laser scanning microscope, assessing total number of monocytes with lipid droplets, and number of lipid droplets per monocyte, in 6–10 field of views (FOVs). To assess lipid content, droplets were extracted from monocytes, followed by neutral lipid extraction and HPLC analyses. To study interdependency of CCR2 expression and lipid accumulation, $CCR2^{high}$ monocytes were sorted by flow cytometry (see Supplementary material online, methods), plated and stained with Nile Red. For representative images, co-immuno fluorescence with Nile Red and CCR2 was performed (see Supplementary material online, methods).

RNA isolation and quantitative PCR analysis

RNA was isolated with High Pure RNA Isolation kits (Roche, Basel, Switzerland) from 500 000 cells. 400 ng of RNA was used for cDNA synthesis with iScript (BioRad, Veenendaal, The Netherlands). qPCR was performed with 4 ng cDNA using Sybr Green Fast on a ViiA7 PCR machine (Applied Biosystems, Bleiswijk, The Netherlands). Gene expression was normalized to the mean of two housekeeping genes (*B2M*, *GAPDH*) (all primer sequences outlined in see Supplementary material online, *Table S2*).

Ex vivo lipopolysaccharide (LPS) challenge for cytokine production

Cells were untreated or stimulated with 10 ng/mL LPS in triplo (see Supplementary material online, material). After 24 h, the medium was stored at -80 °C. Production of cytokines was measured in a panel consisting of TNF and interleukin (IL)-10 using luminex (Bioplex, BioRad, Veenendaal, The Netherlands).

Statistical analyses

Data are mean (standard deviation), median (inter-quartile range) or number (percentage), unless stated otherwise. Differences in clinical characteristics and monocyte phenotype and function between FH patients and controls were assessed with Student's *t*-tests or Mann–Whitney *U* tests. Correlations were assessed using univariate linear regression. Data baseline and post-PCSK9 mAb treatment combined with

Table 1 Clinical characteristics of control subjects and familial hypercholesterolaemia patients not using statin therapy

	Control $n = 18$	FH (no statin) $n = 22$	*P*-value
Age, years	51 ± 11	55 ± 14	0.260
Gender, *n*, male (%)	11 (61)	12 (55)	0.625
BMI, kg/m^2	23 ± 3	27 ± 4	0.002
Smoking, (% active)	0 (0)	1 (0.04)	0.810
CVD history, (%)	0 (0)	10 (46)	<0.001
SBP, mmHg	126 ± 12	132 ± 15	0.239
DBP, mmHg	80 ± 8	81 ± 10	0.749
CRP, mg/L	0.75 [0.53–1.8]	1 [0.4–3.0]	0.522
Total cholesterol, mmol/L	5.2 ± 1.0	9.0 ± 1.9	<0.001
LDL-C, mmol/L	2.9 ± 0.8	6.8 ± 1.9	<0.001
HDL-C, mmol/L	1.8 ± 0.5	1.4 ± 0.6	0.048
Triglycerides, mmol/L	0.72 [0.6–1.1]	1.6 [1.1–2.1]	0.010
Leucocytes, 10^9/L	5.1 ± 0.9	5.8 ± 1.6	0.126
Neutrophils, 10^9/L	2.8 ± 0.7	3.5 ± 1.1	0.022
Lymphocytes, 10^9/L	1.6 ± 0.3	1.7 ± 0.6	0.464
Monocytes, 10^9/L	0.4 ± 0.1	0.4 ± 0.2	0.671

Values are *n* (%), mean ± SD or median [IQR] for skewed data.
BMI, body mass index; CRP; c-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; FH, familial hypercholesterolaemia; HDL-C, high density lipoprotein cholesterol; IQR, inter-quartile range; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure, SD, standard deviation.

stable statin users were assessed with a one-way ANOVA using a dunnett post hoc test. A two-way ANOVA with bonferroni post hoc analysis was performed for all flow cytometry analysis. A two-sided *P*-value <0.05 was considered statistically significant. Data were analysed using Prism version 5.0 (GraphPad software, La Jolla, CA, USA) or SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Study population

Baseline characteristics of 22 FH patients not using statins due to SAMS and 18 matched control subjects are listed in *Table 1*. FH patients had higher cholesterol levels, body mass index (BMI), and a higher prevalence of CVD. CRP levels were comparable between control subjects and FH patients (0.75 [0.53–1.84] vs. 1.00 [0.4–3.0] mg/L, respectively, $P = 0.522$). Disease specifications and medication use are outlined in the Supplementary material online, *Table S3*.

