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Chemokine receptors CXCR2 and CX3CR1 differentially regulate functional responses of bone-marrow endothelial progenitors during atherosclerotic plaque regression

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Aims Atherosclerosis manifests itself as arterial plaques, which lead to heart attacks or stroke. Treatments supporting plaque regression are therefore aggressively pursued. Studies conducted in models in which hypercholesterolaemia is reversible, such as the Reversa mouse model we have employed in the current studies, will be instrumental for the development of such interventions. Using this model, we have shown that advanced atherosclerosis regression occurs when lipid lowering is used in combination with bone-marrow endothelial progenitor cell (EPC) treatment. However, it remains unclear how EPCs home to regressing plaques and how they augment atherosclerosis reversal. Here we identify molecules that support functional responses of EPCs during plaque resolution.

Methods and results Chemokines CXCL1 and CX3CL1 were detected in the vascular wall of atheroregressing Reversa mice, and their cognate receptors CXCR2 and CX3CR1 were observed on adoptively transferred EPCs in circulation. We tested whether CXCL1–CXCR2 and CX3CL1–CX3CR1 axes regulate functional responses of EPCs during plaque reversal. We show that pharmacological inhibition of CXCR2 or CX3CR1, or genetic inactivation of these two chemokine receptors interfered with EPC-mediated advanced atherosclerosis regression. We also demonstrate that CXCR2 directs EPCs to regressing plaques while CX3CR1 controls a paracrine function(s) of these cells.

Conclusion CXCR2 and CX3CR1 differentially regulate EPC functional responses during atheroregression. Our study improves understanding of how chemokines and chemokine receptors regulate plaque resolution, which could determine the effectiveness of interventions reducing complications of atherosclerosis.

Keywords Atherosclerosis regression • Endothelial progenitor cell • Chemokine receptor CXCR2 • Chemokine receptor CX3CR1

1. Introduction

Atherosclerosis is a major cause of death and disability globally. It is a chronic inflammatory disease of the arterial wall triggered by deposition of LDL in the vascular subendothelium, which results in development of multifocal atherosclerotic plaques. Most plaques are asymptomatic; however, some become thrombosis-prone and may rupture, causing complications including acute myocardial infarction and stroke.[1]

Current therapies for atherosclerosis, including lifestyle and pharmacological control of risk factors through use of statins or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, have demonstrated beneficial effects on atherosclerotic vasculature. However, statins do not completely reverse this disease, leaving patients prone to complications.[2,3] Thus, new approaches that would induce complete plaque resolution are sought. New treatment modalities could be developed in animal models in which cardiovascular risk factors, especially

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hypercholesterolaemia as the main inducer of plaque development, are reversible.

Reversa mice represent such a model. They lack low-density lipoprotein receptor ($Ldlr^{-/-}$) and express atherogenic apolipoprotein B (ApoB) 100 ($Ldlr^{-/-}ApoB^{100/100}Mttp^{fl/fl}Mx1\text{-}Cre$). These genetic modifications make this strain susceptible to plaque development, especially if mice are fed the atherosclerosis-inducing western diet. In this strain, plasma lipid levels can be normalized upon Cre-dependent inactivation of microsomal triglyceride transfer protein (*Mttp*), which is required for transport of neutral lipids to nascent ApoB lipoproteins and for assembly of atherogenic LDL in the liver.[4] Reversa mice are well-suited for studies identifying mechanisms supporting plaque regression in humans because reversal of hypercholesterolaemia in these mice mimics statin-mediated lowering of plasma lipids in cardiovascular patients.

The initial event triggering plaque development is endothelial dysfunction, which can be corrected by postnatal neovascularization mediated by endothelial progenitors that reside mostly in the bone marrow (bone marrow EPCs). Following vascular injury, EPCs mobilize to circulation (circulating EPCs) forming a scarce population of peripheral blood mononuclear cells (PBMCs) that mediates vascular repair. EPCs directly contribute to endothelial regeneration by homing to and differentiating to endothelial cells. EPCs may also indirectly contribute to re-establishment of endothelial homeostasis by producing cytokines and growth factors that promote survival and proliferation of resident endothelial cells.[5,6]

EPCs are especially important in cardiovascular patients because their circulating numbers are used as a marker of vascular function and cumulative cardiovascular risk. Hypercholesterolaemia decreases EPC numbers and compromises their survival and functional properties. Conversely, normalization of plasma lipids increases circulating EPCs. Increased circulating EPC numbers are associated with decreased risk of major cardiovascular events and hospitalization of cardiovascular patients.[7,8] This suggests that EPCs contribute to ongoing vascular repair and reduce plaque burden. To this end, we have recently demonstrated that bone marrow-derived EPCs reduce atherosclerosis and repair vascular damage caused by plaques in the Reversa mouse model.[9] However, it remains unclear how EPCs home to regressing plaques and how they reduce atherosclerosis burden.

The chemokine system, composed of chemokines and chemokine receptors, regulates functional responses of bone marrow progenitors including EPCs.[10] We observed increased expression of chemokines CXCL1 and CX3CL1 and their cognate receptors CXCR2 and CX3CR1 in the vascular wall and on adoptively transferred bone marrow EPCs in the circulation of Reversa mice in which plasma lipids were normalized (atheroregressing mice). These results support the hypothesis that CXCL1–CXCR2 and CX3CL1–CX3CR1 axes regulate homing and regenerative responses of EPCs in atheroregressing conditions.

We tested the hypothesis in Reversa mice using pharmacological and genetic approaches. We found that systemic inhibition of CXCR2 or CX3CR1, or genetic ablation of these chemokine receptors on EPCs interfered with accelerated plaque regression. Inhibition or inactivation of CXCR2 or CX3CR1 resulted in increased atherosclerosis burden, poor vascular relaxation, and increased expression of plaque instability markers including matrix metalloproteinase 9 (MMP9), tissue factor (TF), the transcription factor CHOP, and the transcription factor v -ets erythroblastosis virus E26 oncogene homologue 2 (Ets2). These are indicators of apoptosis/necrosis and inflammation in atherosclerotic vessels and are associated with plaque destabilization and rupture in cardiovascular patients.[11] Interestingly, inhibition or inactivation of CXCR2 prevented homing of EPCs to regressing plaques, whereas blockage or genetic ablation of CX3CR1 on EPCs interfered with a paracrine role of EPCs since reduced production and release of transforming growth factor β1 (TGFβ1), a major cytokine involved in regenerative repair, was detected in EPCs lacking this receptor. Neutralization of TGFβ1 impeded EPC-augmented plaque reversal, suggesting that beneficial effects of EPCs during atherosclerosis reversal are supported by this cytokine. Our results are novel and significant because they indicate that CXCR2 and CX3CR1 support EPC-mediated atherosclerosis regression; however, they modulate different functional responses of EPCs. Since inhibition of several chemokine receptors including CXCR2 and CX3CR1 has been proposed to treat atherosclerosis,[12] our study provides evidence that CXCR2 and CX3CR1 functions may not be inhibited during atheroregression as this may decrease the efficacy of treatment.

2. Methods

2.1 Experimental designs

Reversa, Tie2-GFP, $Cxcr2^{-/-}$, and $Cx3cr1^{-/-}$-GFP^{+} mice (all C57BL/6 background) were obtained from Jackson Laboratory. $Cxcr2^{-/-}$Tie2-GFP^{+} mice were generated by crossing Tie2-GFP with $Cxcr2^{-/-}$ mice. Mice of both sexes were used for all experiments.

To evaluate how pharmacological inhibition of CXCR2 or CX3CR1 affects plaque regression, Reversa mice fed western diet (21% anhydrous milkfat/butterfat, 46% carbohydrate, 0.2% cholesterol; Harlan Teklad) for 84 days were treated with polyinosinic–polycytidylic acid (pI–pC; 500 μg/mouse; Sigma) four times at 2-day intervals to inactivate hepatic lipoprotein production necessary for normalization of plasma lipids. Atheroregressing mice were treated with 1×10^6 wild-type Tie2-GFP^{+} EPCs 10, 20, and 30 days after *Mttp* was inactivated in presence or absence of highly specific CXCR2 antagonist SB 265610 (2 mg/kg body weight; Tocris Bioscience; Supplementary material online, *Figure S1*) or anti-mouse rabbit polyclonal CX3CR1 antibody (Ab; 1 mg/kg body weight; eBioscience; Supplementary material online, *Figure S2*). To provide genetic evidence for the role of CXCR2 or CX3CR1 in EPC-mediated atheroregression, mice were treated with 1×10^6 $Cxcr2^{-/-}$Tie2-GFP^{+} (Supplementary material online, *Figure S3*) or $Cx3cr1^{-/-}$-GFP^{+} (Supplementary material online, *Figure S4*) EPCs 10, 20, and 30 days after plasma lipids were lowered.

To investigate how neutralization of TGFβ 1 affects plaque reversal, atheroregressing mice untreated or treated with Tie2-GFP^{+} EPCs 10, 20, and 30 days following normalization of plasma lipids received neutralizing mouse monoclonal TGFβ1 1D11 Ab (R&D Systems) 3 times per week at 5 mg/kg body weight for 3 weeks. Mice were then switched to a regimen of 5 mg/kg body weight of TGFβ1 1D11 Ab once per week for 9 weeks (Supplementary material online, *Figure S5*). The 1D11 antibody is well-characterized and has been used in a wide-range of applications, including *in vitro* and *in vivo* neutralization of TGFβ1. Inhibition of TGFβ1 using the 1D11 antibody was demonstrated to accelerate atherosclerosis in $Ldlr^{-/-}$ mice.[13]

Mice in all groups were anaesthetized with intraperitoneal injection of ketamine/xylazine (80 mg/kg/5 mg/kg body weight, Henry Schein Animal Health) and terminated by exsanguination 70 days after plasma lipids were normalized. All animal procedures were performed in accordance with NIH guidelines (Guide for the Care and Use of Laboratory Animals), and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation (approval numbers 12–52, 13–52).

