Role of CXCL12 and endothelial HIF-1α in atherosclerotic lesion stabilization

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To my parents
The result of this work was in part published in:

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<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Apoe</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>AT-2</td>
<td>Angiotensin-2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>β-gal</td>
</tr>
<tr>
<td>bHLP-PAS</td>
<td>basic helix-loop-helix-Per/Arnt/Sim</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-Diamidino-2-phenylindol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>for example (from Latin: exempli gratia)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EvG</td>
<td>Elastica van Gieson</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FC</td>
<td>Fibrous cap</td>
</tr>
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<td>FCS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF-1α</td>
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<td>FITC</td>
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<td>Forward scatter</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HFD</td>
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</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>HIF response elements</td>
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<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<td>Definition</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
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<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte-derived chemokine</td>
</tr>
<tr>
<td>LCA</td>
<td>Left carotid artery</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density-lipoprotein</td>
</tr>
<tr>
<td>lin</td>
<td>Lineage</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPA$_1$</td>
<td>Lysophosphatidic acid receptor 1</td>
</tr>
<tr>
<td>LPA$_2$</td>
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</tr>
<tr>
<td>LPA$_3$</td>
<td>Lysophosphatidic acid receptor 3</td>
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<tr>
<td>LPA20:4</td>
<td>1-arachidonoyl-2-lyso-sn-glycero-3-phosphate</td>
</tr>
<tr>
<td>LPA18:0</td>
<td>1-stearoyl-2-lyso-sn-glycero-3-phosphate</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAECs</td>
<td>Mouse aortic endothelial cells</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MM6</td>
<td>Mono mac6</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>moxLDL</td>
<td>Mildly oxidised LDL</td>
</tr>
<tr>
<td>MPC</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>nLDL</td>
<td>Native LDL</td>
</tr>
<tr>
<td>ND</td>
<td>Normal diet</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>ODDDD</td>
<td>Oxygen dependent degradation domain</td>
</tr>
<tr>
<td>ON</td>
<td>Overnight</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor receptor-β</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<td>Paraformaldehyde</td>
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<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<tr>
<td>RBC</td>
<td>Red blood cells</td>
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<tr>
<td>RCA</td>
<td>Right carotid artery</td>
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<tr>
<td>RNA</td>
<td>Ribonucleotide acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen-1</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium-dodecylsulphate</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SPC</td>
<td>Smooth muscle progenitor cell</td>
</tr>
<tr>
<td>SR-A</td>
<td>Scavenger receptor A</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>vHL</td>
<td>von Hippel-Lindau tumour suppressor protein</td>
</tr>
<tr>
<td>VLA4</td>
<td>Very late antigen 4</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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1 Introduction

1.1 Atherosclerosis

Atherosclerosis, a chronic inflammatory disease, is the main pathologic process responsible for several cardiovascular diseases (CVD), such as myocardial infarction and stroke.\textsuperscript{1, 2} CVD continues to be the principle cause of death in the developed countries but its incidence is increasing alarmingly in under-developed and developing countries due to decreasing mortality from infectious disease and to the increasing adoption of western lifestyles.\textsuperscript{3, 4} Over 80% of CVD cases take place in low- and medium-income countries.\textsuperscript{4} According to the World Health Organization (WHO) approximately 17.3 million people died from CVDs in 2008 globally, while in 2030 the incidence of CVD is predicted to increase to approximately 23.3 million people, thus projecting it to be the leading cause of death world-wide (http://www.who.int/mediacentre/factsheets/fs317/en/).

The term atherosclerosis originates from the Greek words “athero” (gruel or paste) and “sclerosis” (hardness), which reflects the typical macroscopic lesion morphology in the vessel wall characterized by intimal lipid deposition and fibrosis. The lesion formation is triggered by the accumulation of lipids and inflammatory cells in medium- and large-sized arteries.\textsuperscript{5} Atherosclerosis can begin early in life and progresses with age.\textsuperscript{6} The lesion progression leads to thickening of the arterial wall, which can impair blood flow and oxygen supply due to luminal narrowing or thrombotic occlusion of the lumen following lesion rupture. The impaired oxygen supply results in tissue ischemia, which is the cause for many clinical manifestations of atherosclerosis, such as myocardial infarction, stroke or peripheral arterial occlusion.\textsuperscript{7} Arterial branch points are particularly prone to atherosclerotic lesion formation due to the presence of sustained low shear stress.\textsuperscript{8-10} Moreover, various modifiable (\textit{i.e.}, smoking, stress, physical inactivity, and obesity) and non-modifiable (\textit{i.e.}, hypercholesterolemia, hypertension, diabetes, age, and gender) risk factors can also influence the progression of atherosclerosis.\textsuperscript{11-17}

The structure of a normal artery consists of three layers: the tunica intima (inner layer), the tunica media (middle layer), and the tunica externa (outer layer) (Figure 1). The tunica intima is lined by endothelial cells (ECs), which rest upon a basement membrane containing non-fibrillar collagen types, and is separated from the tunica media by the internal elastic membrane. The tunica media is the thickest layer consisting of mainly elastic fibers and concentric layers of smooth muscle cells (SMCs). The tunica externa (also known as
adventitia) consists of mainly collagen fibrils. Vasa vasorum and nerve endings are localized in this outermost layer.

Figure 1: Structure of a normal artery.
A large artery consists of 3 morphologically distinct layers. The innermost layer, the intima, is bordered by a monolayer of ECs at the luminal side and a sheet of elastic fibers, the internal elastic lamina, on the peripheral side. The normal intima is a very thin region and consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The middle layer, the media, consists of SMCs. The outer layer, the adventitia, consists of connective tissue with interspersed fibroblasts and SMCs (Taken from Lusis AJ. Atherosclerosis. Nature. 2000;407:233-241).

The initiation of atherosclerosis is marked by the onset of endothelial dysfunction triggered by atherogenic factors, such as high levels of low density lipoprotein (LDL) or Angiotensin-2 (AT-2). Lipoprotein particles bind to proteoglycans in the subendothelial space, aggregate, and thus become more susceptible to oxidation or chemical modification. Moreover, the endothelial permeability of LDL is increased at predilection sites of atherosclerosis, which results in the accumulation of LDL in the sub-endothelial space where it is oxidized by enzymes, such as myeloperoxidase and lipoxygenase, or by reactive oxygen species (ROS). This modified LDL activates ECs to increase the expression of adhesion molecules and chemokines. These activated ECs induce the atherogenic recruitment of monocytes by the initial E- and P-selectin-mediated rolling of monocytes, which subsequently adhere firmly to the endothelium via vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1. The firm adhesion of monocytes to the endothelium requires activation of integrins on the leukocyte surface by chemokines, which
can be derived from the ECs (e.g., CXCL1). Adherent monocytes migrate through the endothelial layer into the intima via diapedesis in response to chemokines secreted by the activated endothelium, such as CCL2. In the intimal space, monocytes differentiate into macrophages in response to M-CSF. Macrophages take up modified lipids that have accumulated within the intima via scavenger receptors CD36 and SR-A, and transform into foam cells. Foam cell formation involves the unregulated internalization of lipoprotein particles to generate a lipid-engorged cell, a process, which is primarily due to an imbalance between lipoprotein uptake and cholesterol efflux. In addition to monocytes, T cells also adhere and migrate into the sub-intimal space. These initial lesions are characterized primarily by macrophage-foam cells and are classified as fatty streaks.

**Figure 2: Initiation of atherosclerosis.**

LDL is oxidatively modified in the sub-endothelial space and progresses from minimally modified LDL (mmLDL) to extensively oxidized LDL (oxLDL). Monocytes attach via adhesion molecules to the endothelium activated by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the sub-endothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation. OxLDL cholesterol taken up by scavenger receptors is esterified and stored in lipid droplets (Taken from Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell. 2001; 104(4):503-16).

During lesion progression, SMCs migrates from the media into the intima towards the endothelium in response to growth factors, such as fibroblast-derived growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β secreted by
macrophages and T-cells. The migrating SMCs proliferate and secrete extracellular matrix thus forming a fibrous cap (FC). The more advanced lesion or fibroatheroma harbors a necrotic/lipid core that contains cholesterol esters, free cholesterol, phospholipids, and triglycerides and develops from apoptotic foam cells or from the retention of lipoproteins. The presence of a lipid core results in thickening of the arterial wall, but usually does not compromise the vascular lumen diameter due to the capacity of the vessel for outward remodeling. At this stage, the FC consists of SMCs in a proteoglycan-collagen matrix and lesions contain small capillaries that most likely originate from the vasa vasorum in the adventitia. These “unstable” or “vulnerable” lesions are susceptible to physical disruption.

Figure 3: Formation of advanced lesions.
Interactions between macrophage foam cells, Th1 and Th2 cells establish a chronic inflammatory process. Cytokines secreted by Th1 lymphocytes and macrophages exert pro-atherogenic effects on each of the cellular elements of the vessel wall. SMCs migrate from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous lesion (Taken from Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell. 2001; 104(4):503-16).

Atherothrombosis is primarily due to rupture and/or erosion of the atherosclerotic lesions. Lesion rupture accounts for 75% of acute myocardial infarctions and is facilitated by an impaired collagenous matrix production by SMCs in the FC, which reduces the biomechanical stability of the lesions. Growth factors, such as TGF-β, PDGF-β, IGF-I and FGF increases the collagen type I synthesis in SMCs, whereas interferon (IFN)-γ secreted by activated T-cells in the lesions inhibits the basal and the TGF-β- or PDGF-β-stimulated
collagen type I synthesis in SMCs. In addition to decreased collagen synthesis by SMCs in the FC, matrix metalloproteinases (MMPs) and cathepsins secreted by activated macrophages and non-macrophage cells degrade collagen type I and extracellular matrix. Moreover, the expression of tissue inhibitors of metalloproteinase (TIMP), which limits collagen degradation by MMPs, is reduced in rupture-prone lesions. The disequilibrium between matrix synthesizing and matrix degrading factors determines the tensile strength of the FC and thus the vulnerability of the lesion. Loss of the endothelial coverage of the lesions (known as erosion) caused by EC apoptosis or shedding of ECs exposes sub-endothelial collagen and von Willebrand factor (vWF), which trigger thrombus formation. Although lesion erosion is most often asymptomatic, it causes approximately 25% of all fatal coronary thromboses.

Thrombi formed as a result of lesion erosion or rupture can be either occlusive or non-occlusive. Occlusive thrombi severely reduce the blood flow and can lead to unstable angina or myocardial infarction. Non-occlusive thrombi can be dislodged from the rupture site and embolize causing stroke or acute limb ischemia. Non-occlusive thrombi can also increase lesion growth by triggering a repair process of the arterial wall characterized by infiltration of the thrombi by SMCs, which synthesize extracellular matrix. The repeated formation of non-occlusive thrombi and their organization can rapidly lead to severe luminal narrowing. Hence, preventing the incidence of lesion rupture or erosion would not only reduce the occurrence of clinically important events, such as myocardial infarction, unstable angina, stroke, but would also reduce the development of highly stenotic lesions. Therefore, stabilization of vulnerable lesions is a crucial therapeutic goal in the treatment of atherosclerosis.
Figure 4: Lesion rupture.
Necrosis of macrophage- and SMC–derived foam cells leads to the formation of a necrotic core and accumulation of extracellular cholesterol. Secretion of MMPs by macrophages and neovascularization contributes to the weakening of the fibrous lesion. Lesion rupture exposes blood components to tissue factor, initiating coagulation, recruitment of platelets, and formation of a thrombus (Taken from Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell. 2001; 104(4):503-16).

1.2 Chemokines

Chemokines (chemotactic cytokines) are a group of small (8–14 kDa) structurally related heparin-binding proteins that are important for the organization of tissues during development and direct the movement of circulating leukocytes to the sites of inflammation or injury. In humans, approximately 50 chemokines have been identified and are classified into 4 families (CC, CXC, CX3C and C chemokines) based on the arrangement of the first 2 cysteine residues within their amino-terminal polypeptide sequence. CC chemokines lack an amino acid between the 2 amino-terminal cysteine residues and constitute the largest family of chemokines known to attract mononuclear cells in response to inflammation. The CXC chemokines contain a single amino acid between the two amino-terminal cysteine residues. CXCL8 is a typical CXC chemokine known to attract polymorphonuclear leukocytes to the sites of acute inflammation. CX3CL1 (fractalkine) is the only member of the CX3C chemokine family in which 3 amino acids separate the 2 amino-terminal cysteine residues. CX3CL1 triggers the firm arrest of leukocytes under physiological flow conditions. The C chemokines lack one of the amino-terminal conserved cysteine residues.
Two subfamilies of the CXC chemokine family can be discerned based on the presence of a glutamate-leucine-arginine (ELR) motif in the N-terminal end.\textsuperscript{48-51} The ELR\textsuperscript{+} CXC chemokines harbor the ELR motif in the N-terminal end and are chemoattractant for neutrophils. They induce secretion of neutrophil granules and trigger respiratory burst, thus contributing to the early phase of wound healing. Moreover, ELR\textsuperscript{+} chemokines are also known to play a role in angiogenesis. In contrast, the ELR\textsuperscript{−} chemokines are chemoattractant for lymphocytes and play a role in angiostasis.

Chemokines can also be classified according to their primary function into inflammatory chemokines, homeostatic chemokines and dual function chemokines.\textsuperscript{51} Inflammatory chemokines control the recruitment of effector leukocytes during infection, inflammation and tissue injury. In contrast, homoeostatic chemokines direct leukocytes during hematopoiesis in bone marrow (BM) and thymus, during the initiation of adaptive immune responses in the spleen and lymph nodes, and in the immune surveillance of healthy tissues. The dual function chemokines can participate in both inflammatory as well as homeostatic function.

Chemokines mediate their actions by binding to a subset of seven-transmembrane, G protein-coupled receptors (GPCRs).\textsuperscript{52} The receptors are classified as CXCRs (bind to CXC chemokines), CCRs (bind to CC chemokines), XCRs (bind to C chemokines), and CX3CRs (bind to CX3C chemokines) based on the chemokine class they bind (Table 1).\textsuperscript{50, 51}
Various chemokines, such as CXC chemokines, play a significant role in different steps of atherogenesis, like the recruitment of inflammatory cells to the artery wall and the migration and proliferation of SMCs in atherosclerotic lesions.\textsuperscript{53-55}

### 1.2.1 CXCL12 and atherosclerosis

CXCL12 [also known as stromal cell-derived factor (SDF)-1\(\alpha\)] is a ubiquitously expressed ELR\textsuperscript{-}CXC chemokine composed of 68-amino acids (8 kDa) with homeostatic functions.\textsuperscript{56} In humans, the CXCL12 gene is located on chromosome 10, whereas the genes of other CXC chemokines are clustered on chromosome 4.\textsuperscript{56} The circulating levels of CXCL12 in the blood range between 1 and 4 ng/ml in humans.\textsuperscript{57} CXCL12 acts as a growth factor for B cells and progenitor cells, and as a chemotactic factor for T lymphocytes, monocytes, CD34\textsuperscript{+} cells, and
hematopoietic progenitor cells.\textsuperscript{58-60} Although CXCL12 does not contain an ELR motif, it can promote angiogenesis.\textsuperscript{50} Six isoforms of CXCL12 derived from alternative splicing exist in humans.\textsuperscript{61} The 2 major splice forms are CXCL12-\(\alpha\) and CXCL12-\(\beta\), which have identical amino acid sequences except for the presence of 4 additional amino acids at the carboxy-terminus of CXCL12-\(\beta\).\textsuperscript{56} CXCL12-\(\alpha\) is the prevalent isoform found in all organs; however, it undergoes rapid proteolysis in the blood. CXCL12-\(\beta\) is expressed in highly vascularized organs, such as liver and spleen, and is more resistant to degradation.\textsuperscript{62} The other isoforms include CXCL12-\(\gamma\), CXCL12-\(\delta\), CXCL12-\(\epsilon\), and CXCL12-\(\Phi\).\textsuperscript{63} CXCL12-\(\gamma\) is located in metabolically active organs susceptible to infarction, such as the heart and brain, while CXCL12-\(\delta\), CXCL12-\(\epsilon\), and CXCL12-\(\Phi\) are abundantly expressed in the pancreas. Although the homology of chemokines in humans and mice is approximately at 69\%, CXCL12 is identical between these species except for a single amino acid change from valine to isoleucine at position 18.\textsuperscript{56} In mice, only 2 isoforms of CXCL12 exist, CXCL12-\(\alpha\) and CXCL12-\(\beta\).\textsuperscript{62, 63} The function of CXCL12 is mediated by binding to two different CXC receptors, CXCR4 and CXCR7.\textsuperscript{64, 65} In ECs, the expression of CXCL12 can be induced by the transcription factor hypoxia-inducible factor-1 \(\alpha\) (HIF-1\(\alpha\)) via hypoxia response elements (HRE) in the CXCL12 promoter.\textsuperscript{66}

CXCL12 is highly expressed in human atherosclerotic lesions, especially in SMCs, ECs and macrophages, but not in normal vessels.\textsuperscript{67} Patients with acute coronary syndromes have lower plasma CXCL12 levels as compared to healthy controls. Interestingly, plasma CXCL12 levels are lower in patients with unstable angina than in patients with stable angina.\textsuperscript{68} Treatment of peripheral blood mononuclear cells (PBMCs) isolated from patients with unstable angina with CXCL12 reduces the expression of inflammatory mediators, such as CCL2, CXCL8 and MMPs, suggesting a protective role of CXCL12 in coronary disease.\textsuperscript{68} In contrast, platelet-bound CXCL12 is increased in patients with unstable compared to stable angina and correlates with platelet P-selectin expression and systemic platelet activation implicating a pro-atherogenic and pro-thrombotic role of CXCL12.\textsuperscript{69} It remains unclear whether plasma CXCL12 and platelet-bound CXCL12 have differential roles in atherosclerosis. Moreover, the SNPs rs1746048 and rs501120 located on chromosome 10q11 downstream of the CXCL12 gene are associated with higher plasma levels of CXCL12 and increased CVD severity.\textsuperscript{70} In contrast to the results of Mehta et al., SNP rs501120 has been reported to result in reduced plasma CXCL12 level and increased carotid atherosclerosis.\textsuperscript{71} In a mouse model of atherosclerosis, long-term disruption of the CXC12-CXCR4 axis by treatment with the CXCR4 antagonist AMD3465 aggravates atherosclerosis by enhanced
neutrophil release from the BM. Moreover, microvesicle-mediated transfer of miR-126 reduces atherosclerosis by up-regulating CXCL12 expression in ECs. Thus, these studies suggest an anti-atherogenic role of CXCL12 in mice. However, the role of CXCL12 in human atherosclerosis is still unclear.

During vascular repair following mechanical injury, plasma CXCL12 plays an important role in neointima formation by the recruitment of circulating SPCs. Treating Apoe mice with a neutralizing CXCL12 monoclonal Ab after carotid injury reduces the neointimal area and the lesional SMC content. Furthermore, CXCL12 bound to platelets at the site of injury promotes vascular remodeling by recruiting a subset of SMC progenitors via CXCR4. Although increased CXCL12 mediated SPC recruitment in vascular injury model augments neointimal growth, this mechanism may be harnessed to stabilize the rupture prone lesions by increasing the SMC and extracellular matrix content.

1.2.2 CXCL1 and atherosclerosis

The ELR+ chemokine CXCL1 [also known as keratinocyte-derived chemokine (KC) in mice and growth-related oncogene (Gro)-α in humans] is 73 amino acids long (approximately 8 kDa) and is chemoattractant for neutrophils and T-lymphocytes. CXCL1 is approximately 60% identical between mouse and human. CXCL1 was initially identified in transformed Chinese hamster and human fibroblasts, and is highly expressed by different melanoma and carcinoma cells. Different cells such as monocytes, ECs, fibroblasts, and synovial cells express CXCL1 after stimulation with lipopolysaccharide, IL-1, or TNF-α. The biological effects of CXCL1 are mediated by its receptor CXCR2, which also binds to CXCL2 [also known as macrophage inflammatory protein-2 (MIP-2)]. CXCL1 shares sequence similarity with CXCL2 (approximately 63%).

The CXCL1-CXCR2 axis plays a crucial role in macrophage accumulation in atherosclerotic lesions. In BM chimeric Ldlr mice harboring Cxcr2 BM cells, the lesional macrophage accumulation and atherosclerosis lesion development are reduced. ECs activated in vitro as well as in vivo, up-regulate CXCL1 and CCL2 at the mRNA and protein level. CCL2 is secreted in a soluble form, whereas CXCL1 is immobilized to endothelial heparan sulphate proteoglycans. Blocking CXCL1 by an Ab or CXCR2 by pertussis toxin or CXCL1 peptide analogue (8-73) inhibit monocyte adhesion and arrest to the endothelium under physiological flow conditions in vivo. In contrast to CXCL1, the CCL2 mediates transendothelial migration by establishing a soluble gradient. Increased recruitment of monocyte by endothelial CXCL1 is induced by the interaction of VLA-4 on monocytes and
VCAM-1 on ECs.\textsuperscript{84, 85} Moreover, mildly oxidized LDL (moxLDL) and unsaturated lysophosphatidic acids (LPAs), which play a central role in hypercholesterolemia-induced atherosclerosis, up-regulate CXCL1 in ECs via the LPA receptors LPA\textsubscript{1} and LPA\textsubscript{3} and thus participate in monocyte adhesion and in the progression of atherosclerosis.\textsuperscript{27, 89}

### 1.3 SPCs and atherosclerosis

SPCs have been reported to play a role in vascular repair and atherosclerosis.\textsuperscript{90, 91} In adults, SPCs are derived from various sources such as BM, circulation, adventitia, and from tissue resident cells, and are characterized by the expression of cell surface markers.\textsuperscript{92} BM-derived and tissue-resident SPCs express c-kit, Sca1, but lack the expression of hematopoietic lineage markers (Lin\textsuperscript{-}), whereas SPCs in the adventitia c-kit\textsuperscript{+}Sca1\textsuperscript{+}CD34\textsuperscript{+}. In humans, circulating SPCs have been identified in the CD34\textsuperscript{+} cell population.\textsuperscript{92} SPCs differentiate into SMCs \textit{in vitro} demonstrating the typical hill and valley morphology when treated with growth factor PDGF-\textbeta.\textsuperscript{93, 94} Recently, BM-derived SPCs have been detected in human atherosclerotic tissue\textsuperscript{95} as well as in \textit{in vivo} mouse models\textsuperscript{90, 96, 97} of vascular diseases and several studies have described a role of BM-derived SPCs in vascular repair\textsuperscript{74, 75, 98} and atherosclerosis.\textsuperscript{94, 99}

Mechanical vascular injury leads to vascular remodeling involving accumulation of BM-derived SPCs, which differentiate to SMCs at the site of injury.\textsuperscript{91, 98} Immediately after vascular injury, the CXCL12 concentration in circulation increases rapidly, which mobilizes SPCs from the BM to the circulation.\textsuperscript{75} Mobilized SPCs require immobilized CXCL12 present on the luminal side of blood vessel for their recruitment to the injury site.\textsuperscript{74} LPA-mediated up-regulation of HIF-1\textalpha in SMCs of injured vessels induces CXCL12, which forms a major source of immobilized CXCL12.\textsuperscript{100, 101} Moreover, activated platelets adherent to the injured vessel wall express CXCL12 and are the second source of immobilized CXCL12.\textsuperscript{74} BM-derived SPCs are recruited to the lesion site via CXCR4, but not by CXCR7, since blocking CXCR4 receptor completely blocks neointima formation by SPCs.\textsuperscript{74, 102, 103} Recruited SPCs differentiate to lesional SMCs and contributes to neointimal growth.\textsuperscript{75}

Similar to the vascular injury model, SPCs also play a role in allograft vasculopathy.\textsuperscript{104, 105} In human cardiac allograft vasculopathy, circulating SPCs (CD34\textsuperscript{+} CD140b\textsuperscript{+}) increase with disease progression.\textsuperscript{104} Moreover, plasma CXCL12 levels correlate with disease severity and SPC count.\textsuperscript{104} Similarly, increased intragraft CXCL12 expression mobilizes CXCR4-expressing hematopoetic stem cells (HSCs) in mouse aortic transplant arteriosclerosis, which migrate into the allograft via microvessels in the adventitia.\textsuperscript{105} Within the graft, CXCR4\textsuperscript{+} HSCs differentiate into SMCs and contribute to the neointima formation.
In contrast to mechanical injury and allograft vasculopathy, contribution of SPCs to native atherosclerosis is rare and a debatable issue.\textsuperscript{90, 99, 106} However, selective apoptosis of lesional SPCs limits atherosclerosis and reduces pro-inflammatory cytokine expression indicating a pro-atherogenic role of SPCs in atherosclerosis.\textsuperscript{99} In contrast, Zoll and co-workers have shown that treatment with \textit{ex vivo} expanded human CD34\(^+\) SPCs reduces early atherosclerosis by reducing macrophages and increasing the SMCs and collagen content of the lesions in murine atherosclerosis.\textsuperscript{94} This study indicates a lesion-stabilizing role of SPCs in atherosclerosis. Although studies by Yu et al.\textsuperscript{99} and Zoll et al.\textsuperscript{94} depict opposite roles of SPCs in the progression of atherosclerosis, manipulation of the mobilization and local recruitment of SPCs might be a valuable strategy for the treatment of unstable atherosclerotic lesions.

