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Atherogenic Lipids Induce High-Density Lipoprotein Uptake and Cholesterol Efflux in Human Macrophages by Up-Regulating Transmembrane Chemokine CXCL16 without Engaging CXCL16-Dependent Cell Adhesion

Jana Barlic,*† Wenjia Zhu,* and Philip M. Murphy2*

Atherosclerosis is a complex pathologic process in which chemokine-mediated leukocyte accumulation in arterial walls is thought to be an important mechanism of pathogenesis. An interesting exception to this paradigm is the chemokine CXCL16, also known as the scavenger receptor for phosphatidylserine and oxidized low density lipoprotein, which is highly expressed in mouse and human atherosclerotic lesions, yet appears to be atheroprotective. In this study, we address potential mechanisms responsible for this activity. Consistent with its presence in atherosclerotic plaque, we found that atherogenic lipids up-regulated CXCL16 in primary human monocyte-derived macrophages. However, the same lipids down-regulated the CXCL16-targeted protease ADAM10, resulting in preferential expression of CXCL16 as the transmembrane form, not the shed form. Although transmembrane CXCL16 is known to mediate cell-cell adhesion by binding its receptor CXCR6, and atherogenic lipids are known to stimulate macrophage adhesion to coronary artery smooth muscle cells, we found that heterotypic adhesion of these cell types occurred in a CXCL16-independent manner. Instead we found that in macrophages, CXCL16 promoted internalization of both oxidized low density lipoprotein and high density lipoprotein, as well as release of cholesterol. Moreover, CXCL16 deficiency in macrophages interfered with oxidized low density lipoprotein-induced up-regulation of atheroprotective genes: adenosine triphosphate-binding cassette transporter A1 and G1 as well as apolipoprotein E. Thus, our findings support the hypothesis that CXCL16 mediates atheroprotection through its scavenger role in macrophages and not by cell-cell adhesion. *The Journal of Immunology, 2009, 182: 7928–7936.

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Abbreviations used in this paper: oxLDL, oxidized low density lipoprotein; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; acLDL, acetyl low density lipoprotein; ApoE, apolipoprotein E; CASMC, coronary artery smooth muscle cell; Dil, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; HDL, high density lipoprotein; 9-HODE, 9-hydroxy-10E,12Z-octadecadienoic acid ester; 13-HODE, 13-hydroxy-9Z,11E-octadecadienoic acid ester; LDL, low density lipoprotein; Mo, monocyte; M, macrophage; PGPC, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine; P/O, palmitoyl-2-(5-oxo valeryl)-sn-glycero-3-phosphocholine; RCT, reverse cholesterol transport; SMC, smooth muscle cell; SR-A, scavenger receptor-A; SR-BI, scavenger receptor-BI; sRNAi, small RNA interference.
CXCL16 is undetectable in normal aorta, but is expressed in mouse and human coronary and carotid atherosclerotic lesions, colocalizing with lipid-laden intimal macrophages (Mo) and smooth muscle cells (SMCs) (17, 18). Studies evaluating soluble CXCL16 as a potential biomarker of coronary artery disease have been inconsistent (19, 20). A polymorphic variant of CXCL16 named CXCL16-A181V has been reported to be associated with increased coronary artery stenosis in postinfarction patients (21), suggesting that wild-type CXCL16 may be atheroprotective. Consistent with this, genetic inactivation of cxcl16 in hyperlipidemic low density lipoprotein (LDL) receptor knockout mice (dlpr−/−) results in accelerated atherosclerosis due to enhanced Mo recruitment to the aortic arch (8). Although the CXCL16-CXCR6 relationship is considered monogeneous, cxcr6 inactivation in atherosclerosis-prone apolipoprotein E (ApoE) knockout mice (apoE−/−) decreased susceptibility to atherosclerosis, and was accompanied by reduced homing of lymphocytes into the aorta. These data suggest that cxcr6 is a proatherogenic chemokine receptor (22).

To investigate how CXCL16 may oppose atherosclerosis, we tested how lipid components of the atheromatous microenvironment affect CXCL16 expression and function on primary monocyte-derived Mo. We focused on oxLDL and its lipid derivatives, including 9-hydroxy-10E,12Z-octadecadienoic acid ester (9-HODE) and 13-hydroxy-9Z,11E-octadecadienoic acid ester (13-HODE), the two major bioactive oxidized linoleic acid metabolite components of LDL (23, 24), and arachidonic acid-containing phospholipids 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5-oxo valeryl) sn-glycero-3-phosphocholine (POV-PC) (25), all of which are present in high amounts in human atherosclerotic lesions (23–25).