2.2 EPC isolation, culture, and identification

EPCs were first described by Asahara *et al.*[5] in 1997 as cells expressing CD34 and vascular endothelial growth factor receptor 2 (VEGFR2 or Flk1 in mice)

that stimulate angiogenesis and vascular repair *in vivo*. EPCs therefore co-express surface markers of cellular immaturity and endothelial origin. In the mouse, the minimal antigenic profile for EPCs includes at least one immaturity marker such as CD34, CD133, c-kit, or Sca-1 plus one marker of endothelial commitment such as Flk1. In parallel with the antigenic identification, these cells also internalize 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labelled acetylated LDL (Dil-AcLDL) and are able to bind fluorescently labelled *Ulex europaeus* lectin. It is well-established that both identification methods used in combination provide the most accurate approach to identify EPCs.[6]

Bone-marrow EPCs were isolated using the method previously reported by Yao *et al.*[9] Briefly, Tie2-GFP$^+$, *Cxcr2*$^{-/-}$Tie2-GFP$^+$, *Cx3cr1*$^{-/-}$-GFP$^+$ donor mice were anaesthetized with intraperitoneal injection of ketamine/xylazine (80 mg/kg/5 mg/kg body weight, Henry Schein Animal Health) and terminated by exsanguination (the protocol approval numbers 12–52, 13–52). Bone marrow was flushed from long bones with sterile Hanks' BSS, low-density cells were collected by centrifugation over Histopaque 1083. Mononuclear lineage-negative (Lin$^-$) cells were enriched by lineage-depletion cocktail containing monoclonal antibodies to mouse CD3, CD11b, CD45/B220, Ly-6G, Ly- 6C, and TER-119 (BD Biosciences), and the Lin$^-$GFP$^+$ population deprived of leucocytes was isolated by fluorescence-activated cell sorting (FACS) using a FACSAria cell sorter (BD Biosciences). Lin$^-$GFP$^+$ cells were plated on fibronectin-coated 24-well plates at 5×10^5 cells/well and cultured for 4 days in endothelial growth factor-containing medium (Lonza) supplemented with FBS Gentamycin/Amphotericin, vascular endothelial growth factor, insulin growth factor, and fibroblast growth factor (all from Sigma). Culturing Lin$^-$GFP$^+$ cells in this selection medium supports the outgrowth of EPCs and prevents the survival of contaminant cell types including leucocytes and/or haematopoietic progenitors. Prior to injection into recipient Reversa mice, *ex vivo*-expanded GFP$^+$ EPCs were evaluated by flow cytometry for concurrent expression of immaturity surface antigens CD133 and/or Sca1, and endothelial commitment markers Flk1. The flow cytometry-based antigenic identification was followed by the microscopic evaluation assessing cell uptake of Dil-AcLDL and TRITC-lectin binding.

2.3 Atherosclerosis evaluation

Atherosclerotic plaques were evaluated by *en face* analysis of pinned-open aortas and by cross-sectional analysis of the proximal aorta. For *en face* analysis, aortas were opened longitudinally and stained in Oil Red O (Newcomer Supply). Aortic images were captured with a Canon EOS-1 Mark II digital camera and analysed using Adobe Photoshop CS. For cross-sectional analysis, aortas were fixed, serially cryosectioned, stained with Oil Red O, and counterstained with haematoxylin. Eight sections per site, collected at 40-μm intervals, were examined under light microscope. Images were obtained with a Zeiss AxioCam MRC 12-bit color digital camera (Carl Zeiss Microimaging). The lesional surface was measured using NIH Image J software and is expressed in μm^2.

2.4 Immunofluorescence

CXCL1 and CX3CL1 expression were evaluated by staining aortic cross-sections obtained from hypercholesterolaemic and atheroregressing Reversa mice using primary rabbit polyclonal anti-CXCL1, anti-CX3CL1 Abs, or isotype control IgG Abs (all from Abcam) followed by incubation in secondary goat anti-rabbit IgG Alexa Fluor 568 Ab (Life Technologies).

To examine incorporation of Tie2-GFP$^+$, *Cxcr2*$^{-/-}$Tie2-GFP$^+$, or *Cx3cr1*$^{-/-}$-GFP$^+$ bone-marrow EPCs into recipient endothelium, aortic cross-sections obtained from recipient atheroregressing Reversa mice were stained with primary rabbit VE Cadherin (Santa Cruz Biotechnology) or isotype control IgG Ab (Abcam) and secondary goat anti-rabbit IgG Alexa Fluor 568 Ab.

To evaluate expression of MMP9, TF, CHOP, and Ets2, frozen aortic cross-sections from hypercholesterolaemic, atheroregressing, and EPC-treated atheroregressing mice were stained with polyclonal rabbit anti-MMP9 (Abcam), anti-TF (Abcam), anti-Ets2 (Santa Cruz Biotechnology), or monoclonal mouse anti-CHOP (Abcam) Abs, or their respective isotype controls followed by staining with the secondary Alexa Fluor 568-conjugated Ab.

2.5 Masson's trichrome stain

Collagen deposition was evaluated in aortic cross-sections obtained from hypercholesterolaemic or atheroregressing Reversa mice untreated or receiving Tie2-GFP$^+$, *Cxcr2*$^{-/-}$Tie2-GFP$^+$, or *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs using the Masson's Trichrome stain kit according to manufacturer's instructions (Polysciences Inc.).

2.6 Flow cytometry

To evaluate CXCR2 or CX3CR1 expression on cultured bone-marrow EPCs, Tie2-GFP$^+$ cells were co-stained for Sca-1 (BD Biosciences), Flk1 (BioLegend), and CXCR2 (BioLegend) or CX3CR1 to determine the percent of CXCR2$^+$ or CX3CR1$^+$ cells in the Tie2-GFP$^+$Sca-1$^+$Flk1$^+$ population.

To examine expression of CXCR2 or CX3CR1 on adoptively transferred bone-marrow EPCs in circulation, Tie2-GFP$^+$ cells were separated from PBMCs by fluorescence-activated cell sorting and co-stained for CD133 (BioLegend), Flk1, and CXCR2 or CX3CR1. Percent of CXCR2$^+$ or CX3CR1$^+$ cells was determined in the Tie2-GFP$^+$CD133$^+$Flk1$^+$ population.

Leucocyte CXCR2 is found mostly on monocytes and T lymphocytes, whereas the leucocytes expressing CX3CR1 in peripheral blood are primarily monocytes. Thus, PBMCs were co-stained with Sca1-, CD45-, CD115-, CD11b-, CD3-, CD4- (BD Biosciences), and CXCR2- or CX3CR1-directed Abs, and percent of CXCR2$^+$ or CX3CR1$^+$ cells in the Sca1$^-$CD45$^+$CD115$^+$CD11b$^+$ population or on CD3$^+$CD4$^+$ PMBCs were determined.

Flow cytometry was performed on a BD LSRII (BD Biosciences), correcting for non-specific staining with isotype controls. Dead cells were excluded by propidium iodide stain. FlowJo software (Tree Star) was used for data analysis.

2.7 Vascular relaxation

Vascular relaxation was used as a measure of overall cardiovascular health. Aortas were removed from Tie2-GFP$^+$ EPCs-treated regressing Reversa mice that received CXCR2 antagonist SB 265610, CX3CR1 Ab, isotype control IgG Ab, or vehicle. Aortas were cut into 3 mm-wide rings and precontracted with 30 nmol/L U45519 in organ chambers (PowerLab ADInstruments). Endothelium-dependent acetylcholine (ACh)-induced vasodilation responses were monitored. Vascular relaxation was also assessed for aortic rings obtained from regressing mice treated with *Cxcr2*$^{-/-}$Tie2-GFP$^+$, *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs and animals that received Tie2-GFP$^+$ EPCs plus neutralizing TGFβ1 Ab.

2.8 Enzyme-linked immunosorbent assay (ELISA)

CXCL1, CX3CL1, TF, or MMP9 levels in plasma were determined by ELISA (R&D Systems).

2.9 CX3CL1 stimulation of bone-marrow EPCs and TGFβ1 evaluation

Bone-marrow Tie2-GFP$^+$ or *Cx3cr1*$^{-/-}$- GFP$^+$ EPCs were isolated and expanded *ex vivo* as detailed in 'EPC isolation, culture, and identification'. After 4 days in culture, cells were stimulated with 10, 25, 50, or 100 ng/mL of soluble CX3CL1 for 12 h, and cells and supernatants were collected. Cells were lysed and TGFβ1 amounts in cell lysates and culture supernatants were determined by ELISA (eBioscience).

2.10 Proliferation assay

Proliferation of bone marrow-derived EPCs in culture was evaluated using bromodeoxyuridine (BrdU) cell proliferation ELISA kit as recommended by the manufacturer (Abcam).

2.11 Quantification of GFP$^+$ cell luminal coverage

GFP$^+$ cells were counted on the luminal side of the vessel wall and their numbers were recorded. The total number of cells on the luminal side was determined by counting DAPI$^+$ nuclei. Percent GFP$^+$ cell coverage on the luminal side was calculated: GFP$^+$ cell number/total number of cells $\times 100$. At least three cross-sections from each mice and a minimum of five mice in each group were analysed this way.

2.12 Statistical analysis

Experiments were repeated two times using at least five mice per each group. Data are presented as median $\pm$ SEM. Statistical comparisons between two groups were performed by Student's *t*-test (*Figures 1B, C* and *6A*). The statistical significance of differences among multiple groups was tested by ANOVA (*Figures 2C*, *3D*, *4D*, *5B–G*, *6B*, *6C*, *7C*, *F*), and where applicable with a repeated-measures approach (*Figures 3A* and *C*, *4A* and *C*, *7D*). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 CXCL1–CXCR2 and CX3CL1–CX3CR1 axes are up-regulated in atheroregresing reversa mice

We have recently showed that bone-marrow EPCs accelerate atherosclerotic plaque reversal[9]; however, signals directing these cells to regressing plaques and mechanisms of EPC-mediated vascular repair remain unknown. EPC recruitment to sites of vascular injury is thought to be controlled by the chemokine system.[10] To address whether this system shapes functional responses of bone-marrow EPCs in atheroregressing conditions, we investigated how normalization of plasma lipids affects expression of chemokines in the vascular wall and determined which chemokine receptors are expressed on cultured bone marrow EPCs and on adoptively transferred bone marrow EPCs in circulation.