\subsection*{1.4 Hypoxia inducible factor (HIF)-1}

Hypoxia inducible factor (HIF)-1 is a transcription complex, which responds to the changes in cellular oxygen levels and metabolic stress.\textsuperscript{107} HIF-1 is also important in embryonic development and blood vessel formation.\textsuperscript{108} HIF-1 is a heterodimeric protein complex consisting of one \(\alpha\)- and one \(\beta\)-subunit [the latter is also known as aryl hydrocarbon receptor nuclear translocator (ARNT)]. Both the \(\alpha\)- and \(\beta\)-subunit belong to a protein superfamily called basic helix-loop-helix-Per/Arnt/Sim (bHLH-PAS). When oxygen tension drops below the physiological level, the HIF-1\(\alpha\) (120 kDa) subunit translocates to the nucleus and dimerizes with the HIF-1\(\beta\) (91-94 kDa) subunit. The dimer combines with other cofactors to form a functional transcriptional complex, which binds to the HRE (5'-RGCTG-3') located in the promoter or enhancer regions of hypoxia-inducible genes and thereby activates transcription of the target genes. The \(\beta\)-subunit of HIF-1 is constitutively expressed, while the \(\alpha\)-subunit is increased especially in response to hypoxia; however, other stimuli, such as growth factors, cytokines or chemical compounds, may contribute to its induction as well. The HIF-1\(\alpha\) protein has a short half-life (\(t_{1/2} = 5\) min) and is tightly regulated by oxygen.

The stability and activity of the \(\alpha\)-subunit of HIF-1 are regulated by its post-translational modifications, such as hydroxylation, ubiquitination, acetylation, and phosphorylation.\textsuperscript{107, 108} Under normoxia, hydroxylation of 2 proline residues and acetylation of a lysine residue at the oxygen-dependent degradation domain (ODDD) of HIF-1\(\alpha\) triggers its association with the pVHL E3 ligase complex, leading to HIF-1\(\alpha\) degradation via the ubiquitin-proteasome pathway. Under hypoxia, the HIF-1\(\alpha\) subunit becomes stable and
interacts with coactivators, such as the cAMP response element-binding protein (CEBP)/p300, and regulates the expression of target genes (Figure 5).

Insulin and IGF-1 induce the up-regulation of HIF-1α in a different way compared to the hypoxic pathway. Moreover, growth factors, such as PDGF, endothelial growth factor (EGF), FGF-2, IGF-2, TGF-1β, hepatocyte growth factor (HGF), TNF-α, and IL-1β increase HIF-1α mRNA levels through receptor-mediated pathways. AT-2 and thrombin also enhance HIF-1α protein levels and its transcriptional activity. These factors stimulate HIF-1α synthesis by activating similar signaling transduction cascades, like the phosphatidylinositol 3- kinase (PI3K) and/or the mitogen-activated protein kinase (MAPK) pathways. Mammalian target of rapamycin (mTOR), a known energy sensor and downstream target of PI3K in growth control, is implicated in hypoxic signaling and HIF-1α regulation. Taken together, various growth factors up-regulate HIF-1α by transcriptional and post-transcriptional mechanisms (Figure 5).
Figure 5: Structure and regulation of HIF-1α.

(A) Schematic representation of human HIF-1α and HIF-1β proteins. Both proteins are related to the bHLH-PAS transcription factor family that contains an N-terminal bHLH domain and two PAS domains. HIF-1α contains an ODDD that mediates oxygen-regulated stability and a C-terminal transactivation domain (C-TAD) whose transcriptional repression in normoxia is controlled through hydroxylation of the asparagine 803 by FIH. 

(B) Under normoxia, HIF-1α is subjected to oxygen dependent hydroxylation on proline 402 and 564 in ODDD. Ubiquitination by the VHL protein targets HIF-1α to proteosomal degradation. Under conditions of hypoxia, UV-irradiation, and activation by growth factors, HIF-1α is stabilized, translocates to the nucleus, interacts with the HRE and finally promotes activation of target genes (Taken from Rezvani et al., Hif-lalpha in epidermis: Oxygen sensing, cutaneous angiogenesis, cancer, and non cancer disorders. J Invest Dermatol. 2011;131:1793-1805).
1.4.1 Role of HIF-1α in Atherosclerosis

HIF-1α is expressed in human and mouse atherosclerotic lesions. In humans, approximately 49% of carotid and 60% of femoral endarterectomy specimens express HIF-1α in macrophages, macrophage-derived foam cells, and to a lesser extent in SMCs. HIF-1α knockdown blocks the formation of lipid-loaded macrophages indicating a role for HIF-1α in lipid accumulation during foam cell formation. Similarly, deletion HIF-1α in myeloid cells can be beneficial for atherosclerosis as knockout of HIF-1α in myeloid cells decreases cutaneous inflammation in mice due to reduced monocyte infiltration. The lymphocytic cytokine profile in atherosclerotic lesions is also modulated by HIF-1α. Overexpression of HIF-1α in T-lymphocytes decreased interferon-γ (Th1) and increased IL-10 (Th2) expression, thus reducing lesion size and the lipid core. Moreover, hypoxic activation of HIF-1α up-regulates β2 integrin expression (CD18 specifically) in neutrophils, which promotes their extravasation. In SMCs, hypoxia- and ROS-mediated HIF-1α activation increases proliferation and migration, which may contribute to lesion stability by increasing the FC thickness. Following vascular injury, HIF-1α induces up-regulation of CXCL12 in SMCs, which increases the recruitment of BM-derived cells to the injury site and thereby contributes to neointimal growth. Moreover, Karshovska et al. reported that the expression of HIF-1α is up-regulated in neointimal ECs 2 weeks after injury. However, the expression of HIF-1α in atherosclerotic ECs has not yet been studied.
1.5 Aims of the study

Stabilization of vulnerable atherosclerotic lesions is crucial to reduce the incidence of lesion rupture. Although clinically approved drugs for the treatment of atherosclerosis, such as statins, have lesion-stabilizing effects and improve the outcome of CVD patients, CVD continues to be the leading cause of the death worldwide. Moreover, new treatment strategies to stabilize lesions are required.

Lesional SMCs are the major determinant of stability in advanced lesions. Following vascular injury, the transient increase in plasma CXCL12 mobilizes BM-derived SPCs, which are recruited to the injury site and differentiate into neointimal SMCs. Moreover, the transcription factor HIF-1α up-regulates CXCL12 in the injured vessel, which plays a crucial role in the recruitment of circulating SPCs. Furthermore, treatment with ex vivo expanded BM-derived SPCs induces a stable lesion phenotype with an increased lesional SMCs and collagen content. Therefore, the hypothesis was studied that mimicking the rapid but transient mobilization of BM-derived SPCs by increased plasma CXCL12 levels via repeated injections with CXCL12 in atherogenesis might increase the SMC content of the FC and thus, stabilize the rupture prone lesions. In addition, the hypothesis was tested that endothelial HIF-1α plays a protective role in atherosclerosis by up-regulating CXCL12 expression in ECs. Therefore, the following aims were defined to test these two hypotheses:

- To identify the effect of exogenous CXCL12 on BM-derived SPC mobilization, recruitment and lesion phenotype.
- To elucidate the molecular mechanism of CXCL12-mediated lesion stabilization.
- To determine the long-term effects of CXCL12 on lesion phenotype.
- To find out the role of endothelial-specific expression of HIF-1α in atherosclerosis.

Hence, CXCL12-mediated recruitment and differentiation of BM-derived SPCs to SMCs in lesions can stabilize the unstable lesions, which can be used as a novel treatment strategy for stabilizing vulnerable lesions and thereby reducing the incidence of CVD.
2 Materials and methods

2.1 Materials

2.1.1 General equipment

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### 2.1.2 Antibodies

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<td>1:200</td>
</tr>
<tr>
<td>anti rabbit IgG (H+L) Dylight 549</td>
<td></td>
<td>Goat</td>
<td>KPL</td>
<td>042-04-15-06</td>
<td>1:400</td>
</tr>
</tbody>
</table>
### Materials and Methods

#### 2.1.3 Buffers / Solutions/ Media

<table>
<thead>
<tr>
<th>Buffers/Solutions/Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose gel</td>
<td>2 g agarose, 6 µl ethidium bromide, 100 ml 1x TAE buffer (Tris base, acetic acid and EDTA)</td>
</tr>
<tr>
<td>Anesthesia solution</td>
<td>0.1 ml of ketamine hydrochloride (80 mg/kg), 0.05 ml of xylazine (5 mg/kg) and 0.85 ml of 0.9 % NaCl solution</td>
</tr>
<tr>
<td>Blocking solution</td>
<td>PBS containing 1 % BSA and 2.5 % horse serum, or PBS containing 10 % goat serum</td>
</tr>
<tr>
<td>FACS staining buffer</td>
<td>PBS, 2 % BSA, (2 % mouse, 2 % rabbit, 2 % human) serum</td>
</tr>
<tr>
<td>HH medium</td>
<td>1x HBSS, 1 % FCS, 0.5 % BSA</td>
</tr>
<tr>
<td>RBC lysis buffer</td>
<td>150 mM NH₄Cl, 10 mM potassium hydrogen carbonate (KHCO₃), 0.1 mM EDTA, pH 7.4</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>4 % PFA dissolved in PBS, pH 7.4 - 8</td>
</tr>
<tr>
<td>Sodium citrate buffer</td>
<td>Dissolve 12.6 ml of 0.1 M citric acid, 57.4 ml of 0.1 M sodium citrate dehydrate and 0.35 ml Tween20 in 630 ml of distilled water. Adjust pH to 6.0 with 5 M NaOH.</td>
</tr>
<tr>
<td>X-gal staining solution</td>
<td>Dissolve 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂, 0.02 % Nonidet P-40 (diluted from 10 % stock), 0.01 % sodium deoxycholate (diluted from 10 % stock) and 1 mg/ml X-gal in PBS.</td>
</tr>
<tr>
<td>X-gal stock solution</td>
<td>40 mg/ml X-gal in dimethylformamide</td>
</tr>
<tr>
<td>X-gal rinsing solution</td>
<td>PBS containing 2 mM MgCl₂</td>
</tr>
<tr>
<td>Hexazonium solution</td>
<td>Dissolve 0.4 g of parafuchsin-HCl (Sigma) in 30 ml of 1 N HCl. Dissolve 0.345 g of sodium nitrite in 5 ml of distilled water. Mix well both the solutions and wait for 5 min, then aliquot in 0.5 ml and freeze in -20°C.</td>
</tr>
<tr>
<td>Napthol-AS-D-Chloroacetate solution</td>
<td>Dissolve 90 mg of Napthol-AS-D-Chloroacetate (Sigma) in 30 ml of Tincture solution. Aliquot in 1 ml and freeze in -20°C.</td>
</tr>
<tr>
<td>Tincture solution</td>
<td>Mix 80 ml of DMSO and 10 ml of Triton-X-100. Store the solution at RT.</td>
</tr>
<tr>
<td>ASDCL solution</td>
<td>Freshly mix 0.4 ml of hexazonium solution in 50 ml of PBS and adjust the pH to 6.5 - 6.6 with PBS. Then add 1 ml of Napthol-AS-D-Chloroacetate solution and mix well.</td>
</tr>
<tr>
<td>EvG solution A</td>
<td>Dissolve 10 g of hematoxylin in 100 ml of 96 % ethanol</td>
</tr>
<tr>
<td>EvG solution B</td>
<td>Add 29 % iron (III) chloride solution (145 g iron (III) chloride dissolved in 500 ml of Millipore water) and 7.5 ml of 37 % HCl to 950 ml of Millipore water.</td>
</tr>
<tr>
<td>Resorscin Fuchsin solution</td>
<td>Dissolve 0.2 g of resorcin-fuchsin (Roth) in 70 ml of methanol, then add 30 ml of distilled water and 1 ml of concentrated HCl.</td>
</tr>
<tr>
<td>Picrofuchsin solution</td>
<td>Mix 400 ml of cold filtered aqueous picric acid and 20 - 40 ml of 1 % aqueous fuchsin acid solution</td>
</tr>
<tr>
<td>0.5 % HCL-alcohol</td>
<td>Mix 1000 ml 70 % ethanol with 5 ml of 37 % HCl</td>
</tr>
<tr>
<td>3x DMEM</td>
<td>6 ml of 10x DMEM + 14 ml of sterile H₂O</td>
</tr>
<tr>
<td>1x DMEM with 30% FCS</td>
<td>14 ml of 1x DMEM (with high glucose/ L-glutamin/ sodium pyruvate) + 6 ml of 100% FCS</td>
</tr>
</tbody>
</table>
2.1.4 **Cell culture**

<table>
<thead>
<tr>
<th>Item/Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryotubes</td>
<td>Simport, Beloeil, Canada</td>
</tr>
<tr>
<td>Fetal calf serum, Gold</td>
<td>PAA, Pasching, Austria</td>
</tr>
<tr>
<td>Endothelial cell growth medium</td>
<td>Promo Cell, Heidelberg, Germany</td>
</tr>
<tr>
<td>Gentamicine</td>
<td>Gibco, Life Technologies Inc., Darmstadt, Germany</td>
</tr>
<tr>
<td>Rat tail type collagen-I</td>
<td>Millipore, MA, USA</td>
</tr>
<tr>
<td>DMEM cell culture medium</td>
<td>PAA, Pasching, Austria</td>
</tr>
<tr>
<td>PBS (sterile)</td>
<td>PAA, Pasching, Austria</td>
</tr>
<tr>
<td>Collagenase Type V</td>
<td>Sigma, St. Louis, MO, USA</td>
</tr>
<tr>
<td>Trypsin EDTA</td>
<td>PAA, Pasching, Austria</td>
</tr>
<tr>
<td>ACCELL siRNA delivery medium</td>
<td>Dharmacon, Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>T25, T75 tissue culture flask</td>
<td>Greiner Bio One, Frickenhausen, Germany</td>
</tr>
<tr>
<td>6 well, 12 well, 24 well TC plates</td>
<td>BD Falcon, NJ, USA</td>
</tr>
<tr>
<td>10 ml, 25 ml sterile pipettes</td>
<td>Corning, New York, USA</td>
</tr>
<tr>
<td>2 cm TC dish</td>
<td>BD Falcon, NJ, USA</td>
</tr>
</tbody>
</table>

2.1.5 **Kits**

<table>
<thead>
<tr>
<th>Kit</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription M-MLV Reverse Transcriptase</td>
<td>Promega, Madison, USA</td>
</tr>
<tr>
<td>Real-time PCR Maxima SYBR Green qPCR Master Mix</td>
<td>Fermentas, St. Leon-Rot, Germany</td>
</tr>
<tr>
<td>RNA isolation RNeasy Mini/Micro Kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>Mouse CXCL12 Elisa kit</td>
<td>Ray biotech Inc., GA, USA</td>
</tr>
<tr>
<td>Mouse CXCL1 Elisa kit</td>
<td>Ray biotech Inc., City, USA</td>
</tr>
<tr>
<td>DC Protein Assay Kit</td>
<td>Bio-Rad, Munich, Germany</td>
</tr>
<tr>
<td>BioSprint 15 DNA Blood Kit</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>mirVana kit</td>
<td>Ambion, Darmstadt, Germany</td>
</tr>
<tr>
<td>Total RNA isolation using PAXgene fixed tissue</td>
<td>Qiagen, Hilden, Germany</td>
</tr>
<tr>
<td>TUNEL <em>In Situ</em> Cell Death Detection Kit, TMR red</td>
<td>Roche, Basel, Switzerland</td>
</tr>
</tbody>
</table>
2.1.6 *Miscellaneous*

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml, 2.0 ml microcentrifuge tubes</td>
<td>Sarstedt, Numbrecht, Germany</td>
</tr>
<tr>
<td>15 ml, 50 ml centrifuge tubes</td>
<td>Greiner Bio One, Frickenhausen, Germany</td>
</tr>
<tr>
<td>Polypropylene FACS tube</td>
<td>Becton Dickson, Franklin Lakes, USA</td>
</tr>
<tr>
<td>EDTA tubes</td>
<td>Sarstedt, Numbrecht, Germany</td>
</tr>
<tr>
<td>Serum tubes</td>
<td>Sarstedt, Numbrecht, Germany</td>
</tr>
<tr>
<td>96 well flat round bottom plate</td>
<td>Nunc, Roskilde, Denmark</td>
</tr>
<tr>
<td>10 ml, 25 ml serological pipettes</td>
<td>Corning, New York, USA</td>
</tr>
<tr>
<td>Sterile filter tips</td>
<td>Starlab, Ahrensburg, Germany</td>
</tr>
<tr>
<td>2 ml, 5 ml, 10 ml, 20 ml syringes</td>
<td>Terumo, Leuven, Belgium</td>
</tr>
<tr>
<td>1 ml syringe</td>
<td>Braun, Melsungen, Germany</td>
</tr>
<tr>
<td>23 G, 27 G cannulas</td>
<td>Becton Dickson, Franklin Lakes, USA</td>
</tr>
<tr>
<td>Disposable scalpel</td>
<td>Feather Safety Razor Co., Osaka, Japan</td>
</tr>
<tr>
<td>Glassware</td>
<td>Schott, Mainz, Germany</td>
</tr>
<tr>
<td>40 µm and 70 µm cell strainer</td>
<td>BD Falcon, NJ, USA</td>
</tr>
<tr>
<td>Sterile 0.2 mm acrodisc filters</td>
<td>Corning, New York, USA</td>
</tr>
<tr>
<td>Superfrost® Plus Glass slides</td>
<td>Thermo Scientific, Braunschweig, Germany</td>
</tr>
<tr>
<td>Glass coverslip</td>
<td>Automat Star, Edermunde, Germany</td>
</tr>
<tr>
<td>Neubauer Hemocytometer</td>
<td>Blau Brand, Wertheim, Germany</td>
</tr>
<tr>
<td>Vectasheild with DAPI</td>
<td>Vector Laboratories Inc., Burlingame, USA</td>
</tr>
<tr>
<td>Whatman 3 mm filter paper</td>
<td>Whatman International Ltd., Kent, UK</td>
</tr>
</tbody>
</table>
### 2.1.7 Oligonucleotides for real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse Hif1a</td>
<td>5‘AGAAACGACCACCTGCTAAGGC3’</td>
<td>5‘GTGGCAGACAGGTTAAGGCTC3’</td>
<td>132</td>
</tr>
<tr>
<td>mouse Cxcl12</td>
<td>5‘AGAGCCAACGTCAGGCATCT3’</td>
<td>5‘TAATTTCGGGTCAATGAC3’</td>
<td>108</td>
</tr>
<tr>
<td>mouse Cxcl1</td>
<td>5‘ACCGAAGTCATAGCCACACT3’</td>
<td>5‘TCTCCGGTTATGGGACAC3’</td>
<td>114</td>
</tr>
<tr>
<td>mouse Ccl5</td>
<td>5‘CTGCTGCTTTGGCCTACCTCT3’</td>
<td>5‘TCTTCCAGTGACAAACACG3’</td>
<td>110</td>
</tr>
<tr>
<td>mouse Mif</td>
<td>5‘TTAGCCGCACGAACGATTC3’</td>
<td>5‘CGTTGGCAGCGTTCATGTC3’</td>
<td>172</td>
</tr>
<tr>
<td>mouse Ccl2</td>
<td>5‘CTTCTGGGCTGTGCTTCA3’</td>
<td>5‘CCAGCCTAATCGTGGGATC3’</td>
<td>127</td>
</tr>
<tr>
<td>mouse Tnfa</td>
<td>5‘CCACCACGCTCTTCTGTC3’</td>
<td>5‘AGGGCTGCTGGGATAGA3’</td>
<td>103</td>
</tr>
<tr>
<td>mouse Gapdh</td>
<td>5‘CAGCACATTGTCAGGC3’</td>
<td>5‘CCACACCTTGGCAGC3’</td>
<td>225</td>
</tr>
<tr>
<td>mouse β-actin</td>
<td>5‘CAAAGCGCCCGGGGTGATG3’</td>
<td>5‘GCCACAGGATCATTCCAC3’</td>
<td>67</td>
</tr>
<tr>
<td>E.coli lacZ</td>
<td>5‘ACGCGCAAGCGGTTGCTGATTC3’</td>
<td>5‘AGCAGGATATCCTGACCAC3’</td>
<td>100</td>
</tr>
</tbody>
</table>
2.2 Animal experiments

2.2.1 Mice

Wild type C57BL/6, Apoe<sup>−/−</sup>, SM22-lacZ/Apo<sub>e</sub><sup>−/−</sup> (SM22-lacZ mice provided by Prof. Merticskay, Paris, France)<sup>120</sup>, VECadCre-ER<sup>T2</sup>/Hif1a<sup>floxflo</sup>/Apo<sub>e</sub><sup>−/−</sup>, and VECadCre-ER<sup>T2</sup>/Hif1a<sup>WT/WT</sup>/Apo<sub>e</sub><sup>−/−</sup> mice were used for experiments in this study. All mice were housed in a barrier facility and exposed to a 12 hours light and a 12 hours dark period within the Institute for Laboratory Animal Science of the University Hospital, RWTH Aachen. The mice were fed either a ND (mouse chow) or a HFD (21 % crude fat, 0.15 % cholesterol and 19.5 % casein, Altromin, Germany) and watered ad libitum. C57BL/6 mice were purchased from Janvier (Saint Berthevin Cedex, France) and were allowed a 7-day acclimatization period after their arrival on site prior to being used for experiment. Inducible, EC-specific Hif1a knockout mice were generated by first crossing mice that carry a floxed Hif1a allele (purchased from Jackson Laboratories) with Apoe<sup>−/−</sup> mice to obtain Hif1a<sup>floxflo</sup>/Apo<sub>e</sub><sup>−/−</sup> mice. These Hif1a<sup>floxflo</sup>/Apo<sub>e</sub><sup>−/−</sup> mice were then crossed with VECadCre-ER<sup>T2</sup> mice (courtesy provided by Prof Iruela-Arispe, Los Angeles, USA)<sup>121</sup>, which express a tamoxifen-inducible Cre recombinase under the endothelial cell-specific VE-Cadherin promoter, to generate VECadCre-ER<sup>T2</sup>/Hif1a<sup>floxflo</sup>/Apo<sub>e</sub><sup>−/−</sup> mice. VECadCre-ER<sup>T2</sup>/Hif1a<sup>floxflo</sup>/Apo<sub>e</sub><sup>−/−</sup> mice (EC-Hif1a<sup>−/−</sup>) and VECadCre-ER<sup>T2</sup>/Hif1a<sup>WT/WT</sup>/Apo<sub>e</sub><sup>−/−</sup> mice (EC-Hif1a<sup>+/+</sup>) were used throughout this study. All of the animal experiments were reviewed and approved by the local authorities (LANUV NRW) in accordance with the German animal protection law.

