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NEUTROPHILS IN ATHEROSCLEROSIS

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Cover illustration: A photograph of the abdominal aorta in mice with a visible plaque. The plaque area is visualized by immunofluorescence in a confocal image showing neutrophils (pink) and monocytes (green). The grey scale image aquired by transmission electron microscopy shows high magnification of neutrophils within an atherosclerotic lesion.

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My first book was bad.
It concentrated more on form than content,

Yevgeny Yevtushenko

To all those who passed away during my years in research

My father, my grandmother,
Sören, Carin, Biggan, and Petter

PREFACE

My first steps towards this thesis were taken already 1999 when I took a course in advanced physiology. Since then many years have passed and many things have happened both on a personal level as well as in my field of science. Besides the mandatory parts of a thesis I decided to write an additional part, in order to attract as many readers as possible. For those of you not in the field of science or medicine, I would recommend reading the first part named "*Basics*", and then continue with the chapter I chose to call "*My Scientific Journey*". Herein, I try to recount the path of my scientific years. If you after reading this part would like to know more about this field, I suggest that you return to the *Introduction* and continue with the *Results and Discussion*. For those of you who would like to dig in deep into the data, please read **Paper I, II and III**.

Pierre Rotzius

Feb 2011

ABSTRACT

Atherosclerosis is a complex inflammatory disease localized in medium-sized and large arteries and it is the most important contributor to cardiovascular disease. Complications of atherosclerosis such as myocardial infarction and stroke are leading causes of mortality in many countries. Leukocyte recruitment to the arterial intima is crucial for the development of atherosclerotic lesions. The roles of macrophages and T-lymphocytes in promoting atherosclerotic plaque development and destabilization have been extensively studied. However, the most abundant white blood cell in the circulation, the neutrophil, has until recently rarely been associated with disease pathogenesis. The work of this thesis aimed at investigating the potential presence and roles of neutrophils in atherosclerosis.

Previous *in vivo* studies of leukocyte recruitment in atherogenesis have not been able to selectively detect individual subpopulations of leukocytes. In order to study neutrophils more specifically, we aimed at introducing a system, by which we could selectively study the roles of monocytes and neutrophils by microscopy. By crossing mice deficient in apolipoprotein E ($ApoE^{-/-}$ mice) with mice homozygous for a knock-in mutation for enhanced green fluorescent protein (EGFP) in the lysozyme M (lys-M) locus, we generated lysozyme M-deficient atherosclerosis-prone mice with endogenously fluorescent neutrophils and monocytes ($ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice, *Paper I*). In order to address whether absence of lys-M and replacement with EGFP influence atherogenesis, we compared the generated mice with their littermate $ApoE^{-/-}$ mice and found no differences in white blood cell count, cholesterol profile, plaque composition or lesion area between the two strains. The generated mouse strain enabled us to use intravital microscopy to efficiently detect fluorescent monocytes and neutrophils that were interacting with atherosclerotic endothelium *in vivo*, and to use confocal microscopy to observe individual cells within lesions.

In order to specifically study neutrophil presence in, and recruitment to, atherosclerotic lesions, we used $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice in several experiments. By use of intravital microscopy we showed that a vast majority of leukocytes interacting with endothelium on lesion shoulders are neutrophils, suggesting a significant recruitment of these cells to plaque (*Paper II*). Furthermore, flow cytometry and confocal microscopy showed that neutrophils make up for 1.8% of $CD45^{+}$ leukocytes in the aortic wall of $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice and that their contribution relative to monocyte/macrophages within lesions is approximately 1:3. Interestingly, we could show that neutrophils accumulate at sites of high density of monocytes and preferentially in shoulder regions of plaques. In some regions of plaque neutrophils actually outnumber monocytes/macrophages.

Atherosclerosis is known to aggravate during systemic inflammatory diseases, and common infections can trigger acute cardiovascular events. In *Paper III*, we investigate the potential for systemic inflammatory stimuli to induce recruitment of leukocytes to the walls of large arteries in normal and atherosclerotic mice. $ApoE^{-/-}$ and control C57Bl/6 mice were challenged with cytokines (TNF-α and IL-1β), LPS or infection with Influenza A in order to induce a systemic inflammatory response. The stimulation triggered a rapid systemic cytokine release and an increase in the relative number of peripheral neutrophils. Interestingly, there was a significant increase in the number of leukocytes adherent to atherosclerotic endothelium as detected with scanning electron microscopy on aortic endothelium. Furthermore, flow cytometry on aortic cells revealed a marked recruitment of neutrophils following inflammatory challenge.

Altogether, this thesis demonstrates that neutrophils are recruited to atherosclerotic lesions and that neutrophils represent the principal subset of leukocytes that interact with atherosclerotic endothelium. Furthermore, neutrophils invade lesions in significant numbers under baseline conditions and are found especially in shoulder regions and at sites of high inflammatory activity. Neutrophil invasion is significantly increased during systemic inflammation. These findings establish neutrophils as potentially important players in the pathogenesis of atherosclerosis.

LIST OF PUBLICATIONS

The thesis is based on the following articles, which are referred to in the text by their roman numerals:

I. **Pierre Rotzius**, Oliver Soehnlein, Ellinor Kenne, Lennart Lindbom, Kristofer Nystrom, Sebastian Thams and Einar Eriksson (2009). ApoE$^{-/-}$/Lysozyme M$^{EGFP/EGFP}$ mice as a versatile model to study monocyte and neutrophil trafficking in atherosclerosis. *Atherosclerosis 202:111-118*

II. **Pierre Rotzius**, Sebastian Thams, Oliver Soehnlein, Ellinor Kenne, Chi-Nan Tseng, Niklas K. Björkström, Karl-Johan Malmberg, Lennart Lindbom and Einar E. Eriksson. (2010). Distinct infiltration of neutrophils in lesion shoulders in ApoE$^{-/-}$ mice. *The American Journal of Pathology 177:493-500*

III. **Pierre Rotzius**, Chi-Nan Tseng, Lilian Walther Jallow, Johan Thyberg and Einar Eriksson. Inflammatory challenge triggers systemic cytokine cascade and rapid infiltration of neutrophils in atherosclerosis. *Manuscript*

OTHER PUBLICATIONS BY THE AUTHOR WHICH ARE NOT INCLUDED IN THE THESIS:

Eriksson EE, Karlof E, Lundmark K, **Rotzius P**, Hedin U, Xie X. Powerful inflammatory properties of large vein endothelium in vivo. *Arterioscler Thromb Vasc Biol.* 2005 Apr;25(4):723-8. Epub 2005 Jan 27.

Soehnlein O, Xie X, Ulbrich H, Kenne E, **Rotzius P**, Flodgaard H, Eriksson EE, Lindbom L. Neutrophil-derived heparin-binding protein (HBP/CAP37) deposited on endothelium enhances monocyte arrest under flow conditions. *J Immunol.* 2005 May 15;174(10):6399-405.

Ulbrich HK, Luxenburger A, Prech P, Eriksson EE, Soehnlein O, **Rotzius P**, Lindbom L, Dannhardt G. A novel class of potent nonglycosidic and nonpeptidic pan-selectin inhibitors.
J Med Chem. 2006 Oct 5;49(20):5988-99

Soehnlein O, Kenne E, **Rotzius P**, Eriksson EE, Lindbom L. Neutrophil secretion products regulate anti-bacterial activity in monocytes and macrophages. *Clin Exp Immunol.* 2008 Jan;151(1);139-45. Epub 2007 Dec 7.

Kenne E, Soehnlein O, **Rotzius P**, Eriksson EE, and Lindbom L. Immune cell recruitment to inflammatory loci is impaired in mice deficient in basement membrane protein Laminin-411
J Leukoc Biol. 2010 Sep;88(3):523-8. Epub 2010 May 18.

CONTENTS

LIST OF ABBREVIATIONS

ANS	Anti neutrophil serum
ApoE	Apolipoprotein E
BM	Bone marrow
BMT	Bone marrow transfer
CAM	Cell adhesion molecule
CD	Cluster of differentiation
CRP	C-reactive protein
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EGFP	Enhanced green fluorescent protein
FACS	Fluorescent-activated cell sorting
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IL	Interleukin
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
Lys M	Lysozyme M
mAb	Mouse antibody
MCP-1	Monocyte chemotactic protein-1
MMP	Matrix metallo protease
MPO	Myeloperoxidase
NO	Nitric oxide
oxLDL	Oxidized low density lipoprotein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphnuclear leukocyte
PSGL-1	P-selectin glycoprotein ligand 1
RA	Rheumatoid Arthritis
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
WD	Western diet
WT	Wild-type

BASICS

THE CIRCULATORY SYSTEM

The circulatory system consists of the heart and blood vessels and the main function is transportation of essential substances to different parts of the body through movement of blood. The heart is divided into a right and left half and these two halves act as separate pumps in different vessel circuits where arteries transports blood from the heart and veins transports blood back to the heart. The right half pumps blood to the pulmonary circuit where the blood release carbon dioxide to and pick up oxygen from the lungs, and subsequently transports the oxygenated blood back to the left heart. The left heart distributes blood into the systemic circuit where the blood delivers oxygen and nutrients to the tissues. Exchange between the blood and tissues occurs in the capillaries, the smallest vessels in the body, and after releasing oxygen and nutrients to the tissues the blood can take up carbon dioxide and other waste products and return to the right heart through veins.

BASIC COMPONENTS OF BLOOD:

Blood consists mainly of plasma and cells. Plasma consists of about 90% water and account for more than 50% of the blood volume under normal conditions. Its main purpose is to transport other components of the blood such as nutrients, proteins, vitamins, electrolytes, hormones, cholesterol and other chemicals throughout the body. There are 3 major types of cells in the blood.

1) Red blood cells, *erythrocytes*, containing haemoglobin to carry oxygen to the tissues in the body.
2) White blood cells, *leukocytes*, which perform crucial functions in response to injury and act as the body's host defence. There are five main types of leukocytes; monocytes, lymphocytes, *neutrophils*, eosinophils and basophils.
3) Platelets, *thrombocytes*, with their primary function in blood hemostasis and clotting.

BLOOD VESSELS

Blood vessels are among the simplest structures in the body and consist basically of only 3 cell types; endothelial cells, smooth muscle cells and fibroblasts. The innermost layer is called the intima and consists of a thin layer of endothelial cells (ECs) in all vessels. The middle layer, the media, has different constitution depending on the function of the vessel and consists mainly of smooth muscle cells (SMCs) and elastic fibers allowing for contraction and expansion of the vessel. The outermost layer is the adventitia which has mainly a supporting function and consists of connective tissue, SMCs and fibroblasts. Exceptions to this anatomy are the capillaries that consist only of a thin layer of ECs that are in some cases supported by pericytes.

INFLAMMATION

Inflammation is the body's reaction to injury or infection. The classical clinical signs of inflammation are *rubor* – redness, *calor* – heat; both due to increased local blood flow, *tumor* – swelling due to leakage of plasma, *dolor* – pain and *functio laesa* – loss of function. The recruitment of leukocytes is a key event in the process of inflammation. Leukocytes continuously circulate all tissues in the body searching for injured cells or invasion of pathogens and when encountering such stimuli, these cells respond by invading the injured tissue. Leukocytes are recruited by rolling on the endothelial layer, which is followed by arrest at the site inflammatory stimulation, transmigration through the vessel wall and migration towards the provoking stimulus. However, under certain conditions the inflammatory response can be directed towards the host organism causing chronic inflammatory diseases such as Rheumatoid Arthritis, SLE, inflammatory bowel disease and atherosclerosis.

ATHEROSCLEROSIS AND ITS COMPLICATIONS

Disruption of the blood flow to tissues leads to deficit of oxygen and nutrients which within minutes to hours dependent on type of tissue may cause damage and cell death in the affected area. The major cause of failure in the cardiovascular system is a chronic inflammatory disease named atherosclerosis that is characterized by lipid deposition, leukocyte invasion and accumulation of fibrous elements within the walls of medium-sized and large arteries. Cardiovascular disease related to atherosclerosis is one of the leading causes of death in most parts of the world. The development of atherosclerotic plaques starts early in life, and progress occurs slowly and asymptomatically for decades and affects primarily the intima of the vessel wall thereby causing narrowing of the artery that results in reduced blood flow. Impaired blood movement may result in complications such as angina pectoris or intermittent claudication; however the most feared complications -myocardial infarction and stroke- occur when an atherosclerotic lesion becomes unstable and rupture, causing rapid formation of thrombi that occludes the artery.