Chemokines CXCL1 and CX3CL1 were detected in vascular walls of hypercholesterolaemic and atheroregressing mice. Interestingly, normalization of plasma lipids increased expression of CXCL1 and CX3CL1 in the vascular wall (*Figure 1A*) and in circulation (*Figure 1B* and *C*). Bone marrow EPCs proliferated in culture (Supplementary material online, *Figure S6*). Furthermore, as shown in *Figure 1D*, 93 and 54% of bone marrow Tie2-GFP$^+$Sca- 1$^+$Flk1$^+$ cells expressed CXCR2 and CX3CR1, respectively. The two chemokine receptors were also detected on $\sim$26 and $\sim$10% of Tie2-GFP$^+$CD133$^+$Flk1$^+$ adoptively transferred cells in circulation (*Figure 1E*), respectively. Lipid lowering decreased expression of CXCR2 and CX3CR1 on leucocytes including on Sca1$^-$CD45$^+$ CD11b$^+$CD115$^+$ and CD3$^+$CD4$^+$ cells by 62, 47, or 61%, respectively (Supplementary material online, *Figure S7*). Thus, data suggest that CXCL1–CXCR2 and CX3CL1–CX3CR1 interactions regulate functional responses of EPCs during plaque resolution.

3.2 Pharmacological inhibition of CXCR2 or its genetic ablation in EPCs impairs their homing to regressing plaques

CXCR2 is expressed in the brain, craniofacial tissues, lungs, spinal cord, liver, kidney, endothelial cells, bronchoepithelial cells, and immune cells including neutrophils, lymphocytes, and monocytes.[14] CXCR2 plays a critical role in regulating neutrophil homeostasis,[15] increases cellular senescence in early tumourigenesis and creates circumstances favouring metastasis formation in late tumourigenesis.[16] CXCR2 expression is important in angiogenesis[17] and in wound healing.[18] CXCR2 is also known to promote plaque formation.[19] The CXCL1–CXCR2 interaction recruits human peripheral blood EPCs to sites of arterial injury in athymic nude mice.[20] Inhibition of CXCL1 prevents endothelial recovery and enhances plaque formation after arterial injury in mice.[21] This interaction also facilitates homing of mouse EPCs to the blood vessel endothelium during chronic allergic inflammation in lungs.[22] Detection of CXCL1 in the vascular wall and peripheral blood of regressing Reversa mice and expression of CXCR2 on adoptively transferred bone-marrow EPCs (*Figure 1*) suggest that the CXCL1–CXCR2 interaction is an important regulator of EPC functional responses. We have tested this by pharmacological and genetic approaches.

The CXCR2 antagonist SB 265610 inhibits CXCR2 *in vitro* and *in vivo*. The antagonist blocks neutrophil influx into lungs in hyperoxia-induced newborn lung injury, a model of bronchopulmonary dysplasia in rats and in mice.[23] Since SB 265610 inhibits CXCR2 at the systemic level and not in a tissue-specific manner such that CXCR2 inhibition would only affect CXCR2 on EPCs, we also evaluated whether EPC-specific deletion of CXCR2 affects atherosclerosis regression.

The experimental design in Supplementary material online, *Figure S1* was followed to assess how inhibition of CXCR2 affects atherosclerosis burden. Endothelium-dependent acetylcholine (ACh)-induced vascular relaxation and incorporation of GFP$^+$ cells into recipient endothelium were also evaluated.

Inhibition of CXCR2 diminished EPC-augmented atherosclerosis resolution (*Figure 2*). SB 265610 treatment of mice receiving Tie2- GFP$^+$ EPCs also resulted in vascular relaxation of only 62% relative to 98% relaxation detected following treatment of aortas from control regressing recipients infused with Tie2-GFP$^+$ EPCs but not treated with SB 265610 at 10^{-5} mol/L ACh (*Figure 3A*). Furthermore, significantly lower vascular reactivity was observed for aortas from SB 265610 plus Tie2- GFP$^+$ EPC-treated group following stimulation with lower concentrations of vasodilating agent ($P < 0.0001$ for 10^{-7} and 10^{-6} mol/L ACh, *Figure 3A* and Supplementary material online, *Table S1*) In addition, only a few Tie2-GFP$^+$EPCs incorporated into the endothelium of SB 265610-treated recipients (*Figure 3B* and *D*). The GFP$^+$ cell luminal coverage ranged from 1 to 11.1%, with an average of 3.84% relative to the coverage detected in controls treated with vehicle plus Tie2-GFP$^+$ EPCs that ranged between 12.5 and 61.1%, with an average of 39.08% (*Figure 3D*). These data suggest that CXCR2 is critical for homing of EPCs to the vascular wall.

The role of CXCR2 in recruitment of EPCs to regressing lesions was also assessed following adoptive transfer of *Cxcr2*$^{-/-}$Tie2-GFP$^+$ or control Tie2-GFP$^+$ EPCs into atheroregressing mice (Supplementary material online, *Figure S3*). The *Cxcr2*$^{-/-}$ strain is viable, normal in size, and fertile. *Cxcr2*$^{-/-}$ mice show impaired leucocyte recruitment in response to pathogen challenge, delayed wound healing, and impaired angiogenesis.[18,24] *Cxcr2*$^{-/-}$ mice were crossed with Tie2-GFP$^+$ mice to generate the *Cxcr2*$^{-/-}$Tie2-GFP$^+$ strain that was used as the EPC donor, so that adoptively transferred cells could be monitored.

We observed that treatment with EPCs lacking CXCR2 did not improve atherosclerosis resolution since regressing Reversa mice receiving *Cxcr2*$^{-/-}$Tie2-GFP$^+$ EPCs and mice co-treated with Tie2 -GFP$^+$ EPCs and SB 265610 displayed a similar plaque burden (*Figure 2C*). Increased lesion size in *Cxcr2*$^{-/-}$Tie2-GFP$^+$ EPC-treated mice was also associated with poor vascular relaxation of only 50% when aortas were exposed to 10^{-5} mol/L ACh (*Figure 3C*). Interestingly, a significant reduction in vascular

