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Novel experimental therapies for atherosclerosis : a genomics based approach

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Novel Experimental Therapies for Atherosclerosis

A Genomics Based Approach

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A Genomics Based Approach

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Eva Josephine Anna van Wanrooij

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Chapter 1

General Introduction

EJA van Wanrooij

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1.1 Pathogenesis of atherosclerosis

Cardiovascular diseases, including myocardial infarction and stroke, are the main cause of death in the Western world¹. The main underlying pathology leading to these clinical manifestations is atherosclerosis. It is a progressive disease of the middle and large arteries characterized by lipid deposition, inflammation, cell death and fibrosis²⁻⁵. Atherosclerotic plaque development will lead to narrowing of the vessel, eventually resulting in downstream hypoxia and nutrient deprivation. Finally, the rupture of a destabilized atherosclerotic plaque leads to more dramatic clinical events, and may induce sudden arterial occlusion due to thrombus formation. Depending on the affected artery, the ischemia resulting from the thrombus formation will lead to myocardial infarction, stroke or peripheral artery diseases such as intermittent claudication. In the last decades several risk factors for atherosclerosis have been identified including smoking, hyperlipidemia, hypertension, physical inactivity, diabetes and male sex⁶. Today, treatments are mostly based on the elimination of these identified risk factors and consist of lowering of serum lipid levels by statins, reduction of blood pressure and changes in life style.

Adhesion and transmigration of leukocytes

A healthy arterial wall consists of a tight endothelial cell layer covering the inner surface, elastic lamina and a surrounding layer of smooth muscle cells. Atherosclerotic lesion formation is initiated by increased adhesiveness of the endothelial cell layer⁷. Normally functioning healthy endothelium produces anti-inflammatory and anti-thrombotic agents that prevent large scale infiltration of leukocytes and thrombus formation^{8, 9}. So-called “injury” of the endothelium due to risk factors discussed above, results in an increased permeability and adhesiveness of the vessel wall⁹. Endothelial cells enhance the expression of adhesion molecules and produce pro-inflammatory mediators including chemokines and interleukins¹⁰.

The attracted leukocytes transmigrate through the endothelial layer in a multi-step process in which the following stages can be identified; rolling on the endothelium, firm adhesion and transmigration to the sub-endothelial space^{11, 12}. A schematic overview of this process is shown in figure 1.

Deceleration of leukocyte rolling on the vessel wall is mediated by the selectin family, mainly E- and P-selectin¹³. The expression of these molecules is induced after vascular stress, and is the result of NFκB induced activation¹⁴⁻¹⁶. Firm adhesion of the now slowly rolling leukocyte is facilitated by intercellular adhesion molecule (ICAM)-1 and -2 and vascular adhesion molecule (VECAM)-1^{17, 18}. Due to release of chemo-attractants and pro-inflammatory molecules, the attracted leukocytes get activated and upregulate the surface expression of integrins such as very late antigen (VLA)-4 and lymphocyte function associated antigen (LFA)-1. These integrins function as counter-ligands for ICAM-1 and

VCAM-1¹⁹⁻²¹. Additional molecules with adhesive properties on leukocytes are L-selectin, CD14 and P-selectin glycoprotein ligand (PSGL)-1²². Shortly after firm adhesion, passage of the leukocyte through the endothelial junction is facilitated by the expression of platelet/endothelial cell adhesion molecule (PECAM)-1, junctional adhesion molecule (JAM)-1 and CD99²³⁻²⁷. This process is also known as diapedesis.

The process of leukocyte infiltration is accompanied by the release of chemokines and interleukins that modulate the activation state of both the endothelium as well as the entering leukocyte. The complex function and regulation of interleukins and chemokines is addressed in separate chapters below.

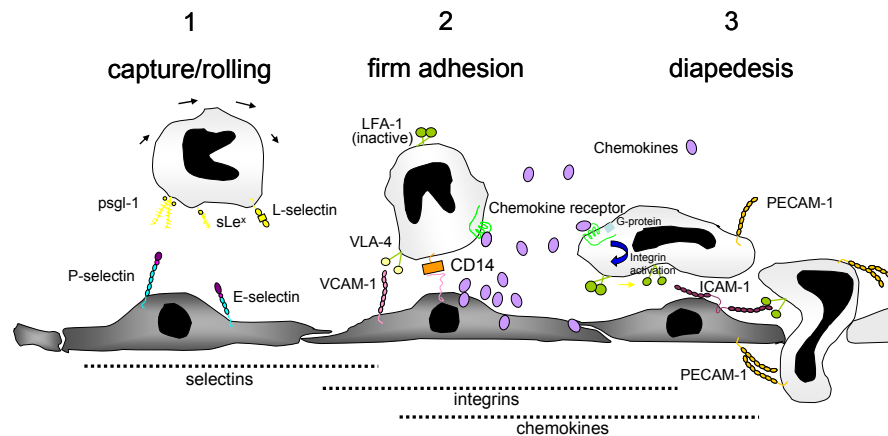


Figure 1: schematic overview of leukocyte egress during atherogenesis

Upon activation, the vascular wall increases the expression of proteins with adhesive properties such as E- and P-selectin and cell adhesion molecules (CAM's). Transmigration of the leukocytes is mediated by endothelial junction proteins such as PECAM-1, junctional adhesion proteins (JAM's) and CD99. (adapted from J. Twisk and minimally modified)

Plaque progression

A high serum cholesterol level is one of the predominant risk factors associated with atherosclerosis and lipid deposition within the arterial wall is a characteristic of atherosclerotic plaques. As mentioned, leukocytes and in particular monocytes enter the sub-endothelial space after endothelial activation due to "injury". Once invaded, they differentiate into macrophages and consistent with their role as scavenging cells, start to take up the modified lipoproteins present in the neo-intima²⁸. In normal cells, cholesterol homeostasis is a tightly regulated process mainly controlled by feedback regulation of low density lipoprotein (LDL) receptor to balance cholesterol intake, and intracellular cholesterol level sensors in combination with sterol-regulatory binding proteins (SREBP's)²⁹⁻³¹. Scavenger receptor mediated uptake of modified lipoproteins is however responsible for the uncontrolled uptake of

cholesterol within a high cholesterol environment²⁸. This excessive accumulation of cholesterol esters (CE) eventually leads to foam cell formation³².

These foamy macrophages are the primary constituents of the initial (non-obstructive) atherosclerotic plaque, also called fatty streak. Next to macrophages, T lymphocytes, that further drive the inflammatory response, are also present.

After the formation of a fatty streak, a more complex lesion can form. This process involves the additional influx of more leukocytes, small extra-cellular lipid deposits, and proliferation of vascular smooth muscle cells. Vascular smooth muscle cells migrate to form a fibrous cap on top of the growing lipid core³³. The lipid core and fibrous cap are hallmarks of the so-called advanced atherosclerotic lesion. Ultimately, plaques form complex lesions that are partially necrotic, contain cholesterol crystals and calcified material. These advanced plaques are prone to rupture due to increased expression of matrix metalloproteinases and other cathepsins that are able to destabilize the fibrous cap. The increasing amount of apoptosis and necrosis within the plaque also contributes to destabilization³⁴⁻³⁷. Plaque rupture and the subsequent release of the pro-thrombogenic plaque content is the direct underlying cause of the majority of acute clinical manifestations such as MI and stroke.