Materials and Methods

Materials

LDL, oxLDL, high density lipoprotein (HDL), acetylated LDL (aLDL), 1,1’-dioctadecyl-3,3,3’,3’-tetramethyl indocarbocyaninet (Dil)-oxLDL, and Dil-HDL were purchased from Intractel. The 9-HODE, 13-HODE, PGPC, and POV-PC were from Cayman Chemical. mAbs included the following: rat anti-human CX3CR1 (Caltag-MedSystems), rat anti-human CXCL16 (R&D Systems), mouse anti-human CD14, CD36, CD68 (BD Biosciences), and scavenger receptor-A (SR-A; CosmoBio). Isotype-matched Abs were used for flow cytometry and scavenger receptor-blocking studies. Some untransfected cells were incubated for 45 min before the adhesion assay.

Characterization of a model of foamy macrophage in vitro

To study potential mechanisms by which human CXCL16 may regulate atheroprotection at the cellular and molecular levels, we first used atherogenic lipids to establish a foamy macrophage model in vitro from cultured mouse and human coronary and carotid atherosclerotic lesions, (23–25), all of which are present in high amounts in human atherosclerotic lesions (23–25).

Atherosclerotic lesions (23–25).

mRNA quantification

RNA was extracted by RNeasy kit (Qiagen) and reverse transcribed with RETROscript (Applied Biosystems/Ambion). cDNA was serially diluted and amplified in triplicate for standard curves for each primer/probe set. Relative target quantification was calculated with the 2−ΔΔCT (CT, cycle threshold) method (28) and normalized to GAPDH.

CXCL16 knockdown

Seven CXCL16-specific small RNA interference (sRNAi) were designed using BLOCK-iT RNAi Designer Program from Invitrogen, and sRNAi with a sequence 5’-CCCGAAACACCTGAGCTTACCAT-3’ which silenced CXCL16 expression by ≥80%, was used for all knockdown experiments. The negative control sRNAi had medium GC content (~Cm) that matched CXCL16-specific sRNAi GC composition. Fluorescein-labeled dsRNA oligomer (F), used to estimate transfection efficiency, had the same length as CXCL16-specific sRNAi. Mo (5 × 10⁶) were nucleofected (Amax) with 50, 150, or 300 nM fluorescein-labeled dsRNA, negative control, or CXCL16-specific sRNAi, resuspended in 2 ml of prewarmed RPMI 1640 supplemented with 20% vol of autologous serum, and then cultured with or without lipids for 24 h.

Soluble CXCL16 and ApoE quantitation using ELISA

Soluble CXCL16 in the supernatants of unstimulated or stimulated Mo was measured using the Quantikine colorimetric ELISA (R&D Systems), recommended by the manufacturer. Soluble ApoE in the supernatants of sRNAi-transfected or untransfected Mo was measured using ApoE4/Pan-ApoE ELISA kit from MBL.

Adhesion assay

Mo (5 × 10⁶/ml), either untransfected or transfected with negative control or CXCL16-specific sRNAi, were cultured with or without lipids for 24 h. Some untransfected cells were incubated for 45 min before the adhesion assay with anti-CX3CR1 or anti-CXCL16 mAbs or isotype controls. Cells were washed with prewarmed RPMI 1640, loaded for 30 min with 5 μM calcein AM at 37°C, resuspended at 0.5 × 10⁵/100 μl, and incubated at 37°C for 60 min with primary human CASMCs cultured with oxLDL for 24 h. Nonadherent cells were removed by washing, and end-point fluorescence (in U/ml) was measured with a fluorescein filter set (absorbance 494 nm; emission 517 nm) using the multifunctional microplate reader FLUOstar Galaxy (BMG Labtech).