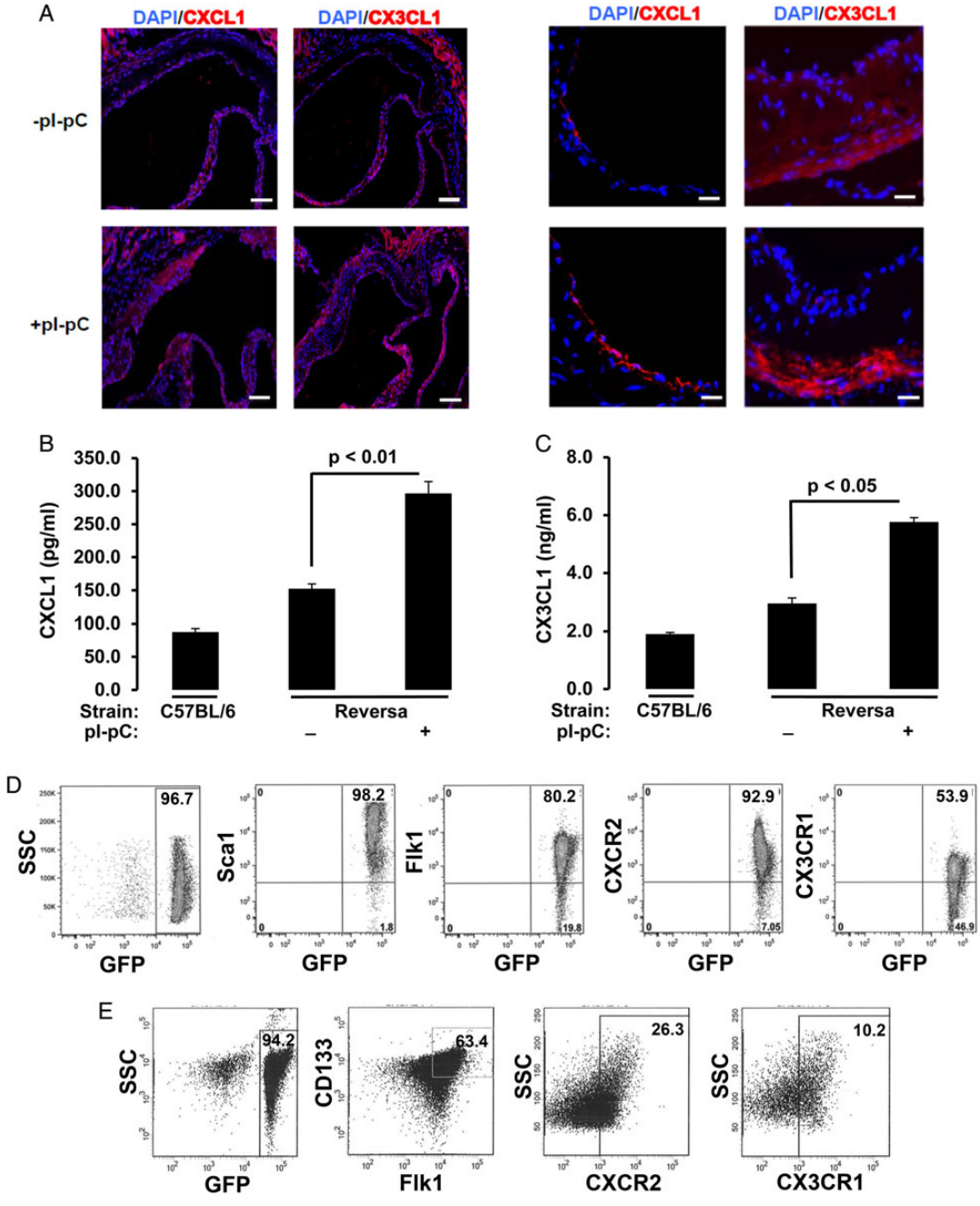

Figure 1 CXCL1 and CXCR2 expressions, and CX3CL1 and CX3CR1 expressions, in hypercholesterolaemic and atheroregressing conditions. (*A*) Representative aortic sections from regressing Reversa mice (+pI–pC, $n = 6$) and hypercholesterolaemic controls (−pI–pC, $n = 6$) were stained with anti-mouse Abs (red) directed against CXCL1 or CX3CL1. Samples were mounted with (DAPI) medium. Nuclei appear blue. Original magnification ×10 (left panel, the scale bar equals 100 μm) or ×40 (right panel, the scale bar, 10 μm). (*B*) Plasma levels of CXCL1 or (*C*) CX3CL1 measured by ELISA. (*D*) Bone-marrow EPCs were obtained from $n = 6$ Tie2-GFP$^+$ mice, cultured as in *Methods* and expression of CXCR2 or CX3CR1 was determined by flow cytometry. (*E*) CXCR2 or CX3CR1 expression on bone marrow Tie2-GFP$^+$CD133$^+$ Flk1$^+$ cells in circulation was evaluated 6 h after adoptive transfer of these cells into atheroregressing recipients ($n = 6$). Percent of cells with the indicated phenotype is present in the upper right corner.

relaxation of aortas from *Cxcr2*$^{-/-}$Tie2-GFP$^+$ EPC-treated mice was detected starting 10^{-9} mol/L ACh ($P < 0.05$, *Figure 3C* and Supplementary material online, *Table S1*). Moreover, the GFP$^+$ luminal coverage in *Cxcr2*$^{-/-}$Tie2- GFP$^+$ EPC recipients ranged between 1 and 8.3%, with an average of 2.49%, indicating that CXCR2-deficient EPCs inefficiently recruited to regressing plaques (*Figure 3B* and *D*). Pharmacological and genetic approaches collectively demonstrate that CXCR2 directs EPCs to regressing atherosclerotic plaques.

3.3 Inhibition or inactivation of CX3CR1 on EPCs inhibits augmented plaque resolution but does not affect EPC recruitment

CX3CR1 is expressed on several cell types including monocytes, macrophages, lymphocytes, neurons, smooth muscle cells, endothelial cells, and EPCs. It has been detected in mouse and human atherosclerotic lesions,[25,26] and has been shown to be pro-atherogenic.[27,28] Roles for

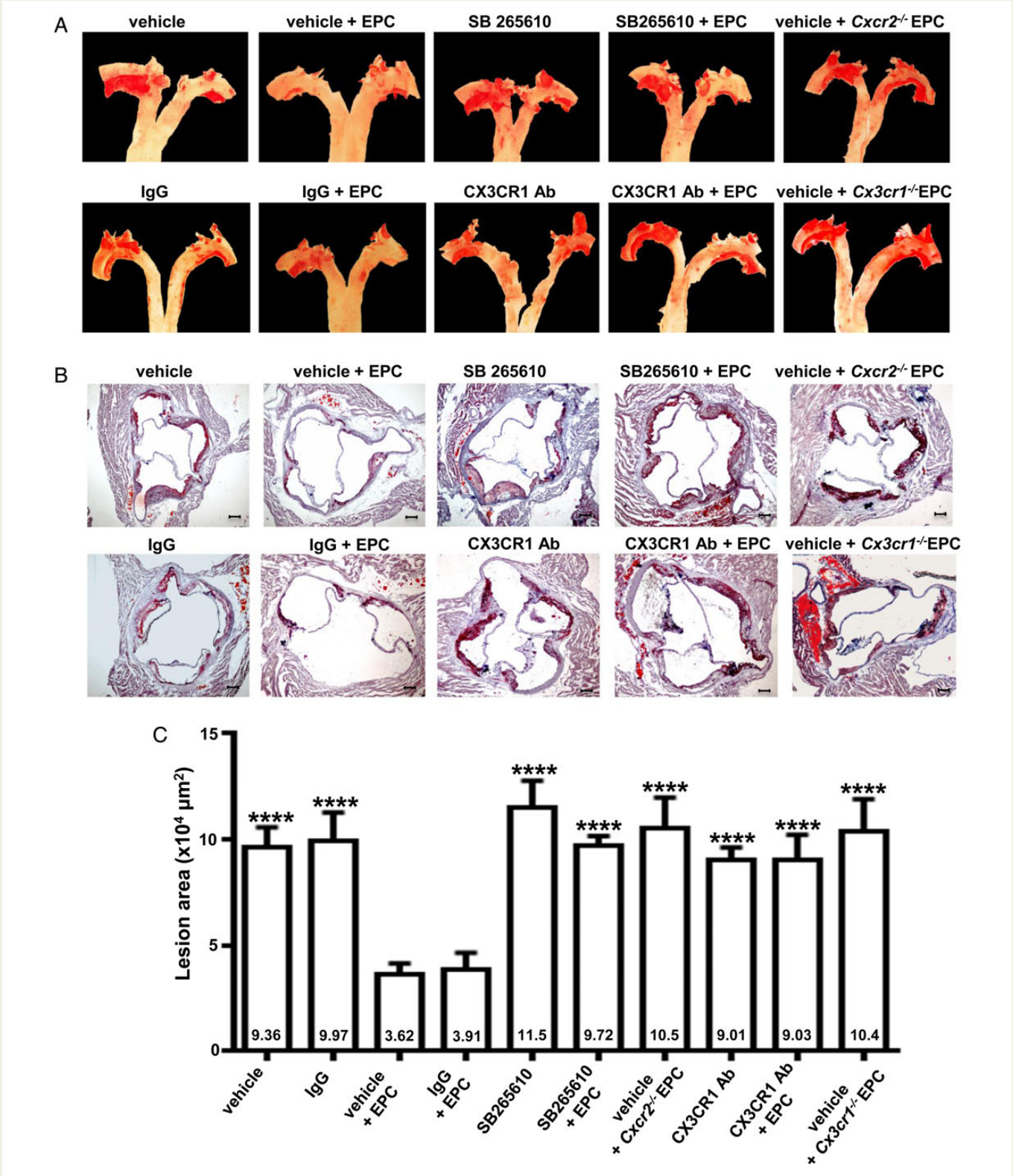

Figure 2 Inhibition or inactivation of CXCR2 or CX3CR1 impedes advanced plaque regression. Hypercholesterolaemia was reversed after 84 days of western diet as outlined in Supplementary material online, *Figures S1–S4*. Mice ($n = 7$ per group) were sacrificed 70 days after normalization of plasma lipids and atherosclerosis burden was assessed. (*A*) Representative *en face* staining of aorta. (*B*) Oil Red O staining of aortic cross-sections. In (*B*), original magnification $\times 4$, the scale bar, 100 µm. (*C*) Quantification of lesion size in the aortic root. Numerical values indicate average lesion surface in each group expressed in μm^2. ****$P < 0.0001$ vs. the corresponding value for the vehicle plus EPC or where applicable isotype control IgG plus EPC-treated group.