Hypercholesterolaemia enhances CCR2 expression and migratory capacity of monocytes

Monocytes were gated based on their CD14 and CD16 expression (*Figure 1A*). Subset distribution was comparable between FH patients and control subjects (*Figure 1B*). Monocyte CCR2 expression was increased on classical monocytes in FH patients (ΔMFI 605 ± 214 vs. 236 ± 155, $P < 0.001$) (*Figure 1C and D*), with concomitant increases in the chemokine receptor CX3CR1, and integrins CD11b and CD18 (see Supplementary material online, *Figure S1A–E*). Plasma LDL-C levels correlated with monocyte CCR2 expression in FH patients ($r = 0.709$, $P = 0.005$) (*Figure 1E*). To address functional significance of the increased CCR2 expression,[10] we assessed TEM rate of monocytes in FH patients and controls (seven FH patients and seven controls; see Supplementary material online, *Table S4*). We observed a 1.6-fold increase in monocyte migration in FH patients compared with control subjects ($P = 0.008$) (*Figure 1F*). In a complementary chemotaxis assays ($n = 3$), monocytes of a representative FH patient showed strong directional movement towards MCP-1, the ligand for CCR2, whereas monocytes of a control subject showed non-directional, random migration (see Supplementary material online, *Video, Figure S4*).

Circulating monocytes of familial hypercholesterolaemia patients accumulate lipids

To evaluate the interaction between lipid accumulation and functional changes, we assessed monocyte intracellular lipid content with Nile red dye, and genes involved in lipid handling in FH patients and controls (seven FH patients and 13 controls; see Supplementary material online, *Table S4*). FH patients had a higher fraction of lipid positive monocytes (FH: 76 ± 12% vs. controls: 62 ± 12%, $P = 0.02$; *Figure 2*), with increased numbers of lipid droplets per cell (8 ± 1 in FH vs. 5 ± 1 in controls, $P = 0.02$) (*Figure 2A–D*). Content analyses by HPLC revealed that lipid droplets consist of both cholesterol esters (CE) and TAGs (see Supplementary material online, *Figure S2A* and *B*). LDLR expression was not detectable on circulating monocytes (see Supplementary material online, *Figure S1F*). However, higher lipid content coincided with increased surface expression of CD36 on classical monocytes, and SRA on intermediate monocytes (*Figure 2E and F*). *ABCA1* and *ABCG1* were also up-regulated (*Figure 2G*), most likely due to induction by increased intracellular lipid content. Next, we assessed the interdependency of increased lipid content and CCR2 expression. FACS sorting revealed that CCR2^{high} cells (MFI cutoff 3500) had a higher number of lipid droplets (CCR2^{high} 33 ± 3 vs. CCR2$^{intermediate/low}$ 10 ± 1, $P < 0.001$) (see Supplementary material online, *Figure S2C* and *D*). Visually, using immunofluorescent imaging, cells without lipid droplets had low levels of CCR2, whereas monocytes with high numbers of lipid droplets had higher CCR2 expression (see Supplementary material online, *Figure S2E*).

PCSK9 monoclonal antibodies lower LDL-C levels in FH patients

Seventeen FH patients not using statins due to SAMS received PCSK9 mAbs: ten received Alirocumab (treatment allocation and dosing outlined in see Supplementary material online, *Table S5*), and seven received Evolocumab. For comparison, we also evaluated 14 FH patients with stable statin use (*Table 2* and see Supplementary material online, *Table S7*). FH patients receiving PCSK9 mAbs had an adverse CV risk profile compared with statin-treated subjects, with higher BMI, SBP, and TAGs. Following PCSK9 mAbs, LDL-C dropped 49% (±14%), from 6.7 ± 1.3 mmol/L to 3.4 ± 1.3 mmol/L ($P \leq 0.001$). Post-PCSK9 mAbs levels were comparable with statin treated FH patients (2.8 ± 0.6; $P = 0.130$ vs. post-PCSK9 mAbs).

