

Role of endothelial CXCR4 in neointimal hyperplasia after wire injury

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ABBREVIATIONS

Apoe	apolipoprotein E
APC	allophycocyanin
APC-Cy7	allophycocyanin-cyanine 7
Arg	arginase
Bmx	bone marrow x kinase
BSA	bovine serum albumin
CAD	coronary artery disease
CCA	common carotid artery
CCL	CC chemokine ligand
CD	cluster of differentiation
CVD	cardiovascular disease
CXCR4	Chemokine (C-X-C motif) receptor 4
CXCL12	Chemokine (C-X-C motif) ligand 12
Cy3	cyanine 3
DAPI	4',6-diamindino-2-phenylindole
DC	dendritic cell
EC	endothelial cell
ECA	external carotid artery
EDTA	ethylenediaminetetraacetic acid
ELC/CCL19	Epstein-Barr virus induced molecule 1 ligand chemokine
ELISA	enzyme-linked immunosorbent assay
ELR	glutamatic acid-leucine-arginine
EPC	endothelial progenitor cell
ER	estrogen receptor
EVG	elastic van Gieson
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
Flk1	fetal liver kinase 1
Flt3	Fms-like tyrosine kinase 3
FSC	forward scatter
FMO	fluorescence minus one
G-CSF	granulocyte colony-stimulating factor
GPCR	G protein-coupled receptor
GM-CSF	granulocyte monocyte colony-stimulating factor
gMFI	geometric mean fluorescence intensity

Gr-1	granulocyte antigen 1
h	hour
HAoEC	human aortic endothelial cell
HBSS	Hank's balanced solution
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HPC	hematopoietic progenitor cell
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
ICA	internal carotid artery
IgG	immunoglobulin G
IL-8	interleukin-8
i.p.	intraperitoneal injection
IP-10	interferon- γ inducible protein-10 (CXCL10)
kDa	kilo-dalton
KO	knockout
Lin	lineage
LDL	low-density lipoprotein
LSA	left subclavian artery
Mac2	macrophage galactose-specific lectin-2 (Galectin-3)
MCP-1/CCL2	monocyte chemoattractant protein-1
MIF	macrophage migration inhibitory factor
min	minute
MIP	macrophage inflammatory protein
MV	microvascular
NK	natural killer
PARC/CCL18	pulmonary and activation-regulated chemokine
PBS	phosphate buffered saline
oxLDL	oxidized LDL
PARC	T-cell chemoattractant pulmonary and activation-regulated chemokine
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy7	phycoerythrin-cyanine 7
PF-4	platelet factor-4
PFA	paraformaldehyde
RANTES	regulated on activation normal T cell expressed and secreted
RSA	right subclavian artery
RT	room temperature

RT-PCR	real time PCR
S1P	sphingosine-1-phosphate
Sca1	stem cell antigen-1
SDF-1 α	stromal cell-derived factor-1 α
SEM	standard error of mean
Sma	smooth muscle actin
SMC	smooth muscle cell
SNP	single-nucleotide polymorphism
SPC	smooth muscle progenitor cell
SSC	side scatter
TMB	tetramethylbenzidine
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor 2
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

1. Introduction

1.1 Atherosclerosis

Cardiovascular disease (CVD) is a group of disorders of the heart and blood vessels, including stroke, heart failure and coronary heart disease, which affect the cardiovascular system. The major cause of morbidity and death worldwide is coronary heart disease, also called coronary artery disease (CAD). The underlying pathology of CAD is atherosclerosis¹.

Atherosclerosis is characterized by a chronic inflammatory process in the vessel wall with an initial endothelial dysfunction, triggering the formation of fatty streaks that can evolve in more progressed atherosclerotic plaques^{2, 3}. This inflammatory response is driven by many cells, growth factors, cytokines and chemokines³.

The development of atherosclerosis is initiated by an endothelial dysfunction of the arterial wall²⁻⁴, which leads to an increased infiltration of lipid components, like low-density lipoprotein (LDL), into the vessel wall and induces the adhesion of leukocytes and activated platelets to the damaged endothelial area⁵ (Figure 1A). Triggered by a local gradient of chemoattractant cytokines, monocytes are then recruited to the damaged sites of the arterial wall and migrate into the subendothelial space, where they differentiate into macrophages. By taking up oxidized LDL (oxLDL), macrophages transform into foam cells, which accumulate to form early lesions (fatty streaks) in the intima³ (Figure 1B). With time, plaques become bigger due to an increasing accumulation of inflammatory cells (mainly macrophages and T cells)⁶ and extracellular lipids. A necrotic core is formed by dying macrophages that have taken up too many lipids and apoptotic cell fragments. At the same time, smooth muscle cells (SMCs) begin to proliferate and migrate to the plaque area. They produce extracellular matrix components as collagen, triggering the

formation of a fibrous cap, which covers the plaque beneath the endothelium (Figure 1C). The progression of an atherosclerotic plaque by further deposition of extracellular matrix components can induce an arterial luminal narrowing (stenosis)⁴. Additionally, neovascularization and hemorrhage can occur in progressed atherosclerotic lesions. The continuing influx and activation of macrophages cause a thinning or degradation of the fibrous cap by the release of metalloproteinases and other proteolytic enzymes, characteristic for an unstable plaque or rupture-prone lesion^{2, 4}. Such unstable lesion can eventually result in plaque erosion or rupture and trigger the formation of a thrombus (Figure 1D), which can block the blood vessel and induce stroke, myocardial infarction or coronary syndrome^{4, 5}.

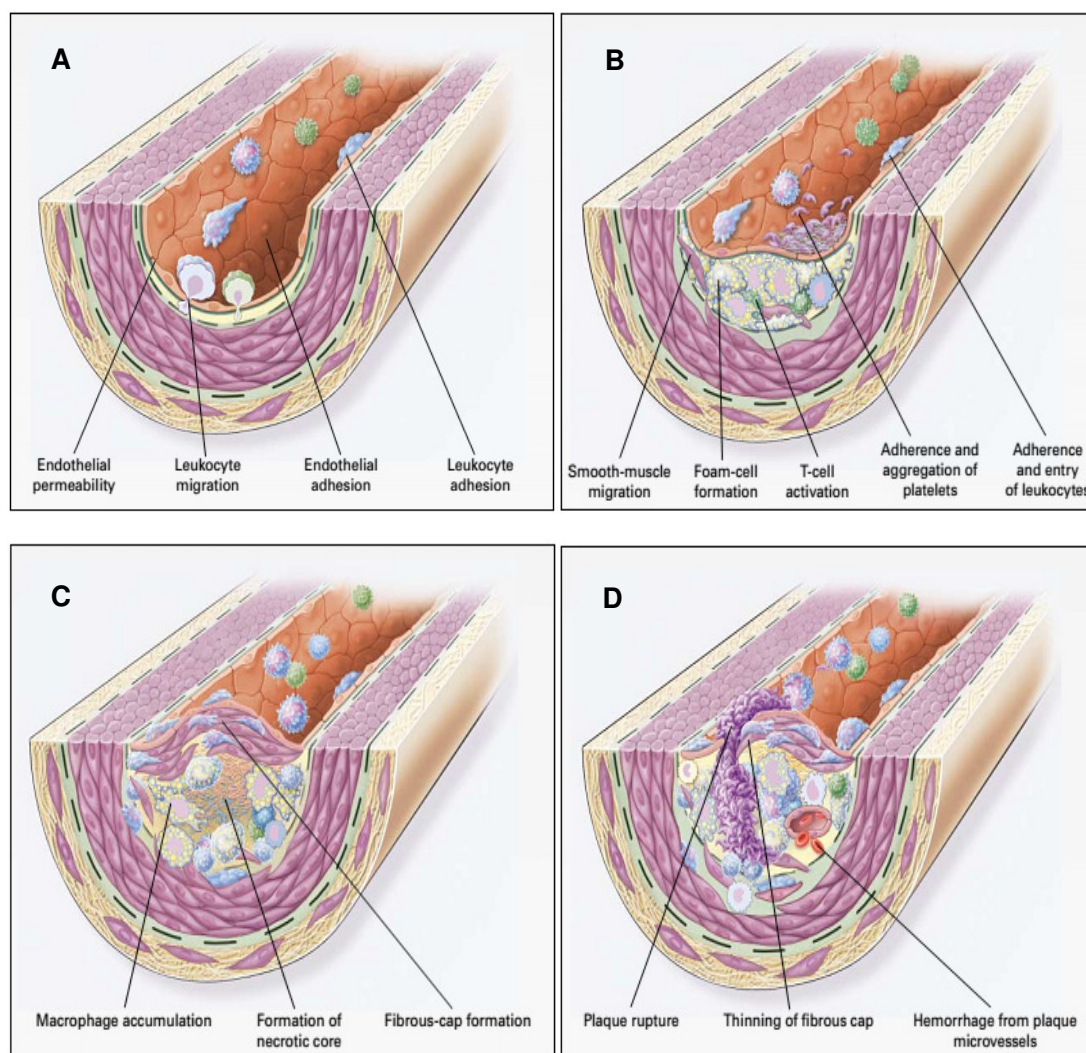


Figure 1: **Evolution of atherosclerosis.** **A**, Endothelial dysfunction of the arterial wall is the initial step of atherosclerosis, inducing the adhesion of leukocytes and activated platelets to the damaged endothelial area and increased endothelial permeability to lipoproteins. **B**, Monocytes are recruited to the damaged site of the arterial wall and differentiate into macrophages. Macrophages transform into foam cells by taking up oxLDL, leading to the formation of early lesions (fatty streaks). **C**, By accumulation of inflammatory cells, e.g. foam cells and T cells, and extracellular lipids, a necrotic core is formed. Proliferation and migration of SMCs to the plaque area, together with the deposition of a collagen-rich matrix, contribute to the formation of a fibrous cap. **D**, Neovascularization and hemorrhage can occur in a progressed atherosclerotic lesion. A thinning or degradation of the fibrous cap characterizes an unstable or rupture-prone lesion, which can eventually result in plaque erosion or rupture and formation of a thrombus. (Figure from Ross R. *et al.*²)

1.2 Restenosis and Mechanical Denudation

Since atherosclerosis is the main cause inducing cardiovascular disease, including myocardial infarction and stroke, management of patients with severe atherosclerotic plaques has evolved over the years. Treatment options differ depending on the grade of severity and include lifestyle changes, medicines, and medical procedures or surgery. Surgical treatments as angioplasty and stenting techniques, are widely used for treating patients with atherosclerotic disease^{7, 8}. Balloon angioplasty and stent implantation aim to remove the vascular obstruction in order to expand the inner diameter of the artery. With this method the blood flow to the heart is improved. However, limitations occur after the treatment, one of which is “restenosis”^{7, 8}.

Restenosis is the re-narrowing of a dilated artery initiated by mechanical denudation of the endothelium during intervention. It is a major limitation of surgical intervention techniques, and often occurs in the first 3-6 months after treatment⁷⁻⁹. Figure 2 shows restenosis of a stent-widened coronary artery. With an expanded stent, a plaque in the coronary artery is compressed to

widen the artery and allow normal blood flow. However, stent insertion is associated with endothelial injury, triggering apoptosis of medial SMCs followed by a hyperproliferative response and the formation of an SMC-rich neointima. Over time, the neointimal lesion grows through the stent, causing re-narrowing of the artery, which is called restenosis.

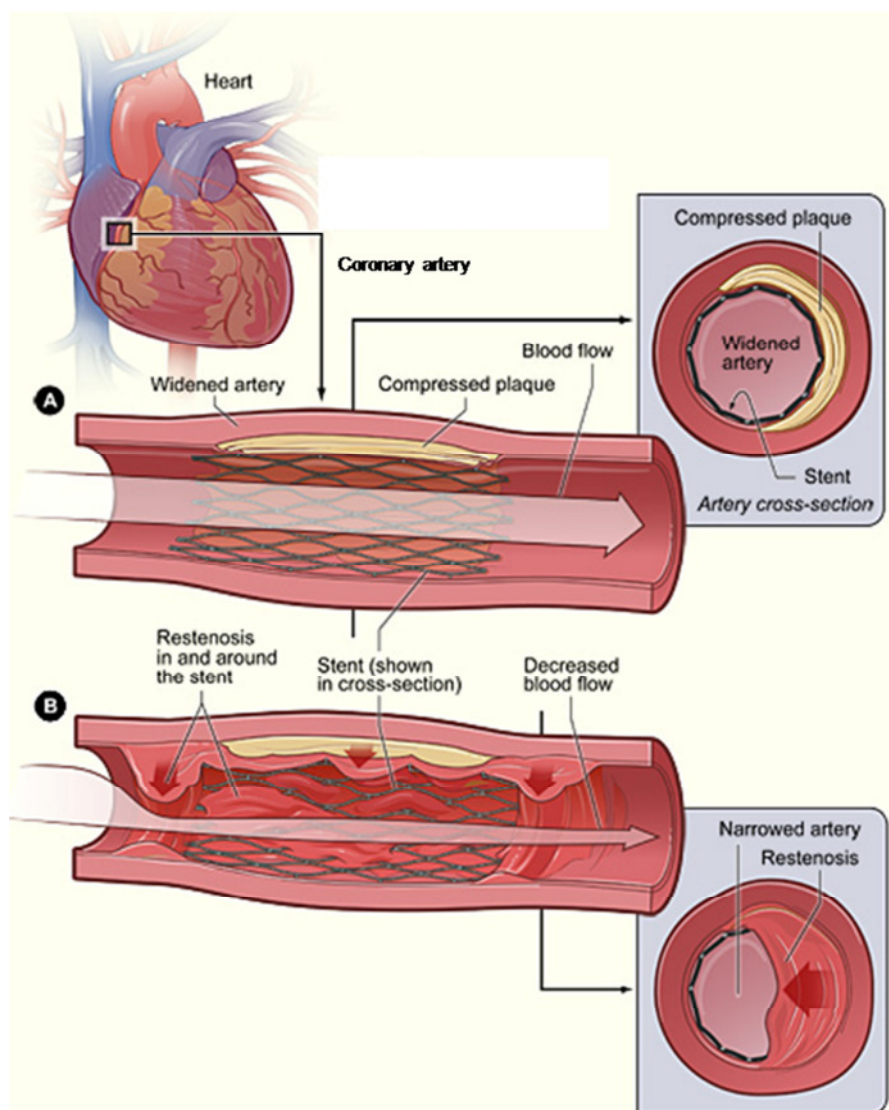


Figure 2: **Restenosis of a stent-widened coronary artery.** **A**, An atherosclerotic plaque is compressed by an expanded stent to widen the artery lumen, allowing normal blood flow. **B**, Triggered by endothelial injury during stent insertion, a neointimal lesion develops and grows through the stent over time, causing re-narrowing of the artery lumen (Figure from <http://www.nhlbi.nih.gov/health/health-topics/topics/angioplasty/risks.html>).

Although restenosis entails a chronic inflammation of the vessel wall in response to injury⁸, the underlying pathological process triggering restenosis is different compared with native atherosclerosis⁹. A native atherosclerotic plaque is formed by a chronic process of lipid accumulation through endothelial dysfunction, inducing inflammatory cell recruitment. Vascular SMCs contribute to plaque stabilization through the formation of a protective fibrous cap. In contrast, restenosis is triggered by intervention-induced endothelial injury or denudation during mechanical dilatation of the blood vessel, triggering the formation of an SMC-rich neointimal lesion^{9, 10}. After endothelial disruption and an initial apoptotic response of vascular SMCs, SMCs from the media start to proliferate and migrate to the subendothelial space^{10, 11}. Thus, SMCs drive injury-induced neointima formation, whereas they are considered protective in native atherosclerosis by forming a protective fibrous cap over a native atherosclerotic lesion.

However, similarly to native atherosclerosis, mechanical denudation also induces vascular inflammation. The presence of an injured and incomplete EC layer allows leukocytes from the blood stream to easily accumulate at the site of injury. Chemokines and inflammatory cytokines are produced by the accumulating inflammatory cells as well as by vascular SMCs and endothelial cells (ECs). These mediators trigger a further recruitment of inflammatory cells (e.g. monocytes), which contribute to vascular inflammation and neointima formation.

Also, chemokines and cytokines mobilize bone marrow-derived progenitor cells, as endothelial and smooth muscle progenitor cells, to the site of injury¹². The role of progenitor cells in injury-induced neointimal hyperplasia is discussed in more detail in chapter 1.4.

1.3 Reendothelialization

The endothelium is the innermost layer of a vessel and consists of a monolayer of ECs. Healthy vascular endothelium modulates vascular tone and SMC proliferation¹³ and protects against subendothelial lipid accumulation, inflammatory cell infiltration and thrombus formation¹⁴. In pathological conditions or after vessel intervention, the normal regulatory functions of ECs are altered or disrupted, which is described as “endothelial dysfunction” and commonly initiates atherosclerosis or restenosis¹³.

Mechanically-induced damage of the endothelial layer of the vessel wall is followed by proliferation and migration of viable, neighboring ECs to the injured site, with the aim to restore the damaged endothelium. This process is called reendothelialization and is triggered by a lot of biological factors such as growth factors, cytokines and chemokines.

Reendothelialization plays a crucial protective role against neointima formation. It has been reported that EC seeding on an injured vessel wall of atherosclerotic rabbits significantly reduces the neointimal area and enhances endothelial recovery, followed by a reduction of restenosis¹⁵. Also EC implants after vascular injury in pigs revealed a delayed neointima formation¹⁶. Moreover, restoration of the endothelium is inversely related to neointimal thickness. An accelerated regeneration of the endothelium protects against neointima formation, whereas an increased neointimal hyperplasia is often associated with a delayed reendothelialization^{17, 18}.

In addition to neighboring ECs contributing to endothelial repair, reendothelialization is also influenced by circulating endothelial progenitor cells (EPCs, more details in Chapter 1.4). EPCs are derived from bone marrow after vessel injury¹⁹⁻²¹ and contribute to endothelial repair through differentiation/maturation into mature ECs²² or by stimulating migration/proliferation of local, mature ECs through cytokine production (Figure 3). Studies have shown that transplantation of EPCs enhances

reendothelialization in prosthetic vascular grafts in human^{23, 24} and in injured vessels of rabbits associated with an inhibitory effect on neointimal hyperplasia²⁴. Also, statin therapy enhances the number of circulating EPCs²², and simultaneously promotes reendothelialization²⁵. Although EPCs may contribute to endothelial repair by differentiating locally into ECs, the current view rather supports a paracrine role for EPCs, in which EPCs produce various proangiogenic cytokines and growth factors that promote proliferation and migration of preexisting local ECs²⁶.

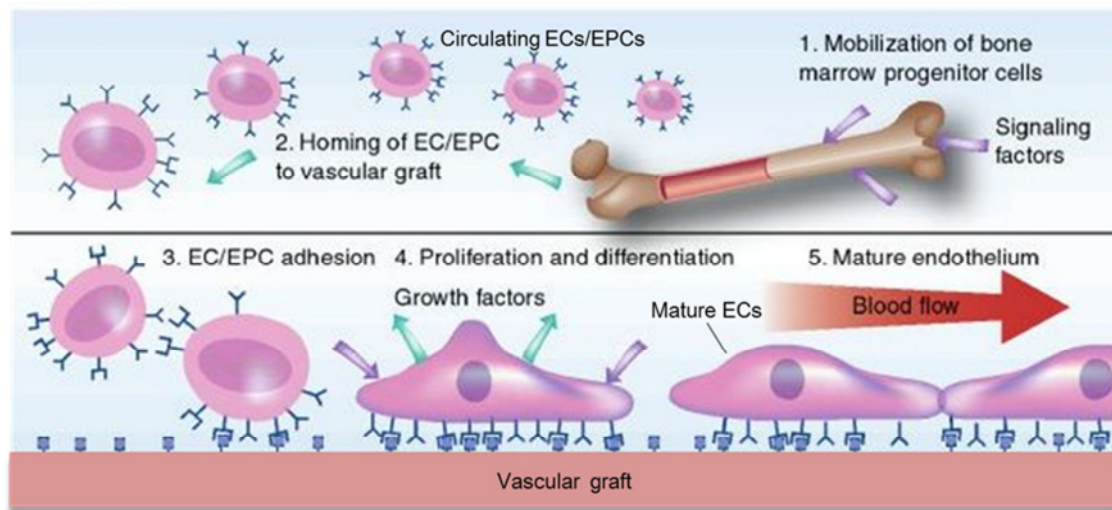


Figure 3: **Reendothelialization.** Reendothelialization is a process of EC recovery driven by migration and proliferation of local mature ECs. Furthermore, bone marrow-derived circulating EPCs play an important role in reendothelialization, either by differentiation into mature ECs or through paracrine stimulation of local ECs. Mobilization of EPCs to an injured vascular site and their differentiation is driven by multiple signaling molecules, e.g. chemokines and growth factors (Figure from Kabir AM *et al.*²⁷).

Mobilization and recruitment of EPCs to injured sites are mediated by chemokines and their receptors (which are discussed in more detail in Chapter 1.5) Together with its ligand CXCL12, the chemokine receptor CXCR4 is important for the mobilization of progenitor cells from the bone marrow²⁸⁻³⁰.

Furthermore, CXCR4 has been shown to be involved in mediating the recruitment of EPCs to injury-induced neointima^{21, 31}, contributing to reendothelialization of denudated vessels^{20, 21}. Also, the chemokine receptor CXCR2 on EPCs contributes to endothelial recovery³², as inhibition of CXCR2 on EPCs reduced the incorporation of injected EPCs in an injured carotid artery with 30 percent³¹. Finally, also chemokine receptor CXCR7, a second chemokine receptor for CXCL12, is expressed on the EPC surface, and a declined expression of CXCR7 on EPCs from patients with hypertension was recently related to a reduced *in vivo* reendothelialization capacity of EPCs³³.

In summary, reendothelialization is a complex process of EC recovery, driven by the proliferation and migration of mature ECs and the recruitment of circulating EPCs. Multiple signaling molecules are involved in this process, e.g. chemokines. Reendothelialization plays an important role in protection against neointimal proliferation after vascular denudation, and in reducing the risk of thrombosis.

1.4 Endothelial Progenitor Cells (EPCs)

EPCs originate from the bone marrow, can be mobilized by chemokines and cytokines and recruited to sites of injury, where they can differentiate into mature ECs or drive proliferation and migration of resident mature ECs through paracrine effects. Human EPCs were first described by Asahara et al. in 1997. They showed that human CD34⁺ mononuclear blood cells could adopt an endothelial phenotype after specific culturing conditions *in vitro*, and these cultured “EPCs” contributed to active angiogenesis in animal ischemic models³⁴. Since this discovery, EPCs have been widely investigated. Mainly, 2 categories of EPCs have been studied. “Culture-derived EPCs” are EPCs that are derived after culturing of specific isolated blood cells under specific defined

conditions. On the other hand, “circulating EPCs” refers to circulating progenitor cells in the blood, which can differentiate into mature ECs in specific conditions³⁵⁻³⁷. However, connection between these culture-derived and circulating EPCs is still unclear.

Two kinds of culture-derived EPCs have been reported, being early and late EPCs, the latter also called late outgrowth EPCs/ECs^{35, 38}. Early EPCs can influence EC regeneration through still unclear cellular and paracrine effects, but do not have the ability themselves to directly mediate vasculogenesis and EC regeneration. In contrast, late outgrowth ECs mostly express endothelial lineage markers without significant amounts of hematopoietic surface markers and possess the ability to form intact vascular networks³⁵.

The surface markers of circulating EPCs are not well defined, and various surface markers are being used by different researchers to quantify this cell population, severely complicating EPC research. Human circulating EPCs have mostly been characterized by using vascular endothelial growth factor receptor 2 (VEGFR-2 or FLK1) and CD34 as markers. Whereas CD34 is shared by human circulating EPCs and hematopoietic stem cells (HSCs), VEGFR-2 was shown to be only present on circulating EPCs and not HSCs³⁶. CD34 is also expressed on mature ECs, but to a lower extent. Furthermore, CD133 is often included as an additional human progenitor cell marker, both present hematopoietic as endothelial progenitor cells, but its expression is lost when EPCs differentiate into mature ECs³⁹. Also in mouse, the definition of “circulating EPCs” is really vague and undefined. Several mouse studies used Sca1⁺Flk1⁺ as cell markers in flow cytometric analysis for quantifying circulating EPCs^{40, 41}.

It has been reported that dysfunction and reduced levels of EPCs in clinical disease states was associated with poor wound healing, *e.g.* in diabetes⁴². Interestingly, subjects with increased numbers of circulating EPCs show a better endothelial function as measured by increased brachial reactivity, independent of their Framingham risk score quantifying cardiovascular risk²⁶.

Furthermore, a reduction of circulating EPC number revealed endothelial dysfunction and also high risk factors of atherosclerotic disease^{43, 44}.

Mechanistically, bone marrow-derived EPCs have the ability to be mobilized into the peripheral blood and migrate to damaged sites to induce endothelial recovery. As such, EPCs have been indicated to play a crucial role in reendothelialization after vessel injury^{19, 45} as well as neovascularization in ischemic tissue^{19, 46}. For example, transplantation of EPCs in rats showed a significant enhancement of new blood vessel formation after myocardial infarction⁴⁷ and hind limb ischemia^{48, 49}. In context of vessel injury, reendothelialization of denudated vessels could be accelerated by the same strategy, i.e. EPC transplantation, which was associated with a reduction in neointimal hyperplasia²⁴, as already discussed in more detail in Chapter 1.3. Furthermore, studies have shown that through statin treatment or physical exercise the number of circulating EPCs is increased, leading to a significant enhancement of endothelial recovery and a declined neointima formation after vascular injury^{40, 50, 51}. In conclusion, EPCs contribute to endothelial recovery and vascular repair and in that way may reduce the risk of cardiovascular disease^{19, 44}.

EPCs can be mobilized by cytokines and chemokines from the bone marrow into the peripheral blood and damaged tissue in response to injury^{21, 52, 53}. Vascular endothelial growth factor (VEGF) can regulate EPC mobilization and recruitment through its receptor VEGFR-2 expressed on the EPC surface^{52, 54, 55}. Similarly, it has been reported that hematopoietic progenitor cell (HPC) mobilization was induced by granulocyte colony-stimulating factor (G-CSF)⁵⁶. Furthermore, granulocyte monocyte colony-stimulating factor (GM-CSF) has the ability to direct circulating EPCs to ischemic hind limb tissue, triggering enhanced neovascularization⁵⁷. Also, chemokines like CXCL12 and its receptor CXCR4 contribute to EPC mobilization^{15, 28, 29}, as is discussed in more detail in Chapter 5.2. In addition, macrophage migration inhibitory factor (MIF), a ligand for the chemokine receptors CXCR4 and CXCR2⁵⁸, has been

reported to trigger EPC recruitment and migration to ischemic myocardial cells⁵⁹.