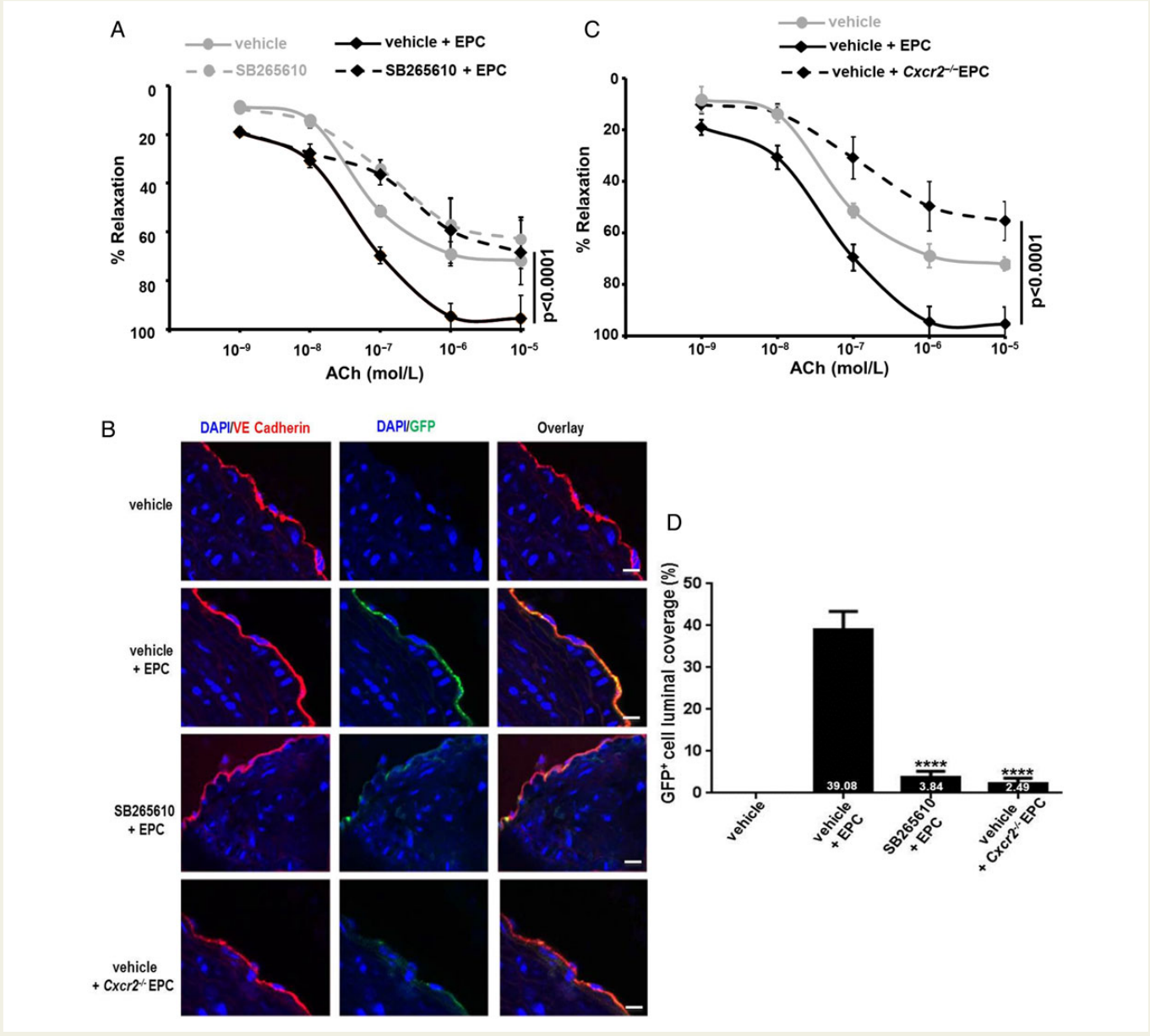

Figure 3 Inhibition or inactivation of CXCR2 reduces vascular relaxation and prevents homing of adoptively transferred bone-marrow EPCs to regressing plaques. (*A*) Acetylcholine (ACh)-induced vascular relaxation was evaluated in vehicle-, SB 265610-, vehicle plus EPC-, and SB 265610 plus EPC-treated groups. Each group consisted of seven mice. (*B*) Representative images of aortic root cross-sections from vehicle-, vehicle plus EPC-, SB 265610 plus EPC-treated mice, or vehicle plus *Cxcr2* $^{-/-}$ EPC-treated mice stained with primary anti-mouse VE Cadherin and secondary Alexa Fluor 568 Abs to identify GFP^{+}VE Cadherin^{+} endothelial cells. Slides were mounted with DAPI medium. Nuclei (blue), GFP (green), VE Cadherin (red), red + green emission overlap = yellow. Original magnification ×60, the scale bar, 25 μm. (*C*) Acetylcholine (ACh)-induced vascular relaxation was evaluated in vehicle- , vehicle plus EPC-, and vehicle plus *Cxcr2*$^{-/-}$EPC- treated groups. Each group consisted of seven mice. $P < 0.0001$ for (*A*) SB 265610 plus EPC- or (*C*) *Cxcr2*$^{-/-}$EPC-treated groups vs. the vehicle plus EPC-treated group at 10^{-5} mol/L ACh. Vehicle and vehicle + EPC data in (*A*) and (*C*) are matched repeats. (*D*) Evaluation of GFP^{+} cell luminal coverage. ****$P < 0.0001$ vs. the corresponding value for the vehicle plus EPC-treated group.

CX3CR1 in various leucocyte subsets are well-characterized; however, CX3CR1 function(s) in non-leucocyte cells including EPCs remain unclear. Thus, we inhibited or inactivated CX3CR1 to investigate how this receptor affects EPC functional responses during plaque reversal.

Rabbit polyclonal anti-mouse CX3CR1 Ab has been shown to ameliorate cardiac allograft rejection in mice[29] and crescentic glomerulonephritis in rats.[30] We therefore used this Ab to determine how pharmacological inhibition of CX3CR1 affects atherosclerosis regression (Supplementary material online, *Figure S2*). However, the Ab treatment inhibits CX3CR1 signalling in all CX3CR1^{+} cells, and so *Cx3cr1*$^{-/-}$-GFP^{+} EPCs were adoptively transferred to regressing Reversa mice to investigate how CX3CR1 deficiency affects EPC-mediated atheroregression (Supplementary material online, *Figure S4*). Mice homozygous for the *Cx3cr1*$^{-/-}$GFP^{+} mutation express GFP instead of CX3CR1 from the endogenous *Cx3cr1* promoter, mimicking the endogenous expression of CX3CR1 in monocytes, NK cells, brain microglia, and bone-marrow progenitors.[31] *Cx3cr1*$^{-/-}$-GFP^{+} mice are viable, normal in size and fertile.

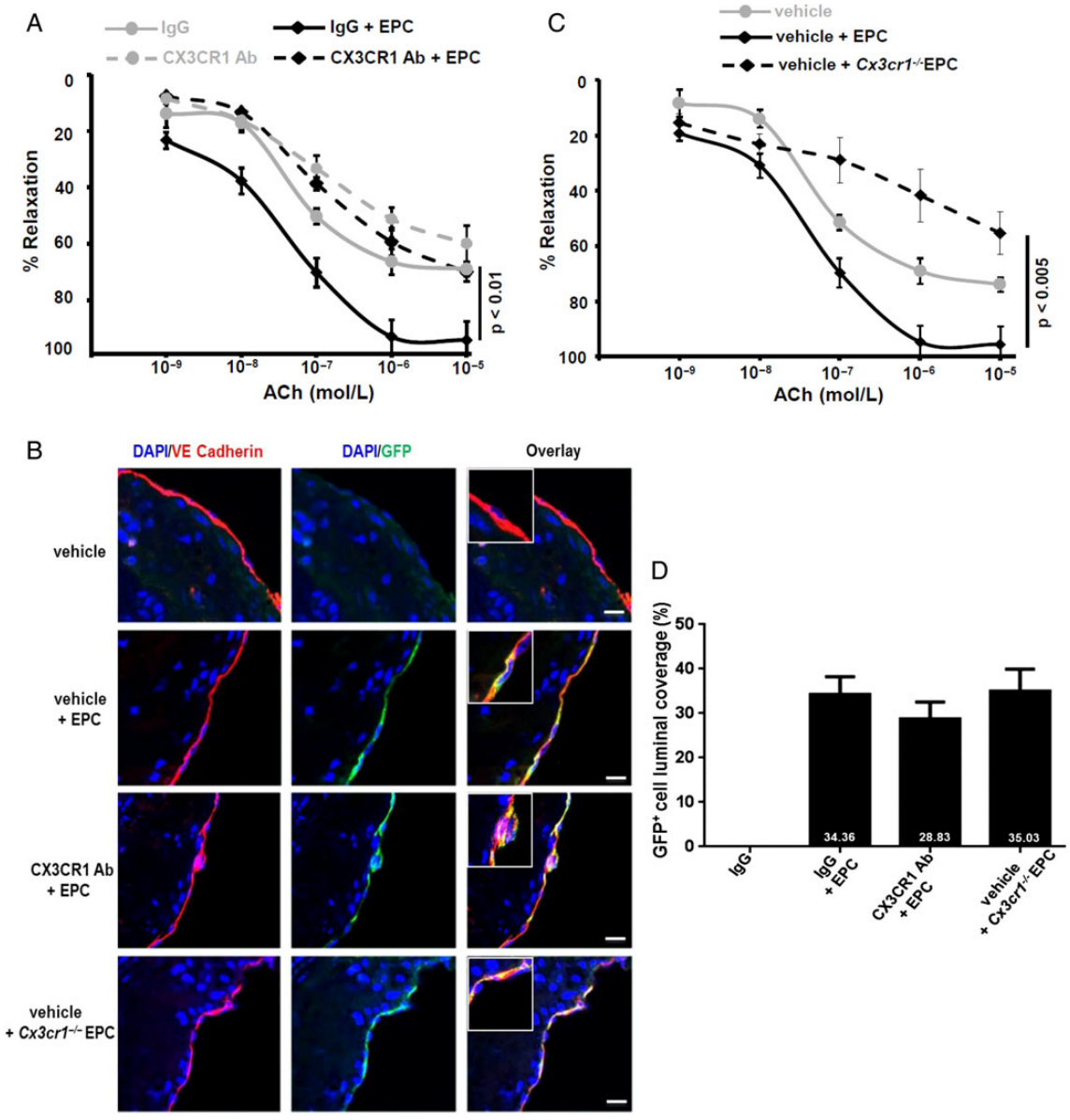

Figure 4 Inhibition or inactivation of CX3CR1 on EPCs hinders augmented plaque resolution without affecting EPC migratory capacity. (*A*) Acetylcholine (ACh)-induced vascular relaxation was evaluated in isotype control IgG-, CX3CR1 Ab-, IgG plus EPC-, and CX3CR1 Ab plus EPC-treated groups. Each group consisted of seven mice. (*B*) Representative images of aortic root cross-sections from IgG-, IgG plus EPC-, CX3CR1 Ab plus EPC-treated mice, or vehicle plus *Cx3cr1*$^{-/-}$ EPC-treated mice stained with primary anti-mouse VE Cadherin and secondary Alexa Fluor 568 Abs to identify GFP^{+}VE Cadherin^{+} endothelial cells. Slides were mounted with DAPI medium. Nuclei (blue), GFP (green), VE Cadherin (red), red + green emission overlap = yellow. Original magnification ×60, the scale bar, 25 μm. Upper left corner images, original magnification ×250. (*C*) Acetylcholine (ACh)-induced vascular relaxation was evaluated in vehicle-, vehicle plus EPC-, and vehicle plus *Cx3cr1*$^{-/-}$ EPC-treated groups. Each group consisted of seven mice. $P < 0.0001$ for (*A*) CX3CR1 Ab plus EPC- or (*C*) *Cx3cr1*$^{-/-}$ EPC-treated groups vs. the IgG plus EPC- or vehicle plus EPC-treated group at 10^{-5} mol/L ACh. Vehicle and vehicle + EPC data in *3A*, *3C*, and *4C* are matched repeats. (*D*) Evaluation of GFP^{+} cell luminal coverage.