RISK FACTORS FOR ATHEROSCLEROSIS

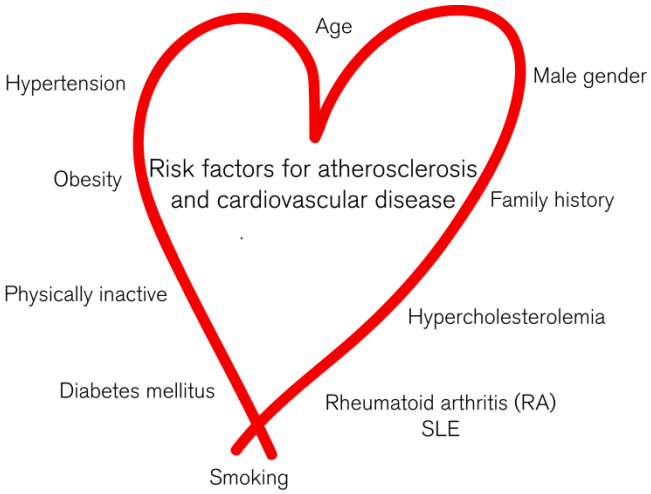

Figure 1: Typical Risk factors for atherosclerosis. There are several risk factors that contribute to athero-slcerosis and cardiovascular disease. Chances of developing atherosclerosis increases with the number of risk factors. Some of these can be controlled such as smoking, physical activity and diet, whereas others such as autoimmune diseases, age, gender and family history cannot.

INTRODUCTION

INFLAMMATION

Inflammation can be triggered by both endogenous and exogenous stimuli and cause a sequence of events which purpose is to serve as host defence and to initiate tissue repair. The vascular response to inflammation occurs in the microvasculature and comprises of rapid arteriolar vasodilation, increased vessel permeability and recruitment of circulating leukocytes in postcapillary venules (Ley 2001).

VASODILATION AND PLASMA LEAKAGE

Arterioles immediately respond to inflammatory stimuli by dilation in order to increase local blood in the inflamed area and thereby facilitate leukocyte recruitment and plasma exudation (Wedmore and Williams 1981). Inflammatory mediators produced at the site of injury will mediate both arteriolar dilation by relaxation of smooth muscle cells in the vessel media and increased vascular permeability by inducing contraction of endothelial cells. The formation of endothelial gaps allows for passage of plasma components such as antibacterial antibodies, complement factors and numerous other proteins that assist in clearing the inflammatory stimulus. When the concentration of extracellular proteins increases, following plasma leakage, osmotic pressure in the interstitial space rises leading to tissue fluid accumulation known as edema (Rubin *et al.* 2008). Under certain pathological conditions, such as influenza-induced pneumonia, severe burn injury or head trauma, the edema formation can lead to life threatening situations.

LEUKOCYTE RECRUITMENT IN INFLAMMATION

Recruitment of leukocytes to sites of injury or infection is essential in the inflammatory response and the sequential steps leading to leukocyte extravasation has been extensively studied during the past decades. The recruitment process depends on a cross-talk between endothelial cells and leukocytes. Inflammatory stimuli leads to activation of venular endothelial cells that upregulate cell adhesion molecules on the surface of the endothelial lining allowing for interaction with leukocytes in the blood stream (Butcher 1991; Lawrence and Springer 1991; Carlos and Harlan 1994; Buckley *et al.* 1998; Ley *et al.* 2007). The sequential steps allowing the recruitment of leukocytes to the extravascular space and site of inflammation include mechanisms as margination, rolling along the endothelium, firm adhesion and transmigration (Figure 2).

The first contact between the leukocytes and the endothelial lining is facilitated by flow dynamics in which erythrocytes are centralized in the blood stream forcing leukocytes in a position closer to the endothelial surface, a mechanism know as margination (Langer and Chavakis 2009). Chemoattractants such as cytokines, chemokines, arachidonic acid metabolites and components of the complement system are produced at the site of inflammation. These chemoattractants induce several different mechanisms that regulate the recruitment of leukocytes. Cytokines can for instance directly activate the endothelium that responds with upregulation of cell adhesion molecules (CAMs). Chemokines on the other hand can, besides affecting the endothelium, diffuse through the endothelial lining and affect leukocytes within the vessel (Robbins and Kumar 2009). Rolling of leukocytes along the endothelium lining is primarily mediated by selectins (E-, L- and P-selectin) and their specialized counterreceptors to which they can transiently adhere. The rolling velocity is partially dependent on the rate of which these bonds are broken. An important ligand for all three selectins, P-selectin glycoprotein ligand 1 (PSGL-1), is expressed on most leukocytes (Ley *et al.* 2007). Rolling velocity decreases in the vicinity of the inflammatory stimulus, due to higher concentration of endothelial cell adhesion molecules and chemoattractants. This reduction in velocity allows for integrin-dependent firm arrest of leukocytes on endothelium. Integrins bind to members of the immunoglobulin superfamily such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which are expressed by activated endothelial cells. Integrins can apart from inducing firm adhesion also affect rolling, cell motility, apoptosis and proliferation (Ley *et al.* 2007; Langer and Chavakis 2009). Once the leukocyte has attached firmly to the endothelium, it starts to migrate along the endothelium to find the optimal place for transmigration (Phillipson *et al.* 2006). Transmigration of leukocytes through postcapillary venular walls into the extravascular space includes passing through the endothelium, the basement membrane and the pericyte sheet (Nourshargh *et al.* 2010).

OUTCOME OF INFLAMMATION

The desired result with the inflammatory response is the elimination of the pathogenic exposure or the source of injury and the restoration of tissue structure and function. Adverse effects of inflammation include formation of abscesses, scarring due to irreversible injuries or persistent inflammation in cases where the pathological stimuli cannot be cleared. These situations can cause cell-mediated immune responses leading to chronic inflammation. Known chronic inflammatory diseases include autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and multiple sclerosis, as well as vasculitis and atherosclerosis.

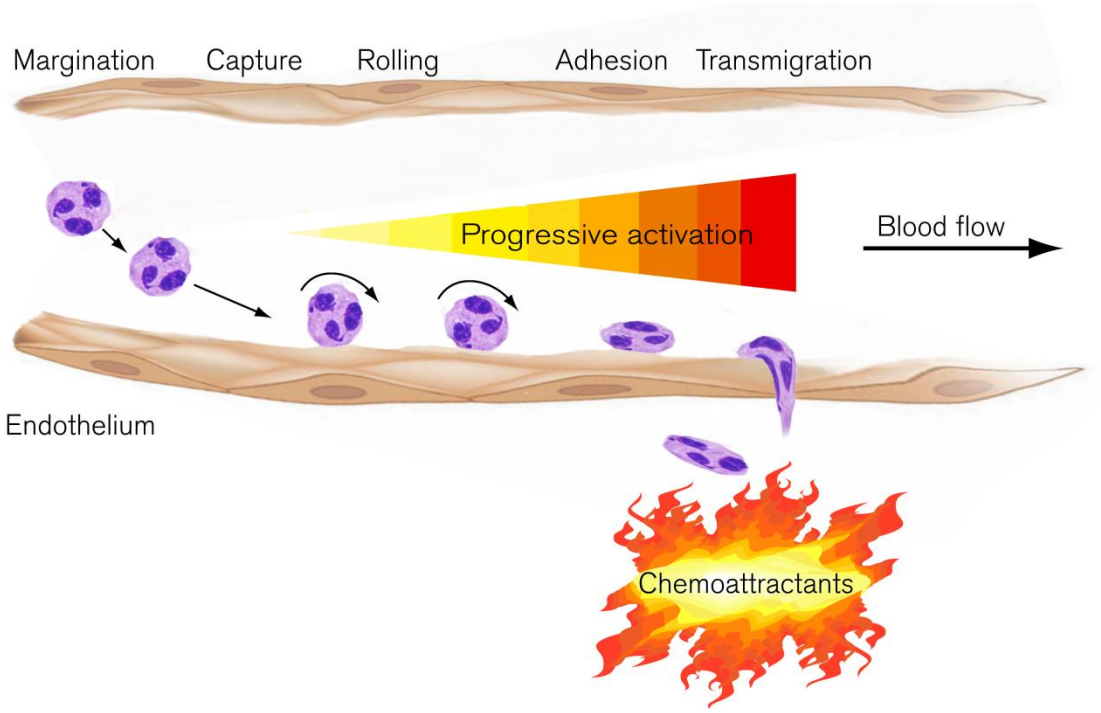

Figure 2: Schematic figure of the multistep leukocyte recruitment cascade. Leukocytes are recruited from the blood stream to sites of inflammatory stimuli through sequential interactions between leukocytes and endothelial cells. These steps include capture to, rolling along, firm adhesion to, and transmigration through the vascular endothelium.

ATHEROSCLEROSIS

Atherosclerosis is a complex chronic inflammatory disease of the walls of medium-sized and large arteries that forms the basis for many cardiovascular diseases. The formation of atherosclerotic plaques is predisposed at sites of branches, bifurcations and curvatures where the flow is disturbed (Lusis 2000). Lesions at predisposed sites have been found already in Egyptian mummies and have the same characteristics as examined plaques in present time. A German-born French pathologist, Jean Lobstein, introduced the term arteriosclerosis already in 1829 which he simply described as a hardening of arteries (Mayerl *et al.* 2006). Atherosclerosis on the other hand is the most common form of arteriosclerosis and consists of focally distributed plaques consisting of accumulated modified lipids, leukocytes, endothelial cells, smooth muscle cells, calcified regions and a necrotic core (Ross 1999). The presence of immune cells within atherosclerotic lesions was described more than 30 years ago when monocytes/macrophages and the formation of foam cells were observed (Gerrity *et al.* 1979). However, retention of low density lipoproteins (LDL) within the arterial wall is one of the first observed changes during early atherogenesis. A subendothelial preservation of LDL particles facilitates oxidative modifications by enzymes such as myeloperoxidase (MPO) and lipoxygenase as well as by radical oxygen species (ROS). Oxidation leads to cleavage of double bonds of fatty acids residues in triglycerides, phospholipids and cholesteryl esters generating

reactive aldehydes and shortened lipids. These lipids can in turn activate endothelial cells to induce cell adhesion molecules (CAMs) on their surface and activate macrophages to produce chemokines (Hansson and Hermansson 2011). CAM expression on the endothelium leads to subsequent recruitment of immune cells - preferentially monocytes- which upon stimulation by macrophage colony stimulating factor (M-CSF) can differentiate into macrophages. Macrophages express scavenger receptors such as SR-A and CD36 that allows for rapid uptake of oxLDL leading to formation of foam cells (Lusis 2000). Early plaques consist mainly of monocyte-derived foam cells, lipids and T-cells. These early stages of atherosclerosis are visible as white areas on the luminal side of arteries and are known as fatty streaks, such lesions can be seen already in human fetal aortas (Napoli *et al.* 1997).

Atherosclerotic progression occurs slowly over years with subsequent invasion of leukocytes and accumulation of lipids. Migration and proliferation of SMCs from the media to the intima occurs in response to cytokines and growth factors released due to the ongoing unsolved inflammation. It's suggested that necrosis and apoptosis of foam cells and smooth muscle cells (SMCs) and accumulation of lipids creates an acellular area. This area known as the lipid/necrotic core is surrounded by extracellular matrix proteins and collagen originating from SMCs (Ross 1999; Libby *et al.* 2009; Hansson and Hermansson 2011). A plaque grows progressively and can cause a stenosis (narrowing of lumen) that contributes to ischemia in the affected tissues, which is the case for Angina pectoris. However, most plaques do not occlude the lumen, instead the arterial wall adapts and expands outwards, a phenomenon known as positive remodelling. Some plaques can evolve into vulnerable plaques, these rupture prone plaques are associated with a large necrotic core and a thin fibrous cap in combination with neovascularization and high inflammatory activity (Ross 1999; Lusis 2000; Hansson 2005). If the plaque ruptures, the necrotic core and the subendothelial space are exposed to the blood stream which induce thrombus formation. These thrombi are responsible for occlusion of the artery that leads ischemia in the affected tissue and is the cause of the majority of clinical events such as myocardial infarction and stroke (van der Wal *et al.* 1994; Hansson and Hermansson 2011).

For many years monocytes were thought to be the only subclass of leukocytes involved in the process of atherogenesis. Later, other leukocyte subclasses were introduced (Jonasson *et al.* 1985; Kovanen *et al.* 1995; Zhou and Hansson 1999; Bot *et al.* 2007). Today it is well accepted that atherosclerosis is an inflammatory disease involving various leukocyte subsets that contribute to lesion formation and play different roles in the pathogenesis of the disease.