1.2 Finding new biological targets using genomics

The genome of mammals consists of thousands of genes, and these genes result in a vast amount of RNA molecules (20000-25000) and their related proteins. The regulation of this important machinery is a complex and multi-factorial process, as the eventual expression level of the functional protein can depend on transcription rate, translation rate, and post translational modification such as phosphorylation.

Common experiments in molecular biology are based on the “one gene, one experiment” design. This setup severely limits the amount of information generated per experiment as it only addresses the regulation of a chosen target. In the past decade, micro-arrays were developed that enabled the simultaneous monitoring of several genes at a time by spotting of cDNA or RNA probes on glass slides. Unravelling the total mouse and human genome facilitated the construction of whole genome arrays that were able to visualize the expression of thousands of genes in one experiment (for a schematic overview, see figure 2).

Expression profiles of different metabolic situations can be easily compared, leading to insight in the underlying mechanisms. These experiments however produce vast amounts of data that have to be analyzed in a reliable and orderly fashion, and generate new and complex statistical challenges.

In the field of atherosclerosis, several micro-array analyses have been performed to identify signature gene expression patterns for different disease stages. In the majority of these studies, entire vessels from animal models of atherogenesis were used to compare diseased to healthy vessel wall³⁸⁻⁴². Clearly, this approach has a drawback with respect to the fact that the tissue used does contain multiple cell types, especially when comparing the healthy vessel wall with advanced atherosclerotic plaques with large leukocyte infiltrates. The observed regulatory profile is therefore hard to address, as it may predominantly result from the difference in sample composition only.

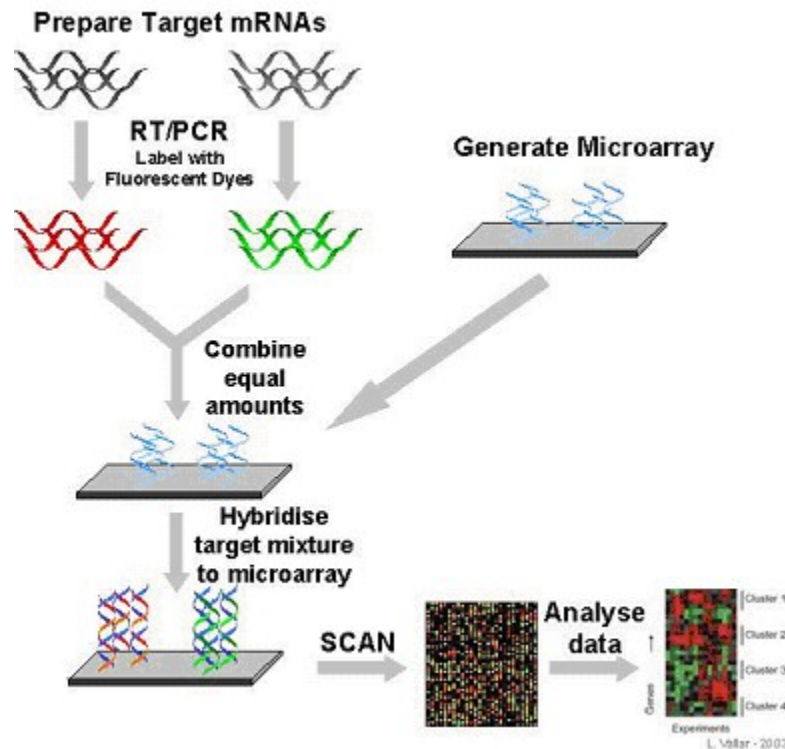


Figure 2: Basic design of a micro-array study

RNA is isolated from target tissue and labelled with fluorescent dyes. Samples from two different situations or 1 sample and a common reference are hybridized and subsequently the expression levels of all the transcripts present on the array can be determined.

Laser capture micro-dissection (LCM) can be used to isolate specific cell types or specific regions from an atherosclerotic plaque. In this way, the transcriptome of distinct cells can be compared in different situations, limiting the amount of variables in the equation. This method has been used to isolate RNA from the smooth muscle cell rich cap, macrophages and endothelial cells from both human and mouse atherosclerotic plaques⁴³⁻⁴⁵.

In general, micro-array studies provide an attractive and fast method to detect transcriptome differences between healthy and diseased phenotypes. However, attention must be paid to the experimental setup, the sample isolation and preparation and the subsequent statistical analyses.

1.3 Immunity in atherosclerosis

Immune cells such as T cells were already discovered in the atherosclerotic plaque in 1986⁴⁶ and certainly during the last decade it has become clear that atherosclerosis is an inflammatory disease⁴⁷⁻⁵⁰. Normally, an inflammatory reaction is evoked against a pathogen or chemical toxin, in order to facilitate the eventual removal of the harmful agent. During this inflammatory response, blood flow is increased and vascular permeability is induced, leading to leukocyte migration to the damaged or infected site. After the initial infiltration of leukocytes, the inflammatory reaction is enhanced by secretion of interleukins, growth factors and chemokines by the now resident immune cells. This in turn attracts more leukocytes, leads to activation of the adjacent tissue and results in a pro-inflammatory feedback loop. The immune reaction ends when the pathogen or stimulus is removed by scavenging cells and the tissue returns to its non-activated state.

The immune system is able to distinguish self from non-self, resulting in tolerance for self-proteins. A problem arises when the harmful agent is not a removable pathogen, but an endogenous stimulus. This will result in the breakdown of tolerance and an auto-immune reaction against self-peptides. The inflammatory reaction then has no clearly defined end point because removal of the stimulus is impossible as it is constitutively expressed in the body.

Atherosclerosis is considered an auto-immune disease since it has features of auto-immune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS). The inflammatory reaction is most likely to be directed against antigens such as oxidized lipoproteins and heat shock proteins that are presented in the atherosclerotic plaque. An immune response against these antigens is demonstrated by the elevated levels of (auto-) antibodies against these epitopes in patients with severe atherosclerotic lesions^{51, 52}. As elevated cholesterol levels and increased vascular permeability continuously lead to the deposition and modification of LDL, total removal of the inflammatory component by scavenging cells is never achieved. Macrophages and dendritic cells take up the oxidized LDL, but due to the constant supply, they end up as foam cells and further contribute to the growing lesion. The immune system has developed ways to repress an ongoing inflammatory response by specific regulatory mechanisms, for example by specific anti-inflammatory cytokines and T cell subsets that suppress the ongoing (auto-) immune reaction.

In this section, the role of leukocyte activation and migration in the initiation and progression of atherosclerosis is addressed. Firstly, the role of chemokines and other molecules associated with leukocyte migration will be discussed. Secondly, molecules with a role in activation and differentiation functions (mostly interleukins) are addressed, followed by a section on co-stimulatory molecules and their role in orchestrating the inflammatory response during atherogenesis.

Chemokines

Chemokines are small proteins with chemoattractant properties and are secreted to induce leukocyte growth and regulation of leukocyte trafficking. Next to these effects on leukocytes, chemokines also mediate platelet activation and aggregation. Chemokines are classified in four sub-families based on the structural arrangement of the N-terminal conserved cysteine residues, being C, CC, CXC, and CX₃C. The receptors for chemokines contain 7 transmembrane loops coupled to heterotrimeric G-proteins and ligand binding to the receptor generally induces cAMP mediated calcium release and subsequent activation of downstream signalling cascades⁵³. An overview of the chemokine receptors and their ligands is shown in figure 3. Chemokine and chemokine receptor expression within the atherosclerotic plaque has been well documented^{12, 54}. The following section will focus on the chemokine families that have a role in atherogenesis and therefore the C-family is not discussed.