No difference in plaque burden was observed between vehicle plus Tie2-GFP^{+}EPC- and isotype control IgG plus Tie2-GFP^{+}EPC-treated regressing animals (*Figure 2*). However, CX3CR1 Ab-treated regressing mice receiving Tie2-GFP^{+}EPCs showed significant atherosclerosis burden (*Figure 2A* and *B*) and a 2.5-fold increase in lesion size relative to the control group which received a combinational treatment with IgG plus Tie2-GFP^{+}EPCs (*Figure 2C*). This suggests that systemic inhibition of CX3CR1 prevents accelerated plaque resolution. Furthermore, CX3CR1 Ab-treated Tie2-GFP^{+}EPC-injected regressing mice showed vascular relaxation of only ~65% relative to control atheroregressing mice treated with Tie2- GFP^{+}EPCs and isotype control Ab that relaxed up to 98% when aortas were stimulated with 10^{-5} mol/L ACh (*Figure 4A*). Significantly reduced vasorelaxation was also observed in aortas from Tie2-GFP^{+}EPC-injected regressing mice treated with the CX3CR1 Ab that were stimulated with low ACh concentrations (10^{-9}–10^{-6} mol/L ACh, $P < 0.001$–$P < 0.0001$, *Figure 4A* and Supplementary material online, *Table S2*). Interestingly, GFP^{+} cell luminal surface was similar in control Tie2-GFP^{+}EPC- and Tie2-GFP^{+}EPC

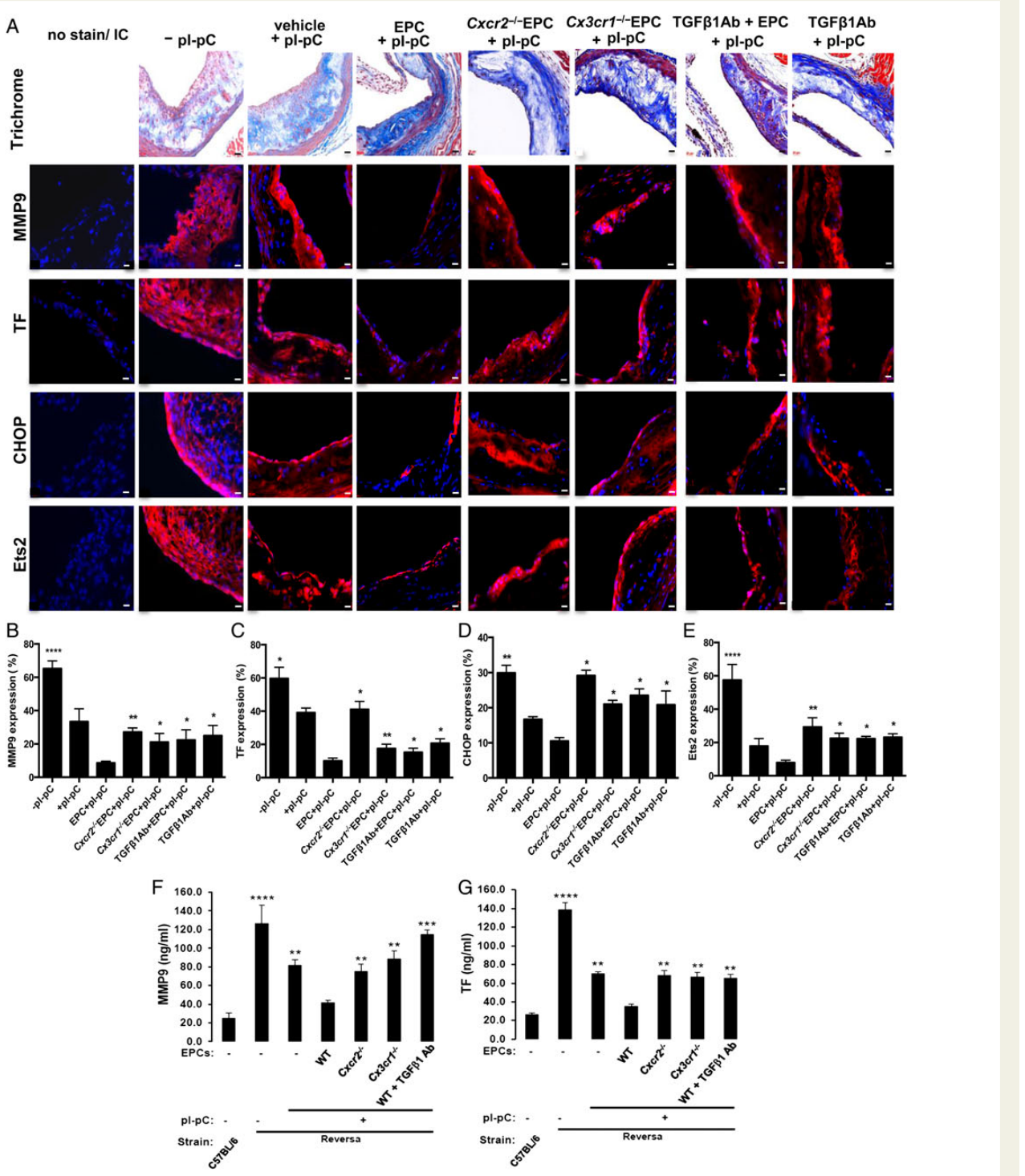

Figure 5 Deficiency in CXCR2 or CX3CR1, or neutralization of TGFβ1 inhibit EPC-mediated decreases in expression of plaque instability markers. Collagen content and expression of plaque vulnerability markers were evaluated in: (*A*) hypercholesterolaemic (−pI−pC, $n = 6$) and atheroregressing mice (+pI−pC) that did ($n = 6$) or did not receive EPCs ($n = 6$), regressing recipients of *Cxcr2*$^{-/-}$EPC ($n = 7$) or *Cx3cr1*$^{-/-}$EPCs ($n = 7$), regressing mice not treated ($n = 6$) or treated with TGFβ1 Ab ($n = 6$) or TGFβ1 Ab plus EPCs ($n = 6$). Collagen content was assessed by Trichrome stain, whereas expression of MMP9, TF, CHOP, and Ets2 were determined by immunofluorescence staining of aortic cross-sections, which were incubated with primary anti-mouse Abs followed by secondary Alexa 568-conjugated Abs (red). Slides were mounted with DAPI medium. Nuclei (blue). Original magnification ×40, the scale bar in Trichrome stain images equals 20 μm, whereas in immunofluorescence images it equals 10 μm. (*B*–*E*) Red channel fluorescence was quantified. Fluorescence was calculated by the following equation: fluorescence of the lesion area staining positively for the instability marker/florescence of the whole lesion area × 100, and is expressed as percent (%) of the total lesion area. (*F*) MMP9 and (*G*) TF plasma levels were evaluated by ELISA. For *B* through *E*, *,**,***, or ****, $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$ vs. the corresponding MMP9, TF, CHOP, or Ets2 fluorescence in Tie2-GFP^{+} EPC-treated atheroregressing Reversa mice (WT). For *F* and *G*; *,**,***, or ****, $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$ vs. the corresponding MMP9 or TF amounts in plasma of Tie2-GFP^{+} EPC-treated atheroregressing Reversa mice (WT). IC, isotype control Ab stain.

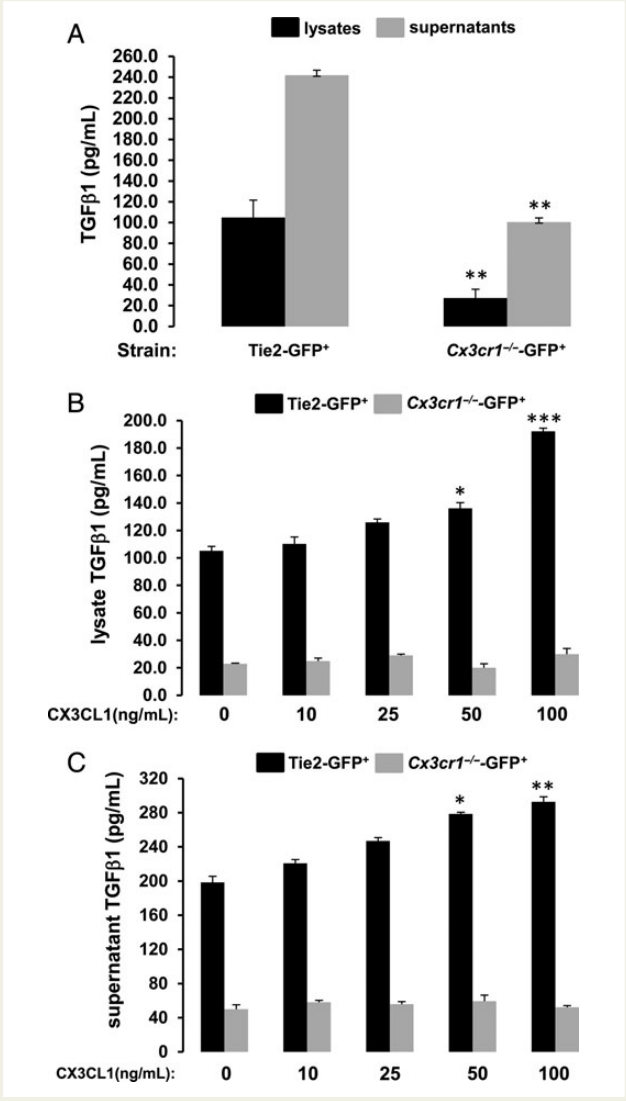

Figure 6 Ablation of CX3CR1 reduces production of TGFβ1 in EPCs. Bone-marrow Tie2-GFP$^+$ or *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs were isolated and expanded *ex vivo* as detailed in 'EPC isolation, culture and identification'. After 4 days in culture, cells were either left unstimulated (*A*) or were stimulated with 10, 25, 50, or 100 ng/mL of soluble CX3CL1 for 12 h (*B*) and (*C*), and cells and supernatants were collected. Cells were lysed and TGFβ1 amounts in cell lysates and culture supernatants were determined by ELISA. Experiment was performed twice with EPCs isolated from $n = 5$ mice per group. In (*A*) $^{**}P < 0.01$ vs. the corresponding TGFβ1 amounts in Tie2-GFP$^+$ EPCs. In (*B*) and (*C*), *,**, or ***, $P < 0.05$, $P < 0.01$, $P < 0.001$ vs. the corresponding TGFβ1 amounts detected in cell lysates or supernatants of Tie2-GFP$^+$ EPCs not stimulated with soluble CX3CL1.