Cholesterol efflux assay

Mo (5 × 10⁶) were incubated with or without 50 μg/ml oxLDL for 24 h, lipid loaded, and labeled for 24 h in the presence of 50 μg/ml acLDL and
2 μCi/ml 1α,2α-(N-14)H)cholesterol (PerkinElmer) in 1 ml of 0.2% BSA in RPMI 1640, and then equilibrated for 4 h in 0.2% BSA-RPMI 1640. After the labeling and equilibration procedures, cells were washed twice with PBS (pH 7.4), incubated for 6, 12, or 24 h at 37°C in 1 ml of 0.2% BSA-RPMI 1640 containing 50 μg/ml human HDL (density 1.063–1.21

g/ml) or 50 μg/ml purified human apolipoprotein A1 (ApoA-1) (Bio-
Vision) as cholesterol-poor acceptors. At different time points, 100 μl of
medium was harvested, and after precipitation at 6000 × g for 10 min to
remove cell debris, radioactivity in the supernatant was measured by liquid
scintillation counting. The cells were lysed in 0.5 ml of 0.1 M NaOH and
0.1% SDS, and cell-associated radioactivity was determined. Cholesterol
efflux was expressed as percentage of radioactivity released from cells into
the medium relative to the total radioactivity in cells plus medium.

Dil-oxLDL/Dil-HDL internalization

Mo (2.5 × 10^5) were differentiated into Mφ in the presence of oxLDL (50
μg/ml) for 24 h. For the Dil-oxLDL internalization assay, cells were pre-
treated with isotype control or mAbs recognizing CD36, SR-A, CXCL16,
or CD68, and then incubated for 2 h at 37°C with 5 μg/ml Dil-oxLDL. For
the Dil-HDL internalization assay, cells were pretreated with isotype con-
trat rat IgG2a or anti-CXCL16 mAb, and then incubated for 2 h at 37°C
with 5 μg/ml Dil-HDL. The efficiency of Dil-HDL uptake in cells was also
measured in the presence of a 40-fold excess of unlabeled LDL, HDL, or
oxLDL. After incubation, Dil-oxLDL or Dil-HDL accumulation in cells
was determined by isopropanol extraction of Dil, and fluorescence was
measured at absorbance 520 nm and emission 564 nm. Results were nor-
malized to total cell protein concentration. Dil-HDL uptake was also mea-
sured, as described above, in Mo that were either untransfected or trans-
fected with the negative control or CXCL16-specific siRNA.

Statistical analysis

All conditions were performed in duplicate or triplicate, and each experi-
ment was performed using cells from three to eight different Mo or
CASM C donors. Values for each condition were averaged, and data are
presented as mean ± SEM. The statistical significance of differences
among matched groups was tested by the nonparametric Friedman two-
way ANOVA by ranks, followed by Dunn’s posttest, using the GraphPad
Prism 3.0 program (GraphPad). Values of p < 0.05 were considered sta-
tistically significant.

Results

To determine how CXCL16 may protect against atherosclerosis,
we first investigated how atherogenic lipids regulate
CXCL16 expression on Mφ, the main CXCL16+ cell type in
atherosclerotic lesions (17, 18). Although standard in vitro Mo-
to-Mφ differentiation protocols use extended culturing of Mo in
various growth factors, including GM-CSF and M-CSF, we
used and optimized previously described protocols that support
rapid differentiation of Mo to Mφ as determined by the immu-
nophenotype of cultured cells (29–33). As expected, freshly
isolated human peripheral blood Mo were ~85% CD14+, but
expressed very low levels of the classical macrophage scavenger
receptors CD68, SR-A, and CD36 (data not shown). Consis-
tent with this, the cells stained very poorly with lipid-specific
Oil Red O (supplemental Fig. 1).4 In contrast, freshly isolated
Mo expressed high levels of CXCL16, a nonclassical scavenger
receptor for oxLDL and a chemokine (~43%; data not shown).
CXCL16 expression on Mo remained constant for 6 h ex vivo
(Fig. 1A and supplemental Fig. 2A),4 but declined thereafter in
the absence of atherogenic lipid stimulation to 25 and 18% by
12 and 24 h, respectively. Compared with these measures,
stimulation of cells with the atherogenic lipids oxLDL,
9-HODE, or 13-HODE for 6, 12, or 24 h markedly increased
CXCL16+ cell frequency (Fig. 1A and supplemental Fig. 2, C,
F, and G),4 which strongly correlated with induction of the
macrophage differentiation marker CD68 (Fig. 1B). CXCL16
expression was also associated with expression of two scavenger
receptor subtypes crucial for foam cell formation: CD36

(Fig. 1C, top panel, and supplemental Fig. 2, C, F, and G)4 and
SR-A (Fig. 1C, bottom panel, and supplemental Fig. 2, C, F,
and G),4 as well as with a significant increase in Oil Red O