LEUKOCYTE RECRUITMENT IN ATHEROGENESIS

Leukocyte recruitment is of great importance in atherogenesis and occurs in sequential steps of rolling, adhesion and transmigration, similar to those occurring in postcapillary venules at sites

of inflammation (Figure 2). The route by which invasion of leukocytes in lesions occur has not been specifically investigated, but the current concept describes that the majority of cells are recruited from the luminal side (Libby *et al.* 2010). However, an alternate recruitment route through the vasa vasorum is possible, and may differ between different subclasses of leukocytes. Several cell adhesion molecules (CAMs) such as P-selectin, ICAM-1 and VCAM-1 are expressed on atherosclerotic endothelium (Cybulsky and Gimbrone 1991; Iiyama *et al.* 1999; Ramos *et al.* 1999). Blocking of any of the contributing CAMs involved in leukocyte-endothelial interactions in atherosclerosis can inhibit leukocyte invasion. Results from gene-targeted mice deficient of either of P-selectin, ICAM-1, or VCAM-1 show that lesion area is reduced with more than 40% in all these models indicating functional importance of leukocyte recruitment to lesions (Nageh *et al.* 1997; Collins *et al.* 2000; Cybulsky *et al.* 2001). VCAM-1 has perhaps gained most attention since it is upregulated during pro-atherogenic conditions and not during baseline conditions (O'Brien *et al.* 1993; Nakashima *et al.* 1998). Furthermore, it has the ability to specifically mediate mononuclear cell accumulation by binding to α4β1 integrin (Berlin *et al.* 1995; Galkina and Ley 2007). Investigation of functional roles of CAMs *in vivo* has been introduced by the use of intravital fluorescence microscopy. By the use of this model it has been shown that the majority of rolling of leukocytes occur predominately in shoulder regions of plaques and is critically dependent of P-selectin. E-selectin on the other hand are suggested to mainly decrease the velocity of rolling cells (Eriksson *et al.* 2001a) whereas L-selectin is important in secondary capture of free flowing leukocytes (Eriksson *et al.* 2001b).

CHEMOATTRACTANTS IN ATHEROSCLEROSIS

A large number of cytokines can be detected in atherosclerotic lesions, and several chemokines and chemokine receptors have been shown to modulate the inflammatory response in plaques (Galkina and Ley 2009). Chemokines such as CCL2, CXCL1, CXCR2, macrophage migration inhibition factor (MIF), CX3CL1 and their receptors have overlapping functions but are primarily involved in regulating the recruitment of leukocytes (Kraaijeveld *et al.* 2007). Cytokines are important regulators of inflammation and can affect endothelial and leukocyte CAM expression, lipid metabolism, endothelial cell permeability, proliferation of smooth muscle cells, extracellular matrix composition and also induce angiogenesis. Cytokines found in atherosclerotic lesions include TNF-a, IL-1, IL-2, IL-6, IL-12, IFN-y and TGF-B (Tedgui and Mallat 2006). Other important chemoattractants include oxidized LDL, reactive oxygen species and leukotrienes.

IMMUNE CELLS IN ATHEROSCLEROSIS

MONOCYTES AND MACROPHAGES

Monocytes are continuously recruited to lesions from the blood stream and differentiate into macrophages upon extravasation. Macrophages containing internalized lipids, foam cells, were the first immune cell associated with atherosclerosis and the number of these cells is correlated to lesion size (Gerrity *et al.* 1979; Hansson 2005; Swirski *et al.* 2007). It has during the past decade become evident that monocytes comprise a heterogeneous population of cells explaining their complex contribution to atherogenesis. In mice, monocytes can be distinguished in the circulation by their differential expression of Ly6C- antigen, CX3CR1 and CCR2. They are regarded as either resident (CX3CR1^{hi}, CCR2-, Ly6C^{low}) or inflammatory (CX3CR1^{low}, CCR2+, Ly6C^{hi}) subsets (Geissmann *et al.* 2003). Similar subsets have also been described in humans depending on their expression of CD14 and CD16 (Passlick *et al.* 1989). The resident subset that express high levels of chemokine CCR5 have been shown to contribute to atheroprogression. Likewise, high fat diet in mice significantly increases the inflammatory subset of monocytes in lesion indicating that hyperlipidemia influences the phenotype of monocytes (Swirski *et al.* 2007; Tacke *et al.* 2007).

LYMPHOCYTES

Lymphocytes are present both inside atherosclerotic lesions and in the surrounding adventitia; however in lesser numbers than monocytes and macrophages. B- and T- lymphocytes constitutively home to the adventitia in healthy mouse aortas and T-cell recruitment is enhanced in both early and advanced atherosclerosis (Galkina *et al.* 2006; Galkina and Ley 2007). T-cells are known to aggravate atherosclerosis (Zhou *et al.* 2000), but the impact on atherogenesis is dependent on which subtype of T-cell investigated. For instance, TH_1-cells clearly increase atherosclerosis possibly by the release of IFN-γ and influence the function of B-cells (Buono *et al.* 2005). TH_2- cells, on the other hand, reduce plaque burden by the release of IL-10 that suppresses vascular inflammation and atherosclerosis (Schulte *et al.* 2008). Regulation between TH_1 and TH_2 response is dependent on regulatory T-cells. Regulatory T-cells produce transforming growth factor (TGF-β) with anti-atherogenic properties, thus decreasing lesion formation (Taleb *et al.* 2008). Likewise, B-cells have until recently been widely accepted with an atheroprotective effect most likely due to the production of antibodies. However, recent studies have shown that B-cell depletion reduces atherosclerotic development and it has been suggested that different subsets of B-lymphocytes (B1 and B2) have opposing effects on atherogenesis (Ait-Oufella *et al.* 2010; Kyaw *et al.* 2010). Atherosclerotic mice show increased levels of antibodies towards oxLDL presumably produced in the spleen since splenectomy aggravates atherosclerosis (Shaw *et al.* 2000; Binder *et al.* 2005). Antibodies have been identified within atherosclerotic lesions but whether or not these antibodies are locally produced remains unknown. Other subsets of lymphocytes such as γδ-T-cells, natural killer

cells and natural killer T-cells have all been suggested to play roles in atherogenesis (Galkina and Ley 2009).

DENDRITIC CELLS AND MAST CELLS

Dendritic cells (DCs) are characterized as cells presenting antigen to naive T-cells in the immune system. DCs form a network within the intima of arteries but not veins, and accumulate in athero-prone regions (Bobryshev and Lord 1995; Millonig *et al.* 2001). The presence of DCs is confirmed in advanced atherosclerosis and they are co-localized with T-cells in shoulder regions and rupture prone plaques. However their function remains unknown (Yilmaz *et al.* 2004; Bobryshev 2005).

Mast cells are known to play a key role in allergy and host defence and have been described both in the adventitia and in atherosclerotic plaques (Galkina and Ley 2009). Mast cells contain proinflammatory cytokines, proteolytic enzymes and vasoactive substances, which they upon activation can release. Activation of mast cells are believed to be of importance in plaque destabilization (Kovanen 2007).

NEUTROPHIL GRANULOCYTES

An increasing body of evidence supports roles for neutrophil involvement in atherosclerosis, and an association with acute coronary events, has grown during the years. Neutrophils are the most prominent cells in the innate immune system and are the first phagocytic cells to invade sites of inflammation. They can efficiently sense and destroy pathogenic intruders by secreting enzymes or by phagocytosis.

White blood cell count, and specifically systemic neutrophil count have been associated with increased atherosclerotic lesion size, more complex lesions, increased risk for myocardial infarction, re-infarction, congestive heart failure and death (Kostis *et al.* 1984; Grimm *et al.* 1985; Ernst *et al.* 1987; de Labry *et al.* 1990; Gillum *et al.* 1993; Thomson *et al.* 1995; Kyne *et al.* 2000; Huang *et al.* 2001; Avanzas, Quiles *et al.* 2004). Atherosclerotic lesions and particularly rupture prone plaque or sites of erosion have an especially high level of inflammatory activity (Kragel *et al.* 1990; Davies *et al.* 1993; Kaartinen *et al.* 1994; Moreno *et al.* 1994; van der Wal *et al.* 1994). Studies have shown the presence of a substantial number of neutrophils at these rupture prone sites (Naruko *et al.* 2002; Avanzas, Arroyo-Espliguero *et al.* 2004). Therefore, inflammation and neutrophils have been suggested to increase the vulnerability and cause destabilization of plaques (Liuzzo *et al.* 1999; Sugiyama *et al.* 2001; Buffon *et al.* 2002; Rioufol *et al.* 2002). Neutrophils can mediate degradation of the basement membrane and cause endothelial cell damage and promote recruitment of monocytes and other inflammatory cells (Dinerman *et al.* 1990; Schratzberger *et al.* 1998; Sugano *et al.* 2005). Furthermore, neutrophils produce reactive oxygen species (ROS) and have been associated with lipid peroxidation

(Katsura *et al.* 1994; Couderc *et al.* 1997). Myeloperoxidase (MPO) an abundant protein in primary granules of neutrophils, is found in atherosclerotic lesions and has been suggested to be important in atherogenesis related to its role in inflammation (Daugherty *et al.* 1994; Podrez *et al.* 2000; Sugiyama *et al.* 2001). MPO binds to LDL in plasma and have been suggested to catalyze the reaction leading to oxidation of LDL (Hazen and Heinecke 1997; Podrez *et al.* 1999; Carr *et al.* 2000; Hazen *et al.* 2000; Heller *et al.* 2000). MPO can also be secreted from monocytes and macrophages, although 95% of MPO in plasma originate from neutrophils and correlates with neutrophil count (Zhang *et al.* 2001).

Detection of neutrophils in atherosclerotic lesions has previously been reported to be rare. This may, however, have plausible explanations other than excluding the involvement of this cell type in the process of atherogenesis. One reason is that the first histological analyses only found monocytes and macrophages, therefore the focus was turned elsewhere. Another reason is that it has been technically difficult to specifically label neutrophils in experimental models. Furthermore, the life span of neutrophils in inflamed tissue is short (Squier *et al.* 1995) and even a small number of detected neutrophils may indicate a significant turnover of these cells in atherosclerotic plaques. Recent findings on this topic will be further discussed in the *Results and Discussion* later in this thesis.

METHODOLOGY

The studies included in this thesis are mainly based on different *in vivo* models adapted to the specific research questions. The most important methods are explained in this chapter; however a detailed description of all methods and material can be found in the individual articles included in this thesis.

GENETICALLY MODIFIED ANIMALS USED IN THIS THESIS

The following genetically modified mouse strains on C57BL/6 background were used in this thesis:

$ApoE^{-/-}$ mice	Mice deficient in apolipoprotein E. It exhibits five times normal serum plasma cholesterol and develops spontaneous atherosclerosis.
$hApoB100/LDLR^{-/-}$ mice	Mice transgenic for human ApoB100 and deficient in the LDL-receptor. Hypercholesterolemic and develops spontaneous atherosclerosis.
$LysM^{EGFP/EGFP}$ mice	Mice are homozygous for enhanced green fluorescent protein (EGFP) in the lysozyme M locus creating mice with endogenously fluorescent myelomonocytic cells (monocytes and neutrophils).
$CX3CR1^{EGFP/EGFP}$ mice	Mice with a knock-in mutation of EGFP in the in fractalkine receptor (CX3CR1) locus creating mice with endogenously fluorescent monocytes, brain microglia cells and a subsets of NK and Dendritic cells.
$ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice	Cross breeding of $ApoE^{-/-}$and Lys M $^{EGFP/EGFP}$ mice creates this atherosclerotic prone mouse strain with endogenously fluorescent monocytes and neutrophils.
$ApoE^{-/-}/CX3CR1^{EGFP/EGFP}$	Cross breeding of ApoE-/- and $CX3CR1^{EGFP/EGFP}$ mice. Atherosclerotic prone mice with endogenously fluorescent monocytes.

PCR on genomic tail DNA was used on each mouse strain to determine their genotype during breeding and before conducting experiments using one forward and two reverse primers each binding either of wild type or knockout DNA segments.