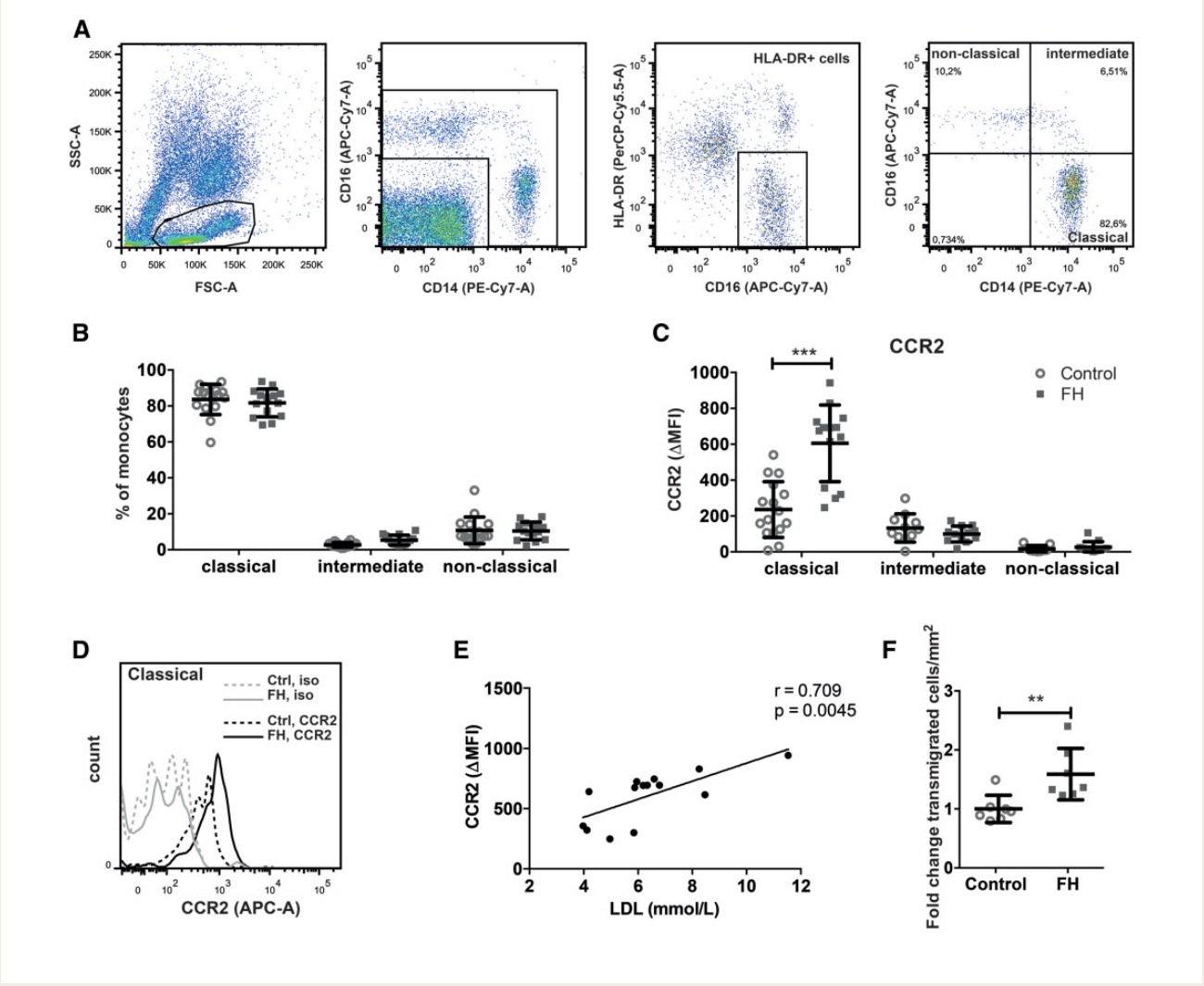

Figure 1 Monocytes of familial hypercholesterolaemia patients show enhanced CCR2 expression and migratory capacity. Flow cytometry on whole blood was performed to study monocyte surface expression. (*A*) CD14+ and/or CD16+ cells were gated, and only HLA-DR positive cells were considered to be monocytes. (*B*) Percentage of monocyte subsets (classical (CD14++/CD16-), intermediate (CD14++/CD16+), or non-classical ($CD14^{dim}$/CD16+)) in FH patients ($n = 22$, filled squares) vs. controls ($n = 18$, open circles). (*C*) Surface expression of monocyte CCR2 represented as delta median fluorescence intensity. (*D*) Histogram of CCR2 expression in classical monocytes of an FH patient (solid, black) or controls (dashed, black) with isotype controls (gray). (*E*) Correlation between plasma LDL-C levels and CCR2 surface expression of FH patients. (*F*) Transendothelial migratory capacity presented as the fold change transmigrated cells/mm^2 relative to controls. For each subject, transmigrated cells are calculated of independent counts of five fields of view. Data represent mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *P* values are unadjusted. CD, cluster of differentiation; CCR, chemokine receptor; FH, familial hypercholesterolaemia; HLA-DR, Human Leucocyte Antigen-antigen D Related; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation.

CRP was unaffected by PCSK9 mAbs (baseline: 1.4 [0.7–3.4] mg/L, post-PCSK9 mAbs: 1.2 [0.7–2.6] mg/L, $P = 0.916$). CRP levels in statin treated FH patients were 0.6 [0.3–1.0] mg/L, ($P = 0.028$ vs. post-PCSK9 mAbs and 0.052 vs. baseline). Other baseline and post-treatment lipid levels are summarized in *Table 2*. Separate baseline characteristics for Alirocumab and Evolocumab treatment groups are provided in the Supplementary material online, *Tables S4, S6* and *S7*.

Lipid lowering by PCSK9 monoclonal antibodies reduces monocyte CCR2 expression, migratory capacity and lipid accumulation

PCSK9 mAbs did not affect monocyte subset distribution (see Supplementary material online, *Figure S3A*), but resulted in a 60% reduction of monocyte CCR2 surface expression (ΔMFI: baseline 607 ± 209, post-PCSK9 mAbs: 207 ± 180, $P < 0.001$), to levels