2.2.2 Genotyping

EC-Hif1a<sup>−/−</sup> and EC-Hif1a<sup>+/+</sup> mice were genotyped by PCR for (a) the loxP sites in the Hif1a gene, (b) the VECadCre gene, and (c) the Apoe gene. Primers used for genotyping were synthesized by Sigma-Aldrich (Germany). Sequences of the primers are listed in Table 2. The primers were stored upon delivery at a stock concentration of 100 µM in TE buffer at -20°C.
Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VECadCre</td>
<td>Forward (VE A)</td>
<td>5’- CCAAAATTTGCTGATTACCGGTGATGC-3’</td>
<td>970 bp product: positive; No product: negative</td>
</tr>
<tr>
<td></td>
<td>Reverse (VE B)</td>
<td>5’- ATCCAGGTACGGATAGT-3’</td>
<td></td>
</tr>
<tr>
<td>Hif1a</td>
<td>Forward (oIMR7068)</td>
<td>5’- TGCTCATCAGTTGCCACCT-3’</td>
<td>655 bp: wild type; 700 bp: knockout Both products: heterozygous</td>
</tr>
<tr>
<td></td>
<td>Reverse (oIMR7069)</td>
<td>5’- GTTGGGGCACTGGAAAG-3’</td>
<td></td>
</tr>
<tr>
<td>Apoe</td>
<td>Forward (oIMR0180)</td>
<td>5’-GCCTAGCCGAGGGAGGAGCCG-3’</td>
<td>155 bp: wild type; 245 bp: knockout</td>
</tr>
<tr>
<td></td>
<td>WT Reverse (oIMR0181)</td>
<td>5’-TGTGACTCTGGAGCTGTCAGC-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutant Reverse (oIMR0182)</td>
<td>5’- GCCGCC CGACATGCATCT-3’</td>
<td></td>
</tr>
</tbody>
</table>

Genomic DNA isolated from mice tail tips was used for genotyping (see section 2.4.2). The PCR reaction was carried out in a thermalcycler (My Cycler, Bio-Rad, Hercules, USA) using the following conditions:

<table>
<thead>
<tr>
<th>VECadCre</th>
<th>Hif1a</th>
<th>Apoe</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C 2 min</td>
<td>94°C 3 min</td>
<td>94°C 3 min</td>
</tr>
<tr>
<td>94°C 20 sec</td>
<td>94°C 30 sec</td>
<td>94°C 30 sec</td>
</tr>
<tr>
<td>58.5°C 20 sec</td>
<td>× 35</td>
<td>×35</td>
</tr>
<tr>
<td>72°C 45 sec</td>
<td>72°C 1 min</td>
<td>72°C 1 min</td>
</tr>
<tr>
<td>70°C 5 min</td>
<td>72°C 2 min</td>
<td>72°C 2 min</td>
</tr>
<tr>
<td>10°C hold</td>
<td>10°C hold</td>
<td>10°C hold</td>
</tr>
</tbody>
</table>

PCR products were electrophoresed on a 2% agarose gel (see section 2.1.3) at 100 V and visualized using a gel documentation system (Genoplex, VWR, Germany). Product sizes were determined by comparison using a 1 kb DNA ladder (New England Biolabs).

2.2.3 Mouse models of atherosclerosis

2.2.3.1 Partial ligation of a carotid artery

Male Apoe<sup>-/-</sup> mice (Jacksons Laboratory, 6 weeks old) or tamoxifen-treated (see section 2.2.9) EC-Hif1a<sup>+/+</sup> and EC-Hif1a<sup>-/-</sup> (6-8 weeks old) mice were subjected to partial ligation of the LCA as described earlier. Male mice were anesthetized by injection (i.p.) of 200 µl anesthesia solution (see section 2.1.3). The epilated area of the frontal neck was disinfected and a ventral midline
Incision was made in the left lateral side of the neck. The LCA was exposed by blunt dissection using a stereo-microscope (Olympus SZX7). The external, internal and the occipital arteries were completely ligated using sutures (Seraflex, USP 7/0, Naila, Germany) allowing blood outflow only via the superior thyroid artery (Figure 6). The incision was closed with wound clips and mice were fed a HFD after recovery from anesthesia. The disturbed flow and HFD led to formation of atherosclerotic lesions in the LCA. The RCA and LCA were harvested 4 weeks, 6 weeks, or 8 weeks after ligation of the LCA following perfusion fixation with 4% PFA (see section 2.2.4) and embedded in paraffin (see section 2.3.3.1). Blood was sampled intracardially for serum lipid measurements (see section 2.3.6).

![Diagram of partial ligation of carotid artery](image)

**Figure 6: Diagrammatic representation for partial ligation of carotid artery.**

Three branches of the LCA [external carotid artery (ECA), internal carotid artery (ICA), and occipital artery (OA)] were ligated, while leaving the superior thyroid artery (STA) open. The RCA was left unligated. RSA: right subclavian artery; LSA: left subclavian artery.

### 2.2.3.2 Diet-induced atherosclerosis

Female EC-Hif1a+/+ and EC-Hif1a–/– mice (6 weeks old) were treated with tamoxifen (see section 2.2.9) and subsequently fed a HFD diet for 3 months. After 3 months of the high fat feeding period, the carotid arteries, the aortic roots, and the thoraco-abdominal aortas were harvested following perfusion fixation with 4% PFA (see section 2.2.4). The thoraco-abdominal aortas including the aortic arch were en face-prepared and stained with oil red O (see section 2.3.3.4). The RCA, LCA and aortic root were embedded in paraffin. Blood was taken intracardially for serum lipid measurements (see section 2.3.6).

### 2.2.4 Micro-computed tomography (CT) angiography

Apoe–/– mice were scanned by in vivo dual energy micro-CT (TomoScope DUO, CT Imaging, Erlangen, Germany) at 6 weeks after partial carotid ligation. A blood-pool contrast agent (radiopaque iodinated emulsions) was injected in the tail vein and the luminal diameters of the common carotid arteries were quantified within a distance of 1 mm from the bifurcation with the Imalytics Research workstation (Philips Technologie GmbH, Aachen, Germany). Then, 3D renderings of the carotid arteries were created using the Definiens Developer XD software.
(Definiens, Munich, Germany). These experiments were carried out in collaboration with Prof. Fabian Kiessling, Department of Experimental Molecular Imaging, University Hospital, RWTH University, Aachen, Germany.

2.2.5 Perfusion fixation

Perfusion fixation was performed in anesthetized mice [with ketamine hydrochloride (80 mg/kg, IP) and xylazine (5 mg/kg, IP)] by inserting a catheter into the left ventricle and cutting of the right atrium. PFA (4%) was perfused using a syringe pump (Aladdin-1000, World Precision Instruments, Inc, 336 µl/min) for 10 min through the left ventricle and the solution was allowed to flow out through the right atrium. Tissues were harvested and kept in formalin ON at 4ºC and the samples were dehydrated and paraffin embedded on the following day (see section 2.3.3.1).

2.2.6 Bone marrow transplantation

To assess the role of BM-derived SPCs in the CXCL12-mediated plaque stabilization, Apoe^{-/-} mice (male, 6 weeks old) were whole-body X-ray irradiated (Faxitron CP-160) with an ablative dose of 6.5 Gy twice at an interval of 4 hours and reconstituted with BM cells from SM22-lacZ/Apoe^{-/-} mice, which express the reporter lacZ gene under the smooth muscle cell-specific SM22 promoter. After anesthetizing the mice, the femurs and tibias were aseptically harvested from donor SM22-lacZ/Apoe^{-/-} mice. BM cells were prepared by flushing the marrow cavities with sterile PBS, followed by repeated pipetting to dissolve cell clumps and passing the resulting suspension through a cell strainer (pore size 40-µm) (see section 2.1.6) to obtain a single cell suspension. The recipient Apoe^{-/-} mice were injected with 5 million donor cells via the tail vein 24 hours after the irradiation. After a reconstitution period of 3 weeks, the LCAs of the recipient mice were partially ligated and the mice were fed a HFD (Figure 7). The degree of re-constitution of the recipient’s BM with donor cells was analyzed by quantifying the peripheral blood cells that contain the lacZ gene (see section 2.4.5). The carotid arteries were harvested at 6 weeks after partial ligation for X-gal staining (see section 2.3.3.5) and immunostainings (see section 2.3.4).
2.2.7 In vivo siRNA treatment

siRNA technology is a powerful tool employed for assessment of gene functions and pathway elucidation by specific suppression of post-transcriptional gene expression. Synthetic siRNAs are approximately 21-nt double stranded RNA containing a 19-nt duplexed region with 2-nt 3’ overhangs. To study the role of intra-lesional CXCL12 expression in lesion stabilization mediated by treatment with exogenous CXCL12, partially ligated Apoe⁻ mice (female, 6 weeks old) were treated perivascularly with either CXCL12 siRNA (4 nmol, Accell SMART pool, Dharmacon, Thermo Scientific) or non-targeting siRNA (4 nmol, Dharmacon, Thermo Scientific) dissolved in pluronic gel for 4 weeks with one treatment per week (Figure 8). Pluronic gel is a thermo-reversible gel, which is liquid at low temperatures (4-5°C) and becomes a gel upon warming to 37°C and is used for slow and continuous release of siRNA into the tissue. siRNA was reconstituted in sterile PBS. Pluronic gel (50%) was prepared by dissolving 2.5 g of pluronic-F127 (Sigma-Aldrich) in 5 ml of Millipore water at 4°C with continuous stirring. This solution was then filter sterilized using syringe filters (0.2 µm pore
size, Corning) and kept on ice until use. The transfection reagent (DharmaFECT4, Thermo Scientific) was diluted in sterile PBS (1:50) and kept on ice till use. The diluted transfection reagent (0.5 µl) and 4 nmol of reconstituted siRNA (in a volume of 4 µl) were mixed and incubated on ice for 15 min. Then, 15 µl of 50% pluronic gel was mixed with 4.5 µl of the siRNA-transfection reagent mix to get a final concentration of 35% pluronic gel. This solution was applied perivascularly on the partially ligated LCA. After the final treatment, the carotid arteries were perfusion fixed with PAXgene Tissue Fix solution followed by stabilization in PAXgene Tissue Stabilizer (PreAnalytix, Qiagen) according to the manufacturer’s protocol. The carotid arteries were then dehydrated, embedded in paraffin, and sectioned to study the lesion size and phenotype.

**Figure 8: Schematic representation of the protocol for siRNA treatment of Apoe<sup>−/−</sup> mice.**

Partial ligation of the left carotid arteries of Apoe<sup>−/−</sup> mice was performed at day 0 and the mice were fed a HFD. CXCL12 treatment (500 ng i.v., twice weekly) was started in the 3<sup>rd</sup> week after partial carotid ligation and continued for 4 weeks. Perivascular application of siRNA (CXCL12 siRNA or non-targeting siRNA, 4 nmol) around partially ligated LCAs was performed once between two CXCL12 injections. The mice were sacrificed at day 43 after partial carotid ligation.

### 2.2.8 CXCL12 injection

To study the SPC mobilization in C57BL/6 mice, 100 ng or 500 ng of recombinant mouse CXCL12 (Peprotech) dissolved in 100 µl of PBS was injected once via the tail vein. In Apoe<sup>−/−</sup> mice, treatment with CXCL12 (500 ng per 100 µl, i.v. injection, twice weekly for 4 consecutive weeks) was started 2 weeks after partial ligation of the LCA (see section 2.2.3.1).

### 2.2.9 Tamoxifen injection

Treatment with tamoxifen (an estrogen receptor antagonist) is required for the deletion of genes flanked by loxP sites through an inducible Cre recombinase (CreER<sup>T2</sup>) system in transgenic mice. In order to activate the Cre recombinase in EC-Hif1α<sup>+/−</sup> and EC-Hif1α<sup>−/−</sup>.
mice, 2 mg of tamoxifen (Sigma-Aldrich) dissolved in 100 µl of Miglyol (Casar and Lorentz, Germany) was injected (i.p.) once daily for 5 consecutive days. One week after the last tamoxifen injection, the mice were either put on a HFD for 3 months (see section 2.2.3.2) or partial ligation of the LCA was performed (see section 2.2.3.1).

2.3 Cell characterization and protein assays

2.3.1 Flow cytometry

2.3.1.1 FACS analysis of Sca1+Lin−Pdgfr−β+ cells in mouse whole blood

At different time points (4 hours, 8 hours and 24 hours) after injection of CXCL12 (100 or 500 ng, i.v.) or vehicle (PBS), blood was drawn by retro-orbital puncture, collected in EDTA tubes and kept on ice. RBC lysis was performed by incubating the blood with 3 ml of lysis buffer (see section 2.1.3) for 10 min at RT. Lysis was stopped by adding 2 ml of Hank’s complete solution (see section 2.1.3) and centrifuged at 300 g for 5 min. The cell pellet was washed again in Hank’s complete solution and resuspended in 50 µl of 1:1 FACS staining buffer (see section 2.1.3) and Hank’s complete solution. Approximately 10^6 cells were incubated with 2 µl of each biotinylated lineage Ab (CD3, CD45R, CD11b, Ter-119, Ly-6G, eBioscience) for 30 min on ice. The cells were washed with 2 ml of Hank’s complete solution and resuspended in 50 µl of 1:1 FACS staining buffer and Hank’s complete solution containing 0.2 µl of PE-Cy7-conjugated streptavidin (eBioscience), 1.25 µl of phycoerythrin (PE)-conjugated Sca-1 (Ly-6A/E, clone D7, eBioscience) and 5 µl Allophycocyanin (APC)-conjugated mouse Pdgfr-β/CD140b (eBioscience) Ab for 30 min on ice in the dark. After washing, cells were resuspended in 400 µl of Hank’s complete solution and analyzed by a flow cytometer (FACS CantoII, BD Biosciences) (see section 2.1.1). In the control tubes, only streptavidin-conjugated PE-Cy7 was added. The data were analyzed using FlowJo software (Tree star Inc., Ashland, OR, USA).

First, the mononuclear cell population was identified using FSC and SSC plots and gated. The fluorescence intensity of PE and PE-Cy7 in this gated mononuclear cell population was analyzed by placing a quadrant based on the negative control. To discriminate positive and negative cells a threshold (>95% of the cells in the negative control) was set according to the auto-fluorescence of the negative control. The Sca-1^+Lin^- cells were then gated and the fluorescence intensity of this population for APC was analyzed. APC^+ cells were identified as Sca-1^+Lin^-Pdgfr-β^+ cell population. For each quadrant, the percentage of cells is automatically given by the quadrant statistics. The percentage of Sca-1^+Lin^- cells and Sca-1^+ Lin^-Pdgfr-β^+
cells in the mononuclear cell population was compared between PBS- and CXCL12-treated mice.

### 2.3.1.2 Quantification of lesional macrophages by flow cytometry

To quantify the lesional macrophages in PBS- and CXCL12 (500 ng)-treated mice, the LCA was harvested after perfusion with 10 ml of ice-cold PBS and kept on ice-cold RPMI-1640 medium (PAA) containing 10% FCS. The excised carotid arteries were digested with 100 µg/ml of Liberase™ (Roche) diluted in RPMI-1640 for 1 hour at 37°C on a shaking incubator. The enzyme action was stopped by adding 10% FCS and the digested arteries were pipetted repeatedly to obtain a single cell suspension. The cell suspension was passed through a cell strainer (70 µm pore size) (see section 2.1.6) and macrophages were stained with an APC conjugated–F4/80 Ab (clone BM8, eBioscience) for 30 min on ice in the dark. The cells were washed with 2 ml of RPMI-1640 and the pellet was resuspended in 200 µl of PBS. To exclude dead cells from the analysis, cells were incubated in 10 µl of Propidium Iodide (PI, eBioscience) for 10 min on ice, in the dark and subsequently analyzed using the FACS CantoII flow cytometer. In the control tube, only PI was added. Raw data were analyzed using FlowJo software. First, PI cells were gated (R1 gate) in a dot plot displaying SSC and the fluorescence intensity of PI to exclude dead cells from the analysis (Figure 9). The PI cell population was then analyzed in an SSC/F4/80-APC dot plot. F4/80-APC⁺ cells (R4) were identified based on the negative control staining. The percentage of F4/80-APC⁺ cells in the R1-gated cells was determined (Figure 9) and compared between PBS- and CXCL12-treated mice.

![Figure 9: Gating strategy for flow cytometric analysis of lesional macrophages.](image)

PI cells, representing live cells (R1) were sub-gated for APC⁺ cells (R4), which represent F4/80 expressing macrophages (see text for further details).
2.3.2 ELISA

ELISA is a highly sensitive immunoassay designed for detecting and quantifying substances, such as proteins, peptides, hormones and Abs by utilizing the specificity of the Ag-Ab interaction. The assay measures changes in enzyme activities proportional to the Ag or Ab concentrations. Briefly, microplates are first coated with a capture Ab specific for the Ag of interest. Samples and standards are added to the wells and incubated for 3 hours allowing binding to the capture Ab. After washing, a detection Ab (specific for Ag) is added and incubated for 1 hour. Following a washing step, a horseradish peroxidase (HRP)-linked secondary Ab, directed against the detection Ab, is incubated for 45 min. Then, 3,3,5,5-tetramethylbenzidine (TMB) substrate (specific for HRP) is added and incubated for 15 min. The enzyme reaction leads to the oxidation of TMB. The resulting oxidation product is a cation free radical, which yields a blue color and has an absorption maximum of 653 nm. Adding the stop solution (0.2 M sulphuric acid) completely oxidizes the blue product by 2-electron oxidation to a yellow color product (diimine), which has an absorption maximum at 450 nm. The absorbance, which is proportional to the amount of Ag bound to the capture antibody over a certain range of concentration, was measured using a microplate reader (see section 2.1.1). The protein concentrations were calculated by graphically plotting the values obtained from standards.

The CXCL12 concentration was determined by ELISA (Ray Biotech Inc.) in plasma and BM samples. Plasma was obtained from blood anticoagulated with EDTA by centrifugation at 1000 g for 10 min at 4°C. For the determination of CXCL12 in the BM, femurs of C57BL/6 mice were flushed with 500 µl of ice-cold PBS and the eluent was centrifuged at 1000 g for 10 min at 4°C. The supernatants were used for analysis.

The CXCL1 concentration was determined in cell culture supernatants by a CXCL1-specific ELISA kit (see reaction 2.1.5) according to the manufacture’s protocol. After stimulation of mouse primary ECs with LPA20:4 (see section 2.5.4) or moxLDL (see section 2.5.4), the cell culture supernatants were collected. All samples were stored at -80°C until used.

2.3.3 Histochemistry

2.3.3.1 Tissue fixation, paraffin wax embedding, and sectioning

In situ perfusion fixation (see section 2.2.4) was performed in anesthetized mice with PFA (4%) (see section 2.1.3) for 10 min. Tissues were harvested and either snap-frozen in liquid
nitrogen for cryo-sectioning (see section 2.2.5) or post-fixation was performed in neutral-buffered formalin (10%, ON at 4°C) for subsequent paraffin embedding. A tissue processor (see section 2.1.1) was used to embed the tissues in paraffin according the following protocol:

1. 70% ethanol for 60 min at 20°C
2. 96% ethanol for 60 min at 20°C
3. 100% ethanol for 60 min at 20°C
4. Xylene for 90 min at 45°C under vacuum conditions
5. Paraffin wax for 1 hour at 60°C ON

After the infiltration with paraffin, embedding into paraffin wax blocks was performed by pouring additional liquid paraffin on the tissues that were placed at the bottom of chilled metal grids using a heated paraffin-dispensing module (see section 2.1.1). Paraffin was hardened for 30 min on a cold plate (-5°C) and blocks were stored at RT until required. Serial sections (5 µm thick) were cut from the paraffin blocks using a microtome (see section 2.1.1) and subsequently flattened by placing on the surface of a water bath (37°C). Glass slides (see section 2.1.6) were used to pick up the sections from the water bath. The tissue sections on the slides were dried at 37°C ON and then stored at RT.

For cryosectioning, PFA fixed tissue sample were snap frozen in liquid nitrogen. Then, the tissue was placed in the cryomold in correct orientation, overlaid with OCT (Tissue Tek, Sakura), and frozen quickly in liquid nitrogen. OCT is viscous at RT but freezes into a solid support at −20°C. Sections (7-10 µm thick) were cut in the pre-cooled cryostat (see section 2.1.1) at −20°C. The sections were transferred to a poly-L-lysine-coated microscope slide (Thermo scientific) at RT by touching the slide to the tissue within 1 min of cutting to prevent freeze drying of the tissue. Any unused tissue was covered with a layer of OCT to prevent freeze-drying and stored at −80°C.

2.3.3.2 Elastica van Gieson (EvG) staining

Elastica van Gieson stain is a combination of Weigert’s hematoxylin, van Gieson picrofuchsin and the resorcin-fuchsin solution, which allows differential analysis of nuclei, connective tissue, muscle and elastic fibers in histological sections. EvG staining was used to study the morphology and size of lesions in the carotid artery and aortic root in the current study. First, tissue sections were de-paraffinised and re-hydrated (xylene for 30 min, 100% isopropanol for 2 × 5 min, 96% isopropanol for 5 min, 70% isopropanol for 5 min, Millipore water for 5 min, and PBS for 5 min). Slides were then stained in resorcin-fuchsin solution (see section 2.1.3) for 15 min at 56°C and then slowly dipped in tap water 1-2 times before differentiation in
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80% ethanol. Slides were then incubated in solution A + B (see section 2.1.3) for 5 min, differentiated in 0.5% HCl-alcohol (see section 2.1.3), and rinsed in tap water. Finally, sections were immersed in picrofuchsin solution (see section 2.1.3) for 1 min and rinsed in tap water. The sections were then dehydrated through a series of increasing alcohol concentrations (short incubation in 70% isopropanol, short incubation in 96% isopropanol, and 3 min in 100% isopropanol), immersed in xylene for 5 min, and mounted in Vitro-Clud.

2.3.3.3  Napthol-AS-D-chloroacetate esterase (ASDCL) staining

The ASDCL staining is used to demonstrate the presence of granulocytes and granulocyte precursor cells in histological sections and is based on the ability of cytoplasmic esterases in granulocytes to hydrolyze the substrate Napthol-AS-D-chloroacetate\textsuperscript{129-131}. The hydrolysis separates a free napthol compound, which couples with the diazonium compound, in the reaction forming deep red / violet deposits at the site of the enzyme activity. This staining was used in the current study to detect and quantify neutrophils in carotid lesions. The ASDCL solution (see section 2.1.3) was freshly prepared by first adding 0.4 ml of hexazonium solution (see section 2.1.3) to 50 ml PBS and adjusting the pH strictly to 6.58 - 6.59. Then, 1 ml of Napthol-AS-D chloroacetate solution (see section 2.1.3) was added. The de-paraffinised and re-hydrated sections were incubated in the ASDCL solution for 80 min at RT followed by rinsing in tap water. The nuclei were stained by incubating the slides in hematoxylin (for 30 sec). The slides were mounted with Vitro-Clud, coverslipped, and studied by bright-field microscopy.

