

Neutrophil-derived cathelicidin in arterial inflammation

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"If you think research is expensive, try disease."

Mary Lasker, 1901-1994

To my family

Abstract

Neutrophils are the first immune cells recruited into tissues during inflammatory processes. They orchestrate the progression of inflammation through the release of preformed granule proteins such as cathelicidin, azurocidin and α -defensin. These proteins are well known for their antimicrobial activities. Cathelicidins have been intensively studied for their cytotoxic, chemotactic, regenerative and angiogenic properties. However, their contribution to atherosclerosis and neointima formation is not yet completely understood. Therefore, the aim of this thesis was to identify molecular mechanisms of cathelicidin-mediated vascular inflammation and a possible therapeutic strategy.

Using a mouse model of arterial injury, we were able to show that cathelicidins, which are released from neutrophils, exert a healing effect. Mechanistically, cathelicidins promote adhesion of endothelial early outgrowth cells (EOCs) thus improve re-endothelialization of the injured vascular wall. In addition, cathelicidins can activate EOCs, leading to the release of growth factors, enhancing the regeneration process of vascular endothelium. In a therapeutic-oriented approach, cathelicidins were biofunctionalized by covalently binding to a nitinol stent surface. This cathelicidin-constructed stent reveals protective properties against in-stent stenosis after implantation into the carotid artery of mice.

In the later studies we could show that adhesion of classical monocyte depends on the presence of neutrophils. Neutrophils mediate classical monocyte adhesion through the release of cathelicidins into tissues which are subsequently transported across endothelium and lumenally presented. Endothelial-bound cathelicidins are presented to classical monocytes via formyl peptide receptor 2 (FPR2). Cathelicidin-FPR2 interaction generates intracellular signaling cascades leading to activation of integrins, thus promoting adhesion of monocytes onto endothelium. This mechanism is preferential to classical monocytes. In addition, we further corroborate the significance of neutrophil-mediated monocyte adhesion through the studies of atherosclerosis in a mouse model. Cathelicidin-deficient mice exhibited reduced atherosclerotic lesions, characterized by lower lesional macrophage content and decreased arterial adhesion of classical monocytes.

In summary, we show here for the first time, the role of cathelicidins in neointima formation and atherosclerosis which could be targeted for clinical therapeutic approach.

List of original publications

The results of this thesis were partially based on the following publications:

Soehnlein O*, **Wantha S***, Simsekylmaz S, Döring Y, Megens RT, Mause SF, Drechsler M, Smeets R, Weinandy S, Schreiber F, Gries T, Jockenhoevel S, Möller M, Vijayan S, van Zandvoort MA, Agerberth B, Pham CT, Gallo RL, Hackeng TM, Liehn EA, Zerneck A, Klee D, Weber C. (2011) Neutrophil-derived cathelicidin protects from neointimal hyperplasia. *Sci Transl Med.* **3**:103ra98.

Döring Y*, Drechsler M*, **Wantha S**, Kemmerich K, Lievens D, Vijayan S, Gallo RL, Weber C, Soehnlein O. (2012) Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res.* **110**:1052-6.

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Soehnlein O designed the study, performed intravital microscopy, analyzed the data, wrote the manuscript, and provided funding. Wantha S performed all *in vitro* experiments unless stated otherwise, analyzed the data, and partially prepared the manuscript. Simseyilmaz S acquired stent data. Döring Y performed *in vitro* experiments. Megens RT performed two-photon microscopy. Mause SF performed enzyme-linked immunosorbent assay measurements. Drechsler M performed wire injury experiments. Jockenhoevel S, Smeets R & Weinandy S contributed to acquisition of stent data. Möller M, Schreiber F & Gries T braided stents. Vijayan S contributed *in vitro* data. Van Zandvoort MA supervised two-photon microscopy. Agerberth B provided reagents. Pham CT & Gallo RL provided mouse strains. Hackeng TM synthesized fluorescent LL-37. Liehn EA performed wire injuries and stent implantations, and acquired and analyzed stent data. Zernecke A developed and performed stent implantation. Klee D supervised stent development. Weber C designed the study, wrote the manuscript, and provided funding.

Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice.

Döring Y performed animal experiments, analyzed the data and wrote the manuscript. Drechsler M performed animal experiments. Wantha S performed *in vitro* experiments and data analysis. Kemmerich K performed *in vivo* imaging. Lievens D stained atherosclerotic lesions. Vijayan S contributed to *in vitro* experiment. Gallo RL provided Cramp^{-/-} mice. Weber C provided consumables and contributed to manuscript preparation. Soehnlein O performed intravital microscopy, analyzed the data, wrote the manuscript, and provided funding.

Neutrophil-derived cathelicidin promotes adhesion of classical monocytes.

Wantha S performed all *in vitro* experiments unless stated otherwise, two-photon imaging, analyzed the data, and partially prepared the manuscript. Alard JE performed surface plasmon resonance experiments, and designed mLL-37. Megens RT performed two-photon imaging and image processing. Van der Does AM contributed to *in vitro* experiment. Döring Y performed animal experiments. Drechsler M performed *in vivo* experiments. Pham CT & Gallo RL provided mouse strains. Wang MW provided Quin C7. Wang JM provided FPR2^{-/-} mice. Von Hundelshausen P contributed to *in vitro* data analysis. Lindbom L provided critical data discussion. Hackeng TM synthesized mLL-37, Weber C provided consumables and contributed to manuscript preparation. Soehnlein O designed the study, performed intravital microscopy, analyzed the data, wrote the manuscript, and provided funding.

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List of abbreviations

ADP	adenosine diphosphate
AMP	anti-microbial peptide
APC	antigen-presenting cells
ApoE	apolipoprotein E
ATP	adenosine triphosphate
CAMP	cathelicidin antimicrobial peptide
CAP37	cationic antimicrobial protein 37
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CXCL	CXC-chemokine ligand
CXCR	CXC-chemokine receptor
CX3CL	CX3-chemokine ligand
CX3CR	CX3-chemokine receptor
CRAMP	cathelicidin-related antimicrobial peptide
COX	cyclooxygenase
DAMP	damage-associated molecular pattern
DC	dendritic cell
EC	endothelial cell
ECM	extracellular matrix
EOC	endothelial early outgrowth cell
EPC	endothelial progenitor cell
fMLP	N-formylmethionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
GAG	glycosaminoglycan
HBP	heparin-binding protein
hCAP-18	human cathelicidin protein-18
HDL	high density lipoprotein
HNP	human neutrophil peptide
HSP	heat-shock protein
ICAM	intercellular adhesion molecule
IFN- γ	interferon gamma
IL	interleukin
JAM	junctional adhesion molecule

LAMP	lysosome-associated membrane protein
LDL	low density lipoprotein
LDLR	LDL receptor
LOX	lectin-like oxidized LDL receptor
LPS	lipopolysaccharide
<i>LTB4</i>	<i>leukotriene B4</i>
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinases
MCP	monocyte chemotactic protein
M-CSF	macrophage colony stimulating factor
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NET	neutrophil extracellular trap
NF- κ B	nuclear factor- κ B
NO	nitric oxide
oxLDL	oxidized LDL
PAMP	pathogen-associated molecular pattern
PEG	polyethyleneglycol
PKC	protein kinase C
PMA	phorbol myristate acetate
PRR	pattern-recognition receptor
PSGL	P-selectin glycoprotein ligand
ROS	reactive oxygen species
SMC	smooth muscle cell
SR	scavenger receptor
TIMP	tissue inhibitors of metalloprotease
TF	tissue factor
TLR	toll-like receptor
TNF- α	tumor-necrosis factor - alpha
VCAM	vascular cell adhesion molecule
VE	vascular endothelial
VLA	very late antigen

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Introduction

Inflammation

Inflammation can be defined as complex cascades of humoral and cellular events in response to tissue injury. Once tissue is injured, an immediate reaction to trauma is triggered and propagated to prevent and terminate the spreading of infection or damage as quickly as possible [Nathan, 2002]. The presence of inflammatory mediators in the body activates and recruits multiple cell types of the immune system that express a wide range of receptors on their surfaces. The initiation of inflammation involves several interconnecting mechanisms through the engagement of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS or endotoxin) and peptidoglycan, or damage-associated molecular patterns (DAMPs), to pattern recognition receptors (PRRs) present on many defense cells [Theoharides *et al.*, 2012]. Most cell types involved in inflammation have receptors for cytokines which are abundantly released at sites of inflammation. Binding of cytokine to their cognate receptor initiates gene transcription via NF- κ B pathway, thus amplifying the inflammatory process by progressive recruitment and activation of leukocytes to sequentially secrete and produce their own inflammatory mediators.

Leukocytes play a critical role in inflammation, controlling a balance of pro-inflammatory and anti-inflammatory signals through phagocytosis and release of various inflammatory mediators becoming visible as the five cardinal signs: rubor (redness), calor (heat), dolor (pain), tumor (swelling) and loss of function (function laesa). Acute inflammation is traditionally described as the influx of neutrophils followed by monocyte-derived macrophages. Activated phagocytes produce reactive oxygen species (ROS) which are not only toxic for microorganisms but can also lead to tissue damage [Björkman *et al.*, 2008]. Monocytes, macrophages, and activated platelets are sources of cytokine interleukin-1 (IL-1) that exhibits paracrine and endocrine effects on the release of prostaglandins and tissue factor (TF) to initiate the blood clotting cascade as well as the synthesis and secretion of other interleukins. The acute inflammatory response to tissue damage is often followed by temporally released pro-resolution factors that switch off inflammation.

Atherosclerosis

Atherosclerosis, a multifactorial chronic inflammation of arteries, is the primary cause of heart disease and stroke, which have increasingly become a threat to human health worldwide [Basso *et al.*, 1999]. Numerous atherosclerotic risk factors have been suggested, most of genetic or environmental sources. These factors include elevated levels of low density lipoprotein, increased blood pressure, diabetes, obesity, gender, systemic inflammation, metabolic syndrome, high fat diet, smoking, lack of exercise and infection [Fruchart *et al.*, 2004]. Atherosclerotic lesion results from the accumulation of fatty materials at the innermost layer of the artery wall. The normal arterial structure consists of three morphologically distinct layers (**Figure 1**) that make up the vessel wall; the intima, the very thin innermost layer, is composed of extracellular connective tissue matrix, primarily proteoglycans and collagen, covered by a monolayer of vascular endothelial cells resting on the luminal side and a sheet of elastic fibers, the internal elastic lamina, separating the intima from the media on the abluminal side. The media, the middle layer, consists of vascular smooth muscle cells (SMCs). An external elastic lamina, connective tissues with interspersed SMCs and fibroblasts, separates the media from the adventitia, the outermost layer, which consists mostly of fibroblasts, mast cells, micro vessels, lymphatic vessels and nerves [Lusis, 2000].

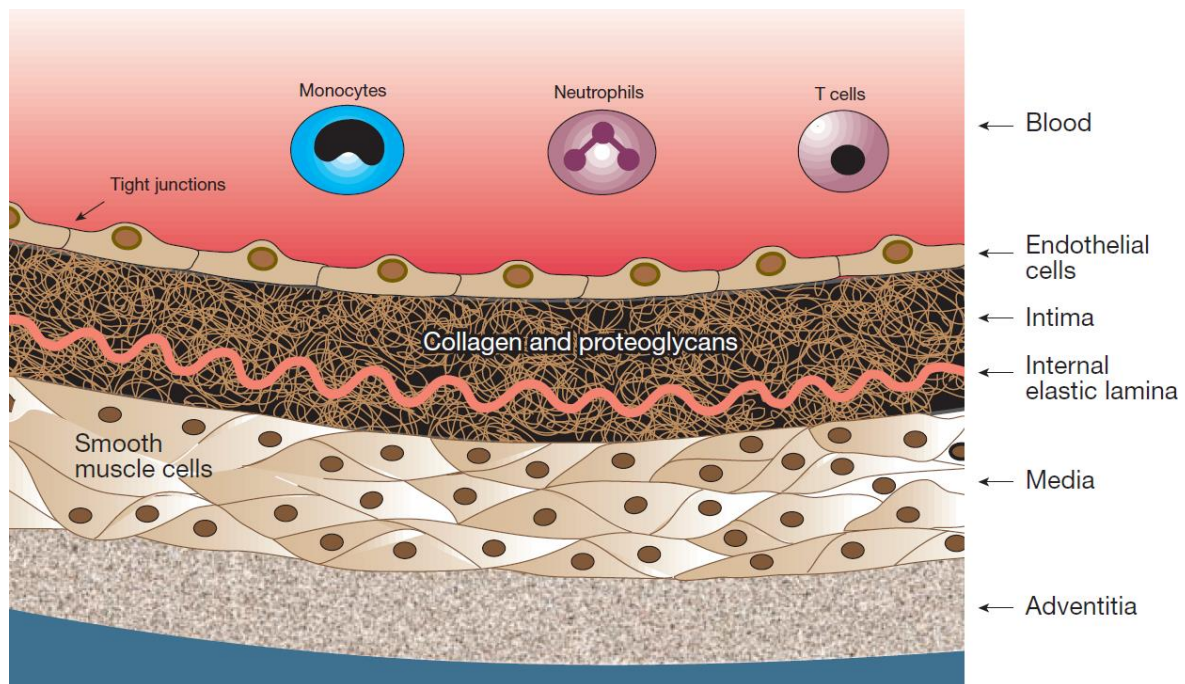


Figure 1. The structure of a normal large artery. The diagram shows a cross-section through a muscular artery depicting a classic trilaminar structure (Lusis, 2000).

Various stages of atherosclerosis

The atherosclerotic plaque is formed within the intima and inner media of large and medium sized arteries, consisting of a modified cholesterol core and debris from dead cells, surrounded by a fibrous cap containing smooth muscle cells and collagen matrix in the arterial intima covered by endothelial cells. Several activated immune cells are present in a plaque, including macrophages, T cells and mast cells. Atherosclerotic lesion formation (**Figure 2**) entails various interconnected complex factors [Faxon *et al.*, 2004], including the dysfunction of endothelial cells, accumulation of lipid droplets in the intima, inflammatory cell recruitment and tissue infiltration, oxidative stress, inflammatory mediators, modification of lipoproteins, foam cell formation, vascular smooth cell proliferation, matrix secretion and degradation, vascular remodeling, cell death and thrombosis.

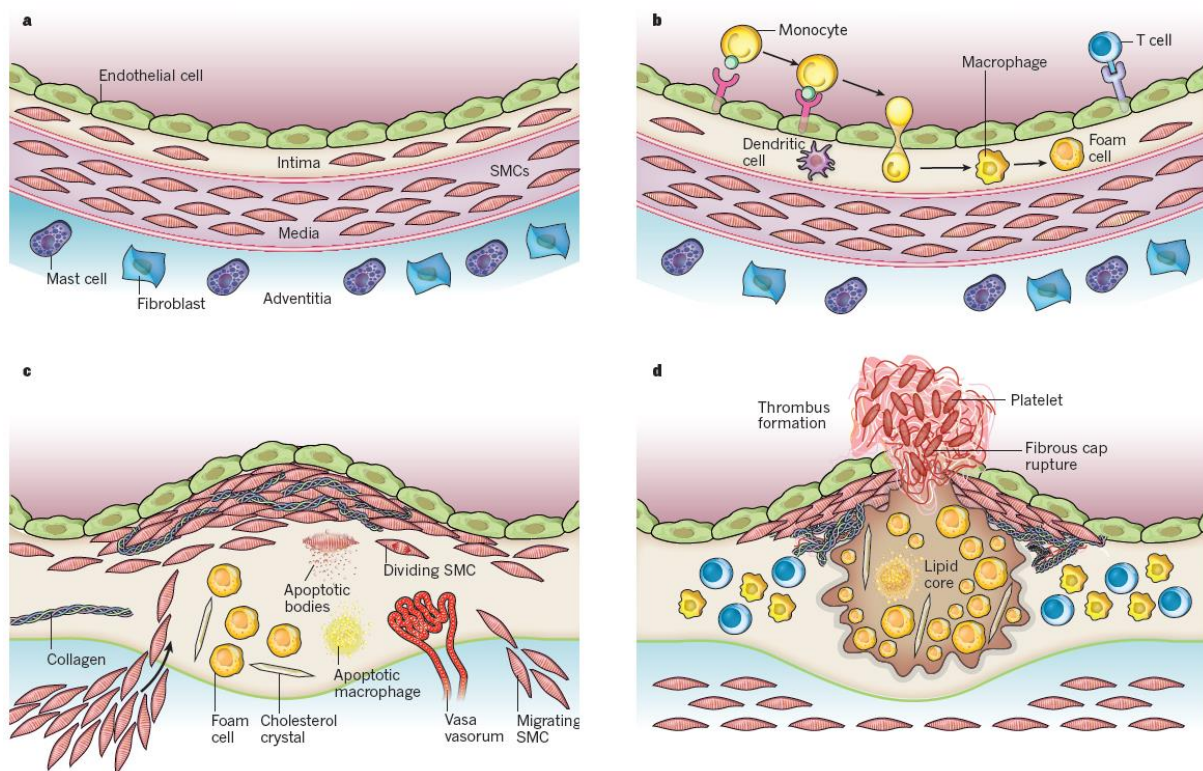


Figure 2. The artery and the cellular changes during the progression of atherosclerosis. *a)* The normal artery contains three layers. *b)* The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes into macrophages, and their uptake of lipid, yielding foam cells. *c)* Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. *d)* Disruption of the atherosclerotic plaque and thrombosis (Libby, Ridker & Hansson, 2011).

On the basis of animal experiments and observations in human specimens, the stages of atherosclerosis can be defined as: Atherogenesis; Plaque progression; Plaque destabilization and Plaque rupture [Libby *et al.*, 2009]. The initial step of atherogenesis involves dysfunctional changes in the endothelial monolayer which leads to up-regulation of adhesion molecules that promote adhesion of leukocytes and increases in endothelial permeability [Deanfield *et al.*, 2005] that allows the entry and retention of low density lipoprotein (LDL) particles in the intima. Oxidatively modified LDLs induce monocyte infiltration and differentiate into macrophages which take up lipid droplets transforming to foam cells, leading to subendothelial cholesterol accumulation. An increase in the number of accumulated foam cells, dead cells and debris accounts for progression of atherosclerosis. Highly proliferative smooth muscle cells migrate into intima layer and synthesize numerous matrix components which form fibrous structures around the lesion. Plaque macrophages and smooth muscle cells undergo apoptosis and contribute to advanced lesions. Large amounts of intracellular cholesterol and dead cells deposited at the central region form a necrotic lipid core. The inflammatory milieu of advanced lesions may induce the secretion of matrix proteases by macrophages and promote the death of matrix-producing smooth muscle cells, both of which accelerate fibrous cap thinning and plaques become unstable leading to vulnerable plaque rupture. At the site of plaque disruption, circulating platelets are exposed to thrombogenic materials, which can lead to acute thrombosis, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow [Libby *et al.*, 2011].

Atherogenesis

Vascular endothelial cells are the first cells reacting to changes occurring in the blood homeostasis. Systemic inflammation, hyperglycemia, changes in shear stress, and hypercholesterolemia all result in endothelial dysfunction thus up-regulating the expression of various adhesion molecules [Sima *et al.*, 2009]. Endothelial cells can also produce many inflammatory mediators which further direct monocyte recruitment through the engagement of endothelial adhesion molecules with integrins expressed on the cell surface of monocytes [Szmitko *et al.*, 2003]. Upon interaction with cell adhesion molecules expressed on the endothelium lining, monocytes undergo capture, rolling, adhesion, and firm arrest and ultimately extravasation towards the arterial intima. Endothelium and its intercellular tight junctions play a central role as a selectively permeable barrier between blood and tissues mediating leukocyte transmigration and the accumulation of oxidized LDL in the intima.

Blood shear stress affects greatly the endothelial cell morphology which shows increased permeability to macromolecules such as LDL when under disturbed flow e.g. in regions of arterial branching [Gimbrone, 1999].

LDL diffuses passively from the blood flow into the intima layer of the arterial wall through endothelial junctions, where the LDL constituent apolipoprotein B (ApoB) associate with proteoglycans of the extracellular matrix (ECM) and are subsequently modified by reactive oxygen radicals and enzymes transforming LDL into oxidized LDL (oxLDL). Native LDL is not taken up rapidly by macrophages unless it does undergo a most significant oxidation, lipolysis, proteolysis and aggregation and such modifications contribute to inflammation. Accumulation of oxLDL in the intima is raised when the level of circulating LDL is elevated and thus is the preferential site for lesion formation. Vascular endothelial cells express leukocyte adhesion molecules VCAM-1, ICAM-1, P-selectin (CD62P) and E-selectin (CD62E) as part of the initial response to oxLDL accumulation in the intima. They also synthesize additional monocyte chemotactic protein 1 (MCP-1) and interleukin-8 (IL-8) which are chemoattractants for monocytes.

The clusters of expressed adhesion molecules on endothelium are correlated with blood flow and shear stress exerted on endothelial cell surface and this usually indicates the site where fatty streaks are formed [Malek *et al.*, 1999]. Experiments using genetically modified mice show that recruitment and adhesion of leukocytes to endothelium are involved in the initiation of atherosclerosis [Eriksson *et al.*, 2001]. In addition, several chemokines produced by vascular cells are suggested to be participating in the recruitment of leukocytes. Lack of CC-chemokine ligand 2 (CCL2; also known as MCP-1) and its receptor, CC-chemokine receptor 2 (CCR2) inhibits monocyte extravasation into the arterial intima and attenuates atherogenesis [Hristov & Weber, 2011]. Absence of CCL5 (also known as RANTES), produced by macrophages and vascular cells, also limits atherosclerosis in mice [Braunersreuther *et al.*, 2010]. Interestingly, humans and mice express the same cell-surface anchored CX3-chemokine ligand 1 (CX3CL1; also known as fractalkine), which is a transmembrane protein preferentially expressed by vascular smooth muscle cells, in atherosclerotic plaques.

Monocytes and macrophages express CX3-chemokine receptor 1 (CX3CR1) that can engage CX3CL1, which stimulates their migration to the arterial intima and contributes to atherogenesis, as indicated by studies using both *ApoE*^{-/-} and *Cx3cr1*^{-/-} mice [Combadière *et*

al., 2003]. Within the intima, monocytes undergo differentiation into macrophages in response to M-CSF and other stimuli, leading to the expression of different classes of pattern-recognition receptors in order to mediate internalization and lysosomal degradation of PAMPs as well as DAMPs, which are associated with modified lipoprotein particles, lipopolysaccharide, fragments of microbes, pathogens, cellular stress or cell components released during cell damage and apoptotic bodies. These receptors include scavenger receptors and toll-like receptors (TLRs). Intimal macrophages take up oxLDL via the binding of scavenger receptors which gives rise to an accumulation of intracellular cholesterol-laden lipoproteins in the cytosol forming foam cells. LDL must be highly oxidized in order to be taken up sufficiently rapidly by macrophages to form foam cells. Myeloperoxidase (MPO), most abundantly expressed in neutrophil granulocytes [Malle *et al.*, 2007], extensively generates reactive oxygen species such as hypochlorous acid and tyrosyl to modify LDL to highly oxidized LDL which can bind to macrophage scavenger receptors.

Foam cell formation is mediated by scavenger receptors, including CD36, CD68, CXCL16, lectin-like oxidized LDL receptor 1 (LOX1), scavenger receptors (SR-A and SR-B1). The expression of macrophage scavenger receptors is controlled by cytokines such as TNF- α and interferon- γ (IFN- γ). One would anticipate scavenger receptors to play a crucial role in atherosclerotic plaque formation as a direct consequence of foam cell development. However, mice deficient in CD36, CXCL16 or SR-A exhibit increased atherosclerosis [Moore *et al.*, 2005]. This might be because the clearance of modified lipoproteins could not take place when foam cells are absent, therefore the removal of such lipids from plaques would be much less efficient and hence the accumulation of extracellular cholesterol might be even higher than the presence of foam cells.

The clearance of modified lipoprotein is regulated by high density lipoprotein (HDL). Macrophage actively secretes apoE which may facilitate the influx of HDL and subsequently the removal of this excess LDL by HDL, thereby limiting the uptake of oxidized LDL by macrophage as well as the transformation into foam cells. An important mechanism underlying the protective role of HDL by inhibiting lipoprotein oxidation is due to antioxidant protein serum paraoxonase on HDL, which can degrade biologically active oxidized phospholipids [Shih *et al.*, 2000].

Plaque progression

Endothelial cells contain both executive and sensory properties which can generate effector molecules to regulate vascular inflammation. Dysfunction or damage of the endothelium stimulates proliferation and migration of smooth muscle cells to the intima which begin to synthesize extracellular matrix macromolecules accumulating at the site where fatty streaks are found and they also develop an occlusive fibrous cap from this matrix secretion. The atherosclerotic plaque is mainly composed of cellular elements and extracellular matrix which constitute more than half of the content of the lesion. The constituents of the plaque's extracellular matrix include glycosaminoglycans, proteoglycans, collagen, elastin, laminin, fibronectin, vitronectin and thrombospondin. The vascular smooth muscle cells play a key role in atherosclerotic plaque development as they are largely responsible for the production of these matrix proteins providing the structural support and scaffold for the atherosclerotic plaque [Raines, 2000].

Initially, the lesions grow towards the adventitia, the area known as intimal thickening, until they reach the critical point where they start to expand towards the lumen. The plaques continue to develop and increase in size by intimal smooth muscle cell proliferation and extracellular matrix enlargement as well as the accumulation of macrophage foam cells. Macrophage-derived-foam cells secrete cytokines, growth factors, tissue factor, IFN- γ , matrix metalloproteinases (MMPs) and ROS which govern the plaque progression by further promoting smooth muscle cell proliferation and migration from the media into the intimal lesion, and in this way contribute to the progression of atherosclerosis.

Smooth muscle cells start to up-regulate the expression of adhesion molecules much later; several weeks after endothelial cells. This expression enhances the recruitment and retention of leukocytes in the arterial intima. Circulating T cells, mostly CD4⁺ and some CD8⁺, are recruited into the atherosclerotic lesions in response to chemokines and cytokines which bind to the cell-specific receptors and undergo activation. Upon recognition of an antigen presented on antigen-presenting cells (APCs) such as macrophages or dendritic cells, T cells differentiate into T helper lymphocytes (Th1), amplifying the inflammatory response accelerating atherosclerosis. Dendritic cells are specialized antigen-presenting cells, present from the early stages till the advanced plaque development, particularly in the rupture-prone shoulder region [Bobryshev, 2005].

Plaque destabilization

Advanced plaques contain extracellular lipid and cholesterol crystals derived from dead foam cells accumulated in the central region of a plaque, often denoted the necrotic core. Macrophages are found abundantly in advanced plaque and contribute greatly to the stability of a plaque as they are necessary for removal of apoptotic cell debris. However, macrophages release several MMPs in the plaque that lead to progressive collagen breakdown and thinning of the fibrous cap. They also produce pro-inflammatory cytokines with pro-apoptotic potential that may contribute to apoptosis of surrounding cells, especially smooth muscle cells that are responsible for matrix production. Accordingly, stable atherosclerotic lesions slowly progress over a period of time to advanced and unstable plaques which are characterized by a thin fibrous cap containing few smooth muscle cells overlying a large necrotic core of lipid and cellular debris. In contrast to stable plaques, advanced atherosclerotic plaques also show neovascularization. These newly formed vessels are fragile and susceptible to leakage, thereby introducing platelets and red blood cells into the plaque [Kolodgie *et al.*, 2003].

Macrophages are the main source of TLRs in plaques [Xu *et al.*, 2001; Edfeldt *et al.*, 2002] which directly activate inflammatory responses. The ligation of macrophage TLRs by oxLDL facilitates the production of various pro-inflammatory cytokines through activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein (MAP) kinase–activator protein 1 signaling pathways, which can further promote recruitment of other cells into atherosclerotic lesion. The downstream response of TLR signaling also involves the secretion of matrix-degrading MMPs that play crucial role in destabilization of vulnerable plaques by promoting matrix destruction, angiogenesis, leucocyte infiltration, and apoptosis. With time, macrophage-derived foam cells die, contributing their lipid contents to enhanced formation of necrotic core. Increase accumulation of free cholesterol can induce macrophage death via caspase signal transduction and activation of endoplasmic reticulum stress pathway. Apoptosis of macrophages lead to secondary necrosis, which in turn promotes plaque destabilization [Tabas, 2004].

Recent studies have shown that interaction of T-lymphocyte surface molecule CD40 ligand (CD40L) regulates the expression of MMPs in human vascular smooth muscle cells which make an important contribution to the advanced plaque destabilization [Lievens *et al.*, 2010; Lutgens *et al.*, 2010]. CD40 is expressed mainly on T and B lymphocytes but also expressed on macrophages, endothelial cells and smooth muscle cells.

Plaque rupture

Thrombosis, the ultimate complication of atherosclerotic plaque rupture, is the most frequent clinical incidence of atherosclerosis caused by disruption of the fibrous cap that covers the lipid core of the plaque which has enabled blood to come into contact with plaque components, including tissue factor, a potent procoagulant expressed by a subpopulation of macrophages present in the plaque [Suades *et al.*, 2012].

Vulnerable plaques are generally characterized by a very thin fibrous cap which is extremely fragile and prone to erosion. The precise plaque rupture mechanism is not well identified, but it includes the thinning of fibrous cap by the accumulation of inflammatory cytokines and proteases that facilitate degradation of the matrix, decreased smooth muscle cell collagen secretion and the elevation of dead cells within the necrotic lipid core. All the cells that are involved in the development of the atherosclerotic plaque are also elicited in the plaque rupture [Simionescu & Sima, 2012].

Plaque disruption frequently appears at the edge where large quantities of foam cells are found. Interstitial collagen fibers, mainly of types I and III, which are the main macromolecular structure in the arterial wall, are produced from vascular smooth muscle cells to stabilize the fibrous cap of the plaque [Rekhter, 1999]. The cytokine IFN- γ strongly inhibits the production of this extracellular matrix collagens and further limits smooth muscle cell proliferation.

In addition, activated macrophages secrete a high amount of proteases that include the interstitial collagenases of the MMP family which can degrade matrix collagen. Thereby the plaque composition becomes de-stabilized and susceptible to rupture when subjected to the forces of arterial blood pressure. Exposure of vulnerable plaque structures and procoagulants such as tissue factor promotes the formation of a thrombus, adherent platelet aggregation and fibrin crosslinks which may lead to arterial thrombotic occlusion thus subsequently causing ischemia results in acute myocardial infarction and strokes.

Neointimal Hyperplasia

Progressively slow formation of atherosclerotic lesion leads to stenosis, a narrowing of blood vessels, that limits blood flow thereby causing many symptoms associated with arterial ischemia. Vascular stenosis, arbitrarily defined as the narrowing of the vessel diameter to more than half of its normal healthy lumen [Marx *et al.*, 2011], is a major clinical complication that has an impact on various cardiovascular disciplines, including cardiology, transplant medicine, cardiothoracic and vascular surgery. Current therapeutic approaches to restore blood flow in stenotic vessels involve the use of percutaneous transluminal interventions and bypass surgery. These have advanced the ability to relieve stenoses and reduce ischemia. During balloon angioplasty and stent placement (**Figure 3**) intervention [Maisel & Laskey, 2007], endothelial denudation and arterial wall fracture are the initial outcomes of mechanical-induced injury which consequently activates the wound healing process in an attempt to contain the damage of injured tissues. However, there is an increased incidence of the re-growth of intimal tissue into a stent, known as re-stenosis which limits the success of the procedures in the long term [Fischer *et al.*, 2012].

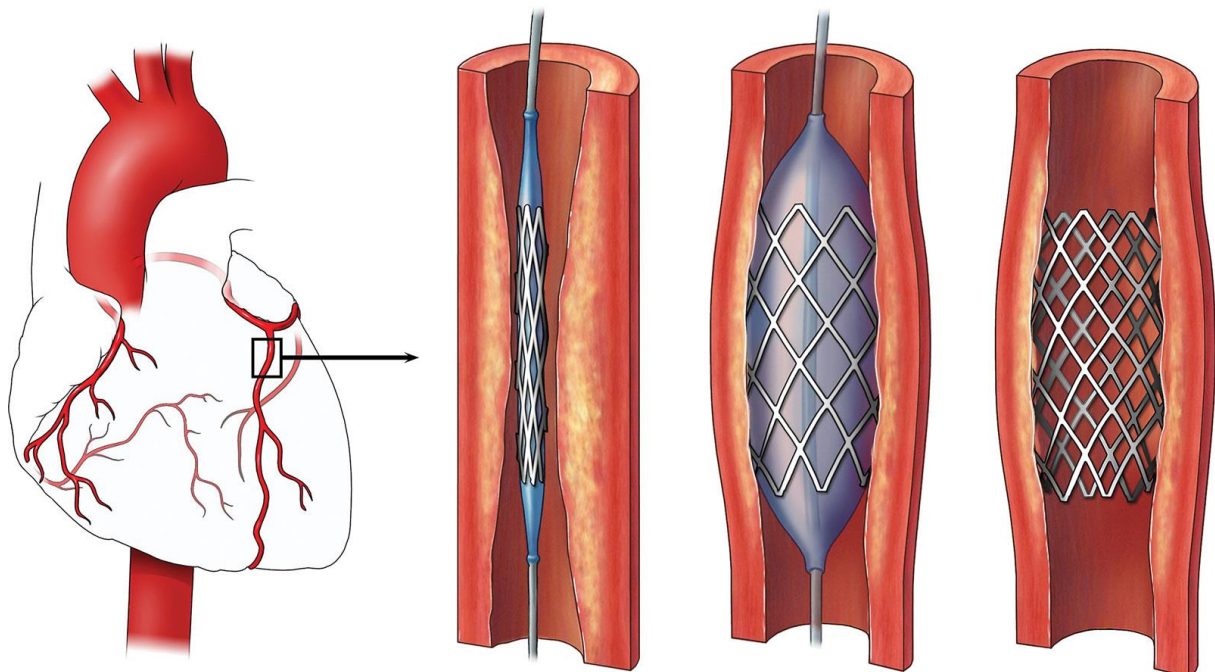


Figure 3. Balloon angioplasty with stent insertion. The insertion of a stent into a coronary artery where a narrowed portion of the vessel has occurred. A stent mounted on a balloon is inserted through a catheter, typically via a groin artery. The balloon is inflated, expanding the stent, and then is removed, leaving the expanded stent embedded in the artery and reestablishing the flow of blood to the heart (Nucleus Medical Art/Getty Images: Encyclopædia Britannica Inc).

Restenosis following percutaneous intervention is characterized by the vascular cells response to injury in which they secrete numerous growth factors modulating infiltration of inflammatory cells, proliferation of medial smooth muscle cell and migration into the intima, and extracellular matrix remodeling as well as platelet aggregation. Arterial re-occlusion often occurs at the site of injured tissues within months after the intervention [Visonà *et al.*, 2009]. This is usually caused by highly proliferative vascular smooth muscle cells migrating into the intima in response to arterial injury which is known as neointimal hyperplasia (in-stent stenosis) [Shah, 2003]. The incidence of re-stenosis has been minimized by the use of stents coated with immunosuppressive substances, such as sirolimus (rapamycin) and cyclosporine which has shown striking effectiveness at reducing in-stent intimal cell proliferation stenosis [McKeage *et al.*, 2003]. However, the occurrence of late stent thrombosis (>6 months) have increased within several months after stent implantation due to vigorous immunosuppressive agents coated on drug-eluting stent causing endothelial dysfunction with the delayed endothelialization of the vascular wall [McFadden *et al.*, 2004].

Re-endothelialization

Endothelium has a key function in the suppression of neointimal hyperplasia through the inhibition of inflammation, thrombus formation and smooth muscle proliferation and migration [Wu *et al.*, 2011]. An injury of the endothelial layer triggers the wound-healing process which is critically involving both proliferation and migration of smooth muscle cells and local endothelial repair. Re-endothelialization at the site of vascular injury has classically been considered to result from the migration and proliferation of adjacent viable endothelial cells. However, the mature resident vascular endothelial cells may not exclusively constitute the whole basis for endothelial repair as they have very limited proliferative capacity [Fang *et al.*, 2012] thus endothelial remodeling process depends pretty much on circulating bone marrow-derived cells that exhibit phenotypic features of endothelial cells [Shi *et al.*, 1998] and are capable to be recruited to sites of endothelial damage and finally incorporating into nascent endothelium, termed endothelial early outgrowth cells (EOCs). Adult hematopoietic progenitor cells can differentiate into an endothelial phenotype that displayed both hematopoietic stem cell markers such as CD34⁺ and an endothelial marker protein such as VEGFR2 [Asahara *et al.*, 1997], that can contribute to improvement of re-endothelialization and neovascularization. Nevertheless, the identification and the origin of endothelial progenitor cells are still controversial as the isolated peripheral blood mononuclear cells can turn into EOCs by cultivation in medium favoring endothelial differentiation.

Several sources for EOCs in peripheral blood exist: (1) the rare number of hematopoietic stem cells, (2) myeloid cells, which may differentiate to endothelial cells under the cultivation selection pressure, (3) other circulating progenitor cells and (4) circulating mature endothelial cells, which are shed off the vessel wall [Urbich & Dimmeler, 2004]. However, circulating EOCs may exert an important function as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer by replacing damaged parts of the endothelium regardless of their origin. By accelerating reendothelialization, the transfusion of EOCs to site of arterial damage enhanced endothelial repair associated with a reduction of neointima formation [Werner *et al.*, 2003].

EOCs contribute to reendothelialization and neovascularization; as a consequence, increasing the number of these cells may be an attractive therapeutic tool. The mobilization of EOCs in the bone marrow is determined by the local microenvironment, the so-called stem cell niche which consists of fibroblasts, osteoblasts, and endothelial cells. Basically, activation of proteinases such as elastase, cathepsin G, and matrix metalloproteinases cleaves adhesive bonds on stromal cell niche and finally allow EOCs to leave the bone marrow via transendothelial migration [Papayannopoulou, 2004]. Physiologically, ischemia is regarded to be the predominant signal to induce mobilization of EOCs from the bone marrow via the up-regulation of VEGF or SDF-1 which in turn activates the cleavage of EOCs via a MMP9-dependent mechanism [Pillarisetti & Gupta, 2001]. Furthermore, granulocyte colony stimulating factor (G-CSF), a cytokine which is typically used for mobilization of CD34⁺ cells also increased the levels of circulating endothelial progenitor cells as well as a related cytokine, the granulocyte monocyte-colony stimulating factor (GM-CSF) and erythropoietin (EPO), which stimulates proliferation and maturation of erythroid precursors, also increases peripheral blood endothelial progenitor cells [Bahlmann *et al.*, 2004; Heeschen *et al.*, 2003].