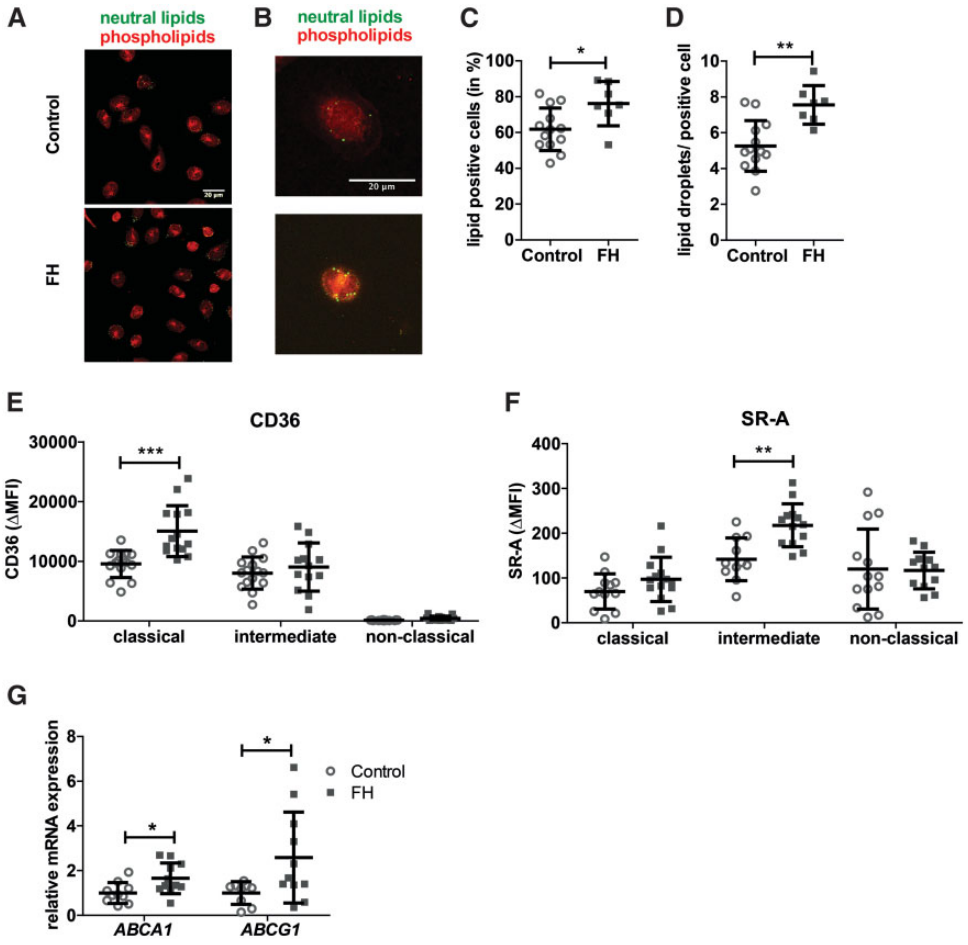

Figure 2 Lipids accumulate in monocytes of familial hypercholesterolaemia patients. Lipid accumulation in FH patients ($n = 7$, filled squares) and control subjects ($n = 13$, open circles) was assessed. (*A,B*) Neutral lipid droplets in green of isolated monocytes. (*C*) Quantification of lipid accumulation presented as the percentage of lipid positive cells (represented in *A*). (*D*) Number of lipid droplets per lipid positive monocytes (represented in *B*). (*E,F*) Surface expression of the scavenger receptors CD36 and SR-A assessed by flow cytometry. Quantified as delta median fluorescence intensity. (*G*) relative mRNA expression, of cholesterol efflux genes *ABCA1* and *ABCG1*, normalized to housekeeping genes (*B2M*, *GAPDH*). Data represent mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *P* values are unadjusted. ABCA, ATP binding cassette transporter; B2M, beta-2-microglubilin; CD, cluster of differentiation; FH, familial hypercholesterolaemia; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NR, Nile Red; SD, standard deviation; SR, scavenger receptor.

comparable to those observed in FH patients using statins (ΔMFI, 271 ± 234, *P* = non-significant vs. PCSK9 mAbs; $P < 0.001$ vs. baseline) (*Figure 3A and B*, separate data for both agents in see Supplementary material online, *Figure S5A*). Moreover, TEM was significantly reduced after PCSK9 mAbs to levels comparable to statin users [fold change in transmigrated cells/mm^2 relative to control subjects: FH baseline 1.6 ± 0.4, FH post-PCSK9 mAbs 0.9 ± 0.2 ($P = 0.002$ compared with baseline). Statin 0.8 ± 0.1 ($P = 0.002$ compared with baseline) *Figure 3C*]. In a representative experiment, directional motility and migration distance of monocytes post-PCSK9 mAb were also lower compared with an untreated FH patient (see Supplementary material online, *Video* and *Figure S4*).

We also found lower levels of intracellular lipid accumulation in monocytes of PCSK9 mAb treated FH patients (fraction of monocytes with lipid droplets post-PCSK9 mAbs: 54 ± 12, $P < 0.001$ vs FH baseline, number of lipid droplets/monocyte: 5 ± 2, $P \leq 0.001$ vs FH baseline) (*Figure 3D and E*, separate data for both agents in Supplementary material online, *Figure S5B* and *C*). Other chemokines and integrins showed non-significant declines upon treatment with PCSK9 mAb and were comparable with those in FH patients using statins (see Supplementary material online, *Figure S3*). Although

Table 2 Clinical characteristics of familial hypercholesterolaemia patients (no statin) pre- and post-PCSK9 monoclonal antibody treatment and FH patients on stable statin therapy