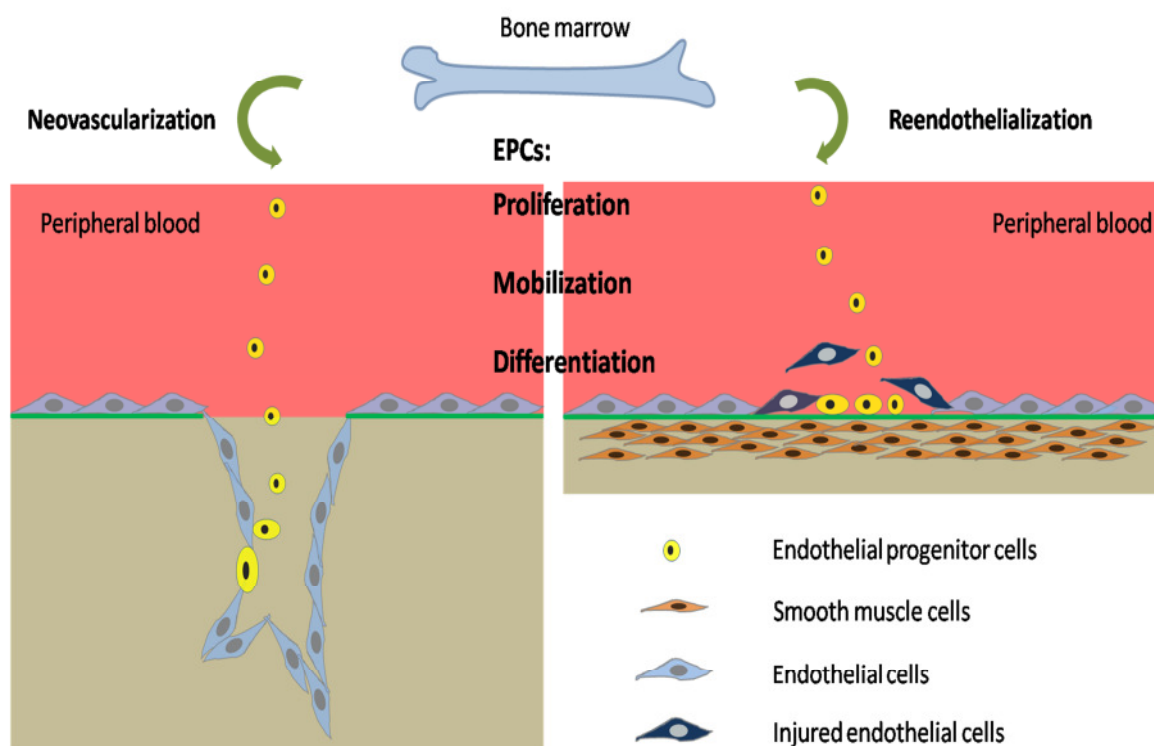


Figure 4: **EPCs**. EPCs are derived from bone marrow and can be mobilized to sites of injury, where they can proliferate and differentiate into mature ECs. In this way, EPCs are involved in neovascularization and reendothelialization.

In conclusion, EPCs are a heterogeneous cell group, which in phenotype and function still need to be defined in more detail. Several cytokines and chemokines are able to promote EPC mobilization from the bone marrow and recruitment to injured sites, where they contribute to reendothelialization after vessel denudation^{20, 21} and neovascularization of ischemic tissue⁵³ (Figure 4). This gives a new view on the therapeutic potential to increase the EPC number by cytokine or chemokine treatment, to accelerate vascularization or recovery of damaged vessels in order to abate the risk on cardiovascular disease.

1.5 Chemokines and Chemokine Receptors

1.5.1 Definition, Classification and Function

In 1992 the term “chemokine” was introduced as an abbreviation of “chemotactic cytokine”. The first chemokine that was characterized, is CXCL8, which is also known as interleukin-8 (IL-8)^{60, 61}. Chemokines or chemoattractant cytokines are a family of structurally related low molecular weight glycoproteins with a molecular weight of the single polypeptides of around 8-10 kDa and a length range from 70-100 amino acids^{3, 62}. Chemokines can guide the movement of leukocytes in homeostatic condition and also direct the migration of immune cells to inflamed or injured sites in pathological condition⁶³⁻⁶⁵. There are approximately 50 known chemokines, which are classified in 4 subgroups based on their structure, and more specifically on the location of the first two cysteine residues. These four subfamilies are termed C, CC, CXC and CX₃C^{3, 62, 64-66} (Figure 5).

Structure of chemokine classes

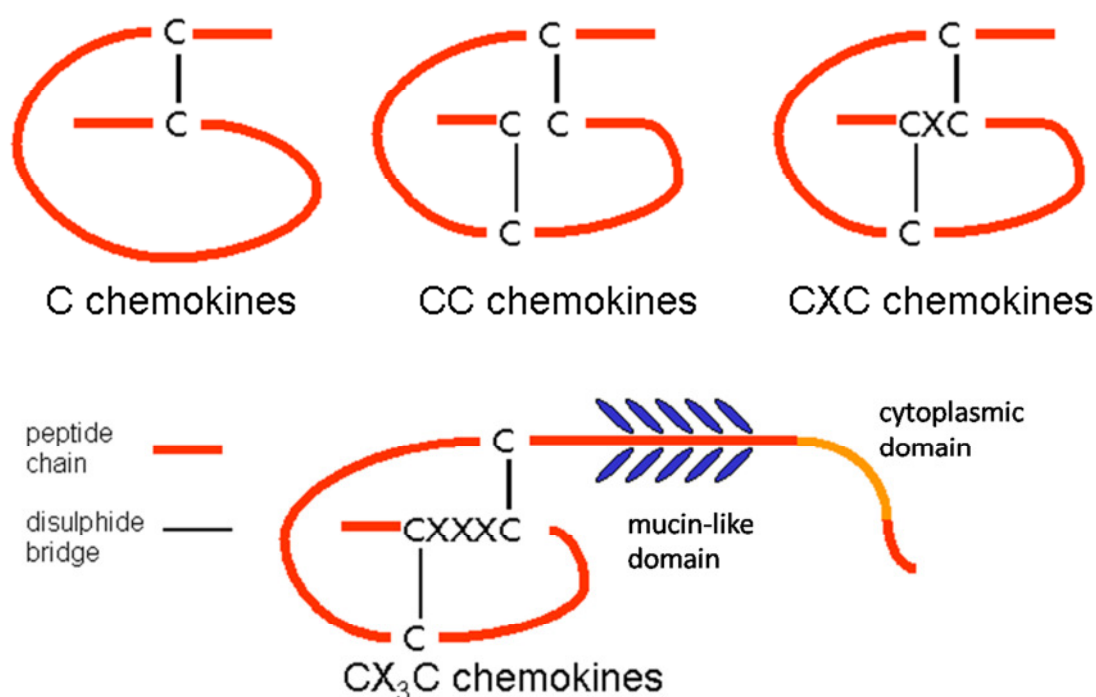


Figure 5: **Classified chemokine structures.** Four different subgroups of chemokines exist: C, CC, CXC and CX₃C. All chemokines exhibit the same basic structural fold known as the “chemokine fold”, consisting of a short, disordered N-terminal region containing the characteristic cysteine motif, an extended N-loop region, 3 antiparallel β -strands linked by turns designated 30s-, 40s- and 50s-loop, and finally a C-terminal α -helix. (Figure from <http://en.wikipedia.org/wiki/File:ChtxChemokineStruct.png>).

The group of “C chemokines” has only one member, Lymphotactin (XCL1), which contains a single cysteine residue with a chemotactic function towards lymphocytes⁶⁷. Similarly as for the C group, only one member has been discovered until now in the CX₃C group, in which the first N-terminal pair of cysteine residues is separated by 3 other amino acids⁶². This CX₃C chemokine is named fractalkine (CX₃CL1), a large chemokine of 373 amino acids that contains in addition to the typical chemokine domain an extended mucin-like domain, which allows its binding to the surface of cells, and a cytoplasmic domain³. This cell-bound CX₃CL1, which is for example found on activated ECs, can enhance the adhesion of leukocytes, whereas a soluble version of the chemokine has the ability to induce chemotaxis of monocytes and T cells⁶⁷.

The CC chemokine family contains two adjacent cysteine residues and consists of around 28 members (CCL1 – CCL28), binding to 10 known chemokine receptors (CCR1 – CCR10)^{65, 68}. The group of CC chemokines plays an important role in mediating chemotaxis of leukocytes, as T cells, monocytes, macrophages and neutrophils, in chronic inflammatory conditions⁶⁵. CC chemokines like CCL1 (I-309), CCL2 (monocytes chemoattractant protein-1, MCP-1), CCL3 (macrophage inflammatory protein, MIP-1 α), CCL4 (MIP-1 β) and CCL5 (regulated on activation normal T cell expressed and secreted, RANTES) have been found to be expressed in atherosclerotic lesions^{65, 69-71}. CCR2, the main receptor for CCL2, is expressed on T-lymphocytes, monocytes and macrophages, and has been shown to mediate monocyte migration into the vascular wall⁷². Deletion of CCL2 or its

receptor CCR2 in mice revealed reduced atherosclerotic lesion size associated with less macrophage deposits^{73, 74}. Similarly, T-cell chemoattractant pulmonary and activation-regulated chemokine (PARC/CCL18) was found to be highly expressed by macrophages in human atherosclerotic lesions⁷⁵. Epstein-Barr virus-induced molecule 1 ligand chemokine (ELC/CCL19), which is also able to recruit T-cells⁷⁶, was expressed not only by macrophages but also by medial SMCs in plaque regions⁷⁵. A neutralizing antibody treatment against CCL12 revealed a decreased lesion size, concomitant with a reduced content of leukocytes and macrophages and an increased content of SMCs and collagen, indicating a relatively stable plaque phenotype in the absence of functional CCL12⁷⁷.

CXC chemokines contain one amino acid residue in between the first and second cysteine residue^{3, 62, 65}. Based on the presence or absence of a glutamatic acid-leucine-arginine (ELR) motif preceding the first conserved cysteine residue, the CXC chemokine group can be divided into two subgroups, namely ELR⁺ and ELR⁻^{78, 79}. ELR⁺ CXC chemokines have the ability to attract neutrophils to inflammatory sites, as for example shown for CXCL8 *in vitro* and *in vivo*⁸⁰ through binding to its receptor CXCR2⁸¹. In addition, it has been reported that CXCL8 acts on ECs and leukocytes through its receptors CXCR1 and CXCR2 to enhance angiogenesis and immune infiltration, respectively, and similarly also enables cancer cells to survive and migrate in an inflammatory microenvironment⁸²⁻⁸⁴. Moreover, ELR⁺ CXC chemokines have the ability to mediate the mobilization of EPCs from the bone marrow and their recruitment to angiogenetic sites⁸⁵. In contrast to ELR⁺ CXC chemokines, ELR⁻ CXC chemokines rather tend to be attractants for natural killer (NK) cells and B and T lymphocytes^{61, 86, 87}, and are mostly not angiogenic, some even angiostatic⁸⁸. For example, ELR⁻ CXC chemokine platelet factor-4 (PF-4/CXCL4) mediates chemoattraction of dendritic cells (DCs), NK cells and T lymphocytes and inhibits angiogenesis through its receptor CXCR3^{89, 90}. Similarly, ELR⁻ CXC chemokine interferon- γ inducible protein-10

(IP-10/CXCL10) functions as an angiostatic factor and can attract anti-tumoral lymphocytes via its receptor CXCR3⁹¹. However, stromal cell-derived factor-1 α (SDF-1 α /CXCL12) is an exception in this ELR⁻ CXC chemokine group; together with its receptor CXCR4 it plays an important role in stimulating angiogenesis^{61, 91, 92}. Taken together, the CXC chemokine family is generally involved in modulation of angiogenesis, either as angiogenic factors as for ELR⁺ CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8)⁸⁵ or as angiostatic regulators as for certain ELR⁻ CXC chemokines (CXCL4, CXCL9, CXCL10, CXCL11 and CXCL14)^{61, 65, 85}.

Chemokine receptors are seven transmembrane G protein-coupled receptors (GPCRs) and are classified according to the chemokines they bind. Around 20 chemokine receptors have been identified⁹³. Since there are more chemokines than receptors, some receptors can bind more than one chemokine. Also the other way around, some chemokines are able to bind to several chemokine receptors^{94, 95} (Figure 6). Cells are influenced by chemokines through activation of the corresponding chemokine receptors⁶⁵.

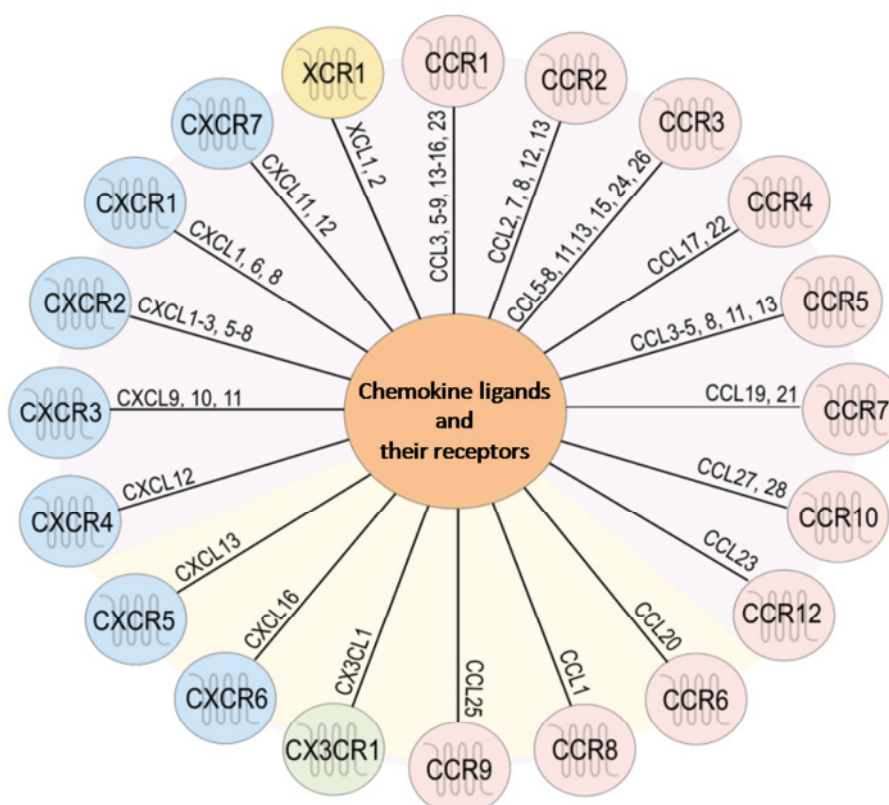


Figure 6: **Chemokine ligands and their receptors.** Since there are more chemokines than receptors, some receptors can bind to more than one chemokine. (Figure from White GE *et al.*⁶⁸)

Chemokines and their receptors are expressed by a lot of cells, such as ECs, SMCs, leukocytes, epithelial cells, tumor cells and fibroblasts⁷⁸. They have an important function in both homeostatic and inflammatory conditions. For example, chemokines and their receptors are involved in directing (progenitor) cell migration during embryonic development, wound healing, adaptive and innate immunity as well as angiogenesis. Chemokines and their receptors also drive cell mobilization and recruitment of inflammatory cells to specific sites of chronic inflammation, potentially leading to tumorigenesis and autoimmune diseases⁸⁵. In addition to inducing cell chemotaxis, chemokines play an important role in cell activation⁹⁶. For example, chemokines can modulate gene expression and trigger the release of inflammatory mediators from *e.g.* macrophages and neutrophils. Furthermore, chemokines play an important role in cell arrest⁹⁶, for example of leukocytes on activated ECs, an important step in atherosclerosis. In this leukocyte adhesion cascade, there are mainly three steps, which are selectin-mediated rolling, chemokine-triggered integrin activation and integrin-mediated arrest. In response to inflammation, EC-secreted chemokines induce activation of leukocytic integrins through chemokine receptor signaling in the leukocytes, allowing strong binding and arrest of rolling leukocytes through their activated integrins⁹⁷. As leukocyte adhesion is strongly mediated by selectins, chemokines and cell adhesion molecules^{98, 99}, these molecules are important in the development of atherosclerosis.

1.5.2 The Chemokine Receptor CXCR4

The chemokine receptor CXCR4 is also known as fusin or CD184. It is expressed on many cell types, including monocytes, platelets, macrophages, SMCs, ECs and notably hematopoietic stem cells¹⁰⁰, and is involved in processes of hematopoiesis, vasculogenesis, neurogenesis and cardiogenesis^{101, 102}. Studies of *Cxcr4*-deficient mice are not possible, as genetic deletion of either *Cxcr4* or its ligand *Cxcl12* in mice induces perinatal death due to defects in bone marrow colonization, B cell lymphopoiesis and cardiac septa development^{29, 101}. Furthermore, CXCR4 is involved in mobilization of bone marrow-derived progenitor cells, including EPCs¹⁰³. In this context, activation of CXCR4 by sphingosine-1-phosphate (S1P), which is a bioactive lipid and involved in EC migration and proliferation, can mediate EPC mobilization and promote neovascularization, suggesting that S1P agonists may have therapeutic potential in progenitor cell therapy to improve EPC function in patients with CAD¹⁰⁴. Furthermore, chronic daily treatment with AMD3100, which is an antagonist of CXCR4, partially inhibited vascular recovery effectively blocking VEGF-mediated revascularization of ischemic hindlimb in mice¹⁰⁵. Thus, these data suggest that activation of CXCR4 plays a pivotal role in revascularization of ischemic tissues by promoting EPC, mobilization chemotaxis and/or function. In context of progenitor cell mobilization during restenosis, CXCR4 seems to play a double-edged role. On the one hand CXCR4 is protective by mediating the recruitment of EPCs. On the other hand, CXCR4 drives neointimal hyperplasia by recruiting smooth muscle progenitor cells (SPCs), as will be explained in more detail in the next chapter.

CXCR4 expression has been found in atherosclerotic plaques. However, the role of CXCR4 in native atherosclerosis remains to be further investigated. Chronic blockade of CXCR4 by systemic treatment with a CXCR4 inhibitor led to leukocytosis and an increased recruitment of neutrophils to atherosclerotic

plaques, inducing a pro-inflammatory plaque phenotype¹⁰⁶, but cell type-specific effects of CXCR4 remain unclear.

In conclusion, CXCR4 seems to play a double-edged role in injury-induced restenosis, whereas its role in native atherosclerosis remains unclear. Further research is required before a potential application of CXCR4 antagonists in context of CAD could be considered.

The chemokine CXCL12, also known as SDF-1 α , is the best-known ligand of CXCR4. Like its receptor CXCR4, CXCL12 is ubiquitously expressed and essential for bone marrow engraftment, homeostasis, vascularization during embryogenesis, as well as (hematopoietic) progenitor cell mobilization¹⁰⁷⁻¹¹⁰. Cxcl12 could trigger the mobilization of EPCs enhancing ischemic neovascularization¹¹¹. Furthermore, CXCL12 expression can be detected in atherosclerotic lesions¹¹², and a decreased CXCL12 plasma levels in patients with unstable angina suggested that CXCL12 may have an anti-inflammatory function and may play a beneficial role in CAD¹¹³. Also, it has been shown in an *Apoe*^{-/-} mouse model that Cxcl12 could promote the stabilization of atherosclerotic plaques by mediating the mobilization of SPCs¹¹⁴. On the other hand, a study has shown that the CAD risk alleles of two single-nucleotide polymorphisms (SNPs), rs1746048 and rs501120, are related to higher CXCL12 plasma levels, suggesting a disadvantageous effect of CXCL12 in CAD¹¹⁵.

Furthermore, CXCL12 seems to play a double-edged role in injury-induced restenosis by mediating the mobilization of EPCs vs. SPCs, as will be discussed in more detail in chapter 1.5.3. The role of CXCL12 in vascular and inflammatory cells in context of CAD remains to be further investigated, similarly as for its receptor CXCR4.

Recently, it has been discovered that MIF is another non-canonical chemokine ligand of CXCR4. MIF was initially known as an important cytokine in acute and chronic inflammatory diseases^{64, 116}. Recent evidence revealed that MIF also harbors a chemokine-like function and can bind to the chemokine

receptors CXCR2 and CXCR4¹¹⁷. This was associated with a pro-atherogenic effect of MIF, as blockade of MIF impaired CXCR4-mediated T cell recruitment, and CXCR2-mediated monocyte recruitment, resulting in atherosclerotic plaque stabilization and even regression¹¹⁷. Comparably, blockade of Mif reduced injury-induced neointima formation¹¹⁸ and reduced neointimal macrophage content¹¹⁹ in an animal model of experimental angioplasty and wire-mediated vascular injury, respectively, again suggesting a pro-inflammatory role of MIF. However, MIF has also been described to mediate EPC chemotaxis⁵⁹, which may be mediated by binding to CXCR2 and CXCR4 on the EPC surface³¹. So similarly to CXCL12 and CXCR4, MIF also seems to be associated with protective roles through EPC mobilization and recruitment, which could play a beneficial role in context of injury-induced restenosis and myocardial ischemia.

1.5.3 Role of CXCR4 in Injury-induced Restenosis

As explained in chapter 1.2, restenosis is caused by intervention-induced endothelial injury, and mediated by arterial remodeling and the formation of neointimal lesions mainly containing inflammatory leukocytes and SMCs^{10, 120}. An accelerated endothelial recovery of the injured vessel is important to reduce the risk on restenosis¹⁸ and EPCs contribute to this reendothelialization step after vessel injury^{19, 45}. In this process, chemokines and their receptors play an essential role. CXCR4 is a key chemokine receptor in regulating homing of bone marrow-derived progenitor cells to an injured vascular wall¹²¹. Blockade of CXCR4 signaling resulted in decreased recruitment of EPCs to injured arteries³¹. Moreover, *Cxcr4* gene transfer increased the reendothelialization capacity of EPCs in mice with denuded carotid arteries¹²². Similarly, *Cxcl12* was shown to be involved in the mobilization and recruitment

of EPCs after arterial injury⁴¹. Thus, these studies reveal a beneficial role of the Cxcl12/Cxcr4 axis in injury-induced restenosis by directing the migration of protective EPCs.

On the other hand, accumulating studies have demonstrated that Cxcr4 mediates the recruitment of SPCs to injury-induced neointima^{21, 49}. In *Apoe*^{-/-} mice, vessel injury induced upregulation of the Cxcr4 ligand Cxcl12 in the injured vessel. Furthermore, the plasma level of Cxcl12 was significantly increased after carotid artery injury, followed by an enhanced mobilization of circulating Lin⁻Sca1⁺ progenitor cells, often referred to as SMC progenitor cells¹²³. A decreased neointimal hyperplasia and neointimal SMC content after arterial injury were observed in *Apoe*^{-/-} mice transplanted with *Cxcr4*^{-/-} bone marrow or after blockade of Cxcl12, and was associated with a reduced mobilization of circulating SMC progenitors²¹. Also, interference with the Cxcl12/Cxcr4 axis by blocking Cxcr4 with a Cxcr4 antagonist induced less mobilization of SMC progenitor cells and reduced neointima formation after arterial injury¹²⁴.

Together, these studies reveal a double-edged role for Cxcr4 and Cxcl12 in injury-induced restenosis by mediating the recruitment of either protective EPCs vs neointima-driving SPCs.

However, specific effects on vascular vs inflammatory cells in context of mechanical injury still remain unknown. Therefore, it is necessary to investigate the CXCL12/CXCR4 axis in more detail, to allow the development of new therapeutic treatment strategies to treat patients with cardiovascular disease.

2 Aims of this Study

It has been reported that the Cxcl12/Cxcr4 axis mediates SPC recruitment to injured vessels, driving injury-induced neointimal hyperplasia²¹. However, the EC-specific functions of Cxcr4 in arterial remodeling remain totally unclear. Nonetheless, reendothelialization is a major step after mechanical injury, with an important role in protecting against neointima formation. This study aimed to investigate the role of endothelial Cxcr4 in neointima formation after vascular injury, using a model of wire-mediated carotid artery injury in mice with a tamoxifen-inducible, EC-specific Cxcr4-deficiency.

The first goal of this study was to evaluate the role of endothelial Cxcr4 in reendothelialization after carotid artery injury. Secondly, the study aimed to investigate the effect of endothelial Cxcr4 on EPC recruitment and endothelial proliferation and migration. Finally, the mechanism underlying the effects of endothelium-specific Cxcr4-deficiency on neointima formation were further studied.