CC family

Until now, 28 member of the CC chemokine family have been identified. MCP-1 (CCL2) was the first chemokine whose function in the formation of atherosclerotic lesions was addressed. It is expressed in atherosclerotic plaques and can be released by all vascular cells. The receptor for CCL2 is CCR2, which is present on monocytes, immature dendritic cells and T cells⁵⁵⁻⁵⁷. Studies with CCR2 and MCP-1 deficient and transgenic animals have shown the pivotal role of this interaction in the formation of atherosclerotic plaques⁵⁸⁻⁶¹. Other chemokines with affinity for CCR2 are MCP-2 (CCL8), -3 (CCL7), -4 (CCL13), and -5 (CCL12). Lutgens *et al.* showed that antibody mediated blockade of MCP-1/5 resulted in attenuated atherosclerotic plaque formation³⁹.

A second well documented CC receptor/chemokine signalling axis in atherogenesis consists of CCR5 and its T cell derived ligands CCL3 (MIP-1 α), CCL-4 (MIP-1 β) and CCL-5 (RANTES). Expression of CCL3, -4 and -5 was shown in atherosclerotic plaques⁶² and antagonizing CCL-5 results in decreased plaque formation^{63,64}. CCL5 has, next to CCR5, high affinity for two other chemokine receptors, CCR1 and CCR3. Reports on the role of CCR1 in the formation of atherosclerotic plaques are controversial. Two recent papers show that neo-intima formation and diet-induced lesion formation are not affected by CCR1 deficiency, but a protective role for bone marrow derived CCR1 is

suggested by Potteaux and colleagues⁶⁵⁻⁶⁷. CCR3 expression is associated with macrophage rich lesions and its main ligand, eotaxin (CCL11) is produced by vascular smooth muscle cells⁶⁸. As CCL11 mainly attracts eosinophils and these cells are not commonly observed in atherosclerotic plaques, more research is needed to address the function of CCR3/CCL11 expression in atheroma.

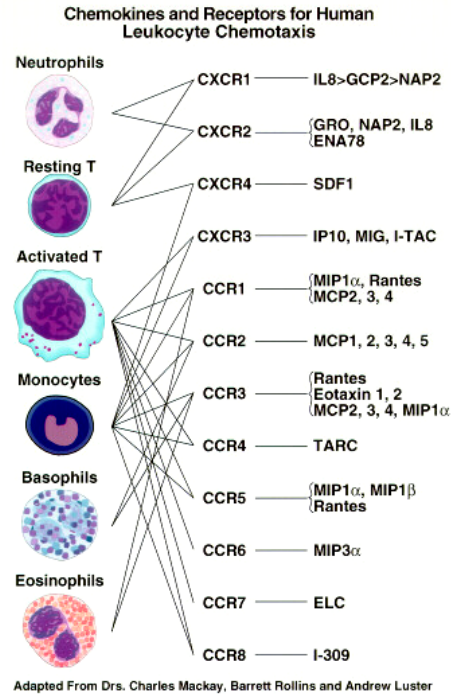


Figure 3: Chemokine receptors and their ligands

The expression of CCR7 and its ligands CCL19 and CCL21 is increased within atherosclerotic lesions of ApoE^{-/-} mice and in human atherosclerotic carotid plaques, and in plasma of patients with coronary artery disease⁶⁹. Furthermore, CCR7 expression is essential in the regression of atherosclerotic plaques due to a decrease in serum lipids, as antibodies against CCR7 and its ligands abolish plaque regression⁷⁰.

A theoretical role for CCR4 and CCR8 signalling has been proposed in literature. Expression of the CCR4 ligands CCL17 (TARC) and CCL22 (MDC) has been established in atheroma and is associated with macrophage rich areas and sites of neo-vascularization⁷¹. CCR8 has a potential role in atherogenesis as it mediates monocyte migration via its ligand CCL1 (I-309) in ApoAI activated human vascular endothelium and has chemotactic effects on vascular smooth muscle cells⁷²⁻⁷⁴.

The CCR6 ligand CCL20 (LARC) is not detected in atherosclerotic plaques on mRNA or protein level indicating that this molecule is not involved in lesion formation or progression⁷⁵. Finally, no clearly defined role for CCR9 and CCR10 signalling has been identified in atherosclerosis so far.

CXC family

The CXC chemokine family has 17 members and several of these molecules have been shown to contribute to atherosclerotic plaque formation.

Confusingly, some interleukins have been directed into the chemokine nomenclature due to their function and molecular structure. One of these molecules is IL-8, also known as CXCL8 (or in mice, KC). CXCR2 is the receptor for IL-8 and is upregulated on monocytes when they are exposed to oxidized LDL⁷⁶. A pathological function for this increased expression was shown by Boisvert *et al*, who showed that deficiency in CXCR2 or (murine) IL-8 decreases plaque formation in apoE deficient mice. They show that CXCR2 is essential for cells to be retained in the advanced plaque⁷⁷⁻⁷⁹. A complicating factor is the rather large number of other CXCL molecules with affinity for CXCR2. These include CXCL1 (GRO α), -2(GRO β), -3(GRO γ), -5 (ENA-78) and -6(GCP-2), and on the other side, the affinity of CXCL6 and -8 for CXCR1. The observed effects in experimental models for atherosclerosis are therefore difficult to interpret.

CXCR3 is expressed on various types of leukocytes and its expression is highly induced upon T cell activation^{80, 81}. CXCR3 has 3 known ligands; CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (ITAC) and the expression of these ligands is highly inducible by interferon- γ (IFN- γ)⁸². Recent publications point towards a prominent role for CXCR3 mediated migration of inflammatory cells in atherosclerosis. Human atherosclerotic lesions express high levels of all three CXCR3 ligands⁸³. Targeted deletion of CXCR3 in ApoE deficient (ApoE^{-/-}) mice results in decreased lesion formation in the abdominal aorta⁸⁴. Furthermore, deletion of the CXCR3 ligand CXCL10 in ApoE^{-/-} mice results in decreased lesion formation by reducing the migration of CD4 effector T cells to the atherosclerotic plaque⁸⁵.

A splice variant of the CXCR3 receptor, called CXCR3B is the receptor for CXCL4, also known as Platelet Factor 4 (PF-4). Elevated serum levels of PF4 are correlated to CAD and peripheral vascular disease and PF-4 release by platelets induced monocyte arrest and oxLDL binding on vascular endothelium^{86, 87}.

CXCL12 (SDF-1) is a chemokine that is a potent inducer of pro-thrombotic events and it is highly expressed by all cell types in the atherosclerotic plaque⁸⁸. In turn, reduced CXCL12 plasma levels have been associated with unstable coronary artery disease, suggesting anti-inflammatory or plaque-stabilizing properties for CXCL12 in atherosclerosis⁸⁹. The receptor for SDF-1 is CXCR4. CXCR4 was shown to be abundantly expressed by atherosclerotic plaque endothelium that is exposed to low/absent shear stress, while it is poorly expressed by healthy endothelium. It is associated with atherogenesis by

favouring the integrity of the endothelial barrier and by inhibiting MCP-1 and IL-8 expression⁹⁰.

Neither CXCR5 nor its ligand CXCL13 is reported to be expressed or differentially regulated during atherosclerotic lesion formation.