	FH, baseline *n* = 17	FH, post-PCSK9 mAbs *n* = 17	*P*-value	FH statin use *n* = 14	*P*-value (*vs. FH, baseline*)	*P*-value (*vs. FH, post-PSCK9* mAbs)
Age, years	57 ± 12	n/a	n/a	49 ± 14	0.162	n/a
Gender, *n*, male (%)	10 (48)	n/a	n/a	7 (50)	0.790	n/a
BMI, kg/m^2	28 ± 5	28 ± 5	0.763	24 ± 3	0.003	0.006
Smoking (% active)	1 (0.06)	n/a	n/a	2 (17)	0.492	n/a
CVD history (%)	9 (53)	n/a	n/a	2 (17)	0.053	n/a
SBP, mmHg	132 ± 15	134 ± 18	0.658	122 ± 16	0.067	0.055
DBP, mmHg	81 ± 10	79 ± 6	0.375	78 ± 8	0.633	0.512
CRP, mg/L	1.4 [0.7–3.4]	1.2 [0.7–2.6]	0.916	0.6 [0.3–1.0]	0.052	0.028
Total cholesterol, mmol/L	8.9 ± 1.8	5.6 ± 1.5	<0.001	4.9 ± 0.7	<0.001	0.220
LDL-C, mmol/L	6.7 ± 1.3	3.4 ± 1.3	<0.001	2.8 ± 0.6	<0.001	0.130
HDL-C, mmol/L	1.4 ± 0.6	1.5 ± 0.5	0.274	1.5 ± 0.5	0.557	0.534
Triglycerides, mmol/L	1.6 [1.1–1.9]	1.2 [0.7–2.6]	0.08	0.9 [0.6–1.2]	0.002	0.077
Leucocytes, 10^9/L	6.2 ± 1.7	6.0 ± 1.4	0.686	5.4 ± 1.6	0.570	0.252
Neutrophils, 10^9/L	3.7 ± 1.0	3.6 ± 1.0	0.589	2.9 ± 1.2	0.096	0.083
Lymphocytes, 10^9/L	1.8 ± 0.7	1.8 ± 0.5	0.440	1.9 ± 0.6	0.461	0.461
Monocytes, 10^9/L	0.5 ± 0.2	0.5 ± 0.2	0.322	0.5 ± 0.1	0.496	0.789

Values are *n* (%), mean ± SD or median [IQR] for skewed data.
BMI, body mass index; CRP, c-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; FH, familial hypercholesterolaemia; HDL-C, high density lipoprotein cholesterol; IQR, inter-quartile range; LDL-C, low density lipoprotein cholesterol; n/a, not applicable; SBP, systolic blood pressure; SD, standard deviation.

surface expression of CD36 and SR-A was unaffected by PCSK9 mAb therapy (*Figure 3F–G*), expression of *ABCA1 and ABCG1* dropped to levels observed in control subjects (*Figure 3H*).

PCSK9 monoclonal antibodies reduce TNF and enhance IL-10 production

Upon entry into the arterial wall, production of (pro-inflammatory) cytokines contributes to plaque destabilization. To assess whether PCSK9 mAbs affected the inflammatory responsiveness of monocytes we measured cytokine production following LPS stimulation in PCSK9 mAb treated subjects (see Supplementary material online, *Table S6*). Production of TNF was reduced (baseline: 896 ± 593 pg/mL, post-PCSK9 mAbs: 471 ± 272 pg/mL, $P < 0.01$), whereas secretion of the anti-inflammatory IL-10 was enhanced (baseline: 950 ± 624 pg/mL, post-PCSK9 mAbs: 2097 ± 772 pg/mL, $P < 0.001$). Cytokine levels of monocytes from PCSK9 mAb treated patients were comparable to those observed in stable statin users (*Figure 3I and J*).

Discussion

We show that monocytes from patients with FH not using statins due to SAMS, display pro-inflammatory and migratory changes, which coincide with an increase in cytoplasmic lipid droplets. The latter implies a direct relation between intracellular lipid accumulation and inflammatory changes in monocytes (*Figure 4*). Following 24 weeks of treatment with a PCSK9 mAb monocyte migratory capacity, lipid content, as well as inflammatory responsiveness decreased towards levels observed in FH patients on stable statin use.

These data imply an LDL-C mediated pro-inflammatory effect on circulating monocytes in patients with FH, which reverses upon LDL-C lowering by PCSK9 mAbs. A potential explanation for these observations is the marked accumulation of lipids in circulating monocytes of FH patients. In contrast to previous findings in macrophages showing that PCSK9 modulates macrophage LDL-C uptake by altering LDL-C receptor expression in macrophages,[21,22] LDLR expression was virtually absent in circulating monocytes in the present study. This data implies that the anti-inflammatory effect observed in monocytes following PCSK9 mAbs is predominantly an indirect effect mediated via lowering of plasma LDL-C. In addition, our findings indicate that the LDL receptor pathway does not play an important role in monocyte lipid accumulation. This is corroborated by a previous report by Mosig *et al.*[23] showing accumulation of lipids in monocytes of patients with homozygous FH, characterized by absence of functional LDL receptors. Potential other pathways contributing to monocyte lipid uptake comprise scavenger receptors. In support, the increased lipid content in FH patients coincided with higher surface expression of CD36 on classical monocytes, and SR-A on intermediate monocytes. As expected, lipid accumulation also resulted in increased expression of efflux mediators (ABCA1 and ABCG1).[24] Nonetheless, the increase in intracellular lipid content in FH subjects supports that this accumulation is dominated by the increased levels of LDL-C in the plasma.