The final vascular repair and remodeling depend solely on the maturation of EOCs to functional endothelial cells integration in the vascular wall. The mechanisms that regulate differentiation in the adult endothelial cells are mostly unknown. Clearly, VEGF and its receptors play a critical role for stimulating EOCs into endothelial cells. Recently, the Genous stent technology enhanced endothelialization by capturing CD34 positive endothelial progenitor cells with antibodies immobilized on the surface of sirolimus-eluting stents [Nakazawa *et al.*, 2010]: the endothelial function remains impaired [Rossi *et al.*, 2009], and its long term fate has to be determined

Neutrophils in atherosclerosis

The recent paradigm of atherosclerosis concentrates on the infiltration of inflammatory cells such as macrophages and T cells into arterial intima corroborating plaque formation and disruption. By contrast, the most abundant leukocytes in the circulation, the neutrophils, have rarely been appreciated for their role associated with the plaque development due to their rare detection in atherosclerotic lesions [Soehnlein, 2012]. Neutrophils, whose role in acute inflammation as the first line of immune response is well established, were for a long time considered of marginal relevance in atherosclerosis. Recently, however, convincing evidence for the presence of neutrophils in atherosclerotic plaques has been provided [Simionescu & Sima, 2012; Soehnlein, 2012; Rotzius *et al.*, 2010] pointing at a significant contribution of neutrophils during multiple steps of atherosclerosis development. Short lived neutrophils can undergo phenotypic change, becoming macrophage-like or dendritic-like cell expressing antigen-presenting cell surface markers [Galligan & Yoshimura, 2003] which makes them difficult to be identified given the lack of sensitive and specific detection methods. Neutrophils were detected in a subendothelial and intimal location in early lesions whereas neutrophils reside within the plaque shoulder and in the adventitia of advanced plaques [Drechsler *et al.*, 2010]. The use of intravital microscopy indicated frequent interactions between neutrophils and the endothelium in regions of high inflammatory activity where neutrophils prominently localized within early and advanced atherosclerotic plaques. In mice, the utilization of antibodies to Ly6G, an antigen specifically expressed on mouse neutrophils, in conjunction with myeloperoxidase staining enabled immuno-histochemical identification of neutrophils in early lesions and similarly in rupture-prone atherosclerotic plaques. CD177 (NB-1) and CD66b are surface markers typically expressed on human granulocytes, whereof the antibody to CD66b has been used to detect neutrophils in human unstable atherosclerotic plaques both eroded and ruptured ones [Naruko *et al.*, 2002].

Neutrophil counts in correlation with atherosclerotic lesion formation

Several studies have demonstrated a close relationship between the number of circulating neutrophils and the fate of atheroma [Rotzius *et al.*, 2010; Soehnlein *et al.*, 2008]. Elevated systemic neutrophil counts are associated with the risk for cardiovascular events in humans while the observations made in the *Apoe*^{-/-} mice showed increased peripheral neutrophil counts correlate with atherosclerotic lesion sizes, an effect reversed by rendering mice neutropenic [Zernecke *et al.*, 2008]. Immunohistochemical analysis of human carotid plaques reveals that high numbers of neutrophils are strongly associated with the histopathologic

features of atherosclerotic lesions with a large lipid core and suggests a role for neutrophils in plaque destabilization [Ionita *et al.*, 2010]. An increase in number of circulating neutrophils can be defined as neutrophilia which may be caused by several mechanisms: increased cell production, accelerated release of cells from the bone marrow into the blood, shift within the circulation from the marginal to the circulating pool, reduced egress of neutrophils from the blood to tissues, or a combination of these mechanisms. It has been known that many inflammatory processes are associated with a rapid and selective mobilization of neutrophils from the bone marrow but the precise mechanism has yet to be determined. However, several chemoattractants released during inflammatory reactions, including leukotriene B₄, C5a and the IL-8, have been shown to induce a rapid mobilization of neutrophils from bone marrow [Jagels & Hugli, 1992; Terashima *et al.*, 1998; Jagels *et al.*, 1995] while CXCL12 increases retention of neutrophils in bone marrow via CXCR4 [Furze & Rankin, 2008]. Interestingly, in hypercholesterolemia-induced neutrophilia, neutrophils infiltrate arteries primarily during early stages of atherosclerosis, suggesting a significant impact of neutrophils in the initiation of atherosclerosis in the murine model [Soehnlein, Lindbom & Weber, 2009]. Moreover, recent evidence indicates that hyperlipidemia-induced mice with the absence of apoE have a tendency to neutrophilia [Weber & Soehnlein, 2011], while the extent of neutrophilia is directly proportional to the degree of early atherosclerotic plaque development, whereas neutropenic mice display reduced plaque sizes at early but not late stages of atherosclerotic lesion formation [Drechsler *et al.*, 2010].

Mechanisms of neutrophil-driven atherogenesis

Circulating neutrophils emigrate towards inflammatory stimuli by adhering to activated vascular endothelium. During these multi-step cascades, activated neutrophils secrete a wide array of granule contents [Soehnlein, 2009] such as cathelicidin, defensin, cathepsin-G and heparin-binding protein (HBP/CAP37/azurocidin) can directly recruit and activate classical monocytes implying a crucial role of the neutrophil extravasation and its secretion products in the subsequent monocyte recruitment [Soehnlein, Zernecke & Weber, 2009]. Neutrophil secretory proteins such as cathelicidin and α -defensins can also enhance macrophage changes towards the M1 phenotype led to elevated phagocytic activities, increased expression of scavenger receptors and internalization of modified lipoproteins which contributes to elevated foam cell formation [van der Does *et al.*, 2010]. Neutrophils also undergo phagocytosis of microbes and present degraded products to lymphocytes amplifying the recruitment and subendothelial accumulation of various cell types. Neutrophil-mediated recruitment of

leukocytes into the intima is a significant step in atherogenesis. Moreover, neutrophils are capable of producing several cytokines and lipid mediators [Baetta & Corsini, 2010], the latter involving oxygenation of arachidonic acid and further processing of the intermediate metabolite leukotriene A₄ (LTA₄) into the potent pro-inflammatory chemoattractant leukotriene B₄ (LTB₄). These mediators play also an essential role in inflammatory processes to promote atherosclerosis [Serhan, Yacoubian & Yang, 2008; Serhan, Chiang & Van Dyke, 2008]. Recently, neutrophil extracellular trap (NET) which can be identified as an extracellular DNA containing granule proteins and histones that are released from neutrophils through a unique cell death pathway termed NETosis, has been reported to locate on the luminal side of human and murine atherosclerotic lesions suggesting a possible role of NET in neutrophil-dependent atherosclerosis development [Megens *et al.*, 2012].

Mechanisms of neutrophil-driven plaque destabilization

Neutrophils are not only rapidly recruited to the vascular wall but also initiate and perpetuate core mechanisms in plaque development as well as plaque instability [Soehnlein *et al.*, 2009]. Activated neutrophils release high amounts of granule proteins, pro-inflammatory mediators, oxidative radical species and proteases (e.g. MMP-2 and MMP-9) which further aggravate endothelial dysfunction, amplify the recruitment of inflammatory cells into the lesion and degrade matrix, leading to fibrous cap erosion and rupture of destabilized plaques [Soehnlein, Weber & Lindbom, 2009]. Furthermore, much of our current knowledge about the potential role of neutrophils in atherosclerosis is based on observations from experimental models in mice. However, it should be pointed out that mouse models have their limitations with regard to modeling plaque destabilization and rupture, and that interventional procedures in mice are of limited availability. Knowledge of the contribution of neutrophils to atherosclerosis will allow for exploration of new avenues in the treatment of atherogenesis and atherothrombosis [Soehnlein, 2012].

Thus far, this intriguing role of neutrophils in the inflammatory conditions associated with atherogenesis and athero-progression suggests the potential importance of modulating neutrophilic inflammation as part of the strategy to prevent and treat atherosclerosis [Soehnlein & Weber, 2009]. However, further research is necessary to unveil the complex mechanisms by which neutrophils contribute to the different phases of atherosclerosis.

Neutrophils & their granule proteins

Neutrophils are major effector cells of innate immunity and the first cell type being recruited to inflammatory sites in response to invading microorganisms, infectious pathogens and foreign substances. The neutrophil population is derived from myeloid progenitor cells in the bone marrow; approximately 100 billion cells are produced daily in a normal adult thus they are the most abundant leukocytes in the circulation constituting up to 80% of all leukocytes [Bagby, 2007]. Neutrophils are characterized by the large number of granules within their cytoplasm. Neutrophils display great heterogeneity in size and shape with a diameter between 12 and 15 micrometers. The nucleus consists of two to five lobes joined together by hair-like filaments. It takes approximately one week for neutrophil precursor cells to become mature, but once in the circulation, the mature neutrophils turn into short-lived cells with limited transcriptional capacities. The life span of mature neutrophils is only a few hours but can be a little longer after migrating into the tissues [Dancey *et al.*, 1976]. Hence, the bone marrow reserves a substantial amount of neutrophils to be mobilized in response to acute inflammation.

Neutrophils contain a wide range of antimicrobial proteins, proteases and membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins and soluble inflammatory mediators stored in the various different granule subsets (**Figure 4**). All the granules have similar structural features, including a bilayer of phospholipid membrane and intragranular matrix proteins. However, the main distinction between these granule subsets is their protein contents. Biosynthesis of neutrophil granules (granulopoiesis) [Elghetany *et al.*, 2004] initiates sequentially during early myeloid cell differentiation with the early-appearing high content of myeloperoxidase granules and consequently named peroxidase-positive granules, also referred to as azurophil granules due to their staining properties to azure A dye or simply primary granules. Subsequently, peroxidase negative granules are formed at later stages of granulopoiesis which can be further subdivided into specific or secondary granules and gelatinase or tertiary granules, based on the time of their appearance and matrix protein contents in the granules, respectively. While secondary granules contain a large amount of lactoferrin and a very low amount of gelatinase, tertiary granules have a high content of gelatinase but are low in lactoferrin. Lastly, secretory vesicles, formed by endocytosis, contain primarily plasma proteins [Faurschou & Borregaard, 2003].

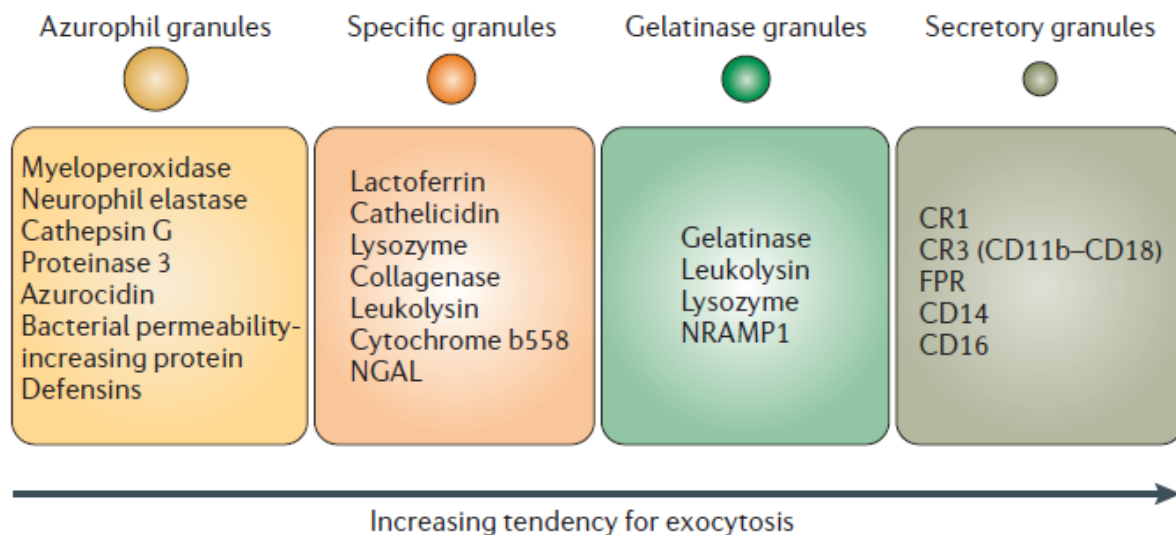


Figure 4. Neutrophil granule contents. Protein constituents of different granules are defined by the timing of their biosynthesis during neutrophil differentiation. In addition, granule contents are also released according to a hierarchy with secretory granules being the most readily exocytosed and azurophil granules only undergoing partial exocytosis. CR, complement receptor; FPR, formyl peptide receptor; NGAL, neutrophil gelatinase-associated lipocalin; NRAMP1, natural-resistance-associated macrophage protein 1 (Pham, 2006).

Peroxidase-positive granules

Azurophilic granules can be subdivided into two subsets: early-appearing defensin-poor azurophil granules and late-appearing defensin-rich azurophil granules. They were thought to be primary lysosomes for many years as they loaded with acidic hydrolases and antimicrobial proteins. In contrast, they do not display the lysosome-associated membrane proteins (LAMPs): instead, they express granulophysin (CD63), CD68, presenilin-1 and stomatin [Cieutat *et al.*, 1998]. Therefore, they are probably best considered as regulated secretory granules or non-specialized lysosomes. Most of the azurophilic proteins are generated as inactive preforms in order to prevent misfolding of the peptide and importantly, autotoxicity [Garwicz *et al.*, 1998]. Azurophil granules undergo specific exocytosis and contribute mainly to the degradation of engulfed microorganisms in the phagolysosome.

Azurocidin is a 29-kDa antimicrobial serine protease homologue found in azurophil granules also known as cationic antimicrobial protein 37 (CAP37) or heparin-binding protein (HBP). Azurocidin is chemotactic for monocytes, fibroblasts and T cells, and increases vascular permeability during neutrophil extravasation. In contrast to elastase, proteinase-3, and cathepsin G, azurocidin is proteolytically inactive because of an amino acid substitution within the catalytic triad of serine proteases [Miyasaki & Bodeau, 1992].

Alpha-defensins are major constituents of azurophil granules. They are small (~3.5 kDa) cationic, potent antimicrobial and cytotoxic peptides active against many Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses. On the basis of their size and pattern of disulfide bonding, mammalian defensins are classified into alpha (α -), beta (β -) and theta (θ -) categories. In humans, the neutrophil contains four closely related α -defensins (human neutrophil peptide (HNP) 1–4) of 29–30 amino acids, which are stored in defensin-rich azurophil granules. The α -defensins exert their antimicrobial effect through the formation of multimeric transmembrane pores. In addition, neutrophil defensins induce chemotaxis of monocytes, CD4⁺ and CD8⁺ T cells [Chaly *et al.*, 2000].

Myeloperoxidase (MPO), a 150-kDa microbicidal member of the haemprotein peroxidase cyclooxygenase superfamily, is abundantly expressed in neutrophils and to a lesser extent in monocytes and certain types of macrophages. MPO is released into the phagosome or extracellular space upon activation of neutrophils. MPO reacts with H₂O₂, formed by the NADPH oxidase, through oxidation of chloride, tyrosine and nitrite, to yield hypochlorous acid (HOCl), other chlorination products, tyrosine radicals and reactive nitrogen intermediates, all of which can attack the surface membranes of microorganisms and also host structures [Malle *et al.*, 2007].

Proteinase-3, cathepsin G, and elastase belong to a class of cationic polypeptides of 25–29 kDa called serine proteases. All human and mouse serine proteases contain histidine, aspartic acid and serine residues in a conserved catalytic triad. Serine proteases display proteolytic activity against a variety of extracellular matrix components, such as elastin, fibronectin, laminin, type IV collagen, and vitronectin. Furthermore, they induce activation of endothelial and epithelial cells, macrophages, lymphocytes, and platelets, and possess antimicrobial properties. Proteinase-3 was recently shown to be responsible for the extracellular cleavage and activation of the specific granule peptide human cathelicidin protein-18 (hCAP-18) to LL-37 [Pham, 2006].

Peroxidase-negative granules

Peroxidase-negative granules can be divided into specific granules and gelatinase granules based on the significant difference in protein content and secretory properties. While the specific granules are loaded with antibiotic agents, majorly involved in antimicrobial activities during phagocytosis, the gelatinase granules contain matrix-degrading enzymes that are important for neutrophils during extravasation and diapedesis [Borregaard *et al.*, 1993].

hCAP-18, an 18-kDa specific granule constituent, belongs to the cathelicidin group of antimicrobial peptides. The 14-kDa N-terminal region of hCAP-18 shares a high degree of homology with other cathelicidins, whereas the C-terminal part, named LL-37, forms an amphipathic α -helix that exerts antimicrobial activity against Gram-positive and Gram-negative bacteria and induces chemotaxis of neutrophils and monocytes. Moreover, hCAP-18 is constitutively expressed by subsets of lymphocytes, monocytes, and by the epithelia of the oral cavity, oesophagus, cervix, vagina, and epidermis [Bülow *et al.*, 2002].

Lysozyme is a cationic antimicrobial peptide of 14 kDa. In agreement with its biosynthetic profile, lysozyme is present in all granule subsets, with peak concentrations in specific granules. Lysozyme cleaves peptidoglycan polymers of bacterial cell walls and exerts bactericidal activity towards the non-pathogenic Gram-positive bacterium *Bacillus subtilis*. Lysozyme has also been shown to bind LPS and reduce cytokine production and mortality caused by LPS in a murine model system of septic shock. In view of the substantial neutrophil production of lysozyme, this antimicrobial peptide is likely to have additional functions that may be revealed by the recently developed lysozyme-deficient mouse [Lollike *et al.*, 1995].

Secretory vesicles

Neutrophil endocytic derived secretory vesicles store a large amount of membrane-associated receptors required during acute inflammatory response and released upon activation by a broad spectrum of inflammatory stimuli. The secretory vesicles are abundant in the β_2 -integrin (CD11b, CD18, and Mac-1), receptors for formylated bacterial peptides (formylmethionyl-leucyl-phenylalanine (fMLP)-receptors), the LPS receptor CD14, the Fc receptor CD16 and the metalloprotease leukolysin, all of which are intercalated into the plasma membrane after the exocytosis process. The mobilization of secretory vesicles followed by shedding of L-selectin forms a key regulation to the establishment of neutrophil firm adhesion on activated endothelium surface.

Cathelicidins

Cathelicidins are major groups of anti microbial peptides (AMPs) found in neutrophils [Izadpanah & Gallo, 2005] that contain both hydrophobic and hydrophilic domains which allows the interactions with aqueous environments and lipid-rich plasma membranes. Cathelicidin was first identified in the skin mediating bacterial killing and wound healing with the peptide sequence of proline/arginine (PR)-39 similar to molecules found in azurophilic granules of neutrophil. The structures of the cathelicidin peptides vary within and between species as they tend to display very little homology to each other. Thus far, around 35 cathelicidin members have been identified in both invertebrate and vertebrate species including mammals [Gallo *et al.*, 1997], however humans and mice synthesize each only one cathelicidin: human cationic antimicrobial protein 18 (hCAP18) and cathelicidin-related antimicrobial peptide (CRAMP), respectively [Lehrer & Ganz, 1999; Agerberth *et al.*, 1995]. Hence, the term cathelicidin refers solely to a group of the similar precursor proteins predominated by the large N-terminal cathelin domain.

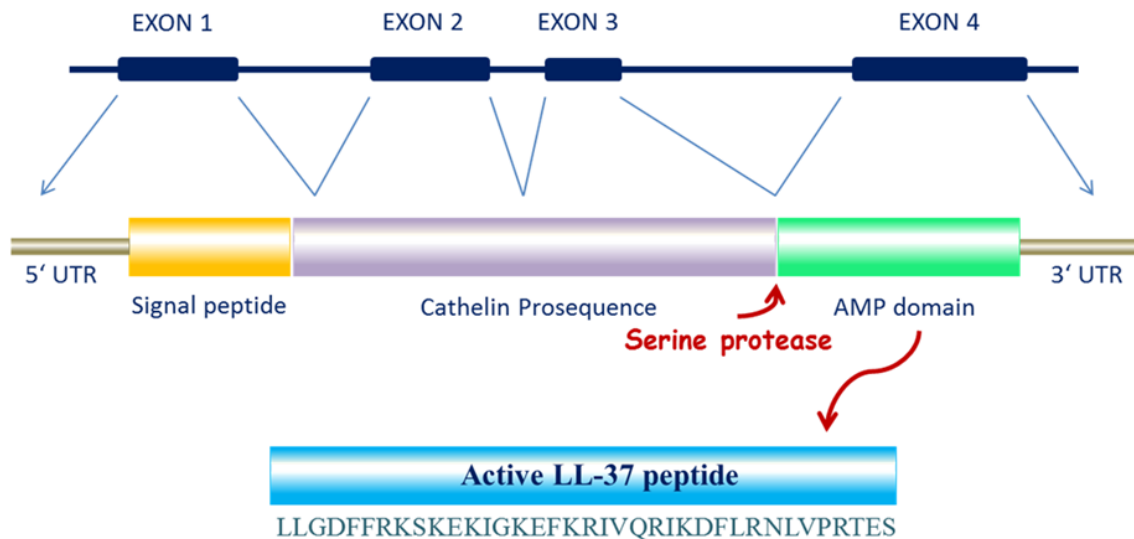


Figure 5. Structural organization of cathelicidin genes and an encoded precursor peptide. Each cathelicidin gene contains four exons separated by three introns. Cathelicidin is synthesized as an inactive precursor protein with an amino-terminal signal sequence, a central cathelin domain, and an inactive carboxy-terminal antimicrobial peptide (AMP) domain. Serine proteases, including proteinase 3, cleave the AMP domain to generate the active antimicrobial peptide LL-37. Further cleavage of LL-37 by serine proteases generates a family of biologically active peptides. UTR = untranslated region (Adapted from Izadpanah & Gallo, 2005).

In humans, cathelicidin is only considered to be a gene family because of the conservation seen in this region of the gene, encoding an inactive conserved cathelin precursor protein with an approximate mass of 18 KDa [Gudmundsson & Agerberth, 1999]. This peptide acts as a cysteine protease inhibitor and also possesses antimicrobial activity consisting of two disulfide bonds between cysteine residues C85-C96 and C107-C124 [Sørensen *et al.*, 1997]. The name cathelicidin is based on the highly conserved prosequence N-terminal putative signal peptide (preregion), a conserved cathelin-like domain (proregion) and a variable C-terminal microbicidal domain that can be released from the precursor protein after cleavage by proteinases [Zanetti *et al.*, 1995]. In human neutrophils, hCAP18 is the only cathelicidin identified to date, stored in specific granule as an inactive proform which usually undergoes processing to the mature peptide by appropriate proteases, mainly by neutrophil-derived serine proteases such as proteinase 3 and elastase to liberate LL37 from its C-terminus [Sørensen *et al.*, 2001]. LL-37 derives its name from the primary peptide structure containing 37 amino acids with two leucine residues. LL37 is mainly expressed by neutrophils and epithelial cells but is also present in other granulocytes as well as NK-cells and mast cells [Agerberth *et al.*, 1995]. The expression of cathelicidin is upregulated by inflammatory components such as LPS or lipoteichoic acid via NF- κ B signaling pathway in an Erk-dependent manner [Schauber *et al.*, 2003]. Comparable to human LL-37, the murine CRAMP forms an amphipathic α -helical peptide. Therefore, studies using CRAMP-deficient mice that show the significant role of cathelicidins in host defense against infection [Nizet *et al.*, 2001] are relevant to understand the role of LL-37 in humans and several studies have shown hCAP-18/LL-37 is increased during inflammation [Schaller-Bals *et al.*, 2002].

Neutrophil-derived cathelicidins in inflammation and atherosclerosis

Inflammation is characterized by an influx of inflammatory cells which is regulated through the production of inflammatory cytokines and chemokines. Because atherosclerosis is an inflammatory disease, understanding the role of neutrophil-derived cathelicidin in inflammation can lead to a novel concept for the treatment of atherosclerosis. Cathelicidin for a long time has been displaying a broad spectrum of various cellular activities related to inflammation including cytotoxicity, cell activation, wound repair and angiogenesis. In addition to their anti-microbial properties, neutrophil-derived cathelicidins exert chemotactic activity towards a variety of cell types, including neutrophils, monocytes, T-cells and mast cells [Niyonsaba *et al.*, 2002], through their interaction with the formyl peptide receptor like - 1 (FPRL-1 or FPR-2) [Yang *et al.*, 2000].

In contrast, LL-37-induced maturation and release of IL-1 β in LPS-primed monocytes was mediated via the P2X7 receptors [Elssner *et al.*, 2004]. LL-37 increases the up-regulation of CXCL8 and CCL2 and their receptors, CXCR2 and CCR2 on phagocytes, therefore LL-37 may indirectly facilitate the recruitment of these phagocytic cells to the site of inflammation through the induction of chemokines and their corresponding receptors [Scott *et al.*, 2002]. LL-37 displays chemotactic activity at concentrations of 0.5-50 μ g/ml (0.1-10 μ M), whereas tissue cytotoxic activities were observed at high concentrations, exceeding 13 μ M during inflammation [Yang *et al.*, 2000; Tjabringa *et al.*, 2006]. It has been reported that LL-37 at low concentration protects from apoptosis in human keratinocytes by decreasing the caspase-3 activity through the up-regulation of cyclooxygenase-2 (COX-2) and prostaglandin E-2 (PGE-2). Moreover, LL-37 induced the expression of inhibitor of apoptosis-2 (IAP-2) facilitating the COX-2/PGE-2 anti-apoptotic pathway [Chamorro *et al.*, 2009]. LL-37 was also shown to activate the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases (ERK) 1/2, JNK and p38 [Scott *et al.*, 2002].

Interestingly, LL-37 was demonstrated to modulate dendritic cell differentiation and dendritic cell-induced T-cell polarization [Davidson *et al.*, 2004]. The modulation of dendritic cell activity suggests that, in addition to its role in innate immunity, LL-37 may also be involved in adaptive immune responses. Recently LL-37 was suggested to be involved in wound repair. Growth factors involved in the process of wound healing were shown to strongly induce LL-37 expression in healing epithelium [Sørensen *et al.*, 2003]. *In vitro*, LL-37 was shown to activate endothelial cells resulting in increased proliferation and formation of vessel-like structures, while *in vivo* CRAMP-deficient mice showed decreased vascularization during wound repair. These results suggest a regulatory function for LL-37 in re-endothelialization after tissue injury [Koczulla *et al.*, 2003], which may help prevent the development of intimal thickening. The development of atherosclerosis with accumulation of necrotic cells in the atherosclerotic core makes the plaques more susceptible to rupture. The immunohistochemical analysis of human atherosclerotic plaque confirms the presence of LL-37 in atherosclerotic lesions [Edfeldt *et al.*, 2006] and its involvement in plaque development. Additionally, LL-37 has been found to induce vascular smooth muscle cell necrosis in atherosclerosis [Ciornei *et al.*, 2006]. Furthermore, LL-37 has an impact on endothelial stiffness that prevents increased pericellular permeability which may help to maintain tissue homeostasis [Byfield *et al.*, 2011]. Taken together, cathelicidin is a promising alternative choice aiming for therapeutics and drug development for inflammatory related cardiovascular disease.

Mechanisms of neutrophil degranulation

Neutrophil granule subsets have different tendencies for the secretion of their contents, with secretory vesicles being released during the interactions between neutrophil and endothelium, while tertiary granules are discharged as a consequence of neutrophil transendothelial extravasation [Borregaard & Cowland, 1997]. Primary and secondary granules are only released once the neutrophil has entered into the vascular tissue. Interestingly, various stimuli have different potency and specificity to induce mobilization of the granule subsets. Inflammatory mediators such as fMLP lead to a rapid discharge of secretory vesicles without significant release of granules, whereas powerful agonists like phorbol myristate acetate (PMA) strongly induces release of gelatinase granules, intermediate release of specific granules but low effect to the release of azurophil granules [Hoult & Nourshargh, 1985].

So far, the degranulation signaling pathways are complex and not fully understood. The elevations in the cytosolic Ca^{2+} levels during which the granule exocytosis takes place have been shown. The ligation of L-selectin, CD11b and the G-protein-coupled fMLP receptors expressed on neutrophil cell surface to corresponding ligands induces activation of cytoplasmic protein tyrosine kinases which further phosphorylate and activate a wide range of effector proteins, including phospholipases. The activated phospholipases transform phosphatidylinositol and phosphatidylcholine into inositol (1,4,5) P₃, diacylglycerol and phosphatidic acid. These molecules, in turn, activate protein kinase C (PKC) and subsequently mobilize Ca^{2+} influx from intracellular endoplasmic reticulum (ER) across the plasma membrane [Lacy, 2006]. Neutrophil degranulation may be mediated by the interactions between Ca^{2+} transient and SNAP receptor proteins. The specific degranulation cascades for each individual granule subset at different phases have yet to be further investigated.

Leukocyte extravasation

Neutrophils are the first line of leukocytes arriving at the site of inflammation and responsible for the early lesion formation. Mechanisms by which neutrophils interact with endothelial cells and subsequently emigrate into vascular tissue may become a therapeutic target to the prevention and treatment of atherosclerosis. Over the past years, the leukocyte extravasation cascade (**Figure 6**) has been intensively studied [Vicente-Manzanares & Sánchez-Madrid, 2004]. The complexity of neutrophil transendothelial mechanisms in the microcirculation entails multiple signaling pathways and adhesion molecules in several sequential cascades.

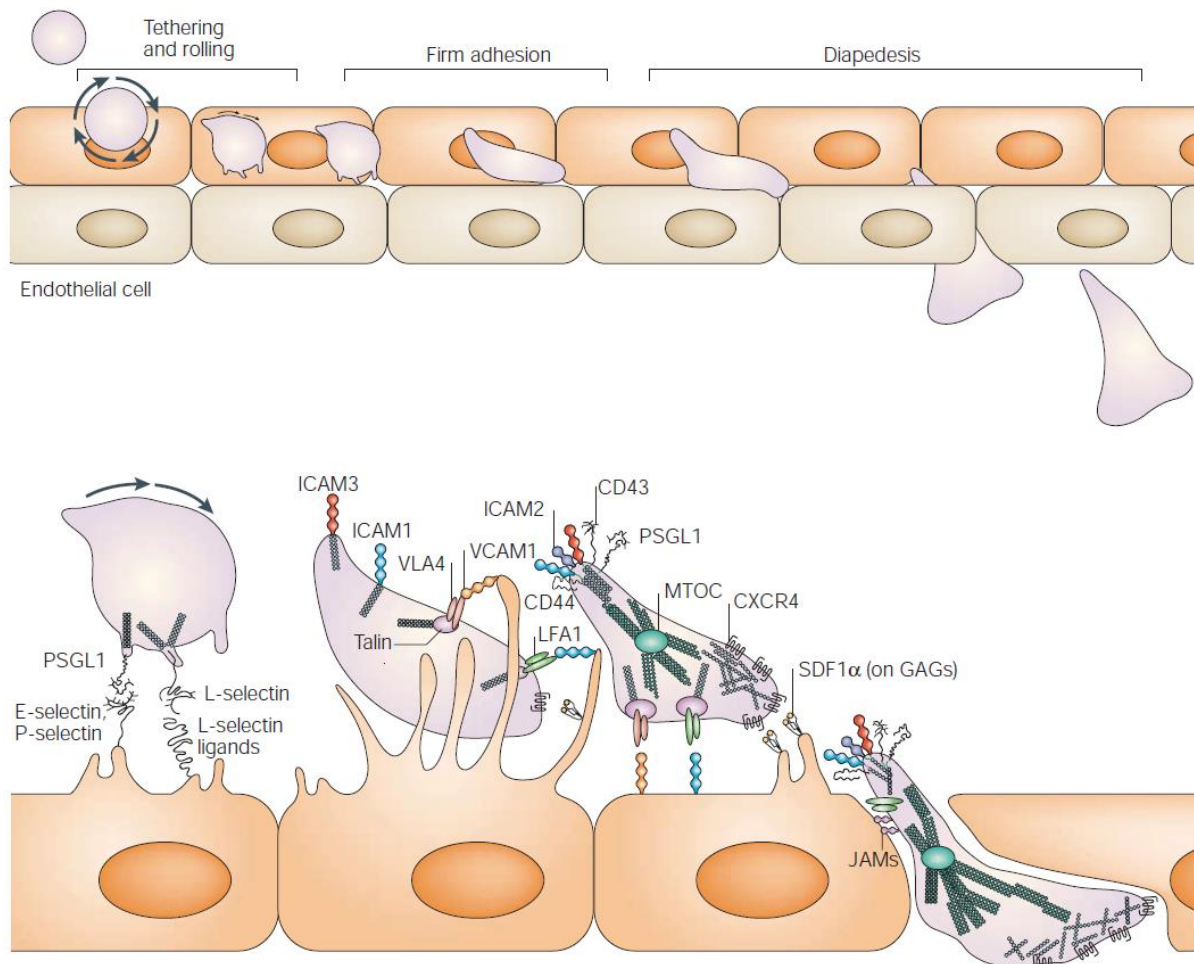


Figure 6. Leukocyte extravasation into target tissues. Tethering of the leukocyte on the surface of activated endothelium occurs through interactions between L-selectin and its endothelial ligands, as well as PSGL-1 mediated contacts. Tethering and rolling precede firm adhesion, which is mediated by interaction of VLA4 ($\alpha_4\beta_1$) and LFA1 ($\alpha_1\beta_2$) with VCAM-1 and ICAM-1 respectively. Integrin-mediated cell adhesion is enhanced by interaction of chemokine receptors with chemokines immobilized on glycosaminoglycans (GAGs) in a mechanism of receptor cross talk. During this step, leukocyte polarization occurs, with clustering of adhesion molecules in the cellular uropod. Finally, leukocytes extravasate and enter the target organ. (Vicente-Manzanares & Sánchez-Madrid, 2004).

Activated endothelial cells express a cluster of adhesion molecules, cytokines and chemokines on their surface [Lodish *et al.*, 2000]. In a similar manner, neutrophils also express various adhesion molecules and receptors. The interaction of these surface-expressed ligands and receptors mediates the different stages of neutrophil infiltration. The first contact made by neutrophils to the endothelium (tethering) is done through the engagement of the selectins with their respective ligands. Neutrophils express L-selectin which binds to sialylated ligands presented on the endothelial cell surface, while P-selectin glycoprotein ligand 1 (PSGL-1) facilitates the conjugations with P-selectin and E-selectin expressed by the endothelium. This initial interaction mediates rolling of neutrophils along the vascular wall. After capture, rolling neutrophils slow down, bringing them into close proximity with endothelial cells where they sense chemokines and cytokines such as fMLP, leukotrienes, CXCL2 and CXCL8 involving the respective receptors such as formyl peptide receptors (FPRs), the low-affinity chemokine receptor CXCR1, which mediates neutrophil activation, and the high-affinity receptor CXCR2, which mediates chemotaxis [Barnes, 2002]. The ligation between chemotactic ligands and their receptors triggers intracellular signaling pathways that activate integrins on the neutrophil surface to undergo conformational changes from a low affinity to a high affinity conformation [Abram & Lowell, 2009; Kinashi, 2005]. Activated high affinity integrins, including β_1 -integrin ($\alpha_4\beta_1$; CD49D; VLA4) and β_2 -integrin ($\alpha_M\beta_2$; CD11B; MAC-1) interacting with endothelial VCAM-1 and ICAM-1, respectively, promote firm adhesion of neutrophils on the surface of the activated endothelium. The interactions between integrins and their intercellular ligands are of crucial importance for leukocyte adhesion [Salmi & Jalkanen, 2005]. The cytoskeleton re-arrangement actively participates in neutrophil attachment on the endothelium through the generation of an actin-based docking structure that concentrates VCAM1 and ICAM1 molecules. The extravasation of neutrophils also involves an interaction of their integrins with junctional adhesion molecules (JAMs), which normally regulate endothelial cell–cell junctions. During this step, cell polarization occurs, with clustering of adhesion molecules in the cellular uropod leading to rear detachment and stretching of the non-transmigrated portion of the cell to gain the passage which finally extravasates through the gap and enter the target tissue. Neutrophils are directed by immobilized chemokines and gradients to migrate across the endothelium. The transmigration process can be enhanced by shear stress, as it activates cytoskeleton regulatory molecules [Weber *et al.*, 2007]. In addition, activated neutrophils also secrete granule proteins such as myeloperoxidase, azurocidin, and proteinase, which induce expression of adhesion molecules and permeability changes which enhance cell arrest and diapedesis.

Integrin activation and leukocyte adhesion

Integrins are cell surface receptors consisting of transmembrane heterodimer α -subunit and a β -subunit (**Figure 7**) which each have a large ectodomain, a single transmembrane domain and a generally short cytoplasmic tail. To date, 18 α - and 8 β -subunits have been identified in mammals which give rise to 24 $\alpha\beta$ pairs [Hemler, 1990].

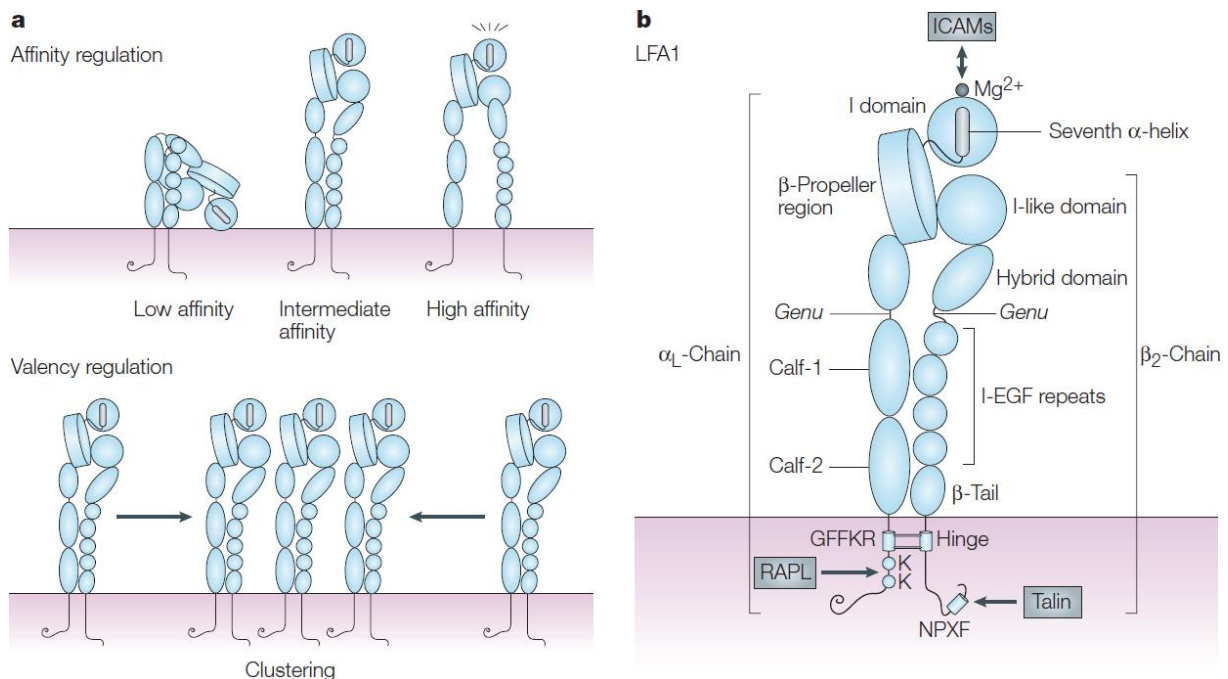


Figure 7. Integrin structure and regulation. (a) Models of affinity and avidity. The upper panel shows the different conformations of an integrin that are associated with distinct affinities: the bent head-piece conformation (low affinity), and the two extended head-piece conformations (intermediate affinity and high affinity). The lower panel shows clustering of integrins on the surface of a cell, which mediates multivalent interactions with ligands. This process is known as valency regulation of integrin avidity. (b) Structural schema of lymphocyte function associated antigen 1 (LFA1). The ligand-binding I domain (inserted domain) of LFA1 binds ICAM-1. The seventh α -helix of the I domain links to the β -propeller region. The linker region between the β -propeller region and the seventh α -helix interacts with the I-like domain of the β_2 -chain, and when triggered, the linker 'pulls' the seventh α -helix, which induces a conformational change that results in the high-affinity of LFA1 (Kinashi, 2005).