3 Material and Methods

3.1 Material

3.1.1 General Equipment

Table 1: List of general equipment

General Equipment	Manufacturer
Autoclave	Systec 2540EL (Systec, Germany)
Balance	Sartorius, Germany
Centrifuges	Eppendorf 5417C (Eppendorf, Germany) Eppendorf 5425 (Eppendorf, Germany)
Flow cytometer	FACSCanto-II (BD Biosciences, USA)
Fluorescence plate reader	Infinite M200 (Tecan, Germany)
Incubator	Hera Cell 240 (Fisher Scientific GmbH, Germany)
Laminar flow hood	Herasafe (Heraeus, Germany)
Microscopes	Leica DM2500 (Leica, Germany) CCD camera (JVC, USA) EVOS FL (Life Technologies, Germany)
Microtome	Leica RM2245 (Leica, Germany)
pH-meter	InoLab level 1 (WTW, Germany)

3.1.2 Consumables

Table 2: List of consumables

Consumable	Manufacturer
6-well plates (flat bottom)	Becton Dickinson, USA
96-well plates (flat bottom)	Becton Dickinson, USA
96-well plates (round bottom)	Nunc, Denmark
Culture flasks (T25, T75)	Greiner Bio-one, Germany
EDTA-coated tubes	Sarstedt, Germany
FACS-tubes	BD Biosciences, USA
Glass microscope slides	Thermo Scientific, Germany
Heparin-coated tubes	Sarstedt, Germany
Serological pipettes (5 ml, 10 ml, 25 ml)	Corning, USA
Syringes (1 ml)	Braun, Germany
Syringes (2 ml, 5 ml, 10 ml, 20 ml)	Terumo, Belgium

3.1.3 Buffers, Solutions and Media

Table 3: List of buffers, solutions and media

Buffer/Solution/Medium	Composition/Manufacturer
0.5% HCl-Ethanol	995 ml 70% ethanol, 5 ml 37% HCL
Antibody diluting solution	Phosphate buffered saline (PBS) containing 10% blocking solution
Anesthesia	0.05 ml Xylazine (final dose: 10 mg/kg), 0.1 ml Ketamine (final dose: 100 mg/kg) and 0.85 ml 0.9% sodium chloride
Blocking solution	PBS containing 1% bovine serum albumin (BSA) and 2.5% horse serum

Collagenase solution	Steril PBS containing 1% of Collagen G (Biochrom, Germany)
Diluting solution	0.9% NaCl
Endothelial cell growth medium	Endothelial cell growth medium MV (Promocell, Germany), 0.1% gentamicin (Gibco, Germany)
FACS staining buffer	2% mouse serum, 2% human serum, 2% rabbit serum, 2% BSA in PBS
Hank's complete solution	1 x HBSS (Invitrogen, USA) containing 0.3 mM EDTA, 0.1% BSA in Millipore water
Paraformaldehyde (PFA) fixation solution	4% PFA, 5% sucrose and 0.02 M EDTA in PBS (pH 7.4)
PBS	with/without 2 mM MgCl ₂ , steril (PAA, Austria)
Red blood cell lysis buffer	0.8% NH ₄ Cl, 10 mM KHCO ₃ and 0.1 mM EDTA in Millipore water (pH 7.4)
Sirius Red solution	0.1% sirius red in picric acid
Sodium citrate cooking buffer	12.6 ml of 0.1 M citric acid, 57.4 ml of 0.1 M natrium-citrate dehydrate and 0.35 ml Tween 20 were dissolved in 630 ml distilled water
Tamoxifen solution	Tamoxifen (Sigma-Aldrich, USA) in Miglyol (Caelo, Germany) at a concentration of 20mg/ml
Vitro-Clud mounting medium	Vitro-Clud (Augenbrinck, Germany) in xylene
Weigert's A solution	10 g hematoxylin, 1000 ml 96% ethanol
Weigert's B solution	40 ml 29% ferric-chloride-solution, 950ml distilled water, 7.5ml 37% HCl
X-gal reaction buffer	PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl ₂ , 0.02% Nonidet P-40, 0.01% Na-deoxycholate

	and 1 mg/ml X-gal
X-gal stock solution	40 mg/ml X-gal in dimethylformamide (stored at -20 °C in dark)

3.1.4 Reagents and Chemicals

Table 4: List of reagents and chemicals

Reagent/Chemical	Manufacturer
Hydrochloric acid (HCl)	Merck, Germany
Human serum	Innovative Research, USA
Isoflurane	Abbott, Germany
Miglyol	Caelo, Germany
Mouse serum	Sigma-Aldrich, USA
Mounting medium with DAPI	Vector Laboratories Inc., USA
Picric acid	Sigma-Aldrich, USA
Picrofuchsin solution	Merck Millipore, Germany
Rabbit serum	Sigma-Aldrich, USA
Resorcin-Fuchsin solution	Carl Roth, Germany
Sirius Red	Polysciences, Inc., USA
Tissue Tek® O.C.T compound	Sakura Finetek Europe B.V., Netherland
X-gal	Invitrogen, USA

3.1.5 Cytokine and Inhibitor

Table 5: List of cytokines

Cytokine	Manufacturer
AMD3100	PeproTech, USA
Recombinant human SDF-1α/CXCL12	PeproTech, USA

3.1.6 Antibodies

Table 6: List of antibodies

Antibody target	Specificity	Conjugate	Application	Manufacturer
Biotin	Rat	Streptavidin APC-Cy7	FC	eBioscience, USA
Cd115	Monocytes, Macrophages	PE	FC	eBioscience, USA
Cd11b	Monocytes, Neutrophils, Macrophages	FITC	FC	eBioscience, USA
Cd31	ECs	PE-Cy7	FC	Abcam, UK
Cd31	ECs	purified	IFS	Santa Cruz Biotechnology, USA
Cd4	T- helper cells	FITC	FC	eBioscience, USA
Cd45	Leukocytes	APC-Cy7	FC	BD Biosciences, USA
Cd8	Memory T-cells	PE-Cy7	FC	eBioscience, USA
Cxcr4		purified	IFS	Abcam, UK
Cxcr4		FITC	FC	BD Biosciences, USA
Cxcl12		purified	IFS	R&D Systems, USA

Goat IgG	Goat IgG	Cy3	IFS	Jackson ImmunoResearch, USA
Gr1 (Ly-6C and Ly-6G)	Neutrophils and inflammatory monocytes	APC	FC	eBioscience, USA
Flk1 (Cd309)	ECs, hematopoietic cells, HPCs	APC	FC	eBioscience, USA
Ki-67	Proliferating cells	purified	IFS	Dako, Denmark
Mac2	Monocytes, Macrophages	purified	IFS	Cedarlane, Canada
Mouse IgG	Mouse IgG	Cy3	IFS	Jackson ImmunoResearch, USA
Rat IgG	Rat IgG	FITC	IFS	Jackson ImmunoResearch, USA
Sma	SMCs	purified	IFS	Dako, Denmark

IFS: Immunofluorescent staining; FC: Flow cytometry

3.1.7 Assay Kits

Table 7: List of assay kits

Assay Kit	Manufacturer
Cobas enzymatic assay	Roche, Germany
Cxcl12 ELISA kit	RayBiotech, USA
Flt-3 ligand ELISA kit (mouse/rat)	R&D System, USA
Lineage Panel	BD Pharmingen, USA
Sphingosine 1 Phosphate assay kit	Lpath, USA
Vegf ELSA kit (mouse)	R&D System, USA

3.1.8 Software

Table 8: List of software

Software	Manufacturer
Diskus	Hilgers, Germany
FACSDiva	BD Biosciences, USA
FlowJo	Treestar, USA
GraphPad Prism 5	GraphPad Software, USA
Image J	National Institutes of Health, USA

3.1.9 Mice

The *Cxcr4*-floxed knock-in mouse model¹²⁵ and the *Bmx-CreER*^{T2} transgenic mouse line were provided by Prof. Y. Zou (Columbia University, New York, US) and by Prof. R.H. Adams (Cancer Research UK-London Research Institute, London, UK), respectively, and were crossed with C57Bl/6 *Apoe*^{-/-} mice to create an atherosclerosis-prone background. The presence of the *Cre* transgene was detected using the primers 5'-AAA TAC CTT CAG TTT TCATCT-3' (Cre-F) and 5'-TTG CGA ACC TCA TCA CTC GTT-3' (Cre-R). The presence of a wild-type or loxP-flanked *Cxcr4* allele was detected using the primers 5'-CAC TAC GCA TGA CTC GAA ATG-3' (5X4 FS) and 5'-GTG TGC GGT GGT ATC CAG C-3' (3X4 FS), as previously described¹²⁵. The inclusion of a third primer 5'-GTG CTC CTC GGA ATG AAG AG-3' allowed to discriminate between a wild-type (330 bp), floxed (430 bp) or deleted (200 bp) *Cxcr4* allele after tamoxifen-induced *Cre* expression. The Cre-reporter mouse line Gt(ROSA)26Sor^{tm1Sor} (Jackson Laboratory), expressing a *lacZ* reporter gene that is transcriptionally silenced by an upstream floxed stop sequence, was crossed with the *Bmx-CreER*^{T2} mouse line to allow investigation of the cellular expression pattern of the *Bmx-CreER*^{T2} transgene through analysis of

β -galactosidase activity after tamoxifen treatment.

3.2 Methods

3.2.1 Mouse Experiments

3.2.1.1 Mouse Model and Tamoxifen Treatment

Five- to 7-week old female *Bmx-CreER^{T2} + Cxcr4-floxed Apoe^{-/-}* mice (indicated as *Cxcr4^{EC-KO} Apoe^{-/-}*) and littermate *Bmx-CreER^{T2} - Cxcr4-floxed Apoe^{-/-}* controls (indicated as *Cxcr4^{EC-WT} Apoe^{-/-}*) were treated with tamoxifen (1mg/20g mouse/day, i.p., tamoxifen dissolved in Miglyol) for 5 consecutive days to induce Cre-mediated deletion of the floxed *Cxcr4*-allele in *Bmx⁺* cells. After a recovery period of minimal 3 weeks after the first tamoxifen injection, a first blood analysis was performed, later referred to as the “baseline control”. Then, the mice were fed a western-type diet containing 21% fat and 0.15% cholesterol (Altromin, Germany). After one week of high-fat diet, wire-induced injury of the common carotid artery was performed. At the indicated time points after injury, the mice were sacrificed and the injured carotid arteries and peripheral blood were collected for further investigation.

The Cre-reporter mouse line *Gt(ROSA)26Sor^{tm1Sor}* (Jackson Laboratory), expressing a *lacZ* reporter gene that is transcriptionally silenced by an upstream floxed stop sequence, was crossed with a *Bmx-CreER^{T2} +* mouse line to allow investigation of the cellular expression pattern of the *Bmx-CreER^{T2}* transgene through analysis of β -galactosidase activity after tamoxifen treatment.

Animal experiments were reviewed and approved by the local authorities in accordance with the German animal protection law.

3.2.1.2 Wire-induced Carotid Artery Injury

Using mechanical denudation of the endothelium, restenosis was simulated in mouse common carotid arteries. A wire-induced arterial injury was performed after one week of high-fat diet.

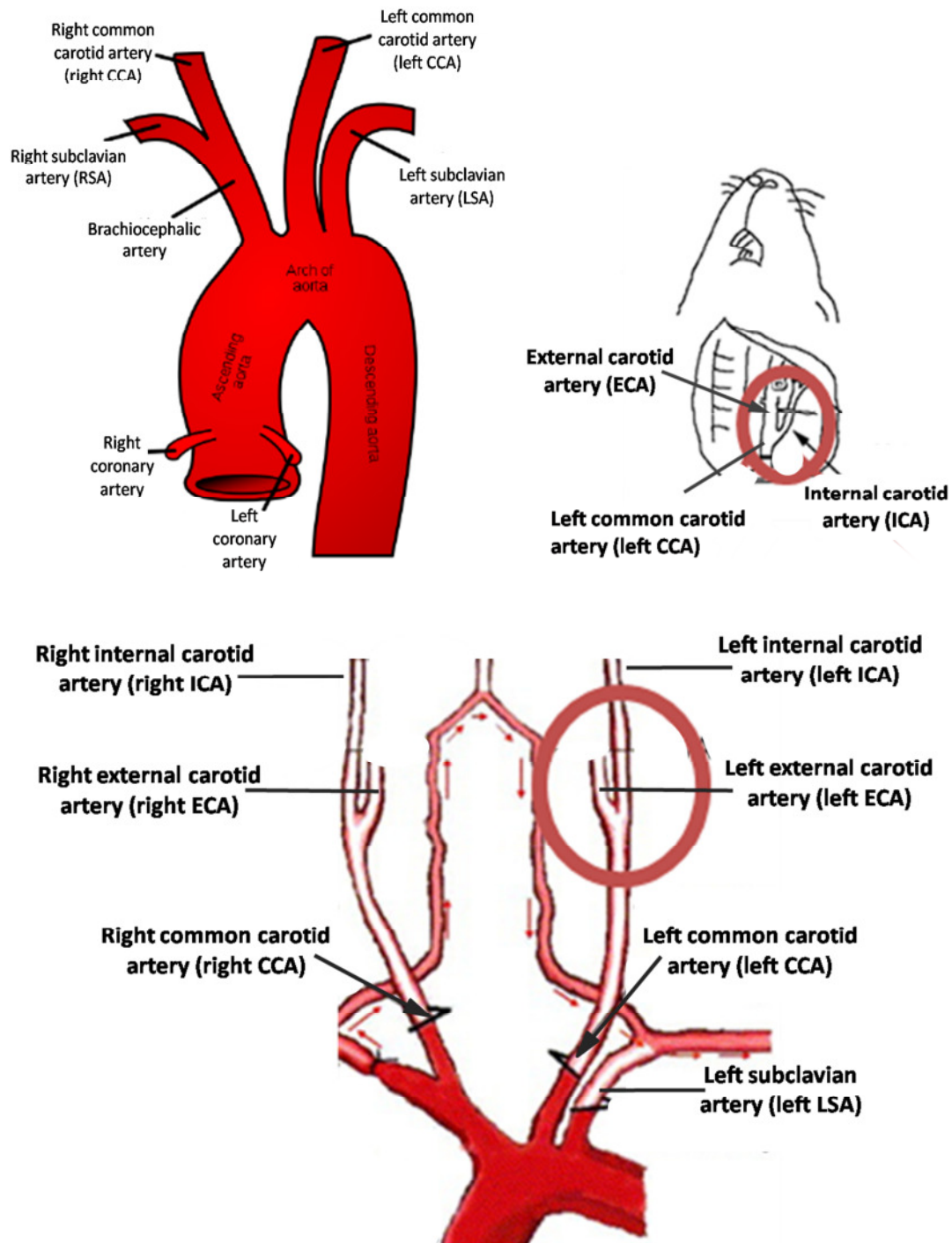


Figure 7: **Anatomy of the mouse carotid artery.** A wire-induced arterial injury of the left common carotid artery is performed by wire insertion through the left external carotid artery (Figure modified from http://0.tqn.com/d/biology/1/0/c/X/aortic_arch.png).

The mice were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine i.p. and an endothelial denudation of the left common carotid artery (CCA) (Figure 7) was induced by a 0.36 mm guide wire through a transverse arteriotomy of the left external carotid artery (Figure 8). After a skin incision at the left lateral side of neck, the bifurcation area and the left CCA were observed by using a dissecting microscope. Loose ligatures were placed around the proximal part of the left CCA, external carotid artery (ECA) and internal carotid artery (ICA). After occluding the ECA with a distal ligature, the blood flow in the left CCA was temporarily interrupted by tying the ligatures around the CCA and ICA. In the ECA, a transverse incision was made between the ligature of the CCA and the distal ligature of the ECA by using a fine scissor. After 1 cm insertion of a flexible 0.36-mm guide wire into the arterial lumen through the incision, endothelial denudation was achieved by a 3-pass rolling movement of the guide wire. The ECA ligature proximal of the incision was then permanently ligated. The ligatures of the ICA and CCA were removed to restore the blood flow and skin incisions were closed with sutures.

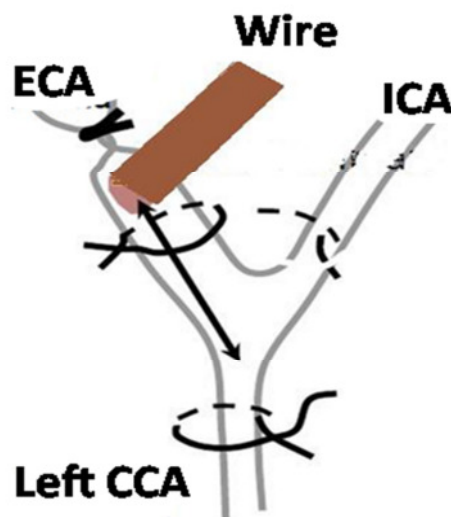


Figure 8: **Scheme of wire-induced left carotid artery injury in mice.** An endothelial denudation of the left common carotid artery (CCA) was induced by a 0.36 mm guide wire, which was inserted through a transverse arteriotomy of the left external carotid artery (ECA). (Figure modified from the dissertation of Dr. rer. nat. S. Simsekyilmaz)

3.2.1.3 Blood Collection, Sacrifice and Carotid Artery Preparation

Peripheral blood was obtained by puncturing the retro-orbital sinus with microhematacrit capillary tubes during short-time isoflurane anaesthesia. Peripheral blood analysis was performed at 1 day, 5 days, 10 days and/or 3 weeks after injury. Differential blood cell counts were determined using a Giemsa staining (Animal facility, University Hospital Aachen).

At the indicated time points after injury, the mice were sacrificed by injection of an overdose of 250 mg/kg Ketamine and 25 mg/kg Xylazine. Then the mice were perfused *in situ* with PBS followed by 4% PFA fixation solution. The injured carotid arteries were carefully isolated, overnight fixed in 4% PFA at 4°C, dehydrated and embedded in paraffin. Within a standardized distance (0 to 400 μm) from the bifurcation, serial 4 μm transversal sections from the

paraffin-embedded carotid arteries were collected on glass microscopy slides by cutting with a microtome.

3.2.2 Histological Stainings

3.2.2.1 Deparaffinization and Dehydration

For paraffin-embedded sections, deparaffinization is needed before histological stainings can be performed. After staining, sections were shortly dehydrated, Table 9 and 10 show general steps for deparaffinization and dehydration.

Table 9: Protocol of deparaffinization

Staining Procedure	Time
Xylol	2 x 15 min
100% isopropanol	2 x 5 min
96% isopropanol	5 min
70% isoprpanol	2 x 5 min
PBS	5 min

Table 10: Protocol of dehydration

Staining Procedure	Time
70% isopropanol	dip
96% isopropanol	dip
100% isopropanol	dip
100% isopropanol	dip
Xylol	5 min

3.2.2.2 Elastica van Gieson Staining

Carotid artery sections (10 sections per mouse, each 40 µm separated) were stained using the Elastica-van Gieson (EVG) stain at room temperature (RT) to determine neointimal lesion size. Images were recorded with a Leica DM2500 fluorescence microscope and CCD camera. The area of neointima, which is the area between lumen and internal elastic lamina, and the area of media, which is the area between internal and external elastic laminae, were measured and quantified by planimetry using Diskus Software. Mean values were derived from the analysis of 10 sections per mouse for neointimal lesion size.

Table 11: Protocol of EVG staining

Staining Procedure	Time
Xylol	2 x 15 min
100% isopropanol	2 x 5 min
96% isopropanol	5 min
70% isopropanol	5 min
Distilled water	5 min
Resorcin-Fuchsin solution	15 min (60 °C)
Distilled water	dip
80% ethanol	dip
Distilled water	—
Weigert's A + B solution	5 min
Distilled water	dip
0.5% HCl-alcohol	dip
Distilled water	—
Picrofuchsin	3 min
Distilled water	dip

70% isopropanol	dip
96% isopropanol	dip
100% isopropanol	dip
Xylol	5 min
Coverslip with Vitroclud mounting medium	—

Results:

Nuclei and elastic fibers: black-brown

Collagen fibers: orange-red

Musculature, cytoplasm and erythrocytes: yellow

3.2.2.3 β -Galactosidase Staining

β -Galactosidase activity was determined using a whole mount X-gal histochemical staining in which the substrate X-gal produces a blue dye when cleaved by β -galactosidase. Mice were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine i.p., exsanguinated and the vasculature was rinsed *in vivo* with cold PBS containing 2 mM MgCl_2 . After *in situ* perfusion with a cold 2% PFA solution, carotid arteries were isolated, fixed for 15 min in the same fixation solution on ice and washed three times in PBS containing 2 mM MgCl_2 . Then, the carotid arteries were incubated overnight at 37°C in a prewarmed β -gal staining solution, containing 1 mg/ml of the substrate X-gal in an X-gal reaction buffer (Table 3). The next day, carotid arteries were rinsed with PBS until the washing buffer no longer turned yellow. The stained carotid arteries were then embedded in Tissue Tek for 2 hours and cryo-sections were prepared. β -Galactosidase staining was visualized using light microscopy.

3.2.2.4 Sirius Red Staining

Collagen content was determined by Sirius Red staining¹²⁶. Sirius Red is used for detecting all types and species of collagen, such as type I to V collagen¹²⁷. Images were recorded with a Leica DM2500 fluorescence microscope and CCD camera. The collagen content was analyzed by Image J software. Mean values were derived from 3-5 sections per mouse.

Table 12: Protocol of Sirius Red staining

Staining Procedure	Time
Xylol	2 x 15 min
100% isopropanol	2 x 5 min
96% isopropanol	5 min
70% isopropanol	5 min
Flowing water	shortly
0.1% Sirius Red staining solution	1h
1% HCl	2 min
Flowing water	shortly
70% isopropanol	dip
96% isopropanol	dip
100% isopropanol	dip
Xylol	5 min
Coverslip with Vitroclud mounting medium	—

3.2.3 Immunohistochemistry and Immunofluorescence Staining

Neointimal macrophages, SMCs and ECs were visualized by immunofluorescent staining for Mac2, Sma or Cd31, respectively, followed by a FITC- or Cy3-conjugated secondary antibody staining. Endothelial expression of Cxcr4 and Cxcl12 was quantified after co-staining for Cd31 and Cxcr4 or Cxcl12, respectively. Endothelial proliferation was visualized by co-staining for Cd31 and the proliferation marker Ki-67¹¹⁴. Appropriate IgG antibodies were used as isotype controls. Nuclei were counterstained with 4',6-diamidino-2-phenylindol (DAPI). Images were recorded with a Leica DM2500 fluorescence microscope and CCD camera. Cellular composition and endothelial Cxcr4 and Cxcl12 expression were analyzed using image analysis software (Diskus Software, Hilgers) without prior knowledge of the genotype. Vessel reendothelialization was calculated as the ratio of the length of luminal Cd31⁺ endothelial immunostaining to the total luminal circumference. Mean values were derived from 3-5 sections per mouse for all these analyses.

Table 13: Protocol of immunostaining

Staining Procedure	Time
Xylol	2 x 15 min
100% isopropanol	2 x 5 min
96% isopropanol	5 min
70% isoprpanol	5 min
PBS	5 min
Cooking in citrate buffer	2 x 10 min
PBS	30 min (cooling)
Blocking solution	30 min
Primary antibody	Overnight (4 °C)

PBS	2 x 5 min
Secondary antibody	1 h
PBS	2 x 5 min
Vectashield mounting medium with DAPI	—

3.2.3 Flow Cytometry

Flow cytometry is a technology for characterizing, sorting and quantifying cells using fluorescent dye labeled biomarkers. By screening for differences in cell size (forward scatter, FSC) and granularity (side scatter, SSC), different cell subsets can be distinguished after passing an argon laser. Furthermore, the cells can be characterized, sorted or quantified by their association with different fluorescent dyes, which are coupled to antibodies recognizing cell type-specific biomarkers.

For this analysis, whole blood obtained from the retro-orbital plexus of mice was EDTA-buffered and subjected to red blood cell lysis (Table 3). After extracellular staining with an antibody cocktail against Sca1, Flk1 and Cd31, flow cytometry was used to analyze circulating Sca1⁺Flk1⁺Cd31⁺ cells. Lin⁻Sca1⁺ progenitor cells were determined after staining with an anti-Sca1 antibody and a Mouse Lineage Panel (Cd3ε, Cd11b, Cd45R, Gr-1, Ter-119, Ly-6G, BD Pharmingen). Cxcr4 surface expression was quantified after staining with an anti-Cxcr4 antibody. Sca1⁺Flk1⁺Cd31⁺ cells and Lin⁻Sca1⁺ cells were analyzed after appropriate fluorescence compensation, using FACSCanto-II and FACSDiva software. The obtained data were analyzed after using gating strategies based on the appropriate FMO (fluorescence minus one) controls with the help of the FlowJo analysis program (Treestar).

3.2.4 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a widely-used technique to detect and quantify a specific target antigen or protein in fluid such as serum, plasma or cell culture supernatants. With a sandwich-based principle (Figure 9), the proteins of interest are bound between capture antibodies and enzyme-linked detection antibodies. After utilizing chromogenic substrates, the enzyme generates a detectable signal, and the target protein can be detected and measured by a spectrophotometric reader at specific wavelengths.

In this study, plasma and bone marrow Cxcl12 concentrations were determined using a mouse Cxcl12 ELISA kit (RayBiotech). This kit employs a 96-well plate coated with an antibody specific for mouse Cxcl12. The standards and samples were pipetted into the wells and incubated for 2.5 hours at RT. After thoroughly washing the wells, a biotinylated anti-mouse Cxcl12 antibody was added and the plate was incubated for 1 hour at RT with gentle shaking. The unbound biotinylated antibody was washed away in a second washing step. Horseradish peroxidase (HRP)-conjugated streptavidin was pipetted into the wells, following with 45 minutes of incubation time at RT with gentle shaking. The wells were again washed and a tetramethylbenzidine (TMB) substrate solution was added to the wells and incubated for 30 minutes at RT in the dark with gentle shaking. After quenching the reaction by adding stop solution to the wells, the color in the wells changed from blue to yellow, with the color in proportion to the amount of Cxcl12 bound. The plate was immediately read by a plate reader (Infinite M200) and the absorbance intensity was measured at a wavelength of 450nm.

Similarly, the plasma concentrations of Vegf, S1P and Flt-3 ligand were determined separately using mouse ELISA kits and measured at demanded wavelengths.

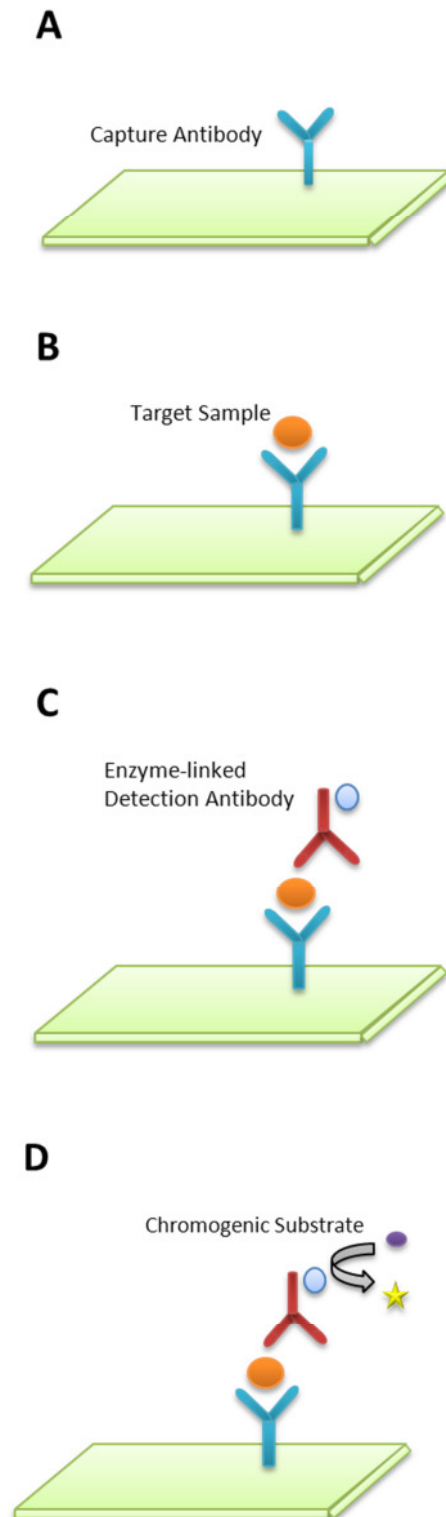


Figure 9: **The sandwich ELISA.** **A**, A plate coated with a capture antibody. **B**, The sample or standard is added and bound to capture antibody. **C**, An enzyme-linked detection antibody is added. **D**, A chromogenic substrate is applied, resulting in a color change and then absorbance can be measured.

3.2.5 *In vitro* Scratch Assay

The scratch assay is a method used for mimicking the endothelial cell response after mouse carotid artery injury and quantifying the speed of wound closure, which is influenced both by cell migration and proliferation. Cells are grown to confluency and a wound (“scratch”) is introduced by dragging a thin pipette tip¹²⁸. Migration of cells into the wounded area is being analyzed under different conditions of cell stimulation.