The increased lipid content in monocytes coincided with elevated CCR2 expression in FH patients. Previous *in vitro* data support the uptake of native LDL in monocytes eliciting increased CCR2 expression and monocyte chemotaxis.[11] The strong correlation between intracellular lipid accumulation and CCR2 expression observed in monocytes of FH patients implies a causal relation between cytoplasmatic lipid increase and pro-inflammatory changes. The functional relevance of these

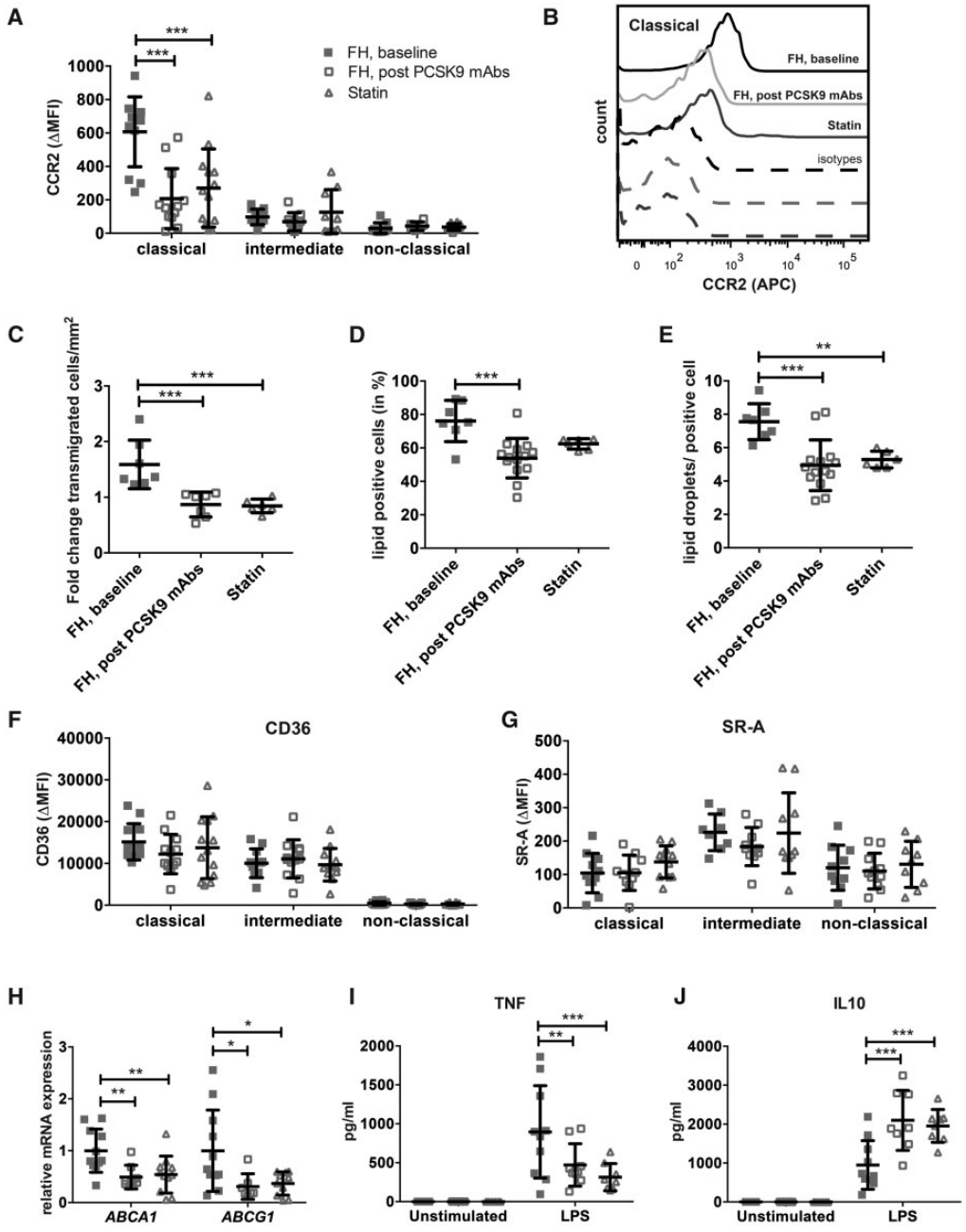

Figure 3 PCSK9 monoclonal antibodies reduce CCR2 expression, lipid accumulation, and pro-inflammatory cytokine production. To assess the effect of lipid lowering with PCSK9 monoclonal antibodies (mAbs), 17 FH patients (baseline filled squares) were treated with PCSK9 mAbs for 24 weeks (post-treatment open squares). A group of stable statin users ($n = 14$, open triangles) was added as a reference. (*A*) Surface expression of monocyte CCR2 represented as delta median fluorescence intensity. (*B*) Histogram of CCR2 expression in classical monocytes of an FH patient, baseline (solid, black), FH patient post-PCSK9 mAbs (solid, light-gray) or stable statin (solid, dark-gray) with isotype controls (dashed lines). (*C*) Trans-endothelial migratory capacity presented as the fold change transmigrated cells/mm^2 relative to controls. For each subject, transmigrated cells are calculated of independent counts of five fields of view. (*D*) Quantification of intracellular lipid accumulation using NR immunohistochemistry presented as the percentage of lipid positive cells. (*E*) Number of lipid droplets per lipid positive cell. (*F,G*) Expression of the scavenger receptors CD36 and SR-A assessed by flow cytometry, quantified as delta median fluorescence intensity. (*H*) relative mRNA expression of the cholesterol efflux genes *ABCA1* and *ABCG1*, normalized to housekeeping genes (*B2M*, *GAPDH*). (*I,J*) Cytokine secretion of TNF and IL-10 by monocytes after 24 h of LPS stimulation measured by luminex. Data represent mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *P* values are unadjusted. ABCA, ATP binding cassette transporter; B2M, beta-2-microglubilin; CCR, chemokine receptor; FH, familial hypercholesterolaemia; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-10, interleukin-10; LPS, lipopolysaccharide; NR, Nile Red; mAbs, monoclonal antibodies; PCSK9, proprotein convertase subtilisin/kexin type 9; SD, standard deviation; SR, scavenger receptor; TNF, tumor necrosis factor.

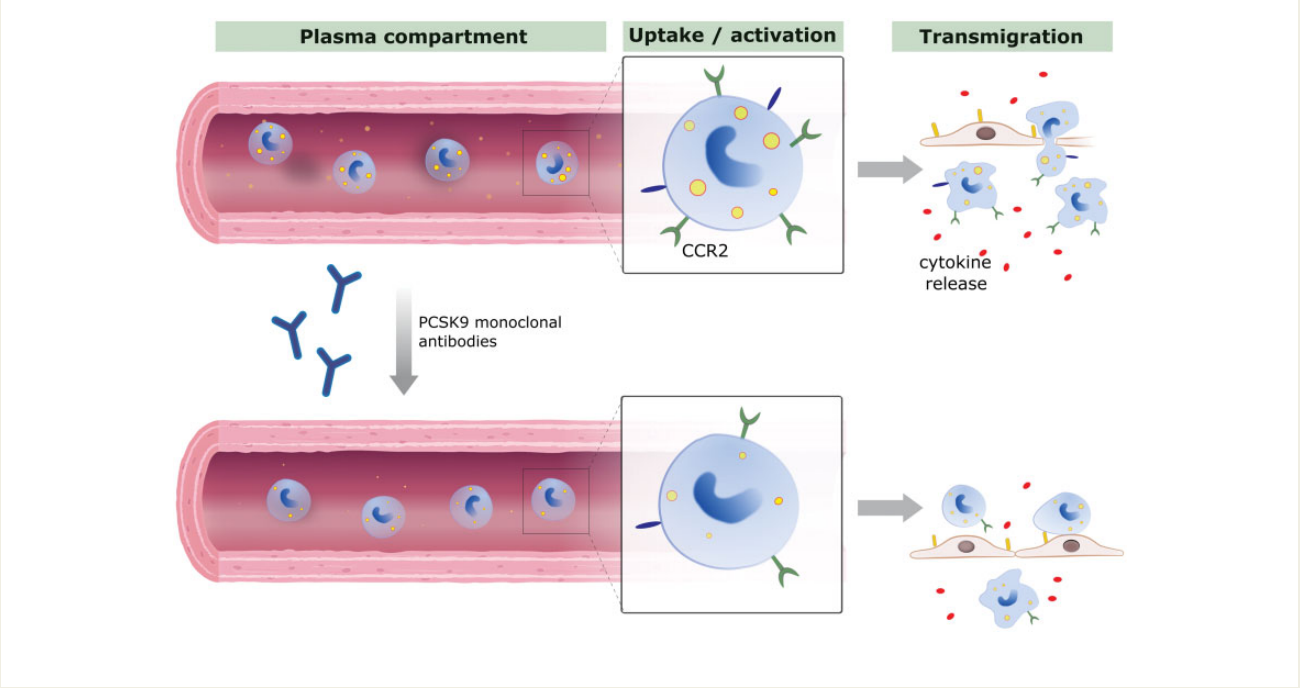

Figure 4 Central illustration: LDL-lowering by PCSK9 monoclonal antibodies reverse lipid-induced monocyte activation and reactivity. Monocytes were isolated from FH patients not receiving lipid lowering treatment. Phenotypic assessment revealed increased lipid uptake, enhanced CCR2 expression with higher migratory capacity ex vivo and increased release of pro-inflammatory cytokines. Lipid lowering with PCSK9 monoclonal antibodies reversed all these parameters. CCR, chemokine receptor; FH, familial hypercholesterolaemia; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9.

changes is substantiated by the marked increase in the *ex vivo* endothelial migration rate of monocytes obtained from FH patients. PCSK9 mAbs reduced plasma LDL-C levels by 49% and also reduced the intracellular lipid content in circulating monocytes. In conjunction, monocyte CCR2 expression was reduced to levels comparable to those observed in FH patients using statins, whereas statin therapy has been previously shown to prospectively lower monocyte CCR2 expression.[25] In parallel, migratory capacity of monocytes as well as the response of circulating monocytes to an inflammatory challenge was also attenuated following PCSK9 mAbs, illustrated by decreased TNF release with increased secretion of the anti-inflammatory cytokine IL-10. These data imply that decreased intracellular lipid accumulation leads to an attenuated inflammatory activity of the monocytes, which is independent of the mechanism by which LDL-C reduction is established.