CXCRL-16, also known as SR-PSOX is a rather peculiar member of the chemokine family. It is membrane bound, and is available for its receptor, CXCR6 upon cleavage by ADAM-10⁹¹. Interestingly the transmembrane form functions as a scavenger receptor and as such mediates the uptake of oxLDL by macrophages⁹²⁻⁹⁴. It is expressed in human atherosclerotic plaques by all vascular cells, and not in healthy tissue. Furthermore, it mediates the adhesion of CXCR6 expressing cells⁹⁵. Recently it was shown that a polymorphism of CXCL16 is associated with aggravated coronary artery stenosis⁹⁶.

CX₃C family

Only one member of the CX₃C family is described, CX₃CL1, and this molecule was only identified in 1997⁹⁷. Like CXCL16, it is expressed as a membrane bound chemokine, and is accessible for its receptor CX₃CR1 only after cleavage by ADAM-17⁹⁸. Despite its recent discovery, a vast amount of evidence exists on the pro-atherogenic role of this chemokine and receptor. It is expressed in macrophages and smooth muscle cells in the advanced human atherosclerotic plaque and provides chemotactic signals to these cells and mediates cell adhesion⁷¹. CX₃CR1 deficient mice reduce less atherosclerosis. Cross breeding with apoE and LDLr deficient mice resulted in a decrease in lesion formation in the brachiocephalic artery, but not in the aortic root⁹⁹⁻¹⁰¹. Next to these findings in animal models, genetic polymorphisms (SNP's) in the gene for CX₃CR1 are linked to increased risk for acute coronary syndromes and unstable plaques¹⁰²⁻¹⁰⁷.

Interleukins

Interleukins are messenger molecules that are used in the communication between (=inter) leukocytes and inflamed tissue to induce or attenuate immune responses (hence the name interleukin). Interleukins are key players in the inflammatory reaction observed in atherogenesis. Both pro-atherogenic as well as anti-atherogenic interleukins have been identified and for most of these factors the expression within the plaque or circulation has been shown. In figure 4 the regulation and expression of these interleukins is shown. In this section, the function and effects of both pro- and anti-inflammatory interleukins will be discussed in respect to their role in atherogenesis. As specific cell types are mostly associated with the specific sub-set of interleukins they produce, these cell types will also be discussed in this section.

Pro-atherogenic signalling routes

A number of cytokines have a clear function in plaque initiation/formation and progression. The first pro-atherogenic interleukins to be studied in detail were IL-1 and IL-6. A strong correlation is observed between the prevalence of CAD, MI and carotid atherosclerosis and the expression of IL-6¹⁰⁸⁻¹¹⁰. The profound effects in patients are attributable to the pro-inflammatory effects of IL-6 on monocytes¹¹¹, smooth muscle cell proliferation¹¹² and endothelial cells¹¹³.

IL-1 β has pro-inflammatory effects on endothelial cells^{114, 115}, vascular smooth muscle cells¹¹⁶ and macrophages, all detrimental in the formation of atherosclerotic lesions¹¹⁷. Increased levels of IL-1 lead to an upregulation of adhesion molecules and induce leukocyte migration to the growing lesion¹¹⁸. Numerous studies in IL-1 or IL-1 receptor knock outs and transgenic mice underlined these findings¹¹⁹⁻¹²¹. The other member of the IL-1 family is IL-18 and the activity of both molecules is dependent on cleavage by caspase-1 (ICE). Binding of IL-18 to its receptor on leukocytes, smooth muscle cells and endothelial cells induces a pro-inflammatory response by the secretion of IFN γ , IL-6 and vascular adhesion molecules^{122, 123}. Furthermore, IL-18 has been shown to decrease plaque stability by the induction of matrix metalloproteinases (MMP's) that degrade the stabilizing collagen content of the plaque¹²⁴.

Interestingly, combined stimulation of T and NK cells with IL-18 and IL-12 leads to activation of Th1 cells and subsequent IFN γ production¹²⁵. The absence or selective blockade of this differentiation route by inhibition of IL-12¹²⁶⁻¹²⁹ or IFN γ ¹³⁰⁻¹³² profoundly inhibits atherosclerosis.

IFN γ is the predominant cytokine produced by T helper type 1 (Th1) cells and the pro-atherogenic role of IFN γ and IL-12/IL-18 indicates that this cell type induces and accelerates atherosclerotic lesion formation. Buono *et al.* underlined this hypothesis by showing severe attenuation of atherogenesis in mice deficient in the Th1 specific transcription factor T-bet¹³³.

The IL-12 family was recently supplemented with 2 new members; IL-23 and IL-27 (see anti-inflammatory section). IL-23 shares the p40 molecule with IL-12 and has p19 as the IL-23 specific subunit¹³⁴. Similar to IL-12, IL-23 is predominantly produced by activated antigen presenting cells (APC's). IL-23 promotes the development of a specific T cell subset that produces high levels of IL-17, the Th17 cell¹³⁵. IL-17 has clearly defined possible pro-atherogenic effects on various atheroma-associated cell types^{136, 137}. In vitro stimulation with IL-23 induces the release of pro-inflammatory cytokines such as IL-6, IL-1, IL-8 and MCP-1 and increases the activity of MMP's that contribute to plaque destabilisation^{138, 139}.

A separate group of interleukins that share a common subunit (in this case of their receptor) is the γ c IL-2 family, comprising of IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21. This interleukin family is less well studied in the context of atherosclerosis, but 2 members are reported to be pro-atherogenic. Although IL-4 is a characteristic Th2 cytokine (thus repressing the above described pro-

atherogenic Th1 pathway), several reports have indicated a pro-atherogenic role for this interleukin^{126, 140}. The unexpected pro-atherogenic effects are possibly mediated by the induction of adhesion molecules such as VCAM-1 and the pro-inflammatory messenger MCP-1 by IL-4^{141, 142}.

IL-15 is expressed in atherosclerotic plaques and autocrine regulation of macrophage cytokine production, such as TNF- α , IL-6 and IL-1 is reported for IL-15¹⁴³⁻¹⁴⁵. For another member of the IL-2 family, IL-7 a pro-atherogenic role was suggested by Damas *et al.* The authors showed that a high levels of IL-7 are associated with unstable angina pectoris¹⁴⁶. However, lower levels of IL-7 mRNA were shown in unstable coronary atherosclerotic plaques¹⁴⁷. These results indicate that the exact role of IL-7 in atherogenesis still needs to be clarified.

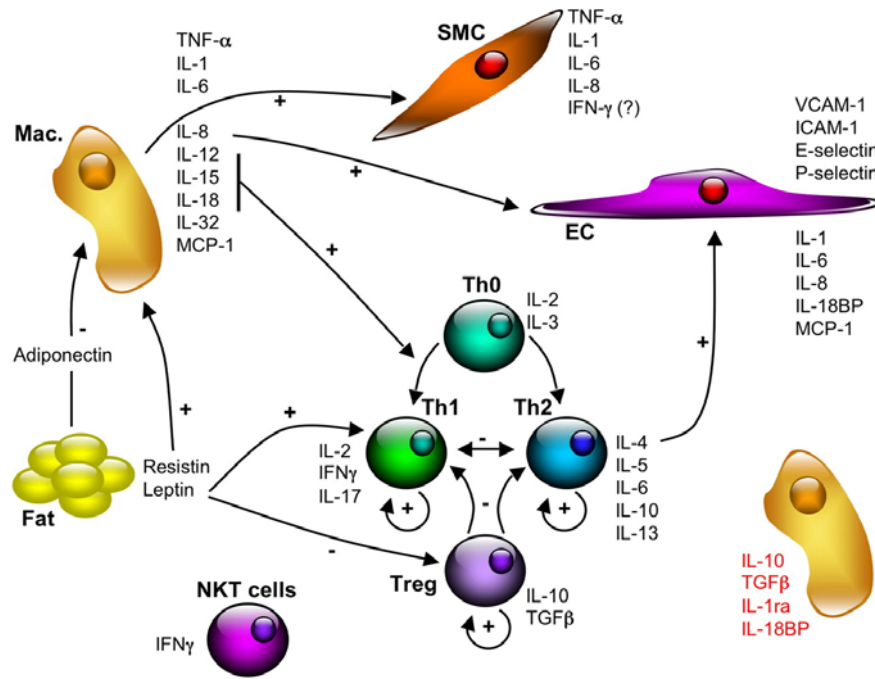


Figure 4: Schematic overview of pro- and anti-atherogenic cytokines

Upon activation of APC's such as macrophages and B cells, these cells secrete cytokines that in turn activate specific subsets of T cells. Activation of T helper cells (Th1 and Th2) can be suppressed by activation of regulatory T cells that secrete IL-10 and TGF β .