Circulating leukocyte firm adhesion on inflamed vascular endothelial surface requires ligation of β_2 integrins LFA-1 ($\alpha_L\beta_2$) and Mac-1 ($\alpha_M\beta_2$) as well as by β_1 integrin VLA-4 ($\alpha_4\beta_1$) to their counter ligands ICAM-1 and VCAM-1 respectively. Neutrophils are more dependent on LFA-1 and Mac-1 mediated arrest, whereas monocytes use primarily VLA-4. The initial contact between G-protein coupled receptors expressed on leukocytes and endothelial-bound chemokines leads to rapid activation of leukocyte integrins.

Integrin affinities for their cognate extracellular ligands are tightly controlled by cellular signaling, resulting in integrin activation through the inside-out and outside-in signaling [Abram & Lowell, 2009]. During the inside-out signaling, intracellular activator proteins bind to the tail of integrin β -subunit, leading to conformational changes that result in increased affinity for extracellular ligands. The outside-in signaling, on the other hand, transmits cellular signal through integrins into cells. Inside-out signaling regulates cell adhesion and migration while outside-in signaling functioning as cell surface receptors. Both signaling pathways are closely associated, firstly inside-out signaling activates integrins which promote ligand binding, and subsequently ligand-bound integrins propagate back the outside-in signaling [Shattil *et al.*, 2010].

Integrins exist in an equilibrium between the inactive, intermediate and highly active states [Kinashi, 2005]. Activated integrin engages several signaling pathways resulting in cytoskeletal organization which controlling cell polarity, adhesion, migration, gene expression, proliferation, survival and apoptosis. The association of integrin α and β subunits maintains the low affinity inactive state and alteration of these transmembrane interactions can destabilize the ectodomain dimer complex. Cytoplasmic domain binding proteins such as talin and kindlin are the detonation for integrin activation and their subsequent conformational changes. Integrins themselves do not possess a kinase domain for enzymatic activity but rely solely on specific binding of ligands which interact with the actin cytoskeleton at focal adhesions on the cell surface where concentrations of integrins are localized.

During the initial stage of integrin activation (**Figure 8**) [Shattil *et al.*, 2010], binding of ligand to its cognate receptor such as G-protein coupled receptor (GPCR) stimulates phospholipid hydrolysis, which results in the generation of inositol triphosphate (IP3) and diacylglycerol (DAG); IP3 also mediates an increase in cytosolic Ca^{2+} . Both DAG and Ca^{2+} further activate calcium and diacylglycerol regulated guanine nucleotide exchange factor I (CALDAG-GEFI; also known as RASGRP1), which in turn converts its encoded Ras subfamily small GTPases protein, RAP1, from an inactive GDP-bound to an active GTP-bound form. DAG and Ca^{2+} also activate certain protein kinase C (PKC) which may facilitate the conversion of RAP1 protein by CALDAG-GEFI. Activation of RAP1 leads to recruitment of its effector, RAP1-GTP-interacting adaptor molecule (RIAM; also known as APBB1IP) which functions as a scaffold that connects the membrane targeting sequences in RAP1 to its binding protein, talin 1, to the plasma membrane.

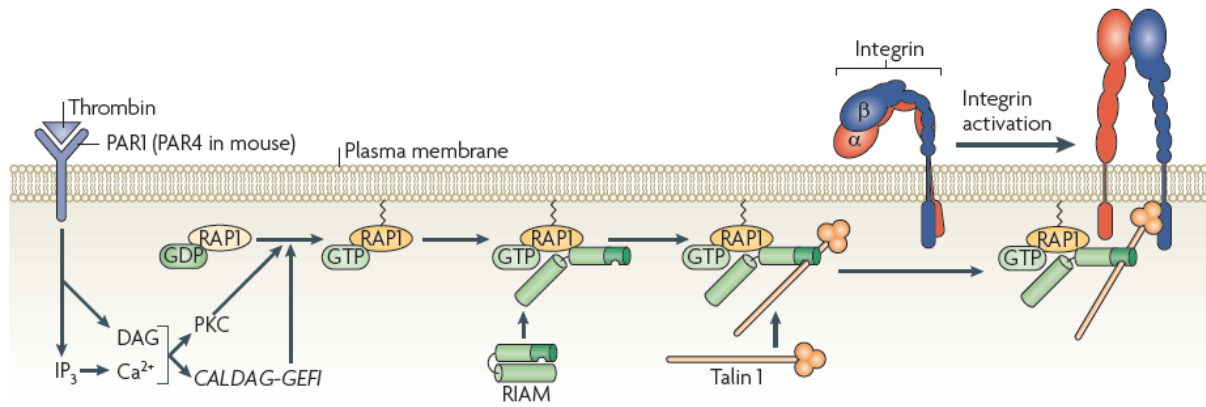


Figure 8. An integrin activation pathway. The schematic represents the minimal elements of one pathway of α IIb β integrin activation by thrombin receptors, which were identified through the synthetic reconstruction of pathway components in Chinese hamster ovary cells and studies of gene-targeted platelets (Shattil, Kim and Ginsberg, 2010).

Talin becomes activated upon binding to phosphatidylinositol 4,5-bisphosphate (PIP₂) and engaging to membrane proximal NPxY motif of integrin cytoplasmic domains and thereby disrupting the connection between $\alpha\beta$ -subunits. This subsequently results in extracellular domain conformational changes from a low affinity bent form to a high affinity extended form [Kinashi, 2005]. Ligation of talin to the integrin cytoplasmic domain leads to subsequent recruitment of the actin-binding proteins and results in the oligomerization of focal adhesion kinase (FAK) lead to clustering of activated integrin heterodimers to form hetero-oligomers. Autophosphorylation of FAK at residue Tyr397 regulates numerous biochemical pathways, including activation of MEKKs (MAPK/ ERK Kinase Kinase), PAK (p21-Activated Kinase) and MEKs (MAPK/ERK Kinases) which in turn promote cell adhesion and migration in a transcription-independent manner.

Integrin clustering and focal complex formation requires a small G-protein - RhoA, a member of the Ras superfamily that is activated by a variety of mitotic stimuli including those for GPCR (G-Protein Coupled Receptor) and cytokine receptors. RhoA is important for the organization of actin filaments and also in the regulation of actin-myosin contractility [Danen *et al.*, 2002]. Integrin activation also stimulates the production of phosphatidyl inositol biphosphate (PIP₂) mediated by RhoA. The increase in PIP₂ is relevant to focal adhesion due to the fact that actin binding activity of several cytoskeletal proteins such as profilin, gelsolin and vinculin is modulated by PIP₂.

Monocytes and macrophages

Monocytes develop from common myeloid progenitor cells in the bone marrow and are subsequently released to the peripheral circulation as non-dividing cells which constitute approximately 10% of peripheral leukocytes in humans and approximately 4% of leukocytes in mice. The half-life of a circulating monocyte has been estimated to be around 3 days in humans and 1 day in mice [van Furth & Cohn, 1968; van Furth *et al.*, 1979, Liu *et al.*, 2007]. This supported the notion that blood monocytes act as a transient reservoir of myeloid precursors enabling the continuous repopulation of peripheral tissue macrophages and dendritic cells in the steady state.

Monocytes are key components of the innate immune system and are circulating precursors of tissue macrophages. Monocytes are a heterogeneous leukocyte subset, both phenotypically and functionally. In humans, three monocyte subsets can be distinguished based on the surface marker expression of CD14 and CD16 receptors: the classical monocytes (also known as inflammatory monocytes) show high CD14 expression but no CD16 (CD14⁺⁺CD16⁻), the intermediate monocytes show a high level of CD14 together with low CD16 (CD14⁺⁺CD16⁺) and the non-classical monocytes (patrolling monocytes) express a low level of CD14 together with high CD16 (CD14⁺CD16⁺⁺). The latter two subsets are often collectively addressed as CD16⁺ monocytes when the intermediate and the non-classical monocytes are not separately defined [Ziegler-Heitbrock *et al.*, 2010]. Similar to humans, three discrete subpopulations of mouse blood monocytes were identified by morphology and the basis differential expression of the antigenic markers; Ly6C and CD43. The classical monocytes show high Ly6C expression and low CD43 (Ly6C^{hi}CD43^{lo}), the intermediate monocytes show a high level of Ly6C together with high CD43 (Ly6C^{hi}CD43^{hi}), and the non-classical monocytes express a low level of Ly6C together with high CD43 (Ly6C^{lo}CD43^{hi}) [Ziegler-Heitbrock *et al.*, 2010]. In addition, expression level of CCR2, CCR5 and CX3CR1 are often used to distinguish monocyte subpopulations [Wong *et al.*, 2012].

During inflammation, circulating monocytes migrate from the blood to lymphoid and non-lymphoid tissues in response to tissue-derived signals caused by infection or tissue damage. Monocytic integrins and the endothelial cell family of adhesion molecules are critically involved in firm adhesion and subsequently endothelial transmigration. Monocytes abundantly express VLA4 (β_1 -integrin) and LFA1 (β_2 -integrin) binding to VCAM-1 and ICAM-1, respectively during the extravasation. Once in the tissue, monocytes differentiate

into macrophages and phagocytose dead cells and toxic molecules such as oxidized LDL, and produce inflammatory cytokines which can turn into foam cells and inflammatory DCs. Tissue macrophages have various functions in the maintenance of tissue homeostasis, including clearance of dead cells, tissue repair and promoting resolution of the inflammatory response. Some studies have demonstrated that under steady state conditions resident tissue macrophage populations are largely maintained by local proliferation [Sawyer *et al.*, 1982].

However, inflammation results in the rapid recruitment of circulating monocyte infiltration into tissue and they subsequently differentiate into macrophages. Monocytes produce various chemokines in response to TLR activation. The ligation of TLR stimulates pro-inflammatory cytokine production such as IL-1, IL6, and TNF- α in classical monocytes, whereas anti-inflammatory cytokines are up-regulated on non-classical monocytes which can promote Th1 response. The non-classical monocytes show a higher level of HLA-DR and this would predict a higher antigen presenting cell activity to T-cells. Monocytes can give rise to macrophages or dendritic cells through distinct pathways [Auffray, Sieweke & Geissmann, 2009]. Interleukin-32 can induce the differentiation of monocytes into macrophages. The precise mechanisms by which monocyte subsets and how they differentiate into macrophage subtypes are not yet well understood and have to be determined.

Migration of monocytes into sites of inflammation is regulated by several chemokine receptors binding with their respective ligands: CCR2 are dominantly expressed on classical monocytes and responsible for chemotactic migration responsiveness for monocyte chemotactic protein-1 (MCP-1/CCL2), whereas non-classical monocytes highly express CCR5 thus migrate in response to macrophage inflammatory protein-1 α (RANTES/CCL5) [Gordon & Taylor, 2005]. Monocytes/macrophages play a crucial contribution in the early stages of atherosclerosis during which monocytes are recruited into intima and differentiate into macrophages. Through the scavenger receptors expressed on the macrophage cell surface, oxLDL and other lipids are taken up and consequently undergo transformation into foam cells to form early intima lesion.

2

Aims

Aims of the study

Neutrophils play a central role in the pathophysiology of inflammation. They are among the first cell types rapidly recruited to the site of inflamed tissues where they discharge a wide array of granule contents upon extravasation, mediating immune defense mechanisms. Neutrophil-derived cathelicidin is known for its broad spectrums of antimicrobial activities during acute infection. Recently, cathelicidins have also been shown to exhibit chemotactic properties towards many cell types involved in inflammatory processes. This new perceptive knowledge comes to our interest to further investigate the participation of neutrophils in immuno-biological modulation of the vascular wall. Although the significance of neutrophils and neutrophil-derived cathelicidins in innate immune response has been well established, their contributions to inflammatory-related vascular diseases are not yet clearly defined.

Therefore the key aims of this thesis were:

- To clarify the role of neutrophils in neointima formation.
- To elucidate the crucial properties of neutrophil-derived cathelicidin in arterial inflammation including intima thickening and restenosis.
- To understand the mechanisms of how neutrophil-derived cathelicidins are released, transported and presented, as well as the signaling pathways of cathelicidin-mediated adhesion of monocytes.
- To ultimately prove the importance of neutrophil-derived cathelicidin in atherosclerosis.

3

Materials

&

Methods

Materials

The general chemicals were obtained from Sigma-Aldrich, Merck or Fluka in analytical grade quality, unless indicated otherwise. All reagents, buffers and solutions were prepared with either double distilled water (Heraeus Destamat, Heraeus, Germany), Millipore water (Milli-Q Plus ultrapure purification, Millipore, MA, USA) or with Aqua Ad Injectabilia (Braun, Melsungen, Germany).

General equipment

Autoclave	Systec VX-150 (Systec, Wettenberg, Germany)
Balances	Sartorius CPA 64 (Sartorius, Göttingen, Germany)
CCD camera	Hamamatsu 9100-02 EMCCD (Hamamatsu, Hamamatsu, Japan)
Centrifuges	Eppendorf 5417C (Eppendorf, Hamburg, Germany), Heraeus Labofuge 400 and Multifuge 3 S-R (Heraeus, Hanau, Germany)
Flow cytometers	FACS Canto II, FACS Aria (BD Biosciences, CA, USA)
Fluorescence plate reader	Infinite M200 (Tecan, Crailsheim, Germany)
Gel electrophoresis	Mini-sub cell GT (Bio-Rad, Hercules, CA, USA)
Laminar flow hood	Herasafe (Heraeus, Hanau, Germany)
Microscopes	Olympus IX71 and BX51 (Olympus Optical, Hamburg, Germany), Leica DMLB (Leica, Wetzlar, Germany)
Multiphoton system	LaVision Trimscope (LaVision, Bielefeld, Germany), Leica TCS SP5 MP (Leica, Wetzlar, Germany), Olympus FV 1000 MPE (Olympus, Hamburg, Germany)
PCR thermocyclers	MyCycler (Bio-Rad, Hercules, CA)
pH-meter	Lab850 (Schott, Mainz, Germany)
Spectrometer	NanoDrop (Pqlab, Erlangen, Germany)

Kits

BrdU cell proliferation	Millipore (Billerica, USA)
DNA digestion	DNase Set (Quiagen, Hilden, Germany)
Monocyte isolation	MACS CD16 ⁺ Monocyte isolation kit and Monocyte isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany)
Reverse Transcription	M-MLV Reverse Transcriptase (Promega, Madison, USA)
Real-time PCR	SYBR Green Master Mix (Fermentas, St. Leon-Rot, Germany)
RNA isolation	RNeasy Mini/Micro Kit (Quiagen, Hilden, Germany)

Consumables

6, 24, 96 well plates	BD (FranklinLakes, USA), Nunc (Roskilde, Denmark), CoStar (Kehl/Rhein, Germany), Greiner Bio One (Frickenhausen, Germany), Corning (New York, USA)
Cannulas 23G, 27G	BD (Franklin Lakes, USA)
Cell strainer	Miltenyi (Bergisch-Gladbach, Germany)
Disposable scalpels	Feather Safety Razor (Osaka, Japan)
Disposable Syringes	Terumo (Leuven, Belgium), Braun (Melsungen, Germany), BD (FranklinLakes, USA)
Eppendorf tubes	Eppendorf (Hamburg, Germany)
FACS-tubes	BD (FranklinLakes, USA)
Falcon tubes	Greiner Bio One (Frickenhausen, Germany)
Glassware	Schott (Mainz, Germany)
Microscope slides	Thermo Scientific, (Braunschweig, Germany)
Microtubes EDTA	Sarstedt (Nümbrecht, Germany)
Serological pipettes	Corning (New York, USA)
Sterile filter-tips	Starlab (Ahrensburg, Germany)
Syringes	Terumo (Leuven, Belgium), Braun (Melsungen, Germany)

Methodology

Bio-Molecular analysis

The genotyping of mice used from in-house breeding was carried out according to laboratory specific standard operating protocols. DNA was obtained from the tail cut tissues using protease digestion and the BioSprint 15 System (Quiagen, Hilden, Germany). Even though mRNA expression levels in cells as well as in tissues is a widely used method to investigate effects and changes within these cells especially after a specific treatment, one has to be aware that mRNA does not necessarily correspond to protein expression thus the physiological relevance has to be further characterized by complementary methods.

RNA isolation

DNA-free total RNA was extracted from endothelial early outgrowth cells (EOCs) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Isolation was followed by digestion of genomic DNA using the RNase-free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

RNA quantification

RNA concentration was determined by measuring the absorbance at 260 nm (A₂₆₀) in a spectrophotometer. The absorbance at 280 nm was also measured to estimate DNA and RNA purity. Pure DNA or RNA has an A₂₆₀/A₂₈₀ ratio of 1.8-2.0 at pH 7.0.

Reverse Transcription

After determination of the RNA amount, up to 2µg total RNA were reverse-transcribed into complimentary DNA (cDNA) at 42°C for 15 min and terminated at 95°C for 1 min using the M-MLV RT Kit (Promega, Madison, WI, USA) and random hexamer primers (Fermentas, St. Leon Rot, Germany).

5µl	5x reverse transcription buffer
0.5µl	dNTP mix (20mM each)
1µl	10µM random hexamer primer
1µl	reverse transcriptase enzyme
up to 2µg template RNA	
H ₂ O to a final volume of 25µl	

Real-time PCR analysis

For estimating relative expression of mRNA within different samples, 20ng of cDNA were amplified and assessed by real-time PCR reaction with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference genes. The following primers were used:

FPR2-forward	5'-TTGGTTTCCCTTTCAACTGG-3'
FPR2-reverse	5'-ACGTAAAGCATGGGGTTGAG-3'
GAPDH-forward	5'-GACCTTCATTGACTCAACTACA-3'
GAPDH-reverse	5'-GCCCCTCCCCTCTTCAAG-3'

2µl cDNA or H₂O
 13µl 2x QuantiTect SYBR Green PCR Master Mix
 Forward primer to a final concentration of 0.5µM
 Reverse primer to a final concentration of 0.5µM
 RNase free water to final volume of 25µl

Program for real-time PCR:

1. Initial activation 95°C, 15 min
 2. Denaturation 95°C, 30 sec
 3. Annealing 60°C, 30 sec
 4. Elongation 68°C, 2 min
 5. Plate read
- Repeat steps 2-5 for 45 times

Relative mRNA expression level of gene of interest compared to a control was calculated using $2^{-\Delta\Delta Ct}$ method [Livak & Schmittgen, 2001].

Binding assay

Binding of LL-37 (AnaSpec, Fremont, CA, USA) to human aortic endothelial cells (HAoECs) was assessed by flow cytometry analysis. LL-37 (1 µg/ml) was incubated with HAoECs for 15 min and stained with rabbit anti-LL-37 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% BSA. HAoECs were pre-treated with heparinase II (50 U/ml), chondroitinase_{ABC} (20 U/ml) or cyclohexamide (500 ng/ml)

Cathelicidin conditioned medium

To prepare cathelicidin conditioned medium (CM), EOCs were washed and cultured in RPMI medium 1% FCS (PAA, Pasching, Austria) with or without LL-37 (1 µg/ml). After 24 h, medium was collected, centrifuged and passed through a filter. Residual LL-37 was immune-depleted using rabbit anti-LL-37 polyclonal antibody coupled to protein G beads.

Cell culture

Cell culture was performed under sterile conditions in a laminar flow hood. Cells were maintained in a CO₂-incubator at 37°C and a humidified 5% CO₂ atmosphere. EOCs were cultured from isolated peripheral blood mononuclear cells (PBMCs) on fibronectin (50 µg/ml, Biochrom, Berlin, Germany) coated tissue culture flask in endothelial growth medium MV2 (PromoCell, Heidelberg, Germany). HAoECs and human umbilical vein endothelial cells (HUVECs) were cultured and propagated on collagen (50 µg/ml, Biochrom) coated tissue culture flask in designated endothelial growth mediums. All growth mediums are supplemented with gentamicin (100µg/ml) (PAA, Pasching, Austria) to avoid contamination with bacteria. Fetal calf serum (FCS) used in the medium was incubated at 56°C for 30 min to inactivate the complement system and stored at -20°C until used. PBMCs were isolated by density gradient centrifugation. Monocyte subsets were then sorted from PBMCs using the CD16⁺ Monocyte Isolation Kit or the Monocyte Isolation Kit II (both Miltenyi Biotech, Bergisch Gladbach, Germany) for non-classical and classical monocytes, respectively. Cultured cells were regularly tested to be mycoplasma free by the VenorGeM Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany).

Cell circularity measurement

Circularity measurements were performed using Image J software. The analysis is based on the following equation: $circularity = 4\pi(area/perimeter^2)$. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.

Cell proliferation and apoptosis

For cell-cycle analysis, cells were fixed in 70% ethanol and stained with propidium iodide (30 µg/ml) in 0.25% triton-X100 in PBS containing RNase A (4 µg/ml). To induce apoptosis, TNF was added to EOC culture medium with or without LL-37. After 12 h, cells were detached and incubated with FITC-conjugated annexin-V.

Flow adhesion assay HAoEC

Dishes were coated with laminin (10 µg/mL), fibrinogen (10 µg/mL) or vitronectin (1 µg/mL; Sigma-Aldrich). Confluent HAoECs were activated with TNF (50 ng/ml) and LL-37 (1 µg/ml) was added 15 min before assembly in a parallel-wall flow chamber. EOCs labeled with calcein were perfused at 1.5 dyne/cm² in the presence or absence of boc-PLPLP (1 µM).

Flow adhesion assay HUVEC

HUVEC cultured in petri dishes were activated with TNF (50ng/ml, 12 h, Peprotech). LL37 (1µg/ml, Anaspec) or mutated LL37 (1µg/ml) were added 15min prior to performance of flow chamber assays. Isolated monocyte subsets were labeled with Calcein AM (Invitrogen) and perfused at 2.5dyne/cm² over HUVEC monolayers. In separate experiments, monocytes were pretreated for 30min with inhibitors detailed in the result section, washed twice and then used for flow chamber assays. Adherent cells were quantified in six randomly chosen fields.

Flow cytometry

Flow cytometry is a method used for cell characterization and quantification. By passing an argon laser the single cells within a fluid stream scatter and fractionize the laser light thus generating a specific fingerprint of the cell including size (forward scatter) and granularity (side scatter). By employing fluorescently labeled antibodies, cells can be further characterized by their expression of marker proteins on the surface. A detailed list of antibodies used for flow cytometry is shown in table 1. Staining of single cell suspensions of EOCs, HAoECs, HUVECs, Monocytes for flow cytometric analysis was conducted using combinations of antibodies (1:100) in 50µl of a 1:1 mixture of Hank's complete and FACS staining buffer as indicated in the result section. Before staining the cells for 30 min on ice, red blood cell lysis has been performed at room temperature using an appropriate volume of lysis buffer. Cells were washed with Hank's complete and directly analyzed by flow cytometry using a FACS Canto II (BD Biosciences, Franklin Lakes, USA). Data were analyzed with FlowJo Software (Tree Star Inc, Ashland, USA). In case the expression level of a protein or receptor was of interest, geometrical mean fluorescence intensity subtracted by the fluorescence minus one (FMO) control was calculated.

Table 1. List of antibodies.

Target	Clone	Isotype	Host	Conjugate	Reactivity	Company
CD11B	CBRM1/5	IgG1, K	Mouse	FITC	Human	eBioscience
CD11B	CBRM1/5	IgG1, k	Mouse	APC	Human	eBioscience
CD14	MφP9	IgG2b	Mouse	PerCP	Human	BD Pharmingen
CD14	MφP9	IgG2b	Mouse	FITC	Human	BD Pharmingen
CD16	3G8	IgG1, K	Mouse	PE	Human	BD Pharmingen
CD16	3G8	IgG1, K	Mouse	PE Cy7	Human	BD Pharmingen
CD29	HUTS-21	IgG2a, k	Mouse	PE	Human	BD Pharmingen
CD31	WM-59	IgG1, k	Mouse	PE	Human	eBioscience
CD45	HI30	IgG1, K	Mouse	PE	Human	Biolegend
CD45	2D1	IgG1, k	Mouse	APC Cy7	Human	BD Pharmingen
CD54	eBioKAT-1	IgG2a, K	Rat	purified	Mouse	eBioscience
CD106	429	IgG2a, K	Rat	purified	Mouse	eBioscience
DARC	# 358307	IgG2A	Mouse	PE	Human	R&D SYSTEMS
FPR1	# 350418	IgG2A	Mouse	PE	Human	R&D SYSTEMS
FPR1	# 350418	IgG2A	Mouse	APC	Human	R&D SYSTEMS
FPR2	# 304405	IgG2B	Mouse	FITC	Human	R&D SYSTEMS
FPR3	# 374822	IgG2B	Mouse	APC	Human	R&D SYSTEMS
LL-37	n/a	Polyclonal	Rabbit	purified	Human	Santa Cruz
LL-37	n/a	Polyclonal	Rabbit	FITC	Human	INNOVAGEN

Immunohistochemistry

Left common carotid arteries were excised after perfusion–fixation with paraformaldehyde (4%) in situ. Serial tissue sections (5 µm) were obtained starting at the bifurcation. Movats-modified pentachrome staining was used to determine neointimal areas. Sections were reacted with antibodies to Mac-2 (Cedarlane), α -SMC actin (Dako), CD3 (Serotec), Ly6G, CD31 (Abcam) or KI-67 (Imgenex). Gomori trichrome stain was used to assess collagen content. The extent of atherosclerosis was assessed by staining of aortic roots and thoracoabdominal aortas by staining for lipid depositions with oil-red-O (Sigma-Aldrich, St. Louis, USA). The 5µm sections were incubated in 0.5% oil-red-O solution for 30min. The percentage of lipid deposition was calculated from dividing the stained area by the total surface. The quantification was performed on computerized image analysis (Diskus Software, Königswinter, Germany) and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Furthermore TUNEL staining has been performed facilitating *In Situ* Cell Death Detection Kit, TMR red (Roche, Basel, Switzerland) to assess the number of apoptotic/necrotic cells within aortic root sections. The assay uses an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with TMR-dUTP.

Integrin activation assay

Human PBMCs were incubated 15min with PBS, PMA (50ng/ml, Sigma-Aldrich), MCP1 (50ng/ml, PeproTech), LL37 (1µg/ml, AnaSpec), or mutated LL37 (1µg/ml) and then stained with antibody against CD45, CD14, and CD16 for 30min at 4°C (BD Pharmingen). Antibodies to CD29 (HUTS-21, BD Pharmingen) and CD11b (CBRM1/5, ebioscience) detecting epitopes of activated integrins only were used to assess integrin activation. Integrin activation was measured by flow using a FACSCanto II (BD Biosciences). The results are analyzed with FlowJo Software (Treestar).

Murine peripheral blood cells were isolated from C57Bl6 mice and suspended in Hanks Balanced Salt Solution containing 1mM CaCl₂ and MgCl₂ (Invitrogen). Cells were exposed to PMA (50ng/ml, Sigma-Aldrich), MCP1 (50ng/ml, PeproTech), CRAMP (1µg/ml, Innovagen) or an equal volume of PBS, in the presence of ICAM-1/Fc (10µg/ml, R&D Systems) or of VCAM-1/Fc (10µg/ml, R&D Systems) and PE-conjugated anti-human IgG1 (Fc-specific, Southern Biotechnology) for 5 minutes at 37°C. After washing, cells were labeled with antibodies to CD45, CD115, and Gr1 to identify monocyte subsets. Binding of ICAM-1 or VCAM-1 was measured by flow cytometry.

Surface plasmon resonance

Interaction between LL37 and chemotactic receptors were studied by Surface Plasmon Resonance on a Biacore X100 system (GE Healthcare). LL37 or mutated LL37 were immobilized on a CM4 sensor chip at a level of 400 response unit (RU) by amine coupling. Receptors FPR1, FPR2, CXCR2 and P2X7 (Abnova) were used as analyte and diluted in a HBS-N running buffer (0.1M HEPES, 1.5M NaCl pH 7.4). Each experiment was performed with a flow of 10µl/min with running buffer. Sensor chip surfaces were regenerated with 50mM NaOH and equilibrated with running buffer prior to the next injection. Results were analyzed with Biaevaluation software.

Stent braiding, coating, implantation, and processing

Stents were braided from sixteen 50µm Nitinol wires yielding an outer diameter of 500µm and heated in a high-temperature oven for shape settings. Stents were cleaned by sonication in acetone, water, and 2-propanol followed by drying in a nitrogen stream. Surface activation was achieved by treatment with UV/ozone before use for amino-functionalization. Substrates were immersed in a solution of N-[3-(trimethoxysilyl)-propyl]ethylenediamine in dry toluene, and the desired amount of isocyanate end group terminated six-arm star-shaped copolymer of 80% ethylene oxide and 20% propylene oxide NCO-sP(EO-*stat*-PO) ($M_n = 12,000$ g/mol; PD=1.15) was dissolved in dry **tetrahydrofuran** under an inert gas atmosphere. Thereafter, the solution was filtered through 0.2 µm syringe filters and used for hydrogel coating. For dip-coating, stents were placed in 300 µl polymer solution.

For biofunctionalization, the following solutions were prepared in PBS: 50 µg/mL RGD + 1µg/ml P-selectin or 50 µg/mL RGD + 1 µg/ml LL-37 + 1 µg/ml P-selectin. Five minutes after hydrogel coating, samples were incubated in corresponding solutions overnight, and washed thoroughly with millipore water. Stents were implanted through an incision into left carotid arteries of *Apoe*^{-/-} mice by insertion into 250 µm silicon tubes, careful forward feeding of the stent whilst retracting the tube to allow for shape memory-based expansion. After 1-4 weeks, stents were harvested, fixed with 4% paraformaldehyde, and embedded in methyl-methacrylate (Technovit 9100, Heraeus). Specimens were sliced and polished using a cutting-grinding system technique (Exakt 400CS) using a diamond-band saw (Exakt 300) resulting in 50 µm sections, and treated with H₂O₂ for 15 min and with Giemsa stain for 30 min.

Images were recorded by 2-photon microscopy (Leica, Wetzlar, Germany) using Diskus software. To assess re-endothelialization, 5 µm cryosections adjacent to the stented carotid artery were stained with a monoclonal antibody to CD31 (Santa Cruz) including nuclear counter-staining. Luminal coverage with CD31⁺ endothelial cells was measured using Diskus software (Hilgers, Germany). The presence of CD31⁺ endothelial cells (red) in the luminal lining within the stented area of carotid arteries was assessed by immunofluorescence staining for CD31 and 2-photon microscopy of arteries prepared one week after stent insertion and re-embedded in agarose gel for analysis.

Transcytosis assay

HUVECs were cultured on 24-well polycarbonate transwell filter inserts (5µm, Corning Costar) coated with collagen and activated with human TNF-α (50ng/ml, 12h, Peprotech). FITC-conjugated LL37 (10µg/ml) was added to the bottom chamber. Alternatively, native (unconjugated) LL37 (10µg/ml) was added to the lower chamber of transwells. In order to detect the transcytosed LL37 on the HUVEC cell surface, a FITC conjugated antibody against human LL37 (Innovagen) was used and the fluorescence intensity assessed by a plate reader. For visual analysis, HUVEC were then counter-stained with DAPI (nuclear dye) and CellMask (plasma membrane stain, Invitrogen) and fixed with 4% PFA at 4°C for 30min. After washing, the samples were analyzed using a Leica SP5 MP system with a water dipping 20x; NA 1.00 objective and a Ti:Sa MaiTai DeepSee laser (Spectra Physics) tuned to 790nm. Three Hybrid detectors (HyD) were spectrally tuned for optimal detection efficiency and low bleed through of signal of the used markers (HyD1: 435-485nm, HyD2: 515-550nm, HyD3: 565-630nm). Furthermore, a dye separation protocol (Leica LAS AF software) was performed for removal of any leftover bleed through of signal. Additional image preparation was performed using ImagePro 3D analyzer 7.0 (Media Cybernetics).

Two-Photon microscopy

LaVision Biotec Trimscope (LaVision Biotec GMBH, Bielefeld, Germany), Olympus FV 1000 MPE (Olympus, Hamburg, Germany) and Leica TCS SP5 MP (Leica, Wetzlar, Germany) multiphoton microscope systems were used in single beam mode with a 20X NA 0.95; water dipping objective (Olympus, Hamburg, Germany). Two-photon excitation was achieved using a pulsed Ti-Sapphire laser (MaiTai HP, Spectra Physics, Mountain View, USA) tuned to 830 nm for visualization of Second Harmonic Generation (SHG) signal. Emitted fluorescence was detected with two photomultiplier tubes (PMTs) of the emission spectra. Series of subsequent xyz-sections were obtained over time at an acquisition rate of 6Hz using the Inspector Pro 4.0.43 acquisition software (LaVision Biotec GMBH). After image acquisition the obtained time series were analyzed and only xy-sections acquired at comparable z-position were selected for further analyses. Overall image quality was improved by resizing the pixel matrix using a bilinear scaling technique. Furthermore, 2D Gaussian weighted filtering was performed to improve the signal-to-noise ratio and thus overall image quality. All image processing was performed using Image-Pro 3D analyzer 7.0 (Media Cybernetics, Silver Spring, USA).

Animal Experiments

All the experiments involving animals were approved by the animal ethical committee of North Rhine Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz) and Bavaria (Sachgebiet 54 - Tierschutz Regierung Oberbayern) and were carried out in accordance to the guidelines for animal experimentation.

Mouse model of atherosclerosis

Mice deficient in Apolipoprotein E as well as different surface receptors were fed normal chow or, starting at 8 weeks of age, an atherogenic diet containing 21% fat (Altromin, Lage, Germany) for either 4 or 8 weeks. The time of high fat diet depended on the experimental setup. To estimate the extent of atherosclerosis, hearts and aorta were harvested after *in situ* perfusion and fixation with 4% PFA. The samples were further used for immunohistochemistry or immunofluorescence analysis.

Blood drawing, animal dissection and organ preparation

Live animals were anesthetized with isofluran (Abbott, Wiesbaden, Germany) before blood was drawn through the retro-orbital plexus and collected in EDTA tubes (Sarstedt, Nümbrecht, Germany). In case mice had to be sacrificed they were anesthetized by injection of 6-8mg Xylazin and 90-120mg Ketamin per kg of body weight and exsanguinated by heart puncture. Whole blood was kept for serum separation. Mice were flushed with 10ml of PBS first and depending on experimental setup furthermore rinsed with 10ml of 4% PFA. Aortas were removed and either kept on ice in RPMI1640 + 10% FCS or used for Oil-red-O staining after overnight-fixation in 4% PFA. For flow cytometry analysis, whole aortas have been digested with 0.25mg/ml Liberase in RPMI1640 + 10% FCS medium. Lysed aortas were then put through a cell strainer and stained with antibodies for further flow cytometry analysis. If bone marrow lavage fluid had to be examined, bones were flushed with 500µl RPMI1640 + 10% FCS. Spleens were mashed through a cell strainer and after red blood cell lysis subsequently used for antibody labeling.

In vivo depletion of leukocyte subsets

Circulating monocytes were eradicated in mice by intravenous injection of 200 µl undiluted chodronate loaded liposomes (Nico van Rooijen, Amsterdam, the Netherlands). Depletion of Neutrophils has been performed by intra-peritoneal injection of 100µg anti mouse Ly6G antibody (1A8, BioXCell, West Lebanon, USA) up to twice a week.

Intravital microscopy of the carotid arteries

Neutrophil rolling, adhesion and extravasation to injured carotid arteries or the cremaster muscles were observed by intravital microscopy in *Lysm^{egfp/egfp}Apoe^{-/-}* mice depleted of monocytes to obtain mice with fluorescent neutrophils only. For luminal detection of CRAMP presented on endothelium, 50µl of protein G Fluoresbrite® YG microspheres (Polysciences) were coupled to 50µg of polyclonal antibody to CRAMP (Innovagen). Beads and antibody were reacted for 30min at room temperature, washed twice and subsequently injected 4 h after injury. Recordings were made 15 min after injection to assess adhesion of 10⁶ DiI-labeled injected EOCs.

Intravital microscopy of the cremaster muscle

An inflammatory response was induced by intrascrotal injection of TNF (50ng/scrotum). The cremaster muscle was exteriorized for microscopic observation and recordings were made using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera (Hamamatsu Photonics) and a 20x water dipping objective. For image acquisition and analysis Olympus cell^r software (Olympus) was used. An antibody to Ly6C (ebioscience) was injected i.v. to detect adhesion of Ly6C-positive CM. Rolling neutrophil flux was determined as the number of neutrophils passing a reference line perpendicular to blood flow within 30 seconds. Neutrophils were considered adherent when no rolling was observed for at least 30 seconds.

Intracellular Cramp detection

For FACS analysis of cremaster muscle single-cell suspensions, mice were injected intrascrotally with TNF (50ng, 4h). An antibody to Ly6G (1A8, 1µg) was injected 5 minutes prior to sacrifice to label adherent neutrophils. The circulation of mice was flushed with cold PBS and the muscle tissue was excised and digested with 0.25 mg/ml Liberase (Roche) in RPMI1640 + 10% FCS medium at 37 °C for 1h. Surface staining for flow cytometric analysis was conducted using combinations of antibodies against CD11b, Gr1, CD31, and CD45 (all ebioscience). Intracellular labeling of CRAMP (rabbit anti-mouse CRAMP) and an appropriate isotype control (rabbit IgG isotype control, GeneTex) was performed with IC fixation buffer and permeabilization buffer (both from ebioscience) according to the manufacturer's protocol for staining of cytosolic proteins. Anti-rabbit IgG (ebioscience) served as fluorescently labeled secondary antibody.

Data illustration and statistical analysis

All data are expressed as mean $\pm$ SD of 3-10 individual experiments, if not stated otherwise. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). After calculating for normality by D'Agostino Pearson omnibus test either unpaired Student's t-test, One-way ANOVA with Newman-Keuls multiple comparison or nonparametric tests such as Mann-Whitney test, or Kruskal-Wallis test with posthoc Dunn test were used. A '*' Indicates a p -value < 0.05 and believed to be statistically significant.

4

Results

Neutrophil-Derived Cathelicidin Protects from Neointimal Hyperplasia

Progression of atherosclerosis causes luminal narrowing and obstruction of blood flow that can usually be treated by mechanical revascularization; however, this intervention creates severe arterial damage and an inflammatory response, leading to restenosis. Implantation of drug-eluting stents reduces the incidence of restenosis with a favorable safety profile and lower cost than surgical procedures [King, Marshall & Tummala, 2010]. However, clinical data implicate an increased risk of late stent thrombosis when using drug-eluting stents compared with bare-metal stents [Daemen *et al.*, 2007]. This risk may partially be attributable to impaired endothelial recovery: the reendothelialization. Coating stents with polymers such as poly ethylene glycol (PEG) can prevent adhesion of serum proteins and platelets to reduce in-stent thrombosis [Hanawa, 2009]. Thus, novel concepts of biofunctionalization may further improve treatment of in-stent restenosis and thrombosis. The initial inflammatory response to arterial injury is characterized by early influx of neutrophils [Soehnlein & Weber, 2009]. These immune cells contribute to the regulation of the inflammatory cascade by discharging preformed granule proteins, such as azurocidin, α -defensins, or cathelicidin, which are crucial mediators of neutrophil-dependent activities in inflammation [Ward *et al.*, 2002; Soehnlein & Lindbom, 2010]. These secretory proteins are potent activators of antigen-presenting cells, but they also activate endothelial cells (ECs), inducing permeability changes and neovascularization [Gautam *et al.*, 2001; Koczulla *et al.*, 2003]. After arterial damage, reendothelialization can regulate inflammation and smooth muscle cell accumulation; thus represents a crucial mechanism to limit neointima formation. Endothelial regeneration primarily relies on migration and proliferation of ECs from adjacent vascular segments. However, angiogenic EOCs can be recruited to sites of injury and accelerate reendothelialization [Urbich & Dimmeler, 2004; Hirschi, 2010]. Notably, infusion of EOCs can attenuate neointimal hyperplasia after arterial injury [Werner *et al.*, 2003, Griesse *et al.*, 2003] which has inspired EOC-based strategies to reduce restenosis. These strategies include elution of cyclic RGD peptides or CXCR2 ligands, which can recruit EOCs to the stent surface or sites of injury [Blindt *et al.*, 2006; Hristov *et al.*, 2007]. Here, we examined the involvement of neutrophil-derived granule proteins in arterial healing. We identified a prominent role for neutrophil-borne cathelicidin (mouse CRAMP, human LL-37) in promoting reendothelialization and limiting neointima formation. Consequently, we translated these findings into a biofunctionalized stent coated with LL-37 and demonstrated that a neutrophil-instructing device can reduce in-stent stenosis in mice.