plus CX3CR1 Ab-treated mice, demonstrating no decrease in incorporation of Tie2-GFP$^+$EPC into recipients' endothelium if mice were treated with CX3CR1 Ab (*Figure 4B, D* and Supplementary material online, *Figure S8*). This indicates that CX3CR1 is not supporting recruitment of endothelial progenitors to regressing plaques. Importantly, treatment with *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs also prevented augmented atherosclerosis resolution, as plaque surface in regressing mice treated with *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs was comparable to that of mice co-treated with Tie2-GFP$^+$ EPC and CX3CR1 Ab (*Figure 2C*). While *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs migrated to and incorporated into recipient endothelium just as efficiently as control Tie2-GFP$^+$EPCs (average GFP$^+$ luminal coverage of ~ 35% in both groups of mice; *Figure 4B, D* and Supplementary material online, *Figure S8*), vascular relaxation in this group was significantly lower compared with vasorelaxation detected in aortas from Tie2-GFP$^+$ EPC-treated control group stimulated with 10^{-7} or 10^{-6} mol/L ACh ($P < 0.0001$, *Figure 4C* and Supplementary material online, *Table S2*). Vascular relaxation was only ~58% when aortas were treated with 10^{-5} mol/L ACh (*Figure 4C*). Thus, pharmacological and genetic approaches suggest that while CX3CR1 does not support recruitment of EPCs to the vascular wall, it controls other responses that advance plaque resolution.

3.4 Endothelial progenitors reduce plaque vulnerability in CXCR2- and CX3CR1-dependent manner

In humans, advanced necrotic atheromata responsible for acute atherothrombotic events has low collagen content with high expression of several vulnerability markers including MMP9, which is directly involved in degradation of the plaque fibrous cap, TF, a major factor determining plaque thrombogenicity and the transcriptional factors CHOP and Ets2, indicators of plaque necrosis and vascular inflammation, respectively.[11] While mice do not provide a good model for spontaneous plaque rupture, which causes myocardial infarction and stroke in humans, they can be used to identify signals and pathways regulating expression of plaque vulnerability markers. Thus, clear understanding of how expression of instability markers is controlled in atherosclerosis-prone mouse models may provide information vital for the development of new interventions preventing atherothrombosis and plaque disruption in humans.

Reversa mice were fed the western diet to promote the formation of advanced lesions containing necrotic cores (Supplementary material online, *Figure S9*). Since these advanced plaques associate with specific plaque vulnerability markers, we evaluated plaques for expression of MMP9, TF, CHOP, and Ets2. We also examined whether reversal of hypercholesterolaemia alone or in combination with EPC treatment affects expression of these markers. Lastly, we determined how deficiency in CXCR2 or CX3CR1 on EPCs affects expression of plaque instability markers.

We found that the vascular wall of hypercholesterolaemic mice had low collagen content with apparent acellular areas and high expression of MMP9, TF, CHOP, and Ets2. Reversal of hypercholesterolaemia alone moderately increased collagen deposition with a limited decrease in MMP9, TF, CHOP, and Ets2 expression, suggesting that normalization of plasma lipids alone is insufficient to eliminate plaque vulnerability. Treatment with Tie2-GFP$^+$ EPCs greatly increased collagen content in the vascular wall and profoundly reduced expression of plaque vulnerability markers (*Figure 5A–E*). Regressing mice receiving *Cxcr2*$^{-/-}$ Tie2-GFP$^+$ or *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs showed no change in collagen deposition relative to untreated mice. Furthermore, plaque instability marker expression remained high in regressing recipients treated with *Cxcr2*$^{-/-}$Tie2-GFP$^+$ or *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs (*Figure 5A–E*). This finding was further supported by detection of high plasma levels of MMP9 and TF in atheroregressing mice treated with *Cxcr2*$^{-/-}$ Tie2-GFP$^+$ or *Cx3cr1*$^{-/-}$-GFP$^+$ EPCs. The highest plasma MMP9 and TF levels were detected in atherosclerotic mice, whereas the lowest MMP9 and TF amounts were recorded in blood of atheroregressing EPC-treated mice (*Figure 5F* and *G*).

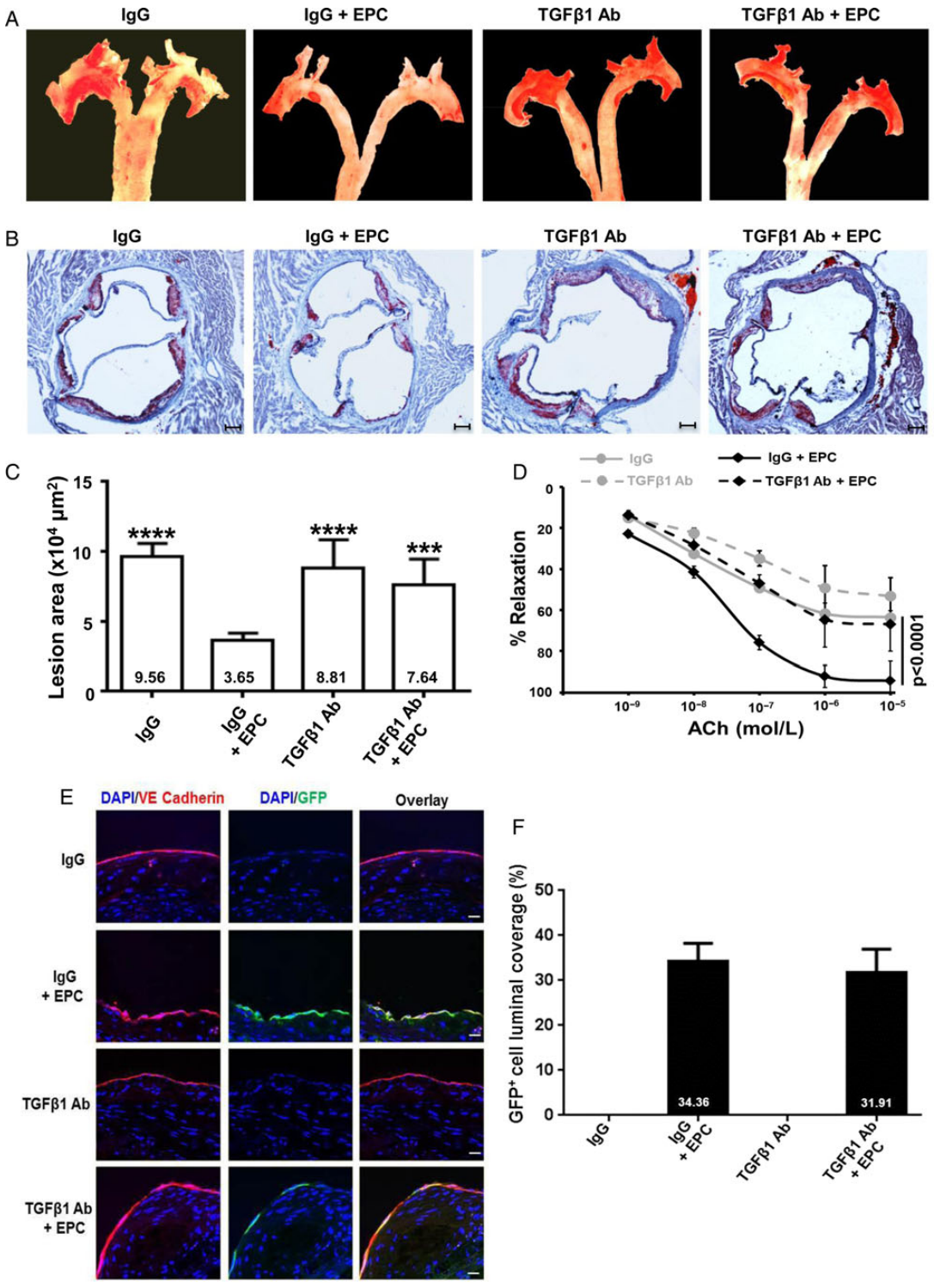

Figure 7 TGFβ 1 neutralization inhibits advanced atheroregression without affecting EPC recruitment to regressing plaques. Mice ($n = 6$ per group) were treated as in Supplementary material online, *Figure S5* and atherosclerosis burden, vascular relaxation, and incorporation of donor cells into recipients' endothelium were evaluated. (*A*) Representative *en face* staining of aorta is shown. (*B*) Oil Red O staining of aortic cross-sections. In (*B*), original magnification ×4, the scale bar, 100 μm. (*C*) Quantification of lesion size in the aortic root. Numerical values represent average lesion surface in each group expressed in μm^2. *** or ****, $P < 0.001$ or $P < 0.0001$ vs. the corresponding value for IgG plus EPC-treated group. (*D*) Acetylcholine (ACh)-induced vascular relaxation was evaluated in IgG-, TGFβ1 Ab-, IgG plus EPC-, and TGFβ1 Ab plus EPC-treated groups. $P < 0.0001$ for TGFβ1 Ab plus EPC-treated vs. IgG plus EPC-treated groups at 10^{-5} mol/L ACh. (*E*) Aortic root cross-sections from IgG-, TGFβ1 Ab-, IgG plus EPC-, and TGFβ 1 Ab plus EPC-treated mice stained with primary anti-mouse VE Cadherin and secondary Alexa Fluor 568 Abs to identify differentiated $GFP^+VE\ Cadherin^+$ endothelial cells mounted in DAPI medium. Nuclei (blue), GFP (green), VE Cadherin (red), red + green emission overlap = yellow. Original magnification ×60, the scale bar, 25 μm. (*F*) Evaluation of GFP^+ cell luminal coverage.