Human aortic endothelial cells (HAoECs) were purchased from PromoCell, plated on cell culture dishes coated with collagen and cultured in Endothelial Cell Growth Medium MV, according to the manufacturer’s recommendations. Cells were grown to confluency and were left untreated or were prestimulated for 8 hours with CXCL12 (Recombinant human SDF-1 α /CXCL12, 100 ng/ml, PeproTech), AMD3100 (1 μ g/ml, PeproTech) or a combination of both. After wound introduction and 20 hours of migration under ongoing stimulation, microscopic pictures were taken using phase contrast microscopy. For each well, 10 consecutive microscopic pictures were taken at the start and end of the migration assay and were overlaid, and the area and number of cells that migrated into the wound area was quantified using Diskus software.

3.2.6 Quantification of Plasma Lipids

Plasma cholesterol and triglyceride levels were quantified using enzymatic assays (Cobas, Roche) according to the manufacturer’s protocol. The plasma and standards were diluted with 0.9% NaCl and after adding a chromogenic reagent for 30 minutes at RT, the absorbance in plasma was measured at a wavelength of 505 nm by a 96-well plate reader.

3.2.7 Statistical Analysis

Data are represented as means $\pm$ SEM and were analyzed using a t-test, 1-way ANOVA with Newman-Keuls post-test or 2-way ANOVA with Bonferroni post-test, as appropriate (GraphPad Prism 5). $P < 0.05$ was considered statistically significant.

4 Results

4.1 Analysis of Endothelial *Cxcr4* Deletion Using the *Bmx-CreER^{T2}* Transgene

To investigate the role of endothelial *Cxcr4* in neointimal hyperplasia following carotid artery injury, *Cxcr4*-floxed *Apoe*^{-/-} mice were crossed with mice expressing the tamoxifen-inducible *Bmx-CreER^{T2}* transgene. A previous study has reported that *Bmx* is highly expressed in arterial ECs and endocardium, but not in venous endothelium¹²⁹. In tamoxifen-treated *Bmx-CreER^{T2}* *ROSA* reporter mice, β -galactosidase activity was detected in the endothelial layer of carotid arteries, showing a specific and intense blue staining (Figure 10A), which confirmed that the *Bmx-CreER^{T2}* transgenic mouse line is suitable to induce a conditional gene deficiency in the endothelium of carotid arteries.

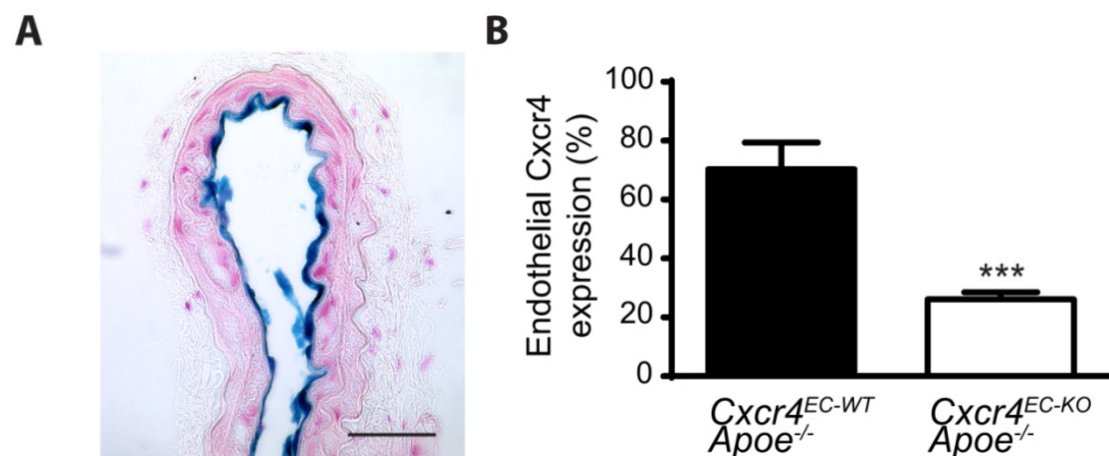


Figure 10: **Efficient deletion of endothelial *Cxcr4* in *Cxcr4*^{EC-KO} *Apoe*^{-/-} mice.** **A**, β -Galactosidase activity in the carotid artery from tamoxifen-treated *Bmx-CreER^{T2}* *ROSA* reporter mice. Scale bar = 100 μ m. **B**, Quantification of *Cxcr4*-expressing endothelium in carotid arteries from *Cxcr4*^{EC-WT} *Apoe*^{-/-} and *Cxcr4*^{EC-KO} *Apoe*^{-/-} mice 3 weeks after injury. The graph displays the *Cxcr4*⁺Cd31⁺ luminal length as % of the total Cd31⁺ luminal length. Represented are means $\pm$ SEM; n=8-13; ****P*<0.0001; two-tailed t-test.

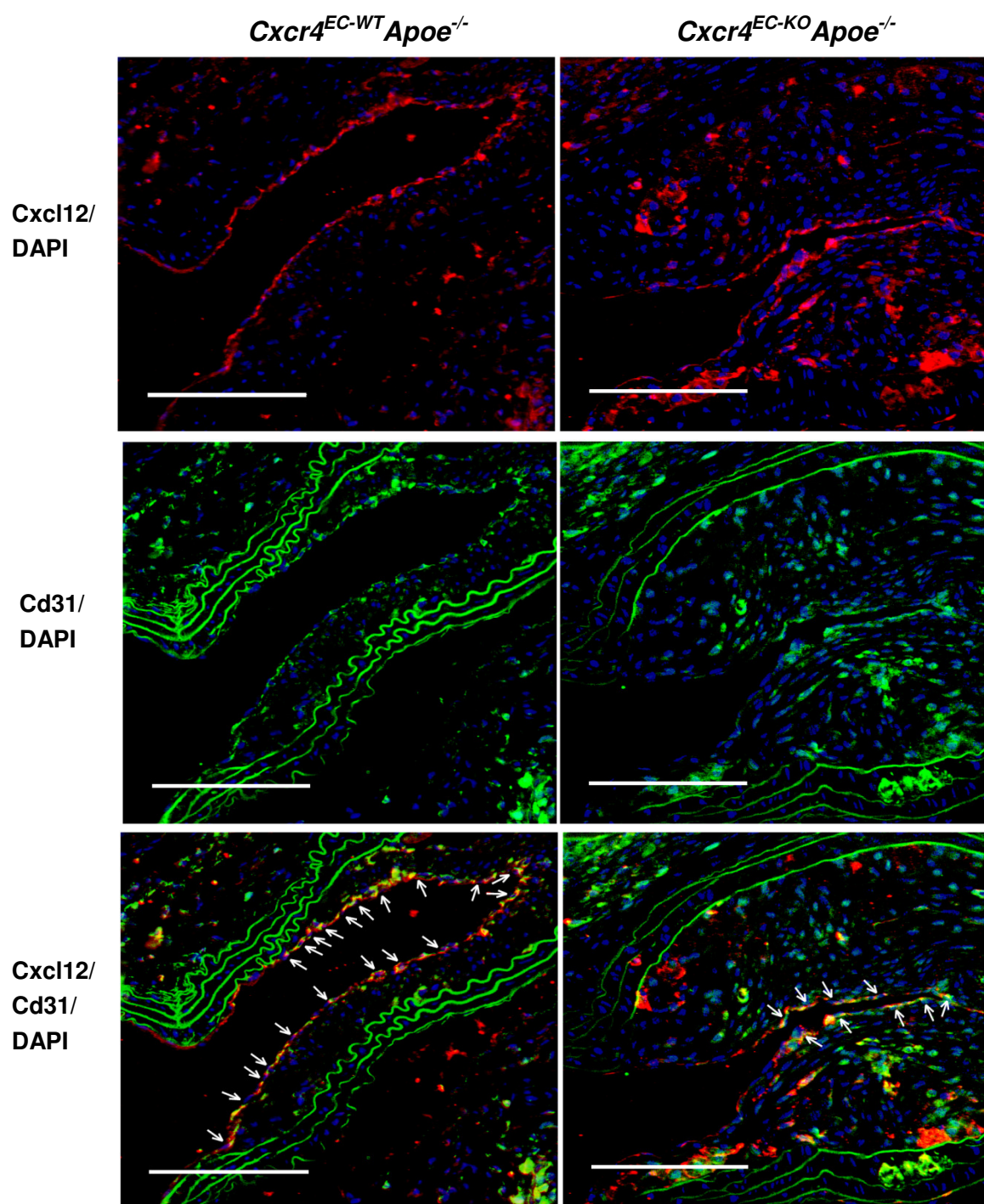


Figure 11: **Representative pictures of endothelial Cxcr4 expression in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice.** Representative images of Cxcr4-expressing endothelium in carotid arteries from *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice 3 weeks after injury, based on staining of carotid artery sections for Cd31 (red), Cxcr4 (green) and DAPI (blue), showing more Cxcr4⁺ ECs (yellow, indicated with arrows) in *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice. Scale bars = 100 μm.

Three weeks after carotid artery injury, endothelial Cxcr4 expression was analyzed in carotid artery sections from *Cxcr4^{EC-KO}Apoe^{-/-}* and *Cxcr4^{EC-WT}Apoe^{-/-}* mice using double immunofluorescence staining for Cd31 and Cxcr4. Cxcr4 expression was evident in around 75% of the endothelial layer of *Cxcr4^{EC-WT}Apoe^{-/-}* mice, comparing with nearly 25% in *Cxcr4^{EC-KO}Apoe^{-/-}* mice (Figure 10 and 11).

4.2 Involvement of Endothelial Cxcr4 in Neointimal Hyperplasia after Carotid Artery Injury

To assess the role of endothelial Cxcr4 in neointima formation, a wire-mediated vascular injury was induced in the left carotid artery of *Cxcr4^{EC-KO}Apoe^{-/-}* mice and *Cxcr4^{EC-WT}Apoe^{-/-}* controls. The mice were fed with a high-cholesterol diet from one week before injury. Wire-induced neointimal lesions were quantified three weeks and four weeks after injury.

Three weeks after arterial injury, concentrations of plasma triglycerides and cholesterol between both groups did not differ (Figure 12), which means that deletion of endothelial *Cxcr4* did not influence plasma lipid levels. Also, numbers of circulating platelets, leukocytes and leukocyte subsets, including monocytes, lymphocytes and neutrophils, were comparable (Figure 13). Furthermore, there were no significant changes in the relative frequencies of Gr1^{low} vs Gr1^{high} subsets, of B-lymphocytes and of T-cells and its Cd8⁺ vs Cd4⁺ subgroups, as quantified by flow cytometric analysis (Figure 14).

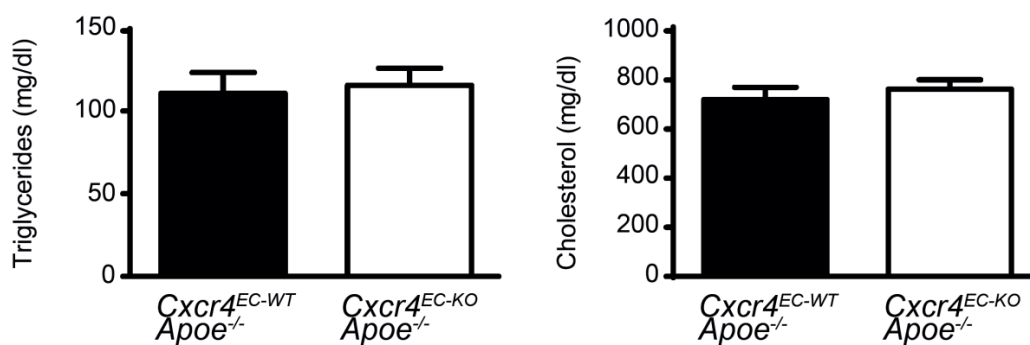


Figure 12: **No differences in plasma lipid levels upon endothelial *Cxcr4* deficiency.** Plasma triglyceride and cholesterol concentrations were measured in *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice 3 weeks after carotid artery injury. The graph shows means $\pm$ SEM; n=8-13.

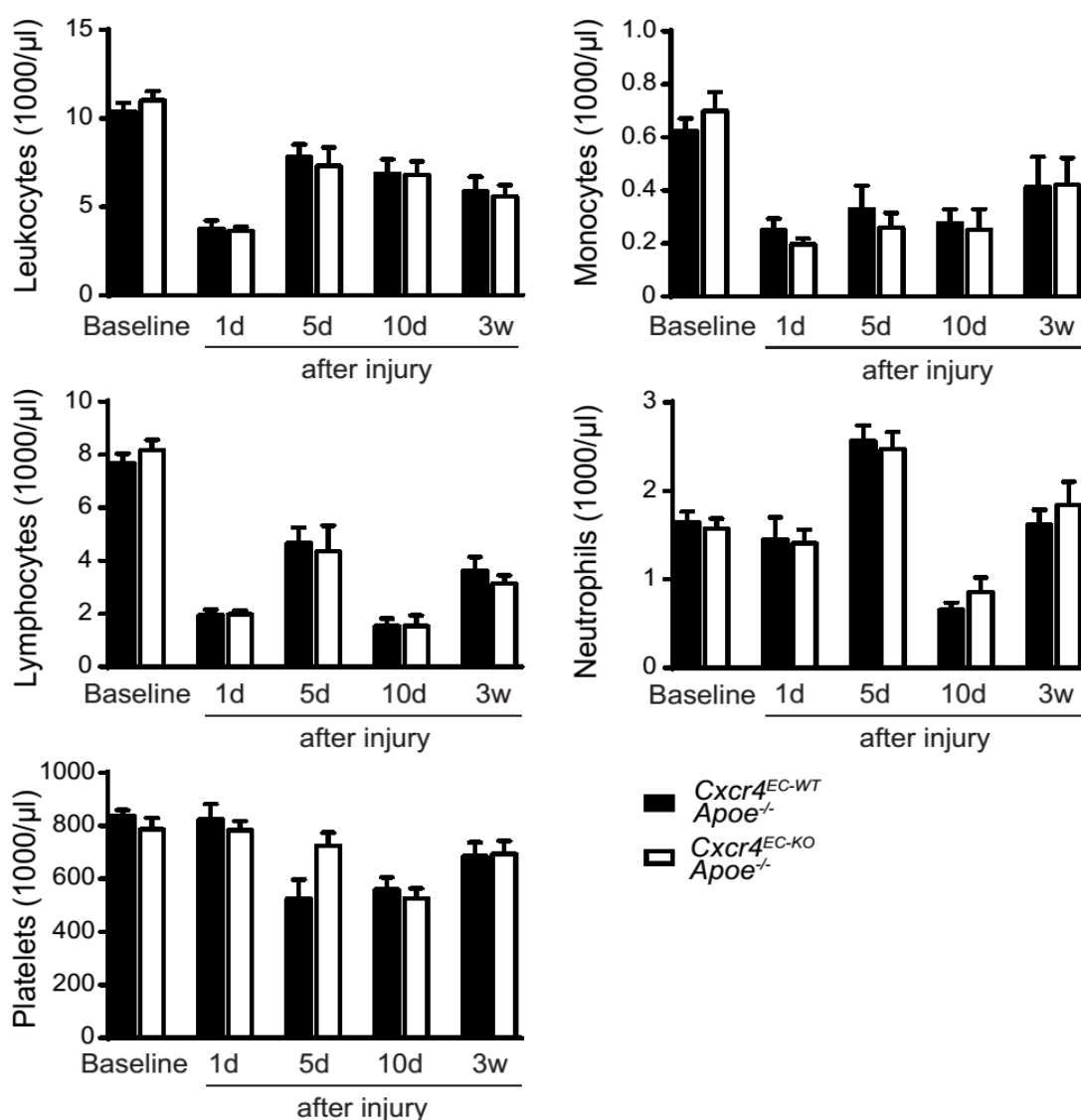


Figure 13: **No significant differences in leukocyte and platelet numbers upon endothelial *Cxcr4* deficiency.** Numbers of leukocytes (including monocytes, lymphocytes and neutrophils) and platelets were quantified in the peripheral blood of *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice at baseline and at different time points after injury, as indicated. “Baseline” was measured at least 3 weeks after the first tamoxifen injection, but before the start of high-fat diet. The graphs represent means $\pm$ SEM; n=13-22 (for baseline, 1 day and 3 weeks); n=3-6 (for 5 and 10 days).

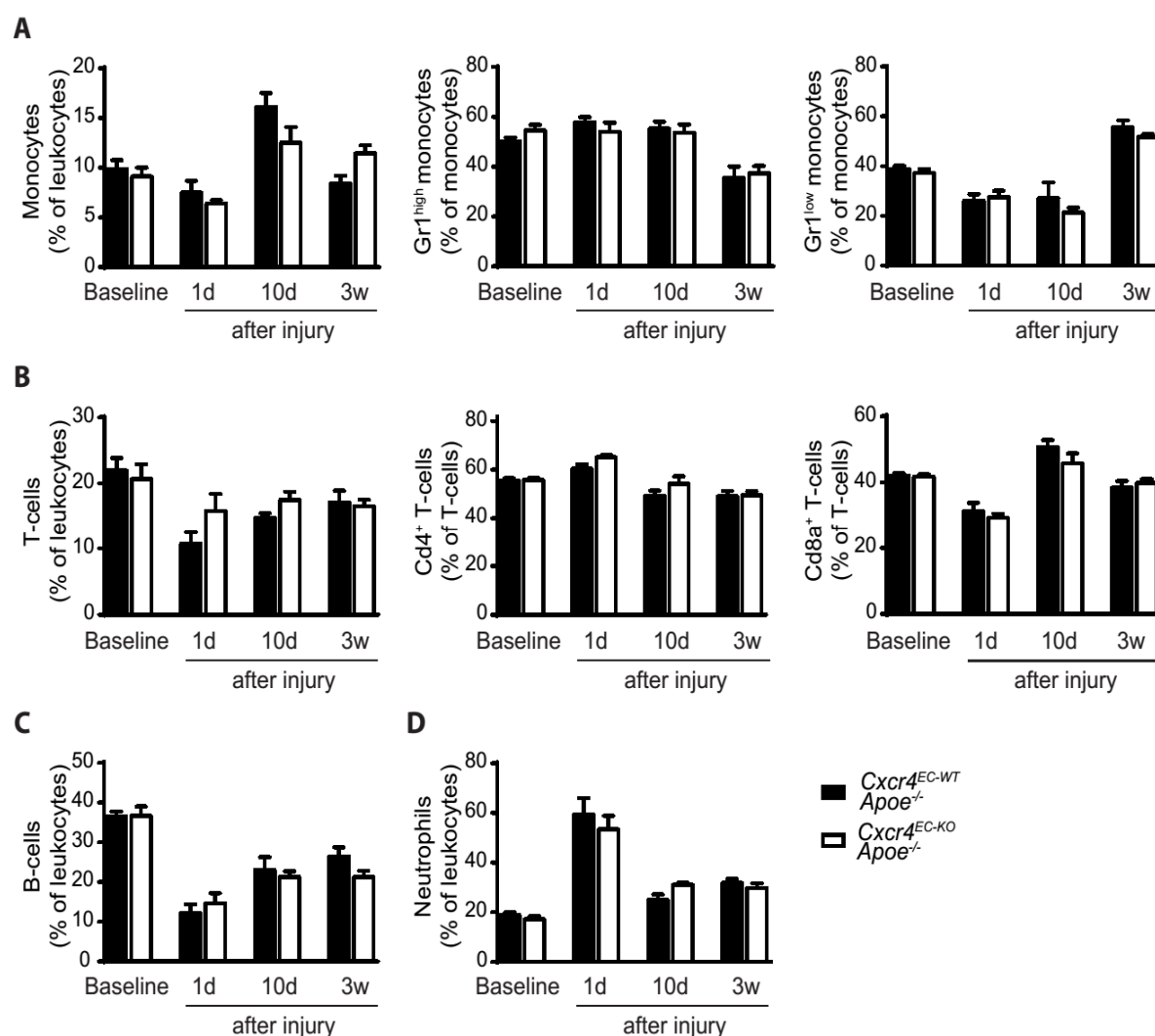


Figure 14: **No significant differences in leukocyte subset frequencies upon endothelial *Cxcr4* deficiency.** A-D, Analysis of monocytes and Gr1^{high} vs Gr1^{low} monocyte subsets (A), T-cells and Cd4⁺ vs Cd8a⁺ T-cell subsets (B), B-cells (C) and neutrophils (D) by flow cytometry of peripheral blood from *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice at baseline and at different time points after injury, as indicated.

“Baseline” was measured at least 3 weeks after the first tamoxifen injection, but before the start of high-fat diet. The graphs represent means $\pm$ SEM; $n=5-13$ (for baseline, 1 day and 3 weeks); $n=2-3$ (for 10 days).

Three weeks after injury, neointimal lesion size of injured carotid arteries showed an increased trend in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice, without reaching statistical significance (Figure 15).

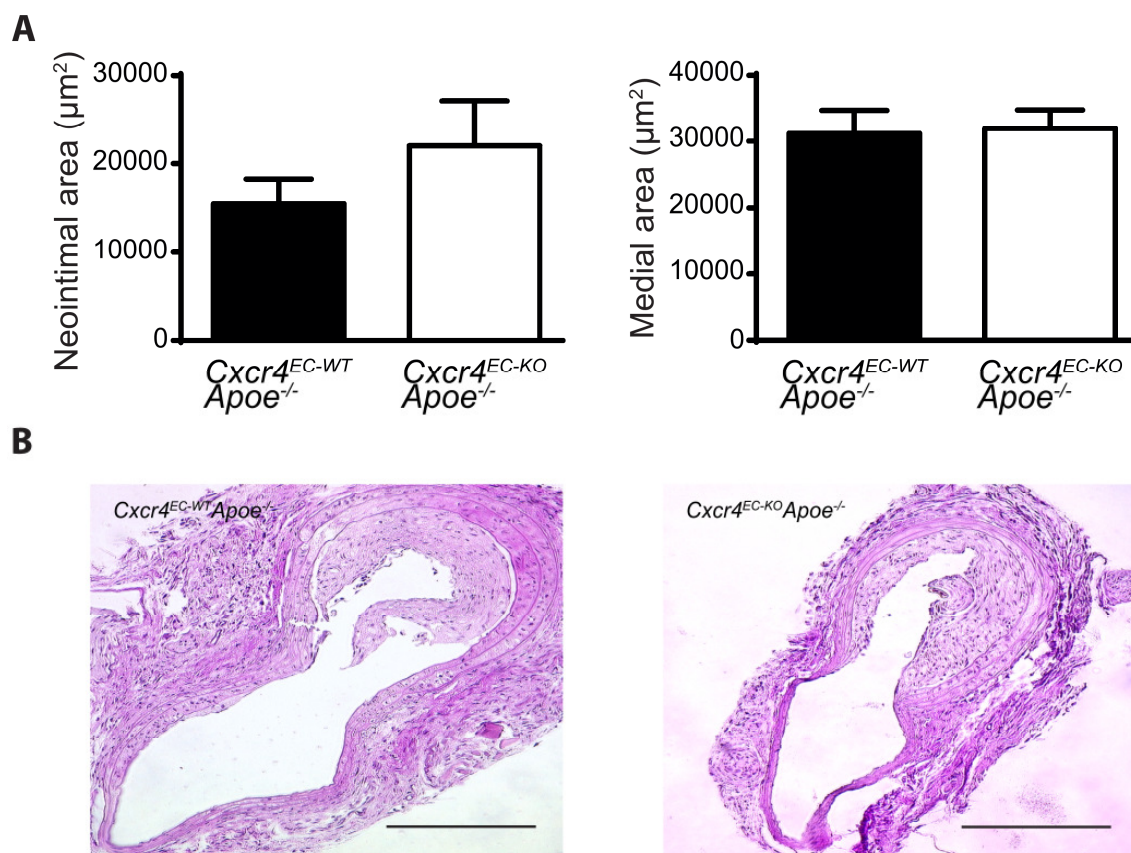
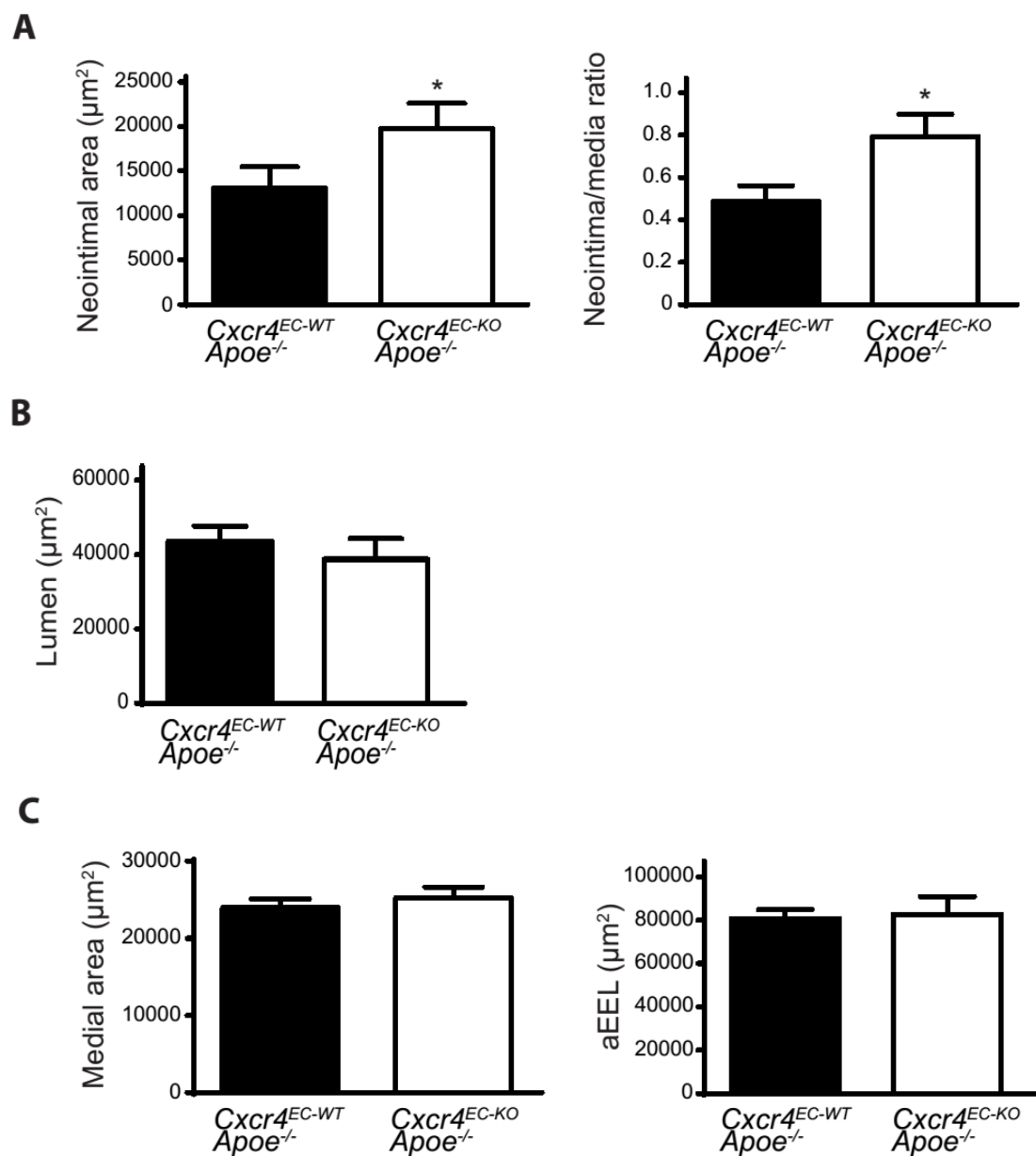


Figure 15: Endothelial-specific *Cxcr4*-deficiency showed an increased trend in neointima formation 3 weeks after wire-induced carotid artery injury. A, Quantification of the neointimal and medial area of carotid arteries 3 weeks after vascular injury ($n=8-12$). Graphs represent means $\pm$ SEM; two-tailed t-test. **B,** Representative images of neointimal lesions, positioned $\sim 90\mu\text{m}$ from the bifurcation. Scale bars = 200 μm .