2.3.3.4  Oil red O staining

Oil red O stains neutral lipids, such as triglycerides and cholesteryl esters, which are the major neutral lipid components in atherosclerotic lesions. The lipid accumulation in the aorta can be quantified after staining with oil red O. For oil red O staining, the complete aorta (aortic arch, thoracic and abdominal aorta) was fixed in 4% PFA ON, cut longitudinally, stripped off the adventitia, and pinned with needles exposing the luminal inside up (\textit{en face}). Pinned aortas were dipped in 60% isopropanol for 15 sec, incubated in 0.3 % oil red O (Sigma) solution for 15 min and again washed in 60% isopropanol for 15 sec. Finally, aortas were washed in water, mounted in glycerin jelly, and coverslipped after removal of the needles. Images were captured with a bright-field microscope and the oil red O$^+$ area was quantified using DISKUS software (Hilgers).
2.3.3.5  X-gal staining

Bacterial β-galactosidase (encoded by the lacZ gene) is commonly used as a reporter for transgene expression. X-gal staining is a common assay used for histochemical detection of β-galactosidase activity in tissues. X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside), an analog of lactose, is hydrolyzed by β-galactosidase into a hydroxyindole product, which dimerizes and oxidizes into an insoluble dark blue product at the site of enzyme activity. To detect β-galactosidase activity in cells of atherosclerotic lesions, X-gal staining was performed. Briefly, the mice were perfused with 10 ml of 2% PFA and the carotid arteries were harvested. The tissue was post-fixed in 2% PFA for another 15 min on ice and washed thoroughly in X-gal rinsing solution (see section 2.1.3). The tissues were then incubated in X-gal staining solution (see section 2.1.3) at 37°C ON. On the next day, the tissues were washed in X-gal rinsing solution twice and immediately processed for cryosectioning (see section 2.3.3.1). Cryosections (7-10 µm thickness) were re-stained in X-gal staining solution followed by counterstaining with nuclear fast red (Vector lab) for 5-10 min. Excess nuclear stain was removed by rinsing in tap water for 5-10 min. The slides were mounted using aqueous mounting medium (IM SOL MOUNT), coverslipped, and microscoped.

2.3.4  Immunofluorescence

Immunofluorescence is an immune staining technique used for demonstrating the presence of Abs bound to Ags in tissues, cultured cells, suspension cells or circulating body fluids. The slides were de-paraffinised in xylene (2 × 10 min) and rehydrated in a graded isopropanol series (100% for 2 × 5 min, 96% for 5 min and 70% for 5 min). After washing in PBS for 5 min, the sections were either cooked in sodium citrate buffer (see section 2.1.3) for 20 min in a microwave oven (Fairline, Düsseldorf, Germany) or directly processed for the blocking of unspecific binding sites. Cooked sections were allowed to cool down for 30 min at RT. After rinsing in PBS for 5 min, the cooked and uncooked sections were then incubated with appropriate blocking solutions (see to section 2.1.3) for 30 min at RT followed by incubation with an un-conjugated primary Ab or an negative control Ab for either 60 min at RT or ON at 4°C (Table 3 and 4). After rinsing in PBS for 5 min, the sections were incubated with appropriate fluorescently labeled secondary Abs for 30 min at RT in the dark. Subsequently, sections were washed twice in PBS for 5 min in dark. For single immunofluorescence staining, the sections were mounted with coverslips using DAPI containing vectashield as mounting medium. For double immunofluorescence staining, the sections were subjected to a second round of incubations with primary and secondary Abs (Table 4), as mentioned above.
Co-immunostaining for β-gal with CD31, calponin or macrophage-specific F4/80 was performed on cryosections by sequential incubation with primary antibodies. Lesional cell apoptosis was assessed by TUNEL assay (Roche) according to the manufacturer’s instructions. Digital images were recorded using a fluorescence microscope (see section 2.1) connected to a CCD camera (see section 2.1.1) and the image acquisition software DISKUS (Hilgers).

Quantification of SM22, α-SMA, calponin, Mac-2, or collagen type I was done by analyzing the positively stained area per lesion area using image analysis software (Image J) and expressed as percentage of lesion area. The analysis was performed by setting the threshold based on the background of the negative control staining. The CD3, β-gal, Ki67, TUNEL, and Mac-2 stainings were analyzed by counting the number of CD3⁺, β-gal⁺, Ki67⁺, TUNEL⁺ and Mac-2⁺ cells identified by a DAPI⁺ nucleus. The results were expressed as the percentage of CD3⁺, β-gal⁺, Ki67⁺, or Mac-2⁺ cells out of the total number of DAPI⁺ nuclei in the lesion area. CD31 and CXCL12 double immunostainings were quantified by counting the CD31⁺ and CXCL12⁺ cells in the lesions. The results were expressed as the percentage of CXCL12 and CD31 double positive cells out of the total number of CD31⁺ cells in the lesions. Similarly, Ki67 and SM22 double immunostaining were quantified by counting the Ki67⁺ and SM22⁺ cells in the lesions and expressed as percentage of SM22 and Ki67 double positive cells out of total number of SM22⁺ cells.
### Table 3: Single immunostaining protocols

<table>
<thead>
<tr>
<th>Ag</th>
<th>Antigen retrieval</th>
<th>Blocking</th>
<th>Primary Ab</th>
<th>Secondary Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM22</td>
<td>None</td>
<td>10% goat serum</td>
<td>1:200, 4°C, ON</td>
<td>goat-anti-rabbit IgG-Dylight549-conjugated Ab, 1:200</td>
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<tr>
<td>Calponin</td>
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<td>goat-anti-rabbit IgG-Dylight549-conjugated Ab, 1:200</td>
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<tr>
<td>Mac-2</td>
<td>None</td>
<td>Blocking solution*</td>
<td>1:400, 4°C, ON</td>
<td>donkey-anti-rat IgG-FITC-conjugated Ab, 1:100</td>
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<td>CD3</td>
<td>Citrate buffer (100°C)</td>
<td>10% goat serum</td>
<td>1:100, 4°C, ON</td>
<td>goat-anti-rabbit IgG-Dylight549-conjugated Ab, 1:400</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Citrate buffer (100°C)</td>
<td>10% goat serum</td>
<td>1:50, 4°C, ON</td>
<td>Donkey-anti-mouse-IgG-FITC-conjugated Ab, 1:100</td>
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<tr>
<td>CD31</td>
<td>Citrate buffer (100°C)</td>
<td>Blocking solution*</td>
<td>1:75, 37°C, 60 min</td>
<td>Donkey-anti-goat-IgG-Cy3-conjugated Ab, 1:200</td>
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<tr>
<td>CXCL12</td>
<td>Citrate buffer (100°C)</td>
<td>10% goat serum</td>
<td>1:50, 4°C, ON</td>
<td>Donkey-anti-mouse-IgG-FITC-conjugated Ab, 1:100</td>
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<td>β-gal</td>
<td>Citrate buffer (100°C)</td>
<td>10% goat serum</td>
<td>1:1000, 4°C, ON</td>
<td>Donkey-anti-rabbit-IgG-Cy3-conjugated Ab, 1:200</td>
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<tr>
<td>Ki67</td>
<td>Citrate buffer (100°C)</td>
<td>Blocking solution*</td>
<td>1:25, 4°C, ON</td>
<td>Donkey-anti-rat-IgG-Cy3-conjugated Ab, 1:300</td>
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*See section 2.3 for more details*
Table 4  Double immunostaining protocols

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<th>1st Secondary Ab</th>
<th>2nd Primary Ab</th>
<th>2nd Secondary Ab</th>
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<td>CD31/ CXCL12</td>
<td>Citrate buffer</td>
<td>Blocking solution*</td>
<td>CD31, 1:75, 37°C, 60 min</td>
<td>Donkey-anti-goat-IgG-Cy3-conjugated Ab, 1:200</td>
<td>CXCL12, 1:50, 4°C, ON</td>
<td>Donkey-anti-mouse-IgG-FITC-conjugated Ab, 1:100</td>
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<td></td>
<td>(100°C)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>CD31/ HIF-1α</td>
<td>Citrate buffer</td>
<td>Blocking solution*</td>
<td>CD31, 1:75, 37°C, 60 min</td>
<td>Donkey-anti-goat-IgG-Cy3-conjugated Ab, 1:200</td>
<td>HIF-1α, 1:50, 4°C, ON</td>
<td>Donkey-anti-mouse-IgG-FITC-conjugated Ab, 1:100</td>
</tr>
<tr>
<td></td>
<td>(100°C)</td>
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</tr>
<tr>
<td>Ki67/ SM22</td>
<td>Citrate buffer</td>
<td>Blocking solution*</td>
<td>Ki67; 1:25, 4°C, ON</td>
<td>Donkey-anti-rat-IgG-Cy3-conjugated Ab, 1:300</td>
<td>SM22, 1:200, RT, 60 min</td>
<td>goat-anti-rabbit IgG-Dylight549-conjugated Ab, 1:200</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>CXCL1/ HIF-1α</td>
<td>Citrate buffer</td>
<td>10% goat serum</td>
<td>CXCL1, 1:100, 37°C, 60 min</td>
<td>Donkey-anti-rabbit-IgG-Cy3-conjugated Ab, 1:200</td>
<td>HIF-1α, 1:50, 4°C, ON</td>
<td>Donkey-anti-mouse-IgG-FITC-conjugated Ab, 1:100</td>
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<td>Blocking solution*</td>
<td>CD31, 1:75, 37°C, 60 min</td>
<td>Donkey-anti-goat-IgG-Cy3-conjugated Ab, 1:200</td>
<td>CXCL1, 1:100, 37°C, 60 min</td>
<td>Donkey-anti-rabbit-IgG-FITC-conjugated Ab, 1:200</td>
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<tr>
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*See section 2.3 for more details

2.3.5  Blood cell count and differentiation

The blood cell count was determined using an automated hematology analyzer (see section 2.1). The differential leukocyte count was obtained manually by a blood film.

2.3.6  Serum analysis

At the end of the mouse experiments, approximately 200 - 300 µl of blood was collected in serum tubes (see section 2.3.6) and allowed to stand at RT for 2 hours. Subsequently, the tubes were centrifuged at 2000 g for 20 min and the serum in the supernatant was collected to measure lipid levels. Serum cholesterol and triglycerides levels were determined in a Vitros 250 automatic analyzer (see section 2.1.1).
2.4 Molecular biology methods

2.4.1 RNA isolation

Total RNA was isolated from ECs using the RNeasy mini kit (see section 2.5) according to the manufacturer’s instructions. Briefly, cells were first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNAases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the samples were then applied to an RNeasy Mini spin column, where the total RNA (> 200 bases) selectively binds to the membrane and contaminants are efficiently washed away. Traces of DNA that may be present are removed by treatment with DNAase I. Total RNA from carotid arteries fixed with PAXgene fixative solution was isolated using PAXgene Tissue RNA kit (PreAnalytix, Qiagen) according to the company’s instructions. The fixating and stabilizing solution used in this method preserves tissue morphology and the integrity of nucleic acids, which is normally lost by cross-linking and degradation in formalin-fixed tissues. Total RNA was isolated from carotid arteries of EC-Hif1a⁺/⁺ and EC-Hif1a⁻/⁻ mice at 2 weeks after partial ligation using mirVana™ miRNA Isolation kit (see section 2.5) according to the manufacturer’s protocol. Briefly, the samples were first lysed in a denaturing lysis solution, which stabilizes RNA and inactivates RNAases. The lysate was then extracted once with Acid-Phenol: Chloroform, which removes most of the other cellular components, leaving a semi-pure RNA sample. This extract was further purified over a glass-fiber filter to yield total RNA.

2.4.2 DNA isolation

Murine genomic DNA was isolated from tail tips and peripheral blood cells of Apoε⁻/⁻ mice using Bio Sprint 15 DNA isolation kit (see section 2.1.5). Magnetic particle technology is applied in this kit for DNA purification. Animal tail tips were lysed in 200 µl of ATL tissue lysis buffer (BioSprint 15) containing 20 µl of proteinase K at 56°C for ON. Lysed samples were processed further for genomic DNA isolation according to the manufacturer’s instructions. Blood cells were lysed in 200 µl of lysis buffer AL (BioSprint 15) containing 20 µl of Proteinase K at 70°C for 10 min. The resulting lysate was used for genomic DNA isolation according to the manufacturer’s instructions. Briefly, DNA in the lysate binds to the silica surface of the magnetic particles in the presence of a chaotropic salt. DNA bound to the magnetic particles is easily attracted to the magnetic rod supplied in the BioSprint workstation.
and separates out. Rapid purification of DNA was achieved by sequential washing of the magnetic particles followed by air-drying. High-quality DNA was eluted in the elution buffer.

2.4.3 **Quantification of DNA and RNA**

DNA and RNA concentrations and purity were determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280) in a spectrophotometer (see section 2.1.1). A A260/A280 ratio of 1.8 and 2.0 at pH 7.0 was considered as pure DNA and RNA, respectively.

2.4.4 **cDNA synthesis - Reverse transcription polymerase chain reaction (RT-PCR)**

RT-PCR involves synthesis of cDNA from a RNA template by the enzyme reverse transcriptase. The reverse transcription is a linear process and thus the cDNA can be used in further applications, such as real time PCR, which directly compare the mRNA level in different samples.

For cDNA synthesis, up to 1 µg of total RNA was mixed with 2.5 µl of random hexamer primers (Applied Biosystems) and supplemented with RNAase-free water to a final volume of 15 µl. To denature the RNA, the reaction mix was incubated at 70°C for 5 min and chilled on ice. Each sample was then supplemented with 10 µl of the reaction master mix containing the following (final concentrations in 25 µl are given): 1× reaction buffer, 1 mM dNTPs mix, and 200 U of the M-MLV RT enzyme (see section 2.1.5). RNAase-free water was added to reach the final volume of 25 µl. The resulting mix was incubated at 37°C for 60 min and finally at 85°C for 5 min to inactivate the reverse transcriptase enzyme. The cDNA sample were diluted in DNAase-free water and stored at -20°C until use.

2.4.5 **Quantitative real-time polymerase chain reaction**

Real-time PCR is a quantitative method to determine transcript expression levels using cDNA synthesized from the sample of interest as a template for gene amplification via PCR.

In this study, synthesized cDNA was used to determine the mRNA expression levels of HIF-1α and various chemokines and cytokines (see section 2.1.7) in stimulated mouse primary ECs and atherosclerotic carotid arteries. Briefly, equal amounts of cDNA (in a volume of 8 µl) were added to 96-well Fast Optical Reaction Plates (Applied Biosystems) in duplicate per biological sample. The SYBR green master mix (10 µl) (see section 2.1.5), 1 µl of forward primer (10 µM) and 1 µl of reverse primer (10 µM) were added to each well. Plates were loaded into a 7900HT PCR machine (see section 2.1.1) and incubated at 95°C for 10 min followed by 40 cycle of incubation at 95°C for 15 sec, 60°C for 30 sec and 72°C for...
Materials and methods

30 sec. Mouse Gapdh and β-actin genes were used either alone or in combination as reference genes. The relative gene expressions were normalized to the reference genes and logarithmically transformed (Qbase, Biogazelle).

The repopulation of the BM of irradiated mice with SM22-lacZ/Apoel BM cells was determined 3 weeks after transplantation by determining the lacZ gene copy number in the peripheral blood cells. The DNA was isolated from the peripheral blood cells using BioSprint (Qiagen, Germany). Standards were made using different concentrations of DNA from the SM22-lacZ/Apoel (lacZ DNA) and Apoel mice (Apoel DNA), while keeping the total DNA content at 40 ng, as follows:

1) 40 ng of lacZ DNA (denoting 100 % repopulation)
2) 30 ng of lacZ DNA + 10 ng of Apoel DNA (denoting 75 % repopulation)
3) 20 ng of lacZ DNA + 20 ng of Apoel DNA (denoting 50 % repopulation)
4) 10 ng of lacZ DNA + 30 ng of Apoel DNA (denoting 25 % repopulation)
5) 5 ng of lacZ DNA + 35 ng of Apoel DNA (denoting 12.5 % repopulation)
6) 40 ng of Apoel DNA (denoting 0 % repopulation)

DNA (40 ng) from peripheral blood cells of BMT mice and the above standards were added to the 96-well Fast Optical Reaction Plates (Applied Biosystems) in duplicate with 10 µl of the SYBR green master mix (Maxima SYBR Green qPCR master mix, Fermentas), 1 µl of lacZ forward primer (10 µM), and 1 µl of lacZ reverse primer (10 µM) (see section 2.1.7). Quantitative real-time PCR was performed using 7900HT Fast-Real Time PCR system (Applied Biosystems) using the following PCR conditions:

Initial Denaturation: 95°C - 10 min
Denaturation: 95°C – 15 sec
Annealing: 63°C – 30 sec (40 cycles)
Extension: 72°C – 30 sec

The percentage of cells harboring the lacZ gene in bone marrow chimeric mice was calculated according to the standard curve using a Microsoft Excel sheet.

2.5 Cell culture

2.5.1 Isolation and culture of mouse aortic endothelial cells (MAECs)

ECs from murine aortas were obtained by outgrowth of ECs from a tissue explant into a collagen matrix in vitro132. First, the collagen gel was prepared by mixing 1.75 ml of 3x DMEM (see section 2.1.3) and 2.625 ml of 1x DMEM with 30% FCS (see section 2.1.3) on
ice. Subsequently, 3 ml of ice-cold rat tail collagen type 1 (3.75 mg/ml) (see section 2.1.5) and 0.5 ml of 0.1 M NaOH were added. This solution was pipetted into the wells (500 µl each well) of a 24-well plate (see section 2.1.6). Following incubation of theses plates for 2 hours at 37°C, EC growth medium was added to each well and incubated ON. The EC growth medium was removed before placing the aortic tissue explants into the wells.

To harvest the murine aortas, C57BL/6 mice were deeply anaesthetized and the chest was opened by a ventral incision to expose the heart. Mice were shortly perfused with ice-cold PBS through a tube inserted into the left ventricle of the heart to remove the blood. Then, the thoracic aorta was harvested aseptically and cut into 6 small pieces, which were placed on the collagen gel with the luminal surface facing towards the gel. The ECs that migrated into the gel after 5 - 6 days were isolated by digesting the gel with 0.3 % collagenase (see section 2.1.4) and seeded to tissue culture flasks pre-coated with the collagen gel. The ECs were cultured in EC growth medium (see section 2.1.4) until they reached 80 - 90% confluence. The EC growth medium was changed every 2 days. ECs were passaged by trypsinization, splitting (1:3) and seeding in new flasks coated with the collagen gel. After three passages, lectin staining was performed to check the purity of the ECs.

To perform lectin staining, ECs were cultured in culture dishes (2 cm diameter) until confluent. After removing the medium, cells were washed in PBS and fixed with 4 % PFA (see section 2.1.3) for 10 min at RT. Unspecific binding sites were blocked by incubation with 0.1% BSA for 15 min. After a washing step, cells were incubated with 40 µg/ml of FITC-conjugated lectin (*Lycopersicon esculentum*, Sigma) for 30 min at RT and then rinsed with PBS (3 × 5 min). The cells were mounted in a DAPI containing mounting solution (Vectashield, Vector Labs), coverslipped, and analyzed by a fluorescence microscope. The purity of the cells was assessed by determining the percentage of lectin+ cells in 5 different microscopic visual fields.

### 2.5.2 Culturing of MonoMac-6 (MM6) cells

MM6 is a human monocytic cell line derived from the peripheral blood of a patient with acute monocytic leukemia\textsuperscript{133}. To culture MM6 cells, frozen cells were thawed and 9 ml of the cell culture medium [RPMI 1640 (Gibco), 2 mM L-Glutamine (Gibco), Penicillin 200 U/ml and Streptomycin 200 µg/ml (Gibco), non essential amino acids (Gibco), OPI supplement containing oxaloacetic acid, sodium pyruvate and insulin (Sigma) and 10 % FCS] was added to 1 ml of the cell suspension. Following centrifugation at 300 g for 5 min, MM6 cells were resuspended in culture medium. Then, $10 \times 10^5$ cells were seeded in a 24-well cell culture
plates and cultured ON before seeding the MM6 cells at a density of $2 \times 10^5$ cells/ml per well (2 ml in each well) in a 24-well plate. MM6 cells were cultured in an incubator (5% CO$_2$, 37°C) for 3 - 4 days before they were used for experiments.

### 2.5.3 Preparation of moxLDL and nLDL

MoxLDL was prepared by incubation of human native LDL (nLDL, 1 mg/ml, Calbiochem, Darmstadt, Germany) in the presence of 5 µM CuSO$_4$ at 37°C for 4 hours. The oxidation of the sample was stopped by the addition of 10 µM EDTA (final concentration). The copper-treated LDL was passed through PD-10 desalting column (GE Healthcare, Uppsala, Sweden) to remove the salts. MoxLDL was finally eluted by sterile PBS (1.5 ml). To prepare nLDL for experimental use as a negative control, all the above steps were performed except the addition of CuSO$_4$. The protein concentration was measured using DC protein assay kit (see section 2.5) using BSA as a standard. The extent of oxidation was evaluated by quantifying the formation of thiobarbituric acid-reactive species (TBARS) spectrophotometrically (532 nm) using TBARS assay kit (Cayman). The moxLDL and nLDL preparations were stored at 4°C for no longer than 14 days.

### 2.5.4 Stimulation of MAECs

MAECs were seeded into 6-well plates and grown to a density of $2 \times 10^5$ cells per well in complete endothelial cell growth medium. Following serum starvation for 18-20 hours, the culture medium was replaced with fresh endothelial cell growth medium. LPA20:4 (10 µM), LPA18:0 (10 µM), moxLDL (50 µg/ml), nLDL (50 µg/ml), or AT-2 (10 nM, dissolved in sterile water) were added to the medium and incubated at 37°C for 2, 4, or 6 hours. Cells in the control group were cultured in either normal endothelial cell growth medium only or containing equal volumes of sterile PBS. In another experiment, MAECs were stimulated with either LPA20:4 (10 µM) or moxLDL (50 µg/ml), and the LPA receptor antagonist Ki16425 (100 µM) for 4 or 6 hours. The cell culture medium was collected and stored at -80°C until the CXCL1 concentration was determined in these samples by CXCL1 ELISA (see section 2.3.2). The cells were harvested for RNA isolation (see section 2.4.1).

### 2.5.5 In vitro siRNA treatment of MAECs

MAECs were seeded in 24-well plates with 500 µl of endothelial cell growth medium per well. Endothelial cell growth medium was replaced by the ACCELL siRNA delivery medium (see section 2.1.4) with or without non-targeting control siRNA or HIF-1α siRNA (1 µM
Materials and methods

Each, SMART POOL, Dharmacon) and incubated at 37°C for 72 hours. Following transfection with siRNA, the cell monolayer was washed and incubated with endothelial cell growth medium with or without LPA20:4 (10 µM) or moxLDL (50 µg/ml) for 4 or 6 hours. The cell culture medium was collected and stored at -80°C. The cells were harvested for RNA isolation (see section 2.4.1).

2.6 Functional assays

2.6.1 Flow chamber assay

Flow chamber assays allow studying the interaction between different types of cells exposed to dynamic fluid flow. These assays have been widely used to examine the adhesion of leukocytes (flowing cells) and the endothelium (adherent monolayer) under definite shear stress conditions. Monocyte adhesion to a confluent monolayer of MAECs activated by moxLDL (50 µg/ml) or LPA20:4 (10 µM) (6 hours) was investigated using a parallel plate flow chamber as previously described. MM6 cells (0.5 × 10^6 cells/ml) in HH-medium (see section 2.1.3) were perfused over MAECs under physiological shear stress conditions using a perfusion pump (WPI-SP100I, Berlin, Germany) adjusted to a constant shear rate of 1.5 dyne/cm². Interactions of monocytes with the endothelial monolayer were visualized by videomicroscopy using 10× magnifications. For each experiment, recordings were made for 15 sec for each of the 5 visual fields studied. After a total observation period of 1 min, firmly attached monocytes (at least 15 sec) were counted per microscopic visual field. At least three independent experiments were performed in each group.