Anti-atherogenic signalling routes

In contrast to the rather long list of pro-atherogenic signalling molecules, only a short list of suppressive molecules is reported.

IL-10 is a pleiotropic molecule that has several athero-protective properties. IL-10 decreases the expression of adhesion molecules in monocytes and reduces antigen presentation and pro-inflammatory cytokine production by macrophages^{148, 149}. It induces a shift towards a protective Th2 phenotype as it inhibits Th1 proliferation of T cells¹⁵⁰. A number of papers provide direct evidence for a link between a decrease in serum IL-10 levels and unstable angina and CAD^{151, 152} and have addressed the protective effects of IL-10 on atherosclerotic lesion formation¹⁵³⁻¹⁵⁵. Next to this direct function of IL-10, adaptive regulatory T cells (Tr1) mainly produce IL-10 upon activation by which they exert an anti-atherogenic effect¹⁵⁶. A similar function is postulated for the Th2 associated cytokine IL-9. This molecule is able to reduce TNF α , IFN γ and IL-12 secretion and induce IL-10 expression after LPS injection¹⁵⁷.

As discussed, Th1 associated interleukins such as IL-12 and IFN γ induce atherosclerotic lesion formation, and the inhibitory effect of the Th2 cytokine IL-10 perfectly fits this hypothesis.

A second Th2 associated interleukin is IL-5. The athero-protective properties of IL-5 were demonstrated by Binder and colleagues¹⁵⁸. They however found that its protective effects were mainly due to increased production of oxLDL specific naturally occurring IgM antibodies, and not per se by the inhibition of Th1 differentiation. That IL-5 is able to induce an anti-atherogenic effect without an accompanying Th2 response was demonstrated by the administration of an OX40L antibody during atherogenesis. This antibody repressed Th2 differentiation, but due to increased IL-5 and IgM levels, atherosclerosis was attenuated¹⁵⁹.

The potent anti-inflammatory molecule TGF β was linked to atherogenesis in a study that showed reduced serum levels of active TGF β in patients with advanced atherosclerosis¹⁶⁰. TGF β has anti-inflammatory properties on macrophages, smooth muscle cells and endothelial cells by reducing the production of pro-inflammatory cytokines, and by increasing IL-10 production^{161, 162}. Interestingly, naturally occurring regulatory T cells identified as CD4⁺CD25⁺FOXP3⁺ positive, exert their protective effects via the release of IL-10 and TGF β ^{163, 164}. This is also observed for the Th3 cells that mediate tolerance and subsequent suppression of inflammation after oral administration of antigens¹⁶⁵.

The most recent addition to the anti-atherogenic cytokine family is IL-27, a member of the IL-12 family. IL-27 is encoded by the Epstein Barr virus (EBV)-induced gene 3 (EBI3) and IL12p35 like protein named IL-27p28¹⁶⁶. Effects on both Th1 and Th2 subsets have been described for IL-27¹⁶⁶⁻¹⁷³. In addition, IL-27 is a suppressor of pro-atherogenic Th17 cells and in this way can provide protection against atherosclerotic lesion formation¹⁷⁴.

T cell activation

Next to chemokines and interleukins, an additional route exists for the modulation of the activity of leukocytes. This route is part of the adaptive immune system and involves the direct cellular contact of T cells with antigen presenting cells such as macrophages, B cells or dendritic cells. Antigen specific T cell activation is mediated via MHC-II presentation by antigen presenting cells. Next to this signalling route, additional signals are required to activate the T cells and induce expansion of the antigen specific T cell subset. Communication via co-stimulatory molecules provides this essential stimulus. The activated T cell subsequently is able to give B cell help and this B-T cell cooperation leads to an antigen specific antibody response. (Schematic overview figure 4). Most of the co-stimulatory molecules described are members of the TNF receptor superfamily (TNFRSF). In this section the known co-stimulatory molecules and their role in the adaptive immunity during atherogenesis are discussed.

One of the best characterized co-stimulatory molecules expressed by T cells is CD28, which interacts with CD80 (B7.1) and CD86 (B7.2) on the membrane of APC's. This pathway is essential in isotype switching to IgG1 and IgG2a in B cells, and formation of germinal centers¹⁷⁵. It is the first stimulatory signal after antigen presentation that is received by T cells and facilitates their survival. Deficiency in both CD80 and CD86 resulted in decreased lesion formation in LDLr^{-/-} mice due to decreased levels of MHCII and IFN γ in the lesion¹⁷⁶. Interestingly, bone marrow transplantation with CD80/CD86 deficient cells to LDLr^{-/-} mice markedly increased lesion formation as shown by Ait-Oufella *et al.* and this was the result of abrogated regulatory T cell differentiation. Also transplantation with CD28^{-/-} bone marrow resulted in an increased lesion area, and this was reversible by the administration of regulatory T cells¹⁶⁴.

CD80 and CD86 have very high affinity for CTLA-4, another co-stimulatory molecule mainly expressed on CD4⁺ T cells. Its functions are however opposite of that of CD28, as CTLA-4 signaling prevents activation and subsequent pro-inflammatory processes¹⁷⁷⁻¹⁸¹. CTLA-4 expression is furthermore increased in T cells with a regulatory phenotype, and in this way can suppress the ongoing inflammatory response. As both CTLA-4 with its inhibitory effects and the pro-inflammatory CD28 are able to regulate the activation and expansion of Tregs, this signaling axis needs further research to delineate the exact role in atherogenesis¹⁸².

After initial activation of T cells via CD28/CD80-CD86 interaction, other signals are provided by the APC. The role of the co-stimulatory couple CD40-CD154 (CD40L) has been well documented in inflammatory disorders and atherogenesis and follows shortly after initial activation. Interaction of CD40 on the APC with CD40L on the T cell results in the expansion and priming of CD4⁺ T cells¹⁸³. The combined exposure of CD40 to CD40L and MHCII to the T cell

receptor (TCR) leads to further activation of the APC and secretion of pro-inflammatory cytokines such as IL-12^{184,185}. As expected, blockade of CD40-CD40L signaling markedly decreased atherosclerotic lesion formation and enhanced plaque stability as shown by numerous authors¹⁸⁶⁻¹⁸⁹.