4.1.1 Neutrophil-derived cathelicidin limits neointima formation

To address the role of neutrophils in inflammatory processes after arterial injury, we studied neointima formation in atherosclerosis-prone *Apoe*^{-/-} mice with intact white blood cell (WBC) count or neutropenia (**Figure 10A**) 4 weeks after wire injury. Neutrophil depletion (**Table 2**) significantly increased neointima formation, suggesting a beneficial function of neutrophils in arterial healing.

Table 2. Differential leukocyte counts in mice with intact WBC or neutropenia.

All values are given in cell count/ml venous blood.

	neutrophils	classical monocytes	resident monocytes	T lymphocytes	B lymphocytes
Intact WBC	8.1x10 ⁵	3.1x10 ⁵	1.9x10 ⁵	2.5x10 ⁶	1.9x10 ⁶
	+/- 1.8x10 ⁵	+/- 0.7x10 ⁵	+/- 0.6x10 ⁵	+/- 0.6x10 ⁶	+/- 0.2x10 ⁶
neutropenic	0.4x10 ⁵	3.3x10 ⁵	1.8x10 ⁵	2.7x10 ⁶	2.4x10 ⁶
	+/- 0.1x10 ⁵	+/- 0.3x10 ⁵	+/- 0.8x10 ⁵	+/- 0.8x10 ⁶	+/- 0.6x10 ⁶
p-value	0.001	0.531	0.980	0.725	0.493

To dissect stage-dependent effects, we depleted neutrophils during the first or the fourth week after injury. Whereas early neutropenia significantly increased the neointimal area, late depletion had no effect (**Figure 10A**). Hence, we restricted subsequent analyses to 1 week after injury (**Figure 9, Figure 10B, and Table 3**).

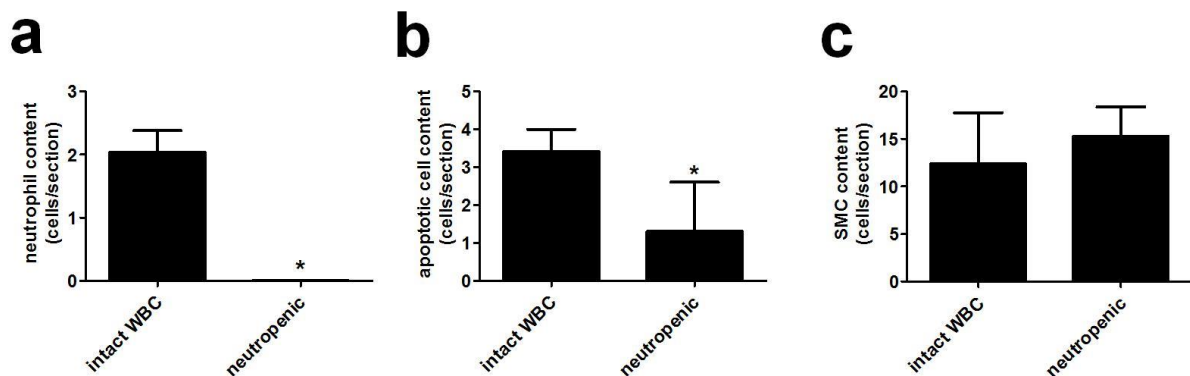


Figure 9. Neointimal cell compositions. Immunohistochemical analysis of neointimal lesions one week after wire-injury in mice with intact white blood cell count (WBC) and in neutropenic mice. Displayed are Ly6G⁺ neutrophils (a), TUNEL⁺ apoptotic cells (b), and α -SMA⁺ smooth muscle cells (SMCs) (c). $n = 7-9$. * $p < 0.05$ versus intact WBC. Mann-Whitney test.

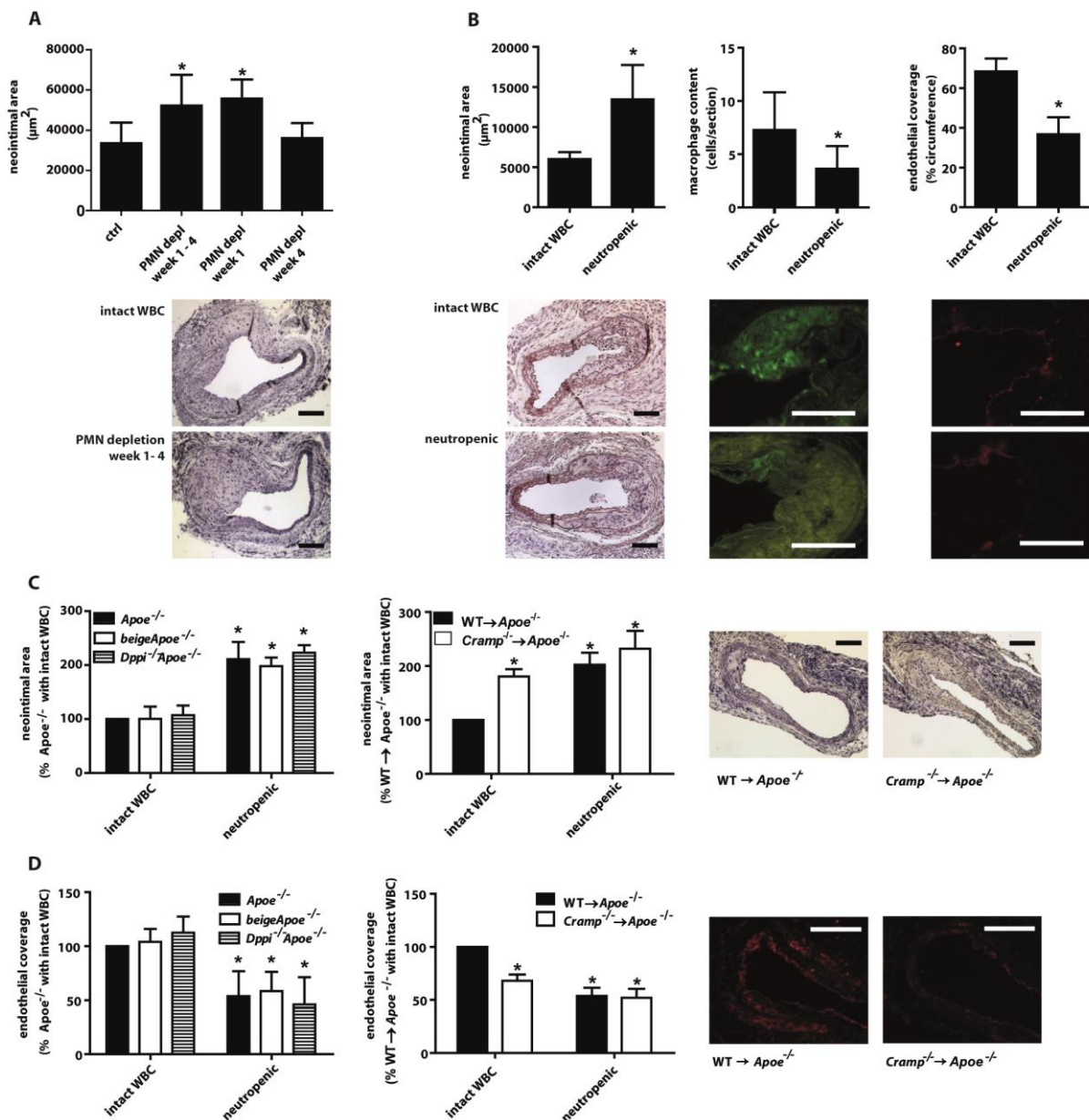


Figure 10. Neutrophil-derived cathelicidin limits neointima formation. (A) Neointima sizes, as quantified in Movat-stained sections of carotid arteries 4 weeks after wire injury in $\text{Apoe}^{-/-}$ mice depleted of neutrophils for indicated periods. Representative images show neointima formation in mice with intact WBC count (top) and in neutropenic mice (bottom). $n = 6$ to 8 . * $P < 0.05$ versus control mice, Kruskal-Wallis test with post hoc Dunn test. (B) Immunohistochemical analysis of neointima 1 week after wire injury in mice with intact WBC count or with neutropenia. Displayed are neointimal area (left), Mac-2⁺ macrophage content (middle), and luminal coverage with CD31⁺ endothelium (right), and representative images (lower panel). Green fluorescence stems from secondary fluorescein isothiocyanate-conjugated antibody; red fluorescence derives from secondary Cy3-conjugated antibody. $n = 7$ to 9 . * $P < 0.05$ versus intact WBCs, Mann-Whitney test. (C and D) Neointima size (C) and endothelial coverage (D) in $\text{Apoe}^{-/-}$, $\text{beigeApoe}^{-/-}$, $\text{Dppi}^{-/-}\text{Apoe}^{-/-}$, $\text{WT} \rightarrow \text{Apoe}^{-/-}$, and $\text{Cramp}^{-/-} \rightarrow \text{Apoe}^{-/-}$ mice 1 week after wire injury. Experiments were performed in mice with intact WBCs or neutropenia. Representative images show neointima formation and CD31 staining in $\text{WT} \rightarrow \text{Apoe}^{-/-}$ and $\text{Cramp}^{-/-} \rightarrow \text{Apoe}^{-/-}$ mice 1 week after wire injury. $n = 6$ to 8 . * $P < 0.05$ versus $\text{Apoe}^{-/-}$ or $\text{WT} \rightarrow \text{Apoe}^{-/-}$ with intact WBCs, Mann-Whitney test (left) and Kruskal-Wallis with post hoc Dunn test (right). Scale bars, 100 μm .

Herein, neutropenic mice exhibited a larger neointimal area than mice with intact WBCs. Paralleling a marked reduction of neutrophils in the neointima, the number of TUNEL⁺ cells was reduced in neutropenic mice (**Figure 9**).

Table 3. Neointimal cell composition in mice with intact WBC or neutropenia.

	total cells	SMC ^a	macrophages ^b	T lymphocytes ^c	neutrophils ^d	collagen ^e
Intact WBC	37.3	32.9	20.7	10.3	7.9	23.1
	+/- 3.8	+/- 8.1	+/- 8.6	+/- 7.8	+/- 5.2	+/- 9.3
neutropenic	32.6	41.7	16.1	17.5	1.2	41.6
	+/- 5.3	+/- 6.2	+/- 4.0	+/- 8.1	+/- 0.8	+/- 8.2
p-value	0.513	0.084	0.068	0.352	0.031	0.029

^a data are displayed as α -SMA⁺ cells in % of total cells

^b data are displayed as Mac2⁺ cells in % of total cells

^c data are displayed as CD3⁺ cells in % of total cells

^d data are displayed as Ly6G⁺ cells in % of total cells

^e data are displayed as collagen⁺ area in % of total neointimal area after Gomori trichrome stain

Analyzing neointimal cell composition revealed that total macrophage content was reduced in neutropenic mice (**Figure 10B**), in line with the role of neutrophils in classical monocyte recruitment [Soehnlein *et al.*, 2008; Soehnlein, Lindbom & Weber, 2009]. Conversely, neutrophil depletion increased neointimal collagen content but did not significantly affect CD3⁺ T lymphocyte numbers, SMC content (Table 4.2), or the number of SMCs undergoing proliferation or apoptosis (**Table 4**), nor did LL-37 alter SMC migration, proliferation, or apoptosis *in vitro* (**Figure 11**).

Table 4. Immunohistochemical analysis of SMC homeostasis one week after wire-injury.

	Proliferating SMCs ^a	Apoptotic SMCs ^b
Intact WBC	1.2	0.8
	+/- 0.9	+/- 0.7
neutropenic	0.6	0.7
	+/- 0.4	+/- 0.3
p-value	0.718	0.913

^a data are displayed as α -SMA⁺KI-67⁺ cells per neointima

^b data are displayed as α -SMA⁺TUNEL⁺ cells per neointima

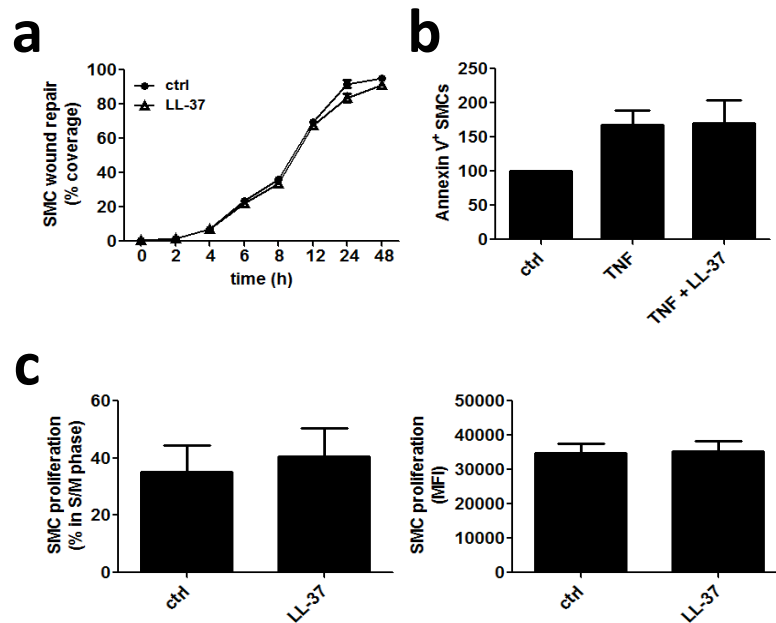


Figure 11. LL-37 does not affect migration, apoptosis, and proliferation of smooth muscle cells (SMCs). (a) Human aortic SMC monolayers were subjected to scratch injury and the recovered wound area was expressed as percentage of the initial wound area. Representative photomicrographs and quantified data are displayed. $n = 5$. (b) SMCs were treated with TNF (50 ng/ml) in presence or absence of LL-37 and apoptosis was quantified by flow cytometry after annexin-V staining. $n = 4$. Kruskal-Wallis-test with posthoc-Dunn-test. (c) SMC proliferation was assessed by cell cycle analysis using propidium iodide (left) or BrdU incorporation assays (right). Analyses were performed in cells treated with vehicle (ctrl) or LL-37. $n = 5$. Mann-Whitney test.

As evident by CD31 staining, endothelial coverage was reduced by 48% in neutropenic mice, indicating diminished reendothelialization (**Figure 10B**). Defective endothelial recovery in neutropenic mice was confirmed by Evans blue staining of en face-prepared carotid arteries (**Figure 12**).

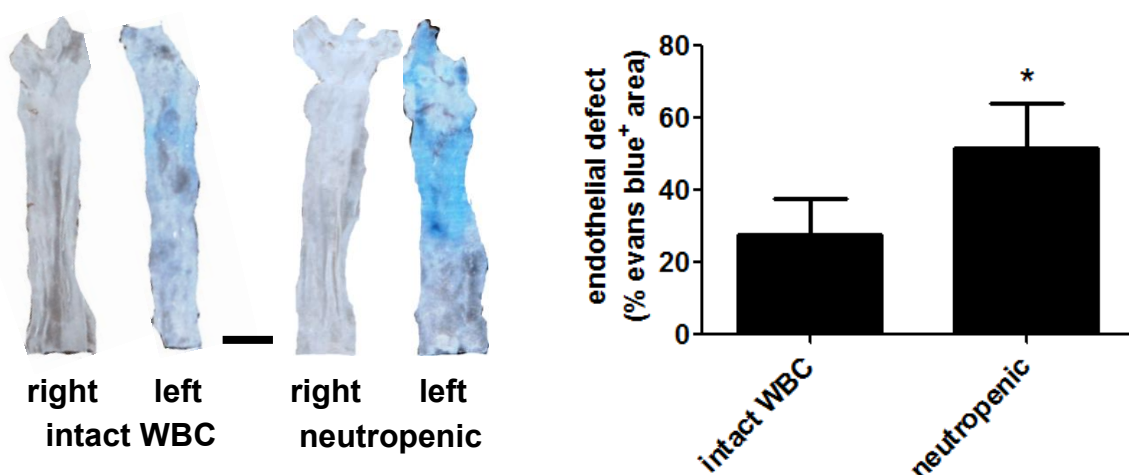


Figure 12. Neutropenia impairs endothelial recovery. Arterial injury was initiated in the left carotid artery of mice with intact white blood cell count (WBC) or neutropenia. After five days, mice were injected with Evans blue and the amount of Evans blue-positive area was assessed in en face preparations. Displayed are representative carotid arteries of mice with intact WBC (left) or neutropenia (middle). Scale bar represents 500 μm . Evans blue-positive area was quantified and expressed as % of total carotid area (right). $n = 8$. * $p < 0.05$ versus intact WBC. Mann-Whitney test.

Neutrophils regulate acute inflammatory responses through discharge of preformed granule proteins [Soehnlein, Weber & Lindbom, 2009; Soehnlein & Lindbom, 2010]. To dissect the role of distinct groups of granule proteins, we used *beigeApoe^{-/-}* and *Dppi^{-/-}Apoe^{-/-}* mice, which have reduced mobilization of primary neutrophil granule contents or lack active forms of neutrophil-derived serine proteases (proteinase-3, cathepsin G, and neutrophil elastase), respectively [Kjeldsen, Calafat & Borregaard, 1998; Adkison *et al.*, 2002]. Neointima formation and luminal CD31+ cell coverage after injury in these mice did not differ from that of *Apoe^{-/-}* mice with intact WBCs (**Figure 10C and D**). Neutropenic mice of all three strains behaved similarly, indicating that primary granule contents and neutrophil-derived proteases are not required for the neutrophil-mediated healing process. Hence, we examined the role of CRAMP, which attracts monocytes to promote wound healing or angiogenesis [Koczulla *et al.*, 2003, Adkison *et al.*, 2002], in neointima formation. Atherosclerotic mice transplanted with *Cramp^{-/-}* bone marrow (BM) (*Cramp^{-/-}→Apoe^{-/-}*) [Nizet *et al.*, 2001] displayed larger neointimal areas and reduced luminal CD31+ lining compared to mice receiving BM from wild-type mice (WT→*Apoe^{-/-}*) (**Figure 10C and D**). Neutropenia in *Cramp^{-/-}→Apoe^{-/-}* mice did not further aggravate this phenotype, which resembled that in neutropenic WT→*Apoe^{-/-}* mice.

4.1.2 Cathelicidins are deposited at sites of endothelial injury

We suspected that CRAMP, a highly positively charged polypeptide, was deposited by adherent neutrophils at sites of injury. Monocyte-depleted *Lysm^{egfp/egfp}Apoe^{-/-}* mice (**Table 5**) carrying only fluorescent neutrophils [Drechsler *et al.*, 2010] were used to monitor the time course of neutrophil adhesion.

Table 5. Differential leukocyte counts in mice depleted of monocytes.
All values are given in cell counts/ml venous blood.

	neutrophils	classical monocytes	resident monocytes	T lymphocytes	B lymphocytes
Monocyte-depleted	9.8x10 ⁵	0.2x10 ⁵	0.1x10 ⁵	2.1x10 ⁶	2.6x10 ⁶
	+/- 2.2x10 ⁵	+/- 0.1x10 ⁵	+/- 0.1x10 ⁵	+/- 0.4x10 ⁶	+/- 0.8x10 ⁶

Although neutrophil adhesion did not occur in uninjured carotid arteries, neutrophils rapidly adhered after injury peaking at 4 to 8h (**Figure 13A**). Hence, we studied arterial deposition of CRAMP 4h after injury. Injection of fluorescent beads conjugated to an antibody to CRAMP allowed for *in vivo* detection of immobilized CRAMP (**Figure 13B**), which was largely abolished in neutropenic mice. The luminal location of CRAMP was corroborated by 2-photon microscopy (**Figure 13C**). To characterize interactions of cathelicidins with ECs, we co-incubated LL-37 with human aortic ECs (HAoECs). LL-37 binding to HAoECs was reduced by pretreatment with heparinase but not chondroitinase (**Figure 13D**). Activation with TNF or induction of apoptosis with cyclohexamide in HAoECs did not modulate LL-37 binding, but vitronectin, laminin, and fibrinogen strongly bound LL-37 when compared to serum proteins alone (**Figure 13E**).

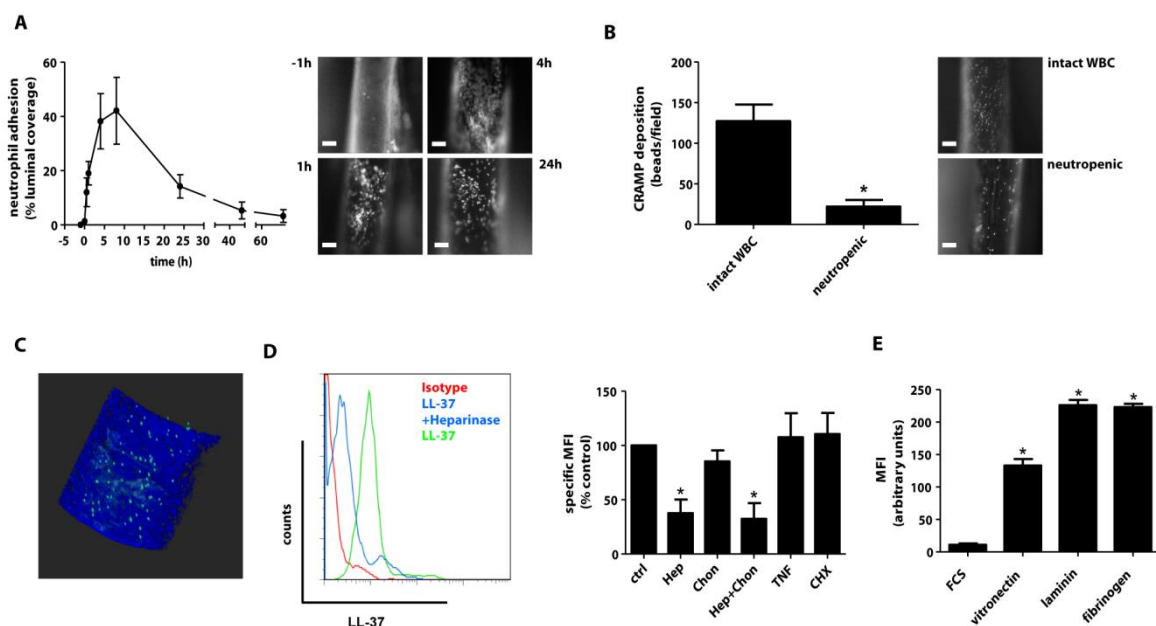


Figure 13. Neutrophils deposit cathelicidin at sites of injury. (A) Adhesion of neutrophils to injured carotid arteries of monocyte-depleted *Lysm^{egfp/egfp}Apoc^{-/-}* mice. Recordings were taken at different time points after injury and quantifications are presented as percent fluorescence of total arterial area. Representative images indicate neutrophil adhesion at indicated time points. *n*=4-5. (B) *In vivo* detection of CRAMP along injured carotid arteries of mice with intact white blood cell count (WBC) or neutropenia, as detected by protein G-coupled fluorescent beads conjugated with an antibody to CRAMP. The number of immobilized beads was quantified. *n*=4. **p*<0.05 versus intact WBC. Mann-Whitney-test. (C) anti-CRAMP bead complexes were detected luminally using intravital 2-photon microscopy. Blue fluorescence indicating collagen in the arterial wall originates from second harmonic generation, green fluorescence derives from beads conjugated with antibodies to CRAMP. (D) Surface binding of LL-37 to human aortic endothelial cells (HAoECs) assessed by FACS analysis. Untreated HAoECs (ctrl), HAoECs pretreated with heparinase (Hep, 50U/ml), chondroitinase (Chon, 20U/ml), or both, activated with TNF (10ng/ml, 4h) or rendered apoptotic with cyclohexamide (CHX, 500ng/ml), as indicated, were incubated with LL-37 (1μg/ml, 15min). MFI, mean fluorescence intensity. *n*=7. **p*<0.05 versus ctrl. Kruskal-Wallis-test with posthoc-Dunn-test. (E) Interaction of LL-37 with extracellular matrix proteins. LL-37 (1 μg/ml) was reacted with indicated extracellular matrix proteins and FCS or FCS alone immobilized on cell culture dishes and detected by immunofluorescence. *n*=8. **p*<0.05 versus FCS alone. Kruskal-Wallis-test with posthoc-Dunn-test. Scale bars, 100μm.

4.1.3 Cathelicidins mediate EOC recruitment

Neointima formation is governed by an ambiguous role of BM-derived cells. Whereas SMC progenitor cells contribute to neointimal hyperplasia, EOCs limit neointima formation [Hirschi, 2010]. As neutropenia and CRAMP-deficiency affected re-endothelialization rather than SMC accumulation, we investigated their role in lesional recruitment of BM-derived EOC-like cells in *Apoe*^{-/-} mice reconstituted with gfp⁺ BM and subjected to arterial wire-injury. After one week, gfp⁺ cells staining for CD31 were quantified in the neointima of mice with neutropenia or intact WBC (**Figure 14**). Neutrophil depletion reduced the number of BM-derived gfp⁺CD31⁺ EOC-like cells in the recovering endothelial lining.

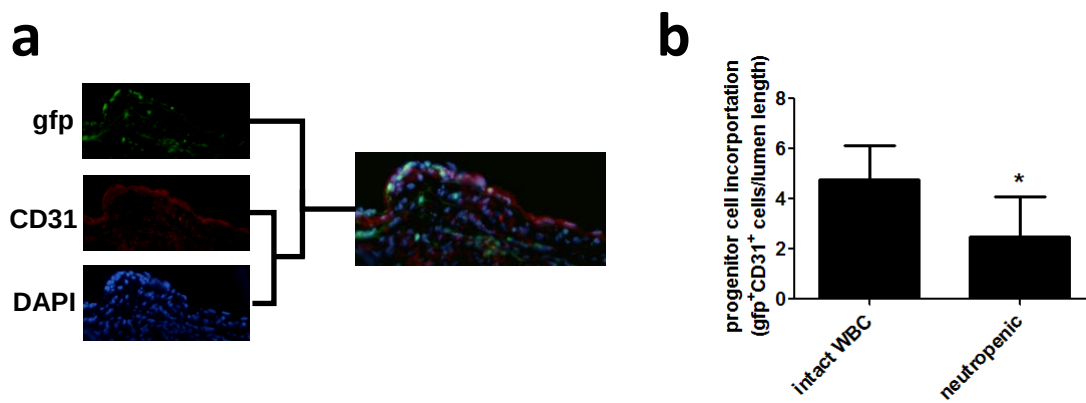


Figure 14. Neutropenia reduces incorporation of bone marrow-derived cells into the endothelial lining. *Apoe*^{-/-} mice were reconstituted with gfp-fluorescent bone-marrow cells. Wire-induced injury was performed in neutropenic mice and in mice with intact white blood cell count (WBC). After one week, the integration of gfp⁺ cells into the endothelial lining was analyzed by immunohistochemistry. **(a)** Representative image of analysis of CD31⁺gfp⁺ cells in serial sections. **(b)** Quantification of CD31⁺gfp⁺ cells. *n* = 8. **p* < 0.05 versus intact WBC. Mann-Whitney test.

Cathelicidins exert multiple immune-regulating functions via formyl-peptide receptor 2 (FPR2) [Soehnlein, Weber & Lindbom, 2009; Yang *et al.*, 2000]. As evidenced by flow cytometry, FPR2 was expressed on human EOCs under basal conditions and up-regulated by VEGF, TNF, or hypoxia at the transcriptional level (**Figure 15A**). In Ca²⁺-flux experiments, stimulation of EOCs with LL-37 resulted in rapid Ca²⁺-mobilization, a response abolished by the FPR-antagonist boc-PLPLP (**Figure 15B**). To explore a link of the FPR2-cathelicidin axis to re-endothelialization, we compared the adhesion of human EOCs to injured carotid arteries in neutropenic mice and mice with intact WBC. Depletion of neutrophils significantly reduced adhesion of injected EOCs (**Figure 15C**). Similarly, pretreatment of EOCs with boc-PLPLP decreased adhesion to levels observed in neutropenic mice receiving untreated EOCs. Notably, boc-PLPLP did not further reduce adhesion of EOCs injected into neutropenic mice (**Figure 15C**).

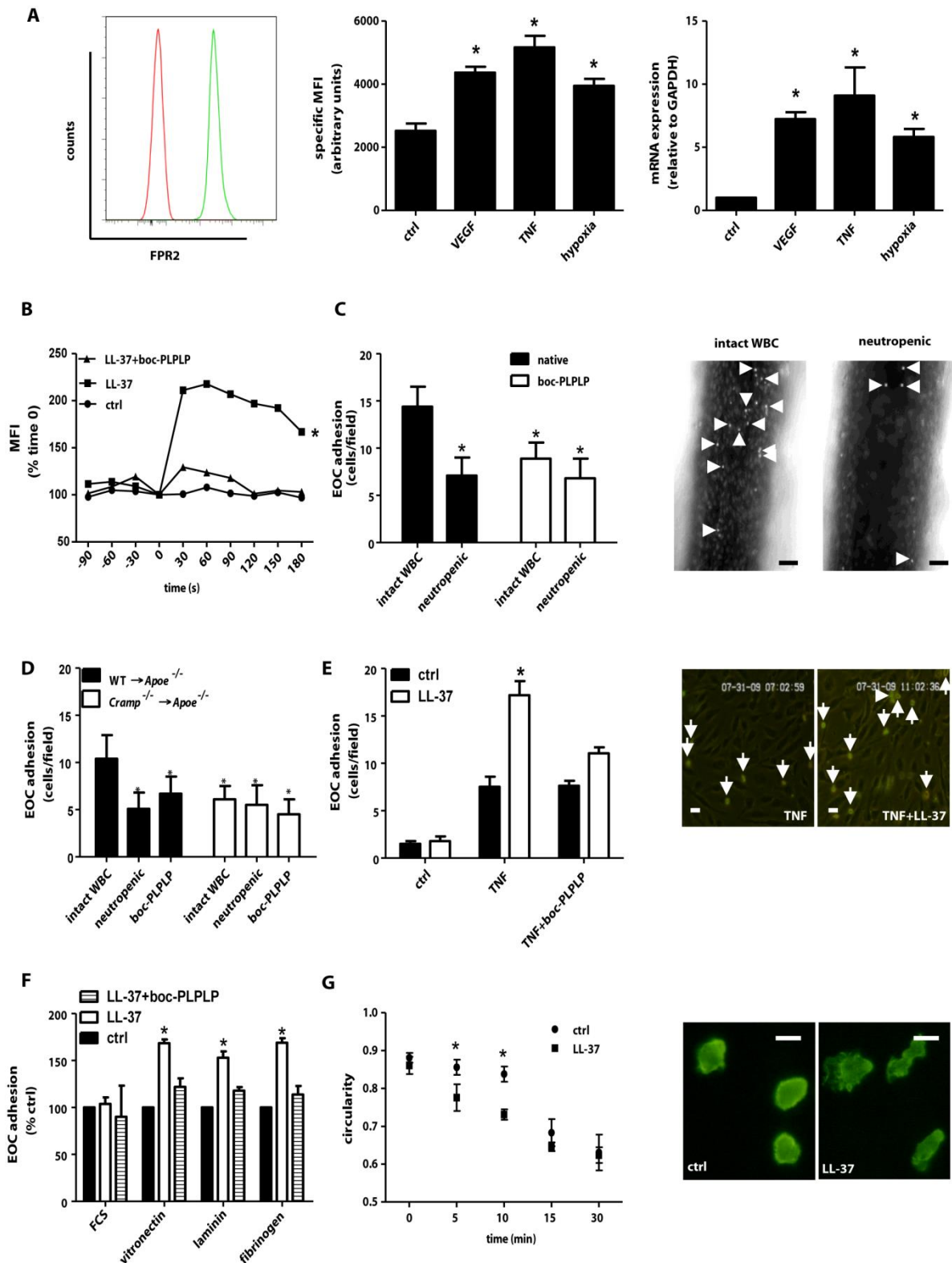


Figure 15. Cathelicidin promotes EOC recruitment. (A) Flow cytometry (left, middle) and real-time PCR (right) analyses of FPR2 expression in early outgrowth cells (EOCs). EOCs were treated with VEGF (20ng/ml), TNF (50ng/ml) or exposed to hypoxic conditions. Representative histograms for FPR2 expression (green) and isotype control (red) at baseline are shown. MFI, mean fluorescence intensity. $n=4$. $*p<0.05$ versus control. Kruskal-Wallis-test with posthoc-Dunn-test. (B) Fluorescence intensity was recorded in EOCs loaded with the Ca^{2+} -sensitive fluo4-AM for 90s before and 180s after stimulation with LL-37 in presence or absence of boc-PLPLP (1 μ M). Shown is the graph of one representative experiment. $n=6$. $*p<0.05$ versus ctrl or LL-37+boc-

PLPLP. One-way analyses of variance followed by Tukey test. **(C,D)** Neutrophil-mediated adhesion of human EOCs to injured carotid arteries involves CRAMP-FPR. Calcein-labeled EOCs pretreated with boc-PLPLP were injected into *Apoe*^{-/-} mice **(C)** or *Apoe*^{-/-} mice transplanted with WT or *Cramp*^{-/-} BM **(D)** with intact WBC or into neutropenic mice 4h after wire-injury. EOCs adherent to injured arteries were counted. Representative images in **C** show adherent EOC (arrows) in mice with intact white blood cell count (WBC) or in neutropenic mice. *n*=6. **p*<0.05 versus untreated EOCs injected into mice with intact WBC. Kruskal-Wallis-test with posthoc-Dunn-test. Scale bars, 100µm. **(E,F)** Adhesion of EOCs to activated HAoECs and matrix proteins is enhanced by LL-37. LL-37 was deposited for 15min on TNF-activated HAoECs. EOCs pre-treated with or without boc-PLPLP were perfused over HAoEC monolayers **(E)** or plates coated with matrix proteins and LL-37 **(F)** and adherent cells were counted. Representative images in **E** show EOC adhesion (arrows) to TNF-activated HAoECs in presence or absence of LL-37. *n*=4-6. **p*<0.05 versus controls and boc-PLPLP treatment. Kruskal-Wallis-test with posthoc-Dunn-test. Scale bars, 20µm. **(G)** Analysis of EOC spreading. EOCs were seeded onto fibrinogen coated with or without (ctrl) LL-37. Circularity was assessed at indicated time points (right). Representative images of F-actin-stained EOCs (left). *n*=4. **p*<0.05 versus control. Mann-Whitney test. Scale bars, 10µm.

A potential interaction of cathelicidin with FPR2 underlying neutrophil-mediated EOC adhesion was addressed in atherosclerotic mice. The adhesion of EOCs was reduced by neutrophil depletion in WT→*Apoe*^{-/-} mice but did not differ between neutropenic and normal *Cramp*^{-/-}→*Apoe*^{-/-} mice (**Figure 15D**). Moreover, EOCs treated with boc-PLPLP before injection into WT→*Apoe*^{-/-} or *Cramp*^{-/-}→*Apoe*^{-/-} mice showed similar adhesion as in neutropenic WT→*Apoe*^{-/-} mice or in *Cramp*^{-/-}→*Apoe*^{-/-} mice with intact WBC or neutropenia, indicating that CRAMP mediates neutrophil-dependent EOC adhesion through FPRs. *In vitro* flow assays confirmed that deposition of LL-37 on TNF-activated HAoECs significantly enhanced EOC arrest, which was abrogated by boc-PLPLP (**Figure 15E**). Moreover, LL-37 co-immobilized with vitronectin, laminin, or fibrinogen enhanced EOC adhesion, which was abolished by the FPR-antagonist (**Figure 15F**). Coordinated cell spreading is essential for effective adhesion, requiring cytoskeletal reorganization and actin polymerization. We therefore analyzed LL-37-dependent spreading of EOCs immobilized on fibrinogen. The presence of LL-37 rapidly decreased circularity (**Figure 15G**), indicating prompt cellular activation and actin reorganization.

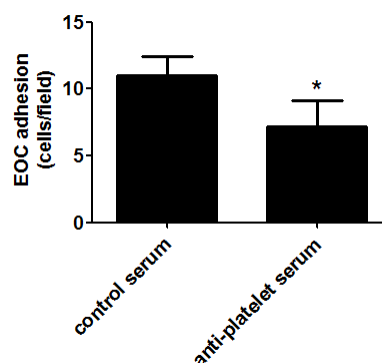


Figure 16. Platelet depletion reduces adhesion of EOCs. Adhesion of injected human EOCs to injured carotid arteries in mice treated with anti-platelet serum or control serum. Data are expressed as number of EOCs adherent to the lumen of the injured artery. *n* = 6. **p*<0.05 versus control serum. Mann-Whitney test.

Moreover, platelet-depleted mice showed reduced EOC adhesion (**Figure 16**), likely attributable to altered EOC interactions with platelets or platelet-derived micro particles [Mause *et al.*, 2010] or secondary effects related to lower neutrophil adhesion in thrombocytopenic mice [Drechsler *et al.*, 2010].

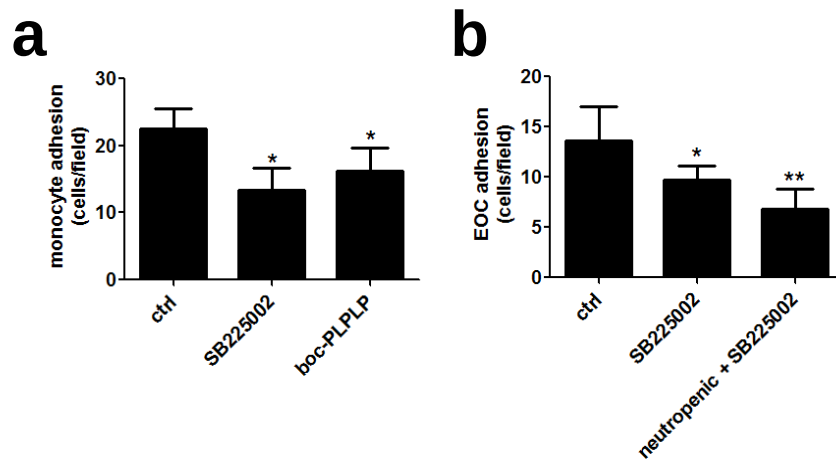


Figure 17. Adhesion of adoptively transferred monocytes and EOCs partially depends on CXCR2. (a) Adhesion of transferred human monocytes to injured carotid arteries following pre-treatment with vehicle (ctrl), a CXCR2 antagonist (SB225002, 100 μ g/ml), or a FPR antagonist (boc-PLPLP, 10 μ M). Data are expressed as cells adherent to the lumen of the injured artery. $n = 6$. * $p < 0.05$ versus control group. (b) Adhesion of adoptively transferred human EOCs to injured carotid arteries following pre-treatment with vehicle (ctrl) or a CXCR2 antagonist (SB225002, 100 μ g/ml). Neutropenia was induced by injection of anti-Ly6G antibody. Data are expressed as cells adherent to the lumen of the injured artery. $n = 9$. * $p < 0.05$ versus control, ** $p < 0.05$ versus SB225002. Kruskal-Wallis-test with posthoc-Dunn-test.