2.7 Data illustration and statistical analysis

Data were expressed as means ± SEM. Statistical analysis was performed using 1-way or 2-way ANOVA followed by the Newman-Keuls, Bonferroni’s post-test or Fischer’s LSD test or an unpaired two-tailed Student’s t-test using Graph Pad Prism 6 software (San Diego, CA). For flow chamber assay, data were analyzed as mean ± SEM of three independent experiments. In each experiment, the mean of 5 randomly chosen microscopic view fields was evaluated. Probability values of p < 0.05 were considered statistically significant.
3 Result

3.1 CXCL12-mediated stabilization of vulnerable lesions by SPCs

3.1.1 CXCL12 treatment mobilized SPCs

To determine whether CXCL12 injection can mobilize Sca-1^+Lin^- cells, a dose-response study was performed. C57BL/6 mice were injected (i.v.) with 100 ng or 500 ng of mouse recombinant CXCL12 or vehicle (PBS) and mobilization of Sca-1^+Lin^- cells was analyzed by flow cytometry (Figure 10). Treatment with 500 ng of CXCL12 increased circulating Sca-1^+Lin^- cells by 6-fold at 4 hours post injection as compared to baseline (Figure 10A, B). Moreover, treatment with 500 ng of CXCL12 increased the circulating Sca-1^+Lin^- cells level by 6-fold compared to 100 ng of CXCL12 and by 2-fold compared to vehicle (Figure 10A, B) at 4 hours. After the peak at 4 hours, the circulating Sca-1^+Lin^- cell count decreased to baseline level gradually after 24 hours in 500 ng CXCL12 treated group (Figure 10B). To further characterize the Sca-1^+Lin^- cell type, the expression of Pdgfr-β was studied. Sca-1^+Lin^- cells, which also express Pdgfr-β were increased at 4 hours after CXCL12 (500 ng) treatment (Figure 10C).

Figure 10: SPC mobilization by injection of CXCL12.
(A) Representative dot plots showing the gated events of Sca1^+Lin^- cells (inserted numbers indicate % Sca1^+Lin^- cells) (B) Time course and dose response analysis of Sca1^+Lin^- cell mobilization in C57BL/6 mice after i.v. injection of CXCL12 and vehicle (PBS) as determined by flow cytometry. (C) Quantification of Sca-1^+Lin^- Pdgfr-β^+ cells after treatment with CXCL12 and vehicle at 4 hours after injecting PBS or 500 ng CXCL12. Differences between the groups were evaluated by 1-way ANOVA (A) and Student-t-test (C). Data are expressed as means ± SEM, N = 3-4 mice per group, *p < 0.05
In addition to hematopoietic progenitor cells, CXCL12 treatment also mobilizes leukocytes to the circulation. To study whether the injection of 500 ng of CXCL12 also expands the mature hematopoietic cell population in the circulation, the peripheral blood count was analyzed. The total leucocyte count, platelet count as well as the differential leucocyte count, such as lymphocytes, neutrophils, monocytes, and eosinophils, were not different between CXCL12 (500 ng)- and PBS-treated mice after 4 hours (Table 5). Taken together, these data suggest that CXCL12 injection transiently, rapidly, and specifically mobilizes SPCs into the circulation in a dose-dependent manner.

### Table 5 Number of differentiated blood cells in C57BL/6 mice at 4 hours after injection of PBS or CXCL12

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>CXCL12 (100 ng)</th>
<th>CXCL12 (500 ng)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WBC (×10^3/µl)</strong></td>
<td>3557 ± 595</td>
<td>3835 ± 613</td>
<td>4312 ± 715</td>
<td>0.3282</td>
</tr>
<tr>
<td><strong>Platelets (×10^3/µl)</strong></td>
<td>560.3 ± 54.3</td>
<td>610.3 ± 55.15</td>
<td>457.7 ± 158.9</td>
<td>0.5880</td>
</tr>
<tr>
<td><strong>Lymphocytes (×10^3/µl)</strong></td>
<td>1937 ± 528.2</td>
<td>2498 ± 482.8</td>
<td>3119 ± 803.1</td>
<td>0.4529</td>
</tr>
<tr>
<td><strong>Neutrophils (×10^3/µl)</strong></td>
<td>430.0 ± 96.0</td>
<td>637.3 ± 125.3</td>
<td>492.0 ± 93.3</td>
<td>0.4185</td>
</tr>
<tr>
<td><strong>Monocytes (×10^3/µl)</strong></td>
<td>96.7 ± 6.4</td>
<td>80.0 ± 2.3</td>
<td>131.3 ± 76.2</td>
<td>0.7173</td>
</tr>
<tr>
<td><strong>Eosinophils (×10^3/µl)</strong></td>
<td>36.3 ± 16.4</td>
<td>70.33 ± 26.1</td>
<td>89.0 ± 34.2</td>
<td>0.4196</td>
</tr>
</tbody>
</table>

N = 3 mice per group; data represent means ± SEM.

### 3.1.2 CXCL12 treatment diminished the physiological CXCL12 gradient between the BM and circulation

An increase in plasma CXCL12 levels or a decrease in BM CXCL12 levels both reduce the physiological difference between the lower CXCL12 concentrations in the circulation and the higher CXCL12 levels in the BM and mobilize hematopoietic progenitor cells from the BM to the circulation. To verify the effect of CXCL12 injection on the CXCL12 gradient, CXCL12 levels were determined after injection of CXCL12 in the plasma and BM by ELISA. Before the injection of CXCL12, the BM-CXCL12 levels were 3-fold higher than in the...
plasma (Figure 11). Following CXCL12 injection (500 ng), the plasma levels of CXCL12 increased to 3-fold after 4 hours and returned to the baseline level after 24 hours (Figure 11). However, the BM CXCL12 levels were not significantly different at 4 hours after CXCL12 injection compared to the baseline (Figure 11). Thus, the CXCL12 levels in the plasma and BM were similar at 4 hours after CXCL12 injection. These results indicate that CXCL12 (500 ng) treatment does not affect the BM but increases the plasma CXCL12 concentration to the level of BM, thus abrogating the physiological CXCL12 gradient between the peripheral blood and the BM.

3.1.3 Partial ligation of carotid arteries led to unstable atherosclerotic lesions

To further investigate the effects of CXCL12 on lesion stabilization, a mouse model of disturbed flow-induced atherosclerosis as described by Nam et al.\textsuperscript{122} was established that is characterized by development of unstable lesions. Partial ligation of the LCA of Apoe\textsuperscript{-/-} mice was performed, which leads acutely to disturbed flow. In vivo analysis by micro-CT angiography revealed a reduction of the luminal diameter of the LCA by approximately 48\% as compared to the unligated RCA in Apoe\textsuperscript{-/-} mice at 6 weeks after partial ligation and feeding a HFD (Figure 12A, B). Although the lesion size in the LCA at 6 weeks tended to be higher than at 4 weeks, the difference was not statistically significant (Figure 12C, D). However, the lesional macrophage accumulation was significantly increased by 60\%, while the SMCs decreased by 50-70 \% in carotid lesions at 6 weeks compared to 4 weeks after partial ligation (Figure 13). In summary, partial carotid ligation and feeding a HFD in Apoe\textsuperscript{-/-} mice resulted in a stenotic and unstable lesion after 6 weeks.
**Results**

Figure 12: Atherosclerotic lesions after partial ligation of the carotid artery.
(A) Representative 3D renderings of a micro-CT angiography scan of the aortic arch (Ao) and the right (RC) and left (LC) carotid arteries from Apoe<sup>−/−</sup> mice 6 weeks after partial ligation of the LCA. Jugular vein, JV. (B) The luminal diameters of the RCA and LCA in Apoe<sup>−/−</sup> mice 6 weeks after partial ligation of the LCA were determined in micro-CT angiography scans. (C) An EvG-stained section of the carotid artery obtained from a stenotic area 6 weeks following partial ligation is shown. Scale bar, 200 μm. (D) Quantification of EvG-stained lesions of the carotid arteries. Differences between the groups were evaluated by Student’s-t-test. Data are expressed as means ± SEM. N= 4 mice per group, ***p < 0.0001.

Figure 13: Phenotype of carotid artery lesions induced by disturbed flow and HFD.
(A, C) The lesional macrophage (Mac-2, A) and SMC content (SM22, C) were determined 4 and 6 weeks after partial carotid ligation by immunostaining. Scale bars, 200 μm. (B, D) Quantification of Mac-2<sup>+</sup> (B) and SM22<sup>+</sup> (D) areas in lesions was performed. Differences between the groups were evaluated by Student’s-t-test. Data are expressed as means ± SEM. **p < 0.01, ***p < 0.001, N = 4 mice per group.
3.1.4 Intermittent CXCL12 treatment mobilized SPCs

To analyze the effect of the CXCL12 treatment on atherosclerotic lesion stabilization, Apoe\textsuperscript{-/-} mice were injected with 500 ng of CXCL12 twice weekly for 4 weeks starting 2 weeks after partial carotid ligation (Figure 14).

![Figure 14: CXCL12 treatment of Apoe\textsuperscript{-/-} after partial carotid ligation.](image)

Partial ligation of the carotid artery of Apoe\textsuperscript{-/-} mice was performed at day 0 and the mice were fed a HFD. CXCL12 (500 ng) or PBS treatment (i.v., twice weekly) was started in the 3\textsuperscript{rd} week after partial carotid ligation and continued for 4 weeks. Before sacrificing, micro-CT angiography was performed at day 43 after partial carotid ligation.

To determine if repeated treatment with CXCL12 still increases Sca-1\textsuperscript{+}Lin\textsuperscript{-} cells in the circulation, flow cytometric analysis of the peripheral blood cells was performed 4 hours after the last CXCL12 injection. The percentage of Sca-1\textsuperscript{+}Lin\textsuperscript{-} cells was increased in the CXCL12-treated mice compared to PBS even after the 8\textsuperscript{th} injection of CXCL12, indicating that each single treatment with CXCL12 mobilized Sca-1\textsuperscript{+}Lin\textsuperscript{-} cells (Figure 15).

![Figure 15: Elevated circulating SPC count after repetitive CXCL12 treatment.](image)

(A) Representative dot plots displaying the gated events of Sca1\textsuperscript{+}Lin\textsuperscript{-} cells by flow cytometry in PBS- and CXCL12-treated mice. (B) Quantification of circulating Sca1\textsuperscript{+}Lin\textsuperscript{-} cells at 4 hours after the last CXCL12 treatment in Apoe\textsuperscript{-/-} mice with partial carotid ligation as compared to PBS. Differences between the groups were evaluated by Student’s-t-test. Data represent means ± SEM, N = 5-7 mice per group, *p < 0.05.
Furthermore, the total leukocyte count and the differential leukocyte count were not significantly different between the groups (Table 6). Together, these results indicate that repeated treatment mobilized SPCs without affecting the circulating mature hematopoietic cells.

Table 6  WBCs and leukocyte subtypes in Apoe\(^{-/-}\) mice at 6 weeks after partial ligation and 4 weeks of treatment with CXCL12 or PBS

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>CXCL12 (500 ng)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count ((\times 10^3/\mu l))</td>
<td>6825 ± 400</td>
<td>5375 ± 175</td>
<td>0.4488</td>
</tr>
<tr>
<td>Lymphocyte count ((\times 10^3/\mu l))</td>
<td>4923 ± 486.6</td>
<td>3791 ± 1409</td>
<td>0.4765</td>
</tr>
<tr>
<td>Neutrophil count ((\times 10^3/\mu l))</td>
<td>1268 ± 303.2</td>
<td>1128 ± 132.9</td>
<td>0.6860</td>
</tr>
<tr>
<td>Monocyte count ((\times 10^3/\mu l))</td>
<td>375.5 ± 82.68</td>
<td>258.0 ± 114.2</td>
<td>0.4365</td>
</tr>
<tr>
<td>Eosinophil count ((\times 10^3/\mu l))</td>
<td>258.5 ± 93.81</td>
<td>198.0 ± 100.5</td>
<td>0.6753</td>
</tr>
</tbody>
</table>

N = 4 mice per group; data represent means ± SEM.

3.1.5  Effect of CXCL12 treatment on lesion size

To examine the effect of CXCL12 on the lesion size of the LCA and the aorta, Apoe\(^{-/-}\) mice were treated with CXCL12 or PBS as described in Figure 14. The Lesion size and the medial area in the LCA were not altered between CXCL12- and PBS-treated groups after 4 weeks as determined by EvG staining (Figure 16).
Furthermore, the *in vivo* luminal diameters of the partially ligated LCA were not different between CXCL12- and PBS-treated mice after 4 weeks of treatment as determined by micro-CT angiography (Figure 17).

![Figure 16: Effect of CXCL12 treatment on carotid lesion size.](image)

(A) Representative micrographs of EvG stained carotid lesions after PBS (left) and CXCL12 (right) treatment. Scale bars, 200 µm. Quantification of the lesion area (B), the medial area (C), and the length of the external elastic lamina (D) after PBS or CXCL12 (500 ng) treatment was determined. Evaluation of data was performed by Student’s-t-test. Data represent mean ± SEM. N = 6-7 mice per group.

**Figure 16: Effect of CXCL12 treatment on carotid lesion size.**

(A) Representative micrographs of EvG stained carotid lesions after PBS (left) and CXCL12 (right) treatment. Scale bars, 200 µm. Quantification of the lesion area (B), the medial area (C), and the length of the external elastic lamina (D) after PBS or CXCL12 (500 ng) treatment was determined. Evaluation of data was performed by Student’s-t-test. Data represent mean ± SEM. N = 6-7 mice per group.

![Figure 17: Effect of CXCL12 treatment on in vivo luminal diameters of partially ligated carotid arteries.](image)

(A) Representative 3D renderings of micro-CT scan of partially ligated Apoe^−/−_mice showing the *in vivo_ luminal diameter of LC and right RC after PBS (left) and CXCL12 (right) treatment (B) Quantification of the carotid luminal diameter in *vivo*. Differences between the groups were evaluated by Student’s-t-test. Data represent means ± SEM, N = 6-7 mice per group.

![Figure 17: Effect of CXCL12 treatment on in vivo luminal diameters of partially ligated carotid arteries.](image)
Moreover, CXCL12 did not affect the serum cholesterol and triglyceride levels (Table 7).

Table 7  Serum cholesterol and triglyceride levels in Apoe<sup>−/−</sup> mice at 6 weeks after partial ligation and 4 weeks of PBS or CXCL12 treatment

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>CXCL12 (500ng)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>18.06 ± 1.9</td>
<td>18.87 ± 1.4</td>
<td>0.7393</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.94 ± 0.1</td>
<td>1.38 ± 0.5</td>
<td>0.4501</td>
</tr>
</tbody>
</table>

N = 5-6 mice per group; data represent means ± SEM.

The effect of CXCL12 treatment on native atherosclerosis was analyzed in the aortic arch of partially ligated Apoe<sup>−/−</sup> mice by oil red O staining. The lipid content in the aortic arch was not significantly changed by the CXCL12 treatment compared with PBS treatment (Figure 18).

![Figure 18: Effect of CXCL12 on aortic arch lesion size.](image)

(A) Representative images of oil red O (red) stained aortic arches of PBS- (left) and CXCL12- (right) treated Apoe<sup>−/−</sup> mice. (B) Quantification of oil red O<sup>+</sup> area in aortic arch. Data were analyzed by Student’s-t-test. Data represent means ± SEM. N = 6-7 mice per group.

To evaluate the long-term effect of CXCL12, treatment of Apoe<sup>−/−</sup> mice after partial carotid ligation with CXCL12 or PBS (twice weekly) was stopped after 4 weeks and the lesions were analyzed after more 2 weeks without treatment (Figure 19).
Results

Figure 19: Experimental scheme for evaluating long-term effects of CXCL12 treatment.
Partial ligation of the LCA in Apoe\(^{-/-}\) mice was performed on day 0 and mice were subsequently fed a HFD. CXCL12 (500 ng) or PBS treatment (i.v., biweekly) was started in the third week after partial carotid ligation and continued for 4 weeks. Mice remained untreated for additional 2 weeks and were sacrificed 8 weeks after partial carotid ligation.

The lesion size of the LCA was comparable between both groups as quantified by EvG staining (Figure 20A). Similarly, the oil red O\(^{+}\) area in the complete aorta was not significantly different between both groups (Figure 20B). In conclusion, there was no long-term effect of repetitive CXCL12 treatment on atherosclerotic lesion size.

Figure 20: Long-term effects of CXCL12 on lesion size.
Quantification of lesion areas in EvG-stained carotid lesions (A) and in oil red O-stained whole aortas (B) was performed. Differences between the groups were evaluated by Student’s-t-test. Data represent means ± SEM, N = 6-7 mice per group.

3.1.6 Effect of CXCL12 treatment on FC thickness

Next, the effect of CXCL12 treatment on the FC thickness and the lipid core area was analyzed in EvG-stained lesions. CXCL12 treatment increased the FC thickness by 3-fold as compared to PBS-treated mice. However, the lipid core area was not significantly different between CXCL12- and PBS- treated mice (Figure 21).
Figure 21: Effect of CXCL12 on FC thickness and lipid core size.

(A) Representative micrographs showing FC thickness (black bars) and the lipid core area (black circle) in PBS- (left) or CXCL12 (500 ng, right)-treated Apoe<sup>−/−</sup> mice after partial carotid ligation. Scale bars, 200 µm. Quantification of the FC thickness (B) and the lipid core area (C) in CXCL12- or PBS-treated Apoe<sup>−/−</sup> mice after partial carotid ligation. Differences between the groups were evaluated by Student’s-t-test. Data represent means ± SEM. *p < 0.05, N=5-7 mice per group.

To further investigate the cell density (cell no./ matrix ratio) in the FC, the ratio between the cell number (estimated by DAPI<sup>+</sup> staining) and the extracellular matrix in the FC region (as determined by collagen-I<sup>+</sup> immunostaining) was evaluated. Cell density in the FC was substantially increased in CXCL12-treated mice compared to PBS (Figure 22).

Figure 22: Effect of CXCL12 treatment on cell density in the FC.

(A) Micrographs representing collagen-I immunostaining with DAPI overlay in carotid lesions of PBS- or CXCL12-treated mice. Arrows delineate the FC region. Scale bars, 100 µm. (B) Quantification of the cell density in the FC region of lesions in partially ligated carotid arteries in CXCL12- and PBS-treated Apoe<sup>−/−</sup> mice. Scale bars, 50 µm. Differences between the groups were evaluated by Student’s-t-test. Data represent means ± SEM. *p < 0.05, N = 4–5 mice per group.

Further evaluation of long-term effects on the FC after CXCL12 application revealed a 2.5-fold increase in FC thickness in CXCL12-treated as compared to PBS-treated mice (Figure 23 A, B). However, no effect on lipid core size was observed between both groups (Figure 23 A, C). Taken together, these data indicate that systemic application of CXCL12
Results

resulted in a sustained increase of the FC thickness of in partially ligated carotid arteries due to an increase in the cell number without affecting the lipid core size.

Figure 23: Increased FC thickness as a longstanding effect of CXCL12 treatment.
(A) Representative EvG-stained lesions of PBS- (left) and CXCL12- (left) treated mice displaying FC (black line) and lipid core (black circle). Scale bars, 200 μm. Analysis of the FC thickness (B) and the lipid core area (C). Differences between groups were evaluated by Student’s-t-test. Data represent means ± SEM. **p < 0.01, N = 6 mice per group.

3.1.7 Effect of CXCL12 on lesion cell proliferation and cell death

Cellular proliferation and apoptosis has been linked to CXCL12.136 To investigate the effects of CXCL12 on lesional cell apoptosis and proliferation, TUNEL assays and Ki67 immunostainings were performed, respectively. No difference in the percentage of TUNEL+ cells was observed between the groups (Figure 24).

Figure 24: Effect of CXCL12 on lesional cell apoptosis.
(A) Representative TUNEL staining (red) in lesions of partially ligated carotid arteries. Nuclei were counterstained with DAPI. The arrows indicate TUNEL+ nuclei. Scale bars, 100 μm. (B) Quantification of TUNEL+ cells in the lesions. Data were evaluated by Student’s-t-test. Data represent means ± SEM. Data represent means ± SEM. N=6-7 mice per group.

However, percentage of lesional Ki67+ cells in lesion was significantly increased by 60% in CXCL12-treated compared to PBS-treated mice (Figure 25 A, B). Moreover, the
Ki67$^+$ cells were significantly increased in the FC region of CXCL12-treated compared to PBS-treated mice (Figure 25C) implicating that CXCL12 treatment increased proliferation but not apoptosis of lesional cells.

![Figure 25: Effect of CXCL12 on lesional cell proliferation.](image)

(A) Representative Ki67 immunostaining shown. Nuclei were counterstained with DAPI (blue). The arrows indicate Ki67+ nuclei. Scale bars, 100 µm. Quantification of Ki67$^+$ cells in (B) lesions and the FC region (C). Differences between groups were evaluated by Student’s-t-test. Data represent means ± SEM, * p< 0.05, N=6-7 mice per group.

### 3.1.8 Effect of CXCL12 on inflammatory cell content

CXCL12 increases recruitment of inflammatory cells, such as monocytes, neutrophils and T-lymphocytes, to the site of injury.\textsuperscript{137, 138} Therefore, the effect of CXCL12 on the lesional inflammatory cell content was studied by immunostaining and histology. Quantification of lesional macrophages by immunostaining (with Mac-2 Ab) and by flow cytometry (with F4/80 Ab) showed a significant reduction in the relative Mac-2$^+$ area and F4/80$^+$ cell number (Figure 26). Similarly, analysis of long-term effect of CXCL12 application on lesional macrophages demonstrated a substantial reduction of the relative Mac-2$^+$ area in lesions of the partially ligated carotid arteries of CXCL12-treated compared to PBS-treated mice (Figure 27).
Figure 26: CXCL12 treatment decreased lesional macrophages.
(A) Typical photomicrographs of sections from partially ligated carotid arteries immunostained for Mac-2 after PBS (left) or CXCL12 (right) treatment to determine the lesional macrophage content. Arrows (white) delineate the lesions. Scale bars, 200 µm. (B) Quantification of the relative lesional Mac-2^+ area. (C) Characteristic dot plot showing the analysis of F4/80^+ cells in lesions. (D) Quantification of F4/80^+ cells in lesional PI cells. Data were analyzed by Student’s-t-test. Data represent means ± SEM, N=6-7 mice per group, *p < 0.05.

Figure 27: Long-term effects of CXCL12 on lesional macrophages.
(A) Representative images of Mac-2 immunostainings of partially ligated carotid arteries harvested 2 weeks after the termination of a 4-weeks CXCL12 or PBS treatment. Nuclei were counterstained with DAPI (blue). Scale bars, 200 µm. (B) Quantification of the relative macrophage content in lesions of PBS- or CXCL12-treated mice. Data were analyzed by Student’s-t-test. Data represent means ± SEM, N=6 mice per group, *p < 0.05.
The number of neutrophils and CD3+ T-cells in the lesions of the partially ligated carotid artery was low compared to other cell types and treatment with CXCL12 did not significantly affect the lesional neutrophil and CD3+ T-cell number compared to PBS (Figure 28). In conclusion, CXCL12 treatment specifically reduced the lesional macrophage content, but not the number of other immune cells, such as neutrophils and T-cells, in the lesions.

Figure 28: Effect of CXCL12 on lesional neutrophils and CD3+ cells.
(A) Cholinesterase stainings (red) of lesions in partially ligated carotid arteries showing lesional neutrophils (arrows). Scale bars, 50 µm. (Right panel) Quantification of cholinesterase+ cells in lesions. (B) Immunostainings of CD3 (red, white arrows) is shown. Nuclei were counterstained with DAPI (blue). Scale bars, 100 µm. (Right panel) Quantification of the CD3+ cells in the lesions. Data were analyzed by Student’s-t-test. Data represent means ± SEM, N=6-7 mice per group.

3.1.9 Effect of CXCL12 on lesional SMCs and collagen
To analyze the effect of CXCL12 treatment on lesional SMCs, SM22 immunostainings were performed. The relative lesional SMC content was significantly increased at 6 weeks after partial ligation of the carotid artery and 4 weeks of CXCL12 treatment compared with PBS (Figure 29).
Figure 29: CXCL12 increased lesional SMCs.
(A) Representative photomicrographs of sections of the partially ligated carotid artery immunostained for SM22 after PBS or CXCL12 treatment. The arrows (white) delineate the lesions. Scale bars, 200 µm. (B) Analysis of the relative lesional SM22 content in CXCL12- and PBS-treated mice. Differences between groups were evaluated by Student's-t-test. Data represent means ± SEM, N=6-7 mice per group **p<0.01.

In addition, the relative lesional SMC content in partially ligated carotid arteries remained increased 2 weeks after the 4-weeks treatment with CXCL12 had been terminated as compared to PBS treatment (Figure 30).