Approximately three days after the primary activation, T cells induce the expression of the co-stimulatory molecule CD134 (OX40, TNFRSF4) on their surface. A correlation with the expression of OX40 and severity of several autoimmune diseases has been established^{190,191}. The ligand for OX40 (OX40L, TNFSF4) is expressed by APC's and by vascular endothelium^{192, 193}. In this way OX40/OX40L binding leads to activation and migration of OX40 positive activated T cells. Recently, a role for the co-stimulatory molecule CD134 and its ligand in atherogenesis was established by the group of Paigen *et al.* They showed that a specific locus associated with atherosclerosis susceptibility in C57/Bl6 mice contained the OX40L gene, and that a specific allele of this gene in humans leads to enhanced incidence of MI. Furthermore, mice with targeted mutations of OX40L had significantly smaller atherosclerotic lesions than did control mice, and mice over-expressing OX40L had significantly larger atherosclerotic lesions^{194,195}. In the present thesis, chapter 8 describes the athero-protective effects of OX40L antibody administration.

CD30, another member of the TNF superfamily, is expressed by activated T cells and is induced in a CD28 dependent activation pathway. CD30L is reported on B cells as well as T cells resulting in reverse signalling. Expression of CD30 is reported in inflamed unstable coronary plaques¹⁹⁶.

GITR (TNFRSF18) in mice, and AITR in humans is a surface receptor molecule that is upregulated upon T cell stimulation via CD28¹⁹⁷. It has been shown to be constitutively expressed in T-regulatory cells and extending the survival of T-effector cells¹⁹⁸. Recently, Kim *et al* demonstrated that GITR and its ligand are expressed mainly in lipid-rich macrophages within atherosclerotic plaques¹⁹⁹. Furthermore they showed that GITR stimulation increased TNF α and MMP-9 secretion by macrophages, and thus postulated a role for GITR in plaque destabilization. How these effects are to be seen in combination with enhanced expression of GITR on Tregs needs to be clarified. The lymphotoxin β receptor (LT β R), a tumour necrosis factor receptor superfamily member has two identified ligands, Lymphotoxin (LT) and LIGHT. These ligands are expressed on T cells and both provide potent pro-inflammatory signals. For lymphotoxin, expression was detected in smooth muscle cells and macrophages in atherosclerotic lesions. Furthermore it was shown that functional variations in this gene are associated with susceptibility to MI and lymphotoxin deficiency reduced atherosclerosis in an experimental model for atherosclerosis^{200, 201}. For LIGHT, a role in lipid metabolism was described very recently by Lo *et al* and a function for this molecule in atherogenesis can be expected²⁰².

Two members of the TNFRSF that are well described in the activation and regulation of T cell immunity are 4-1BB and CD27 but no direct evidence links these co-activation markers to atherogenesis. Interestingly, both molecules were significantly associated with atherogenesis in the study described in chapter 3.

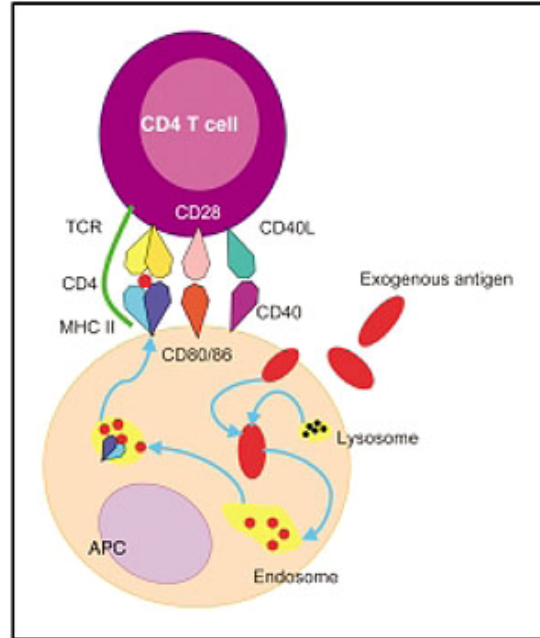


Figure 5: Schematic overview of T cell activation via MHC-II and co-stimulatory molecules

Macrophages process antigens and present fragments of these antigens to T cells via MHC molecules. Additional stimulatory signals are generated by the ligation of CD80/CD86 with CD28 on the T cell. Other co-stimulatory pathways are comprised of CD40/CD40L and OX40/OX40L.

1.4 Treatment strategies

The current treatment of atherosclerosis is based on the reduction of risk factors such as high circulating cholesterol levels or hypertension. Statins are currently clinically the most used drug and exert their effect by lowering cholesterol levels by inhibition of HMGCoA reductase, a rate limiting enzyme in cholesterol synthesis^{203 204}.

Although a role for inflammation has been extensively shown, no treatment directly modulating the inflammatory response that drives atherosclerotic plaque formation has been described. Formulating an ideal strategy to reach immune suppression and subsequent inhibition of plaque progression can be complex.

In general, pharmaceutically active compounds are relatively small, hydrophilic compounds that show high affinity for a receptor or enzyme. In this way, the interaction of a ligand with the targeted receptor is inhibited, or the enzymatic activity is decreased. The generation of such compounds is a time consuming and costly process that needs structural knowledge of the target protein. Next to this, functional dosing of these pharmaceutically active compounds mostly requires daily intake by the patient and in the case of atherosclerosis this will result in (life) long dependency due to the progressive nature of the disease.

Vaccination can provide an alternative for the treatment of atherosclerosis. The onset of lesion formation starts already at a very young age (<20) and removal or blockade of pro-atherogenic factors in childhood could be very effective in the prevention of lesion formation. Active immunization using a vaccine results in the life long immunity against a chosen endogenous factor by inducing an antibody or cytotoxic response and is currently used in the prevention of numerous diseases such as diphtheria, chicken pox and tetanus. In atherosclerosis, several strategies can be defined to attenuate the formation of lesions by using vaccination.

The most ideal strategy would be to develop a vaccine against the actual protein or sugar moiety that induces the immune response in atherogenesis. However, this molecule has not been defined yet. Interesting candidates are indicated by the presence of antibodies against oxLDL or heat shock proteins in patients with CAD or carotid artery occlusion. Furthermore, it was shown that antibodies against modified apoB-100, a protein moiety in LDL correlate with the extent of carotid artery disease and that these antibodies show protective inhibitory effects on atherogenesis²⁰⁵⁻²⁰⁷. Another approach would be to specifically block the signalling of pro-atherogenic molecules that are identified, such as pro-inflammatory interleukins and subsets of chemokines.

In normal vaccination protocols, immunity is raised against a part of, or a complete attenuated pathogen. This method has some practical drawbacks when vaccinating against a protein and not an efficiently multiplying pathogen. Large quantities of protein (antigen) have to be synthesized or isolated, and its storage and production are rather expensive processes.

An attractive way to induce immunity is provided by DNA vaccination. This method uses isolated eukaryotic expression vectors that encode the antigen and administrate this DNA into the body by injection²⁰⁸. Recent studies using this method in patients have shown that the administration of naked DNA alone sometimes does not result in an immune response that is strong enough to induce protection²⁰⁹.

The addition of immuno-modulatory components provides a way to increase the chance that DNA vaccination will result in protection. Examples of this technique are the addition of bacterial motifs, coding regions for interleukins, or

co-stimulatory molecules and the addition of other adjuvants during immunization²¹⁰. Furthermore, the plasmid can be administered to the body inside an attenuated pathogen that will induce an immune response and thus functions both as an adjuvant and a carrier at the same time. An example of such a bacterium is an attenuated strain of *Salmonella Typhimurium* that has been described for vaccination purposes²¹¹. An advantage of *Salmonella* is that it egresses from the gut lumen via the M cells to Peyer's patches, and in this way the vector (via the pathogen) is efficiently transported to the immune system for translation and processing²¹²⁻²¹⁴.