However, after one additional week, the neointimal lesion size of injured carotid arteries was significantly increased in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice, whereas the medial area, luminal area and the area within the external elastic lamina (aEEL) were unaltered, compared with control *Cxcr4*^{EC-WT}*Apoe*^{-/-} mice (Figure 16A-D). No effects of endothelial *Cxcr4* deficiency on vessel dimensions were observed in the absence of injury (Figure 17). These data indicated that deletion of *Cxcr4* on arterial ECs enhances neointimal hyperplasia after wire-induced arterial injury.



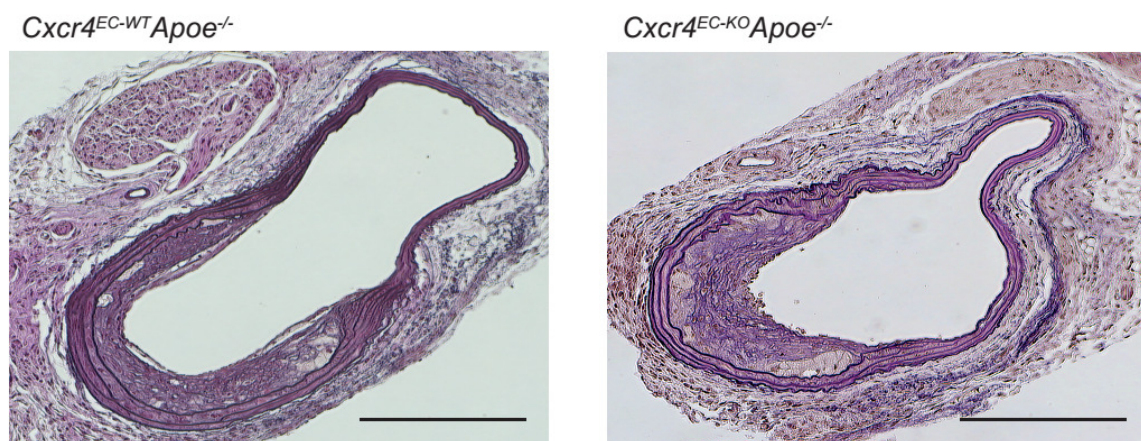
D

Figure 16: **Endothelial-specific *Cxcr4*-deficiency enhances injury-induced neointima formation.** **A-C**, Quantification of the neointimal and neointima-to-media ratio (A), luminal area (B), medial area and the area within the external elastic lamina (aEEL) (C) of carotid arteries 4 weeks after vascular injury (n=7-11). Graphs represent means $\pm$ SEM; * $P < 0.05$; one-tailed t-test. **D**, Representative images of neointimal lesions, positioned $\sim 90 \mu\text{m}$ from the bifurcation. Scale bars = $200 \mu\text{m}$.

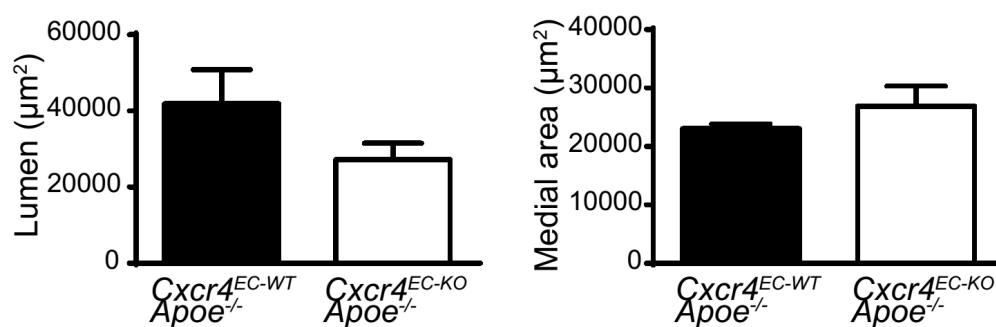
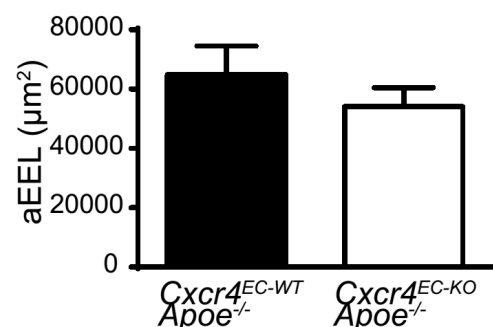
A**B**

Figure 17: **Endothelial *Cxcr4* deficiency does not show significant effects on vessel dimensions in the absence of injury.** A-B, Quantification of luminal area, medial area and the area within the external elastic lamina (aEEL) in carotid artery sections from uninjured *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice. Graphs represent means $\pm$ SEM; n=5-6.

4.3 Cellular Content of Neointimal Lesions in Response to Arterial Injury

We then analyzed the cellular content in the injured carotid artery sections. To determine the response of macrophages and SMCs in the neointima after arterial injury, carotid artery sections harvested 3 weeks after injury were labeled with an antibody recognizing the macrophage marker Mac2 for macrophage quantification and with an antibody directed against the SMC marker Sma for SMC quantification. Analysis of percentage and absolute number of macrophages in neointimal lesions demonstrated an increase in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice compared with *Cxcr4*^{EC-WT}*Apoe*^{-/-} mice (Figure 18).

Conversely, the SMC content in the neointimal lesions was significantly decreased in injured *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice (Figure 19). This was accompanied by a marked reduction in neointimal collagen-rich extracellular matrix content in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice (Figure 20). Thus, endothelial *Cxcr4* deficiency triggered enhanced injury-induced neointimal lesion formation with a more unstable or inflammatory phenotype.

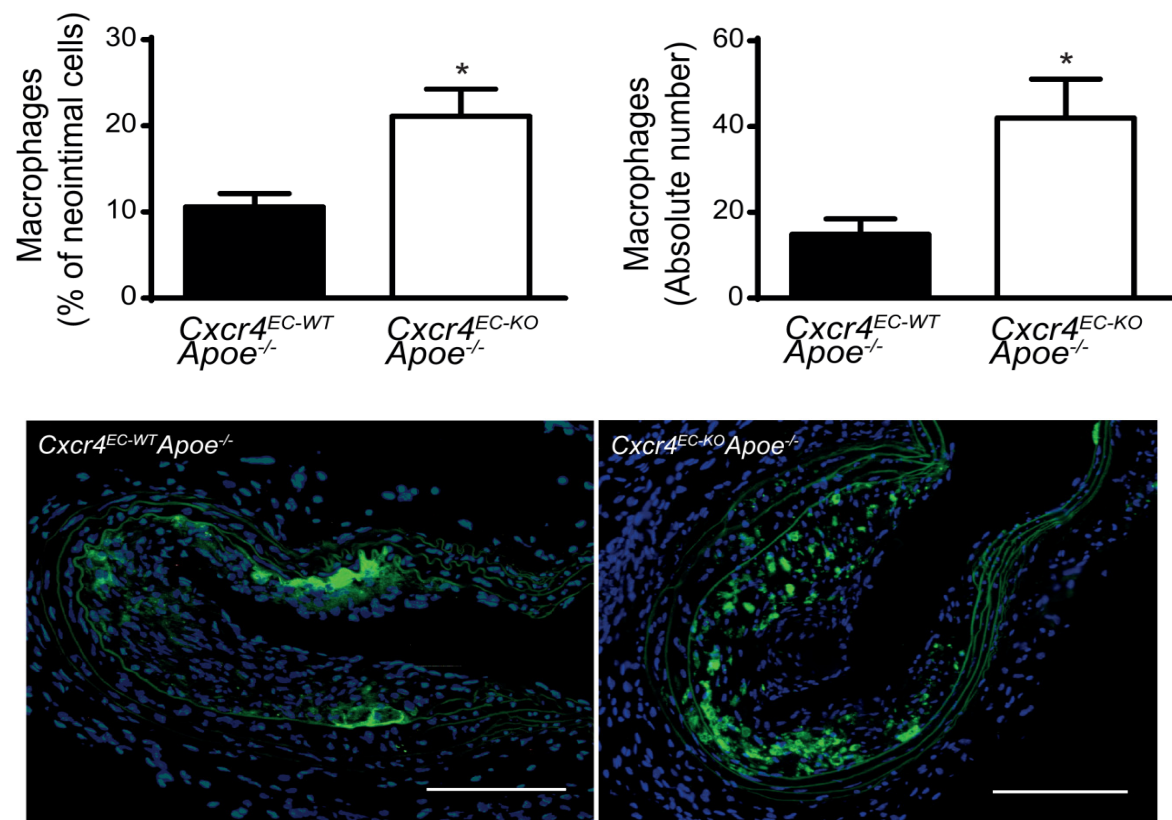


Figure 18: **Endothelial-specific *Cxcr4*-deficiency increases the macrophage content of the neointimal lesions.** Quantification of neointimal macrophages in carotid artery sections from *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice after staining for Mac2 (green) and DAPI (blue) 3 weeks after injury (n=8-12). Scale bars = 100 μm. Graphs represent means ± SEM; **P*<0.05; two-tailed t-test. Representative images are shown.

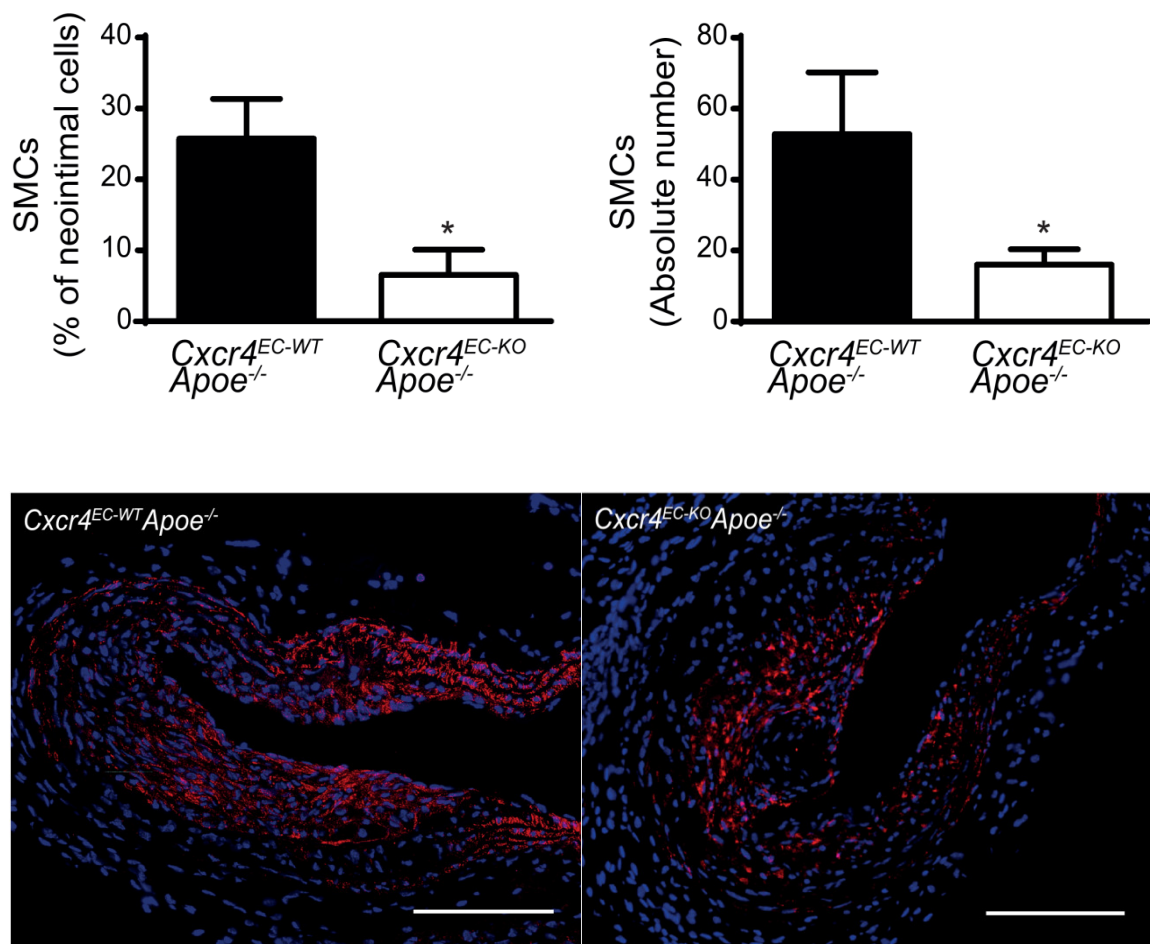


Figure 19: **Endothelial-specific *Cxcr4*-deficiency reduces the smooth muscle cell content of the neointimal lesions.** Quantification of the neointimal SMC content in carotid artery sections from *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice after staining for Sma (red) and DAPI (blue) 3 weeks after injury (n=8-12). Scale bars = 100 μm. Graphs represent means ± SEM; **P*<0.05; two-tailed t-test. Representative images are shown.

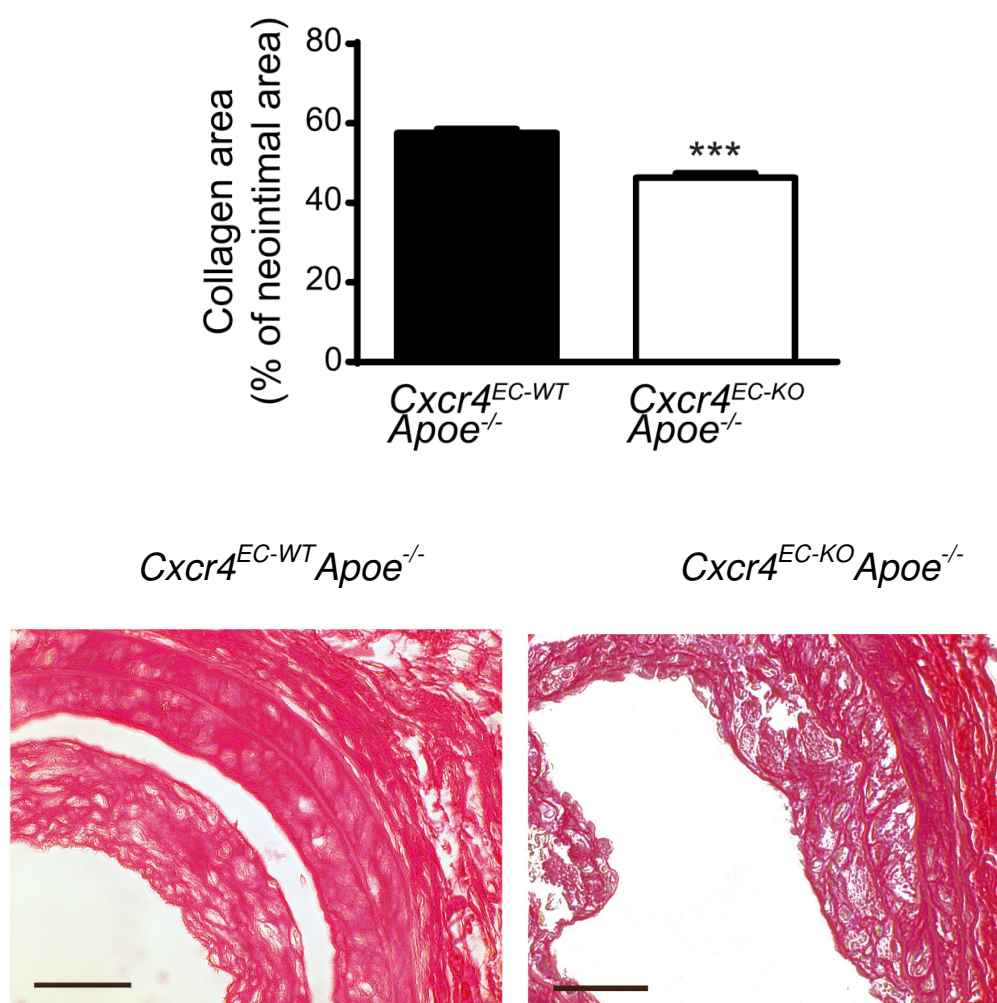


Figure 20: **Endothelial-specific *Cxcr4*-deficiency alters the collagen content of the neointimal lesions.** Analysis of the neointimal collagen content in carotid artery sections from *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice by Sirius Red staining 3 weeks after injury (n=8-13). Scale bars = 50 μm. A-C, Graphs represent means ± SEM; ****P*<0.0001; two-tailed t-test. Representative images are shown.

4.4 Importance of Endothelial Cxcr4 for Reendothelialization of Denudated Arteries

As reendothelialization plays an important role in limiting injury-induced restenosis¹⁵, carotid artery sections of injured carotid arteries were stained for Cd31, in order to have a view on the influence of endothelial *Cxcr4*-deficiency on the endothelial recovery process after endothelial injury of carotid arteries. Three weeks after injury, reendothelialization in the *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice reached only 49%, whereas reendothelialization in the *Cxcr4*^{EC-WT}*Apoe*^{-/-} control mice was 64% (Figure 21).

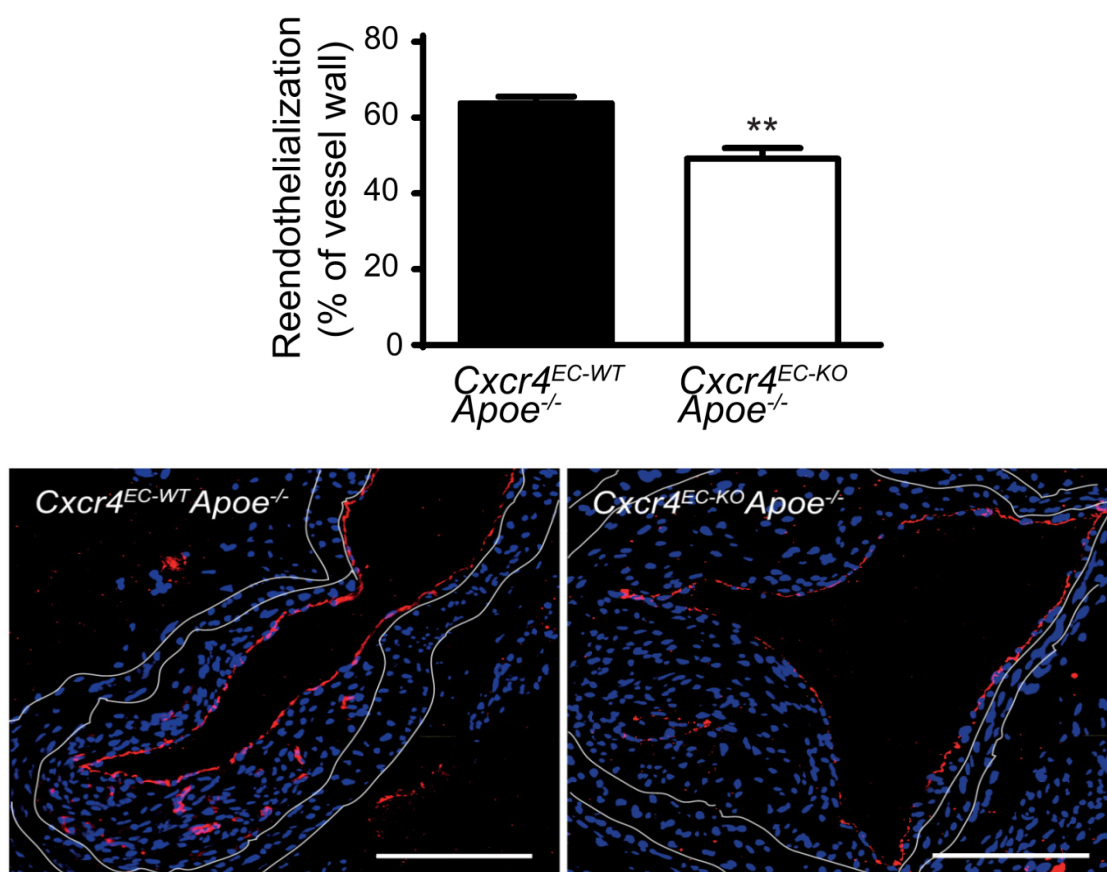


Figure 21: **Endothelial-specific *Cxcr4*-deficiency reduces reendothelialization.** Reendothelialization of the vessel wall, quantified after Cd31 staining (red) of carotid artery sections from *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice 3 weeks after injury (n=8-12). Nuclei were counterstained with DAPI (blue). Scale bar = 200 μ m. Graphs represent means $\pm$ SEM; ***P*<0.01 with two-tailed t-test.

To clarify the role of Cxcr4 in the wound-healing capacity of ECs, a scratch migration assay was performed *in vitro*. HAOECs were cultured *in vitro* as described. Flow cytometry revealed HAOECs to express CXCR4 and respond to the CXCR4 ligand CXCL12 as shown by a decreased CXCR4 surface expression in response to CXCL12 stimulation (Figure 22).

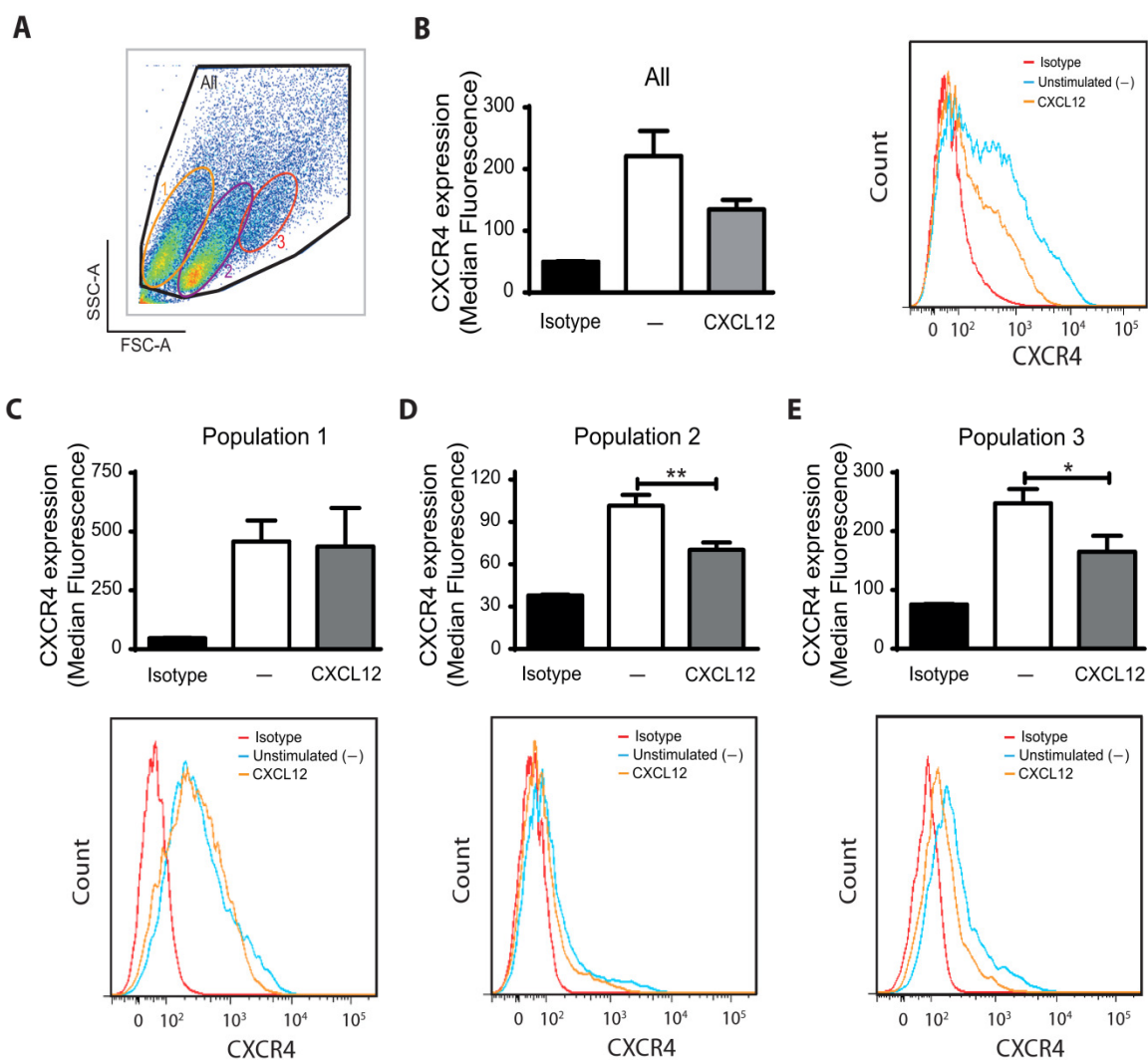


Figure 22: Heterogeneous CXCR4 expression on human aortic endothelial cells. HAOECs, untreated or stimulated with 100 ng/ml CXCL12 for 22 h as indicated, were analyzed for surface CXCR4 expression using flow cytometry and an appropriate isotype control. Different cell populations were identified in the FSC-SSC blot (A), showing dissimilar levels of CXCR4 expression on their cell surface (B-E). Shown is 1 representative experiment of 2, with $n=2-4$. Graph represent means $\pm$ SEM. Representative histograms are shown. * $P<0.05$ and ** $P<0.01$; one-way ANOVA with Newman-Keuls post-test.

HAoECs were stimulated with the CXCR4 inhibitor AMD3100, the CXCR4 ligand CXCL12 or AMD3100 together with CXCL12, respectively, from 8 hours before scratching. Twenty hours after scratching and ongoing stimulation, more CXCL12-stimulated HAoECs were migrated into the scratched site in comparison with control and AMD3100-stimulated cells. Moreover, AMD3100 could slightly block migration of CXCL12-stimulated HAoECs compared with CXCL12 stimulation alone (Figure 23 and 24). Thus, the scratch migration assay showed an increased migratory activity of ECs under activating CXCR4 by its ligand CXCL12. However, CXCL12 could not enhance proliferation of HAoECs *in vitro* (Figure 25).