Figure 30: Long-term effects of CXCL12 on lesional SMCs.
(A) Representative micrographs demonstrating SM22 immunostaining of lesions from partially ligated carotid arteries 2 weeks after the 4-weeks treatment with CXCL12 or PBS had been stopped. Scale bars, 200 µm. (B) Quantification of the relative SM22 immunopositive area in the lesions of the carotid artery. Differences between groups were evaluated by Student’s-t-test. Data represent means ± SEM, N=6-7 mice per group, **p<0.01.

Moreover, the lesional collagen-I content was also substantially increased in CXCL12-treated as compared to vehicle-treated mice as determined by immunostaining (Figure 31). The lesional collagen-I was still increased 2 weeks after the CXCL12 treatment had been terminated (Figure 32).
**Figure 31: CXCL12 increased the lesional collagen-I content.**

(A) Representative immunostainings demonstrating collagen-I (red) expression in lesions of the carotid arteries 6 weeks after partial ligation and a 4-weeks treatment with CXCL12- or PBS. Nuclei were counterstained with DAPI (blue). Scale bars, 200 µm. (B) Quantification of the collagen-I+ area in the lesions of the partially ligated carotid arteries. Differences between groups were evaluated by Student’s-t-test. Data represent means ± SEM, N=6-7 mice per group, **p<0.01.

**Figure 32: Long-term effects of CXCL12 on the lesional collagen-I content.**

(A) Representative immunostainings demonstrating collagen-I (green) expression in lesions of the carotid arteries 8 weeks after partial ligation and 2 weeks after the 4-weeks treatment with CXCL12- or PBS had been terminated. Nuclei were counterstained with DAPI (blue). Scale bars, 200 µm. (B) Quantification of the collagen-I+ area in the lesions of the carotid arteries. Differences between groups were evaluated by Student's-t-test. Data represent means ± SEM, N=6-7 mice per group, ***p<0.001.

Double immunostaining of the proliferation marker, Ki67, and the SMC marker, SM22, showed increased proliferation of SMCs in the lesions of the carotid arteries 6 weeks after partial ligation in CXCL12-treated as compared to PBS-treated mice (Figure 33).

Taken together, these results demonstrate that CXCL12 increased the lesional SMC and collagen-I content and the proliferation of lesional SMCs. This effect of CXCL12 on the lesional SMCs and collagen-I was maintained even after termination of the CXCL12 treatment.
Figure 33: CXCL12 treatment increased SMC proliferation in lesions.
(A) Representative double immunostainings of Ki67 (pink) and SM22 (green) in lesions of the carotid artery 6 weeks after partial ligation and treatment with CXCL12 or PBS. Nuclei were counterstained with DAPI. The arrows indicate Ki67+ SMCs. Scale bars, 50 μm. (B) Quantification of Ki67+ and SM22+ cells in lesion. Differences between the groups were evaluated by Student’s-t-test. Data represent means ± SEM, N=6-7 mice per group, *p<0.05.

3.1.10 CXCL12-mediated recruitment of BM-derived SPCs to lesion site

Recruitment of BM-derived SPCs after the CXCL12 treatment into the lesions was determined after BMT of irradiated Apoe−/− mice with un-fractioned BM cells from SM22 LacZ/Apoe−/− mice. The efficiency of the BMT was approximately 50-55% as determined at 3 weeks after transplantation by real-time PCR. The β-gal activity in lesions of the carotid artery was determined 6 weeks after partial ligation and treatment with CXCL12 or PBS by X-gal staining. Positive X-gal staining (blue chromogen) was detected in the lesion of CXCL12-treated mice (right), while lesions of PBS- (left) treated mice did not show any X-gal staining (Figure 34).

LacZ+ cells in the lesions were also studied by β-gal immunostainings. In CXCL12-treated mice, β-gal immunopositive cells were detected in the lesions of the carotid artery 6 weeks after partial ligation (Figure 35). In contrast, β-gal immunopositive cells were hardly
detectable in the partially ligated carotid artery of PBS-treated mice or in the unligated RCA of CXCL12-treated mice suggesting that treatment with CXCL12 recruited BM-derived SPCs specifically to atherosclerotic lesions (Figure 35).

**Figure 35: CXCL12 treatment recruited BM-derived SPCs specifically to the lesion site.** Representative photomicrographs showing immunostainings of β-gal (red) in the partially ligated LCA of PBS- (left) or CXCL12- treated mice (middle) and in the unligated RCA (right) of CXCL12-treated mice following BMT of SM22-LacZ/Apoε−/− BM cells. Nuclei were counterstained with DAPI (blue). The arrows delineate the lesion area (left and middle panel) or the lumen (right panel). Scale bars, 100 μm.

Quantification of β-gal+ cells in lesions of the partially ligated carotid arteries showed an increase in the recruitment of BM-derived SPCs following CXCL12 treatment compared to PBS treatment in Apoe−/− mice harboring SM22LacZ/Apoε−/− BM (Figure 36).

**Figure 36: CXCL12 treatment increased lesional SPC accumulation.** Quantification of β-gal+ cells in the lesions of partially ligated carotid arteries in PBS- and CXCL12-treated Apoe−/− mice harboring SM22-LacZ/Apoε−/− BM cells. Data were evaluated by Student’s t-test. Data represent means ± SEM. **p < 0.01, N = 4-5 mice per group.

In CXCL12-treated mice, β-gal+ cells were primarily localized in the FC region as compared to non-FC regions of lesions in the carotid artery following partial ligation (Figure 37). To confirm that BM-derived β-gal-expressing cells only differentiate to SMCs in the lesions, double immunostaining of β-gal with EC-specific CD31, SMC-specific calponin, or macrophage-specific F4/80 was performed. β-gal immunostaining was co-localized with calponin immunostaining, while co-localization of β-gal with CD31 or F4/80 was not found (Figure 38). Together, these data implicate that BM-derived SPCs mobilized by CXCL12...
were recruited specifically to the lesion site and were mainly localized in the FC region where they differentiated into lesional SMCs.

Figure 37: CXCL12 increased SPC accumulation in the FC of lesions. Evaluation of β-gal⁺ cells by immunostaining in the FC and non-FC region of lesions in the carotid artery 6 weeks after partial ligation and treatment with CXCL12. Data were evaluated by Student’s t-test. Data represent means ± SEM. *p < 0.05, N = 4-5 mice per group.

Figure 38: BM-derived SPCs differentiate to lesional SMCs.
Double immunostaining of β-gal with endothelial-specific CD31 (A), SMC-specific calponin (B), and macrophage-specific F4/80 (C) was performed in lesions of the carotid artery 6 weeks after the partial ligation in irradiated Apoe⁻/⁻ mice transplanted with SM22-LacZ/Apoe⁻/⁻ BM cells that were treated with CXCL12. Scale bars, 20 µm.
3.1.11 Effect of repetitive CXCL12 treatment on lesional CXCL12 expression

In addition to their mobilization, CXCL12 is required for the local recruitment of SPCs to the injured arteries.74, 75 Proteoglycans are expressed on ECs and bind to the chemokine CXCL12.139 The transcription factor HIF-1α directly up-regulates CXCL12 by binding to its promoter site.66 Moreover, HIF-1α-mediated up-regulation of CXCL12 at the injury site leads to the recruitment of SPCs after vascular injury.101 Therefore, the hypothesis was tested that repetitive CXCL12 treatment might augment CXCL12 expression in the lesions via HIF-1α. To study whether systemic treatment with CXCL12 increases the lesional CXCL12 content as a prerequisite for the efficient recruitment of SPCs, CXCL12 expression was analyzed by immunostaining. CXCL12 expression was detectable in a subset of the cells within the lesions and in a subset of the ECs covering the lesions in PBS-treated mice, as determined by double immunostaining for CXCL12 and CD31. Following CXCL12 treatment, almost all of the lesional ECs expressed CXCL12, and prominent CXCL12 staining was detectable in the cell layers underneath the endothelium (Figure 39). Moreover, the number of CXCL12-immunopositive ECs was substantially greater in mice that were treated with CXCL12 as compared with PBS (Figure 39). These results indicate that CXCL12 expression was increased in the lesions of CXCL12-treated mice.

![Figure 39: Administration of CXCL12 increased endothelial-specific CXCL12 expression in lesions.](image)

(A) Representative micrographs demonstrating co-immunostaining of CXCL12 and CD31 in the lesions of the partially ligated carotid arteries after 4 weeks of CXCL12- (500 ng) or PBS-treatment. Scale bars, 100 µm. (B) Quantification of CXCL12+ ECs (CD31+) in carotid artery lesions following treatment with PBS or CXCL12. Data were evaluated by Student’s-t-test. Data represent means ± SEM. *p < 0.05, N = 3 mice per group.

Moreover, the relative CXCL12 mRNA expression in CXCL12-treated carotid arteries was significantly higher than in PBS-treated mice (Figure 40A). Stimulating ECs with
CXCL12 (100 ng/ml) in vitro significantly increased CXCL12 mRNA levels compared to vehicle (Figure 40B).

Figure 40: Autoinduction of CXCL12 in carotid arteries and ECs.
(A) Quantification of CXCL12 mRNA expression in partially ligated LCAs and unligated RCAs of Apoe−/− mice treated with CXCL12 or PBS by qRT-PCR. (B) Analysis of the relative CXCL12 mRNA expression in ECs stimulated with CXCL12 (100 ng/ml) or PBS for 2 hours. Data were evaluated by 2-way ANOVA with Bonferroni’s posttest or Student’s-t-test. Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, N = 3-4 per group.

The HIF-1α mRNA expression was compared between CXCL12 and PBS treatment in the lesions of the carotid artery and in ECs. The HIF-1α mRNA expression was significantly increased in carotid lesions of CXCL12-treated mice (Figure 41A). Stimulation of MAECs with CXCL12 for 4 hours increased the HIF-1α mRNA expression as compared to PBS (Figure 41B).

Figure 41: CXCL12 treatment increased HIF-1α expression.
(A) Quantification of Hif1a mRNA expression in partially ligated LCAs and unligated RCAs of Apoe−/− mice treated with CXCL12 or PBS. (B) Relative expression of Hif1a mRNA in ECs stimulated with CXCL12 (100 ng/ml) or PBS for 4 hours. Data were evaluated by 2-way ANOVA with Bonferroni’s posttest and Student-t-test. Data represent means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, N = 3-4 per group.

Together these data suggest that repetitive treatment with CXCL12 auto-induced its own expression in ECs presumably via up-regulation of HIF-1α transcription factor.
3.1.12 Effect of CXCL12 siRNA on lesion size and phenotype

To study whether increased CXCL12 expression in the partially ligated carotid arteries plays a role in the lesion stabilization following CXCL12 treatment, the CXCL12 expression in lesions was silenced by perivascular application of CXCL12 siRNA or non-targeting siRNA once weekly for 4 weeks. The lesion size following partial ligation and treatment with CXCL12 was substantially increased in CXCL12 siRNA-treated as compared to control siRNA-treated mice (Figure 42A). Moreover, the lesional macrophage content increased by 41% in CXCL12 siRNA-treated mice (Figure 42B). In contrast, the lesional SMC content was decreased by 30% in CXCL12 siRNA-treated mice as determined by SM22 immunostaining (Figure 42C). Silencing of lesional CXCL12 expression increased lesion size due to an increased lesional macrophage and decreased lesional SMCs content implicating that the increased lesional CXCL12 expression in CXCL12-treated mice contributes to the lesion stabilization. Moreover, up-regulation of CXCL12 during lesion formation seems to have athero-protective effect independent of the recruitment of SPCs.
Figure 42: Role of lesional CXCL12 expression on lesion formation during CXCL12 treatment.
(A) Lesion size was determined in EvG-stained carotid sections 6 weeks after partial ligation in Apoe−/− mice treated systemically with CXCL12 and perivascularly with non-targeting siRNA (siCTRL) or an siRNA against CXCL12 (siCXCL12). The lesional macrophage (B) and SMC (C) content was assessed by Mac2 and SM22 immunostaining, respectively. Scale bars, 200 µm. *p < 0.05, **p < 0.01, N=4 mice per group.
3.2 Role of endothelial HIF-1α in the development of atherosclerosis

3.2.1 HIF-1α expression in atherosclerotic lesions

Evidence for the expression of HIF-1α in early atherosclerotic lesions is lacking. To study the regulation of HIF-1α expression during the initiation of atherosclerosis in Apoe<sup>−/−</sup> mice, the level of Hif1a mRNA was quantified in different regions of the aorta, such as the aortic root, aortic arch, thoracic aorta and abdominal aorta and in the carotid arteries by qRT-PCR. The Hif1a mRNA expression was higher in the aortic root than in the other parts of the aorta in Apoe<sup>−/−</sup> mice fed a ND. After 4 months of a HFD, the expression of Hif1a mRNA was upregulated in the aortic arch, the thoracic aorta, the abdominal aorta and in the carotid arteries compared to Apoe<sup>−/−</sup> mice on a ND (Figure 43). Interestingly, the expression of Hif1a mRNA was not increased after feeding a HFD in the aortic roots of Apoe<sup>−/−</sup> mice (Figure 43). Thus, the mRNA expression of Hif1a is up-regulated by hyperlipidemia in most parts of the aorta.

![Figure 43: Expression of Hif1a mRNA in murine atherosclerotic lesions.](image)

Hif1a mRNA expression in aortic roots, aortic arch, thoracic aortas, abdominal aortas and carotid arteries of Apoe<sup>−/−</sup> mice after 4 months of a HFD or ND was quantified by qRT-PCR. Data were evaluated by 2-way ANOVA with Bonferroni’s post-test. Data represent means ± SEM. *p < 0.05, ***p < 0.001, N=3-4 mice per group.

To further scrutinize the discrepancy between the Hif1a mRNA expression in the aortic root and in large arteries, immunostaining for HIF-1α was performed on aortic root and carotid artery sections of Apoe<sup>−/−</sup> mice fed a ND and on aortic root sections of Apoe<sup>−/−</sup> mice fed a HFD for 3 months. Interestingly, HIF-1α was highly expressed in nuclei of the luminal and medial cells of normal aortic roots (Figure 44, left), but only weak HIF-1α stainings was detectable in the media of normal carotid arteries (Figure 44, middle) of Apoe<sup>−/−</sup> mice. Similar
to the normal aortic root, HIF-1α was also highly expressed in luminal, intimal and medial cells of the aortic root after 3 months of HFD (Figure 44, right). This high protein expression of HIF-1α in aortic roots of ND and HFD fed Apoe<sup>−/−</sup> mice is in line with the results of the Hif1a mRNA expression in both groups, implicating a significant role of HIF-1α in the tissue homeostasis of the aortic root.

![Image](https://via.placeholder.com/150)

**Figure 44: HIF-1α expression in the aortic root and in carotid arteries.**
Representative images displaying HIF-1α<sup>+</sup> immunostaining (green) in the aortic root of Apoe<sup>−/−</sup> mice fed a ND (left). Weak HIF-1α<sup>+</sup> immunostaining was detectable in the media of non-atherosclerotic carotid arteries of Apoe<sup>−/−</sup> mice fed a ND (middle). Nuclear localization of HIF-1α<sup>+</sup> immunostaining in aortic lesions of Apoe<sup>−/−</sup> mice fed a HFD for 3 months (right). Nuclei were counterstained with DAPI (blue). Arrows delineate the lumen (left, middle) or lesion area (right). Scale bars, 20 μm (A, B) and 100 μm (C).

Next, the endothelial expression of HIF-1α was studied 6 weeks after partial ligation of the LCA and feeding a HFD. For this purpose, double immunostaining of HIF-1α and EC-specific CD31 was performed. Nuclear localization of HIF-1α was found in CD31<sup>+</sup> cells demonstrating that ECs of atherosclerotic lesions express HIF-1α (Figure 45). In conclusion, HIF-1α was also activated in ECs of atherosclerotic lesions in addition to other cell types.

![Image](https://via.placeholder.com/150)

**Figure 45: EC-specific expression of HIF-1α in atherosclerotic lesions.**
Co-immunostaining of HIF-1α and CD31 demonstrates nuclear localization of HIF-1α (green) in CD31<sup>+</sup> ECs (red). Nuclei were counterstained with DAPI (blue). Arrows indicate the nuclear HIF-1α expression in ECs. Scale bar, 20 μm.
3.2.2 Regulation of Hif1α expression by disturbed flow

Branching points of blood vessels are predilection sites of atherosclerosis and are characterized by disturbed flow. Therefore, a model of disturbed flow-induced atherosclerosis in the carotid arteries of Apoe<sup>−/−</sup> mice was adopted, which mimics the flow pattern at blood vessel branch points, to study the expression of Hif1α. Following partial ligation of the left carotid artery, Hif1α mRNA was significantly down-regulated at 1 day and after 1 week as compared to the uninjured RCA (Figure 46). However, Hif1α mRNA was substantially up-regulated at 2 and 4 weeks in the LCA as compared to the RCA (Figure 46). The Hif1α mRNA levels tended to be higher at 6 weeks in the LCA as compared to the RCA. Thus, acutely disturbed blood flow reduced Hif1α expression, whereas prolonged flow disturbances seemed to increase Hif1α expression.

Figure 46: Hif1α mRNA expression in partially ligated carotid arteries.

Time course of Hif1α expression in partially ligated LCAs compared to non-ligated RCAs of Apoe<sup>−/−</sup> mice fed a ND. Data were evaluated by 2-way ANOVA followed by Bonferroni’s posttest. Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.0001, N=3-4 mice per group.

3.2.3 EC-specific deletion of Hif1α in Apoe<sup>−/−</sup> mice

To unravel the role of HIF-1α in ECs in the progression of atherosclerosis, EC-specific knockout of Hif1α was induced in Apoe<sup>−/−</sup> mice by treatment of EC-Hif1α<sup>−/−</sup> mice with tamoxifen (see section 2.2.9). In the control group, EC-Hif1α<sup>++</sup> mice were also treated with tamoxifen. One week after the last injection of tamoxifen, mice were subjected to partial ligation of the LCA and subsequently fed a HFD for 6 weeks. The knockdown of HIF-1α in ECs was verified 6 weeks after partial ligation in carotid lesions by co-immunostaining of CD31 and HIF-1α. The number of HIF-1α-expressing ECs was reduced from 66% in EC-Hif1α<sup>++</sup> mice to 34% in EC-Hif1α<sup>−/−</sup> mice (Figure 47). Moreover, there was no substantial difference in the HIF-1α-expressing CD31<sup>−</sup> cells in the lesion and media (Figure 47). These findings indicate that the tamoxifen treatment specifically knocked out Hif1α in ECs.
3.2.4 Role of endothelial Hif1α in disturbed flow-induced atherosclerosis

Next, the effect of endothelial HIF-1α on the lesion size was analyzed in carotid arteries after partial ligation by EvG staining. The lesion size was reduced by 45% in EC-Hif1α−/− compared to EC-Hif1α+/+ mice, whereas the difference in the medial areas between both groups was not statistically significant (Figure 48).

Despite the change in the lesion size, the in vivo luminal diameter of the partially ligated carotid arteries was not substantially altered in EC-Hif1α−/− compared to EC-Hif1α+/+ mice as determined by micro-CT angiography (Figure 49).
In addition, the serum lipid levels were not significantly different between the groups (Table 8). In summary, these results suggest that reduced HIF-1α expression in ECs limits atherosclerosis induced by disturbed flow in carotid arteries.

Table 8  Serum cholesterol and triglyceride levels in EC-Hif1a+/+ and EC-Hif1a−/− mice after 6 weeks of a HCD.

<table>
<thead>
<tr>
<th></th>
<th>EC-Hif1a+/+ (mmol/L)</th>
<th>EC-Hif1a−/− (mmol/L)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>18.16 ± 1.409</td>
<td>16.64 ± 1.880</td>
<td>0.5268</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.97 ± 0.098</td>
<td>0.84 ± 0.101</td>
<td>0.3770</td>
</tr>
</tbody>
</table>

N = 8 mice per group; data represent means ± SEM.

3.2.5 Effect of endothelial HIF-1α on lesion phenotype

Partial ligation model of carotid arteries in Apoe−/− mice fed a HFD induces lesions composed of mainly macrophages, SMCs, and extracellular matrix proteins, such as collagen (refer to section 3.1). To determine whether the decreased lesion formation in EC-Hif1a+/− mice is accompanied by changes in the composition of the lesions, analysis of the SMC, macrophage, and collagen content was performed by quantitative immunofluorescence. The SMC (SM22α area; Figure 50) and collagen content (Collagen+ area; Figure 51) were not altered in the carotid lesions, whereas a significant decrease in the macrophage content (Mac-2+ area;
Figure 52A, B) and macrophage cell number (Mac-2⁺ cells; Figure 52A, C) was observed in EC Hif1a⁻/⁻ mice, suggesting that elevated endothelial HIF-1α expression induces macrophage rich lesions by increasing the lesional macrophage cell number.

![Figure 50: Effect of endothelial HIF-1α on lesional SMCs.](image)

(A) Representative immunofluorescence stainings of SM22 (red) in lesions of the carotid artery 6 weeks after partial ligation in EC-Hif1a⁺/+ and EC-Hif1a⁻/⁻ mice are shown. Arrows indicate the lesion area. Scale bars, 100 µm. (B) Quantification of the relative SM22 immunopositive area in the lesions was performed. Differences between the groups were evaluated by Student’s-t-test. Data are expressed as means ± SEM, N = 6-7 mice per group.

![Figure 51: Effect of endothelial HIF-1α on lesional collagen-I content.](image)

(A) Representative micrographs demonstrating collagen-I immunostaining (red) in lesions of the carotid artery 6 weeks after partial ligation in EC Hif1a⁺/+ and EC Hif1a⁻/⁻ mice are depicted. Arrows indicate the lesion area. Scale bars, 100 µm. (B) Quantification of the collagen-I immunopositive area in the lesions was performed. Differences between the groups were evaluated by Student’s-t-test. Data are expressed as means ± SEM, N = 6-7 mice per group.
Figure 52: Deletion of HIF-1α in ECs reduced lesional macrophages.
(A) Representative micrographs demonstrating macrophage-specific Mac-2 immunostaining (green) in lesions of the carotid artery 6 weeks after partial ligation in EC-Hif1α+/+ and EC-Hif1α−/− mice are shown. Arrows indicate the lesions. Nuclei were counterstained with DAPI (blue). Scale bars, 100 μm. Quantification of the Mac-2 immunostainings revealed a significant reduction of the Mac-2+ area (B) and Mac-2+ cell number (C) in lesions of EC-Hif1α−/− compared to EC-Hif1α+/+. Differences between the groups were evaluated by Student’s-t-test. Data are expressed as means ± SEM, *p < 0.05, **p < 0.01, N = 6-7 mice per group.