DNA vaccination by using oral administration of *Salmonella* can lead to different immune responses, based on the T cell subset that is activated by APC's present in the Peyer's patch²¹⁵. By activation of CD8⁺ cells, a cellular immune response is initiated. This leads to the specific killing of cells that express and present the antigen via MHC I. A humoral response is characterized by the production of antibodies against the chosen antigen. This is the result of specific T cell help from activated CD4⁺ T cells to antigen specific B cells. The following two paragraphs will discuss the mechanisms leading to cellular or humoral immunity.

Cellular immunity

Atherosclerosis is a multi-factorial disease and numerous cell types contribute to the growing lesion. The specific removal of pro-atherogenic cell types that drive the atherogenic response could be a potential way to attenuate lesion formation. Vaccination strategies using *S. Typhimurium* transformed with plasmid DNA encoding pathogen proteins have been shown to result in cytotoxic responses to the live pathogens upon infection. This strategy induces protection against a lethal challenge with several bacterial strains^{216, 217}. Not only bacterial strains can be the target of CTL mediated apoptosis, also eukaryotic cells expressing high levels of the protein via MHC I can be targeted via vaccination. This was elegantly shown by Niethammer and colleagues, who used this technique to target tumour associated proteins that enhanced angiogenesis. They induced cellular immunity against vascular endothelial growth factor (VEGF) receptor 2 and carcinoembryonic antigen (CEA). In this way they attenuated tumour growth and prolonged survival in mouse models for cancer²¹⁸⁻²²¹.

This experimental setup can also be used in the treatment of atherosclerosis as several cell types show clear pro-atherogenic effects. Most likely, removal of these cells would lead to attenuation of disease progression. Our lab has demonstrated that vaccination against VEGFR2 inhibits lesion formation in LDL receptor deficient mice by the generation of CD8⁺ VEGF specific CTL's that remove activated endothelial cells from the damaged vascular wall²²².

Humoral immunity

Vaccination against membrane bound proteins is mediated via the above described cellular vaccination. A problem arises when the pro-atherogenic moiety is not a cell, but a secreted protein. As there is no cell type that will be targeted, a different vaccination strategy is needed. Induction of antibody secretion is an attractive strategy, because antibodies are highly efficient in mediating removal of the unwanted proteins from the circulation and at sites of infection. Studies that used *Salmonella* mediated DNA vaccination in C57/Bl6 mice showed that without any additional stimulants the antibody production in this animal was negligible upon DNA vaccination²¹⁵. This indicates that a moiety has to be inserted that drives activation of the T helper subset, instead of the activation of the CD8⁺ cytotoxic response mediated by the *Salmonella* alone.

Several peptides and DNA sequences have shown to induce a profound T cell help to B cells, and when these epitopes are coupled to the protein or DNA sequence in a vaccination strategy, a robust antibody production is the result. Examples of these sequences are Hen Egg Lysosyme (HEL), PanDRepitope (PADRE) and ovalbumine (OVA)²²³. The mechanism by which these epitopes break tolerance against the attached protein/DNA sequence is as follows. After administration of the plasmid by using oral administration of transformed attenuated *Salmonella*, or injection of the plasmid DNA, the fusion protein is synthesized. B cells process the complex after binding to the B cell receptor. As the complex encodes a self-protein, no T cell help is available to start antibody production. This essential T cell help, and the resulting loss of tolerance, is generated by the inserted epitope (HEL/PADRE) by HMC-II restricted T cell activation. In this way, antibodies can be generated against identified pro-atherogenic factors and an attractive therapeutic possibility for the treatment of atherosclerosis is generated.

1.5 Scope of the thesis

The research described in this thesis has two major focus points. Firstly, we used micro-array experiments to find new molecules or genes that provide attractive therapeutic possibilities to treat or prevent atherogenesis. This is described in the first two research chapters. **Chapter 2** shows a study in which the transcriptome of vascular tissue from LDL receptor deficient mice on a Western type diet is compared to healthy mice. In this way, new genes and transcription factors are identified that have a possible role in atherogenesis. **Chapter 3** focuses on CD4 positive T cells during atherosclerotic lesion formation, as this cell type is identified as the predominant T cell type in atherosclerosis. CD4 positive T cells are isolated from mice on a Western type diet at two different time points. The transcriptional regulation in these cells is compared to cells from healthy mice and relevant pathways and genes are identified.

The second part of this thesis illustrates several novel therapeutic strategies to affect atherosclerotic lesion formation. A number of these chapters is based on the targets identified in the above described micro-array experiments. Both the migration (chapters 4-7) and activation (chapters 7-8) of leukocytes are subject of research.

Chapter 4 identifies the HIV entry inhibitor TAK-779 as a CCR5/CXCR3 antagonist that reduces atherosclerotic lesion formation in LDLr^{-/-} mice. Attenuated lesion formation is the result of reduced migration of Th1 cells into the atherosclerotic plaque. In **Chapter 5** it is shown that atherogenesis is inhibited by administration of the selective CXCR3 inhibitor NBI-74330. The observed reduction in plaque formation is the result of increased migration of regulatory T cells to the plaque and the draining lymph nodes. **Chapter 6** focuses on the functional role of the recently identified molecule CD99 during atherosclerotic lesion formation. This molecule, which is expressed both in the endothelial junction as well as on leukocytes, has a pro-atherogenic role, as vaccination against CD99 results in reduction of lesion formation in LDLr^{-/-} mice. **Chapter 7** shows for the first time that IL-16 has anti-atherogenic properties. This interleukin is the first described chemotactic factor for T cells. Vaccination against interleukin-16 results in accelerated lesion formation, especially after longer periods of high fat diet feeding.

Next to the above described interference in leukocyte migration, 2 chapters are incorporated that indicate the possible therapeutic possibility of leukocyte activation modulation. **Chapter 8** describes the antibody mediated blockade of CD134 (OX40 ligand) during atherogenesis. This molecule is the ligand for OX40, a T cell activation marker. Blockade of OX40/OX40 ligand signalling results in reduced lesion formation by the generation of protective OxLDL specific IgM antibodies due to inhibited Th2 mediated isotype switching. **Chapter 9** shows that CD127 is essential for macrophage and regulatory T cell function in atherosclerotic lesion formation. Vaccination against this receptor for IL-7 severely increases the lesion burden in LDLr^{-/-} mice by inducing macrophage apoptosis and decreasing the locally present regulatory T cell number. **Chapter 10** reviews the combined results of the earlier chapters. Clinical implications of the described novel therapeutic candidates and strategies are discussed and implications for possible future studies are suggested.

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Chapter 2

Transcriptional Profiling of Initial Atherogenesis in LDL Receptor Deficient Mice Identifies Diet Induced Upregulation of FOXO1 controlled Genes

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Abstract

Atherosclerosis is a multi-factorial disease associated with elevated plasma cholesterol levels, lipid deposition and inflammation within the vessel wall. Several molecules and transcriptional pathways have been identified in established atheroma, but initial factors contributing to the onset of disease are less well described.

In this study, we compared the transcriptome of the aortic arch of LDL receptor deficient mice that were fed a Western type diet with chow fed mice by using two different statistical approaches. We identified transcription factor binding sites overrepresented within similarly regulated gene clusters and found that FOXO1 mediated transcription is enhanced during Western type diet feeding. This was further demonstrated by the significant upregulation of the FOXO1 downstream gene HMG-CoA reductase. In addition, we used Gene Set Enrichment Analysis to identify genes and pathways enriched in initial and early atherogenesis. We confirmed the specific upregulation of ABCB10 in initial lesion formation, and CCL8 in early lesion formation.