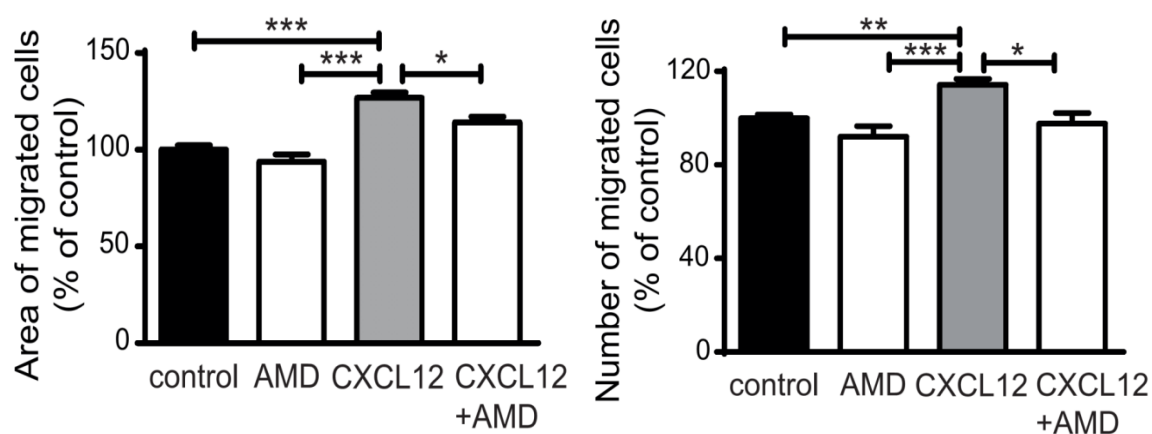


Figure 23: **Analysis of scratch migration assay.** CXCL12 increases the wound healing capacity of ECs in an *in vitro* scratch assay. Human aortic ECs, untreated or prestimulated for 8 h with CXCL12 or AMD3100 as indicated, were scratched and cell migration into the wounded area was quantified 20 h later. *Left:* Analysis of 8-14 scratches from 5 or 2 (for CXCL12 + AMD) independent experiments; *Right:* Analysis of 5-10 scratches from 3 or 1 (for CXCL12 + AMD) independent experiments. Graphs represent means $\pm$ SEM; * P <0.05, ** P <0.01 and *** P <0.001 with one-way ANOVA with Newman-Keuls post-test.

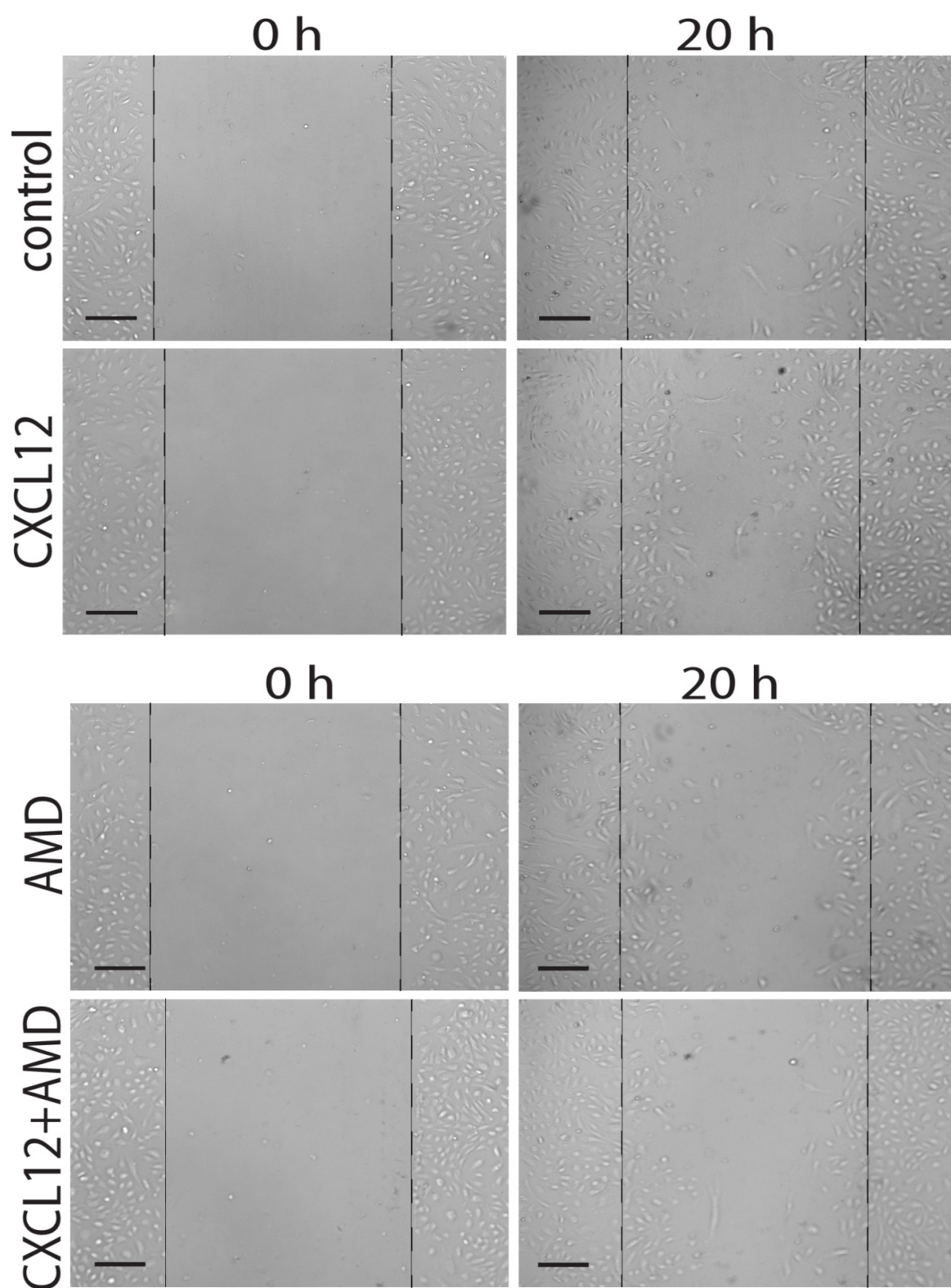


Figure 24: **Representative pictures of a scratch migration assay.** Human aortic ECs, untreated or prestimulated for 8 h with CXCL12 or AMD3100 as indicated, were scratched and cell migration into the wounded area was quantified 20 h later. Quantification of migrating cells was accomplished by following with the dash lines. Scale bar = 250 μm .

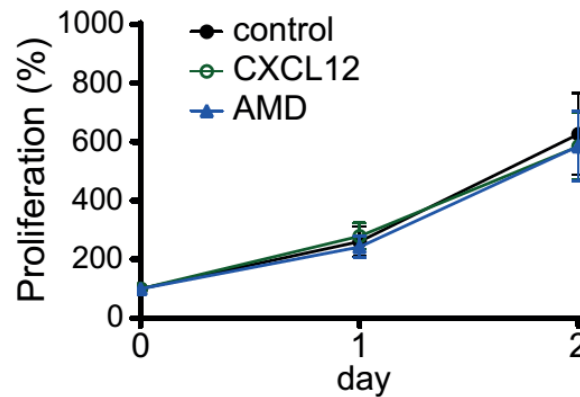


Figure 25: **CXCL12 does not directly enhance basal proliferation of ECs *in vitro*.** Human aortic ECs were left untreated or were stimulated with CXCL12 or AMD3100 for the indicated amount of time, after which proliferation was quantified (n=3 independent experiments, each performed in duplicate).

Nonetheless, 4 days after carotid artery injury, we took the carotid arteries out for quantification of neointimal endothelial proliferation. The carotid artery sections were stained for Cd31 in combination with the proliferation marker Ki67. The number of endothelial proliferating cells, revealed as Ki67⁺Cd31⁺ cells, was significantly reduced in injured carotid arteries from *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice compared with *Cxcr4*^{EC-WT}*Apoe*^{-/-} control mice (Figure 26 and 27).

Together, these results reveal an important role of the chemokine receptor *Cxcr4* in the proliferative and migratory response of ECs following vascular denudation *in vivo*.

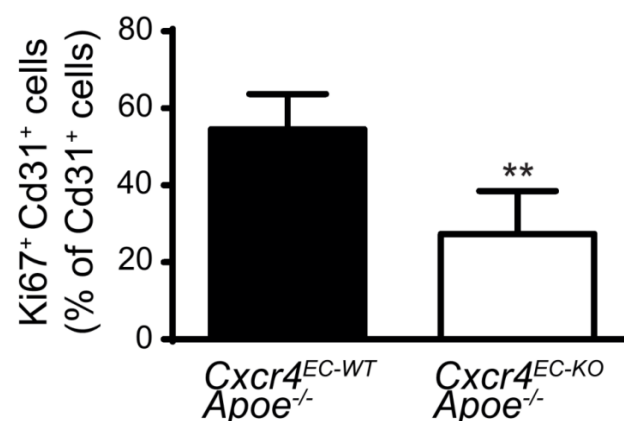


Figure 26: **Endothelial-specific *Cxcr4*-deficiency reduces endothelial proliferation in injured carotid arteries.** Quantification of Ki67⁺ ECs in carotid arteries of *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice 4 days after injury (n=4 each). Graphs represent means $\pm$ SEM; ***P*<0.01 with two-tailed t-test.

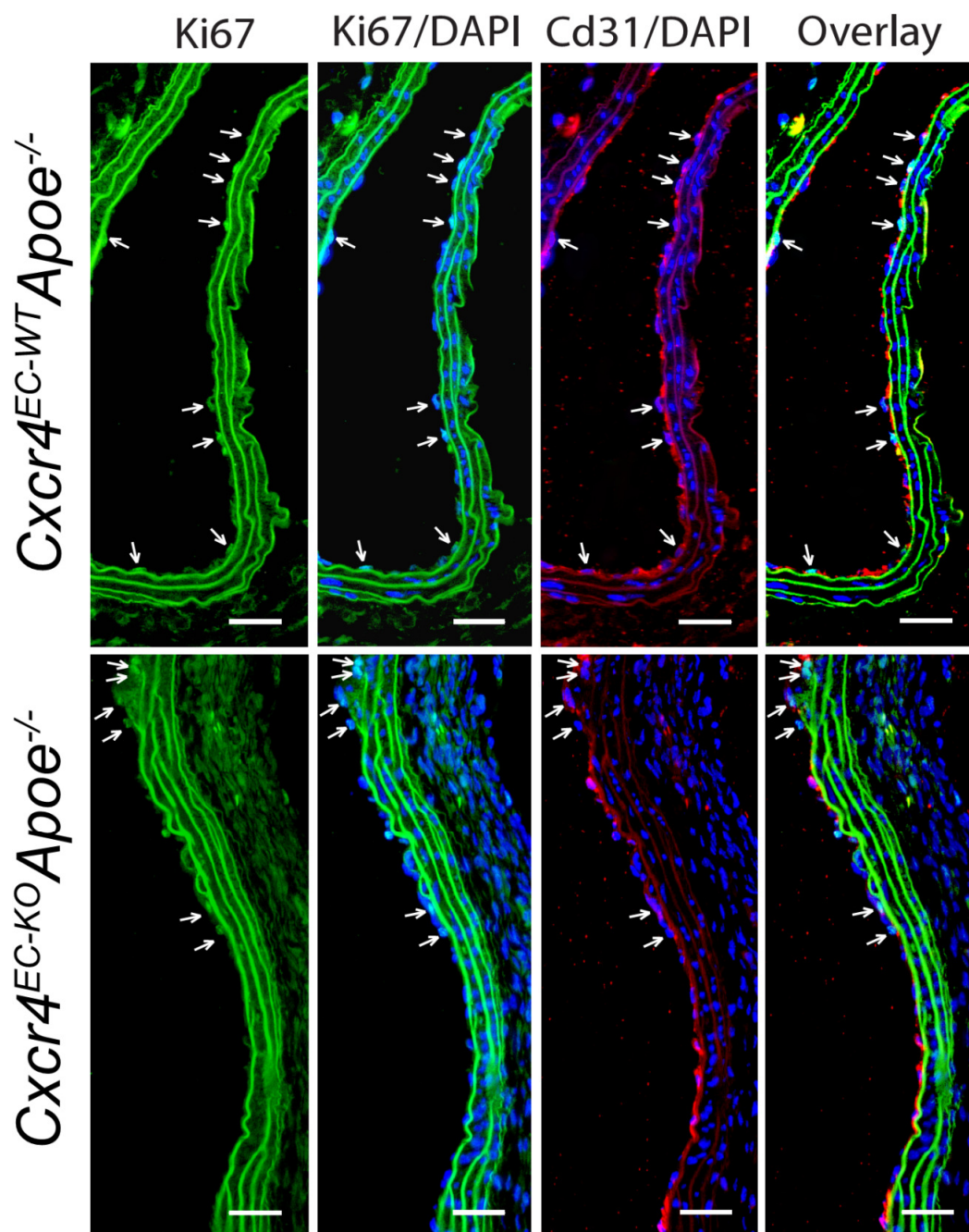


Figure 27: **Representative pictures of endothelial proliferating cell staining.** Representative images of carotid artery sections co-stained for Cd31 (red), Ki67 (green) and DAPI (blue) indicate less proliferating ECs (Ki67⁺Cd31⁺, light blue, indicated with arrows) in *Cxcr4*^{EC-KO}*Apoe*^{-/-} vs *Cxcr4*^{EC-WT}*Apoe*^{-/-} mice. Scale bar = 25 μ m.

4.5 Influence of Endothelial *Cxcr4* Deletion on the Mobilization of Sca1⁺Flk1⁺Cd31⁺ Cells after Arterial Injury

As explained in the introduction, previous studies have demonstrated that EPCs contribute to an efficient reendothelialization of denudated vessels^{20, 42}. Although markers of EPCs are not well characterized, the analysis of circulating EPCs in mice with flow cytometry is regularly performed using Sca1⁺Flk1⁺ expression^{35, 40, 41}. Moreover, we checked that mouse Sca1⁺Flk1⁺ mononuclear cells were also Cd31⁺ (Figure 28). Thus, we performed studies to examine the level of circulating Sca1⁺Flk1⁺Cd31⁺ cells in our mouse models before and after carotid artery injury. At least 3 weeks after the first tamoxifen injection but before the start of high-fat diet, 6-8 drops of blood were taken from the mice for analysis, which were considered as baseline results. The gating strategy of Sca1⁺Flk1⁺Cd31⁺ cell analysis is shown in Figure 27. As presented in Figure 28, no differences in circulating Sca1⁺Flk1⁺Cd31⁺ cells were observed between *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice at baseline conditions.

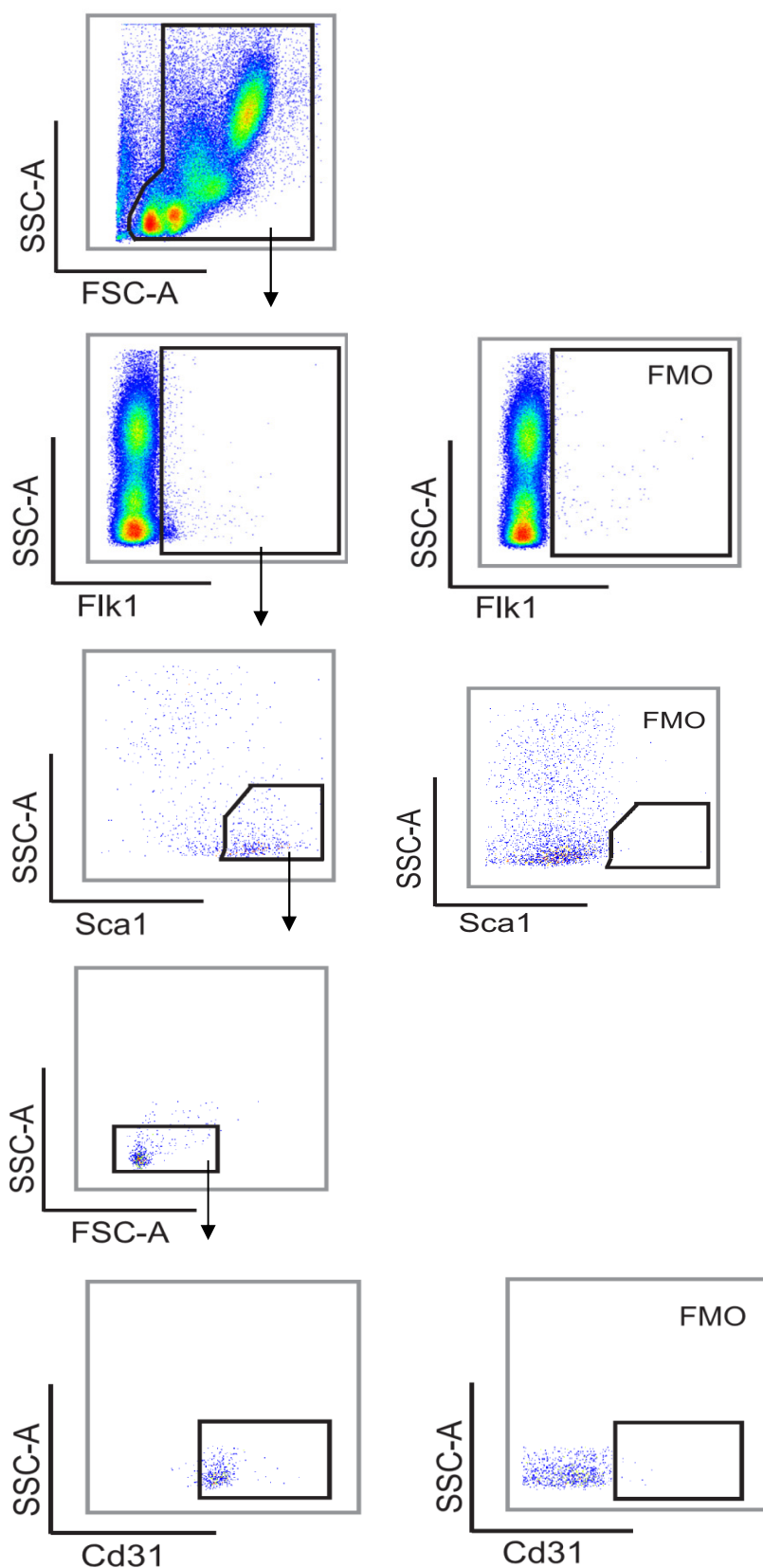


Figure 28: **Analysis of EPCs.** Gating strategy of Sca1⁺Flk1⁺Cd31⁺ cells by flow cytometry, including the appropriate FMO controls. Representative dot plots are shown.

The level of circulating Sca1⁺Flk1⁺Cd31⁺ cells was increased in *Cxcr4*^{EC-WT}*Apoe*^{-/-} mice 3 weeks after vascular injury (Figure 29). Surprisingly, circulating Sca1⁺Flk1⁺Cd31⁺ cells were significantly reduced in injured *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice compared to injured *Cxcr4*^{EC-WT}*Apoe*^{-/-} controls and did not show an increased trend over a three weeks recovery period after vascular injury (Figure 29).

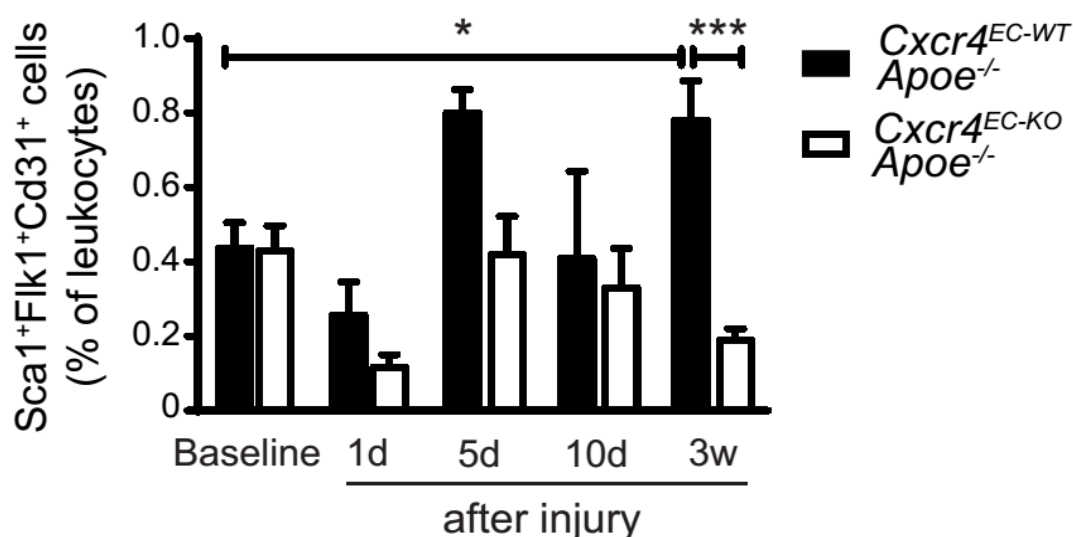


Figure 29: **Less circulating Sca1⁺Flk1⁺Cd31⁺ cells in injured mice with an endothelial-specific *Cxcr4*-deficiency.** Quantification of Sca1⁺Flk1⁺Cd31⁺ progenitor cells in the peripheral blood of *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice before or at different time points after carotid artery injury, as indicated. The graph represents means $\pm$ SEM; n=12-20(for baseline, 1 day, 3 weeks) and n=5-6 (for 5 and 10 days); **P*<0.05 and ****P*<0.001; one-way or two-way ANOVA with Bonferroni post-test, as appropriate.

Comparably, in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice there was a significantly reduced mobilization of circulating Lin⁻Sca1⁺ progenitor cells after injury, compared with *Cxcr4*^{EC-WT}*Apoe*^{-/-} controls (Figure 30 and 31).

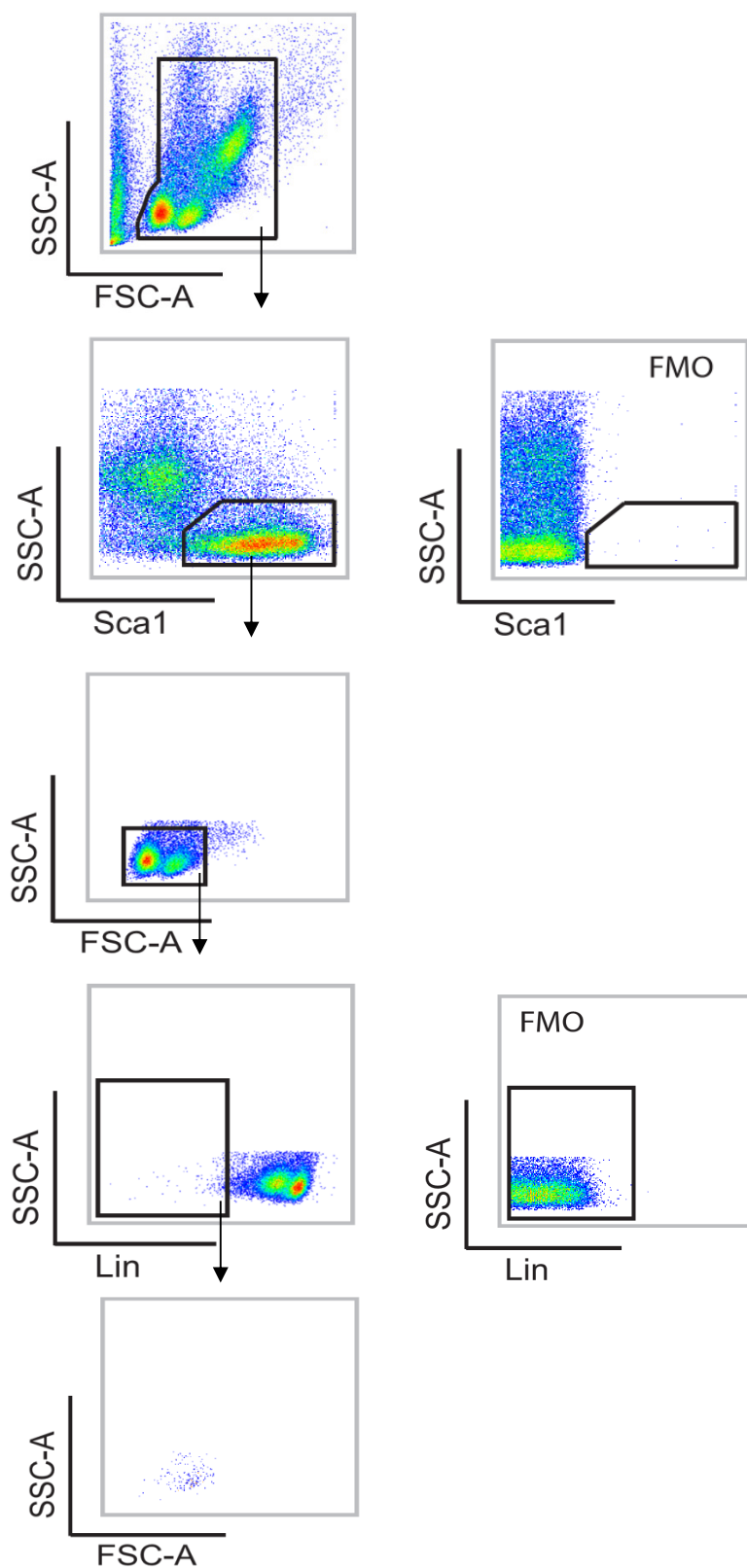


Figure 30: **Analysis of $\text{Lin}^- \text{Sca1}^+$ progenitor cells.** Gating strategy of $\text{Lin}^- \text{Sca1}^+$ progenitor cells by flow cytometry, including the appropriate FMO controls. Representative dot plots are shown.

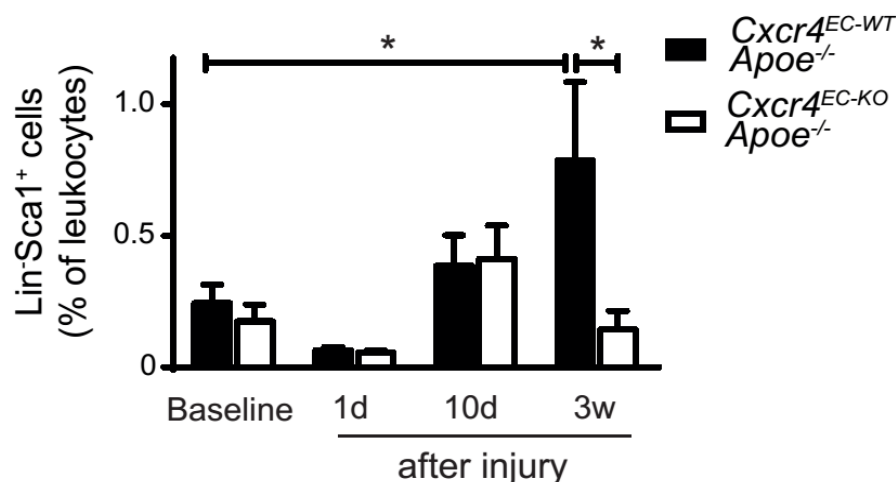


Figure 31: **Less circulating Lin⁻Sca1⁺ progenitor cells in injured mice with an endothelial-specific *Cxcr4*-deficiency.** Quantification of Lin⁻Sca1⁺ cells in the peripheral blood of *Cxcr4*^{EC-WT}*Apoe*^{-/-} vs *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice before or at different time points after carotid artery injury, as indicated. The graph represents means $\pm$ SEM; n=11-21 (for baseline, 1 and 10 days) or n=5-6 (for 3 weeks); **P*<0.05; one-way or two-way ANOVA with Bonferroni post-test, as appropriate.