4 The online version of this article contains supplemental material.
FIGURE 1. Blockage of CD36 resulted in the greatest decrease (50%) in Dil-oxLDL internalization (Fig. 5A). Conclusion 1. Treatment of Mφ with specific siRNAi (supplemental Fig. 5A) affected adhesion of Mφ to CASMCs. As we have shown previously (26, 27), when both Mφ and CASMCs were stimulated with oxLDL or oxidized linoleic acid-containing lipids before assay, heterotypic adhesion increased dramatically (Fig. 4A). Preincubation of Mφ with anti-CX3CR1 mAb specifically reduced adhesion (Fig. 4A), indicating, as previously shown (26, 27), that Mφ-SMC interaction is in part dependent upon CX3CL1-CX3CR1 interaction. Surprisingly, neither pretreatment of Mφ with CXCL16-directed mAb (Fig. 4A) nor silencing endogenous CXCL16 with specific sRNAi (supplemental Fig. 5) affected adhesion of Mφ to CASMCs (Fig. 4B).

CXCL16 is the only known chemokine that functions as a scavenger receptor for phosphatidylserine and oxLDL (16-18, 35). In this regard, Mφ from cxcl16−/− mice internalize significantly less oxLDL (34%) than wild-type controls (8), suggesting that CXCL16 may mediate a significant portion of oxLDL uptake in vivo. These results are supported by our study in which we measured the contribution of known scavenger receptors toward oxLDL uptake in human Mφ. Blockage of CD36 resulted in the greatest decrease (~50%) in Dil-oxLDL internalization (Fig. 5A), which confirms that CD36 is the main scavenger receptor for ox-LDL on foam cell Mφ (36). Although SR-A is the major scavenger

FIGURE 2. Oxidized lipids inhibit release of the CXCL16 chemokine domain from monocytes. Mo were cultured with or without the stimuli listed on the x-axis (concentrations as in Fig. 1). Cell culture supernatants were collected at indicated time points and analyzed for the presence of CXCL16 by ELISA. Data represent the mean ± SEM from three independent experiments using three different donors with each condition tested in triplicate.

Because CXCR6 and CXCL16 mediate cell-cell adhesion (11), we next tested whether they mediate adhesion of Mφ to CASMCs, the two cell types known to be in close proximity in atherosclerotic lesions (34). Neither CXCR6 nor CXCL16 has been detected in healthy coronary arteries (17, 18). However, we observed low constitutive expression of CXCR6 on the surface of CASMCs cultured in vitro, and this increased 4.6-fold if CASMCs were stimulated with 25 μg/ml oxLDL for 24 h. The adhesion assay was performed, as described in Materials and Methods. A, Before the adhesion assay, Mo or Mφ were blocked for 45 min at 37°C with the following agents: 5 μg/ml isotype control rat IgG2b or CX3CR1 mAb or 4 μg/ml rat IgG2a or CXCL16 mAb. B, Mo were nucleofected with 150 nM indicated CXCL16-specific (CXCL16) or negative control (−Cmed) sRNAi, and then cultured for 24 h in the presence or absence of atherogenic lipids. Data in A are from three independent experiments using Mo from three different donors with each condition tested in duplicate. Results in B are from four independent experiments using four different donors, each condition tested in triplicate. Data are expressed as the mean ± SEM.

FIGURE 3. Atherogenic lipids down-regulate ADAM10 surface expression on CXCL16IR cells. Mo were cultured with or without the indicated stimuli. Summary data are shown for the percentage of CXCL16IR/ADAM10IR cells as a function of cell stimulus. Data are from three independent experiments using three different donors and are presented as the mean ± SEM.

FIGURE 4. CXCL16-CXCR6 axis does not promote adhesion of Mφ to CASMCs. Static adhesion of Mφ to CASMCs, which were cultured with 25 μg/ml oxLDL for 24 h. The adhesion assay was performed, as described in Materials and Methods. A, Before the adhesion assay, Mo or Mφ were blocked for 45 min at 37°C with the following agents: 5 μg/ml isotype control rat IgG2b or CX3CR1 mAb or 4 μg/ml rat IgG2a or CXCL16 mAb. B, Mo were nucleofected with 150 nM indicated CXCL16-specific (CXCL16) or negative control (−Cmed) sRNAi, and then cultured for 24 h in the presence or absence of atherogenic lipids. Data in A are from three independent experiments using Mo from three different donors with each condition tested in duplicate. Results in B are from four independent experiments using four different donors, each condition tested in triplicate. Data are expressed as the mean ± SEM.