As seen for monocytes, blocking CXCR2 on EOCs reduced their adhesion (**Figure 17**), an effect partially independent of neutrophils, implying a non-redundant role of this putative LL-37 receptor [Zhang *et al.*, 2009]. Antagonists to P2X7 or TLR4, both involved in cathelicidin-dependent cell activation, failed to alter EOC adhesion (**Figure 18A**).

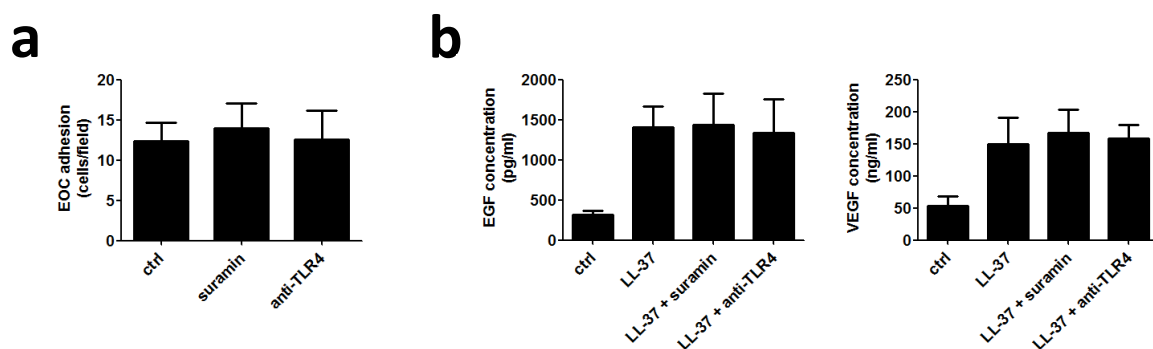


Figure 18. Role of P2 receptors and TLR4 in LL-37-mediated EOC activation. (a) Adhesion of injected human EOCs to injured carotid arteries following pre-treatment with vehicle (ctrl), suramin (100 μ M) or a TLR4-neutralizing antibody (W7C11, 10 μ g/ml). Data are expressed as EOCs adherent to the lumen of the injured artery. $n = 6$. (b) EOCs were treated with medium (ctrl) or LL-37 (1 μ g/ml) in presence or absence of suramin (100 μ M) or a TLR4-neutralizing antibody (W7C11, 10 μ g/ml). $n = 4$. Kruskal-Wallis-test with posthoc-Dunn-test.

4.1.4 Cathelicidins promote survival of EOCs

Once immobilized, EOCs may facilitate re-endothelialization by proliferation, maturation, or secretion of growth factors. We assessed the relevance of LL-37 in the repopulation of scratch wounds inflicted in EOC monolayers. Compared to controls, incubation with LL-37 promoted EOC re-growth into the wounded area (**Figure 19A**). Mechanistically, we investigated the effects of LL-37 on EOC apoptosis, proliferation, and maturation.

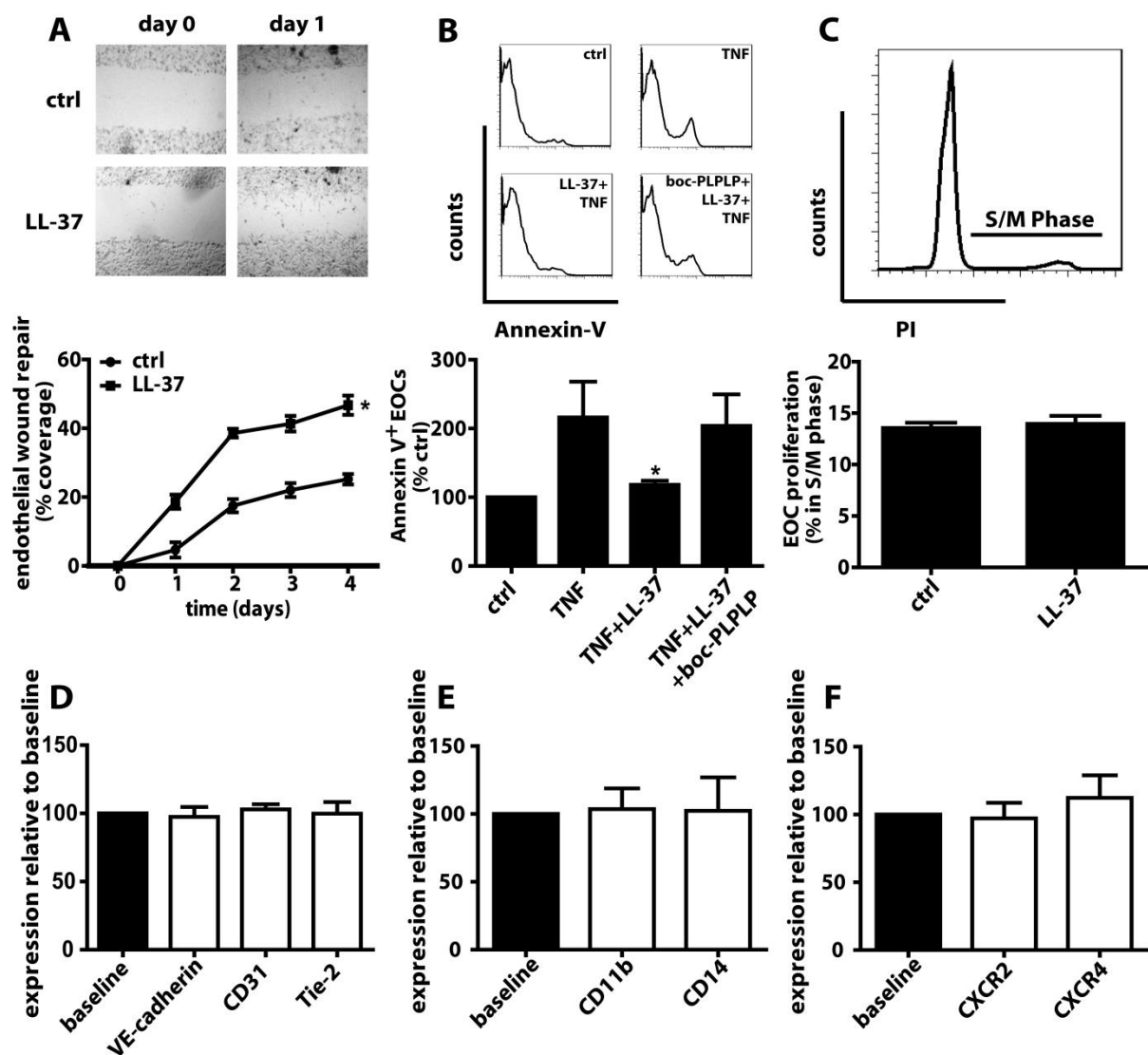


Figure 19. LL-37 promotes EOC survival. (A) EOC monolayers were subjected to scratch injury and the recovered wound area is expressed as percentage of the initial wound area. Representative photomicrographs are displayed. $n=4$. $*p<0.05$ versus ctrl. One-way analyses of variance followed by Tukey test. (B) EOCs were treated with TNF (50ng/ml) in presence or absence of LL-37 or boc-PLPLP and reacted with Representative histograms and percentage of annexin-V⁺ EOCs are displayed. $n=3$. $*p<0.05$ versus TNF and TNF+LL-37+bocPLPLP. Kruskal-Wallis-test with posthoc-Dunn-test. (C) Cell cycle analysis based on propidium iodide incorporation of EOCs treated with or without LL-37. $n=3$. (D-F) FACS analysis of endothelial (D) and myeloid (E) markers, and chemokine receptors (F). Baseline expression was set to 100% (black bars), and expression after LL-37 stimulation (white bars) is shown relative to baseline. $n=3$.

LL-37 prevented TNF-induced EOC apoptosis in a FPR-dependent manner (**Figure 19B**) but did not affect proliferation (**Figure 19C**) or maturation, as evidenced by unaltered expression of endothelial (**Figure 19D**) and myeloid (**Figure 19E**) markers. Moreover, LL-37 did not modulate expression of CXCR2 and CXCR4 (**Figure 19F**), and serum levels of CXCL12 and mCXCL1, ligands of CXCR4 or CXCR2, respectively, were unaltered in neutropenic mice after wire-injury (**Table 6**).

Table 6. mCXCL1 and CXCL12 serum levels in mice with intact WBC or neutropenia.
All values are given in pg/ml venous blood.

	mCXCL1 (KC)	CXCL12 (SDF-1 α)
Intact WBC	107.3	20.1
	+/- 20.2	+/- 8.3
neutropenic	112.7	18.7
	+/- 25.6	+/- 9.7
p-value	0.782	0.851

4.1.5 Cathelicidins modulate secretory properties of EOCs

The low numbers of circulating EOCs and the contribution of endothelial re-growth to arterial healing led us to explore whether the cathelicidin-EOC axis exerts paracrine effects on ECs. As potent sources of growth factors [Mause *et al.*, 2010, Urbich *et al.*, 2005], EOCs exposed to LL-37 showed increased release of VEGF and EGF, which was abolished by boc-PLPLP (**Figure 20A**) but not antagonists to P2X7 or TLR4 (**Figure 18B**). To examine functional properties of EOC secretory products, confluent HAoECs were subjected to scratch injury and incubated with conditioned medium harvested from LL-37-treated EOCs (LL-37/CM), untreated EOCs (CM) or control medium with 1% FCS. To exclude effects of LL-37 in CM, LL-37 was immuno-depleted, as confirmed by dot-blot analysis (**Figure 20B**). Moreover, LL-37 failed to induce or enhance proliferation of HAoECs (**Figure 21**), indicating that direct effects on local EC proliferation are unlikely. Injured HAoEC monolayers in control medium recovered to about 20% within 24 h, and endothelial recovery was slightly improved by CM but markedly enhanced by LL-37/CM (**Figure 20C**).

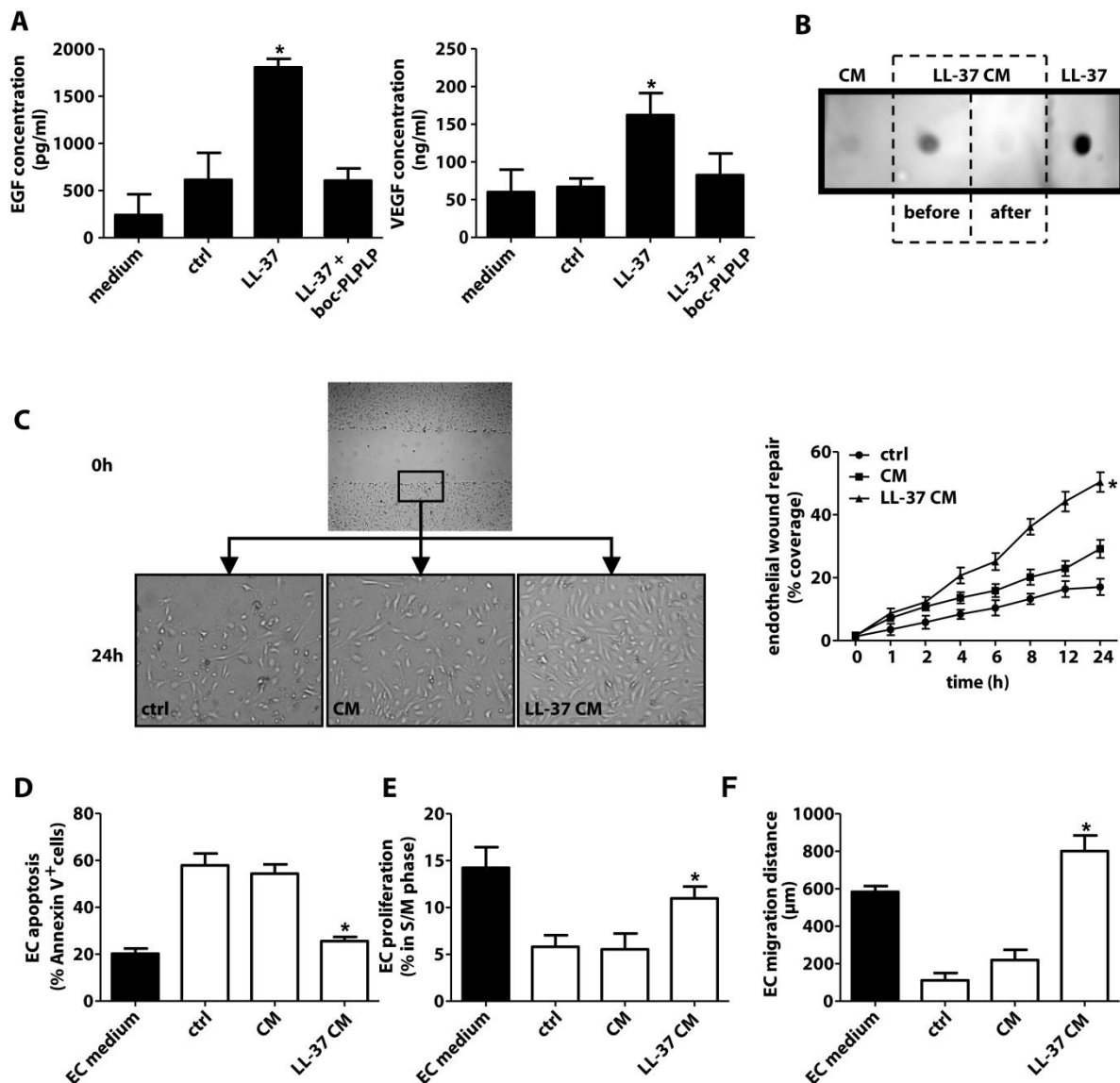


Figure 20. LL-37-treated EOCs exert paracrine proendothelial effects. (A) EOCs were treated with medium (ctrl) or LL-37 (1μg/ml) in the presence or absence of boc-PLPLP. EGF and VEGF were measured in medium or supernatant of non-activated EOCs (ctrl) or LL-37-treated EOCs. $n=4$. * $p<0.05$ versus all bars. Kruskal-Wallis-test with posthoc-Dunn-test. (B) LL-37 was detected in conditioned medium harvested from untreated EOCs (CM), LL-37-treated EOCs before (LL-37 CM, before) or after (LL-37 CM, after) immuno-depletion of LL-37, or synthetic LL-37 by dot blot. (C) Human aortic endothelial cell (HAoEC) monolayers were wounded linearly and the recovered area is expressed as percentage of the initial wound area. Monolayers were treated with medium containing 1% FCS (ctrl), CM from untreated EOCs or LL-37-immuno-depleted CM from LL-37-treated EOCs (LL-37 CM). $n=3$. * $p<0.05$ versus other groups. One-way analyses of variance followed by Tukey test. (D-F) HAoEC apoptosis (D), proliferation (E), and migration (F) is promoted by supernatant of LL-37-treated EOCs. Apoptosis is expressed as percentage of annexin-V⁺ HAoECs. Proliferation was assessed by cell cycle analysis using propidium iodide. The migration distance was measured after time-lapse tracking of HAoEC movement. HAoECs were treated as in (C). EC growth medium (solid bar) served as control. $n=5$. * $p<0.05$ versus ctrl and CM-treatment. Kruskal-Wallis-test with posthoc-Dunn-test.

We analyzed alterations of apoptosis, proliferation or migration, possibly underlying improved recovery with LL-37/CM. Control medium and CM but not LL-37/CM induced apoptosis and reduced proliferation and migration in HAoECs, as compared to EC medium (Figure 20D-F).

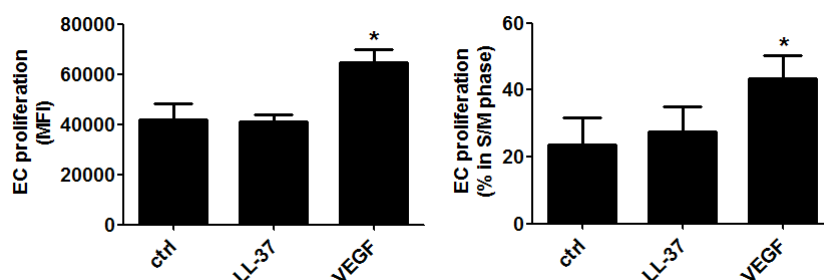


Figure 21. LL-37 does not induce endothelial cell (EC) proliferation. Human aortic ECs were treated with medium (ctrl), LL-37 (1 $\mu\text{g/ml}$, 24 h), or VEGF (20 ng/ml , 24 h) and proliferation was assessed by BrdU incorporation (left) or cell cycle analysis using propidium iodide (right). $n = 5$. * $p < 0.05$ versus control and LL-37 treatment. Kruskal-Wallis-test with posthoc-Dunn-test.

4.1.6 Cathelicidin-biofunctionalization reduces in-stent stenosis

Due to its biocompatibility, corrosion resistance, and elasticity, Nitinol is frequently used for stents. The role of the LL-37-EOC axis in protecting against neointima formation prompted us to develop biofunctionalized stents exploiting this principle. We assessed the biofunctional properties of surface-modified Nitinol in EOC adhesion assays. First, Nitinol foils were aminosilanized (AS) with N-[3-trimethoxysilyl-propyl]ethylenediamine and coated with six-arm star-shaped polyethylene glycol (star-PEG) creating an inert and anti-adhesive surface, as evidenced by minimal unspecific background adhesion of EOCs, when compared to uncoated or fibronectin-coated plastic surfaces or untreated Nitinol foils (Figure 23A). The aminosilanized, star-PEG-coated Nitinol foils were biofunctionalized by dip-coating with recombinant P-selectin, RGD, LL-37 individually or in combination. The adhesion of EOCs to Nitinol foils was unaffected by coating with any proteins alone but markedly enhanced by the combination of P-selectin, RGD, and LL-37 (Figure 23B). This prompted us to explore the effectiveness of LL-37-coated stents in mice. Miniaturized stents were braided from Nitinol wire, aminosilanized, and coated with star-PEG and biofunctionalized by covalently binding P-selectin/RGD without or with LL-37, which was well retained on stent surfaces, as shown by 2-photon microscopy and fluorescent LL-37 (Figure 22).

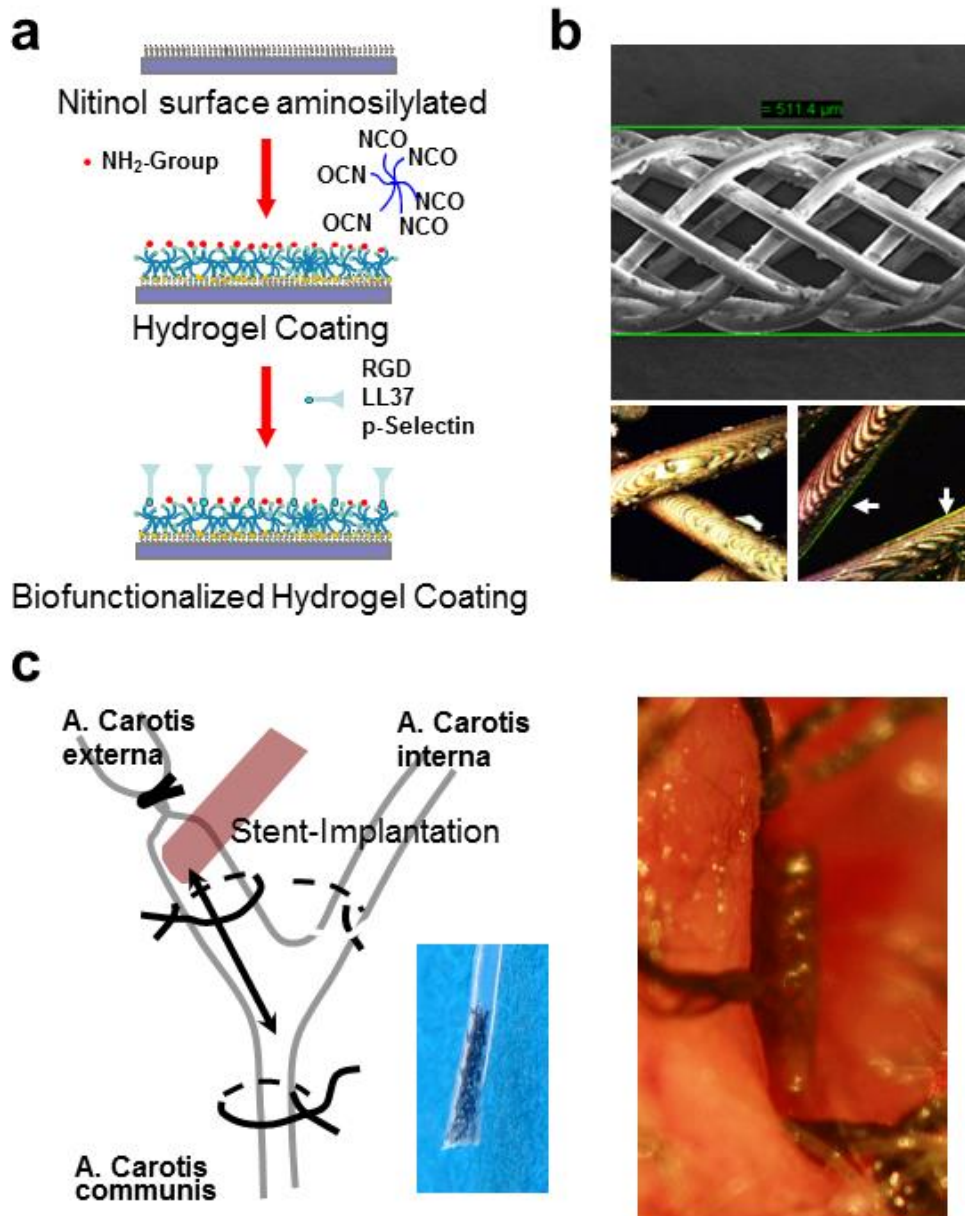


Figure 22. Development of a LL-37-coated Nitinol stent. (a) Schematic overview of Nitinol coating. Nitinol wires were aminosilylated with N-[3-trimethoxysilyl-propyl] ethylenediamine, coated with six-arm star-shaped polyethylene glycol, and biofunctionalized by covalently binding P-selectin and RGD-peptide or a combination of P-selectin, RGD-peptide, and LL-37. (b) Scanning electron microscopy image of a Nitinol stent (top). LL-37 coating was evidenced by use of green fluorescent LL-37 (bottom right, arrows) employing 2-photon microscopy. (c) Schematic overview of stent implantation procedure (left) and in situ image of implanted stent (right).

Stents were implanted into the left carotid artery of *Apoe*^{-/-} mice through insertion into a silicon tube, careful forward feeding of the stent whilst retracting the tube to allow for shape memory-based expansion (**Figure 22**). After one week and 4 weeks, we performed histological analyses to assess lumen and neointima by Giemsa staining. Whereas neointima size was only reduced in mice receiving LL-37-coated stents compared to bare-metal stents,

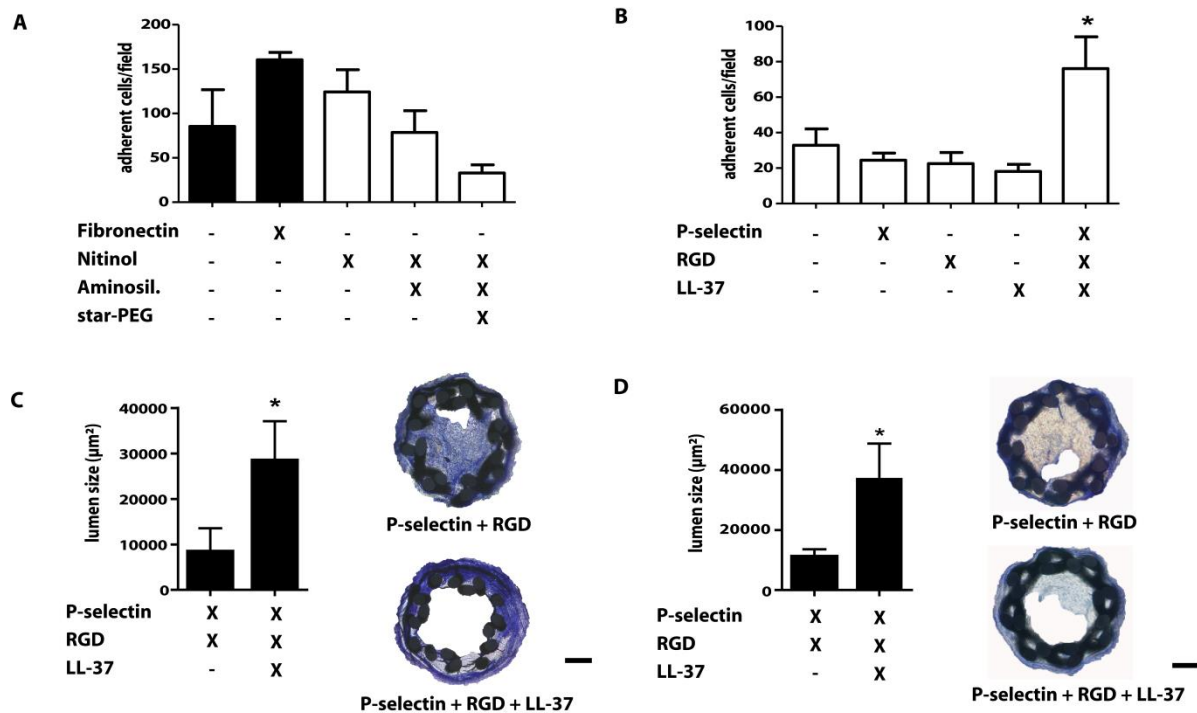


Figure 23. LL-37 coating reduces in-stent stenosis. (A,B) EOCs were seeded onto Nitinol foils coated as indicated for 15min and the number of adherent EOCs was quantified. * $p < 0.05$ versus aminosilanized, star-PEG-coated foils. $n = 4$. Kruskal-Wallis-test with posthoc-Dunn-test. (C,D) RGD/P-selectin- or RGD/P-selectin/LL-37-coated stents were implanted into *Apoe*^{-/-} mice and after 1 (C) or 4 weeks (D) luminal areas were analyzed by Giemsa staining. Representative images are displayed. Scale bars, 100μm. $n = 9$. * $p < 0.05$. Mann-Whitney test.

aminosilanized, star-PEG- and P-selectin/RGD-coated stents without LL-37 (Figure 24), the luminal area was markedly increased in LL-37-coated stents (Figure 23C and D), revealing protective effects against in-stent stenosis. Improved endothelial coverage of the luminal lining with CD31⁺ endothelial cells was observed by immunofluorescence staining in carotid arteries that had received LL-37-coated stents, as compared to control stents, and an abundance of CD31⁺ endothelial cells within the stented area but no significant thrombosis was detected in carotid arteries with LL-37-coated stents (Figure 25).

As seen in other models of arterial injury, for example wire-induced denudation, in mice a period of 4 weeks is commonly sufficient to obtain a maximal degree of neointimal hyperplasia. Hence, it is conceivable that long-term efficiency and patency of LL37-coated stents would persist beyond 4 weeks. To corroborate that CRAMP limits in-stent stenosis, we implanted uncoated Nitinol stents in *Apoe*^{-/-} mice reconstituted with WT or *Cramp*^{-/-} BM. Mice lacking leukocyte CRAMP displayed larger neointima but reduced in-stent lumen size (Figure 26).

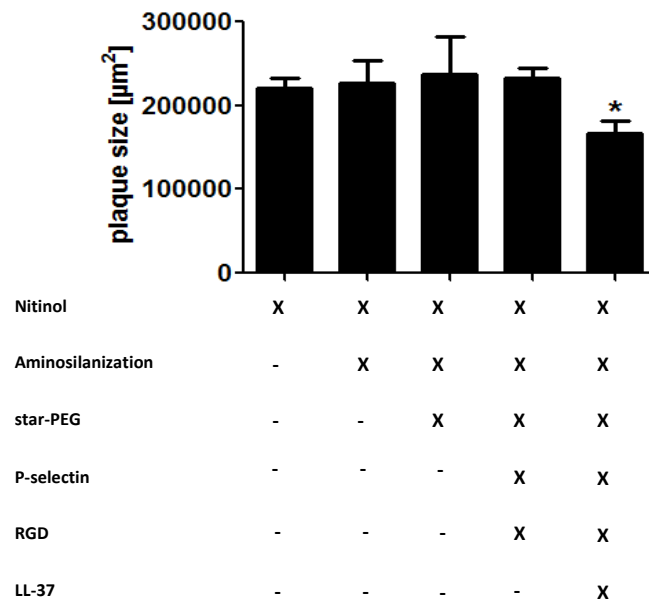


Figure 24. Neointima sizes are reduced in mice receiving LL-37-coated stents. Stents prepared as indicated were implanted into *Apoe*^{-/-} mice and the neointimal area was analyzed one week later after Giemsa staining. *n* = 7-10. **p* < 0.05 versus all other groups. Kruskal-Wallis-test with posthoc-Dunn-test

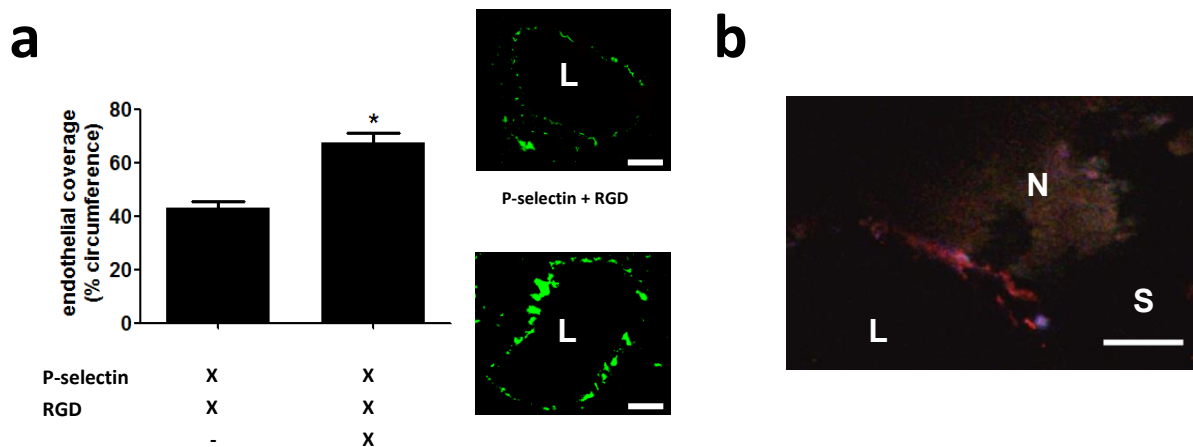


Figure 25. Re-endothelialization is improved in arteries with LL-37-coated stents. **A:** Biofunctionalized stents coated as indicated were implanted into *Apoe*^{-/-} mice and the luminal endothelial coverage was assessed by immunofluorescence staining for CD31 in cryosections directly adjacent to the proximal end of the stent after one week. *n* = 8. **p* < 0.05 versus P-selectin/RGD-coated stent. Mann-Whitney test. Scale bar, 25 μ m. L, lumen. **B:** High abundance of CD31⁺ endothelial cells (red) in the luminal lining within the stented area of carotid arteries that had received LL-37-coated stents, as evidenced by immunofluorescence staining and 2-photon microscopy of agarose-embedded arteries prepared one week after stent insertion. Scale bar, 25 μ m. L, lumen; N, neointima; S, stent strut.

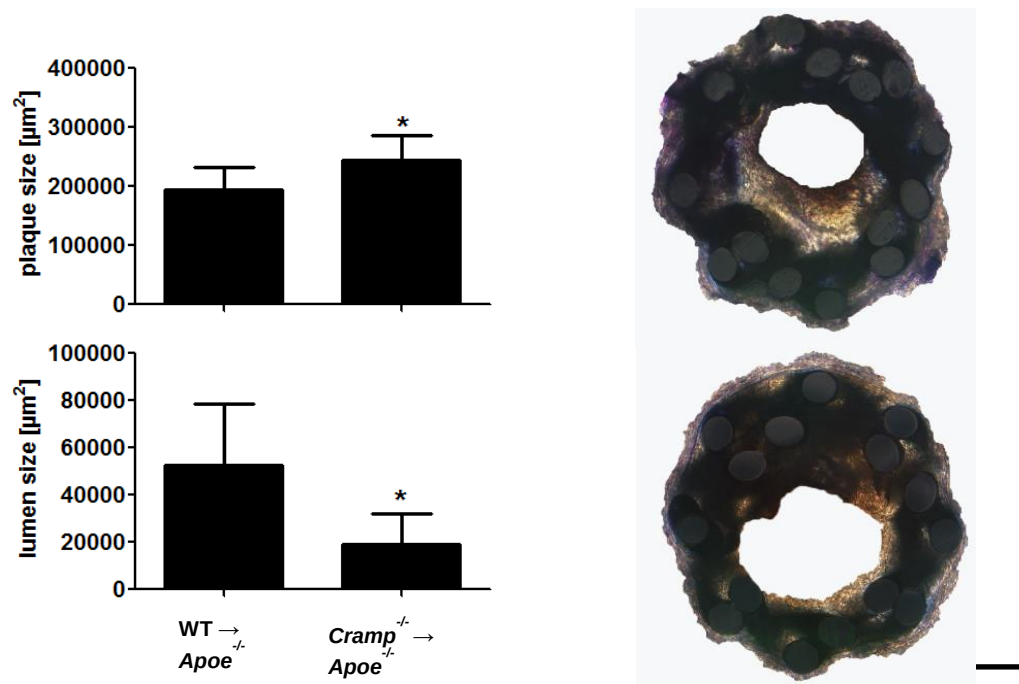


Figure 26. CRAMP prevents in-stent stenosis. $Apoe^{-/-}$ mice were reconstituted with bone marrow from C57Bl/6 mice ($WT \rightarrow Apoe^{-/-}$) or from $Cramp^{-/-}$ mice ($Cramp^{-/-} \rightarrow Apoe^{-/-}$). Nitinol stents were implanted and based on Giemsa staining (right) neointimal area (top left) and lumen size (bottom left) were analyzed one week later. Scale bar, 100 μm . $n = 8$. * $p < 0.05$ versus $WT \rightarrow Apoe^{-/-}$. Mann-Whitney test.

Neutrophil-derived cathelicidin promotes adhesion of classical monocytes

Neutrophils dominate the initial wave of leukocytes emigrating into inflamed tissues [Witko-Sarsat *et al.*, 2000]. This first wave of neutrophil extravasation precedes a second wave of monocyte extravasation. Recruited neutrophils are thought to contribute to this cellular switch by several mechanisms [Soehnlein, Lindbom & Weber, 2009; Soehnlein & Lindbom, 2010] including the release of soluble factors like neutrophil granule proteins which are deposited at the site of inflammation [Soehnlein *et al.*, 2010]. Indeed, supernatants of activated neutrophils from patients with specific granule deficiency show a reduced capacity to attract monocytes despite normal monocyte exhibiting chemotaxis activity to other stimuli *in vitro* [Gallin *et al.*, 1982]. Following this observation, several neutrophil granule proteins with monocyte-attracting activity were identified. Among them are cathelicidin (human: LL37, mouse: CRAMP) [Yang *et al.*, 2000], cathepsin G [Chertov *et al.*, 1997], α -defensins [Territo *et al.*, 1989], and azurocidin [Chertov *et al.*, 1997].

Peripheral blood monocytes constitute a heterogeneous population of circulating leukocytes in both humans [Passlick *et al.*, 1989] and mice [Geissmann, Jung & Littman, 2003]. Based on the expression of Gr1, classical (inflammatory, Ly6C^{hi}) and non-classical (resident, patrolling, Ly6C^{lo}) mouse monocytes can be discriminated. Functionally, non-classical monocytes are thought to contribute to phagocytosis, wound healing, and tissue remodeling, whereas classical monocytes dominate inflammatory processes such as the early response in infections, but also sterile inflammatory conditions such as myocardial infarction or atherosclerosis [Woollard & Geissmann, 2010]. In humans, monocyte subsets are discriminated based on their CD14 and CD16 expression profile with classical monocytes being CD14⁺⁺CD16⁻, intermediate monocytes being CD14⁺⁺CD16⁺, and non-classical monocytes being CD14⁺CD16⁺⁺ (12).

While several studies have shown the important chemotactic activities of neutrophil granule proteins towards monocytes, the role of neutrophil secretory products in early steps of monocyte recruitment have not been studied. Hence, we here investigated the role of neutrophils and their granule constituents in their capacity to differentially induce adhesion of monocyte subsets.

4.2.1 Cathelicidin induces firm adhesion of classical monocytes

To study the importance of neutrophils in the adhesion of monocytes we injected TNF into the cremaster of $Cx_3cr1^{egfp/wt}$ mice carrying green fluorescent monocytes and recorded the adhesion of monocytes *in vivo*. To discriminate between classical monocytes and non-classical monocytes, an antibody to Ly6C was introduced which labels classical monocytes (Figure 27).