As previous studies have shown that *Cxcr4* mediates the recruitment of progenitor cells to neointimal lesions after carotid artery injury^{21, 31} and that the *Cxcl12/Cxcr4* axis plays an important role in progenitor cell mobilization from the bone marrow to the periphery^{130, 131}, we questioned whether the reduced mobilization of Sca1⁺Flk1⁺Cd31⁺ cells in injured *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice could be related to a potential deletion of *Cxcr4* in Sca1⁺Flk1⁺Cd31⁺ cells mediated by the *Bmx-Cre* transgene. Analysis of *Cxcr4* expression on the surface of circulating Sca1⁺Flk1⁺Cd31⁺ cells, as detected by FACS, showed that Sca1⁺Flk1⁺Cd31⁺ cells did not show significant differences in surface *Cxcr4* expression between *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice (Figure 32), as represented by the geometric mean fluorescence intensity (gMFI). Thus, these data indicate that the reduced Sca1⁺Flk1⁺Cd31⁺ cell level in injured *Cxcr4*^{EC-KO}*Apoe*^{-/-} is not caused by a *Cxcr4* deficiency in the Sca1⁺Flk1⁺Cd31⁺ cells themselves. Comparably, there was no statistically significant difference in the *Cxcr4* expression level on circulating Lin⁻Sca1⁺ progenitor cells (Figure 33).

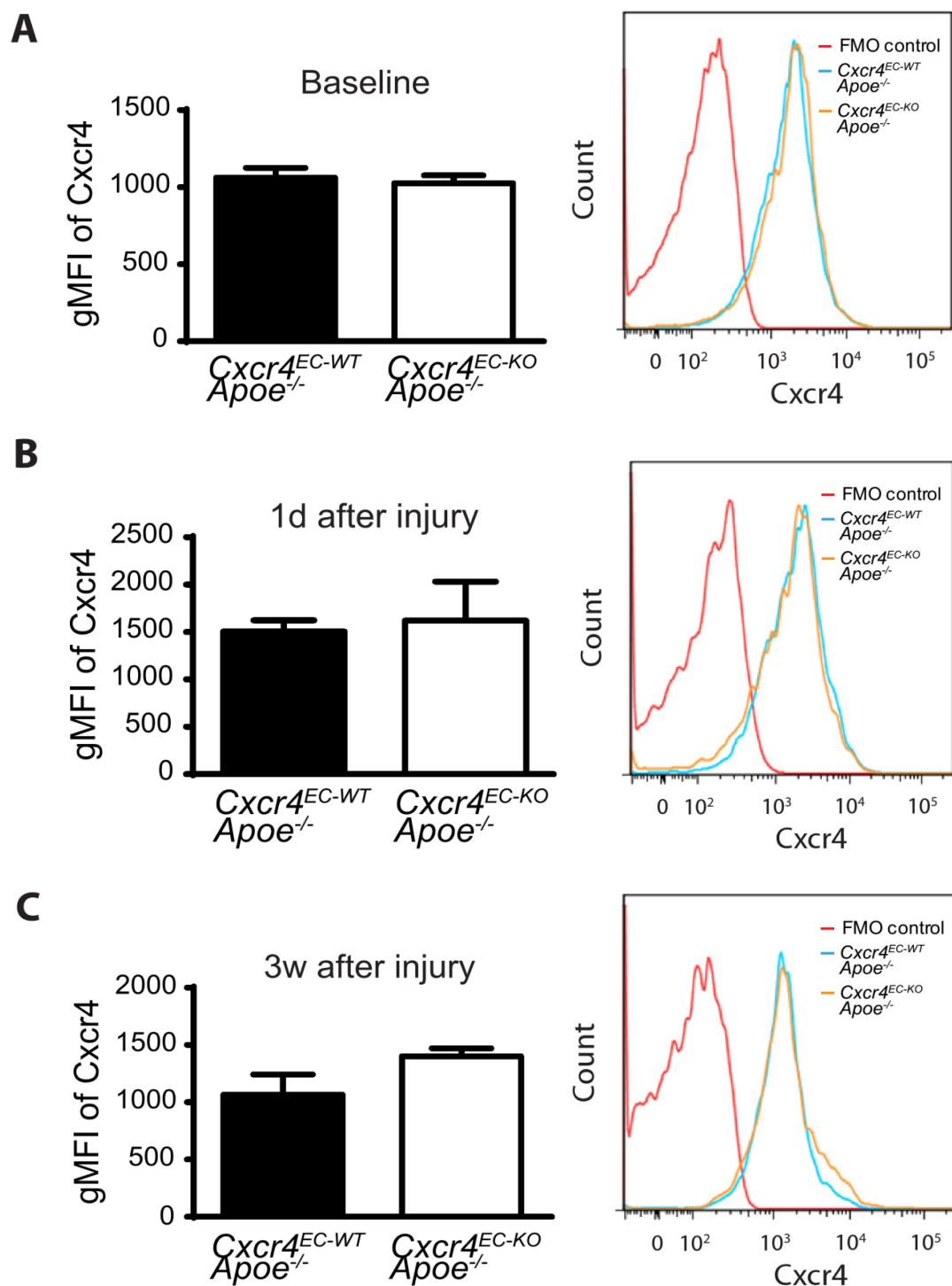


Figure 32: **Comparable expression of Cxcr4 on circulating Sca1⁺Flk1⁺Cd31⁺ cells upon endothelial Cxcr4 deficiency.** Cxcr4 expression on the surface of circulating Sca1⁺Flk1⁺Cd31⁺ cells was analyzed by flow cytometry in *Cxcr4^{EC-WT}Apoe^{-/-}* and *Cxcr4^{EC-KO}Apoe^{-/-}* mice before and at different time points after vascular injury, as indicated. Displayed is the geometric MFI (gMFI) of surface Cxcr4 after subtraction of the FMO control. The graphs represent means $\pm$ SEM; n=4-10. Representative histograms are shown.

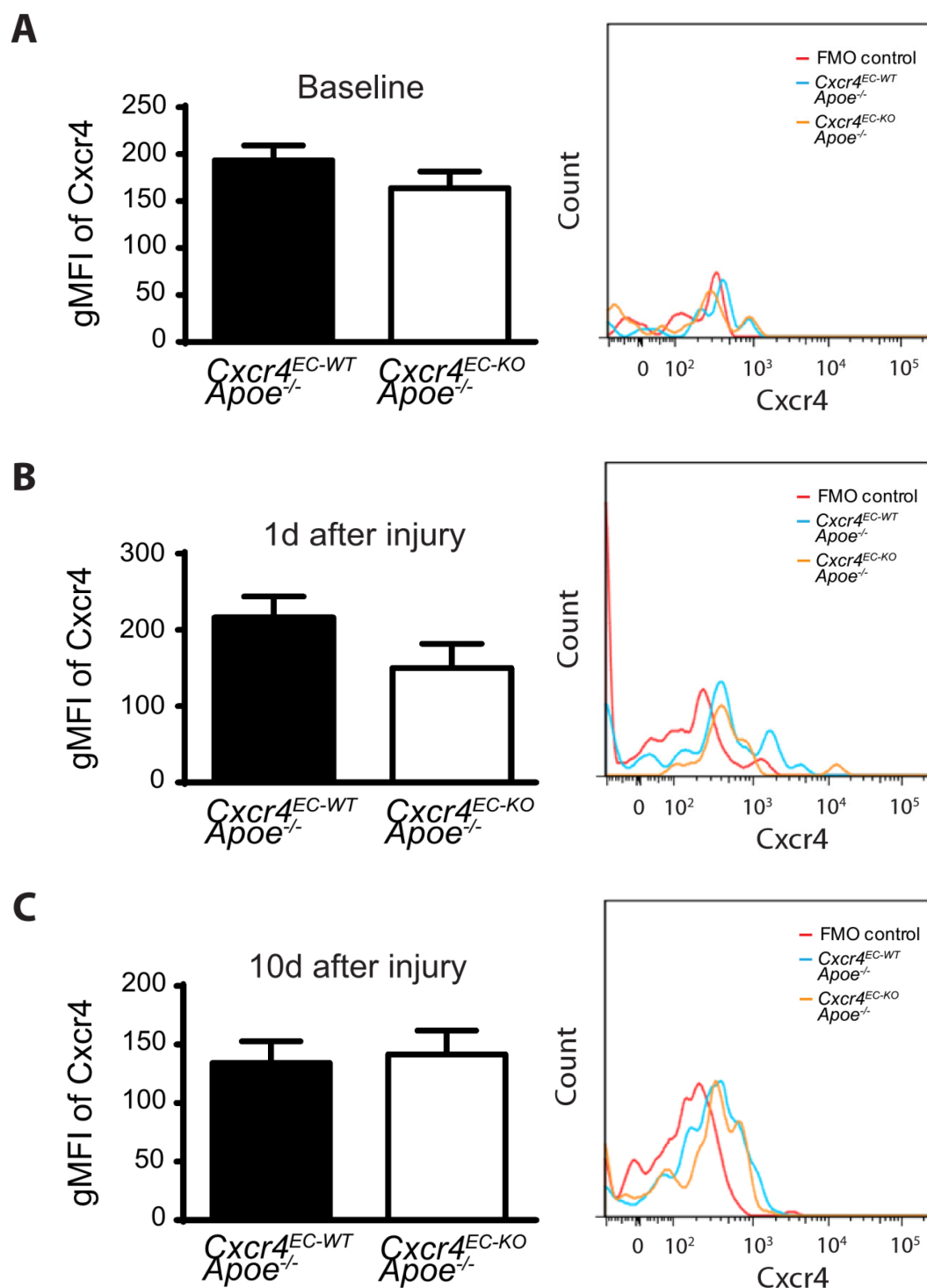
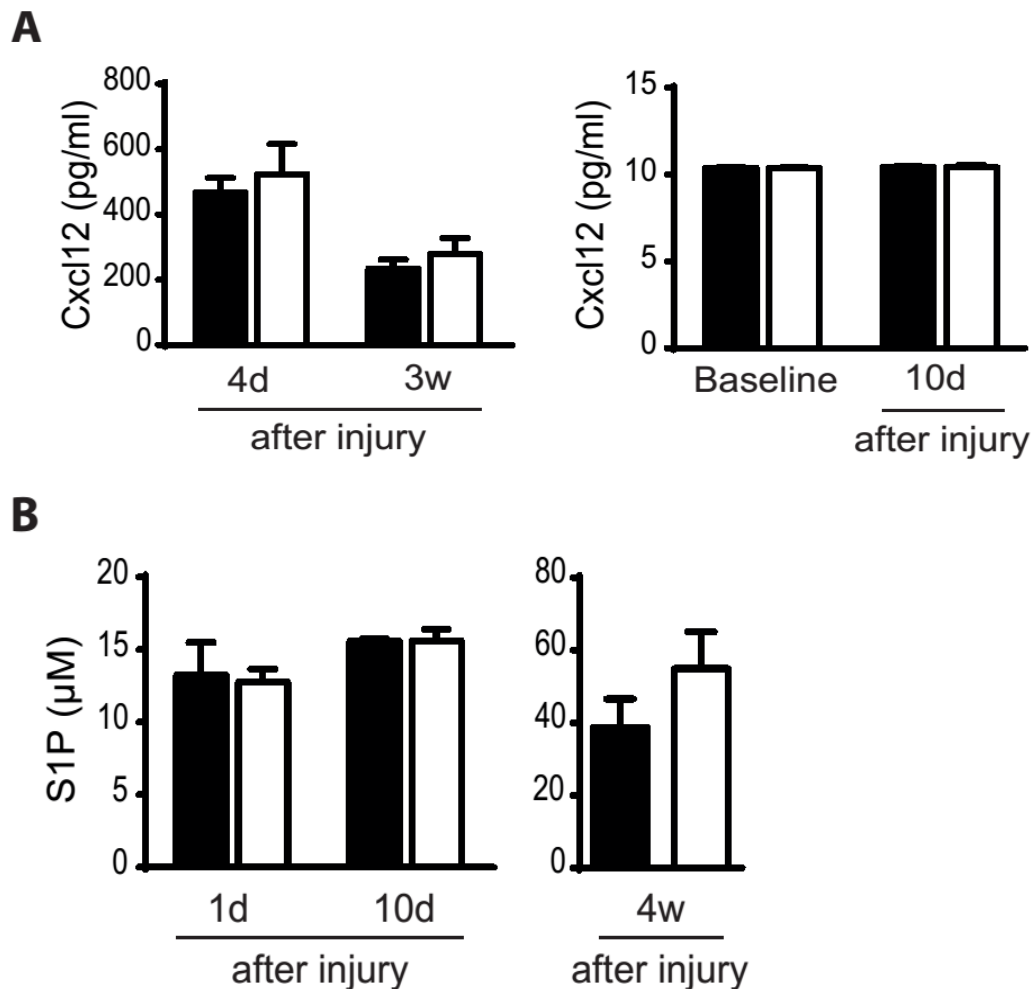


Figure 33: **No significant effect on Cxcr4 expression on circulating Lin⁺Sca1⁺ progenitor cells upon endothelial Cxcr4 deficiency.** A-C, Cxcr4 expression on the surface of circulating Lin⁺Sca1⁺ progenitor cells was analyzed by flow cytometry in *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice before and at different time points after vascular injury, as indicated. Displayed is the gMFI of surface Cxcr4 after subtraction of the FMO control. The graphs represent means $\pm$ SEM; n=9-12. Representative histograms are shown.

As Cxcl12 is an important ligand for Cxcr4 and ECs have been shown to produce Cxcl12 through a Cxcl12/Cxcr4 positive feedback loop¹³², we then investigated whether the reduced Sca1⁺Flk1⁺Cd31⁺ cell level could be caused by a reduced Cxcl12 expression in the injured *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice, *i.e.* we investigated the regulation of Cxcl12 expression after endothelial deficiency of Cxcr4 in the injured mice. The Cxcl12 level was assessed by ELISA after carotid artery injury. No significant differences in the Cxcl12 level in plasma or bone marrow was observed in *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice after vascular injury (Figure 34A).



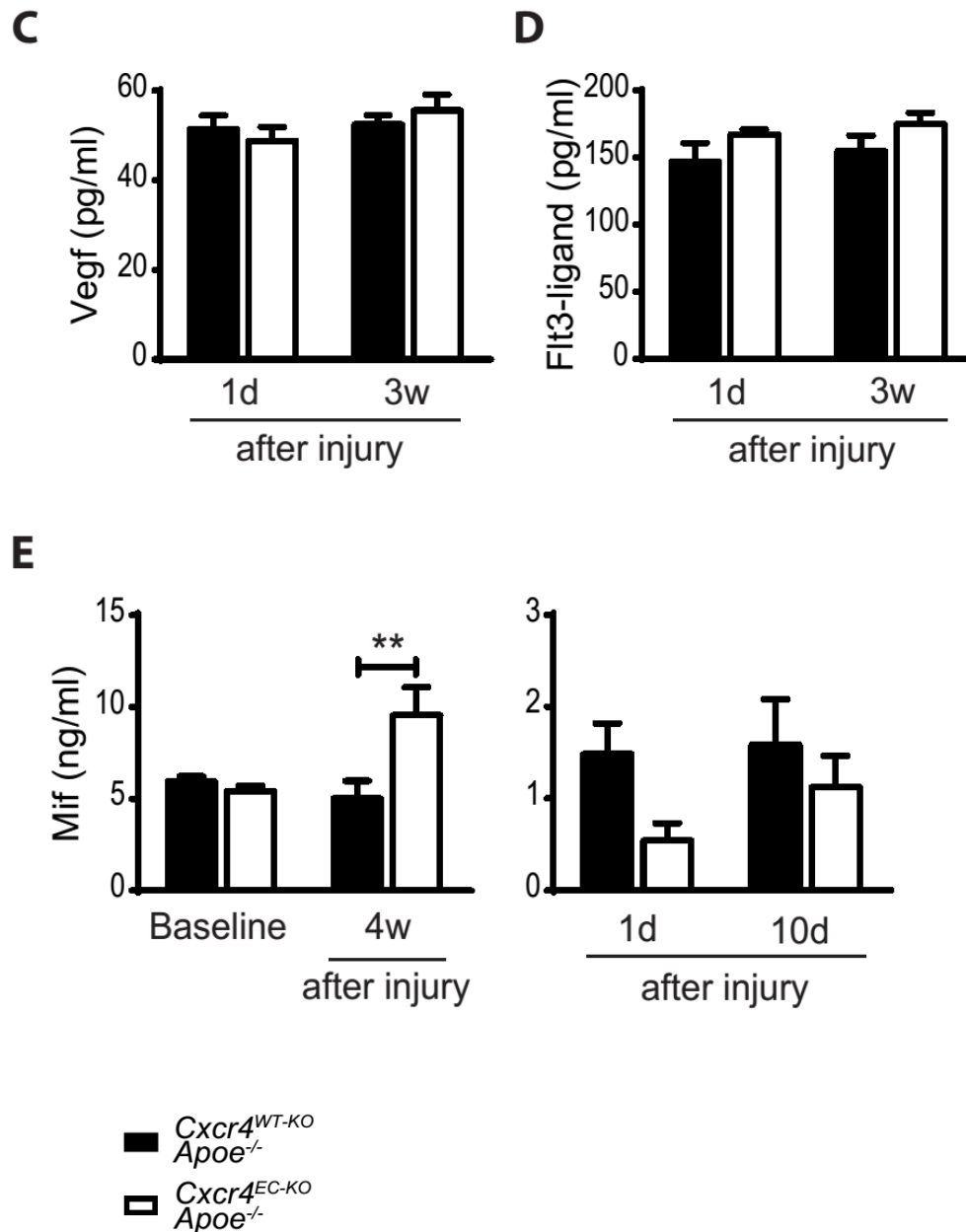


Figure 34: **Plasma levels of different stimuli upon endothelial *Cxcr4* deficiency.** **A**, *Cxcl12* concentration in plasma (*left panel*) or bone marrow (*right panel*) before or after injury, as indicated; $n=5-6$ (for bone marrow) or $n=8-13$ (for plasma). **B-E**, Plasma concentrations of Vegf, Flt3-ligand, S1P and Mif, before or after injury, as indicated. Measurements of heparin-plasma are presented in separate graphs (D and E, left panels) as EDTA-plasma (all other panels), as heparin- vs EDTA-plasma can differentially affect absolute ELISA read-outs. $n=2-4$ (for Mif baseline and 1 day) or $n=5-8$ (for all others).

To explore other potential mechanism underlying the observed effect on mobilization of Sca1⁺Flk1⁺Cd31⁺ progenitor cells, plasma concentrations of different stimuli involved in this process were quantified. Plasma concentrations of Vegf, Flt3-ligand and S1P, which can all influence progenitor cell mobilization, were unaltered at different time points after injury (Figure 34B-D). We then investigated Mif, as an alternative ligand of Cxcr4 known to mediate EPCs *in vitro* through Cxcr4⁵⁹. Plasma concentrations of Mif were unaltered until 10 days after vascular injury. Surprisingly, at later stage the plasma level of Mif was significantly increased in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice (Figure 34E), which can thus also not explain the decreased mobilization of Sca1⁺Flk1⁺Cd31⁺ progenitors. Finally, we investigated GM-CSF as another cytokine involved in progenitor cell mobilization, but its plasma level stayed below the detection limit.

Subsequently, Cxcl12 expression on the endothelial layer of the neointima was evaluated 3 weeks after vascular injury in both groups of mice. Immunostaining of injured carotid artery sections for Cxcl12 and Cd31 revealed that Cxcl12 level in vascular endothelium was lower in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice compared to *Cxcr4*^{EC-WT}*Apoe*^{-/-} mice after injury (Figure 35 and 36).

Together, these results suggest that endothelial deficiency of *Cxcr4* in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice reduces the lumenally-exposed Cxcl12 level in injured vascular endothelium. This may then contribute to the observed reduction in circulating Sca1⁺Flk1⁺Cd31⁺ cells, although the underlying mechanism of declined mobilization currently remains unclear. The reduced mobilization of Sca1⁺Flk1⁺Cd31⁺ progenitor cells upon endothelial *Cxcr4* deficiency may be involved in the observed reduction in reendothelialization.

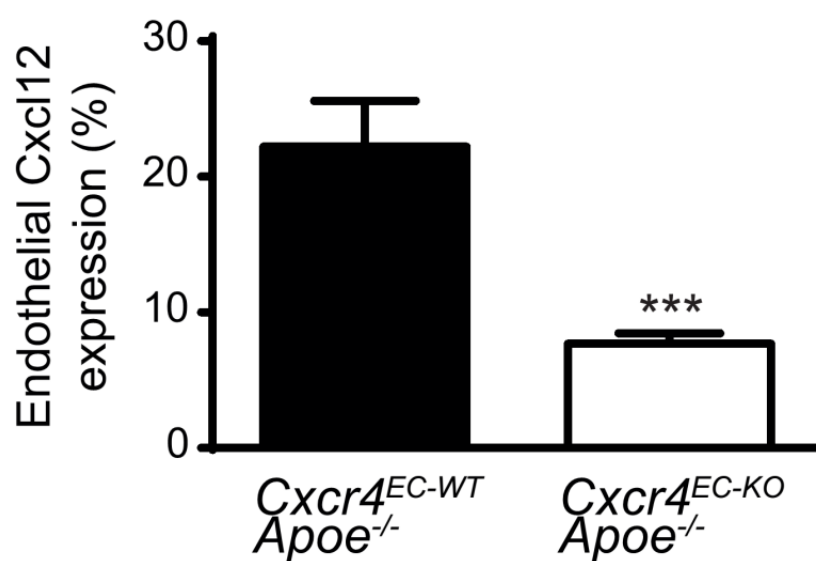


Figure 35: **Reduced Cxcl12 expression in *Cxcr4*-deficient endothelium.** Quantification of endothelial Cxcl12 expression in carotid arteries from *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice 3 weeks after injury. The graph displays the Cxcl12⁺Cd31⁺ luminal length as % of the total Cd31⁺ luminal length. Represented are means $\pm$ SEM; n=8-13, ****P*<0.0001; two-tailed t-test.

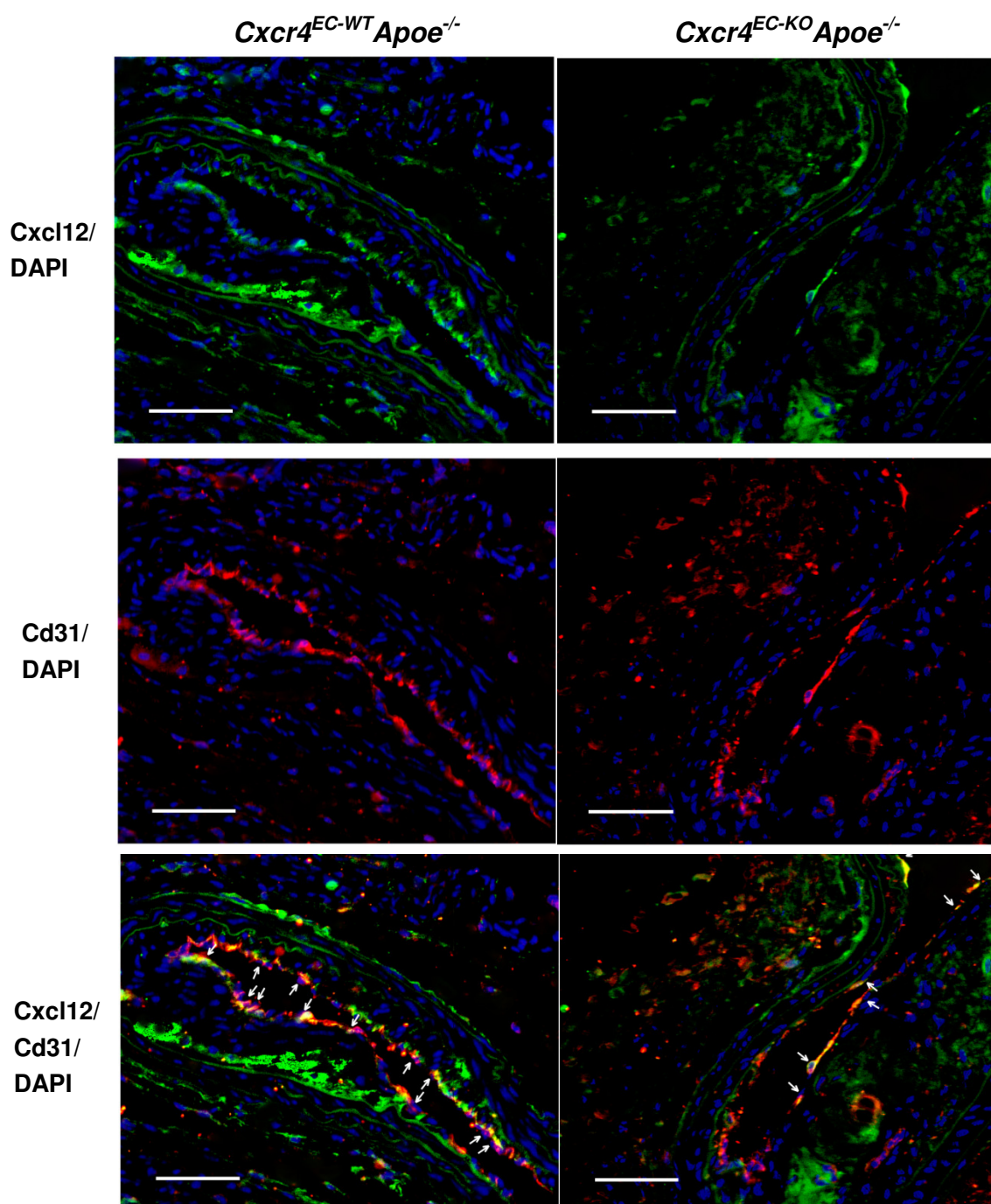


Figure 36: **Representative pictures of Cxcl12 expression in endothelium.** Representative images of endothelial Cxcl12 expression in carotid arteries from *Cxcr4*^{EC-WT}*Apoe*^{-/-} and *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice 3 weeks after injury, based on staining of carotid artery sections for Cd31 (red), Cxcl12 (green) and DAPI (blue). Cxcl12⁺Cd31⁺ cells (yellow) are indicated with arrows. Scale bars = 50 μm.