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uptake (supplemental Fig. 1).4 The effect was atherogenic lipid specific, because unmodified LDL, PGPC, or POV-PC had no significant effect on CXCL16 expression (Fig. 1A and supplemental Fig. 2, B, D, and E).4

CXCL16 is a multimodular chemokine, which may be either membrane anchored or soluble (9, 10); therefore, we tested whether atherogenic lipids that specifically up-regulated membrane-bound CXCL16 (Fig. 1) may also regulate release of the CXCL16 chemokine domain. Stimulation of Mo with LDL or arachidonic acid derivatives for 6, 12, or 24 h promoted release of CXCL16. In contrast, oxLDL, 9-HODE, or 13-HODE promoted a rapid and time-dependent decrease of ADAM10 surface expression on CXCL16 (Fig. 3)4. Thus, atherogenic lipid-induced up-regulation of CXCL16 on human Mφ favors transmembrane localization over release due to concomitant down-regulation of the CXCL16-specific protease ADAM10.

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Dil-oxLDL by a milligram of total cellular protein. Cell-associated fluorescence was expressed as nanograms of Dil-oxLDL per milligram of total cellular protein. A, Before Dil-oxLDL internalization assay, Mφ were incubated for 45 min at 37°C with 4 μg/ml isotype control or mAbs recognizing CD36, SR-A, CXCL16, or CD68. B, Mo were nucleofected with 150 nM indicated CXCL16-specific (CXCL16) or negative control (∼Cmed) sRNAi, and then cultured for 24 h with or without 50 μg/ml oxLDL, 10 μg/ml 9-HODE, or 10 μg/ml 13-HODE. Values shown are the mean (±SEM) of n = 4 donors for each condition.

Among the scavenger receptors for acLDL (37), this receptor also internalizes oxLDL (36). We were able to confirm this because blockade of SR-A decreased Dil-oxLDL internalization by ∼20% (Fig. 5A). Although the contribution of CD68 toward Dil-oxLDL internalization was minor, blockade of CXCL16 resulted in a strong decrease of Dil-oxLDL scavenging (Fig. 5A). Furthermore, silencing of endogenous CXCL16 in Mφ decreased the amount of cell-associated Dil-oxLDL by ∼25% (Fig. 5B), confirming our data shown in Fig. 5A. These results imply that CXCL16 is the second major scavenger receptor for oxLDL on human Mφ ex vivo.

Exacerbation of atherosclerosis in ldlr<sup>−/−</sup> mice by inactivation of cxcl16 was unexpected and suggested that CXCL16 is atheroprotective, even though cxcl16<sup>−/−</sup> Mφ exhibited marked reductions in uptake of oxidized lipids in vitro (8). This prompted us to analyze CXCL16 scavenger functions further; thus, we examined whether CXCL16 may promote uptake of HDL in Mφ. Internalization of Dil-HDL in Mφ was dose dependent (Fig. 6A). Neutralization of CXCL16’s scavenger function resulted in reduced Dil-HDL uptake in Mφ (Fig. 6A). Furthermore, CXCL16-silenced Mφ internalized significantly less Dil-HDL compared with untransfected or negative control CXCL16 sRNAi-transfected cells (Fig. 6B). We confirmed a role for CXCL16 in HDL uptake by incubating cells in excess amounts of unlabeled LDL, HDL, or oxLDL. Internalization of Dil-HDL by Mφ was greatly reduced in the presence of unlabeled HDL or oxLDL, but not LDL (supplemental Fig. 6A),<sup>4</sup> indicating that HDL and oxLDL may be internalized through the same set of macrophage scavenger receptors. This pattern of Dil-HDL uptake remained unchanged if Mφ were treated with isotype control rat IgG2a Ab before the assay; however, Dil-HDL internalization in Mφ decreased by 36% if cells were pretreated with anti-CXCL16 mAb before the assay (supplemental Fig. 6B).<sup>4</sup> Although our data do not identify CXCL16 as a physiologic HDL scavenger receptor, they demonstrate that CXCL16 expression is important in the process of HDL internalization in Mφ.