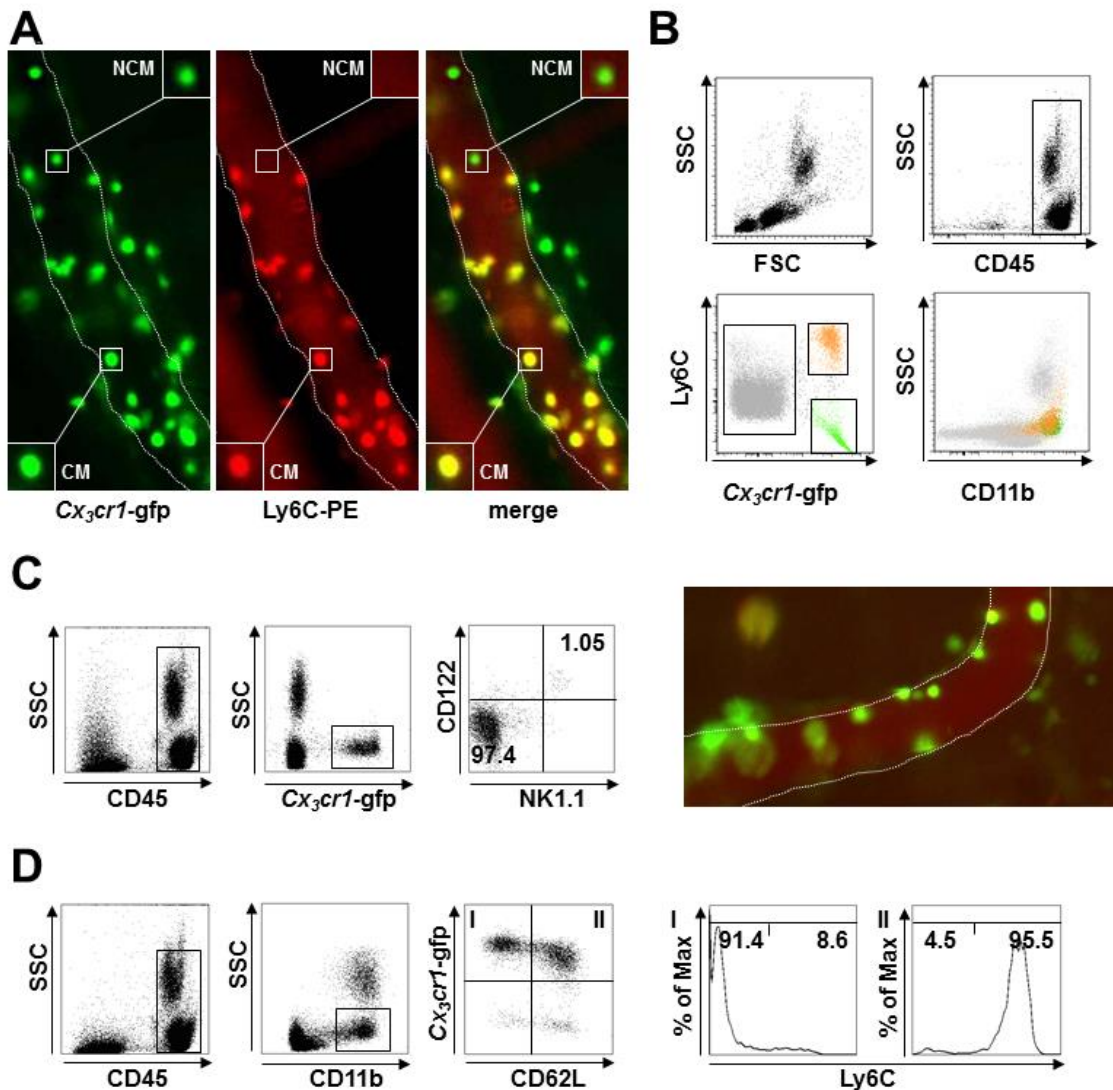


Figure 27. In vivo labeling of classical monocytes. To discriminate between monocyte subsets an antibody to Ly6C (1 μ g) was introduced i.v. into $Cx_3cr1^{egfp/wt}$ mice. **A:** Example of intravital microscopy of the cremaster muscle using a beam splitter to allow recording of two emission wavelengths. CM, classical monocyte; NCM, non-classical monocyte. **B:** Blood was drawn from $Cx_3cr1^{egfp/wt}$ mice injected with an antibody to Ly6C and stained with antibodies to CD45 and CD11b. Classical monocytes (Ly6C⁺gfp⁺, orange) and non-classical monocytes (Ly6C⁻gfp⁺, green) also appear CD11b⁺SSC^{lo}. In contrast, gfp⁻ leukocytes (grey) are either CD11b⁺SSC^{hi} (neutrophils) or CD11b⁻SSC^{lo} (lymphocytes). **C:** $Cx_3cr1^{egfp/wt}$ mice were injected i.v. with a PE-conjugated antibody to NK1.1. Labeling of NK cells was confirmed by counterstaining with CD122. In vivo, the antibody to NK1.1 did not label adherent gfp⁺ cells. **D:** Intravenous injection of an antibody to Ly6C efficiently labels classical monocytes. Anti-Ly6C-PE (1 μ g) was introduced i.v. into $Cx_3cr1^{egfp/wt}$ mice. To identify labeling efficiency, white blood cells were counterstained with antibodies to CD45, CD11b, and CD62L.

In these experiments, neutropenic mice displayed reduced adhesion of classical monocytes ($\text{Ly6C}^+/\text{gfp}^+$) to postcapillary venules, whereas rolling flux of classical monocytes was increased. In contrast, adhesion of non-classical monocytes ($\text{Ly6C}^-/\text{gfp}^+$) remained unaffected (**Figure 28A-C**). With the reported importance of neutrophil granule proteins in the recruitment of monocytes [Lai & Gallo, 2009] we aimed at addressing the role of granule proteins in monocyte adhesion. Neutrophil-derived azurocidin, cathepsin G, α -defensins, and cathelicidins have been suggested to be involved in neutrophil-dependent monocyte activation [Gleissner, von Hundelshausen & Ley, 2008; Soehnlein & Lindbom, 2010]. As azurocidin and defensins are absent in mouse neutrophils [Belaaouaj *et al.*, 1999; Eisenhauer & Lehrer, 1992], we focused on the role of cathepsin G and cathelicidin in

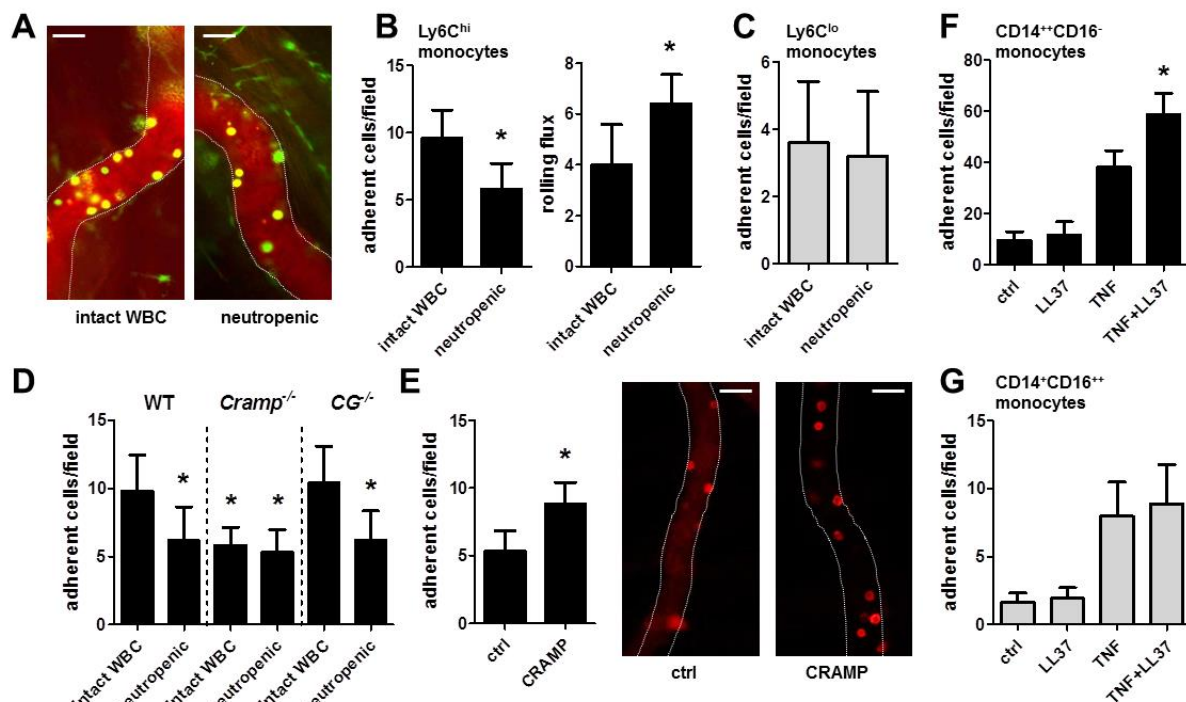


Figure 28. Neutrophil-derived cathelicidin enhances adhesion of classical monocytes. **A-C:** Neutropenia reduces adhesion of classical monocytes. $\text{Cx}_3\text{cr1}^{\text{egfp}/\text{WT}}$ mice with intact white blood cell count or neutropenia (1A8, 50 μg i.p., 12h prior to experiment) were injected intrascrotally with TNF (50ng, 12h). To discriminate between monocyte subsets an antibody to Ly6C (1 μg) was introduced i.v. **A:** Representative images, scale bar 20 μm . **B:** Adhesion and rolling flux of classical monocytes. * Significant difference from respective control. **C:** Adhesion of non-classical monocytes. $n=6$. **D:** Comparison of adhesion of Ly6C-labeled classical monocytes in indicated mouse strains with intact white blood cell count or neutropenia following intrascrotal TNF injection (50ng, 12h). * Significant difference compared to wild type (WT) mice with intact white blood cell count, $n=6$. **E:** Intrascrotal injection of CRAMP in $\text{Cramp}^{-/-}$ mice restores adhesion of classical monocytes. $\text{Cramp}^{-/-}$ were intrascrotally injected with TNF (ctrl) or with TNF and CRAMP (CRAMP, 1 μg). Adhesion of Ly6C-labeled classical monocytes was assessed by intravital microscopy. $n=5$. Representative images are displayed. Scale bar 20 μm . **F/G:** Human classical $\text{CD14}^+\text{CD16}^-$ (F) or non-classical $\text{CD14}^+\text{CD16}^{++}$ monocytes (G) were perfused over TNF-activated (20ng, 12h) HUVEC monolayer and the number of adherent cells was quantified. LL37 (1 μg) was deposited 15 min prior to perfusion. $n=6$. * Significant difference compared to TNF-activated HUVEC.

neutrophil-mediated monocyte adhesion. Adhesion of classical monocytes in TNF-stimulated cremaster muscles was impaired in cathelicidin-deficient (*Cramp*^{-/-}) mice when compared to wild type (WT) control animals, a response not further aggravated by neutrophil depletion (**Figure 28D**). In addition, intrascrotal injection of CRAMP restored the defect in *Cramp*^{-/-} mice (**Figure 28E**). In contrast, cathepsin G-deficient (*CG*^{-/-}) mice exhibited no impaired adhesion of classical monocytes (**Figure 428D**). In line, neutrophil depletion reduced classical monocyte adhesion in *CG*^{-/-} mice indicating that not cathepsin G but CRAMP is relevant for neutrophil-dependent monocyte adhesion.

To further corroborate our findings with human monocyte subsets, we performed *in vitro* flow chamber assays. Human classical CD14⁺⁺CD16⁻ (**Figure 28F**) and non-classical CD14⁺CD16⁺⁺ monocytes (**Figure 28G**) were perfused over human umbilical vein endothelial cells (HUVEC). Adhesion of either subset was enhanced when HUVEC were activated with TNF. However, deposition of LL37 on HUVEC 15 minutes prior to monocyte perfusion specifically enhanced adhesion of classical CD14⁺⁺CD16⁻ monocytes. As treatment of HUVEC with LL37 did not impact the expression of endothelial adhesion molecules (**Figure 29**), we concluded that the LL37-mediated increase in monocyte adhesion is not achieved by modulating endothelial cell adhesion molecule expression.

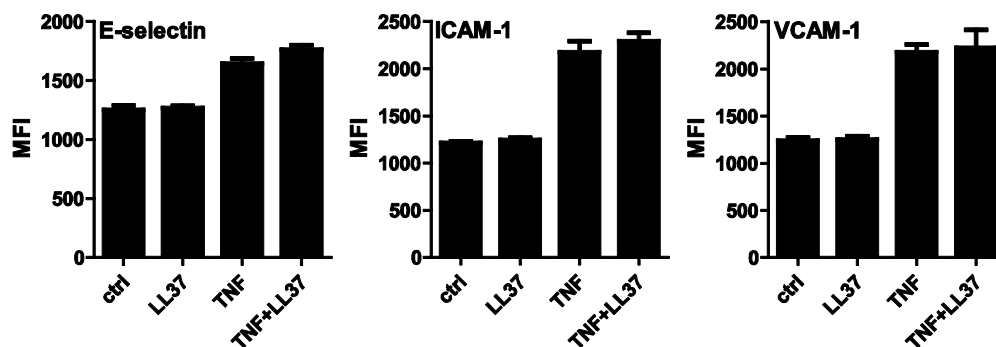


Figure 29. LL37 does not induce endothelial cell adhesion molecule expression. Human umbilical vein endothelial cells were treated with buffer (ctrl), LL37 (1 μ g, 15 min), TNF (20 ng, 12 h), or a combination of both. Expression of E-selectin, ICAM-1, or VCAM-1 was measured by use of a fluorescence plate reader and directly conjugated antibodies towards the respective adhesion molecule. $n = 3$ for each bar.

4.2.2 Neutrophil-derived cathelicidin is transported in a baso-apical direction across the endothelium

To study the dynamics of cathelicidin release and *in vivo* presentation, we injected monocyte-depleted *Lysm^{gfp/gfp}* mice (thus creating mice with fluorescent neutrophils only) with TNF intrascrotally and recorded the adhesion and extravasation of neutrophils. In addition, microbeads conjugated with an antibody to CRAMP were injected to visualize endothelial bound CRAMP (**Figure 30A**). While adhesion of neutrophils occurred rapidly and peaked at 2h, endothelial-bound CRAMP was not detected until 4h and peaked between 4 and 12h after TNF stimulation thus correlating more closely with the number of emigrated neutrophils. During this time, neutrophil adhesion decreases, whereas the number of emigrated neutrophils reaches its maximum (**Figure 30B, C**).

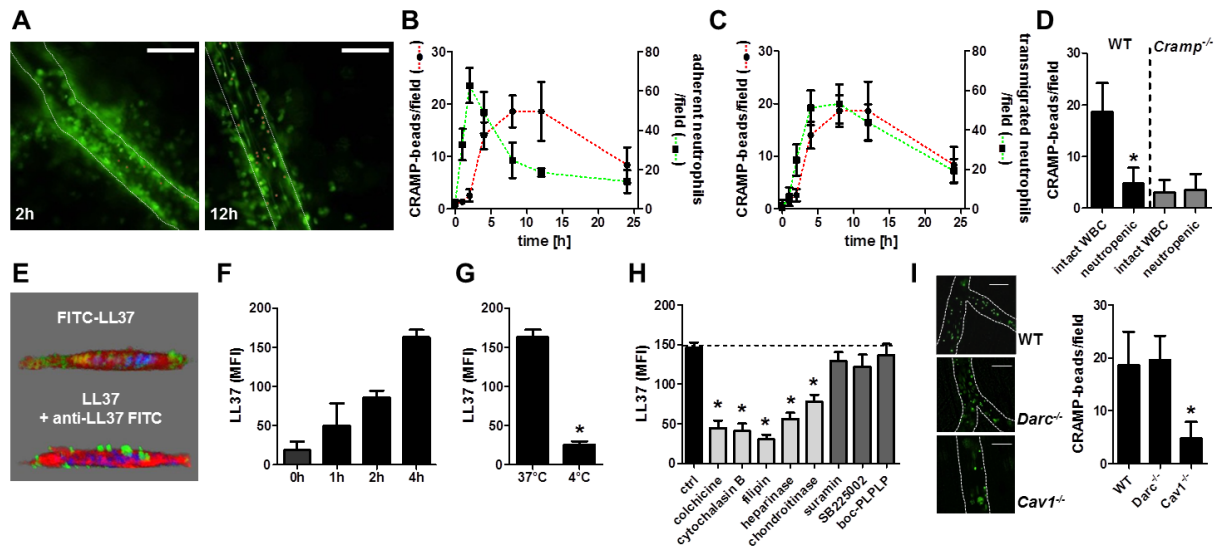


Figure 30. Neutrophil-derived cathelicidin is transported in a baso-apical direction across the endothelium. **A-C:** Luminal CRAMP accumulation parallels with neutrophil extravasation but not neutrophil adhesion. *Lysm^{gfp/gfp}* mice were intrascrotally injected with TNF (50ng) and the adhesion (B, green line) and transmigration (C, green line) of neutrophils was quantified by intravital microscopy at indicated time points. To assess luminal CRAMP presentation, immobilization of G-protein coupled fluorescent beads conjugated with an antibody to CRAMP was analysed (red line in B and C). *n*=4 for each data point. Representative images for indicated time points are displayed in A. For better visibility, immobilized beads were marked with red dots. Scale bar, 50µm. **D:** Wild type (WT) or *Cramp^{-/-}* mice with intact white blood cell count (WBC) or neutropenia were intrascrotally injected with TNF (50ng, 12h). Luminal CRAMP presentation was assessed after injection of G-protein coupled fluorescent beads conjugated with antibodies to CRAMP. * indicates significant difference compared to respective mouse strain with intact WBC. *n*=5. **E:** LL37 is transported across HUVEC grown on transwell filter inserts. LL37 was added to the bottom well and analyses were made 4h later by 2-photon microscopy. LL37 (green) was visualized by a fluorescent tag (top) or by antibody staining of non-permeabilized endothelial cells (bottom). Cell membrane, red; Nucleus, blue. **F:** Antibody detection of transcytosed LL37 on the apical side of endothelial cells in time course experiments. **G/H:** Transcytosis experiments performed at 4 °C (G) or by pretreatment of endothelial cells with indicated inhibitors (H). *n*=6 for each bar. * Significant difference from ctrl. **I:** CRAMP transportation is impaired in *Cav1^{-/-}* mice. Neutropenic WT, *Darc^{-/-}*, or *Cav1^{-/-}* mice were intrascrotally injected with TNF (50ng, 4h) and CRAMP (1µg). Luminal CRAMP presentation was assessed after injection of G-protein coupled fluorescent beads conjugated with an antibody to CRAMP. Scale bar is 20µm in representative images. * Significant difference compared to wild type mice. *n*=4.

Depletion of neutrophils reduced the number of adherent anti-CRAMP-conjugated microbeads to a level observed in *Cramp*^{-/-} mice thus confirming that CRAMP in this location is neutrophil derived (**Figure 30D**). To further investigate alternative CRAMP sources, we analyzed intracellular CRAMP expression in both endothelial cells and in neutrophils obtained from enzymatically digested cremaster muscles. To discriminate between adherent and extravasated neutrophils, an antibody to Ly6G was injected intravenously 5 minutes prior to sacrifice [Grommes *et al.*, 2012]. In these experiments, minor amounts of CRAMP were detected in endothelial cells (**Figure 31**). In contrast, we could evidence large amounts of CRAMP in adherent neutrophils which were decreased in emigrated neutrophils suggesting discharge of CRAMP from emigrating neutrophils (**Figure 31**).

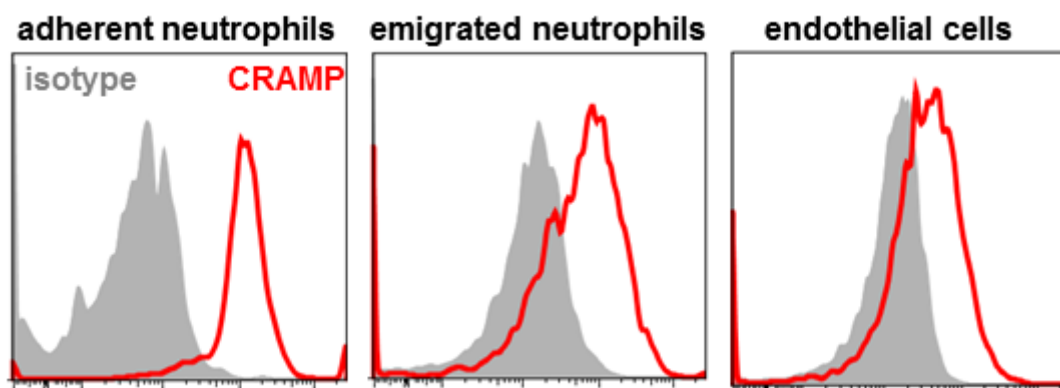


Figure 31. Neutrophils release CRAMP during extravasation. Mice were injected intrascrotally with TNF (50ng, 4h) and an antibody to Ly6G (1A8, 1µg) was injected 5 minutes prior to sacrifice to label adherent neutrophils. Cremaster muscles were excised and enzymatically digested. Intracellular CRAMP was measured in adherent and emigrated neutrophils as well as in endothelial cells. Representative histograms of three independent experiments display specific CRAMP staining as well as the respective isotype control fluorescence.

Since our *in vivo* experiments suggested that cathelicidins are transported across the endothelium in a baso-apical direction, we aimed at studying such mechanism *in vitro*. HUVEC were grown on tissue culture filter inserts and stimulated with TNF. FITC-LL37 was added to the bottom well and LL37 transportation was assessed by two-photon microscopy. In these experiments LL37 was found to accumulate on the endothelial cell surface (**Figure 30E**). Transcytosed LL37 was assessed by fluorescence resulting from antibody-stained, surface-bound LL37. In time course studies we found that LL37 accumulated on the HUVEC surface not until 2 h (**Figure 30F**). The transendothelial transportation was abrogated at 4°C (**Figure 30G**) and by pretreatment with colchicine or cytochalasin B (**Figure 30H**). Cathelicidins were reported to act through FPR2, CXCR2, and P2X7 [Nagaoka, Tamura &

Hirata, 2006; Zhang *et al.*, 2009]. However, pretreatment of HUVEC with boc-PLPLP (antagonist to FPRs), SB225002 (antagonist to CXCR2), or suramin (antagonist to P2X7) did not impact the transendothelial LL37 transportation (**Figure 30H**). As highly cationic polypeptide, we suspected that LL37 might interact with endothelial proteoglycans. To this end we cleaved endothelial heparansulfate or chondroitinsulfate side chains by use of heparinase or chondroitinase and recorded its effect on LL37 transcytosis. Interestingly, pretreatment with either enzyme markedly reduced the transendothelial transportation across the endothelium. Finally, targeting caveolae-mediated transportation by pre-treatment with filipin resulted in clear-cut decreases of LL37 transcytosis. Since Duffy antigen/receptor for chemokines (DARC) facilitates transport of chemokines across the endothelium [Pruenster *et al.*, 2009] we also wanted to address its involvement in the transendothelial transportation of CRAMP. However, in our hands HUVEC activated with TNF did not express DARC and we could not detect an interaction between DARC and LL37 (**Figure 32**).

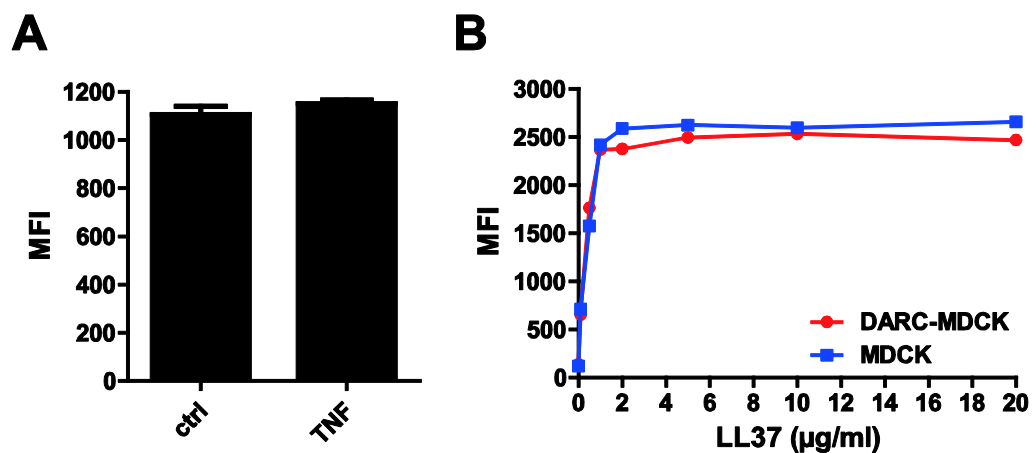


Figure 32. Cathelicidin does not interact with duffy antigen/receptor for chemokines (DARC). **A:** TNF does not induce expression of DARC on human umbilical vein endothelial cells (HUVEC). HUVEC were treated with TNF (20 ng, 12 h) and the expression of DARC was analyzed by fluorescence plate reader. $n = 3$. **B:** LL37 does not bind to DARC. DARC-expressing Madin-Darby canine kidney (DARC-MDCK) or control MDCK cells were incubated with fluorescent LL37 at various concentrations and binding was assessed by flow cytometry. Displayed is one representative experiment.

We next injected CRAMP intrascrotally into neutropenic WT mice and detected its luminal appearance by intravital microscopy. In WT mice luminal CRAMP was detected 4h after intrascrotal CRAMP injection, a response abrogated in caveolin 1-deficient (*Cav*^{-/-}) mice (**Figure 30I**). Immobilization of anti-CRAMP microbeads was not seen when PBS was used instead of CRAMP (not shown). However, the transportation of CRAMP across the endothelium was maintained in *Darc*^{-/-} mice.

4.2.3 Cathelicidin-dependent recruitment requires FPR2 activation on monocytes

Cathelicidins were reported to activate target cells through various receptors including FPR2, CXCR2, and P2X7. To find out which of these receptors is relevant in cathelicidin-mediated adhesion of classical monocytes, we performed *in vitro* flow chamber assays (**Figure 33A**). Monocytes were pre-treated with suramin, SB225002, or boc-PLPLP and perfused over TNF-activated HUVEC. While all antagonists were without effects when classical CD14⁺⁺CD16⁻ monocytes were perfused over TNF-activated HUVEC, boc-PLPLP specifically abrogated

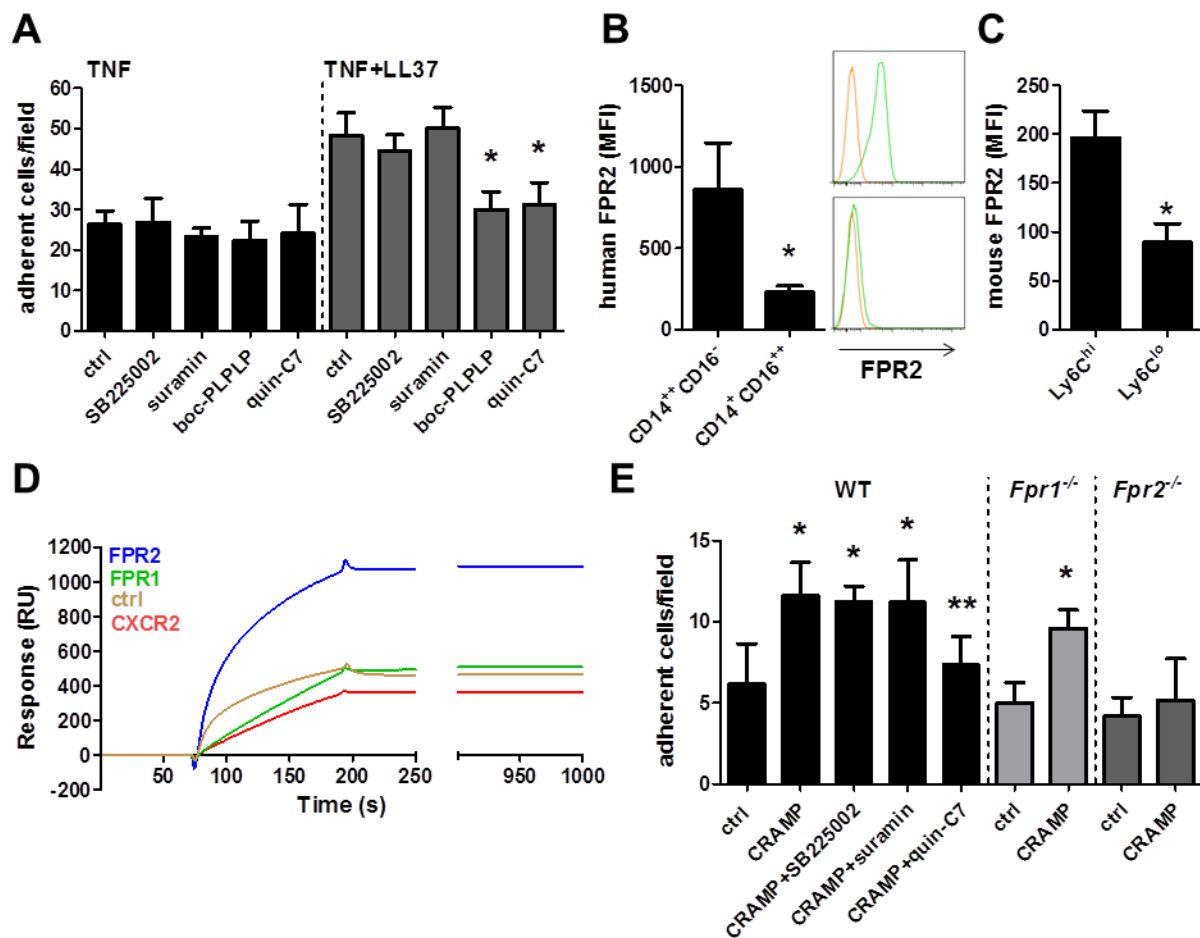


Figure 33. Cathelicidin-induced monocyte adhesion requires FPR2. **A:** Human classical CD14⁺⁺CD16⁻ monocytes were perfused over TNF-activated (20ng, 12h) HUVEC and the number of adherent cells was quantified. LL37 (1μg) was deposited 15 min prior to perfusion. Monocytes were left untreated (ctrl) or were pretreated for 15 min with SB225002 (blocks CXCR2, 100nM), suramin (blocks P2X7, 100nM), boc-PLPLP (blocks FPRs, 100nM), or quin-C7 (blocks FPR2, 1μM). * significant difference compared to respective control. n=8. **B/C:** FPR2 expression was analysed on human (B) or mouse (C) classical and non-classical monocytes using flow cytometry. Representative histograms in B display staining with isotype control IgG (orange) and a FPR2 antibody (green). **D:** Representative surface plasmon resonance sensorgrams of binding of indicated receptors expressed in proteoliposomes to immobilized LL37 on a CM4-sensorchip. Empty proteoliposomes were used as control. **E:** Wild type (WT), *Fpr1*^{-/-}, or *Fpr2*^{-/-} mice were rendered neutropenic and injected with TNF (ctrl) or with TNF and CRAMP (CRAMP, 1μg). Antagonists to CXCR2 (SB225002, 5mg/kg), P2X7 (suramin, 10mg/kg), and FPR2 (quin-C7, 10mg/kg) were administered i.v. 30 minutes prior to recording. Adhesion of Ly6C-labeled classical monocytes was assessed by intravital microscopy. n=6 for each bar. * Significant difference compared to respective control group, ** significant difference compared to CRAMP-treated WT mice.

LL37-mediated increases in classical CD14⁺⁺CD16⁻ monocyte adhesion. Since boc-PLPLP blocks activation of all FPR family members we further used specific antagonists to FPR1 and FPR2. In these experiments, the FPR2 specific antagonist quin-C7 (**Figure 33A**) but not the FPR1-specific antagonist spinorphin (not shown) abrogated LL37-dependent adhesion of classical CD14⁺⁺CD16⁻ monocytes.

We further suspected that differences in FPR2 expression levels between monocyte subsets may explain the specificity of LL37 in enhancing adhesion of classical monocytes but not of non-classical monocytes. Analysis of FPR2 expression revealed that FPR2 is expressed to a higher degree on classical CD14⁺⁺CD16⁻ monocytes as compared to non-classical CD14⁺CD16⁺⁺ monocytes (**Figure 33B**), thus offering an explanation for the specificity of cathelicidin-dependent adhesion. Furthermore, the FPR2 expression on CD14⁺⁺CD16⁺ monocytes (MFI 614±27) was found to be in between the FPR2 expression on classical and non-classical monocytes. In line, classical monocytes in mice carry higher levels of FPR2 when compared to their non-classical counterpart (**Figure 33C**). As few data exist on direct interaction of LL37 with its putative receptors, we aimed at analyzing the interaction of LL37 and FPR1, FPR2, CXCR2, and P2X7 biochemically. For this we performed surface plasmon resonance experiments employing recombinant receptors expressed in proteoliposomes thus maintaining the three dimensional chemokine receptor structure. LL37 was immobilized on a CM4 chip and superfused with receptor-containing proteoliposomes (**Figure 33D**). With this approach we were able to detect a clear binding of LL37 to FPR2, while the interaction with FPR1 or CXCR2 was not different when compared to empty liposomes. For P2X7 we found no interaction with LL37 (not shown).

To further investigate if cathelicidin-mediated adhesion *in vivo* depends on employment of FPR2, we injected CRAMP along with TNF into the scrotum of neutropenic WT mice. Receptor utilization was initially studied by use of antagonists to FPRs, P2X7, and CXCR2. In these experiments inhibition of FPRs by quin-C7 clearly reduced CRAMP-mediated adhesion, while the other antagonists were without effects (**Figure 33E**). To further corroborate this, we injected neutropenic *Fpr1*^{-/-} or *Fpr2*^{-/-} mice with TNF and CRAMP. While CRAMP injection resulted in increased adhesion in *Fpr1*^{-/-} mice, this response was not observed in *Fpr2*^{-/-} mice (**Figure 33E**).

4.2.4 Cathelicidins induce integrin activation on classical monocytes

Thus far we have shown that neutrophil-derived cathelicidins resemble a functional similarity with arrest chemokines [Ley, 2003]. The latter mediate firm adhesion through inside-out integrin signaling and subsequent changes in integrin conformation towards an extended, high affinity state. Classical ligands for VLA4 (CD49d/CD29, $\alpha_4\beta_1$ integrins) and Mac1 (CD11b/CD18, $\alpha_M\beta_2$ integrin) are VCAM-1 and ICAM-1, respectively. To test the importance of these two ligands for LL37-mediated adhesion, we perfused classical CD14⁺⁺CD16⁻ monocytes over plates coated with P-selectin/ICAM-1 or P-selectin/VCAM-1 (**Figure 34A,B**). In these experiments, co-immobilization of LL37 or MCP1 strongly enhanced monocyte adhesion. This response was fully abrogated by a blocking antibody to CD11b or CD49d. While the LL37-dependent monocyte adhesion was abolished in presence of an

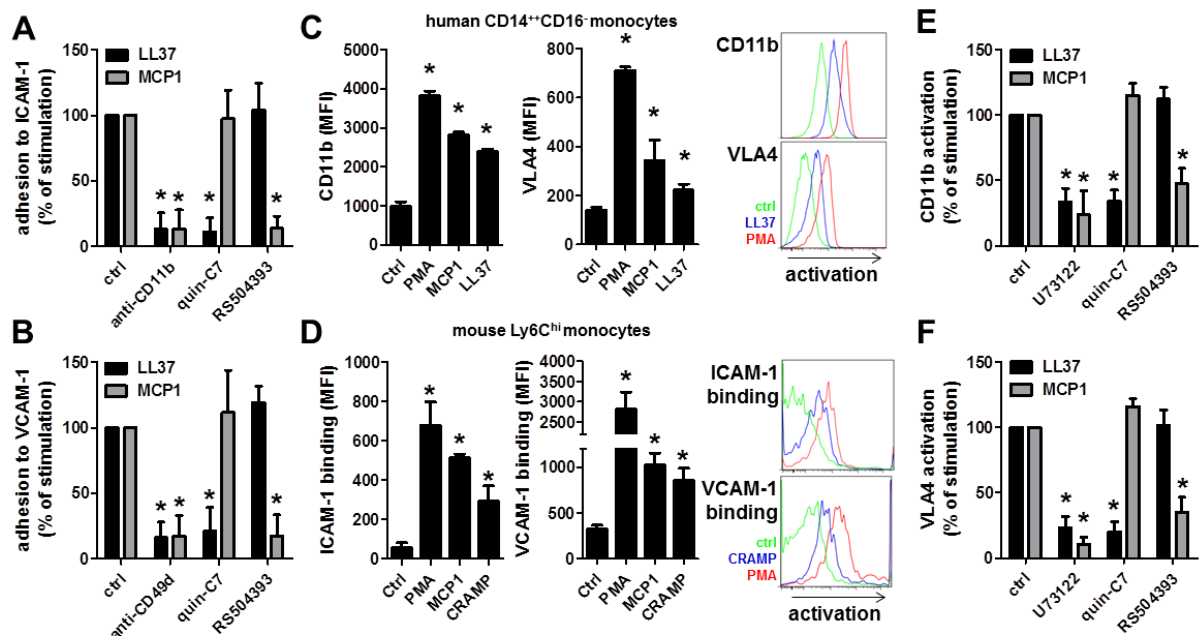


Figure 34. Cathelicidins induce integrin activation. A/B: LL37 mediates Mac1 (CD11b/CD18) and VLA4 (CD49d/CD29) dependent adhesion. Classical CD14⁺⁺CD16⁻ monocytes were perfused over P-selectin/ICAM-1 (A) or P-selectin/VCAM-1 (B) coated dishes. The increase in adhesion by co-immobilization of LL37 or MCP1 was set to 100%. Classical CD14⁺⁺CD16⁻ monocytes were pretreated with anti-CD11b (1 μ g/ml), anti-CD49d (1 μ g/ml), or antagonists to FPR2 (quin-C7, 1 μ M) or to CCR2 (RS504393, 1 μ M). *n*=8. * Significant difference compared to ctrl. C: Based on the CD16 and CD14 staining properties, human monocyte subsets were identified within peripheral blood mononuclear cells. Moreover, antibodies to activation epitopes of VLA4 (HUTS-21) and CD11b (CBRM1/5) were added. Cells were treated with PMA (50ng/ml), MCP-1 (50ng/ml), or LL37 (1 μ g/ml) for 15 min and the expression of activated CD11b (left) or VLA4 (right) was assessed on classical CD14⁺⁺CD16⁻ monocytes. * Significant difference from control group. *n*=6. D: CRAMP activation enhances ICAM-1 and VCAM-1 binding to classical monocytes. Mouse peripheral leukocytes were treated with PMA (50ng/ml), MCP-1 (50ng/ml), or CRAMP (1 μ g/ml) in the presence of ICAM-1-Fc (left) or VCAM-1-Fc (right) and an anti-Fc antibody. Monocyte subsets were identified by additional antibody staining (CD45, CD11b, CD115, Gr1). * Significant difference from control group. *n* = 5. E/F: Expression of activated CD11b (E) or VLA4 (F) on classical CD14⁺⁺CD16⁻ monocytes in response to LL37 (1 μ g/ml) or MCP1 (50ng/ml) was set to 100 %. PBMCs were pretreated with antagonists to PLC (U73122, 100nM), FPR2 (quin-C7, 1 μ M) or CCR2 (RS504393, 1 μ M). *n*=4. * Significant difference compared to respective control group

inhibitor to FPR2, the MCP1- mediated adhesion of monocytes was blocked by an antagonist to CCR2. To further investigate the activation of β_1 - and β_2 -integrins by LL37, we used an antibody that specifically recognizes the extended conformation of these integrins. Monocytes were incubated with PMA, MCP1, or LL37 and stained for the expression of active integrins. While PMA induces integrin activation on both monocyte subsets, MCP1 and LL37 only induce significant integrin activation on classical $CD14^{++}CD16^{-}$ (Figure 34C) and intermediate $CD14^{++}CD16^{+}$ monocytes but not on non-classical $CD14^{+}CD16^{++}$ monocytes (Figure 35).