The following primers were used:

ApoE	5'-GCCTAGCCGAGGGAGAGCCG-3' 5'-TGTGACTTGGGAGCTCTGCAGC-3' 5'-GCCGCCCCGACTGCATCT-3'
$LysM^{EGFP/EGFP}$	MLYSUP, 5'-AAGCTGTTGGGAAAGGAGGG-3' EGFPDWN, 5'-GTCGCCGATGGGGGTGTTCT-3' mlp1, 5'-TCGGCCAGGCTGACT-3'
$CX3CR1^{EGFP/EGFP}$	5′-AAGATAGGATGAGTGAAGAC-3' 5'-TAC CGGTGGATGTGGAATGTGTGCG-3' 5'-GGTTGTTCATGGAGTTGGCGG-3'

Atherosclerotic mice were fed either a standard chow diet or a western diet containing 21% triglycerides and 0.15% cholesterol which gives significantly higher cholesterol levels and faster development of atherosclerotic lesions.

SURGICAL PREPARATIONS

Aortic en face histology, confocal microscopy, scanning electron microscopy, transmission electron microscopy, flow cytometry and whole mount preparations of mouse aortas.

Mice were anesthetized with Forene® (isoflurane) and perfused through the left ventricle with 2-3% glutaraldehyde in phosphate buffer, 1% paraformaldehyde or heparinized PBS (20U/ml) for 1-20 minutes at 100mmHg. Outflow was through the right ventricle. The whole aorta, the carotid arteries and the iliac arteries was excised and dissected free from perivascular tissue. For en face preparations, aortas were opened longitudinally and pinned out on paraffin plates, cork or mounted on glass slides. For analysis by confocal microscopy, transmission electron microscopy and immunohistochemical analyses different sections of the aortas were excised. Flow cytometric analyses were conducted on the whole aorta after enzymatic digestion.

INTRAVITAL MICROSCOPY

Aorta: Under isoflurane anaesthesia a catheter was placed in the left jugular vein which allowed for administration of flurochrome, drugs and fluids. The core temperature was kept at 37°C using an infrared heat lamp and a heating pad. The abdomen was opened through a midline incision from the xiphoid process to the genital region. The intestines were a-traumatically retracted and kept moist during the whole experiment. The aorta was visualized and separated from the vena cava without direct manipulations. Areas of visible plaques were identified and an ultrasonic flow probe was placed around the artery proximal to area of interest allowing for continuous blood flow measurements. The exposed tissue was superfused with a thermostated

(37°C) bicarbonate-buffered saline solution. The mouse was placed under the microscope and direct leukocyte-endothelial interactions in shoulder regions of atherosclerotic plaques were recorded on videotape (Figure 3).

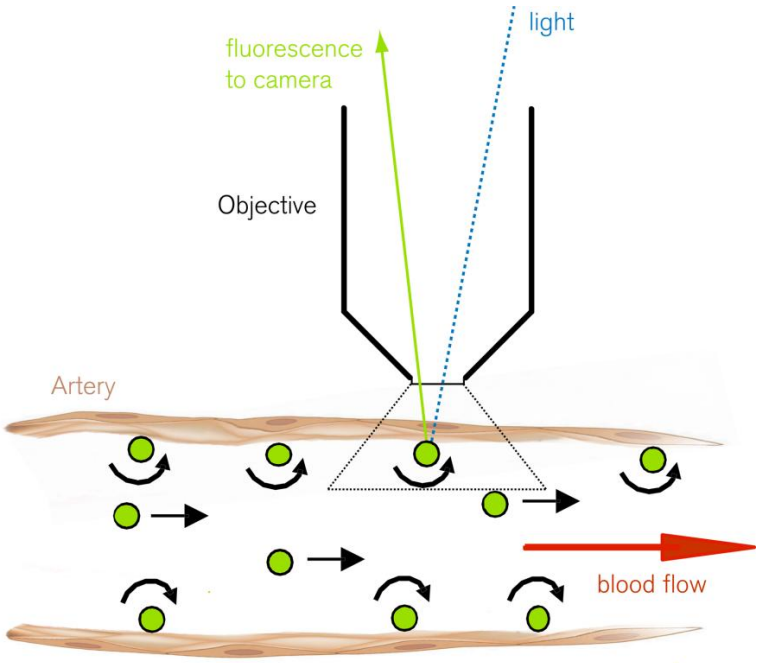

Figure 3: Intravital microscopy. A schematic picture showing intravital fluorescence microscopy. Leukocytes rolling on the endothelium facing the microscope were subjected to light of a certain wavelength allowing for excitation of the cells. Excitated cells emit light of a different wavelength that can be seen through the microscope and recorded with a video camera.

FLOW CYTOMETRY OF ENZYMATICALLY DIGESTED AORTAS

Mice were anesthetized and perfused through cardiac puncture (PBS with heparin; 20U/ml). The aorta and the carotid arteries were harvested, carefully isolated from adjacent tissue. After microdissection, the vessel was enzymatically digested with collagenase XI (125 U/ml), collagenase I (450U/ml), hyalorinidase I-s (60 U/ml) and DNase I (60 U/ml) in PBS with 20mM HEPES buffer. Aortas were incubated at 37°C on a desk shaker for 90 minutes and the obtained cell suspension was filtered through a 40µm strainer before incubation with Fc-receptor blockage (anti-CD16/CD32 ;BD Pharmingen). After subsequent wash staining with primary conjugated antibodies was performed for 20 minutes at room temperature and then washed twice. Data were acquired immediately after staining using the CyAn™ ADP instrument (Dako, Denmark) and later analyzed and compensated with FlowJo Software (Three Star Inc.).

CONFOCAL MICROSCOPY ON WHOLE-MOUNTED PLAQUES

Atherosclerotic lesions from ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice were isolated and fixed in 1% formaldehyde. Subsequently, lesions were mounted *en face* and blocked with 5% donkey serum and incubated overnight at 4°C in a humid chamber with primary antiserum diluted in a solution containing bovine serum albumin, PBS and Triton X-100. For detection of neutrophils we used the neutrophil specific antibody Ly6G/1A8 (BD Pharmingen). The following day,

specimens were rinsed in PBS and incubated with a Cy3 or Cy5-conjugated polyclonal donkey secondary antibody (1:250 and 1:500 respectively) for 45 minutes in a humid chamber at room temperature. Specimens were then rinsed in PBS and mounted in a mixture of glycerol/PBS and examined in a laser scanning confocal microscope (Zeiss, LSM meta 510; Carl Zeiss, Germany). 100 µm optical stacks with a section interval of 1-2µm were created in order to study fluorescent cells. Importantly, neutrophils were readily labeled by antibodies at a depth of at least 50µm in plaques. Analyses of whole mounted plaques was performed in LSM image browser (Carl Zeiss, Germany) by creating a grid overlaying each plaque and manually label each fluorescent cell with arrows. Cells positive for both EGFP and Ly6G were regarded as neutrophils whereas cells fluorescent only in EGFP were regarded as monocytes. The whole plaque was analyzed with 1-2µm steps to a depth of 40 µm in areas of 100x100µm defined as volume units (Figure 4).

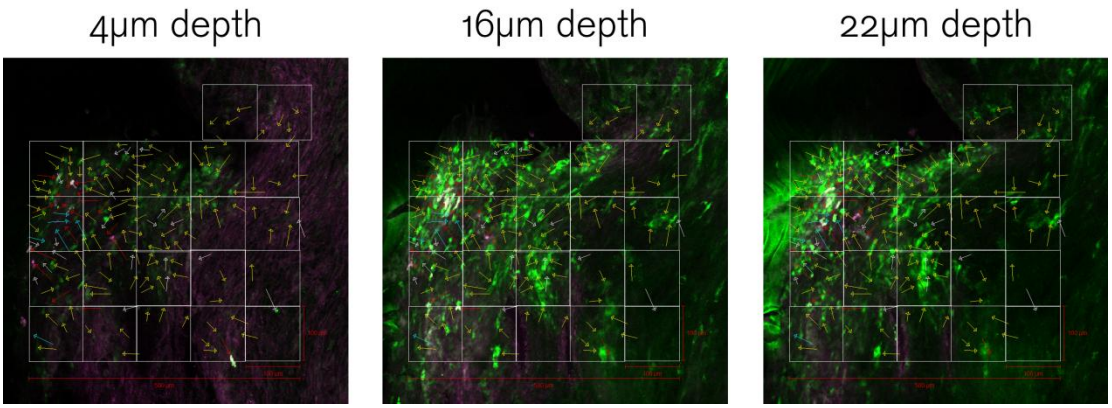

Figure 4: Analysis of neutrophil invasion in atherosclerotic lesions. Representative images from confocal microscopy on whole-mounted atherosclerotic plaques at labeled depth. Each plaque was examined to a depth of 50µm with 1-2µm section intervals. Each analysis was divided in 100µm squares and cells were labeled with arrows of different colors dependent on their fluorescence for EGFP (green) or for a Ly6G antibody (pink).

ELECTRON MICROSCOPY

For analysis of leukocyte adhesion in aortas scanning electron microscopy (SEM) was performed. Fixation was conducted by perfusion with glutaraldehyde (2.5%) through the left ventricle under physiological conditions (100mmHg) for 20 minutes with outflow through the right atrium. The aorta was mounted *en face* on cork plates, dehydrated in ethanol and carbondioxide. After coating the vessels were examined in a Philips SEM 515 microscope. Digital pictures were taken over the thoracic part of the aorta and later analyzed in number adherent leukocytes/mm^2.

In order to investigate leukocyte subsets within lesions at a high magnification we performed transmission electron microscopy (TEM). Sections of the distal part of the aorta (from renal

arteries to proximal part of the iliac arteries) where excised and immediately fixed in 3% cacodylate-buffered glutaraldehyde. Postfix was conducted using 1.5% cacodylate-buffered osmium tetroxide, followed by dehydration in graded ethanol from 70% to 100%. Samples were embedded in Spurr epoxy resin and cut with a diamond knife using an LKB Ultratome IV. Thin sections were subsequently stained with uranyl acetate and lead citrate. Examination of sections was performed in a Philips CM1 20-Twin electron microscope. Identification and localization of neutrophils was conducted based on nuclear shape and staining, the presence of typical granules, and the presence of glycogen particles in the cytoplasm.

MONOCYTE AND NEUTROPHIL DEPLETION

In order to selectively study monocytes and neutrophils by intravital microscopy in $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice we employed different depletion protocols. For selective depletion of neutrophils we treated mice with an intraperitoneal injection of 20µg Gr-1 mouse antibody (mAb) 24 hours prior the experiments. Circulating monocytes were depleted by an intravenous injection of 200µl dichloromethylene-bisphosphonate (clodronate) liposome. Blood samples were analyzed for white blood cell count and flow cytometry prior to and after depletion.

INFLAMMATORY CHALLENGE

In order to induce an inflammatory response we stimulated mice 4 hour prior to experiments by an intraperitoneal injection with cytokines (0.5µg TNF-α and 0.125µg IL-1β in 0.5 ml in PBS) or bacterial lipopolysaccharide (LPS) from E.coli (0.015mg/mouse). For viral infection with influenza virus, a virus highly virulent for mice (Influenza A/PR/8/34; H1N1) was administered nasally by spontaneous inhalation during metophane anesthesia. Blood samples were taken 3 days post infection for differential leukocyte count and cytokine/chemokine concentrations.

AIMS

The aim of this thesis was to investigate the potential involvement of neutrophil granulocytes in the development of atherosclerotic lesions.

The specific objectives were:

- To establish a genetically modified mouse strain that allows for specific investigation of neutrophils in atherosclerosis.
- To use intravital microscopy to investigate if neutrophils interact with arterial endothelium in atherosclerosis.
- To establish techniques allowing sensitive investigation and quantification of neutrophils within arteries and individual atherosclerotic lesions.
- To investigate if neutrophils have the potential of invading atherosclerotic lesions and examine how inflammatory challenge may influence this phenomenon.

RESULTS AND DISCUSSION

Our understanding of the pathophysiological events that cause atherosclerosis has exploded over the past decades, much thanks to development of genetically modified animal models. For instance, mice deficient in apolipoprotein E ($ApoE^{-/-}$) become hypercholesterolemic and develop atherosclerotic lesions of similar phenotype and at sites commonly seen in humans (Plump and Breslow 1995; Lusis 2000). At the time of initiating this study neutrophils retained little or no attention in regard to atherosclerosis development, despite the fact that neutrophil granulocytes were observed in fatty streaks in cholesterol fed African monkeys already in the early eighties (Trillo 1982). Instead, most if not all attention was focused at the roles of monocytes/macrophages and T-lymphocytes. This thesis addresses the potential for neutrophil invasion in atherosclerotic lesions.