Through a process known as reverse cholesterol transport (RCT), excess cholesterol may be released from extrahepatic lipid-loaded cells, such as foam cells in the arterial wall, to lipid-poor acceptors such as HDL and Apo-A1, and this in turn promotes formation of mature HDL-cholesterol particles that transport the excess cholesterol to the liver and intestine for excretion. By reducing accumulation of cholesterol in the arterial wall, cholesterol efflux and RCT may interfere with atherosogenesis (38). Because genetic evidence suggests that CXCL16 is atheroprotective (8, 21), we investigated CXCL16’s role in macrophage cholesterol efflux. [3H]Cholesterol egress to lipid-poor acceptors was time dependent

**FIGURE 5.** CXCL16 promotes oxLDL internalization in Mφ. Uptake of Dil-oxLDL was performed, as detailed in Materials and Methods. Cell-associated fluorescence was expressed as nanograms of Dil-oxLDL per milligram of total cellular protein. A, Before Dil-oxLDL internalization assay, Mφ were incubated for 45 min at 37°C with 4 μg/ml isotype control or mAbs recognizing CD36, SR-A, CXCL16, or CD68. B, Mo were nucleofected with 150 nM indicated CXCL16-specific (CXCL16) or negative control (∼Cmed) sRNAi, and then cultured for 24 h with or without 50 μg/ml oxLDL, 10 μg/ml 9-HODE, or 10 μg/ml 13-HODE. Values shown are the mean (±SEM) of n = 4 donors for each condition.

**FIGURE 6.** CXCL16 promotes HDL uptake in Mφ. Uptake of Dil-HDL was performed, as described in Materials and Methods. Cell-associated fluorescence was expressed as nanograms of Dil-HDL per milligram of total cellular protein. A, Mφ, which were differentiated in presence of 50 μg/ml oxLDL for 24 h, were incubated with 4 μg/ml rat IgG2a or CXCL16 mAb before Dil-HDL internalization assay. B, Mo were nucleofected with 150 nM CXCL16-specific (CXCL16) or negative control (∼Cmed) sRNAi, and then cultured for 24 h with or without 50 μg/ml oxLDL, 10 μg/ml 9-HODE, or 10 μg/ml 13-HODE. Values shown are the mean (±SEM) of n = 4 donors for each condition.

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Transfection with CXCL16-specific sRNAi specifically knocked down expression of CXCL16 and significantly decreased the rate of \[^{3}H\]cholesterol efflux to HDL (Fig. 7A) and Apo-A1 (Fig. 7B) 24 h after addition of lipid-poor acceptors.

Macrophage uptake of oxLDL leads to profound changes in gene expression and lipid metabolism that collectively influence the development of atherosclerotic lesions. In this regard, cholesterol-enriched Mφ show marked induction of anti-inflammatory and anti-atherogenic ApoE as well as the ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), two principal molecules involved in cholesterol efflux from foam cells. Effects of oxLDL on Mφ gene expression are in part scavenger receptor dependent and mediated by transcriptional activation of nuclear receptors (39). Although oxLDL-induced activation of nuclear receptors results in foam cell formation that is detrimental if unopposed, it also provides a mechanism for lipid clearance from the arterial wall if coupled to RCT (39, 40). Because CXCL16 is a receptor for oxLDL (17, 18), CXCL16 could, like other scavenger receptors, regulate nuclear receptor activity and thus control macrophage cholesterol efflux and RCT as well as the severity of atherogenic inflammation through the coordinate regulation of apoE, ABCA1, and ABCG1 expression (39–41).

We tested this hypothesis and, as shown in Fig. 8, lack of macrophage CXCL16 significantly decreased oxLDL-induced up-regulation of ABCA1 (A), ABCG1 (B), and ApoE (C) mRNA. Furthermore, CXCL16 deficiency affected ABCA1 and ApoE expression immediately, whereas ABCG1 expression was affected at later time points (Fig. 8). Moreover, silencing of endogenous CXCL16 significantly reduced expression of macrophage (Fig. 9B), but not monocyte ApoE (Fig. 9A). These data correlate with a decrease in ApoE mRNA accumulation (Fig. 8C). Thus, our data suggest that at least one possible mechanism by which CXCL16 opposes atherosclerosis is by mediating the uptake of oxLDL, which in turn affects expression of atheroprotective molecules at the transcriptional level.