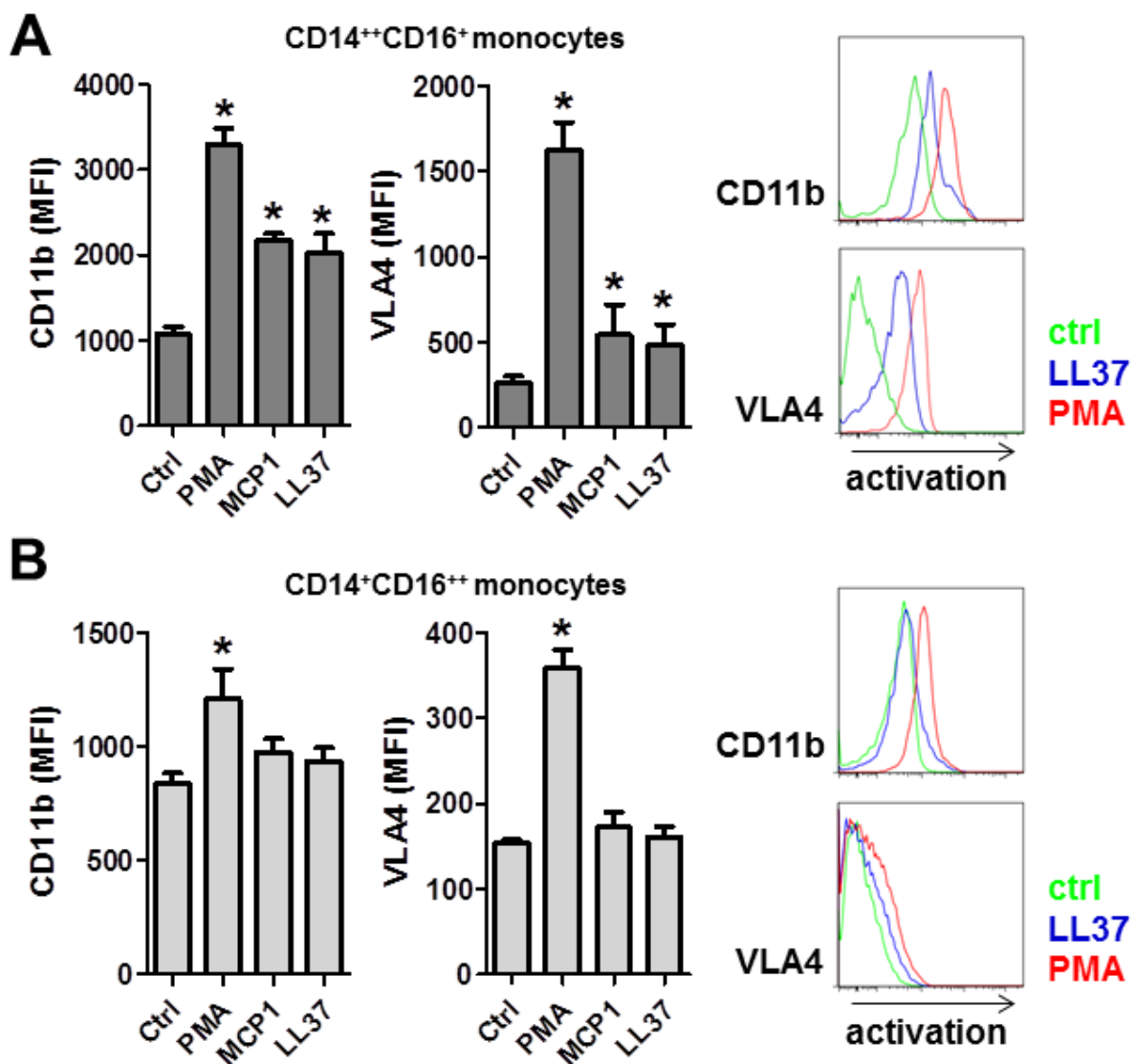


Figure 35. LL37 activates integrins on human intermediate but not on non-classical monocytes. Based on the CD16 and CD14 staining properties, $CD14^{++}CD16^{+}$ intermediate monocytes (A) and $CD14^{+}CD16^{++}$ non-classical monocytes (B) were identified within peripheral blood mononuclear cells. Moreover, antibodies to activation epitopes of VLA4 (HUTS-21) and CD11b (CBRM1/5) were added. Cells were treated with PMA (50 ng/ml), MCP-1 (50 ng/ml), or LL37 (1 μ g/ml) for 15 minutes and the expression of activated CD11b (left) or VLA4 (middle) was assessed on intermediate and non-classical monocytes. Representative histograms are displayed to the right. * indicates significant difference compared to ctrl.

Similarly, PMA, MCP1, or CRAMP induced integrin activation on murine classical but not non-classical monocytes leading to increased binding of ICAM-1 or VCAM-1 (**Figure 34D**). In line with the adhesion experiments, antagonists to P2X7, CXCR2, and FPR1 failed to block LL37-dependent integrin activation (not shown), whereas antagonists to FPR2 fully abrogated integrin activation induced by LL37 (**Figure 34E, F**). In addition, we tried to elucidate if LL37 and MCP1 employ similar intracellular pathways that lead to integrin activation. To this end, monocytes were pre-treated with inhibitors to phospholipase C (PLC), which is crucial in chemokine-mediated activation of β_2 -integrins [Lefort & Ley, 2012]. PLC blockage reduced LL37- and MCP1-induced integrin activation on classical monocytes (**Figure 34E, F**). In contrast, blockade of bruton-tyrosine kinase (BTK) or Syk kinase, which are required for selectin-dependent integrin activation [Lefort & Ley, 2012], failed to block ligand-induced integrin activation (not shown).

4.2.5 Mutation of cathelicidin abrogates its arrest function

To identify the motif within LL37 that interacts with FPR2, we compared the amino acid sequences of known FPR2 ligands including MKYMVM, MMK1, and humanin with clustalW software. Despite the absence of any strict amino acid homology, we found a pattern of chemical properties present in all FPR2 ligands. More precisely, an aromatic amino acid and two hydrophobic residues separated by two amino acids were the only similarities between the molecules (**Figure 36A**). LL37 and the other FPR2 agonists are linear peptides which simplifies the comparison of their three-dimensional structure. In their helicoil structure with a turn of 3 amino acids, the aromatic and the hydrophobic residues were nearby and can form a hydrophobic domain. To test the involvement of this pattern in LL37-dependent monocyte adhesion, we synthesized a mutated LL37 (mLL37) with an alanine instead of the phenylalanine in position 17 (F17A) and glycine in replacement of the isoleucine and the valine in position 20 and 21 (I20A and V21A) (**Figure 36A**).

To test the functionality of mLL37 we performed adhesion and integrin activation assays. Interestingly, mLL37 did not induce adhesion to TNF-activated HUVEC (**Figure 36B**) or to immobilized recombinant cell adhesion molecules (**Figure 36C,D**). In addition, mLL37 failed to enhance monocyte adhesion *in vivo* (**Figure 36E**) and to induce conformational changes of β_1 - or β_2 -integrin (**Figure 36F**). Thus, these data indicate the importance of the central FKRIV motif within LL37 to induce FPR2-mediated monocyte adhesion.

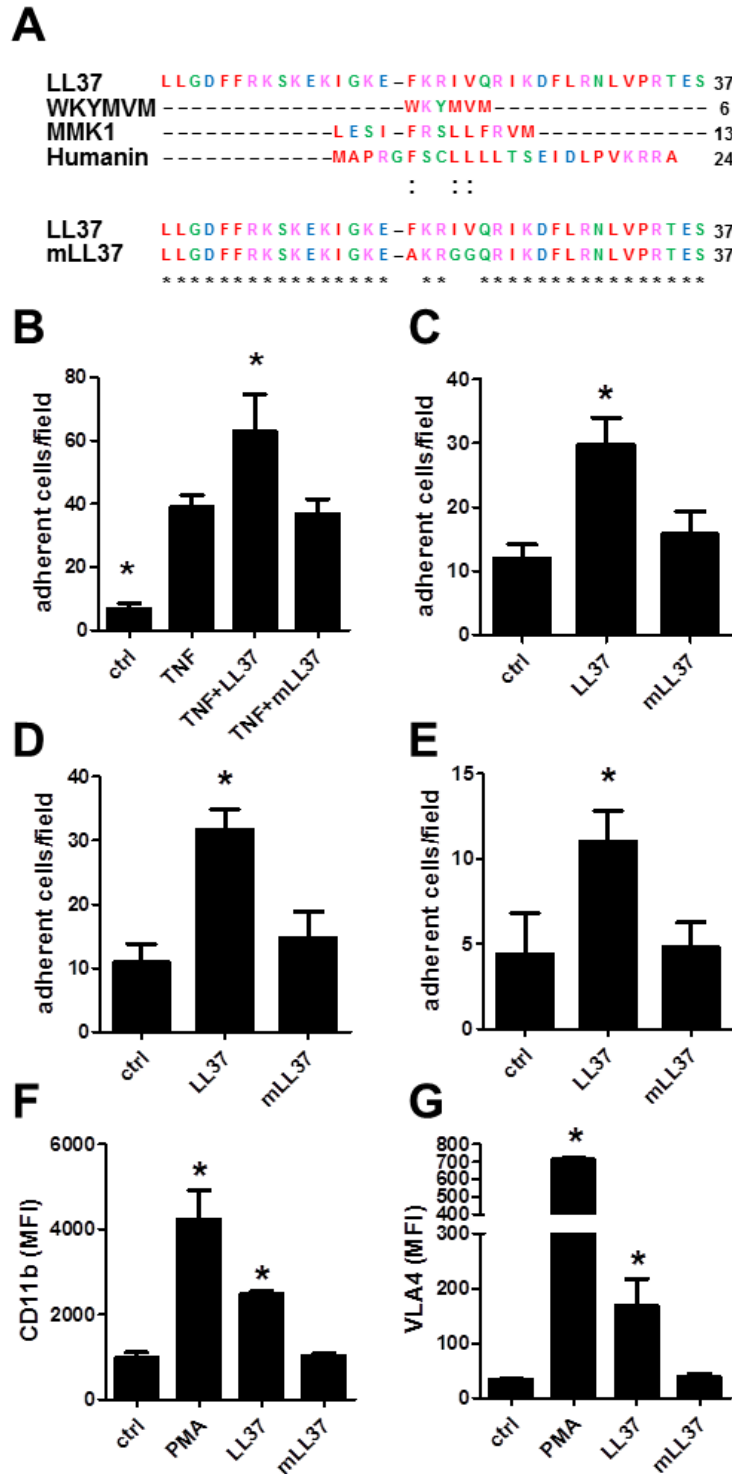


Figure 36. Mutation of LL37 abrogates its adhesive capacity. **A:** Alignment of amino acid sequence of known FPR2 ligands, LL37, and mutated LL37 (mLL37). **B:** Human classical CD14⁺⁺CD16⁻ monocytes were perfused over TNF-activated (20ng, 12h) HUVEC and the number of adherent cells was quantified. LL37 (1 μ g) or mLL37 (1 μ g) were deposited 15 min prior to perfusion. * Significant difference compared to TNF-treated sample. n=5. **C/D:** Classical CD14⁺⁺CD16⁻ monocytes were perfused over P-selectin/ICAM-1 (C) or P-selectin/VCAM-1 (D) coated dishes. In addition, LL37 or mLL37 were co-immobilized. n=3. * Significant difference compared to ctrl. **E:** Wild type mice were rendered neutropenic and injected with TNF (ctrl) or with additional LL37 (1 μ g) or mLL37 (1 μ g). Adhesion of Ly6C-labeled classical monocytes was assessed by intravital microscopy. n=5. * Significant difference compared to control. **F/G:** Activation of CD11b (F) or VLA4 (G) in response to PMA (50ng/ml), LL37 (1 μ g/ml), or mLL37 (1 μ g/ml) was assessed by flow cytometry. n=6. * Significant difference compared to control.

Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice

Neutrophils have been reported to contribute to early atherosclerotic lesion formation; however the mechanisms of neutrophil-driven atherosclerosis remain unclear so far. Here, we investigated the role of the neutrophil granule protein, cathelicidin CRAMP, in murine atherosclerotic plaque formation.

4.3.1 Lack of CRAMP reduces atherosclerotic lesion size

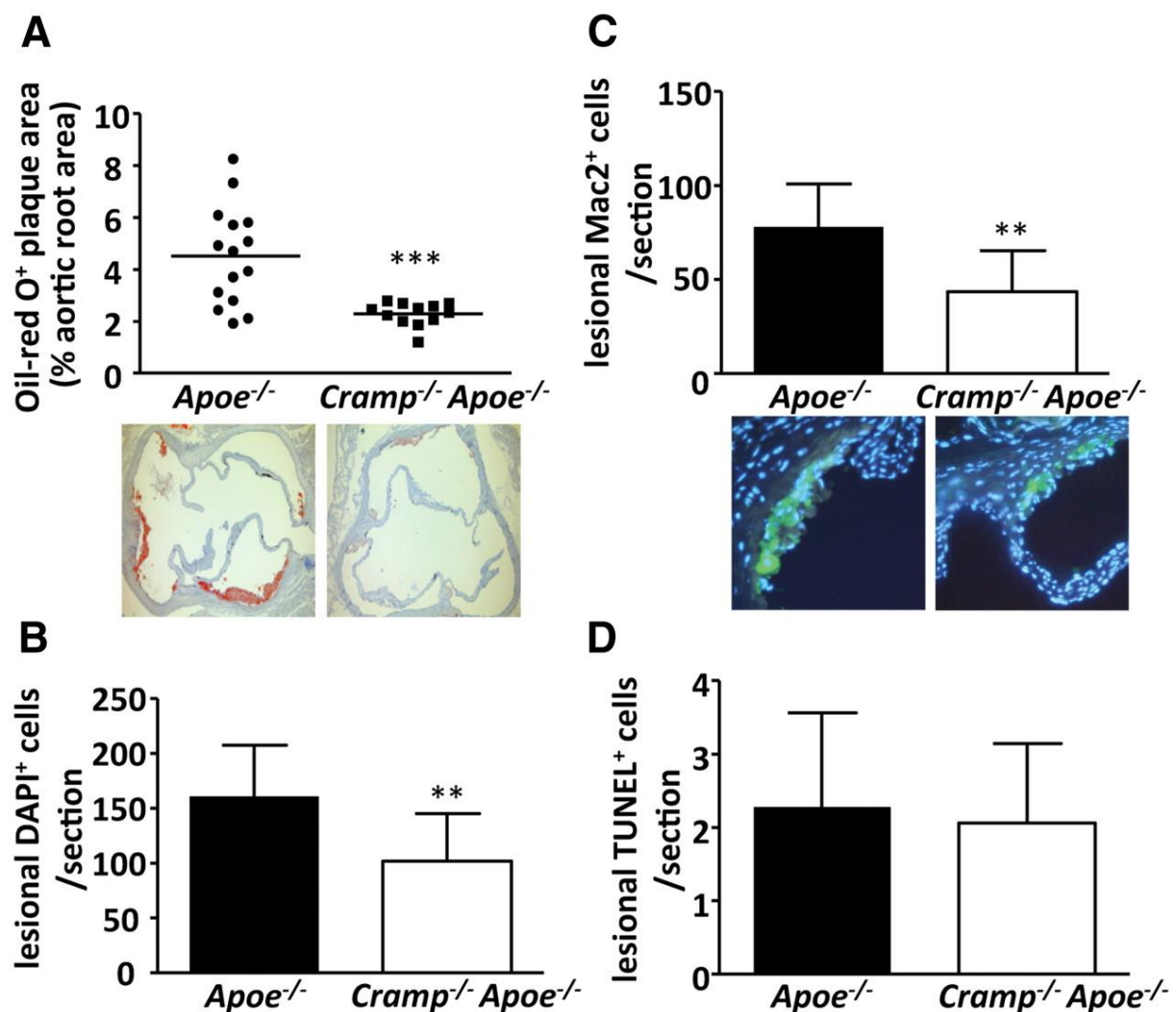


Figure 37. Lack of CRAMP reduces atherosclerotic lesion size. The *Apoe*^{-/-} or *Cramp*^{-/-} *Apoe*^{-/-} mice were fed a high-fat diet for 4 weeks. (A) Quantification of Oil-Red O-stained sections. Representative images are shown. (B) Enumeration of DAPI-positive cells. (C) Quantification of Mac2-positive cells indicating macrophage accumulation. Representative images are displayed underneath. (D) Quantification of TUNEL staining indicating apoptotic cells. ****P*<0.001 between the groups and ***P*<0.01 between the groups as assessed by unpaired *t* test.

To investigate the role of CRAMP in early atherosclerotic lesion formation, we fed *Apoe*^{-/-} and *Cramp*^{-/-}/*Apoe*^{-/-} mice a high-fat diet for 4 weeks and assessed atherosclerotic lesion sizes in aortic root sections. Lack of CRAMP led to significantly decreased lesion sizes (**Figure 37A**) characterized by reduced cellularity and a matching decrease in macrophage content (**Figure 37B, C**). In addition, no difference was found in the number of TUNEL-positive cells in the aortic root (**Figure 37D**). Cholesterol and triglyceride levels did not differ between *Apoe*^{-/-} and *Cramp*^{-/-}/*Apoe*^{-/-} mice (data not shown). Moreover, counts of circulating leukocytes subsets were not different between the strains either before or after 4 weeks of high-fat diet (data not shown).

4.3.2 CRAMP associated with atherosclerotic lesions is neutrophil-derived

Although cathelicidins are largely restricted to neutrophils, previous studies have reported the presence of cathelicidins in macrophages and endothelial cells in human plaque specimens [Edfeldt *et al.*, 2006]. Hence, we aimed at investigating the cellular origin of CRAMP in atherosclerotic aortas. Intracellular staining of CRAMP in single-cell suspensions of aortas of *Apoe*^{-/-} and *Cramp*^{-/-}/*Apoe*^{-/-} mice revealed that cellular CRAMP expression there is specifically confined to neutrophils (**Figure 38A**). In addition, immunohistochemistry showed that CRAMP also is located in the interstitium of aortic root sections of *Apoe*^{-/-} mice receiving high-fat diet (**Figure 38B**), whereas feeding normal chow diet results in markedly reduced detection of CRAMP (data not shown). The interstitial location of CRAMP is in line with previous reports showing that cathelicidins interact with extracellular matrix proteins [Soehnlein *et al.*, 2011]. To evaluate the possibility of endothelial CRAMP expression, we injected *Apoe*^{-/-} mice with microbeads conjugated to an antibody against CRAMP. The immobilization of these beads along the carotid artery was evident in *Apoe*^{-/-} mice after 4 weeks of high-fat diet (**Figure 38C**). In addition, neutrophil depletion clearly reduced the number of immobilized beads to a level close to what was found in *Cramp*^{-/-}/*Apoe*^{-/-} mice (**Figure 38C**), suggesting that CRAMP is released by activated neutrophils and immobilized on the endothelium, a circumstance likely favored by its cationicity [Soehnlein *et al.*, 2011]. Finally, no CRAMP was detected in B and T lymphocytes of spleens and inguinal lymph nodes (data not shown).

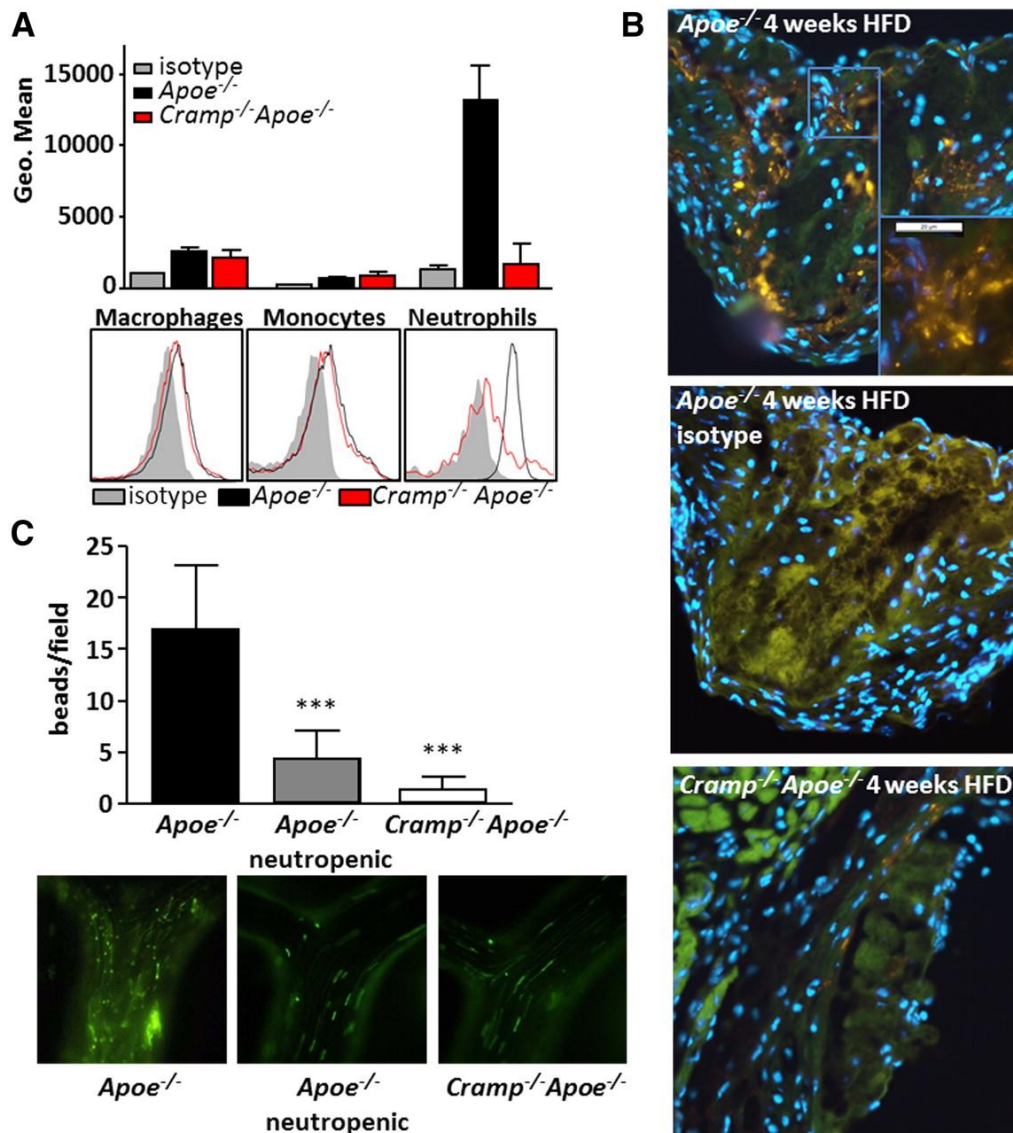


Figure 38. CRAMP associated with atherosclerotic lesions is neutrophil-derived. (A) Intracellular CRAMP staining in monocytes (SSC^{lo}CD45⁺CD11b⁺Ly6G⁻), macrophages (SSC^{hi}CD45⁺CD11b⁺F4/80⁺), and neutrophils (CD45⁺CD11b⁺Ly6G⁺) of enzymatically digested aortas from *Apoe*^{-/-} or *Cramp*^{-/-}*Apoe*^{-/-} mice having received high-fat diet (HFD) for 4 weeks (n=5). (B) Immunohistochemical detection of CRAMP in aortic root sections of *Apoe*^{-/-} (top) or *Cramp*^{-/-}*Apoe*^{-/-} (bottom) mice after 4 weeks of HFD. Stainings also were made by using an isotype control antibody (middle). Pictures were taken with a 25× objective, insert was taken with a 100× objective. (C) Luminal arrest of G-protein-coupled fluorescent beads conjugated with antibodies to CRAMP in the carotid artery of *Cramp*^{-/-}*Apoe*^{-/-} mice or *Apoe*^{-/-} mice with intact white blood cell count or neutropenia. All mice received HFD for 4 weeks (n=8). ***P<0.001 compared to mice with intact white blood cell count as calculated by Kruskal-Wallis test with post hoc Dunn test.

4.3.3 Neutrophil-derived cathelicidin induces adhesion of classical monocytes

With no differences in white blood cell counts in *Apoe*^{-/-} and *Cramp*^{-/-}/*Apoe*^{-/-} mice, as well as equal numbers in TUNEL-positive cells in the aortic root, reduced accumulation of macrophages is likely attributable to impaired recruitment rather than a shift in monocyte/macrophage homeostasis.

Hence, we investigated the *in vivo* adhesion of leukocytes with intravital fluorescence microscopy of the carotid artery. In *Cramp*^{-/-}/*Apoe*^{-/-} mice, we found reduced adhesion of rhodamine 6G-labeled leukocytes (Figure 3A). Injection of antibodies to Gr1 and CD11b revealed that most of these adherent cells were inflammatory myeloid cells. Their adhesion was found to be significantly diminished in *Cramp*^{-/-}/*Apoe*^{-/-} mice compared to *Apoe*^{-/-} mice (**Figure 39A**).

To further investigate whether cathelicidins induce adhesion of a specific myeloid cell subset, we perfused classical monocytes, non-classical monocytes, and neutrophils over tumor necrosis factor-activated monolayers of human aortic endothelial cells. Whereas LL37 induced adhesion of classical monocytes and neutrophils, non-classical monocytes were not affected (**Figure 39B**). The LL37-mediated adhesion was abrogated by an antagonist to formyl-peptide receptors (**Figure 39C**), a class of chemotactic receptors that previously was found to be involved in LL37-mediated leukocyte recruitment [Yang *et al.*, 2001].

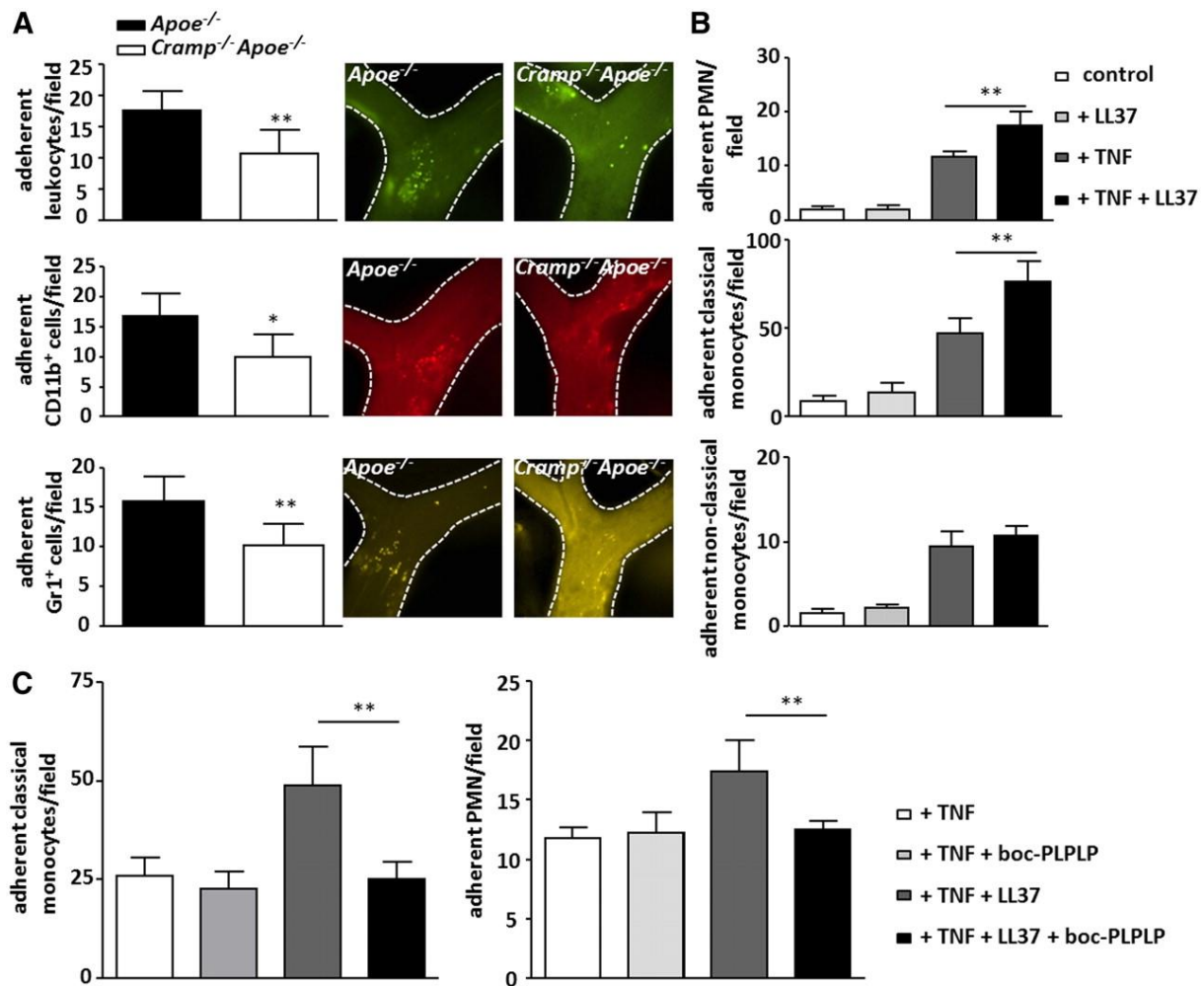


Figure 39. Neutrophil-derived cathelicidin induces adhesion of classical monocytes. (A) Interactions of leukocytes with the carotid artery were visualized in $Apoe^{-/-}$ or $Cramp^{-/-}Apoe^{-/-}$ mice fed high-fat diet (HFD) for 4 weeks by injection of rhodamine (top, labels all circulating leukocytes), an antibody to CD11b (middle, labels myeloid cells), or an antibody to Gr1 (bottom, labels neutrophils and classical monocytes; $n=8$). $**P<0.01$ and $*P<0.05$ as calculated by Mann-Whitney test. (B) Human neutrophils (top), classical monocytes (middle), or non-classical monocytes (bottom) were perfused over tumour necrosis factor-activated (20 ng, 12 hours) human aortic endothelial cells and the number of adherent cells was quantified. LL-37 (1 μ g) was deposited 15 minutes before perfusion ($n=6$). $**P<0.01$. Kruskal-Wallis test with post hoc Dunn test or one-way analysis of variance (ANOVA) with Bonferroni posttest. (C) Human neutrophils (left) and classical monocytes (right) were perfused over tumour necrosis factor-activated (20 ng, 12 hours) human aortic endothelial cells and the number of adherent cells was quantified. LL-37 (1 μ g) was deposited 15 minutes before perfusion. Leukocytes were pre-treated with the formyl-peptide receptor antagonist boc-PLPLP (1 μ mol/L, 30 minutes) when indicated ($n=6$). $**P<0.01$. Kruskal-Wallis test with post hoc Dunn test.

5

Discussion

Cathelicidins limit in-stent re-stenosis

Percutaneous Transluminal Angioplasty (PTA) has become an accepted and widely used intervention for treatment of atherosclerosis with ~90% success rate. However, the procedure usually causes vascular injury by endothelial denudation and stretching of the vessel wall, consequently, results in re-occlusion of the lumen in most of the cases. The damage to vascular endothelial cells exposes the underlying medial vascular smooth muscle cell leading to vascular remodeling [Newsome, Kutcher & Royster, 2008] during which several growth factors and inflammatory cytokines are released. Neutrophils are the first immune cells recruited to sites of injury in response to inflammatory mediators and release a vast amount of granule proteins upon activation. Here we identified neutrophil-borne cathelicidins as potential mediators of vascular repair after injury. The novel therapeutic approach was based on the capacities of cathelicidins to promote EOC recruitment and to facilitate the release of regenerative growth factors, which are crucial mechanisms to enhance re-endothelialization and to substantially limit the extent of neointima formation. We translated this finding into a miniature stent coated with LL-37 peptide, which effectively reduced in-stent stenosis.

Neutrophils were considered insignificant in the pathogenesis of atherosclerosis due to their rare detection in plaques and a short life span. Recently, more and more supporting evidences suggest an involvement of neutrophils in later stages of atherosclerotic plaque progression and acute plaque destabilization [Ionita *et al.*, 2010]. In addition, recent studies revealed convincing data supporting a regulatory function of neutrophils in early stages of atherogenesis. In contrast, we showed in our study that neutrophils provide beneficial properties during acute arterial injury. Previous studies have shown that the trafficking of leukocytes into vascular tissue increase neointimal thickening, whereas inhibition of β_2 -integrins successfully limited neointimal formation [Simon *et al.*, 2000]. β_2 -integrins are responsible for firm adhesion of leukocytes to platelets and fibrinogen on injured endothelium [Li *et al.*, 2008]. The contribution of β_2 -integrins to neointima formation is associated with the activation of platelets and leukocytes, possibly through the interaction of platelet P-selectin and leukocyte β_2 -integrin [Inoue *et al.*, 2003]. On the other hand, targeting the CCL2/CCR2 axis by deletion of CCR2 which is essential for neutrophil tissue infiltration, markedly decreased neointimal hyperplasia while decreasing endothelial recovery [Schober *et al.*, 2004]. Conversely, elevated neutrophil counts after G-CSF applications were found to reduce neointima size and promote re-endothelialization [Yoshioka *et al.*, 2006]. Taken together, neutrophils clearly exert protective effects during early phases of arterial injury.

Bone marrow-derived progenitor cells have been implicated in vascular repair; whereas EOCs enhance re-endothelialization and limit neointimal thickening, SMC progenitor cells promote neointimal hyperplasia [Marx, Totary-Jain & Marks, 2011]. Our analysis revealed that neutropenia affected endothelial recovery but not SMC content in neointimal lesions. Thus, we focused on the interrelation between neutrophils and EOCs. Bone marrow-derived endothelial progenitor cells (EPCs or in our definition: endothelial early outgrowth cells, EOCs), are known to be important in re-endothelialization and vascular repair [Pearson, 2009]. The chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) represents the single natural ligand for the chemokine receptor CXCR4. CXCL12 possesses angiogenic properties while CXCR4 expression is upregulated by angiogenic factors, such as vascular endothelial growth factor (VEGF). CXCL12/CXCR4 interactions were shown to play an important role in the migration of progenitor cells from their retention within the bone marrow. Stromal cell-derived factor-1 alpha (SDF-1alpha; CXCL12) associated with chemokine receptor CXCR4 forms an axis (CXCL12-CXCR4) for EOC mobilization after injury [Morris *et al.*, 2010; Liekens, Schols & Hatse, 2010]. Recent studies indicated a pivotal role of CXCR2 and its ligands, including CXCL1, CXCL2, CXCL5, and CXCL8 in progenitor cell homing, mobilization recruitment and neovascularization [Li *et al.*, 2011]. However, inhibition of CXCR4 and CXCR2 leads to mobilization/entry of neutrophils from bone marrow into the circulation [Eash *et al.*, 2010], which did not interfere with CXCR4 and CXCR2 expression on EOCs and CXCL12 and CXCL1 serum levels were unaffected by LL-37 or neutropenia.

FPR2 (FPRL-1), expressed dominantly by monocytes and mononuclear-derived cells, including EOCs, has been identified as a major receptor for LL-37 [Yang *et al.*, 2000]. In addition, LL-37 may act as a functional ligand for CXCR2 on neutrophils [Zhang *et al.*, 2009]. And yet, neutrophil-mediated recruitment of EOCs relies on LL-37-FPR interactions, as an indication, the LL-37-dependent EOCs adhesion was diminished by antagonizing FPRs. Remarkably, LL-37 was found deposited at sites of injury by adherent neutrophils, possibly through binding to positively charged glycosaminoglycans on endothelial surface. In closely related manners, azurocidin seeded by extravasated neutrophils could promote adhesion of monocytes under flow conditions [Soehnlein *et al.*, 2005], as well as the deposition of various chemokines by activated platelets could potentially provoke monocyte arrest on endothelium [Koenen *et al.*, 2009]. Currently, three membrane receptors - formyl peptide receptor-2 (FPR2), P2X7 and CXCR2 [Tjabringa *et al.*, 2003] - have been reported to be engaged by LL-37. Endothelial cells express both FPR2 and P2X7 receptors but direct FPR2 involvement in

endothelial barrier regulation has not been fully studied. Activation of P2X7 receptors induces signals to the cytoskeleton that result in decrease of EC permeability; however, the FPR2-LL-37 axis may be a key to EC activation and cytoskeleton re-arrangement leading to cell stiffness and barrier permeability [Engel *et al.*, 2011]. Moreover, LL-37-induced angiogenesis was mediated via engagement of FPR2 during EC activation and proliferation [Koczulla *et al.*, 2003]. Activation of embryonic endothelial progenitor cells (eEPCs) with LL-37 *ex vivo* has been found to enhance NF- κ B activity and increases functionally eEPC recruitment to ischemic muscle tissue via induction of PSGL-1 and E-selectin inducing angiogenesis [Pfosser *et al.*, 2010]. Moreover, LL-37 has been implicated in regulating cell survival, with high concentrations being cytotoxic, whereas lower concentrations may prevent apoptosis [Yang *et al.*, 2000; Tjabringa *et al.*, 2006; Chamorro *et al.*, 2009].

Here we demonstrate that LL-37 promoted EOCs recruitment and enhanced EOCs survival in a FPR2-dependent manner, providing a novel mechanism for EOC mobilization to the site of arterial injury. Even though LL-37 prominently induced proliferation in endothelial cells, nonetheless, EOC growth was left unaffected. Consequently, we identified effectors by which LL-37-stimulated EOC function may contribute to re-endothelialization. More precisely, EOCs release pro-angiogenic growth factors such as VEGF and EGF upon activation by LL-37. A similar effect was identified for porcine macrophage-derived cathelicidin PR-39, which inhibits ubiquitin-proteasome-dependent degradation of hypoxia-inducible factor-1 α protein HIF-1 to increase VEGF expression resulting in accelerated formation of vascular structures *in vitro* and increased myocardial vasculature in mice [Li *et al.*, 2000]. In our studies, the secretion of EOC treated with LL-37 significantly intensified paracrine effects exerting on ECs, resulting in enhanced migration and proliferation but reduced apoptosis. Considering the limited number of circulating EOCs directly incorporating into the endothelial lining, this event may be a dominant mechanism in neutrophil-dependent re-endothelialization. Thus, LL-37 improved re-endothelialization through several pathways, including EOC recruitment and survival as well as enhanced endothelial recovery via paracrine activities.

Present evidences supported the use of drug-eluting stents as a preferred choice in primary percutaneous coronary intervention. However, concerns regarding delayed reendothelialization and in-stent thrombosis associated with drug-eluting stents have been raised [Valgimigli, Airolidi & Zimarino, 2009]. As a result, current treatment regimens after

drug eluting stent implantation require long-term dual antiplatelet therapy for at least one year, which may pose further problems in patients prone to bleeding. Therefore, we have developed a stent coated with LL-37, which limits in-stent stenosis in mice. LL-37 is a well known mediator in both pro-[Wan *et al.*, 2011] and anti-[Jönsson & Nilsson, 2012] inflammatory processes, including monocyte recruitment and activation [Soehnlein, Lindbom & Weber, 2009], wound healing and angiogenesis [Brown *et al.*, 2011]. The reduction of in-stent neointima formation in LL-37-coated stents suggests that the anti-inflammatory role of LL-37 dominated in this study model. Furthermore, LL-37-grafted surfaces could exert potent antimicrobial activities [Carretero *et al.*, 2008] but show little pro-inflammatory effect in connection to platelet or complement activation [Gabriel *et al.*, 2006]. Although our *in vivo* study was limited to 4 weeks after stent implantation as the maximum extent of neointima formation in mice is usually obtained within 4 weeks after arterial injury, thrombus formation was not observed in these LL-37-constructed stents. Consequently, stents exploiting pro-endothelial properties of LL-37 may also be suitable to overcome the major problem of stent-induced thrombosis in drug-eluting stents after longer periods of clinical use. It is well established that covalent immobilization of antimicrobial peptides onto biomaterial surfaces have long term high stability profiles withstanding extreme environmental conditions such as severe changes in pH, temperature or fluidic flow shear stress [Gao *et al.*, 2011]. Therefore, we have little reason to believe that the efficiency and exposure of these stents in the long run would be dramatically different beyond our 4 week model. Previously, novel stent developments have been primarily tested and validated in larger animals [Hristov *et al.*, 2007; Ma *et al.*, 2010]. Thus, our implantation of miniaturized and biofunctionalized stents in mouse carotid arteries approach would allow for versatile analysis of novel stent coatings in a disease-related context of genetically modified mice.