5 Discussion

5.1 Endothelial *Cxcr4*-Deficiency Induced Enhanced Neointimal Formation with Neointimal Macrophage Accumulation, but with Reduced SMC Content

Under pathological conditions, the chemokine receptor *Cxcr4* and its ligand *Cxcl12* can mediate bone marrow-derived progenitor cell mobilization to the periphery³⁰. This has also been shown in context of vascular injury, in which the *Cxcl12/Cxcr4* axis is associated with neointimal hyperplasia and mobilization of bone marrow-derived SPCs after arterial injury²¹. Treatment with either *Cxcr4* antagonists or *Cxcl12* blocking antibodies reduced the mobilization of SPCs and neointima formation after vascular injury^{21, 123, 124}. However, the cell type-specific function of *Cxcr4* in injury-induced neointima formation still remains unknown. Especially, the role of *Cxcr4* on vascular ECs in reendothelialization of denudated vessels has not been described so far.

In this study, we demonstrated that deficiency of endothelial *Cxcr4* enhanced neointimal hyperplasia in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice compared to *Cxcr4*^{EC-WT}*Apoe*^{-/-} control mice 4 weeks after carotid artery injury. The increased neointima formation in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice was associated with a higher neointimal macrophage content, a reduced content of neointimal SMCs and a lower neointimal collagen content (Figure 18-20). Additionally, these mice showed a higher plasma level of Mif 4 weeks after vascular injury but no significant differences in early stage (Figure 34E). Previously, expression of Mif could be detected mostly in neointimal foam cells 2 weeks after injury¹¹⁹. Furthermore, it has been reported that blocking Mif in *Ldlr*^{-/-} mice revealed a lower neointimal plaque size after experimental angioplasty¹¹⁸ and blocking Mif in *Apoe*^{-/-} mice significantly reduced neointimal macrophage content of injury-induced lesions¹¹⁹. Therefore, the higher content of

neointimal macrophages in injured *Cxcr4^{EC-KO}Apoe^{-/-}* mice may be at least partly mediated by the observed increased plasma level of Mif in these mice at late stage after arterial injury, driving an increased size and inflammatory phenotype of the neointimal lesions. The mechanisms for enhanced Mif plasma levels in injured *Cxcr4^{EC-KO}Apoe^{-/-}* mice remain currently unclear.

It was previously shown that reduced neointima formation in mice treated with the *Cxcr4* antagonist AMD3465 was associated with a reduced neointimal SMC content and a reduced injury-induced mobilization of Lin⁻Sca1⁺ progenitor cells in the peripheral blood¹²⁴. Lin⁻Sca1⁺ cells have been reported to be increased shortly after injury, and Lin⁻PDGFRβ⁺ cells were shown to be able to transdifferentiate in SMCs *in vitro* in response to PDGF-BB. Furthermore, injection of sorted Lin⁻Sca1⁺ cells from Sm22^{LacZ} mice into injured *Apoe^{-/-}* mice revealed their incorporation into neointimal lesions, and their transdifferentiation into PDGFRβ⁺ SMCs visualized by co-staining for β-galactosidase and PDGFRβ. These results led to the conclusion that circulating Lin⁻Sca1⁺ cells can be recruited to injured vessel giving rise to neointimal SMCs¹⁰. Therefore, the reduced neointimal SMC content (Figure 19) in our injured *Cxcr4^{EC-KO}Apoe^{-/-}* mice could be related to the observed decrease in Lin⁻Sca1⁺ progenitor cell mobilization into the peripheral blood (Figure 31). However, surface expression of *Cxcr4* on Lin⁻Sca1⁺ progenitor cells was not decreased in *Cxcr4^{EC-KO}Apoe^{-/-}* mice compared with *Cxcr4^{EC-WT}Apoe^{-/-}* control mice (Figure 33), suggesting that other factors are involved in the observed reduction in progenitor cell mobilization. As mice treated with a *Cxcl12* blocking antibody revealed reduced neointima formation after arterial injury associated with a decreased mobilization of Lin⁻Sca1⁺ progenitor cells in the peripheral blood²¹, we hypothesized that reduced Lin⁻Sca1⁺ progenitor cells in *Cxcr4^{EC-KO}Apoe^{-/-}* mice may be associated with reduced *Cxcl12* expression levels. This is being further discussed in Chapter 5.3.

Remarkably, there was only a trend for increased neointima in *Cxcr4^{EC-KO}Apoe^{-/-}* mice at 3 weeks after injury, without reaching statistical significance (Figure 15). However, after one additional week, neointimal lesion

size was significantly increased with a still incomplete and significantly reduced reendothelialization in *Cxcr4^{EC-KO}Apoe^{-/-}* mice (Figure 16). In early stage after injury, both the *Cxcr4^{EC-KO}Apoe^{-/-}* mice as controls lack endothelium at the injured site, so in both groups, macrophages and SPCs can enter the vessel wall to contribute to neointima formation. Furthermore, no significant differences were observed in the SPC level in peripheral blood up to 10 days after injury. Thus, a comparable neointimal hyperplasia can be expected in the first stages after injury. Gradually, reendothelialization starts off, but to a lower extent in *Cxcr4^{EC-KO}Apoe^{-/-}* mice compared to controls. However, an enhanced infiltration rate of blood cells (macrophages and SPCs, contributing to neointima formation) in the injured vessel wall of *Cxcr4^{EC-KO}Apoe^{-/-}* mice is counteracted by a reduced mobilization of SPCs at later stage. Whereas the absolute amount of infiltrated macrophages is significantly increased in neointimal lesions of injured *Cxcr4^{EC-KO}Apoe^{-/-}* mice 3 weeks after injury, the absolute amount of SMCs is significantly decreased. As a net balance, only a trend for increased neointima formation is observed in *Cxcr4^{EC-KO}Apoe^{-/-}* mice compared to controls 3 weeks after injury. However, at 4 weeks after injury, neointimal lesions were found to be significantly larger in *Cxcr4^{EC-KO}Apoe^{-/-}* mice compared to controls. With a significant reduction in reendothelialization in these mice at this stage, with control mice reaching 70% of reendothelialization compared to 50% in *Cxcr4^{EC-KO}Apoe^{-/-}* mice, these data suggest an important role for reduced reendothelialization and enhanced macrophage filtration to the observed increase in neointima formation at this stage. Although SPCs were significantly reduced in *Cxcr4^{EC-KO}Apoe^{-/-}* mice compared to controls 3 weeks after injury, this reduction was not able to counteract the increased neointima formation driven by reduced reendothelialization and enhanced macrophage infiltration. This suggests that in this stage, the role of circulating SPCs in neointimal hyperplasia is reduced in comparison to earlier time points. Furthermore, the enhanced MIF plasma levels observed in *Cxcr4^{EC-KO}Apoe^{-/-}* mice at this stage may further stimulate enhanced neointima formation, as explained above in Chapter 5.1, suggesting that from this stage on,

macrophages and SPCs can more readily enter the lesion in *Cxcr4^{EC-KO}Apoe^{-/-}* mice. Although these cell types both contribute to enhanced neointima, a reduced SPC mobilization in *Cxcr4^{EC-KO}Apoe^{-/-}* mice counteracts this effect.

Altogether, these results indicate that endothelial *Cxcr4* is involved in neointimal SMC accumulation, associated with the recruitment of Lin⁻Sca1⁺ progenitor cells. The reduced SMC content in *Cxcr4^{EC-KO}Apoe^{-/-}* mice was associated with a small but significant reduction in collagen content, suggesting a lower plaque stability. Furthermore, endothelial *Cxcr4* deficiency triggered neointimal macrophage accumulation, which could be partially driven by an increased *Mif* level at later stage.

5.2 Endothelial *Cxcr4* Modulates Reendothelialization by EC Proliferation/Migration and Sca1⁺Flk1⁺Cd31⁺ Progenitor Cell Recruitment

EPCs are pivotal in contributing to reendothelialization of denudated vessels^{20, 21}, whereas chemokine receptor CXCR4 plays an important role in regulating EPC mobilization^{15, 29}. In the current study, we observed that reduced reendothelialization after carotid artery injury in *Cxcr4^{EC-KO}Apoe^{-/-}* mice was related to a decreased presence of proliferating ECs in the injured vessel (Figure 26). Moreover, this result was also associated with a lower mobilization of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells after vascular injury (Figure 29). Furthermore, as shown in an *in vitro* scratch experiment (Figure 23 and 24), a lower wound-healing, migratory capacity of HAoECs after blocking CXCR4 with its antagonist AMD3100 could also contribute to the defective reendothelialization after vascular injury in endothelial *Cxcr4*-deficient mice.

Reendothelialization has been investigated in many studies, and has been

shown to play a crucial, protective role against neointima formation. Whereas increased neointima formation has been correlated with decreased reendothelialization after injury¹⁷, neointima formation is reduced by an accelerated regeneration of the endothelium¹⁸. For example, endothelial seeding on an injured vessel wall of atherosclerotic rabbits significantly reduced neointimal hyperplasia and enhanced endothelial recovery, followed by a reduced restenosis¹⁵. Also, in a porcine model, implantation of ECs after vascular injury revealed a delayed neointima formation¹⁶. In summary, these data, together with our observation that deletion of endothelial *Cxcr4* is associated with defective reendothelialization and enhanced neointimal hyperplasia after arterial injury, support the notion that endothelial *Cxcr4* plays a key role in vascular endothelial recovery, named reendothelialization, which is inversely associated with neointimal hyperplasia after arterial injury.

One process contributing to reendothelialization is the proliferation and migration of uninjured neighboring ECs to the injured site³⁴. Deficiency of endothelial *Cxcr4* significantly reduced endothelial proliferation at the injured site *in vivo*, driving a lower reendothelialization level. Furthermore, blocking CXCR4 with its antagonist resulted in a reduced wound-healing capacity of CXCL12-stimulated ECs in an *in vitro* scratch migration assay. Since CXCL12 could not directly influence the proliferation of HAoECs in an *in vitro* proliferation assay (Figure 25), absence of endothelial *Cxcr4*-induced effects on EC proliferation in injured vessels of *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice may be regulated by additional factors, which are not present in the *in vitro* proliferation assay.

Mif, an alternative ligand of *Cxcr4*, binds not only *Cxcr4*, but also *Cxcr2*⁵⁸. It has been reported that *Cxcr2* contributes to endothelial recovery triggered by its ligand KC/Cxcl1 and blockade of *Cxcl1* *in vivo* reduced reendothelialization and increased neointima formation after vascular injury³². Furthermore, *Cxcr2* inhibition reduced improvement of endothelial recovery triggered by EPC injection following vascular injury³¹. Therefore, it is possible that the reduced endothelial proliferative effect *in vivo* upon endothelial *Cxcr4* deficiency is influenced by a

reduced Mif/Cxcr4 or Mif/Cxcr2 stimulatory effect on ECs. The latter could be mediated by a direct interaction between Cxcr4 and Cxcr2, or by an indirect interaction by binding a joint interaction partner as *e.g.* Cd74⁵⁸.

Besides the contribution of migration and proliferation of adjacent ECs to reendothelialization of injured endothelium, also bone marrow-derived EPCs play a pivotal role in reendothelialization after blood vessel injury¹⁹⁻²¹. For example, studies have shown that reendothelialization was enhanced by transplantation of EPCs after prosthetic vascular grafting in human^{23, 24} and vessel injury of rabbits²⁴. Moreover, statin therapy promoted the number of circulating EPCs or the homing of EPCs to injured sites in rats, and thereby enhanced reendothelialization²². Also, through physical exercise the number of circulating EPCs was increased, leading to a significant enhancement of endothelial regeneration with decreased neointima formation in mice^{40, 51}. Thus, transplanted or circulating EPCs seem to contribute to endothelial recovery and vascular repair and in that way may reduce the risk on cardiovascular disease^{19, 44}. Conversely, reduced numbers of circulating EPCs were associated with endothelial dysfunction and also higher risk on cardiovascular disease^{43, 44}. In conclusion, an increased level of (circulating) EPCs has been related to an accelerated reendothelialization after vascular injury. Similar results were observed in this study. Although the precise cell surface markers for definition of mouse circulating EPCs are not well defined, several mouse studies used Sca1⁺Flk1⁺ as cell markers in flow cytometric analysis for quantifying circulating EPCs^{40, 41}. In our study, we similarly gated Sca1⁺Flk1⁺ cells, which were found to be also Cd31⁺, for quantifying mouse circulating EPCs. Our results showed that a lower endothelial recovery in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice was associated with a significantly decreased level of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells compared with *Cxcr4*^{EC-WT}*Apoe*^{-/-} control mice after carotid artery injury.

In summary, endothelial Cxcr4 induces reendothelialization by proliferation or migration of ECs and mediating the mobilization of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells, resulting in decreased neointimal hyperplasia.

5.3 Endothelial Cxcl12/Cxcr4 Axis Involved in the Mobilization of Sca1⁺Flk1⁺Cd31⁺ Progenitor Cells

The chemokine receptor Cxcr4 plays a key role in mediating the recruitment of bone marrow-derived progenitor cells to the injured vascular wall¹²¹. In this context, blockade of Cxcr4 signaling reduced the recruitment of EPCs to injured arteries³¹. In mice with injured carotid arteries, *Cxcr4* gene transfer enhanced reendothelialization capacity of EPCs¹²². Moreover, vascular ischemia and injury induced an upregulation of the Cxcr4 ligand Cxcl12, inducing injury-induced mobilization and/or recruitment of progenitor cells through the Cxcl12/Cxcr4 axis^{21,49}.

Although the level of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells was significantly decreased after carotid artery injury in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice compared with *Cxcr4*^{EC-WT}*Apoe*^{-/-} mice, the level of Cxcr4 surface expression on circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells in both groups of mice did not show a significant difference (Figure 32). This result suggested that Bmx was not expressed on the mouse progenitor cells, when the mice were treated with tamoxifen. It also indicated that the reduced level of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells in the peripheral blood had to be caused by an alteration in the attracting chemokine concentration instead of its chemokine receptor Cxcr4 on the progenitors. It has previously been shown Cxcl12 is produced after endothelial injury¹²³. In this study, the significantly decreased endothelial Cxcl12 staining at the injured site in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice compared with controls (Figure 35) may contribute to the reduced progenitor cell mobilization in these mice, although the concentration of Cxcl12 in plasma and bone marrow was not altered (Figure 34A). This may reveal that the expression level of Cxcl12 in the *Cxcr4*-deficient endothelial layer is downregulated, which fits with the previous observation that endothelial Cxcl12 production is stimulated through a Cxcl12/Cxcr4 positive feedback loop¹³². On the other hand, the observed reduction in endothelial Cxcl12

staining may also merely be a consequence of reduced endothelial Cxcr4 availability in *Cxcr4^{EC-KO}Apoe^{-/-}* mice, leaving less possibility for Cxcl12 to bind endothelial Cxcr4. Since Cxcl12 blocking previously resulted in reduced reendothelialization after injury⁴¹, our observed reduced endothelial Cxcl12 staining at the injured site of carotid arteries in *Cxcr4^{EC-KO}Apoe^{-/-}* mice may be causally associated with decreased reendothelialization. Furthermore, recent findings demonstrate that Cxcl12 is involved in the mobilization of EPCs and their recruitment to sites of injury or stress^{41, 111}. Increased plasma level of Cxcl12 after arterial injury in *Apoe^{-/-}* mice was previously shown to be associated with enhanced mobilization of circulating Lin⁻Sca1⁺ progenitor cells¹²³, and blockade of Cxcl12 decreased the number of circulating Sca1⁺Flk1⁺ EPCs and reendothelialization after carotid artery injury⁴¹. Based on all these data, the reduced staining for Cxcl12 on endothelium at the injured site in *Cxcr4^{EC-KO}Apoe^{-/-}* mice may suggest a contribution of luminal Cxcl12 to reduced reendothelialization through reduced recruitment of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells to the injured carotid artery.

However, the molecular mechanism of reduced mobilization of Sca1⁺Flk1⁺Cd31⁺ progenitor cells from the bone marrow into the peripheral blood in injured *Cxcr4^{EC-KO}Apoe^{-/-}* mice still remains unclear, as the Cxcl12 level in plasma and bone marrow was found to be unaltered in *Cxcr4^{EC-KO}Apoe^{-/-}* compared to control mice. In this study, the Cxcl12 level was quantified using an ELISA-based measurement of the Cxcl12- α isoform, whereas the antibody used to stain for endothelial Cxcl12 on histological sections was able to recognize not only the Cxcl12- α isoform but also the Cxcl12- β isoform. It has been reported that infiltration of leukocytes in cerebral ischemic tissue is associated with an upregulation of endothelial Cxcl12- β , but not of the Cxcl12- α isoform¹³³. Therefore, our observation that no differences could be detected in the Cxcl12- α plasma level between *Cxcr4^{EC-KO}Apoe^{-/-}* and *Cxcr4^{EC-WT}Apoe^{-/-}* mice, might indicate that the reduced recruitment of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells in injured *Cxcr4^{EC-KO}Apoe^{-/-}* mice may be induced by a reduced plasma level of the Cxcl12- β

instead of the Cxcl12- α isoform. Alternatively, other local mobilizing factors could possibly directly or indirectly affect the reduced progenitor cell mobilization upon *Cxcr4* deficiency. VEGF, which is an angiogenic mediator produced by ECs, can regulate mobilization and recruitment of EPCs through its receptor VEGFR2⁵⁵. S1P, which is a bioactive lipid also produced in ECs and involved in EC migration and proliferation, can also mediate EPC mobilization¹⁰⁴. In addition, the CXCL12/CXCR4 axis has the ability to mediate the production of VEGF¹³⁴ and S1P^{104, 135}. Therefore, we investigated whether *Cxcr4* deficiency was associated with reduced systemic levels of these mediators, which may then contribute to reduced progenitor cell mobilization in our *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice. However, the plasma levels of Vegf and S1P revealed no differences in both groups of mice after carotid artery injury, and the same result was obtained for the plasma concentration of Flt3-ligand, which plays a role in mobilization of progenitor cells¹³⁶ (Figure 34B-D). Thus, our data indicate that the Cxcl12/Cxcr4 axis did not influence Sca1⁺Flk1⁺Cd31⁺ progenitor cell mobilization through altered plasma levels of these 3 signaling factors in our *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice. Surprisingly, the alternative ligand of Cxcr4, Mif, which can mediate EPCs *in vitro* through Cxcr4⁵⁹, was not altered in plasma until 10 days after vascular injury but was significantly enhanced at later stage in plasma of injured *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice (Figure 34E). That can thus also not explain the decreased mobilization of Sca1⁺Flk1⁺Cd31⁺ progenitors, but could contribute to the increased content of neointimal macrophages in *Cxcr4*^{EC-KO}*Apoe*^{-/-} mice, as already mentioned in chapter 4.1. It is possible that instead of the investigated signaling molecules, other mediators in the plasma, which can also regulate circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cell mobilization from bone marrow to peripheral blood after arterial injury, are influenced by endothelial *Cxcr4* deficiency. One possibility is G-CSF, which can induce hematopoietic progenitor cell mobilization⁵⁶. Also, GM-CSF has the ability to modulate the recruitment of circulating EPCs to ischemic hind limb in mice⁵⁷, but the plasma level of GM-CSF remained below the detection limit in our study.

In conclusion, based on the observed reduction in endothelial Cxcl12 staining,

a positive Cxcl12/Cxcr4 interaction may influence the recruitment of circulating Sca1⁺Flk1⁺Cd31⁺ progenitors to injured sites after arterial vascular injury. However, further studies are required to identify the signaling molecules and underlying mechanisms contributing to reduced mobilization of progenitor cells into the peripheral blood of injured *Cxcr4*^{EC-KO}*ApoE*^{-/-} mice. Furthermore, a similar restenosis study in mice lacking endothelial Cxcl12 could further clarify the role of endothelial Cxcl12 in the mobilization of Sca1⁺Flk1⁺Cd31⁺ progenitors and in their recruitment to sites of vascular injury.

6 Summary

Balloon angioplasty or stent implantation is one of the therapeutic strategies for treatment of atherosclerosis. However, mechanical denudation of the endothelium and the associated formation of a neointimal lesion are major limitations of these techniques. Injury-induced neointimal hyperplasia can cause a re-narrowing of a dilated artery, called restenosis, and may even be associated with thrombosis in case of an unstable neointimal plaque. Reendothelialization plays an important role in protecting against injury-induced neointima formation, and chemokines and their receptors are also involved. The Cxcl12/Cxcr4 axis has been reported to mediate the mobilization of SMC progenitors, driving injury-induced neointimal hyperplasia. This study focused on the role of endothelial Cxcr4 in neointima formation. The effect of an endothelial-specific *Cxcr4* deficiency on neointimal hyperplasia following carotid artery injury was investigated using *Cxcr4*^{EC-KO}*ApoE*^{-/-} mice, in which absence of endothelial Cxcr4 could be induced by tamoxifen treatment.

Endothelial *Cxcr4* deficiency significantly increased neointima formation after wire-induced carotid artery injury in *Cxcr4*^{EC-KO}*ApoE*^{-/-} mice. A higher content of neointimal macrophages was detected in the neointimal lesions, whereas the neointimal SMC and collagen content were decreased. This was associated with a significant reduction in reendothelialization and EC proliferation in injured *Cxcr4*^{EC-KO}*ApoE*^{-/-} carotid arteries compared to *Cxcr4*^{EC-WT}*ApoE*^{-/-} controls. Moreover, in an *in vitro* scratch assay, treatment of human ECs with CXCL12 significantly increased their wound-healing capacity, which could be reversed with the CXCR4 antagonist AMD3100. Furthermore, flow cytometric analysis revealed a reduced mobilization of circulating Sca1⁺Flk1⁺Cd31⁺ and Lin⁻Sca1⁺ progenitors after vascular injury in *Cxcr4*^{EC-KO}*ApoE*^{-/-} mice, although their surface expression of Cxcr4 was not changed. No difference could be detected in plasma concentrations

of Cxcl12, and also plasma levels of Vegf, S1P or Flt3-ligand, which are able to mediate progenitor cell mobilization, were unchanged. However, immunofluorescent staining demonstrated a significant reduction of local endothelial Cxcl12 in injured carotid arteries from *Cxcr4*^{EC-KO}*ApoE*^{-/-} mice.

In summary, Cxcr4 and the Cxcl12/Cxcr4 axis play a protective role in wire injury-induced neointima formation by promoting reendothelialization and mediating EC migration and proliferation to the site of injury, as well as by mediating the mobilization of circulating Sca1⁺Flk1⁺Cd31⁺ progenitor cells (often referred to as circulating EPCs) to the periphery. For understanding the role of endothelial Cxcl12 in mobilization of circulating Sca1⁺Flk1⁺Cd31⁺ progenitors, a similar restenosis study in mice lacking specifically endothelial Cxcl12 is required in future.

7 Zusammenfassung

Ballon-Angioplastie oder Stent-Implantation ist eine der therapeutischen Strategien zur Behandlung der Atherosklerose. Diese Methoden werden allerdings durch die mechanische Denudation und die damit einhergehende Entstehung einer neointimalen Läsion eingeschränkt. Die neointimale Läsion kann eine erneute Verengung des Lumens, die sogenannte Restenose, induzieren, welche letztlich in einem instabilen neointimalen Plaque sogar zur Thrombose führen kann. In der Neointimalbildung spielt die Reendothelialisierung eine wichtige Rolle, wobei auch Chemokine und ihre Rezeptoren involviert sind. Von der Cxcl12/Cxcr4 Achse wurde berichtet, dass sie die Mobilisierung der glatten Muskelvorläuferzellen vermittelt und dabei die Verletzungs-induzierte neointimale Hyperplasie beeinflusst. Der Fokus dieser Studie lag auf der Rolle des endothelialen *Cxcr4* in der Neointimabildung. Der Effekt der Endothel-spezifischen *Cxcr4* Deletion auf die neointimale Hyperplasie nach Karotidenschädigung wurde in *Cxcr4^{EC-KO}Apoe^{-/-}* und *Cxcr4^{EC-WT}Apoe^{-/-}* Mäusen untersucht. Die Defizienz von endothelialem *Cxcr4* konnte durch eine Tamoxifenbehandlung induziert werden.

In *Cxcr4^{EC-KO}Apoe^{-/-}* Mäusen erhöhte der endotheliale *Cxcr4*-Mangel deutlich die Neointimabildung nach von Draht induzierter Verletzung der Halsschlagader. Ein höherer Anteil von neointimalen Makrophagen wurde in den neointimalen Läsionen beobachtet, während der Anteil von neointimalen glatten Muskelzellen und Kollagen verringert wurde. Im Vergleich zu *Cxcr4^{EC-KO}Apoe^{-/-}* Kontrollen konnte eine signifikante Reduktion der Reendothelialisierung und Endothelzellenproliferation in verletzten *Cxcr4^{EC-KO}Apoe^{-/-}* Halsschlagadern beobachtet werden. Die Stimulation von humanen Endothelzellen mit CXCL12 im in vitro Scratch Assay erhöhte signifikant ihre wundheilende Kapazität, während die mit CXCR4-Antagonist AMD3100 behandelten Endothelzellen einen umgekehrten Effekt zeigten. Weiterhin zeigte die Durchflusszytometrieanalyse eine reduzierte Mobilisierung von zirkulierenden Sca1⁺Flk1⁺Cd31⁺ und Lin⁻Sca1⁺

Vorläuferzellen nach Gefäßverletzung in *Cxcr4^{EC-KO}Apoe^{-/-}* Mäusen, obwohl die Oberflächenexpression von Cxcr4 nicht verändert war. Kein Unterschied konnte in den Plasmakonzentrationen von Cxcl12, und auch Vegf, S1P oder Flt3-Ligand detektiert werden, welche die Mobilisierung der Vorläuferzellen vermitteln können. Allerdings konnte per Immunofluoreszenzfärbung eine signifikante Reduktion des lokalen luminalen endothelialen Cxcl12 in verletzten Halsschlagadern von *Cxcr4^{EC-KO}Apoe^{-/-}* Mäusen gezeigt werden.

Zusammenfassend, spielen Cxcr4 und die Cxcl12/Cxcr4 Achse eine schützende Rolle in der Drahtverletzungs-induzierten Neointimabildung durch die Beförderung der Reendothelialisierung und der EC-Migration und Proliferation an der verletzten Stelle, als auch durch Rekrutierung von zirkulierenden Sca1⁺Flk1⁺Cd31⁺ Vorläuferzellen, die oft als zirkulierende EPCs bezeichnet werden. Zum Verständnis der Rolle des endothelialen Cxcl12 in der Mobilisierung der zirkulierenden Sca1⁺Flk1⁺Cd31⁺ Vorläuferzellen, ist in Zukunft eine ähnliche Restenose-Studie mit Mäusen, die eine Endothel-spezifische Deletion von Cxcl12 haben, nötig.

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