3.2.6 Effect of endothelial HIF-1α on Cxcl12 expression

Results from the current study indicate a role of endothelial Hif1α in the transcriptional and translational up-regulation of CXCL12 after CXCL12 treatment leading to increased recruitment of SPCs, which differentiate into lesional SMCs (see section 3.1). Therefore, the hypothesis was tested that deleting endothelial HIF-1α might affect the lesional SMC content by regulating CXCL12 expression. In contrast to the hypothesis, knockdown of endothelial HIF-1α did not affect the lesional SMCs content in EC-Hif1α+/+ and EC-Hif1α−/− mice. This prompted us to investigate if endothelial HIF-1α could directly affect the Cxcl12 expression in EC-Hif1α+/+ and EC-Hif1α−/− mice. Cxcl12 mRNA expression was analyzed in EC-Hif1α+/+ and EC-Hif1α−/− mice 2 weeks after partial ligation of the LCA and feeding a HFD by qRT-PCR. The Cxcl12 mRNA expression was significantly up-regulated in the LCA as compared to the RCA in both EC-Hif1α+/+ and EC-Hif1α−/− mice. However, the Cxcl2 mRNA expression in the LCAs was not significantly different between EC-Hif1α+/+ and EC-Hif1α−/− mice (Figure 53), suggesting that endothelial-specific HIF-1α did not regulate endothelial Cxcl12 expression.
3.2.7 **Effect of endothelial HIF-1α on pro-inflammatory chemokine expression**

Reduced monocyte influx to atherosclerotic lesions due to impaired adhesion to the endothelium or infiltration to the sub-intimal space reduces the lesional macrophages content\(^1\). EC-derived chemokines or cytokines, such as CXCL1\(^86, 89\), CCL2\(^87, 88, 142, 143\), MIF\(^144\), CCL5\(^145\), and TNF-α\(^33, 146, 147\), are known to promote atherogenic monocyte recruitment. Therefore, it was studied whether endothelial HIF-1α regulates the expression of any of these chemokines and may thus increase the lesional macrophage number by promoting monocyte adhesion or infiltration. The mRNA expression of Cxcl1, Ccl2, Mif, Ccl5, and Tnfa was quantified in the carotid arteries at 2 weeks after partial ligation and feeding of a HFD by qRT-PCR. The expression of Cxcl1, but not that of Ccl2, Mif, Ccl5, or Tnfa, was substantially reduced in EC-Hif1α\(^−/−\) as compared to EC-Hif1α\(^+/+\) mice (Figure 54) indicating that the chemokine Cxcl1 is a potential target regulated by endothelial HIF-1α in *vivo*, which may mediate the effects of HIF-1α on lesional macrophage accumulation.
To further confirm that the reduced Cxcl1 expression in LCAs of EC-Hif1a<sup>−/−</sup> mice is actually due to a reduction of endothelial CXCL1 expression, double immunostaining of CXCL1 and CD31 was performed in left carotid lesions 2 weeks after partial ligation and feeding a HFD in EC-Hif1a<sup>+/+</sup> and EC-Hif1a<sup>−/−</sup> mice. The CD31 and CXCL1 double-positive cells were reduced to approximately 67% in EC-Hif1a<sup>−/−</sup> mice as compared to EC-Hif1a<sup>+/+</sup> mice (Figure 55). Moreover, the CD31<sup>−</sup>/CXCL1<sup>+</sup> cells also seem to decrease in EC-Hif1a<sup>−/−</sup> mice compared to EC-Hif1a<sup>+/+</sup> mice (Figure 55) suggesting deletion of endothelial HIF-1α not only reduces endothelial-specific CXCL1 expression but also reduces the lesional CXCL1 expression.

Figure 55: Endothelial cell-specific HIF-1α regulates CXCL1 expression.
(A) Representative micrographs of CD31 and CXCL1 double immunostained carotid lesions of EC-Hif1a<sup>+/+</sup> and EC-Hif1a<sup>−/−</sup> mice 2 weeks after partial ligation and feeding a HFD. Nuclei were counterstained with DAPI. Scale bars, 50 µm. (B) Quantification of CD31 and CXCL1 double immunopositive cells was performed. Data were evaluated by Student’s-t-test. Data are expressed as means ± SEM. *p < 0.05, N = 3-4 mice per group.
specific siRNA greatly reduced the Hif1a mRNA expression as compared to a non-targeting siRNA (Figure 56). In addition, silencing of Hif1a increased the Cxcl1 mRNA expression, whereas no significant effects of the Hif1a siRNA on the expression of Ccl2, Mif, Ccl5, or Tnfa were observed (Figure 56).

![Graph showing relative expression of Hif1a, Cxcl1, Ccl2, Ccl5, Mif, and Tnfa for siNTC and siHif1a](image)

**Figure 56: Role of Hif1a on the expression of chemokines and cytokines in MAECs.**
The effect of a treatment with a Hif1a-specific siRNA (siHif1a) or a non-targeting siRNA (siNTC) on the expression of Hif1a, Cxcl1, Ccl2, Ccl5, Mif, and Tnfa mRNA was determined after 72 hours in MAECs by qRT-PCR. Differences between the groups were evaluated by Student’s t-test and data were expressed as means ± SEM. ***p < 0.0001; *p < 0.05; N = 3-4 independent experiments per group.

Taken together, these findings indicate that HIF-1α plays a crucial role in the regulation of Cxcl1 in ECs in vitro and in vivo during atherosclerosis.

### 3.2.8 Effect of EC-specific Hif1a deletion on native atherosclerosis

To study whether endothelial HIF-1α also plays a role in the development of diet-induced atherosclerosis, EC-Hif1a<sup>+/−</sup> and EC-Hif1a<sup>−/−</sup> mice that had been injected with tamoxifen were fed a HFD for 3 months. Quantification of the oil red O-stained lipid depositions in en face prepared thoraco-abdominal aortas showed a substantial reduction of the lesion size in EC-Hif1a<sup>−/−</sup> compared to EC-Hif1a<sup>+/−</sup> mice (Figure 57 A, B, C). However, in abdominal aortas the lesion size was not significantly altered between both groups (Figure 57D). To further examine lipid deposits in specific aortic regions, the oil red O+ areas were quantified at vessel branching points (predilection sites) and non-branching points (non-predilection sites). Lipid deposits at predilection site were decreased by 50% in EC-Hif1a<sup>−/−</sup> compared to EC-Hif1a<sup>+/−</sup> mice (Figure 57E). At non-predilection sites, the lipid deposits were comparable between both groups (Figure 57F). Furthermore, the effect of endothelial Hif1a deletion on serum lipid levels was analyzed in EC-Hif1a<sup>+/−</sup> and EC-Hif1a<sup>−/−</sup> mice after 3 months of a HFD. Total serum cholesterol and triglycerides levels were not significantly different between both groups (Table 9).
Figure 57: Endothelial-specific knockout of Hif1a reduced lipid deposition in the aorta. (A) Representative en face prepared aortas of EC-Hif1a+/- and EC-Hif1a-/- mice stained with oil red O after 3 months of a HFD are shown. Quantification of the oil red O+ area in the thoracic aorta (B), the abdominal aorta (C), whole aorta (D), at predilection (E), and non-predilection sites (F) was performed in EC-Hif1a+/- and EC-Hif1a-/- mice. Data were evaluated by Student’s-t-test and expressed as mean ± SEM. *p < 0.05, **p < 0.01, N = 8 mice per group.

Table 9  Serum cholesterol and triglyceride levels in EC-Hif1a+/- and EC-Hif1a-/- mice after 3 months of a HFD.

<table>
<thead>
<tr>
<th></th>
<th>EC-Hif1a+/-</th>
<th>EC-Hif1a-/-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol (mmol/L)</strong></td>
<td>18.74 ± 0.623</td>
<td>16.80 ± 0.730</td>
<td>0.1123</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol/L)</strong></td>
<td>0.81 ± 0.143</td>
<td>0.80 ± 0.075</td>
<td>0.9655</td>
</tr>
</tbody>
</table>

N = 5-7 mice per group; data represent means ± SEM.
However, the lesion size in aortic roots was not significantly different between EC-Hif1a<sup>+/+</sup> and EC-Hif1a<sup>−/−</sup> mice (Figure 58).

![Figure 58: Effect of endothelial HIF-1α on aortic root lesion size.](image)

(A) Representative EvG stainings showing lesion size in the aortic roots of EC-Hif1a<sup>+/+</sup> and EC-Hif1a<sup>−/−</sup> mice after 3 months of a HFD. Scale bars, 200 µm. (B) Quantification of the lesion size in the aortic roots was performed by planimetry in both groups. Data were evaluated by Student’s-t-test and expressed as mean ± SEM. N = 8 mice per group.

In summary, these results indicate that increased HIF-1α expression in ECs also promotes diet-induced atherosclerosis at predilection sites in specific regions of the aorta.

### 3.2.9 Regulation of Hif1a expression in ECs by atherogenic stimuli

To determine the role of atherogenic factors in the transcriptional regulation of Hif1a in ECs, the effect of AT-2, which up-regulates Hif1a mRNA in SMCs<sup>110</sup>, on Hif1a mRNA expression was studied in primary mouse ECs. In addition, LPA derived from moxLDL is a key factor in the release and transcriptional regulation of Cxcl1 in ECs<sup>89, 148</sup> and might therefore be involved in the regulation of HIF-1α.

AT-2 (10 nM) treatment for 2 hours transiently increased the Hif1a mRNA expression compared to control as determined by qRT-PCR (Figure 59). This increased Hif1a expression gradually declined at 4 and 6 hours after AT-2 stimulation (Figure 59), suggesting that HIF-1α is temporarily up-regulated by AT-2 in ECs.
In contrast to treatment with the saturated LPA18:0, treatment with LPA20:4 for 4 hours substantially increased the expression of Hif1a mRNA compared to vehicle (PBS) (Figure 60A). Similarly, stimulation by moxLDL for 4 hours also up-regulated Hif1a mRNA expression in ECs as compared to vehicle (PBS), whereas treatment with nLDL had no effect (Figure 60B). Taken together, these results indicate that Hif1a is transcriptionally regulated by unsaturated LPA and moxLDL in ECs.

3.2.10 Role of HIF-1α in AT-2- and LPA-induced expression of pro-inflammatory mediators in ECs

First, the effect of AT-2 and LPA on the mRNA expression levels of Cxcl1, Ccl2, Ccl5, Mif, and Tnfa was determined in ECs by qRT-PCR. Activation of ECs with AT-2 increased the mRNA expression of Ccl2, whereas the mRNA levels of Cxcl1, Ccl2, Ccl5, Mif, and Tnfa were not significantly different from control (Figure 61A). By contrast, the expression of
Cxcl1 and Ccl5 was substantially increased after stimulation with LPA20:4 as compared to LPA18:0, whereas the expression of Ccl2, Mif, and Tnfa was not different between LPA20:4- and LPA18:0-treated MAECs (Figure 61B).

![Figure 61: Expression of pro-inflammatory factors in AT-2 and LPA20:4-stimulated ECs.](image)

(A) The relative mRNA expression of Cxcl1, Ccl2, Ccl5, Mif, and Tnfa was quantified in MAECs with or without AT-2 (10 nM; for 2 hours) stimulation. (B) Expression of Cxcl1, Ccl2, Ccl5, Mif, and Tnfa mRNA was determined in MAECs stimulated with LPA18:0 (10 µM) or LPA20:4 (10 µM) for 6 hours. Differences between the groups were evaluated by 2-way ANOVA followed by Bonferroni’s post-test. Results are expressed as means ± SEM. N = 3-4 per group, **p < 0.01, ***p < 0.0001.

To study the effect of HIF-1α on the expression of pro-inflammatory factors in AT-2-stimulated ECs, MAECs were isolated from tamoxifen-treated EC-Hif1a−/− and EC-Hif1a+/+ mice. The mRNA expression of Hif1a was clearly reduced in MAECs stimulated with AT-2 as determined by qRT-PCR (Figure 62A). Interestingly, the mRNA levels of Cxcl1, Ccl5, Mif, and Tnfa, but not that of Ccl2, were significantly lower in MAECs from EC-Hif1a−/− than from EC-Hif1a+/+ mice (Figure 62B). These findings indicate that Hif1a regulated the expression of Cxcl1, Ccl5, Mif, and Tnfa in AT-2-stimulated ECs, although AT-2 does not increase their expression beyond the level observed in unstimulated ECs.
Next, the role of HIF-1α in the expression of Cxcl1, Ccl2, Ccl5, Mif, and Tnfa after stimulation with moxLDL or LPA20:4 was examined in MAECs. The expression of Hif1a was silenced in MAECs stimulated with moxLDL or LPA20:4 by treatment with a Hif1a-specific siRNA compared to a non-targeting siRNA (Figure 63A).

In moxLDL-stimulated MAECs, treatment with a Hif1a-specific siRNA reduced the mRNA expression of Cxcl1, Ccl2, Ccl5, and Tnfa compared to a non-targeting siRNA (Figure 63B). Moreover, the expression of Mif mRNA tended to be lower following treatment with a Hif1a siRNA than after a non-targeting siRNA, but this difference did not reach statistical significance (Figure 63B). In MAECs treated with LPA20:4, only the expression of Cxcl1 and Ccl5 mRNA was decreased by silencing of Hif1a, whereas the Ccl2, Mif, and Tnfa expression was not significantly different between MAECs treated with Hif1a siRNA and non-targeting siRNA (Figure 63C). These data suggest that the up-regulation of Cxcl1 and Ccl5 by LPA20:4 is mediated through HIF-1α. Moreover, moxLDL induced pro-inflammatory activation of ECs via HIF-1α, which may at least partly be due to the action of LPA.
Figure 63: Effect of endothelial HIF-1α on the pro-inflammatory activation of ECs by moxLDL and LPA20:4.

(A) Relative expression of Hif1a mRNA in MAECs after treatment with Hif1a-specific siRNA (siHif1a) and non-targeting siRNA (siNTC) and stimulation with moxLDL or LPA20:4. (B) The mRNA expression of pro-inflammatory mediators was studied after stimulation with moxLDL (50 µg/ml) (B) and LPA20:4 (10 µM) (C) in MAECs after treatment with Hif1a-specific siRNA (siHif1a) or non-targeting siRNA (siNTC). Differences between the groups were evaluated by Student’s-t-test (A) or 2-way ANOVA followed by Fischer’s LSD (B and C). Data are expressed as means ± SEM. *p < 0.05, ***p < 0.001, ****p < 0.00001, N = 3-4 per group.

To confirm the effect of HIF-1α on the expression of Cxcl1 in ECs at the protein level, CXCL1 protein was analyzed in the cell culture supernatants by ELISA. The CXCL1 protein expression was substantially increased in LPA20:4- (Figure 64A) and moxLDL- (Figure 64C) treated MAECs compared to LPA18:0 and nLDL, respectively. The up-regulation of CXCL1 by LPA20:4 (Figure 64B) or moxLDL (Figure 64D) stimulation was significantly inhibited through treatment with a Hif1a-specific siRNA compared to non-targeting siRNA. In summary, endothelial HIF-1α promotes the up-regulation of Cxcl1 by LPA20:4 or moxLDL at the mRNA and protein level.
3.2.11 Involvement of the LPA receptors LPA₁ and LPA₃ in endothelial expression of CXCL1

LPA20:4 and moxLDL can activate ECs via the LPA receptors LPA₁ and LPA₃. Moreover, LPA₁ and LPA₃ also mediate the up-regulation of CXCL1 expression in LPA20:4- and moxLDL-activated ECs. Therefore, the role of LPA₁ and LPA₃, in the activation of Hif1α in ECs by moxLDL and LPA20:4 were studied. LPA₁ and LPA₃ were blocked by treating MAECs with the LPA receptor antagonist Ki16425 (100 µM). Blocking LPA receptors by Ki16425 substantially reduced Hif1α mRNA expression in LPA20:4- and moxLDL-treated ECs as compared to vehicle (Figure 65), suggesting that the up-regulation of Hif1α mRNA by LPA20:4 or moxLDL in MAECs is mediated through LPA₁ and LPA₃.
Furthermore, inhibition of LPA₁ and LPA₃ substantially reduced the mRNA expression of Cxcl1 in LPA20:4-stimulated MAECs compared to vehicle treatment as determined by qRT-PCR. Of note, the Cxcl1 mRNA levels were similar in LPA18:0-stimulated MAECs and in LPA20:4-stimulated MAECs treated with Ki16425 (Figure 66A). Accordingly, Ki16425 substantially reduced the CXCL1 protein concentration in the cell culture supernatants of LPA20:4-stimulated ECs compared to vehicle treatment as determined by ELISA (Figure 66B). The CXCL1 protein level was comparable between LPA18:0-stimulated MAECs and LPA20:4-stimulated MAECs treated with Ki16425 (Figure 66B). Similarly, inhibition of LPA₁ and LPA₃ by Ki1645 also reduced the CXCL1 protein expression in cell culture supernatants of moxLDL-stimulated MAECs (Figure 66C). Together, these data indicate a crucial role of the LPA receptors LPA₁ and LPA₃ in the HIF-1α-mediated up-regulation of CXCL1 in LPA20:4- or moxLDL-stimulated ECs.

Figure 65: Role of LPA₁ and LPA₃ in the regulation of Hif1α mRNA expression.
The Hif1α mRNA expression after stimulation with LPA20:4 (A) or moxLDL (B) was assessed in MAECs treated with the LPA receptor antagonist Ki16425 or vehicle by qRT-PCR. Differences between the groups were evaluated by 1-way ANOVA followed by Newman-Keuls post-test. Data are expressed as means ± SEM. N = 3-4 per group, *p < 0.05, **p < 0.01, ***p < 0.0001.
Figure 66: LPA₁ and LPA₃ mediated CXCL1 expression in activated ECs. (A) Quantification of the Cxcl1 mRNA in MAECs stimulated with LPA20:4 (10 µM) with or without Ki16425 (100 µM) or LPA18:0 (10 µM) for 6 hours by qRT-PCR. (B) Measurement of CXCL1 protein levels in the supernatant of MAECs stimulated with LPA20:4 (10 µM) with or without Ki16425 (100 µM) or with LPA18:0 (10 µM) after 6 hours by ELISA. (C) Assessment of CXCL1 protein levels in MAECs stimulated with moxLDL (50 µg/ml) with or without Ki16425 (100 µM) or nLDL (50 µg/ml) for 6 hours by ELISA. Differences between the groups were evaluated by 1-way ANOVA followed by Newman-Keuls post-test. Data are expressed as means ± SEM. N = 3-4 per group. *p < 0.05, ***p < 0.0001.

3.2.12 Role of HIF-1α on monocyte adhesion to LPA20:4 or moxLDL activated ECs

To determine the functional relevance of endothelial HIF-1α on monocyte arrest, in vitro flow chamber assays were performed. Treatment with LPA20:4 significantly increased the adhesion of MonoMac6 cells (MM6) to MAECs as compared to LPA18:0. Silencing Hif1α in ECs by Hif1α-specific siRNA reduced the adhesion of MM6 cells on LPA20:4-activated ECs as compared to a non-targeting siRNA (Figure 67A). Similar to the effect of LPA20:4, stimulation of MAECs with moxLDL increased the adhesion of MM6 cells as compared to nLDL. Treatment with Hif1α-specific siRNA substantially reduced the adhesion of MM6 cells on moxLDL-treated MAECs as compared to a non-targeting siRNA (Figure 67B). In conclusion, these data demonstrate a direct role of endothelial HIF-1α in the adhesion of monocytes to activated ECs.
Figure 67: Endothelial HIF-1α increased monocyte adhesion.

(A). Quantification of MM6 cells adhering to MAECs after stimulation with LPA20:4 (10 µM) or LPA18:0 (10 µM) for 6 hours and on LPA20:4-stimulated MAECs (10 µm for 4 hours) treated with Hif1α-(1 µM) or NTC-siRNA (1 µM) under laminar flow conditions. (B) Assessment of MM6 cell adhesion to MAECs stimulated with moxLDL (50 µg/ml) or nLDL (50 µg/ml) for 6 hours or to moxLDL-stimulated (50 µg/ml for 4 hours) MAECs treated with either Hif1α-(1 µM) or non-targeting control-(NTC) (1 µM) siRNA under laminar flow conditions. Differences between the groups were evaluated by Student’s-t-test. Data are expressed as means ± SEM. N = 3-4 per group, *p < 0.05, **p < 0.01.
4 Discussion

4.1 Stabilization of atherosclerotic lesions by CXCL12

4.1.1 CXCL12 and SPC mobilization

A transient increase in plasma CXCL12 levels after wire injury mobilizes BM-derived Sca-1^Lin^Pdgfr-β^+^ cells into the circulation.\(^75\), \(^101\) This CXCL12-induced mobilization of progenitor cells is due to a reduction of the physiological concentration gradient of CXCL12 between the circulation and the comparatively high CXCL12 levels in the BM, which is crucial for the retention of stem cells in the BM\(^59\), \(^151\), \(^152\). In the current study, injection of CXCL12 increased temporarily the CXCL12 concentrations in the plasma to a similar level as in the BM, where CXCL12 levels did not increase after CXCL12 treatment, and also mobilized Sca-1^Lin^Pdgfr-β^+^ cells into the circulation. Moreover, elevated plasma CXCL12 levels can mobilize mature WBC into the circulation.\(^59\), \(^152\) This effect is achieved by complete reversal of the CXCL12 gradient between the BM and circulation. However, the acute elevation of plasma CXCL12 levels in the current study did not alter the WBC count probably because the CXCL12 concentration gradient was not reversed. Chronic blockade or knockout of CXCR4 in BM cells in mice increases the mobilization of neutrophils and promotes their subsequent recruitment to atherosclerotic lesions, thus exacerbating atherosclerosis.\(^72\) Furthermore, the level of circulating Sca-1^Lin^ cells increases during short-term CXCR4 inhibition but not during long-term CXCR4 inhibition.\(^72\) Therefore, the continuous, long-term disruption of the CXCL12 gradient may have different effects on the mobilization of BM-derived cells compared with intermittent CXCL12 injections.

4.1.2 CXCL12 treatment stabilized atherosclerotic lesions

CXCL12 is highly expressed in SMCs, ECs and macrophages in human atherosclerotic lesions but not in normal vessel.\(^67\) CXCL12 affects the adhesion of T-lymphocytes under flow conditions and induces platelet aggregation, suggesting a pro-atherogenic role of CXCL12.\(^67\), \(^153\), \(^154\) Moreover, coronary artery disease (CAD) risk alleles are associated with increased plasma CXCL12 levels indicating that elevated circulating CXCL12 levels are atherogenic even though the plasma CXCL12 levels between patients with and without CAD were not significantly different.\(^70\) In mouse models of vascular injury or allograft vasculopathy, increased plasma CXCL12 levels mediate the recruitment of SPCs, which contribute to
Discussion

neointimal growth, also implicating a pro-atherogenic role of CXCL12.\textsuperscript{101, 104, 105} In contrast, chronic disruption of the CXCL12/CXCR4 axis in the BM by either treatment with CXCR4 antagonist or BMT of Cxcr4\textsuperscript{-/-} BM cells aggravates atherosclerotic lesion formation by increased recruitment of neutrophils suggesting a protective role of CXCL12.\textsuperscript{72} Similarly, microRNA 126-mediated up-regulation of CXCL12 in ECs reduces atherosclerotic lesion formation and the recruitment of EPCs.\textsuperscript{73} Moreover, patients with unstable angina have lower CXCL12 levels compared with patients with stable angina implicating an anti-atherogenic role of CXCL12.\textsuperscript{58} In the current study, repetitive treatment with CXCL12 reduced the lesional infiltration with inflammatory cells, promoted the accumulation of SMCs and collagen in the lesions, which in turn contributed to an increased FC thickness. Similar to what has been observed after vascular injury; treatment with CXCL12 increased the recruitment of BM-derived SPCs to the lesions and thus enhanced the accumulation of SMCs.

CXCL12 chemoattracts monocytes, neutrophils, T-cells, early stage B-cell precursors, and proangiogenic cells.\textsuperscript{154-156} Therefore, additional cell types, such as mature hematopoietic or proangiogenic cells, might have been recruited to the lesion site due to the increased CXCL12 protein expression in CXCL12-treated mice. However, neutrophils and T-lymphocytes were either absent or very infrequent in lesions regardless of the treatment suggesting that increased lesional CXCL12 expression did not increase lesion inflammation and thus vulnerability. In contrast, the reduced lesional macrophage content in CXCL12-treated mice indicates an anti-inflammatory effect of the CXCL12 treatment.

4.1.3 CXCL12 enhanced the recruitment of SPCs in atherosclerosis

The role of BM-derived SPCs in atherosclerotic lesion formation and stabilization is under debate\textsuperscript{90, 95, 97, 99, 106}. A few studies detected the recruitment of SPCs in murine and human atherosclerosis\textsuperscript{90, 95}; however, the results of other groups indicate that the contribution of SPCs to lesion formation is minimal or not verifiable at all\textsuperscript{90, 99, 106}. In line with the findings of the latter reports, BM-derived SPCs were rarely found in disturbed flow-induced lesions in the current study. In contrast, treatment with CXCL12 greatly increased the number of BM-derived SPCs in the lesions indicating that CXCL12 treatment promotes their recruitment. Moreover, SPCs were more frequently detectable in the FC than in other regions of the lesions suggesting that CXCL12 recruited SPC preferentially to the FC and thereby increased the FC thickness.