**FIGURE 7.** CXCL16 expression increases \[^{3}H\]cholesterol release from Mφ. Mo were nucleofected with 150 nM CXCL16-specific (CXCL16) or negative control (\(-C_{med}\)) sRNAsi, and then cultured for 24 h with 50 \(\mu\)g/ml acLDL in the presence of radioactive cholesterol. Cells were equilibrated and washed, and efflux was stimulated by incubating Mφ in medium containing lipid-poor acceptors for 6, 12, or 24 h. A, Macrophage \[^{3}H\]cholesterol efflux to human HDL. B, The release of \[^{3}H\]cholesterol to purified human ApoA-1. Results represent the mean \(\pm\) SEM and are from three independent experiments with each condition tested in triplicate. *, \(p<0.05\), comparing the indicated value to the corresponding negative control sRNAsi (\(-C_{med}\)) value for the same time point.

**FIGURE 8.** CXCL16-dependent uptake of oxLDL affects expression of atheroprotective genes. Mo were nucleofected with 150 nM CXCL16-specific (CXCL16) or negative control (\(-C_{med}\)) sRNAsi, and then cultured for 12, 24, or 48 h with or without 50 \(\mu\)g/ml oxLDL. Cells were harvested at indicated times and RNA isolated, as described in Materials and Methods, and accumulation of mRNA for ABCA1 (A), ABCG1 (B), and ApoE (C) was examined. Results represent the mean \(\pm\) SEM and are from five independent experiments with each condition tested in triplicate. Donors were the same in A–C. *, \(p<0.05\) and **, \(p<0.01\) vs the corresponding negative control sRNAsi (\(-C_{med}\)) value for the same time point.
MECHANISMS OF CXCL16-MEDIATED ATHEROPROTECTION

Discussion

Within 10 years, cardiovascular disease will be the major cause of mortality worldwide (42), underscoring the intensified need to precisely identify and characterize mechanisms of atherogenesis. Recent investigations suggest that atherosclerosis may be mediated in part by the balance of atherogenic and atheroprotective chemokines. Currently, the sole example of an atheroprotective chemokine is CXCL16, supported by genetic data in mice and humans (8, 21); however, underlying mechanisms have not been delineated. We have addressed this gap at the cellular and molecular level using a model cell system involving primary human Mφ treated with proatherogenic lipids. We found that oxLDL and oxidized linoleic acid derivatives of LDL found in large amounts in atherosclerotic plaque specifically up-regulated expression of CXCL16. CXCL16, one of only two known transmembrane and adhesive chemokines, was expressed in this system preferentially in the transmembrane form, not the shed form, yet did not function as an adhesion molecule promoting interaction between CXCR6+ human SMCs and human Mφ, but rather as a scavenger receptor for oxLDL. It also contributed significantly toward the uptake of HDL in Mφ. Our results also show that CXCL16 expression in Mφ increased the rate of cholesterol efflux to lipid-poor extracellular HDL and ApoA-1 acceptors, which implies that CXCL16 is involved in macrophage RCT, a fundamental mechanism protecting against atherosclerosis (43).

Zhang et al. (44) previously reported that oxLDL at very high concentrations could increase CXCL16 expression using a cultured human macrophage cell line (THP-1). Our results extend this finding to primary human peripheral blood monocyte-derived Mφ under lipid conditions simulating the atherogenic microenvironment. In this system, atherogenic lipids up-regulate CXCL16 on Mφ, which are the predominant inflammatory cell type expressing this chemokine in atherosclerotic lesions (17, 18). The observation that CXCL16 expression accompanies the process of foam cell development in vitro (44) suggested to us that it is not functioning in this context as a classic chemokine, mediating either cell-cell adhesion or chemotaxis, because those functions would be expected to be atherogenic, not atheroprotective. In this regard, our finding that macrophage CXCL16 expression is important in the process of HDL internalization as well as in macrophage cholesterol efflux identifies a mechanism by which CXCL16 may oppose atherogenesis in atheromatosus conditions and provides a potential mechanism of how CXCL16 supports the atheroprotective role of HDL.

Numerous epidemiological studies show an inverse correlation between HDL plasma level and the risk of coronary heart disease. The central anti-atherogenic activity of HDL is its ability to remove cholesterol from foam cells, SMCs, and endothelial cells in the arterial wall by cholesterol efflux (38). Experimental evidence obtained in vitro and in vivo suggests that cholesterol release from foam cells is predominantly receptor mediated and fractional. Scavenger receptor-B1 (SR-B1), the only known physiological HDL receptor, which facilitates the bidirectional flux of free cholesterol between cells and lipoprotein, stimulates ~7% of total cholesterol efflux (45). ABCA1 and ABCG1 together account for ~65% of the net cholesterol efflux to HDL or serum from cholesterol-loaded Mφ, as revealed in studies of Abca1−/− Abcg1−/− Mφ (46, 47). Taken together, our results indicating that CXCL16 increases cholesterol egress to lipid-poor acceptors by ~34% (Fig. 7) complement genetic knockdown studies (46, 47) and suggest the new hypothesis that CXCL16 negatively regulates atherogenesis in vivo by facilitating RCT.