DEVELOPMENT OF AN ATHEROSCLEROTIC MOUSE STRAIN WITH FLUORESCENT MONOCYTES AND NEUTROPHILS (PAPER I)

Leukocyte recruitment to the arterial intima is central in the development of atherosclerosis and targeted deletion of various cell adhesion molecules (CAMs) have indicated important roles for these molecules in atherogenesis (Nageh *et al.* 1997; Nakashima *et al.* 1998; Collins *et al.* 2000). Previous results from our lab have investigated the multistep recruitment process in real time by the use of intravital microscopy on atherosclerotic arteries (Eriksson *et al.* 2001a; Eriksson *et al.* 2001b). However, due to technical limitations, distinction between different subclasses of leukocytes interacting with the endothelium had not been possible *in vivo* at the time when this study was initiated. As a tool to individually study different subclasses of leukocytes and their contribution to plaque development, we crossed $ApoE^{-/-}$ mice with $Lys^{EGFP/EGFP}$ mice (Faust *et al.* 2000), that are homozygous for a knock-in mutation of EGFP (enhanced green fluorescent protein) in the lysozyme M locus. Crossing these strains generated lysozyme M-deficient atherosclerosis prone mice with endogenously fluorescent neutrophils and monocytes, ($ApoE^{-/-}$ /$Lys^{EGFP/EGFP}$ mice) and littermate controls ($ApoE^{-/-}$/$LysM^{wt/wt}$) (Figure 5). Previous findings had shown that mice transgenic for lysozyme with increased expression of the protein in serum have a sustained reduction in oxidant stress and reduced atherosclerosis (Liu *et al.* 2006). Consequently, we therefore needed to explore the possibility that absence of Lysozyme M might influence atherogenesis.

We compared the newly generated mouse strain $ApoE^{-/-}$/$Lys^{EGFP/EGFP}$ with its littermate controls, hereafter only described as $ApoE^{-/-}$ mice, for parameters typically associated with lesion development. We found no significant differences in white blood cell count, differential blood cell count, cholesterol profile, global pressure of reactive oxygen species, plaque composition or

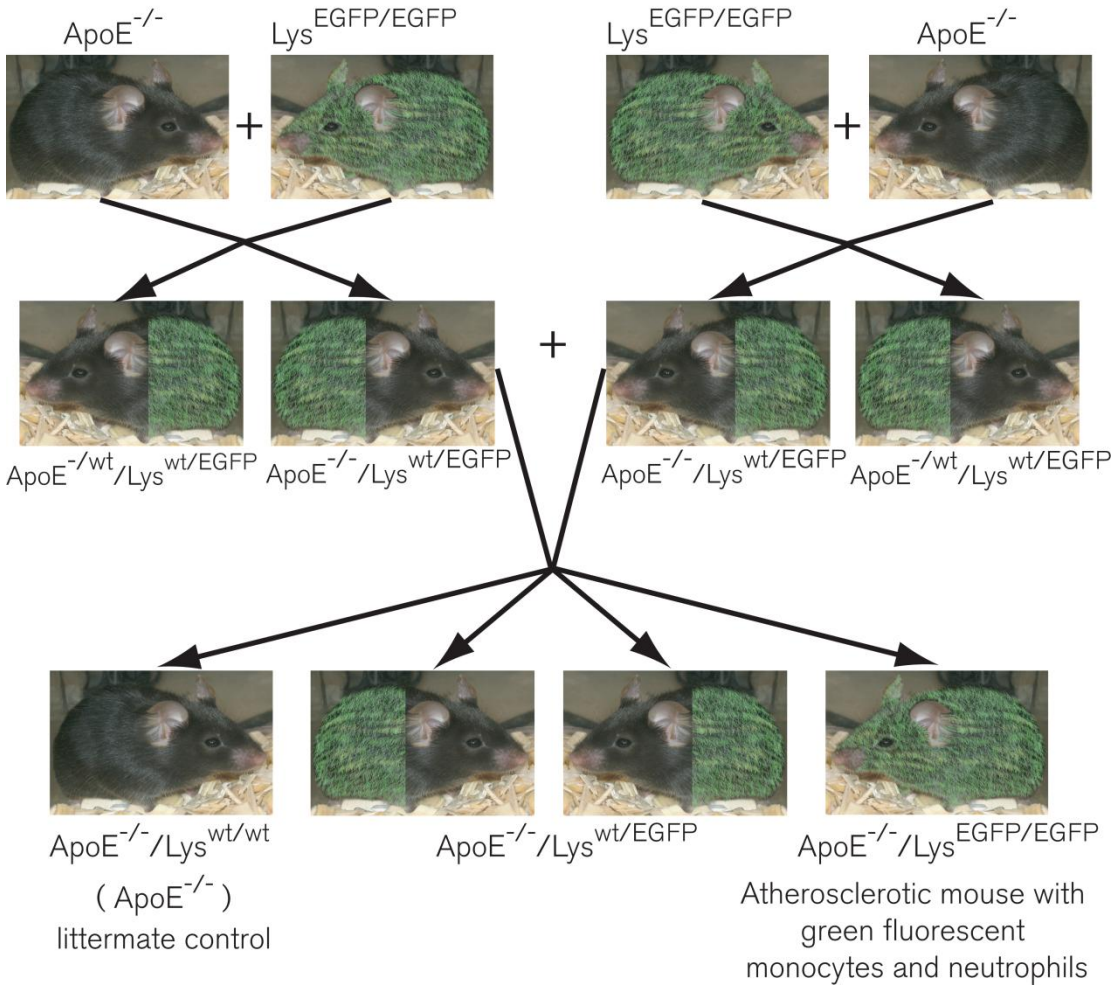

Figure 5: Mouse breeding scheme. Atherosclerotic mice ($ApoE^{-/-}$) were crossed in two generations with $Lys^{EGFP/EGFP}$ mice generating $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice and littermate controls. Genotyping with PCR was conducted on each offspring.

lesion area between the two strains. It was evident from our findings that absence of lysozyme M and replacement with EGFP in its locus does not influence atherogenesis. By flow cytometry we detected three distinct populations of leukocytes with respect to EGFP fluorescence. Lymphocytes were negative in EGFP fluorescence ($EGFP^{-}$) as previously described (Faust, 2000), whereas neutrophils were high ($EGFP^{high}$) and monocytes low in fluorescence ($EGFP^{low}$). Moreover, by the use of confocal microscopy on *en face* prepared atherosclerotic lesions, brightly fluorescent cells were clearly visible within the lesion. Moreover, as seen in Figure 6, EGFP fluorescent cells could be detected while rolling and adhering to atherosclerotic endothelium by the use of intravital microscopy.

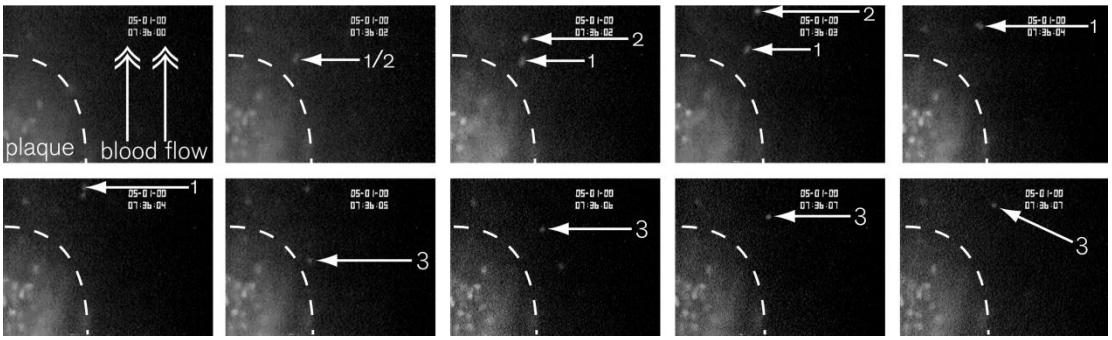

Figure 6: Sequential intravital fluorescence microscopic videoimages from ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice. The blood flow is from the bottom to the top and an atherosclerotic plaque is visible in the bottom left corner as a slightly brighter area with invaded and adherent myelomonocytic leukocytes. Leukocytes rolling on the endothelium are marked with arrows and numbered. Leukocytes are rolling with different velocity where leukocyte **1** has a lower rolling velocity than **2**. Video frame rate was 25 images per second.

NEUTROPHILS REPRESENT THE MAJORITY OF LEUKOCYTES ROLLING ON ATHEROSCLEROTIC ENDOTHELIUM (PAPER II)

Previous experiments using intravital microscopy have shown that the multistep pathway of leukocyte recruitment in inflammation, rolling, firm adhesion and transendothelial migration, also is important in atherosclerosis. Furthermore, leukocyte-endothelial interactions occurred predominately in shoulder regions of lesions (Eriksson *et al.* 2001a). However, previous experiments have been dependent on intravenous administration of flurochrome in order to study these events (Eriksson *et al.* 2001b). Using animals with endogenously fluorescent leukocytes it was now possible to study these events without exogenous manipulation.

In a first approach we studied the number of rolling cells on atherosclerotic endothelium in ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice and compared it with ApoE$^{-/-}$ mice treated with Rhodamine 6G, which labels all circulating leukocytes. Rolling flux (number of rolling cells per minute) was similar in these experiments indicating that cells of non-myelomoncytic origin (i.e. lymphocytes) do not contribute in significant numbers to leukocyte-endothelial interactions on atherosclerotic plaques *in vivo*. To further address this issue we analyzed the number of rolling cells in ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice before and after treatment with Rhodamine 6G. The number of rolling cells on atherosclerotic endothelium did not increase after labelling with Rhodamine. These results may indicate a different path for lymphocyte recrutiment to atherosclerotic lesion other than through the lumen of the artery. Lymphocytes are found to reside both within lesions as well as in the adventitia (Galkina *et al.* 2006), and possibly the majority of lymphocytes are recruited through vasa vasorum or neovasularized venules in plaque.

In order to further dissect the relative contribution of neutrophil and monocyte rolling on atherosclerotic endothelium several different approaches were used. In one set of experiments

we performed bone marrow transplantation (BMT) to ApoE$^{-/-}$ mice after a single dose of irradiation with bone marrow (BM) from Lys$^{EGFP/EGFP}$ mice or CX3CR1$^{EGFP/EGFP}$ mice (a strain with endogenously fluorescent monocytes) (Jung *et al.* 2000). These results showed that the rolling flux was significantly higher in ApoE$^{-/-}$ mice receiving BM from Lys$^{EGFP/EGFP}$ mice than in mice receiving BM from CX3CR1$^{EGFP/EGFP}$ mice. After subsequent treatment with Rhodamine there was no significant difference in rolling flux between the groups indicating that rolling was not impaired in the CX3CR1$^{EGFP/EGFP}$ mice (Figure 7A). In another approach to address the relative contribution of neutrophils to the rolling population we selectively depleted neutrophils or monocytes by intraperitoneal treatment with Gr-1 antibody or intravenous treatment with clodronate liposomes as previously described (Sunderkotter *et al.* 2004; Tacke *et al.* 2006; Soehnlein *et al.* 2008). Clodronate liposomes reduced the number of circulating monocytes by 80%, but it had no reductive effect of the number of rolling cells. In contrast, anti-Gr-1 treatment reduced the number of circulating neutrophils by 90% and the number of rolling cells by more than 80% (figure 7B). Taken together, these data indicate that the majority of leukocytes rolling along atherosclerotic endothelium are in fact neutrophils, and that lymphocytes do not interact with atherosclerotic endothelium in significant numbers.

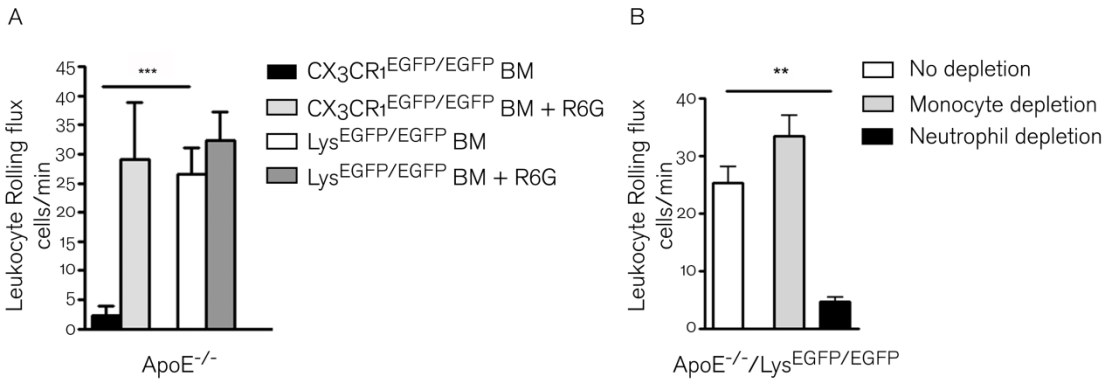

Figure 7: Neutrophils account for most rolling cells in shoulder regions on lesions. (A) Leukocyte rolling flux in ApoE$^{-/-}$ mice after BMT with Lys$^{EGFP/EGFP}$ (fluorescent monocytes and neutrophils) or CX3CR1$^{EGFP/EGFP}$ (fluorescent monocytes) bone marrow before and after treatment with Rhodamine 6G (R6G). (B) Leukocyte rolling on atherosclerotic endothelium after depletion of circulating monocytes or neutrophils. $^{**}p<0.01$, $^{***}p<0.001$.