In contrast to diet-induced atherosclerosis, SPCs are known to play a key role in neointimal growth after vascular injury\textsuperscript{75, 100} and inhibition of SPC mobilization and
recruitment by blocking the CXCL12/CXCR4 axis reduces neointima formation\textsuperscript{74, 103}. Although the functional role of SPCs in atherosclerosis is unclear, inducing apoptosis of lesional SPCs limits native atherosclerosis by reducing inflammatory gene expression suggesting a pro-inflammatory role of BM-derived SPCs\textsuperscript{99}. On the contrary, treatment of Apoe\textsuperscript{−/−}/Rag2\textsuperscript{−/−} mice with \textit{ex vivo} expanded CD34\textsuperscript{+} SPCs derived from human umbilical cord blood reduces atherosclerotic lesion size and increases lesion stability due to a lower macrophage content and a elevated collagen content.\textsuperscript{94} In accordance to the results of Zoll et al.\textsuperscript{94}, CXCL12-induced SPC recruitment also decreased lesional macrophages and increased the SMC and collagen content in the current study. However, the lesion size was not affected by CXCL12-induced SPC recruitment, which may be due to the duration of the treatment or to functional differences between the \textit{ex vivo} expanded human CD34\textsuperscript{+} SPCs and the CXCL12-responsive murine SPCs. Moreover, CXCL12 might also affect the function and phenotype of SPCs.

\textbf{4.1.4 Auto-induction of CXCL12 expression in atherosclerotic arteries promoted SPCs recruitment}

Circulating CXCR4\textsuperscript{+} BM-derived progenitor cells home to the sites of injury by increased local expression of CXCL12\textsuperscript{74}. The expression of CXCL12 in ECs is regulated by the hypoxia-induced transcription factor HIF-1\textalpha.\textsuperscript{157} Following vascular injury, increased HIF-1\textalpha in SMCs can also up-regulate the expression of CXCL12, which is deposited on platelets at the luminal surface of the injured vessel wall and mediates the recruitment of CXCR4\textsuperscript{+}/Pdgfr-\beta\textsuperscript{+} SPCs.\textsuperscript{74, 101} Moreover, endothelial deposition of CXCL12 via proteoglycans is required for the homing of hematopoietic progenitor cells to the BM.\textsuperscript{139} Auto-induction of CXCL12 in ECs is mediated by the activation of CXCR4, which can be unleashed by suppression of RGS16, a negative regulator of GPCR signaling, through the microRNA-126.\textsuperscript{73} In the present study, CXCL12 treatment increased the lesional expression of CXCL12, most notably in ECs, and HIF-1\textalpha. Up-regulation of Cxcl12 and Hif1a mRNA by CXCL12 was verified in MAECs indicating that HIF-1\textalpha may play an important role in the auto-induction of CXCL12. However, increased deposition of circulating CXCL12 to endothelial proteoglycans or microRNA-126-mediated up-regulation of CXCL12 expression in ECs could serve as alternative pathways for the increased lesional CXCL12 expression following CXCL12 treatment. In concert with increased lesional CXCL12 expression, BM-derived cells expressing lacZ under a SMC-specific promoter were also increased in the lesions suggesting
enhanced recruitment of SPCs. These recruited SPCs differentiate specifically into SMCs as determined by co-localization of β-gal-specific and SMC-specific immunostaining.

Silencing of CXCL12 expression in the vessel wall of CXCL12-treated mice decreased the lesional SMC content; this supports the hypothesis that lesional CXCL12 expression is crucial for the recruitment of SPCs mobilized by CXCL12. Therefore, increased CXCL12 both in the circulation and on the lesion surface following repetitive CXCL12 injections coordinates the mobilization and subsequent lesional recruitment of SPCs.

4.1.5 Role of local CXCL12 expression in atherosclerosis

CXCL12 is up-regulated in atherosclerotic lesions. Moreover, CXCL12 is also up-regulated in partially ligated carotid arteries after 48 hours even in the absence of CXCL12 treatment. Although the expression of CXCL12 in lesions and carotid arteries subjected to disturbed flow has been linked to pro-atherogenic effects and inflammation, no direct experimental evidence exists for this hypothesis. In the current study, inhibiting the lesional CXCL12 expression by CXCL12 siRNA increased the lesion size suggesting a protective role of local CXCL12 expression. Moreover, a weaker expression of CXCL12 was found in macrophage-rich lesions of the carotid artery. Inducing CXCL12 expression in these lesions by CXCL12 treatment reversed the lesion phenotype by increasing the SMC and reducing the macrophages content, which also indicates athero-protective role of lesional CXCL12 expression.

4.1.6 Potential effects of long-term CXCL12 treatment

Thinning of the FC is characteristic for rupture-prone, advanced atheromata and is caused by a loss of SMCs and extracellular collagen. Activated macrophages and T-lymphocytes in the lesions release proinflammatory cytokines, which promote FC thinning. For example, T-lymphocytes produce TGF-β and IFN-γ, which suppress SMC proliferation. Moreover, activated macrophages release IL-1 and TNF-α, which increase Fas receptor expression on SMCs. Apoptosis of Fas receptor-expressing SMCs is induced by the binding of the Fas ligand expressed on T-lymphocytes. Furthermore, increased MMP secretion from activated macrophages degrades lesional extracellular matrix proteins, like collagen-I, thus weakening the FC. Therefore, treatment strategies for vulnerable lesions stabilization would ideally reduce lesion inflammation and also increase and maintain the FC thickness and integrity. In the current study, the increased FC thickness and the increased SMC and collagen-1 content after CXCL12 treatment was maintained even after 2
weeks without treatment. In addition, the reduced lesional macrophage content following CXCL12 treatment was preserved after interrupting this treatment, thus indicating a long-term stabilizing effect on the lesion. Moreover, the CXCL12/CXCR4 pathway is also involved in tumour progression and metastasis\textsuperscript{165}. Therefore, the effect of CXCL12 treatment on tumour formation has been investigated. However, micro-CT scanning and tissue histology does not indicate increased carcinogenesis following CXCL12 treatment.\textsuperscript{166} Taken together, CXCL12 treatment increased the FC thickness of rupture-prone lesions and maintained it for long-term even in the absence of further CXCL12 treatment. However, CXCL12 treatment may supports the metastasis of the CXCR4 expressing tumour cells, which may limit the applicability of the CXCL12 treatment strategy to patients without cancer.

In summary, intermittent treatment with CXCL12 mobilized BM-derived SPCs into the circulation and induced a stable lesion phenotype with increased SMCs, collagen, FC thickness, and reduced macrophages. This stabilization was due to an increased recruitment of mobilized SPCs to the lesion site, maybe via the HIF-1α-mediated auto-induction of CXCL12 in lesions. Moreover, the stable lesion phenotype was maintained even after terminating the CXCL12 treatment indicating that long-term treatment with CXCL12 is not required for the beneficial effect.
Figure 66: Model of vulnerable lesion stabilization by CXCL12 treatment.

Increase in plasma CXCL12 after treatment eliminates the CXCL12 gradient between the BM and circulation. This causes mobilization of BM Sca-1$^{+}$linPDGFR-$\beta$ cells (SPCs) into the circulation, which home to the site of vulnerable lesions. Increased plasma CXCL12 stimulates CXCL12 expression in atherosclerotic arteries probably by up-regulation of HIF-1$\alpha$. CXCL12 protein might become immobilized on the endothelial surface via proteoglycans, which may be important in the homing of CXCR4$^+$ SPCs to the lesion site. The adhered SPCs migrate into the lesion, differentiate into lesional SMCs, and increase the FC thickness (modified from$^{[167]}$).
4.2 Role of endothelial HIF-1α in atherosclerotic lesion formation

4.2.1 HIF-1α expression in ECs

The transcription factor HIF-1α is expressed in advanced human and murine atherosclerotic lesions. However, evidence for the expression of HIF-1α in early lesions is lacking. In the current study, Hif1a mRNA is substantially up-regulated in early lesions of the thoraco-abdominal aorta, but not in the aortic root in hyperlipidemic Apoe−/− mice. Notably, the Hif1a mRNA expression is already high in the aortic root of Apoe−/− mice fed a ND indicating a significant role of HIF-1α in the homeostasis of the aortic root tissue.

In advanced human lesions, HIF-1α expression is localized mainly in macrophage-rich areas and to a lesser extent in SMCs. This lesional expression of HIF-1α is closely associated with hypoxia and inflammation. The continual recruitment of leukocytes into the intima increases the arterial wall thickness and may thereby produce a hypoxic milieu due to impaired oxygen diffusion. Moreover, the inflammatory reaction and the increased uptake of modified lipoprotein by macrophages may increase the oxygen demand locally and thereby activates HIF-1α in surrounding cells. Together, these studies link hypoxia directly to the expression of HIF-1α in macrophages and SMCs of atherosclerotic lesions. In contrast, the expression of HIF-1α in ECs during atherosclerosis is less well established. Following vascular injury, HIF-1α is expressed in ECs covering the neointimal lesions, but the functional role of endothelial HIF-1α is unclear. In addition to lesional cells, luminal ECs of atherosclerotic lesions clearly expressed HIF-1α in the current study, indicating that HIF-1α contributes to the activation of ECs.

Transcriptional regulation of HIF-1α in ECs in vitro is mediated mainly via two mechanisms, hypoxia and ROS. However, hypoxia is unlikely to play a role in the activation of HIF-1α in luminal ECs during atherosclerosis, because oxygen availability is not limited in these cells due to their close proximity to the blood stream. Therefore, normoxic activation of HIF-1α by ROS, which are generated in ECs by various atherogenic factors, such as disturbed flow, hyperlipidemia, hypertension, and proinflammatory cytokines, might be more important in atherosclerosis. Partial ligation of the carotid artery is a valuable in vivo model to study the effect of disturbed flow on medium-sized arteries. The shear stress at the carotid artery is acutely reduced due to reduced flow, whereas the circumferential wall stretch may be increased following partial ligation. Although Hif1a has not been found to be regulated within the first 48 hours after partial ligation in a
previous study, Hif1a mRNA was down-regulated early after partial ligation and upregulated after prolonged exposure to disturbed flow in the current study. These changes in the HIF-1α expression in partially ligated carotid arteries may occur in ECs, because they are mechanosensitive and are primarily affected by changes of the blood flow due to their location at the interface between the vessel wall and the bloodstream. High shear stress can suppress, whereas mechanical stretch up-regulates Hif1a mRNA expression in microvascular ECs. Therefore, the results of the current study would be in line with the hypothesis that reduced shear stress decreases Hif1a expression early after partial ligation, whereas increased mechanical stretch up-regulates Hif1a at later time points. Alternatively, the changes in the Hif1a expression after partial ligation may occur in other cell types than ECs, such as SMCs or macrophages. Furthermore, up-regulation of Hif1a at 2 weeks after partial ligation may be induced in ECs by other pro-atherogenic factors related to hypercholesterolemia or hypertension, such as modified LDL, LPA or AT-2, which can up-regulate Hif1a mRNA expression in ECs in vitro.

In hypercholesterolemia-induced atherosclerosis, moxLDL and unsaturated LPAs are important triggers for monocyte recruitment. LPA is produced locally in response to inflammation and is also generated as a by-product of mild oxidation of LDL via autotaxin. LPA mediates its function by binding to surface GPCRs including the Edg family receptors LPA₁₋₃ and the purino cluster receptors LPA₄₋₆. Of all the receptors known, ECs mainly express LPA₁ and LPA₃. Moreover, unsaturated LPAs and moxLDL activate ECs via LPA₁ and LPA₃. 3-(4-[(1-(2-chlorophenyl) ethoxy] carbonyl amino)-3-methyl-5-isoxazolyl] benzyl sulfanyl] propanoic acid (aka Ki16425) is a known antagonist for LPA₁ and LPA₃. Treatment with Ki16425 diminished LPA₂₀:₄- and moxLDL-mediated activation of HIF-1α in ECs, indicating that their effect is mediated through LPA₁ and LPA₃ receptors.

In summary, our results demonstrate that HIF-1α is activated in ECs of early atherosclerotic lesions, which can be mediated by various pro-atherogenic factors, such as disturbed flow, moxLDL, unsaturated LPA, and AT-2. The up-regulation of HIF-1α by moxLDL is most likely due to the generation of unsaturated LPA, because blocking the LPA receptors LPA₁ and LPA₃ can inhibit the effect of moxLDL on Hif1a transcription.

4.2.2 Role of endothelial-HIF-1α in atherosclerosis

In the current study, it was demonstrated that CXCL12 treatment promotes its own expression, probably by up-regulating HIF-1α. CXCL12 treatment increased lesion
stabilization by recruiting BM-derived SPCs and reduced the lesional macrophage content. This effect of CXCL12 was not associated with a change in the lesion size\textsuperscript{166}. Although the regulation of the auto-induction of CXCL12 by HIF-1α was not directly demonstrated, these results indicate a protective role of HIF-1α. In contrast, endothelial-specific deletion of Hif1α reduced lesion size due to a diminished accumulation of macrophages, whereas the lesional SMC content was not affected. Therefore, it may be hypothesized that non-endothelial HIF-1α expression accounts for the increased, atheroprotective CXCL12 expression, whereas endothelial HIF-1α promotes macrophage accumulation by a distinct mechanism. The reduced lesional macrophage content in mice harboring an endothelial-specific Hif1α deletion can be due to reduced adhesion of circulating monocytes or decreased infiltration of adherent monocytes. Pro-inflammatory mediators, such as TNF-α\textsuperscript{146}, CXCL1\textsuperscript{86, 89}, MIF\textsuperscript{144}, and CCL5\textsuperscript{145}, increase monocyte adhesion, while CCL2\textsuperscript{142} primarily affects monocyte migration across ECs under physiological conditions. TNF-α mediates monocyte adhesion by up-regulating the expression of the adhesion molecules VCAM-1 and ELAM-1 on activated endothelium.\textsuperscript{146} Similar to TNF-α, MIF up-regulates the adhesion molecules VCAM-1 and ICAM-1 in ECs via activation of transcription factor NF-κB and thereby induces monocyte arrest.\textsuperscript{144} Moreover, binding of MIF to CXCR2 induces monocyte arrest to ECs by inducing rapid integrin activation on monocytes.\textsuperscript{196, 197} Platelet-derived CCL5 deposited via GAGs on the endothelium triggers monocyte arrest on inflamed and atherosclerotic endothelium.\textsuperscript{145, 198} Furthermore, endothelial CXCL1 is immobilized on the endothelial surface via GAGs after secretion and induces monocyte adhesion via CXCR2\textsuperscript{86, 89}, whereas CCL2 is secreted in a soluble form and contributes to monocyte shape change, spreading, and trans-endothelial migration via CCR2.\textsuperscript{142} Among these pro-inflammatory factors, endothelial deletion of HIF-1α specifically reduced CXCL1 expression \textit{in vivo} implicating a role of endothelial HIF-1α in monocyte adhesion mediated by CXCL1. CXCL1 and its human ortholog IL-8 are expressed mainly in macrophage-rich regions and to some extent in the endothelium of atherosclerotic lesions, while its expression in lesional SMCs remains elusive.\textsuperscript{82, 83} Silencing of HIF-1α reduced the expression of CXCL1 in LPA20:4- and moxLDL-stimulated, but not in AT-2-stimulated ECs indicating that hypercholesterolemia-induced HIF-1α affects CXCL1 expression in ECs. Moreover, the LPA receptors LPA\textsubscript{1} and LPA\textsubscript{3} mediated the up-regulation of HIF-1α by moxLDL and LPA20:4 suggesting that LPA is the main moxLDL component inducing Hif1α expression in ECs. In addition, monocyte adhesion to moxLDL- and LPA20:4-stimulated ECs, which are mediated by CXCL1, required the expression of HIF-1α.
Taken together, these finding corroborate that endothelial HIF-1α promotes atherosclerosis by augmenting CXCL1-mediated monocyte adhesion.

4.2.3 Regulation of CXCL1 expression by endothelial HIF-1α

The transcription of CXCL1 is regulated mainly through interactions of NF-κB with other transcriptional regulatory molecules, such as poly (ADP-ribose) polymerase-1 (PARP-1) and cAMP response element binding protein (CREB)-binding protein (CBP).199 These two proteins interact with NF-κB and other enhancers to form an enhanceosome at the promoter region of CXCL1 and modulate CXCL1 transcription. In addition to these positive cofactors, a negative regulator, CAAT displacement protein (CDP), may also be involved in the transcriptional regulation of CXCL1.199 The interaction of CDP with the CXCL1 promoter down-regulates, whereas PARP and/or CBP enhance CXCL1 transcription. Although NF-κB is the major transcriptional factor regulating CXCL1 expression, other transcription factor, such as Sp1 and HMGI(Y) also play a role.200 Together, these studies implicate that expression of CXCL1 is regulated by more than one transcription factor.

Using different cell types, Rius et al. have shown that NF-κB is a critical transcriptional activator of HIF-1α and that the basal NF-κB activity is required for HIF-1α protein accumulation under hypoxia in cultured cells and in the liver and brain of hypoxic animals.201, 202 Similarly, the transcriptional activation of Hif1a by ROS is dependent on NF-κB in SMCs.201, 203 Conversely, HIF-1α can also activate the transcription of NF-κB proteins.204 However, HIF-1α activation and inhibition of NF-κB can occur during hypoxia via RSUME.205 In conclusion, these studies are compatible with the hypothesis that HIF-1α regulates CXCL1 expression indirectly via increased NF-κB activation.

Moreover, several studies have linked elevated CXCL1 expression to the activation of HIF-1α.115, 206-208 In keratinocytes, HIF-1α stabilization up-regulates CXCL1 expression.206 Similarly, over-expression of HIF-1α in mouse spleenocytes also increases CXCL1.115 Hypoxic up-regulation of HIF-1α in primary human acute myeloid leukemia (AML) cells up-regulates CXCL1 expression.207 Moreover, co-stimulation with nickel (Ni) and the mycoplasma-derived lipopeptide macrophage-activating lipopeptide-2 (MALP-2) synergistically induces CXCL1 and CXCL5 mRNA expression and protein release from human lung fibroblast.208 Accordingly, HIF-1α-dependent transcriptional up-regulation of CXCL1 in LPA20:4- or moxLDL-stimulated ECs was demonstrated in the current study. Blocking NF-κB activation reduces LPA20:4-induced CXCL1 mRNA and protein expression in ECs89, which implicates that NF-κB plays an important role in the expression of CXCL1 in
LPA- or moxLDL-stimulated ECs. This indicates that either HIF-1α regulates the CXCL1 expression in the late phase of stimulation or HIF-1α regulates the CXCL1 expression in a NF-κB dependent mechanism.

In summary, the current study suggests that HIF-1α is activated in the endothelium during early atherosclerosis by LPA, which increases monocyte adhesion through CXCL1 and exacerbates atherosclerosis. Drugs directly targeting endothelial HIF-1α or inhibiting LPA receptor LPA1 or LPA3 might be a promising tool for the treatment and prevention of atherosclerosis.

Figure 67: Graphical summary of the role of endothelial HIF-1α in atherosclerosis. Plasma LPA or LPA produced by mild oxidation of LDL and endothelial autotaxin activates LPA1 and LPA3 receptors on ECs, which mediate the transcriptional up-regulation of HIF-1α. HIF-1α augments CXCL1 transcription and translation leading to increased monocyte adhesion (modified from 89)
5 Summary

Atherosclerosis is a chronic inflammatory process where pro-inflammatory cytokines and chemokines mediate the continuous recruitment of inflammatory cells and thereby increase lesion inflammation.\(^{18, 19, 54, 187}\) The chronic inflammation in atherosclerosis damages the structural components of lesions, such as SMCs and collagen, by increasing degradation and apoptosis, which promotes lesion vulnerability to hemodynamic stress.\(^{5, 18, 19, 187, 209}\) Hence, treatment strategies to stabilize advanced rupture prone lesions should aim at decreasing the lesion inflammation and increasing the structural components, which would increase the biomechanical strength of the lesions. Treatment with ex vivo expanded SPCs induces stable lesions in mice model of atherosclerosis.\(^{94}\) Moreover, elevation in plasma CXCL12 level for short duration mobilizes SPCs from BM.\(^{75}\) Furthermore, LPA-mediated HIF-1\(\alpha\) expression in injured carotid artery up-regulates CXCL12, which leads to recruitment of BM-derived SPCs to the lesion site via CXCR4.\(^{72, 100, 101}\) Recruited SPCs differentiate to SMCs and contributes to the neointimal growth.\(^{75}\) Therefore, the effect of CXCL12 treatment on stabilization of vulnerable lesions was studied.

Application of CXCL12 transiently increased SPCs in the circulation by eliminating the physiological CXCL12 gradient between the BM and circulation. Repeated treatment with CXCL12 reduced the lesional macrophage content without affecting the lesion size. Moreover, FC thickness, and the lesional SMC and collagen-I content were also increased by CXCL12. The induction of a stable lesion phenotype by CXCL12 was due to increased recruitment of SPCs from the circulation, which differentiated specifically to lesional SMCs. Recruitment of SPCs was in part due to increased expression of CXCL12 in lesions after CXCL12 treatment presumably mediated by HIF-1\(\alpha\). Furthermore, CXCL12 treatment had no effect on inflammatory cell mobilization or recruitment. Of note, the stable lesion phenotype with increased SMCs, collagen and decreased macrophages was sustained even in the absence of further CXCL12 treatment, which subjected application of CXCL12 as a potential therapeutic approach for treating unstable lesions.

To study the role of endothelial HIF-1\(\alpha\) in atherosclerosis, Apoe\(^{-/-}\) mice with an EC-specific deletion of Hif1a were studied. Endothelial deletion of HIF-1\(\alpha\) reduced the lesion size and lesional macrophage content in aortas in diet-atherosclerosis and in disturbed flow-induced atherosclerosis in the carotid artery. Interestingly no effect on lesional SMCs and collagen-I was observed, which suggests that endothelial HIF-1\(\alpha\) is involved primarily in
monocyte recruitment to the lesion site. Moreover, HIF-1α was up-regulated in ECs by the pro-atherogenic factors moxLDL, unsaturated LPA, and AT-2. Furthermore, stimulation of ECs with moxLDL and LPA20:4 increased transcription and translation of CXCL1 in an HIF-1α- and LPA receptor-dependent manner. Silencing of HIF-1α reduced monocyte adhesion to moxLDL- and LPA20:4-stimulated ECs. Thus, these findings suggest that endothelial HIF-1α participates in the early recruitment of monocytes by augmenting monocyte adhesion to the endothelium via increased synthesis of CXCL1 and thus drives the inflammatory response in atherosclerosis.
6 References


35. Ball RY, Stowers EC, Burton JH, Cary NR, Skepper JN, Mitchinson MJ. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis.* 1995;114:45-54


98. Han CI, Campbell GR, Campbell JH. Circulating bone marrow cells can contribute to neointimal formation. *J Vasc Res*. 2001;38:113-119


103. Hamesch K, Subramanian P, Li X, Dembowski K, Chevalier E, Weber C, Schober A. The cxcr4 antagonist pol5551 is equally effective as sirolimus in reducing neointima


108. Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol.* 2006;70:1469-1480


References


199. Wood LD, Farmer AA, Richmond A. Hmgi(y) and sp1 in addition to nf-kappa b regulate transcription of the mgsa/gro alpha gene. *Nucleic Acids Res*. 1995;23:4210-4219


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Y.CD34+/CD140b+ cells and CXCL12 in the blood correlate with the angiographically assessed severity of cardiac allograft vasculopathy. Eur Heart J. 2011 Feb; 32(4):476-84.


CONFERENCES

Oral Presentation

Poster Presentation
Shamima Akhtar, Felix Gremse, Fabian Kiessling, Christian Weber, Andreas Schober. Stabilization of atherosclerotic plaques by CXCL12- induced mobilization of smooth muscle progenitor cells at Joint meeting of the European Society of Microcirculation (ESM) and German Society of Microcirculation and Vascular Biology (GfMVB), Munich, Germany (2011).
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