We have shown that oxLDL induces macrophage expression of ABCA1, ABCG1 (Fig. 8), and ApoE in a CXCL16-dependent manner (Figs. 8 and 9). All of these molecules are involved in cholesterol efflux, the initial step in RCT (48). Although the critical role of ABCA1 and ABCG1 in promoting cholesterol release from foam cells has been clearly demonstrated in vivo (46, 47) and in vitro (49), bone marrow transplantation studies indicate that macrophage-derived ApoE may also be anti-atherogenic because it promotes cholesterol efflux from lipid-loaded macrophages in the artery wall by three distinct processes, as follows: ABC independent, and ABCA1 and ABCG1 dependent. Macrophage synthesis of ApoE stimulates cholesterol efflux from cells not loaded with cholesterol, but this process is ABC independent (50). In cholesterol-loaded cells, stimulation of lipid efflux by macrophage ApoE is primarily ABCA1 dependent. This is most likely the predominant pathway in the artery wall that generates lipidated ApoE (51). In contrast to ABCA1, which promotes efflux to lipid-poor acceptors, ABCG1 stimulates efflux to mature HDL particles, which contain ApoE (48). The Apo-E-HDL-containing particles can be cleared from the plasma via SR-B1 (45). Because the uptake of these particles is impaired in the liver and adrenal glands of apoE−/− mice, it has been suggested that ApoE may facilitate HDL presentation to SR-B1 (52). Because CXCL16-dependent ox-LDL internalization affects ApoE, ABCG1, and ABCA1 expression, CXCL16 may oppose atherogenesis by increasing ABC-dependent

**FIGURE 9.** Silencing of endogenous CXCL16 decreases production of macrophage ApoE. Mo were transfected with sRNAi listed on the x-axis and then cultured for 6, 12, or 24 h with (B) or without (A) 50 μg/ml oxLDL. Cell culture supernatants were collected at indicated time points and analyzed for the presence of pan-ApoE by ELISA. Data represent the mean ± SEM from three different donors with each condition tested in triplicate. *p < 0.05 vs the corresponding negative control sRNAi (−Cmed) value for the same time point.
cholesterol efflux in the vessel wall and by stimulating the SR-BI-dependent uptake of mature HDL particles in the liver. This conclusion is supported by experimental evidence demonstrating that targeted disruption of cxcl16 (8) or SR-BI (53, 54) exacerbates atherosclerosis. Thus, effects of CXCL16 and SR-BI may be additive and cooperative, and expression and function of both scavenger receptors may be necessary for sustained protection against atherosclerosis.

Although ABCA1 and ABCG1 are predominantly involved in cholesterol efflux (46, 47), ApoE, which is produced by several tissues and cells, including foamy macrophages, is a multifunctional protein that in addition to facilitating cholesterol release from foamy macrophages in atherosclerotic lesions may also inhibit SMC migration and proliferation, directly modifies lymphocyte-mediated immunity in atherosclerotic lesions and decreases vascular inflammation by suppressing production of proinflammatory and proatherogenic cytokines TNF-α, IL-6, and IFN-γ (55). Thus, CXCL16 may interfere with atherogenesis on multiple levels by regulating expression of this multifunctional protein.

Our results also demonstrate that CXCL16’s scavenger role in our system is supported through lipid-induced down-regulation of surface ADAM10. Although the mechanism through which atherogenic lipids down-regulate ADAM10 remains unclear, our data suggest that these lipids shift the balance between soluble and transmembrane CXCL16 in an ADAM10-dependent manner in favor of a membrane-bound form of CXCL16. Thus, these data suggest a concept that although CXCL16 is a multimodular chemokine that exists in two different forms, soluble and membrane bound (9, 10), the atherogenic environment may dictate preference of a membrane-bound form of CXCL16. Moreover, CXCL16 may interfere with atherogenesis on multiple levels by regulating expression of this multifunctional protein.

Thus, CXCL16 may interfere with atherogenesis on multiple levels by regulating expression of this multifunctional protein.

Disclosures
The authors have no financial conflict of interest.

References

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