3.5 Ablation of CX3CR1 reduces EPC potential to produce TGFβ1

CX3CR1 was recently shown to support insulin secretion from pancreatic islet β-cells.[32] Thus, CX3CR1 could regulate regenerative potential of EPCs by controlling their paracrine function. We evaluated how deficiency in *Cx3cr1* affects paracrine function of bone marrow EPCs.

Cx3cr1$^{-/-}$-GFP^{+} and control Tie2-GFP^{+} EPCs were cultured as detailed in *Methods* and expression of pro-angiogenic cytokines including vascular endothelial growth factor-A, fibroblast growth factor, insulin growth factor, tumour necrosis factor α, angiopoietins, and TGFβ1 were measured in *Cx3cr1*$^{-/-}$-GFP^{+} EPCs and control Tie2-GFP^{+} cells.

We found that while expression of other angiogenic cytokines remained unaltered, *Cx3cr1*$^{-/-}$- GFP^{+} EPCs produced and secreted significantly less pleiotropic immunomodulatory cytokine TGFβ 1 relative to control Tie2-GFP^{+} EPCs (*Figure 6A*). Interestingly, stimulation of Tie2-GFP^{+} EPCs with increasing concentration of soluble CX3CL1 resulted in a dose-dependent increase in TGFβ1 in cell lysates and culture supernatants, whereas *Cx3cr1*$^{-/-}$-GFP^{+} EPCs produced and secreted low amounts of TGFβ1 regardless of CX3CL1 dose used to activate CX3CR1 signalling (*Figure 6B* and *C*). These data indicate that the CX3CL1–CX3CR1 axis controls TGFβ1 production in EPCs.

3.6 Neutralization of TGFβ1 hinders advanced plaque resolution without affecting recruitment of EPCs to regressing plaques

TGFβ1 is synthesized in a latent form which is converted to active form following cell activation. Low concentrations of active TGFβ are found in subjects with advanced atherosclerosis.[33] Heterozygous *Tgfβ1*$^{+/-}$ mice develop vascular lesions when fed a lipid-enriched diet.[34] Inhibition of TGFβ1 signalling by neutralizing Ab increased vascular inflammation in atherosclerosis-prone apolipoprotein E-deficient mice.[13] Since TGFβ1 prevents plaque development, it may function in a similar fashion during atherosclerosis regression. We therefore tested whether this cytokine supports plaque resolution.

TGFβ1 activity was neutralized *in vivo* using the 1D11 Ab (Supplementary material online, *Figure S5*) and parameters including atherosclerosis burden, vascular relaxation, incorporation of Tie2-GFP^{+} cells into endothelium, collagen deposition, and expression of plaque instability markers MMP9, TF, CHOP, and Ets2 were compared between Tie2-GFP^{+}EPC-treated group and regressing mice concurrently treated with Tie2-GFP^{+}EPCs and TGFβ1 Ab.

Mice treated with EPCs that received the 1D11 Ab had bigger plaques (*Figure 7A* and *B*), showing a 2.2-fold increase in lesional surface relative to controls treated with IgG plus EPCs (*Figure 7C*). Vascular relaxation recorded in the 1D11 Ab-treated regressing group was significantly lower compared with the control group starting at 10^{-7} mol/L ACh ($P < 0.0001$, *Figure 7D* and Supplementary material online, *Table S3*), and was only 63% at 10^{-5} mol/L ACh ($P < 0.0001$, *Figure 7D*). Interestingly, regressing recipients receiving a combination of Tie2-GFP^{+} EPC plus CX3CR1 Ab, *Cx3cr1*$^{-/-}$-GFP^{+} EPCs or mice treated either with TGFβ1 Ab alone or with a combination of TGFβ1 Ab and Tie2-GFP^{+} EPCs had similar average GFP^{+} cell luminal coverage of 29, 35, 34, and 32% respectively (*Figures 4D*, *7E* and *F*), indicating that neutralization of this cytokine has no effect on homing of EPCs to regressing plaques. Furthermore, expression of plaque instability markers MMP9, TF, CHOP, and Ets2 (*Figure 5A–E*) and high levels of plasma MMP9 and TF were detected in 1D11 Ab-treated mice (*Figure 5F* and *G*). These data collectively show that while TGFβ1 is not recruiting EPCs to regressing plaques, it facilitates advanced plaque resolution.

4. Discussion

Complete regression of plaques to alleviate and prevent complications of atherosclerosis is a desired clinical goal; however, pathways supporting this process remain obscure. This is in part due to the limited number of preclinical models employed to investigate mechanisms of plaque resolution. We utilized the Reversa mouse model, which mimics statin-induced lipid lowering in cardiovascular patients, to identify cells[9] and molecules that support plaque resolution. We show that chemokine receptors CXCR2 and CX3CR1 are critical molecular determinants in endothelial progenitor-mediated plaque resolution. Interestingly, their roles in this process differ since we demonstrate that CXCR2 mediates recruitment of EPCs to regressing plaques while CX3CR1 controls the regenerative capacity of these cells, determined by production and secretion of paracrine factors including TGFβ1, which facilitates repair of vascular damage caused by plaque development in this model. We also show that CXCR2 and CX3CR1 support EPC-mediated plaque stabilization, indicating that the Reversa model can be used to investigate signals and pathways controlling plaque vulnerability and/or stabilization. Thus, studying atherosclerosis regression in Reversa mice may define new targets that could be explored to develop novel approaches to treat atherosclerosis.

Leucocyte CXCR2 and CX3CR1 were both shown to promote plaque formation. CXCR2 is central to macrophage accumulation in established fatty streak lesions. The repopulation of *Ldlr*$^{-/-}$ mice with bone marrow deficient in CXCR2 interfered with progression of atherosclerosis and substantially reduced advanced atherosclerosis.[19] Antagonism of, or deficiency in, CX3CL1 or CX3CR1 reduces atherosclerosis in mice. Analysis of the Framingham Heart Study Offspring Cohort showed that the two single loss-of-function nucleotide polymorphisms of CX3CR1, V249I, and T280M are associated with markedly reduced risk of coronary artery disease.[27,28] Accumulated evidence indicates that the main mechanism supporting pro-atherogenic effects of the CX3CL1–CX3CR1 axis is entrapment of monocytes in plaques.[35]

The CXCL1–CXCR2 axis has been demonstrated to promote endothelial regeneration in a model of arterial injury.[36] CXCR2 is expressed on circulating EPCs and directs these cells to injured vessels, enhancing endothelial recovery and reducing neointimal hyperplasia.[20] Our study, although conducted in atheroregressing conditions, which are the subject of intense investigation, strengthens the argument that CXCR2 is critical for homing of EPCs to damaged vascular wall and for endothelial regeneration, which are necessary for efficient plaque resolution. However, our findings raise an important question of why EPCs home to regressing plaques in a CXCR2-dependent manner, while other leucocytes including monocytes and lymphocytes that express this chemokine receptor in normal and atherogenic environments fail to do so. To this end, we show that atheroregressing conditions support very low CXCR2 expression on monocytes and lymphocytes. This suggests that CXCR2 is unlikely to mediate large-scale recruitment of these two leucocyte subsets to regressing plaques. Thus, contrary to leucocyte CXCR2, high expression of this chemokine receptor on EPCs supports their homing to residual plaques.

CX3CR1 has been shown to provide paracrine support during initiation, expansion, and maturation of microvascular networks in a model of monocyte/macrophage-mediated neovascularization.[37] The role of CX3CR1 in controlling paracrine functions of monocytes in

this model is consistent with our observation demonstrating that CX3CR1 regulates TGFβ1 production in EPCs, thereby controlling paracrine function and regenerative capacity of these cells. This is not the sole instance where CX3CR1 regulates release of paracrine factors. As reported by Lee *et al.*,[32] CX3CR1 also regulates secretion of insulin from pancreatic β-cells.

TGFβ1 is atheroprotective, especially in early stages of the disease. Evidence shows that this cytokine limits excessive smooth muscle cell proliferation and accumulation in the vascular wall[38] and inhibits plaque rupture by stimulating extracellular matrix synthesis and tissue repair.[39] In addition, TGFβ1 controls vascular inflammation by stimulating activity of Th3 and regulatory $CD3^{+}CD25^{+}$T cells.[40] Our results extend our understanding of TGFβ1 roles in vascular inflammation, as they suggest that this cytokine supports accelerated plaque regression.

In addition to the chemokine system, EPCs also use selectins and integrins to recruit to sites of vascular injury.[41] Whether and how selectins and integrins mediate rolling and adhesion of bone marrow EPCs to vascular endothelium in regressing conditions is under investigation.

Studies have focused on the therapeutic applications of endothelial progenitors such as using EPCs in autologous transplantation to improve neovascularization and tissue repair. An important aspect of EPC-based therapeutic approaches is incorporation of transplanted or mobilized EPCs into the affected/damaged tissues and/or vasculature. It has been validated that treatment with autologous immunologically neutral EPCs obtained from bone marrow is associated with improved therapeutic outcomes.[42] Our study may extend the use of EPCs for therapeutic purposes since we show that advanced regression of atherosclerosis associated with reduced plaque burden, improved vascular reactivity and decreased plaque instability could be achieved by a combinational therapy that would include, in addition to lipid-lowering, an EPC-based approach. Most importantly, our investigation clearly shows an important role of the chemokine system in atheroregression. Thus, maintaining and/or stimulating CXCR2 and CX3CR1 expression on EPCs may be necessary for these cells to home, incorporate into the vascular wall, and stimulate endothelial regeneration that accelerates atherosclerosis resolution.

Conflict of interest: none declared.

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Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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