NEUTROPHILS INVADE ATHEROSCLEROTIC VESSELS (PAPER II)

Our results clearly showed that neutrophils roll along the endothelium in shoulder regions of atherosclerotic lesions. However, the significance of these interactions was unclear. In order to find out whether neutrophils also invade atherosclerotic vessels we performed flow cytometry on cells from enzymatically digested aortas using a modification of a previously described protocol (Galkina *et al.* 2006). We examined aortas from 16 month old ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice fed a western diet and found that 1.8±0.3% and 2.0±0.19% of CD45^{+} (leukocyte common antigen) leukocytes were neutrophils. In contrast, in non-atherosclerotic 12 month old

$Lys^{EGFP/EGFP}$ mice only 0.12±0.03% of $CD45^+$ cells were neutrophils. In order to investigate that neutrophil invasion was not simply an effect of high fat diet we performed additional experiments on 10 month old $ApoE^{-/-}/Lys^{EGFP/EGFP}$ and 12 month old $ApoE^{-/-}$ mice fed a standard chow diet and found that the percentage of neutrophils among the $CD45^+$ leukocytes was 1.6±0.3% and 1.5±0.8% respectively. Interestingly, in 16 month old $ApoE/Lys^{EGFP/EGFP}$ mice, as many as 18±3.0% of myelomonocytic cells were neutrophils. These results clearly indicate that neutrophils invade atherosclerotic vessels in both severely atherosclerotic mice and in mice with less advanced disease. Furthermore, the results indicate that neutrophil invasion is not critically dependent on the type of massive hypercholesterolemia seen in $ApoE^{-/-}$ mice fed a high fat diet.

ACCUMULATION OF NEUTROPHILS IN SHOULDER REGIONS OF LESIONS AND AREAS WITH SUSPECTED HIGH DEGREE OF INFLAMMATORY ACTIVITY (PAPER II)

In order to quantify neutrophils within individual lesions and to investigate their spatial distribution, we took advantage of a novel approach in studying whole mounted atherosclerotic plaques by confocal microscopy. To discriminate neutrophils from monocytes in lesions we used an antibody directed against Ly-6G (1A8) previously shown to selectively label neutrophils (van Leeuwen *et al.* 2008; Zernecke *et al.* 2008). Our results showed that 23±1.6% of all myelomonocytic cells in investigated plaques were neutrophils thus supporting our data retrieved by flow cytometry. During initial investigation of these lesions it became evident that certain areas contained a higher density of invading cells. Due to the knowledge that certain areas of atherosclerotic plaques show a specifically high degree of inflammatory activity (Davies *et al.* 1993; Kaartinen *et al.* 1994; Moreno *et al.* 1994; van der Wal *et al.* 1994), we decided to systematically investigate the regions with a high density of infiltrated EGFP-positive (myelomonocytic) cells in the aspect of neutrophil infiltration. Surprisingly, we found a positive correlation between the number of EGFP positive cells within a defined area and the relative contribution of neutrophils. In areas with high degree of infiltrating cells as many as 54±4.0% of the myelomonocytic (EGFP positive) cells were neutrophils.

Atherosclerotic lesions are in the literature described as early, intermediate or advanced (complex) lesions. Early lesions consist mainly of macrophage derived foam cells, intermediate lesions are characterized as lesions with ongoing smooth muscle cell proliferation. Further invasion of leukocytes can utterly damage the vessel wall and subsequent focal necrosis together with enlargement and protrusion of the lesion into the vessel lumen may occur. During these advanced stages of atherosclerosis, a necrotic core covered with a thin fibrous cap forms within the plaque (Hansson 2005). Based on data presented here we would like to challenge this widely accepted notion and instead suggest that all different stages of plaque development are present simultaneously. According to our findings it is likely that there are different immunological processes that occurr in the core of the plaque compared to shoulder regions or at sites of

erosion. Plaques develop in a both internally and externally manner, but also by spreading longitudinally along the vessel wall. It's possible that these atherosclerotic shoulder regions resemble early atherosclerotic lesions.

We specifically investigated shoulder regions of atherosclerotic plaques with respect to neutrophil invasion. In these regions many infiltrating cells were visualized as a sign of increased inflammatory activity. Interestingly, almost 40% of the myelomonocytic cells were neutrophils compared to the core of the plaques where less than 20% were identified as neutrophils (Figure 8). These results support the previously described findings that most cells rolling on atherosclerotic endothelium in shoulder regions of plaque are neutrophils.

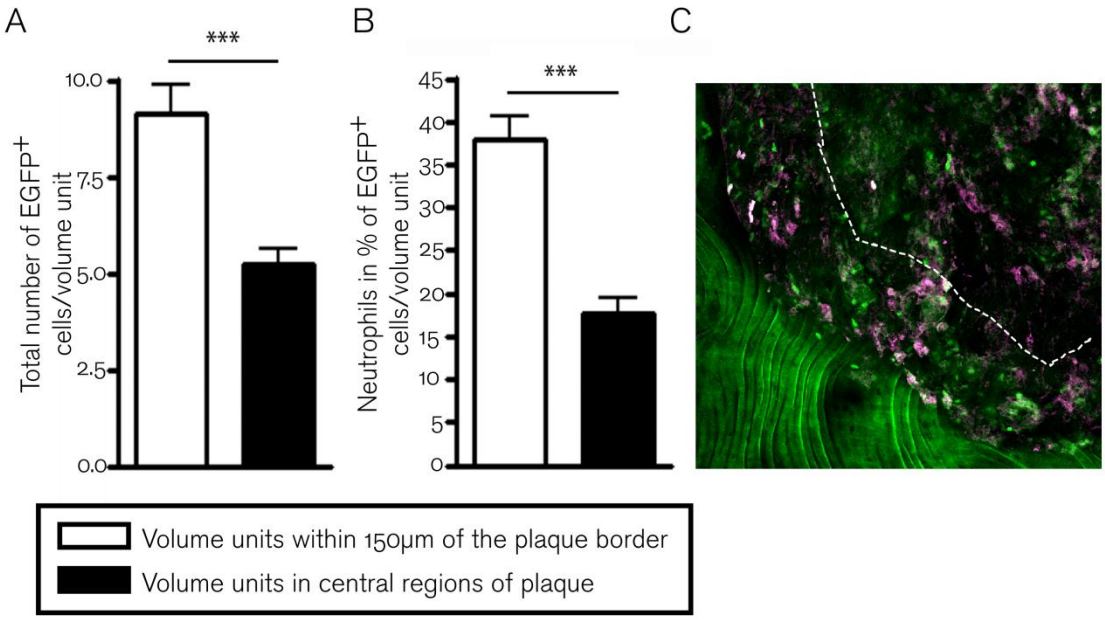

Figure 8: Neutrophils invade lesion shoulders in significant numbers. Graphs show the quantitative contribution of EGFP fluorescent cells (**A**) and relative neutrophil contribution (**B**) in shoulder regions of atherosclerotic plaques. ***$p<0.001$. Representative image of an atherosclerotic lesion stained by Ly6G (**C**). The area between the dashed line and the border is 150µm wide and represents the lesion shoulder.

Our results are also corroborated by findings describing neutrophils to be present in intermediate and advanced lesions of $LDLR^{-/-}$ mice. Neutrophils were found in both the lesional cap and the adventitia, but were rarely observed in the core of the lesion that consisted mainly of macrophage-derived foam cells (van Leeuwen *et al.* 2008). Furthermore, disruption of the Cxcr4/Cxcl12 axis results in an increased atherosclerosis as well as an expansion in the number of circulating neutrophils. Long-term treatment with a Cxcr4 antagonist significantly increased the number of neutrophils within lesions. Moreover, long term depletion of neutrophils by Gr-1 mAb impaired plaque development and significantly reduced the relative content of macrophages within lesions without affecting the peripheral monocyte count (Zernecke *et al.*

2008). Possibly, neutrophil infiltration promotes recruitment of monocytes to the lesion as is the case in tissue inflammation (Soehnlein *et al.* 2008).

INFLAMMATORY STIMULATION INCREASES LEUKOCYTE ADHESION AND NEUTROPHIL INVASION IN ATHEROSCLEROTIC VESSELS (PAPER III)

Previous results have concluded that atherosclerosis is aggravated during systemic inflammatory diseases such as SLE and Rheumatoid Arthritis (Van Doornum *et al.* 2002; Asanuma *et al.* 2003) as well as during infection with circulating levels of bacterial endotoxins (Wiedermann *et al.* 1999). Furthermore, the risk of acute cardiovascular events such as myocardial infarction and stroke increases at times of common infections and auto-immune diseases (Spodick *et al.* 1984; Smeeth *et al.* 2004; Shoenfeld *et al.* 2005). These conditions are known to be associated with a systemic inflammatory response and display elevated levels of proinflammatory cytokines. Several proteins and cytokines such as IL-1, IL-6 and C-reactive protein (CRP) have therefore been used to predict the risk for future cardiovascular events (Biasucci *et al.* 1996; Manten *et al.* 1998; Biasucci *et al.* 1999; Danesh *et al.* 2004). Elevation of certain cytokines have also been correlated to systemic neutrophil count (Nijm *et al.* 2005), which in turn have been widely associated with increased atherosclerotic lesion size, more complex lesions and increased risk for myocardial infarction (Ernst *et al.* 1987; Kyne *et al.* 2000; Huang *et al.* 2001; Avanzas, Arroyo-Espliguero *et al.* 2004; Avanzas, Quiles *et al.* 2004; Gillum *et al.* 2005).

We wanted to investigate how a systemic inflammatory stimulus affects leukocyte recruitment in arteries and especially infiltration of neutrophils in atherosclerotic lesions. After stimulating atherosclerotic mice with inflammatory cytokines TNF-α and IL-1β, or with bacterial endotoxin (LPS) we observed a rapid increase in the levels of several cytokines/chemokines, such as IL-6, IL-12, CXCL1, CCL2 and CCL5, in plasma and the proportional number of peripheral neutrophils. By analyzing the aorta with scanning electron microscopy (SEM) we found that a more than 10-fold increase in the number of leukocytes adherent to the aortic wall compared to non-stimulated ApoE$^{-/-}$ mice. Adhesion appeared to occurred preferentially in the proximal part of the thoracic aorta, and therefore we systematically investigated the area around the two proximal and distal intercostal pairs in ApoE$^{-/-}$ mice. We found that atherosclerotic lesion area was more than doubled in proximal regions compared to distally located regions. Interestingly, four times as many leukocytes were adherent in the proximally located regions upon cytokine stimulation or infection with Influenza A virus. To address the possibility that the increased adhesion was dependent on atherosclerosis we treated C57Bl/6 mice with cytokines, LPS or Influenza A virus. We found no significant preferential leukocyte adhesion in any of the stimulated animals, however it appeared that there might be the same tendency towards proximal adhesion (Figure 9).