Interestingly, the anti-inflammatory changes in monocyte phenotype and responsiveness following PCSK9 mAbs were not accompanied by a decrease in plasma CRP levels. The reduction in CRP following statin therapy[4,26] reflects a direct effect of statins in hepatocytes,[27] which is not present following PCSK9 mAb administration.[18] Whereas the clinical relevance of the absence of a CRP change remains to be established, recent studies have revealed that CRP is not a mediator but merely a marker for CVD risk.[5,28] In parallel, the reduction in arterial inflammation following statin therapy is not correlated to statin-induced changes in CRP.[29,30] Whether the reduction in immune cell activity following PCSK9 mAbs will translate into decreased inflammatory activity in atherosclerotic lesions is currently being addressed in the ANITSCHKOW study (NCT02729025).

Limitations

Several limitations merit attention. First, this was a proof-of-concept study using mAbs against PCSK9 to lower LDL-C without a placebo arm. Since the wide array of laboratory tests in the present study consistently show an anti-inflammatory effect of PCSK9 mAbs on monocytes in conjunction with lowering of both plasma LDL-C and intracellular lipid content in monocytes, this study does support an anti-inflammatory effect of potent LDL-C lowering in patients with elevated LDL-C levels not receiving statins. Future randomized, placebo-controlled trials are recommended to further elaborate on these findings.

Secondly, the interpretation of the findings is limited by the lack of a statin intervention comparison group, which is first-line therapy in FH. To show the independent effect of PCSK9 mAbs, we included FH patients with intolerable SAMS to at least three different statins,[31] which precludes adding an additional statin intervention group. Therefore, we reverted to the inclusion of FH patients on stable statin use. These subjects had a more favourable CVD risk profile compared with PCSK9 mAb treated subjects, with a lower BMI. Nonetheless, the similarity of monocyte inflammatory status as well as LDL-C levels in PCSK9 mAb and statin users implies that LDL-C lowering *per se* is the pre-dominant actor in the pro-inflammatory effects, independent from the pathway by which LDL-C is reduced.

Third, although murine data is consistent, the role of CCR2 expression on monocytes in predicting CVD risk has not been established prospectively in patients.[10] Moreover, final evaluations on the role of PCSK9 lowering on plaque regression (GLAGOV,

NCT01813422) and definitive end point studies (ODYSSEY Outcomes NCT01663403, FOURIER NCT01764633), awaited in 2017 and 2018), are paramount to assess functional relevance of the present mechanistical insights.

Finally, it should be taken into account that besides potentially beneficial effects, lowering of inflammatory responses may also increase vulnerability for infectious diseases. Since CV disease is widely recognized as a pro-inflammatory state, the use of anti-inflammatory strategies is, however, considered to be pre-dominantly beneficial, underscored by ongoing large outcome studies using anti-inflammatory agents (Cantos, NCT01327846, CIRT, NCT01594333).

In conclusion, we show that lipid lowering with PCSK9 mAbs reduces the pro-inflammatory phenotype of monocytes without affecting CRP, implying that potent lowering of LDL-C has an anti-inflammatory impact in hypercholesterolemic patients. These data highlight that inflammation is not solely captured by CRP measurement and warrant future investigations to assess the role of attenuating cellular inflammation in patients at high CV-risk.

Supplementary material

Supplementary *Tables S1–S7* and *Figures S1–S5* are available at *European Heart Journal* online.

Acknowledgements

The authors thank J.P.K., S.R., and S.K. for assisting in patient recruitment. The ODYSSEY CHOICE II study was funded by Sanofi and Regeneron Pharmaceuticals, Inc.

Funding

The Netherlands Heart Foundation (CVON 2011/B019: Generating the best evidence-based pharmaceutical targets for atherosclerosis [GENIUS]) and REPROGRAM grant (European commission Horizon2020-PHC-2015: 667837). J.v.d.B. received a Junior Postdoc grant (The Netherlands Heart Foundation (2013T003)) and a VENI grant (ZonMW (91615052)).

Conflicts of interest: M.B.D. is employed by Sanofi and G.M. is employed by Regeneron; Sanofi-Regeneron is the manufacturor of one of the PCSK9-antibodies used in the study (Alirocumab). E.S. reports having received lecturing fees from Sanofi-Regeneron and Amgen; and served as (principal) investigator in several trials on PCSK9-antibody in patients. The other authors have no relevant conflicts of interest to declare.

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