All percutaneous interventions cause damage and denudation of vascular endothelial cells, which crucially provide hemostasis through the regulation of lipid uptake, inflammatory cell migration and protect against thrombus formation. In general, drug-eluting stents have been implanted in millions of patients worldwide and have achieved significantly reduced restenosis rates compared with bare-metal stents. However, in the long term, drug eluting stents have a higher incidence of late (6 months -1 year post implantation) and very late (> 1 year post implantation) stent thrombosis than bare-metal stents [Ertaş, van Beusekom & van der Giessen, 2009; Pfisterer, 2008]. The underlying mechanisms might be related to a delay in re-endothelialization following stenting, which has been shown in pathology study [Finn *et al.*,

2007]. Observational studies of patients who received first-generation drug-eluting stents (sirolimus or paclitaxel coated) have shown a steady increase in cumulative risk of very late stent thrombosis, whereas impaired re-endothelialization has been identified as the primary cause of this increased risk [Lagerqvist *et al.*, 2009]. While second-generation drug-eluting stents (zotarolimus, everolimus and biodegradable polymer-coated) were associated with a reduced incidence of very late stent thrombosis, suggesting of improved vascular healing, in particular for the newer generation of drug-eluting stents [Stefanini *et al.*, 2011]. Although a link between re-endothelialization and late-stent thrombosis is not firmly established, maintaining a competent endothelium is important for the long-term health of the vessel wall. The regenerated endothelium in stented regions has lost its vascular protective functions [Otsuka *et al.*, 2012]; therefore, an improved understanding of the biological properties of the endothelium is very important. Future studies should concentrate on the long-term effect of newer generations of stents, in particular biocompatibility coatings, as well as biodegradable stents, in order to maintain endothelial competency and functions.

In conclusion, our data clearly establish a robust role for neutrophils during the early healing process after arterial injury by depositing cathelicidins as mediators of endothelial recovery. We indicated that under endothelial injury conditions, neutrophil-derived cathelicidin-FPR2 axis enhanced recruitment and functions of EOCs, promoting re-endothelialization which in turn limited neointima formation. Accordingly, biofunctionalized LL37-coated stents demonstrated effectiveness in a mouse model. These findings clearly warrant investigation of the applicability of this strategy in large animal models and its translation into clinical use.

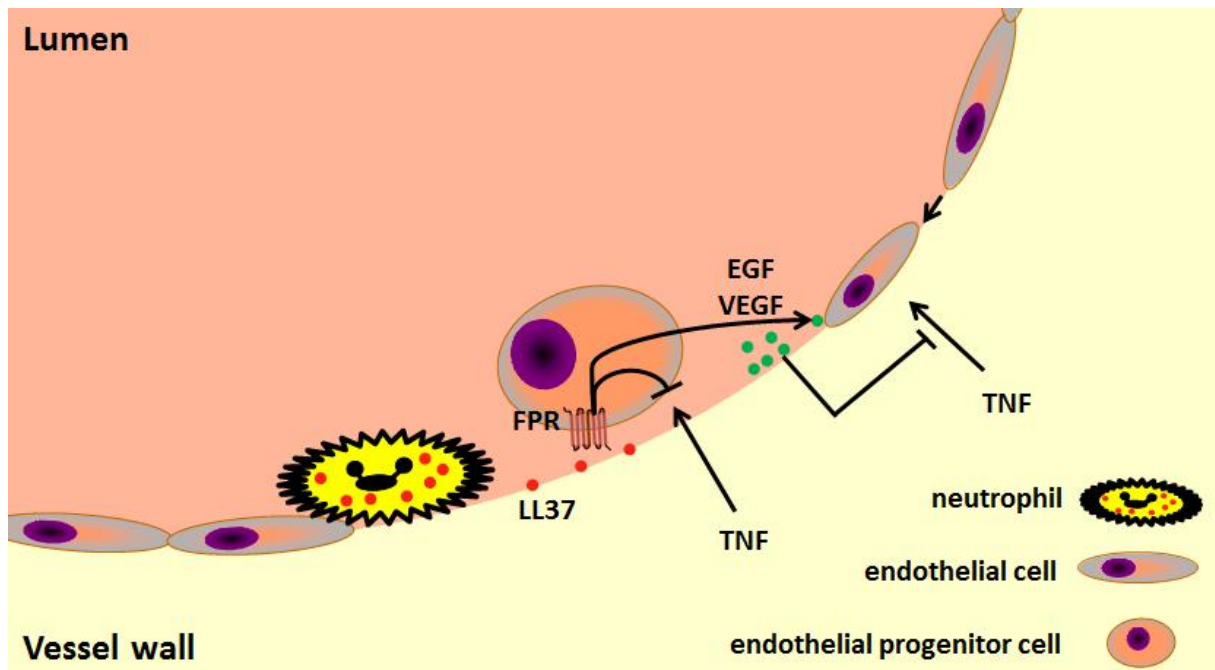


Figure 40. Mechanisms of cathelicidin-mediated reendothelialization. Neutrophils deposit cathelicidins (LL-37) at sites of vascular injury. Circulating bone marrow-derived endothelial progenitor cells (or EOCs) utilize LL-37 through FPR2 leading to inhibition of TNF-induced apoptosis while promoting cell adhesion and survival. LL-37 also exerts growth factor release from EOCs which further enhance proliferation of endothelial cells in vascular repair.

Cathelicidin attracts classical monocytes

The here presented data show a novel mechanism of neutrophil-mediated classical monocyte recruitment to the site of inflammation. Upon tissue infiltration, neutrophils secrete cathelicidins which are subsequently transported across the endothelium, a process facilitated by caveolin-1. Cathelicidins are then deposited on the endothelial surface and hence presented to leukocytes rolling along the endothelium. Classical monocytes recognize endothelial-bound cathelicidins via FPR2. This binding triggers an intracellular signaling cascade involving PLC ultimately leading to monocytic β_1 - and β_2 -integrin activation and subsequent adhesion of classical monocytes on the endothelium.

Distinct subsets of monocytes display crucial but unique functions during onset of inflammation. Classical monocytes originate from the bone marrow and spleen, are recruited into inflamed tissue, where they change their phenotype to macrophages [Chow, Brown & Merad, 2011]. Non-classical monocytes patrol the luminal side of post-capillary venules, where they sense damage or infection and trigger inflammatory reactions [Woollard & Geissmann, 2010]. In a quiescent state, classical monocytes are thought to transform into non-classical monocytes, although this is still under debate [Strauss-Ayali, Conrad & Mosser, 2007; Varol *et al.*, 2007]. Here we identify that classical but not non-classical monocytes sense cathelicidins, which are known to be potent danger signals [Oppenheim & Yang, 2005; Bianchi, 2007], subsequently become activated and adhere to the endothelial lining. Classical monocytes harbor potent pro-inflammatory functions, including release of powerful proinflammatory mediators such as reactive oxygen species, TNF, IL-6, and type I interferons [Kurosaka *et al.*, 2005]. Moreover, classical monocytes disrupt the resolution of inflammation [Panizzi *et al.*, 2010], thus perpetuating inflammatory processes. Hence, our study elucidates a mechanism which may be important in regulating the influx of classical monocytes at early stages of inflammation. The recruitment behavior of monocyte subsets is governed by the relative albeit differential expression of adhesion molecules and chemokine receptors. In this context, it was shown that classical monocytes utilize CCR2, CX₃CR1, CXCR2 as well as CCR5 to adhere and immigrate to inflammatory sites [Tacke *et al.*, 2007]. Similarly, PSGL-1 is expressed to a higher degree on classical monocytes and was hence found to be a major determinant in their recruitment [An *et al.*, 2008]. In the present study we demonstrate that FPR2 is dominantly expressed on classical monocytes and almost absent on non-classical monocytes, thus explaining the exclusive adhesion of classical monocytes in response to immobilized cathelicidins. Recent studies also suggest the importance of FPR1 in the

recruitment of neutrophils towards the sites of inflammation [Phillipson & Kubes, 2011; McDonald *et al.*, 2010]. Thus, it remains to be identified how FPR1 ligands differentially affect adhesion and recruitment of monocyte subsets.

In addition to the enhancement of classical monocyte adhesion, cathelicidins may facilitate monocyte recruitment via direct and indirect chemotactic activities. The direct chemotactic effect is primarily mediated through the ligation to FPR2 with the optimum concentrations of 0.5-50 µg/ml (0.1-10µM) [Yang *et al.*, 2000]. Interestingly, direct chemotactic activities require relatively high concentrations of cathelicidin suggesting a low-affinity interaction between the peptide and receptor. This implies that cathelicidin-mediated recruitment *in vivo* may be achieved only when a threshold concentration of the peptide secreted from neutrophils is reached [Romagnani *et al.*, 2005]. Notably, the direct chemotactic activity of cathelicidin is not affected by serum [Romagnani *et al.*, 2005], thus contradicting the effects of serum on the microbicidal properties of cathelicidin [Johansson *et al.*, 1998]. This contrast may be due to the fact that the chemotactic activity is receptor-mediated and not dependent on a peptide-membrane interaction. Presumably, the part of the LL37 peptide that activates FPR2 is unaltered by association of serum components, indicating that recruitment of leukocytes by LL37 is an important biological mechanism that is maintained along the vascular lumen. Besides its direct chemotactic activities, cathelicidins contribute to the inflammation by activation of chemokine and cytokine production through inflammatory cells. In this context, LL37 induces the production and release of IL8 from monocytes [Bowdish *et al.*, 2004]. Interestingly, the presence of GM-CSF increased the magnitude of this activation, and significantly decreased the threshold amount required for LL37, suggesting possible synergistic effects *in vivo*. In addition, LL37 was also shown to interact with the purinergic receptor P2X7, a receptor that is predominantly expressed on monocytes, macrophages and dendritic cells. LL37-stimulation via P2X7 of LPS-primed monocytes induced processing and release of the potent cytokine IL-1β [Elssner *et al.*, 2004]. This proinflammatory cytokine up-regulates adhesion molecules on endothelial cells and hence promotes leukocyte adhesion.

The accumulation of monocytes and macrophages at sites of inflammation must be tightly regulated in both adhesion and mobilization from the bone marrow and importantly their survival through inflammatory events in order to maintain the hemostasis [Gautier *et al.*, 2009]. As the number of circulating leukocyte subsets are not affected in *Cramp*^{-/-} mice [Döring *et al.*, 2012], it can be assumed that cathelicidins do not involve in the mobilization of

monocytes from their origin. On the other hand, cathelicidins were shown to cast their effects on cell apoptosis. While LL37 promotes apoptosis in various T-cells, smooth muscle cells, and epithelial cells [Lau *et al.*, 2006; Aarbiou *et al.*, 2006], it was shown to inhibit apoptotic cell death in neutrophils. As a consequence, prolonged neutrophil survival is beneficial for the host during bacterial infection. It has been proposed that the underlying mechanism for the inhibition of neutrophil apoptosis by cathelicidin is mediated via FPR2 and P2X7 receptors, resulting in the inhibition of caspase 3 activities. Noteworthy, this inhibition is mediated at a concentration as low as 0.01-5 µg/ml LL-37 [Nagaoka, Tamura & Hirata, 2006], supporting its physiological relevance. Although such data are not readily available for monocytes, one may assume that similar mechanisms could contribute to the viable of cathelicidin-mediated accumulation of monocytes at sites of inflammation.

As described above, cathelicidin-dependent mechanisms of monocyte adhesion, chemotaxis, and homeostasis may promote accumulation of classical monocytes at inflammatory sites. Such mechanisms importantly contribute to monocyte accumulation as indicated by observations from air pouch models where the infusion of cathelicidins results in pronounced accumulation of monocytes [Kurosaka *et al.*, 2005]. In disease models of vascular inflammation, lack of CRAMP reduces the adhesion of classical monocytes as well as the number of macrophages in atherosclerotic lesions. In a model of arterial injury, neutrophil-derived CRAMP was found to promote adhesion of angiogenic monocytes thereby limiting neointima formation [Soehnlein *et al.*, 2011]. In addition, CRAMP-deficient mice exhibit reduced accumulation of classical monocytes into the lungs of mice treated with hypo-chloric acid or LPS (unpublished data) and into the peritoneum upon stimulation with TNF, the latter being rescued by local application of CRAMP (unpublished data). Taken together, CRAMP may be an important facilitator of the accumulation of classical monocytes at sites of inflammation independently of the underlying stimulus. Since the chemotactic activity of cathelicidins is independent of receptors that are typically employed by classical chemokines (e.g. CCR2 for CCL2/MCP-1), we suspect that cathelicidins exert a non-redundant role in the extravasation of monocytes. In line, intracellular Ca^{2+} mobilization in response to neutrophil secretory products is conserved in *Ccr2*^{-/-} classical monocytes [Soehnlein *et al.*, 2008]. This notion along with the fact that many chemokines require *de novo* synthesis, whereas granule proteins are in general preformed, led us to propose a concept where the initial monocyte efflux is primarily mediated by alarmins (*i.e.* preformed proteins with chemotactic activity)

whereas later phases of monocyte emigration rely on chemokines [Soehnlein, Lindbom & Weber, 2009].

The here unraveled process of cathelicidin transendothelial transportation and monocyte activation offers various possibilities for therapeutic interference. We here demonstrate that the transendothelial transportation of cathelicidin depends on caveolin 1 which mediates transportation of chemokines such as MCP1 across endothelium hence being an important facilitator of leukocyte adhesion [Ge *et al.*, 2008]. In atherosclerosis, mice lacking caveolin 1 exhibit strongly reduced atherosclerotic lesion sizes along with lower amounts of MCP1 presented on the endothelium as well as fewer adherent and transmigrating monocytes [Engel *et al.*, 2011] thus implying that caveolin 1-dependent shuttling of chemokines is relevant to inflammatory pathologies such as atherosclerosis. As similar observations regarding monocyte adhesion and recruitment are made in atherosclerotic Cramp-deficient mice, caveolin 1-mediated transportation of cathelicidins may not just occur in the microcirculation as shown here but also in large arteries. Hence, tissue-specific targeting of such process may be an interesting approach to control the accumulation of classical monocytes.

Previous studies have unveiled that the antimicrobial properties of cathelicidin reside within a specific domain sequence of the parent peptide which is different from the immune cell surface binding domains, suggesting that cathelicidin may indirectly stimulate multiple signaling pathways associated with cell surface receptors [Braff *et al.*, 2005]. Cathelicidin establishes an important component of antimicrobial defense, while lack of cathelicidins in humans or mice favors the onset of infections and impairs monocyte recruitment [Nizet *et al.*, 2001]. This study provides evidence for the importance of the central FKRIV motif in FPR2-dependent adhesion of classical monocytes. Several groups have studied the involvement of specific amino acid domains in the LL37 sequence responsible for its antimicrobial [Sigurdardottir *et al.*, 2006] or other immuno-modulatory effects [van der Does *et al.*, 2010] usually resulting in a LL37-derived peptide sequence with the FKRIV motif present in it. Taken together, these observations suggest that a modification to this motif sequence may alter the balance between cathelicidin antimicrobial and host immuno-stimulatory functions. Even though the role of FPR2 was not investigated in these studies, our findings show that strategies specifically preventing the LL37-FPR2 interaction could be applicable in treatment of inflammatory diseases where recruitment of classical monocytes is involved.

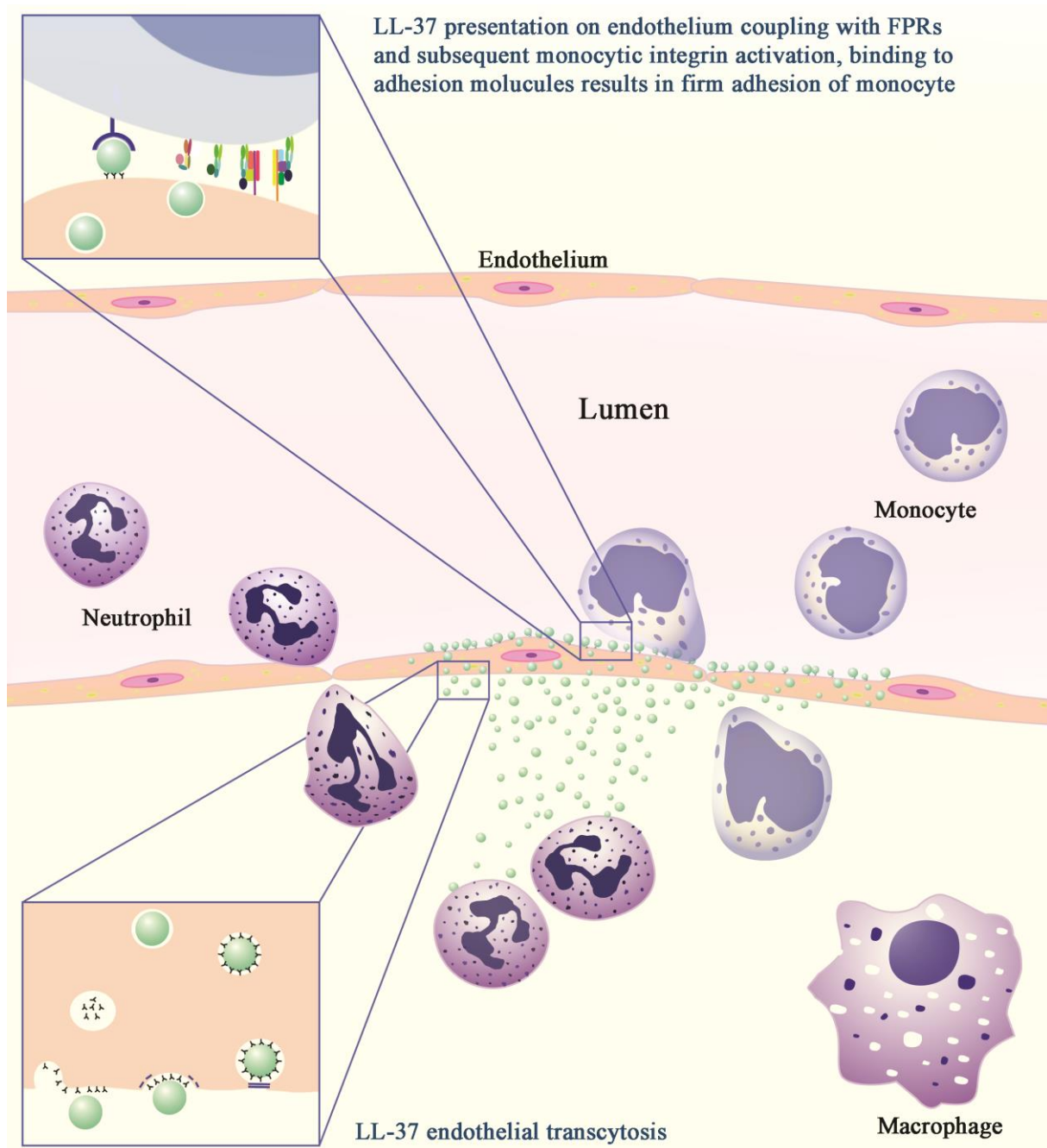


Figure 41. Mechanisms of cathelicidin-mediated classical monocyte recruitment. Neutrophils first arrive at endothelium venue and subsequently extravasate into vascular tissue during which neutrophil-derived cathelicidins (LL-37) are discharged. LL-37 undergoes endothelial transcytosis in a baso-apical direction across endothelium and presented on endothelial lining surface. Circulating classical monocyte expressed FPR2 on the surface which preferentially binds to LL-37 resulting intracellular signaling and activation of integrin which further enhances firm adhesion of monocytes on endothelium.

In summary, we have demonstrated an intriguing mechanism of transendothelial cathelicidin transportation and subsequent cathelicidin-mediated monocyte recruitment at the site of inflammation through the interaction of monocytic FPR2 and neutrophil-borne cathelicidin. Therapeutic interference with cathelicidin-FPR2 axis may be beneficial in circumstances where recruitment of classical monocytes plays an unfavorable role.

Thus far, our recent studies have provided evidence for neutrophil-secreted peptide cathelicidin in activation and accumulation of classical monocytes as well as the contribution of neutrophils during early stages of atherosclerotic lesion formation. Indeed, mechanisms underlying neutrophil-mediated atherogenesis need to be further investigated. In connection with previous study on murine cathelicidin (CRAMP) as an important mediator for neutrophil-driven monocyte recruitment and macrophage accumulation in early atherosclerotic lesions, similar observations regarding monocyte adhesion and recruitment are made in atherosclerotic CRAMP-deficient mice [Döring *et al.*, 2012].

CRAMP is the only cathelicidin in mouse and it shares a homology with the human counterpart LL-37. We have demonstrated in a mouse model of atherosclerosis that neutrophil-derived cathelicidins which are rapidly released by activated neutrophils upon arterial infiltration, promote atherosclerotic lesion formation by enhancing the recruitment of classical monocytes. *CRAMP*^{-/-}*ApoE*^{-/-} double knocked-out mice exhibit significantly reduced atherosclerotic lesion sizes with lower macrophage counts compared to *ApoE*^{-/-} mice. Reduced accumulation of macrophages is likely due to impaired recruitment of monocytes rather than a shift in monocyte to macrophage homeostasis as there are no differences in white blood cell counts in *ApoE*^{-/-} and *CRAMP*^{-/-}*ApoE*^{-/-} mice. Moreover, most of these adherent cells were classical monocytes. By use of intravital microscopy and intracellular staining, CRAMP was found to be deposited on inflamed endothelium of large arteries specifically by activated neutrophils but not monocytes or macrophages. Neutrophil-depleted mice have clearly shown the reduction of Deposited CRAMP in the aortic root sections confirming that CRAMP is released by activated neutrophils.

This study elucidates important mechanisms concerning the key mediators for inflammatory cell recruitment in early stage of atherogenesis. Several studies indicate the important involvement of platelets during vascular inflammation. Activated plates secrete a vast amount of various proinflammatory cytokines and chemokines that affect leukocyte responses

[McEver, 2001; Gleissner, von Hundelshausen & Ley, 2008]. Platelet deficient *ApoE*^{-/-} mice display reduced leukocyte accumulation in the carotid artery and thus attenuated atherosclerotic plaque formation. In the same fashion, neutrophil-derived CRAMPs could initiate monocyte recruitment corroborating the role of neutrophils in early steps of atherosclerosis.

The classical scheme of monocyte recruitment is dominated by the ligation of chemokine receptors, mainly CCR2, CCR5, and CX3CR1 expressed on classical monocytes [Tacke *et al.*, 2007] to their cognate ligands presented on the endothelium. The interaction between chemokines and their receptors modulates the activation of monocytic integrins which subsequently bind to endothelial cell adhesion molecules, establishing firm adhesion of monocytes onto endothelial lining and subsequent tissue extravasation. Our finding suggests that adhesion of classical monocytes may also be highly dependent on the FPR2-cathelicidin axis in a similar manner as those involved in chemokine receptor-mediated recruitment. Neutrophil-derived cathelicidin was previously shown to facilitate direct monocyte attracting activity through FPRs which further activate monocyte β_2 -integrins [Soehnlein *et al.*, 2008]. In addition, cathelicidins can exert the production of several cytokines and chemokines from monocytes through receptor-dependent mechanisms [Lai & Gallo, 2009]. Taken together, cathelicidin enhances recruitment and adhesion of classical monocytes therefore promoting inflammation and accumulation of monocyte-derived macrophage which can be regarded as an important step during early atherosclerotic lesion development. The study in mouse model corroborated the link between cathelicidin and atherosclerosis suggesting that the absence of neutrophil-derived cathelicidin limited recruitment of monocytes and subsequently diminished the accumulation of macrophages and therefore reduced early atherosclerotic lesion formation in mice.

These data add a new perspective to the complexity of neutrophil-mediated leukocyte recruitment mechanisms in arterial inflammation and atherosclerosis. Further investigation confirms the rare detection of neutrophils in murine atherosclerosis as they can only be transiently identified in plaque which is consistent with several studies of their absence histologically. This novel concept leads to the inclusion of neutrophils in early atherosclerotic lesion formation. Understanding the broad spectrum of neutrophil-derived cathelicidin functions in vascular homeostasis and pathogenesis would be beneficial in targeting early stage of inflammation-related diseases.

6

Summary & Perspectives

Neutrophils are the first line of immune defense against acute infections and usually the first phagocytes to arrive at the site of inflammation. Activated neutrophils undergo transendothelial extravasation and subsequently release different granule contents, most of which are potent antimicrobial peptides and some of them are able to activate and recruit immune cells and thus have been coined alarmins. Cathelicidins residing in neutrophil secondary granules were shown to potently activate and recruit monocytes and macrophages, and were identified in atherosclerotic lesions. We have focused on neutrophil-derived cathelicidins and their contribution in chronic inflammatory vascular disorders. Atherosclerosis is known to be associated with an accumulation of lipids and inflammatory cells in the vessel wall leading to occlusion of the arteries. Neutrophils and their granule proteins participate in every stage of atherogenesis from early lesion formation to advance plaque rupture.

Percutaneous transluminal angioplasty with stent implantation is the intervention of choice for treatment of occluded arteries caused by atherosclerotic plaques and atherothrombosis. Commonly used drug-eluting stents release anti-proliferative or anti-inflammatory agents to reduce the incidence of in-stent stenosis. However, these stents may still lead to in-stent stenosis; they also increase the rate of late stent thrombosis, possibly related to endothelial recovery. We here identified the contribution of neutrophil-borne cathelicidin to vascular repair after injury. The study demonstrated the capability of cathelicidin to promote re-endothelialization and thereby limited neointima formation after stent implantation. We evidenced that neutrophil depletion increased neointima formation in atherosclerosis-prone apolipoprotein E-deficient mice, indicating a beneficial effect of neutrophils. The mechanisms can be depicted through various signaling pathways, crucially, the recruitment of EOCs and prolonged survival of these cells. Cathelicidin enhances accumulation of EOCs at the site of vascular damage as well as promotes EOC proliferation and migration while limiting apoptosis through the interactions with FPR2 expressed on EOC surface. In addition, cathelicidin accelerates re-endothelialization by stimulating the release of pro-angiogenic growth factors such as VEGF and EGF from EOC which exhibit paracrine effects on neighboring endothelial cells. We finally translated these findings to an animal study using a biofunctionalized miniature stent coated with cathelicidin that substantially reduced in-stent stenosis in a mouse model of atherosclerosis confirming that cathelicidin enhances vascular healing after interventional therapy. These data highlight the possibility of translating these findings into clinic applications to help prevent restenosis.

Neutrophils are thought to promote atherosclerosis. Nevertheless, mechanistic insights into how neutrophils promote early atherosclerotic lesion formation remain elusive. In general, an initial recruitment of neutrophils in acute inflammation is followed by a second wave of monocytes. We then described the important role of neutrophil-derived cathelicidin as the cellular switch for monocyte recruitment and adhesion during acute inflammation. Cathelicidins are found to preferentially induce adhesion of classical monocytes but not of non-classical monocytes in the mouse cremaster muscle and in *in vitro* flow chamber assays. Classical monocytes highly express FPR2 whereas it is almost absent in non-classical monocytes, indicating that FPR2 is the significant receptor for cathelicidin. Neutrophils only release cathelicidin once they infiltrate into the tissue, then cathelicidin is further transported across the endothelium where it is lumenally presented to rolling monocytes. Endothelial transcytosis of cathelicidin is mediated by caveolin-1 protein as well as proteoglycans and cytoskeleton re-arrangement in endothelial cells. Endothelial-bound cathelicidin activates FPR2 on classical monocytes, resulting in monocytic β_1 - and β_2 -integrin conformational change towards an extended, active conformation that allows for adhesion to their respective ligands VCAM-1 and ICAM-1 on the endothelium. These data elucidate a novel mechanism of neutrophil-mediated monocyte recruitment, which could be targeted in inflammatory conditions where recruitment of classical monocytes is involved.

We also examined atherosclerosis-prone cathelicidin-deficient mice to determine whether this cathelicidin-driven monocyte accumulation is involved during atherosclerotic lesion formation. The study explicitly shows decreased adhesion of classical monocytes on vessel walls, which resulted in reduction of atherosclerotic plaques containing a small proportion of macrophages. Cathelicidin has previously been detected in other cell types including endothelial cells and macrophages, but we were able to show that cathelicidin in atherosclerotic lesion was specifically secreted from neutrophils. Indeed, once again we have confirmed that cathelicidin promotes classical monocyte recruitment through interaction with their membrane receptor FPR2 in mice model. Thus, targeting the cathelicidin-FPR2 axis may be an avenue toward atherosclerosis therapy.

In conclusion, cathelicidin is a multifunctional peptide displaying both antimicrobial activity and a variety of inflammatory modulating functions, suggesting that cathelicidin may regulate both infection and inflammation. Therefore, cathelicidin may be a novel target in the treatment of inflammatory diseases and the development of new anti-infectious agents.

Intriguingly, cathelicidins may act as dual agents and we have clearly demonstrated that neutrophil-derived cathelicidin exerts protective effects against neointima formation through EOC-mediated re-endothelialization. In contrast, cathelicidin-mediated classical monocyte adhesion would exhibit the opposite activity by provoking inflammatory responses thus amplifying inflammation. Questions have been raised: what could be a turning point where cathelicidin divert their properties from one to another? This cannot be answered in one simple direction; however the explanations might just be lying within the unique characters and signaling associated with endothelial cells and monocytes. Numerous studies have shown the ability of human CD14⁺ and mouse monocytes to undergo phenotypic changes and differentiate into functional endothelial or endothelial-like CD34⁺ cells that can secrete angiogenic growth factors given the suitable environment and signal cues [Romagnani *et al.*, 2005; Rehman *et al.*, 2003; Sölder *et al.*, 2012; Sharpe *et al.*, 2006; Ingram *et al.*, 2004]. As for the fact that rare circulating CD34⁺ progenitor cells are outnumbered by CD14⁺ myeloid cells, recent paradigms of tissue repair and regeneration including angiogenesis have been focused on myeloid-derived endothelial progenitor cells, providing that they are a readily available source for cellular therapy.

Despite the recognition of their occurrence, little is known about the molecular signals that regulate this transition. Interestingly, TNF- α has been identified as an important mediator of myeloid cells differentiation into endothelial cells [Li *et al.*, 2011] both *in vitro* and *in vivo* during mouse cancer growth and most efficiently with the presence of fibronectin that promotes firm adhesion and cell differentiation. Consequently, blocking of $\alpha 5\beta 1$ integrin, a fibronectin receptor, inhibits TNF α -enhanced adhesion of monocytes to fibronectin matrix [Li *et al.*, 2011]. This indicates the importance of adhesion processes to the transforming of TNF- α -activated monocytes into endothelial phenotype. Additionally, the expression of $\alpha 5\beta 1$ integrins is known to induce expression of VE-cadherin in endothelial cells and may serve as the differentiation cue for monocytes [Danen *et al.*, 2002].

Cathelicidin promotes adhesion and enhances monocytic $\beta 1$ and $\beta 2$ integrin activation preferentially on CD14⁺ subsets during which various cytokines, namely TNF- α and other growth factors have been released in inflammatory response. Cathelicidin-coated stents may act as a substrate, similarly to fibronectin, that captures monocytes and stimulates integrin expression together with additional signal from TNF- α and probably other angiogenic growth factors such VEGF and EGF, they induce VE-cadherin expression on CD14⁺ monocyte

changing to CD34⁺ endothelial-like cells. When compared to the common monocyte extravasation process, the critical point missing here on cathelicidin-coated stents is the presence of integrin counter ligands, adhesion and junctional molecules, VCAM-1, ICAM-1 and JAMs which are crucial for outside-in signaling for monocytic integrin to mediate cytoskeleton re-arrangement, pseudopod and crawling of monocytes into endothelium junction. Absence of these molecules may be an explanation to activated- β_1 -integrin-mediated VE-cadherin expression leading to phenotypic change and subsequent immobilization of myeloid-derived endothelial progenitor cells on stent surface.

Furthermore, cathelicidin was identified to be the most up-regulated gene during EPC differentiation on fibronectin [Ahrens *et al.*, 2011]. Cathelicidin-induced proliferation is exerted via the activation P2X7 receptor [Tomasinsig *et al.*, 2008]. The fact that cathelicidin is up-regulated in early EPC development generates the hypothesis that it contributes to the paracrine pro-angiogenic effect, directly acting on endothelial cells and inducing neovascularization [Koczulla *et al.*, 2003]. Interestingly, a recent study demonstrated cathelicidin-induced recruitment of embryonic EPCs to ischemic tissue by up-regulation of the adhesion molecule PSGL-1 (CD162) [Pfosser *et al.*, 2010]. In addition, a very recent report indicates that cathelicidin is directly involved in differentiation of monocytes towards a more immature osteoblast/osteoclast-like cell type [Zhang & Shively, 2010].

In summary, protective effects of cathelicidin towards neointimal hyperplasia, despite the pro-inflammatory functions and monocyte recruitment property, may be due to the contribution of chemotactic activity from pro-angiogenic factors that are released during inflammatory stimulation and thus facilitate the phenotypic change of classical monocytes into endothelial-like cells. Cathelicidin is also directly associated with increased atherosclerotic lesion formation. Despite all these remarkable advances in understanding the role of cathelicidin in immunological pathophysiology of inflammatory disease in murine, we still lack definitive evidence of causation for humans. What should now be observed is how cathelicidins affect these conditions in the long term and probably in larger animal models with the specific inhibition of the intracellular signaling. The contribution of this thesis highlights the underlying mechanisms of neutrophil-derived cathelicidin-mediated inflammation and atherosclerosis in murine model. Taken together, the prospective of cathelicidin utilization in inflammation-associated diseases can be a promising clinical concept which may further be developed for urgent therapeutic in humans.

Zusammenfassung

Neutrophile Granulozyten sind die ersten Entzündungszellen, die bei entzündlichen Reaktionen ins Gewebe einwandern. Sie orchestrieren den Verlauf der Entzündung durch die Freisetzung von präformierten Granulaproteinen wie Azurocidin, α -Defensin und Cathelicidin. Ursprünglich wurden diese Proteine wegen ihrer antimikrobiellen Aktivität gewürdigt. In den letzten Jahren konnte jedoch gezeigt werden, dass diese Granulaproteine wichtige Funktionen in der vaskulären Inflammation übernehmen. So besitzen Cathelicidine zytotoxische, chemotaktische, regenerative und angiogene Eigenschaften. Nachdem die Rolle von Cathelicidinen im Gesamtkontext der Atherosklerose und der Neointimbildung bislang völlig unverstanden ist, war es das Hauptziel dieser Doktorarbeit Mechanismen der Cathelicidin-vermittelten vaskulären Inflammation zu identifizieren und nach Möglichkeit einer therapeutischen Strategie zuzuführen.

In einem Mausmodell der arteriellen Verletzung konnten wir nachweisen, dass Cathelicidine, die aus neutrophilen Granulozyten freigesetzt werden, einen protektiven Effekt ausüben. Mechanistisch fördern Cathelicidine die Adhäsion von zirkulierenden endothelialen Vorläuferzellen und fördern somit die Reendothelialisierung der verletzten Gefäßwand. Darüber hinaus setzen Cathelicidine ein Transkriptionsprogramm in diesen Vorläuferzellen in Gang, das zur Freisetzung von Wachstumsfaktoren führt und damit die Regeneration des Gefäßendothels vorantreibt. In einem therapeutischen Ansatz wurden Cathelicidine auf einem Nitinolstent kovalent gebunden. Ein derart modifizierter Stent zeichnet sich durch seine protektiven Eigenschaften nach Implantation in Karotisarterien von Mäusen aus.

In einer zweiten Arbeit konnten wir zeigen, dass die Adhäsion klassischer Monozyten zum Teil von der Rekrutierung aktivierter neutrophiler Granulozyten abhängt. Diese Aktivität basiert auf Cathelicidinen, die von neutrophilen Granulozyten im Gewebe freigesetzt werden und über das Endothel transportiert werden. Über diesen Mechanismus werden Cathelicidine auf dem Endothel präsentiert und von klassischen Monozyten über FPR2 wahrgenommen. Die nachfolgende intrazelluläre Signalkaskade führt zur Aktivierung von Integrinen und in Folge zur Adhäsion klassischer Monozyten. Dieser Mechanismus hat in einem Mausmodell der Atherosklerose eine wichtige Bedeutung. Hier haben Cathelicidin-defiziente Mäuse kleinere atherosklerotische Läsionen, weniger läsionale Makrophagen und eine reduzierte arterielle Adhäsion klassischer Monozyten.

Zusammenfassend zeigen wir hier erstmalig die Rolle von Cathelicidinen in der Atherosklerose und der Neointimafornationen und zeigen Wege der therapeutischen Interferenz auf.

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Curriculum Vitae

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Academic education

2009 – 2013	Graduate student at the Euregio Cardiovascular (EuCAR) International Research Training Group GRK/IRTG 1508
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2006 – 2009	M.Sc. Biomedical Engineering RWTH Aachen University, Germany
1995 – 1999	B.Sc. Industrial Chemistry Rajamangala Institute of Technology, Thailand
1989 – 1995	Secondary school & high school Thanyarat School, Thailand

Professional experiences

2004 – 2006	Halliburton, Thailand
2001 – 2004	M-I Drilling Fluids, Thailand
1999 – 2001	Chevron Offshore, Thailand

Publications

Soehnlein O*, **Wantha S***, Simsekyilmaz S, Döring Y, Megens RT, Mause SF, Drechsler M, Smeets R, Weinandy S, Schreiber F, Gries T, Jockenhoevel S, Möller M, Vijayan S, van Zandvoort MA, Agerberth B, Pham CT, Gallo RL, Hackeng TM, Liehn EA, Zernecke A, Klee D, Weber C. (2011) Neutrophil-derived cathelicidin protects from neointimal hyperplasia. *Sci Transl Med.* **3**:103ra98.

Also see editorial: “Research Highlights” (2011) Medical devices: A protective stent coating. *Nat Rev Drug Discov.* **10**:902.

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Also see Editor’s Picks: “Circulation Research featured articles” (2013) Neutrophils usher monocytes into sites of inflammation. *Circ Res.* (2013)**112**:744-745.

* Equal contribution

Meetings

The Netherlands Heart Foundation's PhD Training Course 'Vascular Biology'

11-15 October 2010, Papendal, the Netherlands

Oral presentation: Neutrophil-derived cathelicidin protects from neointimal hyperplasia

The Leukocyte in Cardiovascular Disease Conference

27-28 January 2011, Geneva, Switzerland

Poster presentation: Neutrophil-derived cathelicidin protects from neointimal hyperplasia

Joint Meeting European Society of Microcirculation (ESM) and Vascular Biology (GfMVB)

13-16 October 2011, Munich, Germany

Oral presentation: Neutrophil-derived cathelicidin protects from neointimal hyperplasia

Frontiers in Cardiovascular Biology 2012

30 Mar 2012 - 01 Apr 2012, London, United Kingdom

Oral presentation: Neutrophil-derived LL-37 mediates adhesion of classical monocytes

The Inflammation and Atherosclerosis Conference

20-21 September 2012, Munich, Germany

Poster presentation: Neutrophil-derived LL-37 promotes adhesion of classical monocytes

47th Annual Scientific Meeting of the European Society for Clinical Investigation

17-20 April 2013, Albufeira, Portugal

Oral presentation: Neutrophil-derived cathelicidin promotes adhesion of classical monocytes

Awards & Grants:

2011 Young investigator award by Japanese Society for Microcirculation

Joint Meeting European Society of Microcirculation and Vascular Biology

2012 Travel grant by European Society of Cardiology Frontiers in Cardiovascular Biology

2013 Best poster presentation award from Scientific Retreat of SFB 914 on "Trafficking of Immune Cells in Inflammation, Development and Disease".