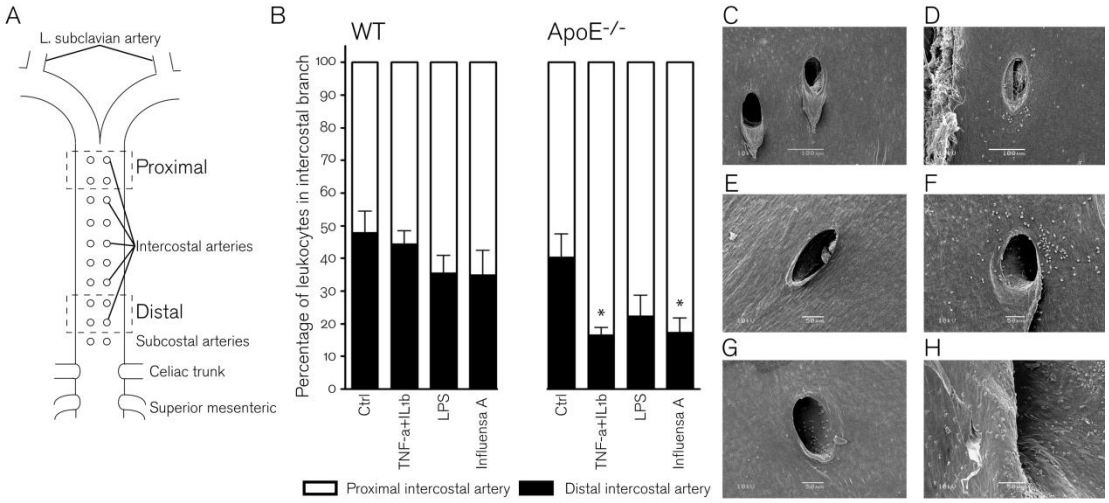

Figure 9: Inflammatory stimuli induce preferential leukocyte adhesion in proximal regions of atherosclerotic aortas. Area of investigation is shown in a schematic picture of a longitudinally opened thoracic aorta (**A**). Graph shows the relative numbers of leukocyte adhesion in the proximal and distal parts of atherosclerotic aortas (**B**). Representative images from SEM are shown in **C-H**. In (**C**) an intercostal pair of an un-stimulated C57Bl/6 with no adherent leukocytes and (**D**) shows leukocyte adhesion around one intercostal branch upon LPS stimulation. An atherosclerotic intercostal branch is shown in a non-stimulated ApoE$^{-/-}$ in (**E**) and a similar branch after cytokine stimulation in (**F**). In some branches, adhesion in the intercostal artery was observed (**G**) and some proximally located atherosclerotic branches had a very high number of adherent leukocytes (**H**). Graphs show mean±SEM (n=4 in each bar). *p<0.05.

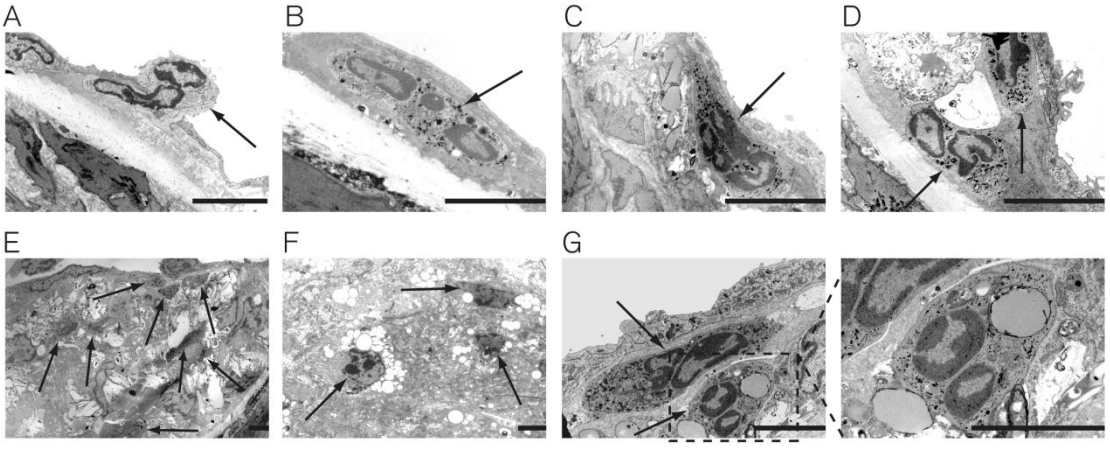

Figure 10: Representative images from transmission electron microscopy (TEM) in normal and atherosclerotic aortas after inflammatory stimulation. Neutrophils transmigrate through the endothelial layer in cytokine treated WT mice (**A**). In (**B**), a neutrophil trapped in the subendothelial space between the endothelial layer and the internal elastic lamina is captured in a WT mouse after infection with Influenza A. In (**C**), a typical neutrophil in an atherosclerotic lesion of an ApoE$^{-/-}$ mouse is displayed. In (**D**), neutrophils are seen in the shoulder region of an atherosclerotic plaque after cytokine stimulation. In (**E**), multiple neutrophils are seen at different levels in an atherosclerotic plaque after LPS stimulation. Neutrophils adjacent to lesion debris are observed in (**F**) and in (**G**), a neutrophil containing a large phagosome is displayed. Arrows indicate neutrophils. Scale bar 10µm.

Furthermore, by the use of flow cytometry on enzymatically digested aortas, we found an increased invasion of neutrophils in atherosclerotic vessels during inflammatory conditions. These findings may, at least in part, explain the increased risk for cardiovascular mortality among patients with ongoing infection or autoimmune diseases. Elevated levels of cytokines among these patients may lead to neutrophilia that in turn leads to increased infiltration of neutrophils in atherosclerotic plaques that accelerate the process of atherogenesis. Notably, transmission electron microscopy revealed that some infiltrated neutrophils had large phagosomes containing lipids indicating a previously undescribed role for neutrophils in atherosclerosis. (Figure 10).

NEW INSIGHTS AND FUTURE STUDIES ON NEUTROPHIL INVOLVEMENT IN ATHEROSCLEROSIS

Recently Dreschler *et al* investigated neutrophil infiltration in atherosclerotic lesions in mice at different time points after introducing a high fat diet. They showed that neutrophils predominately infiltrate in early lesions and as many as 14% of all leukocytes in the aorta were in fact neutrophils. Neutrophil depletion in these mice decreased the number of invaded neutrophils, monocytes/macrophages and reduced atherosclerotic development with almost 50%. In experiments with older animals treated with a high fat diet for 3 or 11 months no reduction of atherosclerotic lesions was observed after 4 weeks of neutrophil depletion. Taken together these data indicate that neutrophils play an important role not only in later stages of atherosclerosis associated with plaque rupture, but in fact contribute to the initiation of atherosclerosis (Drechsler *et al.* 2010).

Despite our and other researchers results showing that neutrophils are present in atherosclerotic lesions, and that they in fact seem to have an impact on atherogenesis, it will require many additional published articles before the roles for neutrophils are defined and generally accepted as important inflammatory cells in the process of atherosclerosis. Future experiments need to investigate the rate of neutrophil invasion in lesions, the lifespan of extravasated neutrophils in plaques and their immunological interplay with other subclasses of leukocytes. Moreover, the chaotic inflammatory process going on within lesions may not be attributed to whole plaque, and requires in depth analysis of different areas within lesions.

SUMMARY

Summary	METHODS	RESULTS	CONCLUSIONS
PAPER I *ApoE$^{-/-}$ /Lysozyme M$^{EGFP/EGFP}$ mice as a versatile model to study monocyte and neutrophil trafficking in atherosclerosis*	Mouse breeding. Genotyping by PCR. Measurement of reactive oxygen species. Flow cytometry Sudan IV staining of lesion area. Fluorescence microscopy. Immunohisto-chemistry. Differential leukocyte count.	No difference was found between ApoE$^{-/-}$ and ApoE$^{-/-}$/LysM$^{EGFP/EGFP}$ mice in following parameters: weight, differential white blood cell count, reactive oxygen species, lesion area. Lesions in the two strains had similar collagen content, expression of VCAM-1 and alpha actin. Similar number of CD68^{+} macrophages and CD3^{+} T-cells were identified.	Deletion of lysozyme M does not influence development of atherosclerosis. Endogenously EGFP fluorescent myelomonocytic cells (monocytes and neutrophils) can be visualized in atherosclerotic mice by intravital- and confocal microscopy.
PAPER II *Distinct infiltration of neutrophils in lesion shoulders in ApoE$^{-/-}$ mice*	Flow cytometry. Confocal microscopy. Intravital microscopy. Leukocyte subset depletion. Bone-marrow transplantation.	Most cells interacting with atherosclerotic endothelium are neutrophils. Neutrophils invade atherosclerotic lesions in significant numbers. There is a great variety of the number of invading cells in different areas of individual plaques. Neutrophil infiltration is higher in areas rich in invaded myelomonocytic cells such as lesion shoulders.	Very few lymphocytes interact with atherosclerotic endothelium suggesting an alternative recruitment route for this subset. Since neutrophils invade lesions in significant numbers it's likely that they also play a role in atherogenesis.
PAPER III *Inflammatory challenge triggers systemic cytokine cascade and rapid infiltration of neutrophils in atherosclerosis*	Infectious and inflammatory stimulation by Influenza virus, LPS or TNF-α and IL-1β. Luminex analysis of cytokines and chemokines. Scanning electron microscopy. Transmission electron microscopy. Flow cytometry.	Inflammatory challenge causes a significant increase in serum levels of cytokines and chemokines in both C57Bl/6 and ApoE$^{-/-}$ mice. Leukocyte adhesion increased more than 10-fold upon stimulation in atherosclerotic aortas. A dramatic increase was observed in the number of invaded neutrophils in atherosclerotic vessels.	Systemic inflammation triggers a major systemic response that induces adhesion and infiltration of activated neutrophils to atherosclerotic lesions. These finding may have implications to atherogenesis in infectious and autoimmune diseases.

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MY SCIENTIFIC JOURNEY

THE EARLY YEARS

At the time of initiating my studies, neutrophils received little or no attention in regard to atherosclerosis development. Instead, most attention was focused at the roles of monocytes/macrophages and T-lymphocytes. Neutrophils, on the other hand, were by some regarded mainly as an "unintelligent" subpopulation of leukocytes that was important in acute inflammation, but had no part in the complex immunological processes in atherosclerosis.

My supervisor, Einar Eriksson, developed an intravital fluorescence microscopy technique allowing direct observations of leukocyte-endothelial interactions in arteries and atherosclerosis *in vivo*. By the use of this newly developed method it was shown that leukocyte rolling on atherosclerotic endothelium occurs in similar ways as that occurring in venules at site of inflammation. In these set of experiments, treatment with anti-neutrophil serum (ANS) caused a rapid parallel decrease in systemic neutrophil count and leukocyte rolling on atherosclerotic endothelium. These results, and the fact that neutrophils account for 2/3 of all circulating leukocytes in humans together with the knowledge of neutrophils abundance in acute inflammation, raised the question why neutrophils would not play a role in atherosclerosis.

MOUSE BREEDING AND TECHNICAL DEVELOPMENT

Since the previous data could not specifically show that neutrophils rolled along atherosclerotic endothelium, my first objective was to investigate and quantify to what extent neutrophil-endothelial interactions occurred on atherosclerotic lesions. At that time, no techniques which allowed for specific labelling of neutrophils in atherosclerotic mice were available. However, a mouse strain with green fluorescent monocytes and neutrophils ($Lys^{EGFP/EGFP}$ mice) had recently been developed and was kindly provided to us by Thomas Graf at the Albert Einstein School of Medicine, New York. In order to generate atherosclerotic mouse strains with green fluorescent monocytes and neutrophils, we crossed $ApoE^{-/-}$ (an atherosclerotic prone strain) mice with $Lys^{EGFP/EGFP}$ mice in two generations creating $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice.

In order to certify that the genetic modification in these mice did not alter the development of atherosclerosis *per se*, we compared the generated $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice with littermate $ApoE^{-/-}$ mice. There was no difference in cholesterol levels in blood, atherosclerotic lesion area or atherosclerotic plaque composition between the two strains and I could start to apply the newly generated mouse strain in different microscopic techniques. First of all we needed to investigate whether the green fluorescent leukocytes, i.e. monocytes and neutrophils, could be visualized while performing intravital fluorescence microscopy on atherosclerotic lesions. It became evident that the fluorescence from the cells was quite bright and easily identified with significantly less artefacts compared to experiments performed with older techniques using intravascular flurochrome labelling. Adherent or invaded cells were observed within atherosclerotic lesions and neutrophils and monocytes were observed rolling along the endothelium in shoulder regions of atherosclerotic plaques.

LOOKING FOR NEUTROPHILS

When the newly generated mouse strain ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ was established as a model to study monocyte and neutrophil trafficking in atherosclerosis, we were ready to investigate if neutrophils actually rolled on atherosclerotic endothelium. In a first approach to address this issue we used the difference in excitation and emission wavelengths between Rhodamine 6G (flurochrome) and EGFP (enhanced green fluorescent protein) by employing different filter settings in the intravital microscope. Our first results were promising since EGFP fluorescent cells could not be seen while using the Rhodamine filter. Our objective was then to treat ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice with Rhodamine 6G, which labels all circulating cells containing a nucleus, and by switching filters estimate the contribution of lymphocytes. However, theory and actual experiments do not always match up and this did not work out as planned since there was a leakage of rhodamine fluorescence into the EGFP channel. All cells were visible in both filter settings, and these first results had to be discarded.

In our secondary approach, we started out from the fact that we could not find any significant difference between the generated mouse strain and its non-fluorescent littermate controls in the aspect of atherogenesis. We performed intravital microscopy on both these strains in shoulder regions of visible atherosclerotic plaques in order to quantify the total number of rolling cells. The ApoE$^{-/-}$ mice were injected with Rhodamine 6G and rolling was recorded and compared with the results with ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice, which has fluorescence in monocytes and neutrophils only, and discovered that there was no difference between the two strains. We could not detect any significant differences in the number of rolling cells and therefore concluded that most of the rolling cells are either monocytes or neutrophils.

The results described above indicated that not many lymphocytes actually roll on atherosclerotic endothelium. To further investigate the lymphocyte-endothelial interactions, we investigated ApoE$^{-/-}$/Lys$^{EGFP/EGFP}$ mice before and after treatment with Rhodamine 6G and recorded the number of rolling cells. Interestingly, the number of rolling cells did not increase with subsequent treatment of Rhodamine 6G further indicating that not many lymphocytes actually roll on atherosclerotic endothelium.

At this point it seemed plausible that neutrophils interact with atherosclerotic endothelium, since many cells actually rolled on the endothelium and neutrophils make up for about 1/3 of all circulating leukocytes in mice, whereas monocytes are only between 5-10%, but we still needed to prove and quantify to what extent neutrophils and monocytes contributed to leukocyte-endothelial interactions. We had recently gained access to a new mouse strain, the CX3CR1$^{EGFP/EGFP}$ mice which had green fluorescence exclusively in monocytes. In a first approach we performed cross-transfusion of whole blood from Lys$^{EGFP/EGFP}$ mice (fluorescent monocytes and neutrophils) and CX3CR1$^{EGFP/EGFP}$ mice to atherosclerotic mice (ApoE$^{-/-}$). Blood shift was allowed through catheters connecting the carotid artery from one mouse to the left jugular vein of the other and vice versa. Immediately after blood-exchange, intravital microscopy was performed and the number of fluorescent leukocytes rolling on atherosclerotic endothelium was significantly higher in ApoE$^{-/-}$ mice cross-transfused when receiving fluorescent monocytes and neutrophils mice than in those transfused fluorescent monocytes only.

Even though these results were promising and constituted a further indication of neutrophil-specific rolling on atherosclerotic endothelium, they were discarded due to difficulties in calculating the number of fluorescent cells that actually passed over into the circulatory system in the recipient mice. Instead, by help from Filip Farnebo at the Department Cell and Molecular Biology (CMB) at Karolinska Institutet, we performed bone marrow transfer (BMT) from $Lys^{EGFP/EGFP}$ or $CX3CR1^{EGFP/EGFP}$ mice into atherosclerotic mice. Intravital microscopy on these mice showed that very few fluorescent cells were interacting with atherosclerotic endothelium after transfer with bone marrow containing fluorescent monocytes only, compared to animals rescued with bone marrow from mice with fluorescent monocytes and neutrophils. These results clearly indicated that neutrophils are responsible for a major part of leukocyte rolling on atherosclerotic endothelium. In a third approach to further discriminate between different subclasses of rolling leukocytes we selectively depleted either monocytes or neutrophils in $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice. In monocyte-depleted animals rolling frequency remained unchanged compared to controls, whereas neutrophil-depleted animals rolling were reduced by more than 80%.

NEUTROPHILS ROLL ON ATHEROSCLEROTIC ENDOTHELIUM – SO WHAT!

At this point we had clearly shown that most leukocytes rolling on atherosclerotic endothelium were in fact neutrophils. As these data were presented at an international congress, our major concerns were reflected in some of the reactions we received from our peers. Okay, neutrophils are indeed rolling - but what does that mean? It does by no means show that they are important in the pathophysiological progress of atherosclerosis. So we needed to develop a method where we could actually investigate if neutrophils invade atherosclerotic plaques and if so, how many that actually infiltrate lesions. Around that time in May 2006 Klaus Ley's group at the University of Virginia in Charlottesville published a paper where they performed flow cytometry on enzymatically digested mouse aortas to investigate lymphocyte homing in arteries. If we could apply this model and specifically label neutrophils, this could give us the answer on whether neutrophils, invade atherosclerotic lesions.

My objective was then to learn multi-color flow cytometry, a technique for counting and examining microparticles and cells, and setup a protocol that could identify neutrophils within atherosclerotic vessels. Unfortunately, no neutrophil specific antibody for mice existed at that time and I knew that critics would be harsh if the certainty of the labelling was not absolute. As a cornerstone in these experiments I decided to use the $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice, since we previously had shown that lymphocytes are not fluorescent in EGFP and neutrophils have brighter fluorescence intensity than monocytes. It soon became apparent that this was not going to be as easy as anticipated, since leukocytes show a greater variety of background fluorescence when extracted from solid tissue than from blood. Furthermore, not many leukocytes reside in the aorta and therefore you only get one chance to get the instrument settings correct. On top of this, atherosclerotic mice take considerable long time to develop lesions (6-12 months) making them quite expensive and scarce in supply. Consequently, I needed to explore the method in healthy mice and different tissues such as spleen, lung and lymph nodes after inflammatory stimulation.

With great help from Kalle Malmberg at the Center for Infectious Medicine (CIM) at the Karolinska Institutet, I decided to label all leukocytes with an antibody directed towards a

common leukocyte antigen (CD45), and then label different leukocytes subsets (T-lymphocytes, B-lymphocytes, monocytes, macrophages) and discard them until I was certain that only neutrophils were left. Overall, I tested more than 50 different antibodies in different color combinations in order to find the optimal combinations and minimal leakage between the different channels in the flow cytometer before I was convinced that I had a sufficiently stringent protocol.

HAPPINESS AND COMPETITION

The results from the flow cytometry experiments came back positive and we could show that around 2% of all leukocytes in atherosclerotic aortas (obtained from atherosclerotic mice fed a high fat diet) were in fact neutrophils compared to non-inflamed aortas where less than 0.15% were neutrophils. We had finally successfully found that neutrophils invade atherosclerotic vessels.

At that time I had no knowledge of any other research group focusing on detecting neutrophils in atherosclerosis. During the period of summarizing and writing a paper on my previously discussed results, two papers from two different groups were published online the same day in November 2007, revealing the first existence of neutrophils in atherosclerotic lesions in mice. One of the papers, originating from Maastricht University, described that neutrophils were present in lesions and the arterial adventitia in both intermediate and advanced stages of atherosclerosis. The other paper originated from Aachen University and described that blocking a specific chemokines receptor increased systemic neutrophil count and caused an increase in plaque formation and a relative increase of neutrophils in atherosclerotic lesions. Furthermore, by depleting neutrophils during four weeks, plaque development in atherosclerotic roots was attenuated indicating that neutrophils promote atherogenesis. Interestingly, both papers used a monoclonal antibody (anti-Ly-6G) as a specific marker for neutrophils which I had lacked in my previous experiments.

DIGGING DEEPER

Since the prospect of being the first to put neutrophils on the atherosclerotic map in modern time was gone, we decided to further examine the potential role for diet-induced neutrophil invasion and to investigate the specific localizations of neutrophils in atherosclerotic lesions. Both the previously described papers used a diet-induced atherosclerosis by feeding the mice a high fat diet. We wanted to explore if neutrophil invasion was strictly induced by diet or if it also occurred in animals fed a standard chow diet. We used atherosclerotic mice that were fed a standard diet and found that around 1.5% of leukocytes within the atherosclerotic aortas were neutrophils, which is not significantly less than from mice fed a high fat diet.

To be able to quantify neutrophils within atherosclerotic lesions and to investigate their spatial distribution in plaques, I took help from Sebastian Thams at the Department of Neuroscience at Karolinska Institutet. We developed a novel approach in studying whole-mounted atherosclerotic plaques by using confocal microscopy, a technique allowing for investigation of tissues in individual focal planes and allowing for 3D reconstructions of the examined object. We used the neutrophil-specific antibody previously described to label neutrophils and could detect neutrophils at a depth of more than 50μm within individual atherosclerotic lesions. We found that neutrophils were present in lesions at a ratio of about

1:3 to monocytes/macrophages. This number was even greater than the results we received from flow cytometry analysis, which may reflect the fact that we with confocal microscopy specifically investigated plaques whereas we studied cells derived from the whole aorta in our flow cytometry approach. Interestingly, the density of immune cells was very variable within plaques and some areas contained a significantly higher number of invaded cells. In areas with a high number of infiltrated cells, more neutrophils than monocytes/macrophages were actually present, thus indicating an acute inflammatory state within chronic atherosclerotic lesions.

THE PROCESS OF PUBLICATION

In order to get recognition for our major findings describing neutrophil rolling on atherosclerotic endothelium and subsequent invasion in lesion we were required to validate the animal strain $ApoE^{-/-}/Lys^{EGFP/EGFP}$ by first publish results showing that the genetic manipulation in fact did not alter the atherosclerotic phenotype. At first we wanted to compile all these data into one publication, but realized early on that the content would be too extensive and lack an appropriate focus. Instead we decided on two papers, the first describing the generated mouse strain and the second on the findings of neutrophil invasion.

The first article is primarily methodological and descriptive in its approach and such articles sometimes tend to receive less scientific attention compared to more mechanistic studies. During this period several papers were published describing how different knockout mice affect the development of atherosclerosis. Another research group showed that mice transgenic for lysozyme, resulting in overexpression of the protein in serum, express reduced atherosclerosis due to a reduction in oxidative stress. This gave us the incentive that absence of Lysozyme M might influence the development of atherosclerosis. This article was sent to three different journals and it took more than twelve months from the first submission before actually receiving comments that we could reply to. Despite mixed reviews regarding the importance of the findings, our study was finally judged suitable for publication in *Atherosclerosis*.

After the long process of getting recognition for the first article, the second paper took even longer. More than 2 years actually passed from initial submission until publication. Our paper did not present a functional approach to what extent neutrophils influence the process of atherogenesis, which could be a likely reason why it was difficult to get it published. Another reason might be the reluctance of the atherosclerotic research community to accept the neutrophil as a player in the complex immunological disease of atherosclerosis. However, we believed that if the presence of neutrophils is not described and recognized, then no functional analysis of their role and importance in atherosclerosis will ever be accepted. Finally, after performing additional control experiments required by the reviewers and extensive text editing, the article was accepted for publication in *American Journal of Pathology*.

INVESTIGATING THE POTENTIAL ROLE FOR NEUTROPHILS IN DISEASES ASSOCIATED WITH PREMATURE DEATH IN CARDIOVASCULAR EVENTS.

Previous results have concluded that atherosclerosis increases during systemic inflammatory diseases such as SLE and Rheumatoid Arthritis. Furthermore, the risk of acute cardiovascular events such as myocardial infarction and stroke increases at times of common infections and auto-immune diseases. These conditions are known to be associated with a systemic inflammatory response and display elevated levels of proinflammatory proteins in blood. Several proteins and cytokines have therefore been used to predict the risk for future cardiovascular events.

We wanted to investigate how a systemic inflammatory stimulus affects leukocyte recruitment in arteries and especially infiltration of neutrophils in atherosclerotic lesions. After stimulating atherosclerotic mice with inflammatory cytokines, or with bacterial endotoxin, we observed a rapid increase in the levels of proinflammatory proteins and relative number of neutrophils in blood. By analyzing the aorta with scanning electron microscopy (SEM) we found an elevated number of leukocytes adhered to the endothelium specifically in regions were atherosclerotic lesions commonly occur. Furthermore, by the use of flow cytometry we found a preferential invasion of neutrophils in atherosclerotic vessels during inflammatory conditions. These findings may, at least in part, explain the increased risk for cardiovascular mortality among patients with ongoing infection or autoimmune diseases. Notably, transmission electron microscopy revealed that some infiltrated neutrophils contained ingested lipids, a function commonly attributed to macrophages indicating a previously unknown role for neutrophils in atherosclerosis.

THE END OF THE ROAD

Despite all the results that introduce and advocate the presence of neutrophils within atherosclerotic lesions, it will take many more experiments and published articles before the role of the neutrophils in atherosclerosis will be established. Future experiments need to investigate the rate of neutrophil invasion in lesions, the lifespan of neutrophils in plaques and their communication with other subclasses of leukocytes. No matter what, the simplistic view of the neutrophils as cells that infiltrate only at sites of acute inflammation too early, and act too blindly and die too rapidly is now surely outdated.

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