This study identifies new transcriptional regulators and genes associated with very early changes in the vascular wall that precede the large scale influx of leukocytes leading to atherosclerotic plaque formation. These new molecules might serve as targets to modulate the initiation of atherosclerotic lesions.

Introduction

Atherosclerosis is a progressive disease of the medium to large arteries that is characterized by lipid deposition and a subsequent influx of leukocytes into the vessel wall¹. It is the primary cause of myocardial infarction (MI) and stroke, and a major cause of death in the Western world². Several risk factors have been identified over the past decade, including hyperlipidemia, smoking, hypertension, obesity and diabetes³. To increase the understanding of the disease, numerous studies have been performed that focused mostly on single genes, for example by using knockout animals, SNP analysis of a single gene or gene silencing via siRNA. Due to the multi-factorial and complex nature of atherosclerosis, a broad, non-biased approach will be highly informative, leading to a better understanding and insight in the biological processes that underlie the disease. Furthermore, not only genes that have previously been associated with atherosclerosis are included, but also unexpected genes that have never been linked to this disease can be identified.

Along this line, efforts have been made to identify disease related gene expression patterns in atherosclerosis via transcriptional profiling using micro-arrays⁴. Several studies are performed to define the transcriptome of human atherosclerosis, but the identified genes were mostly linked to atherogenesis already⁵⁻⁶. They include genes involved in inflammation, lipid metabolism, matrix degradation as well as cell proliferation and differentiation. Due to the difficulties in human sample incorporation and isolation, mouse models for atherosclerosis provide an attractive alternative. The commonly used animal model for these studies is the ApoE deficient mouse. Array studies were performed to identify differentially expressed genes in mice of different ages, and mice fed with different atherogenic diets⁷⁻¹¹. Functional gene groups that have been reported to be differentially expressed are chemokines and chemokine receptors, cathepsins and general pro-inflammatory markers.

The aim of the present study was to identify new genes and/or gene clusters that are differentially expressed during very early lesion formation in the aorta. For this purpose we used LDL receptor deficient (LDLr^{-/-}) animals, which unlike ApoE deficient animals, only develop atherosclerotic lesions when fed an atherogenic diet¹². This provides the possibility to time the onset of disease, and identify very early changes in gene expression in the aortic wall. We used whole mount tissue from the aortic arch of animals at 0-1.5-3-4.5-9 weeks of Western type diet feeding and compared the transcriptome to aortic tissue from mice on chow. By using advanced statistical analyses both on single transcript levels as well as on functional level we identified genes and pathways that are specific for changes in the vessel wall during the initiation of lesion formation.

Material and methods

Animal experiments

Female LDLr^{-/-} mice (n=6 per group) were fed an atherogenic Western type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Service, UK) for 0-1.5-3-4.5 and 9 weeks. After the indicated diet feeding periods, mice were anaesthetized, flushed with PBS and the aortic arch (thoracic) was prepared free of peri-adventitial fat tissue and snap frozen in liquid nitrogen. All animal experiments were approved by the Leiden University animal welfare committee and in concordance with Dutch law. Concentration of serum cholesterol was determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

Micro-array protocol

RNA was isolated from aortic tissue using the GTC method and DNase treated. The RNA was linearly amplified for 1 round (starting material 1 µg total RNA) synthesizing anti-sense cRNAs with an average base length of 500 nucleotides using the Message-Amp kit from Ambion. Aminoallyl-UTP was incorporated with a molar ration of 1:1 to rUTP. Cy3 or Cy5 mono-reactive dyes were coupled according to the manufacturers' instructions (Amersham Bioscience, Piscataway, New Jersey). Labeled cRNA was purified using the RNeasy purification kit (Qiagen, Germany). All aortic RNA samples were hybridized against a common reference containing (in equal amounts and amplified one round); liver, spleen and aorta of LDLr^{-/-} mice 6 weeks on western type diet and LDLr^{-/-} mice treated with LPS, RAW cells treated with LPS, H5V cells treated with TNFα and Strategene universal mouse reference RNA.

Hybridization was performed on glass based micro-arrays representing 22,056 unique murine oligonucleotide sequences (Micro Array Department, University of Amsterdam, The Netherlands (<http://www.microarray.nl/libraries.html>)).

Microarray data were acquired and imported in Rosetta Resolver database and Loess normalized (limma package, Bioconductor). Significant differences in time were calculated by using one-way Bayesian ANOVA with Benjamini-Hochberg multiple testing correction (Cyber-T, R script, UCLA) with genes detectable in all arrays and FDR < 25%.

Data were grouped into control (0 weeks of diet), no lesion formation, 1.5 and 3 weeks of diet), and initial lesion formation (4.5 and 9 weeks of diet). Hierarchical clustering of significant genes was performed using Cluster (Eisen Lab, UCB, <http://rana.lbl.gov/EisenSoftware.htm>)¹³. Conserved transcription factor binding sites in the region of 500 base pairs from ATG of genes overrepresented within the clusters were analyzed with whole genome rVista by using the 2004 mouse/human conserved genome database¹⁴.

Genome wide analysis at group level was performed using GSEA after conversion of mouse to human signature¹⁵. The molecular signature database that was used was a modified version of the original named c2.symbols.gmt

(http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html) set and earlier described by Volger *et al*⁶. The dataset contained 594 gene sets. Analysis was performed with the following settings: 100 permutations on phenotype, gene sets with more than 10 genes were included in the analysis.

Validation of candidate genes by RT-PCR

Quantitative gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (appendix I) were designed using Primer Express software with the manufacturer's default settings (Applied Biosystems). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Statistical analysis

Values are expressed as mean $\pm$ SEM. Two-tailed student's T-test was used to compare normally distributed data. Mann-Whitney test was used to compare lesion area data and other not normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

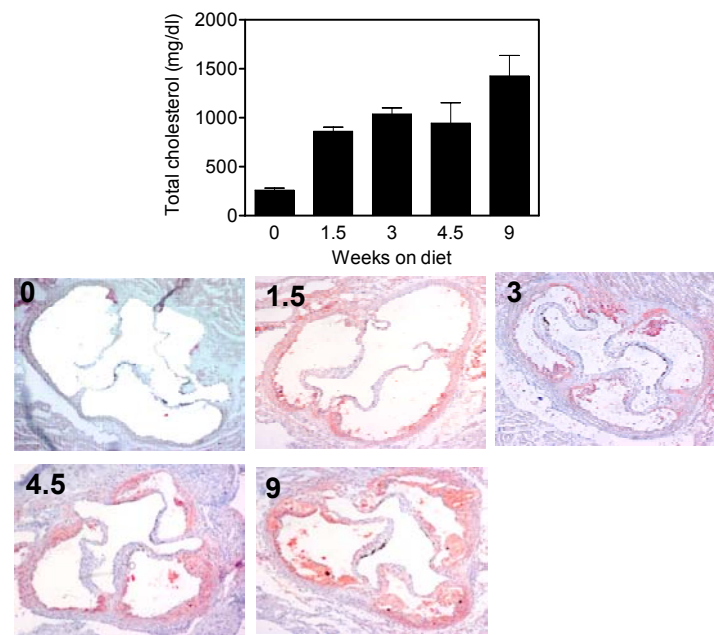


Figure 1: Diet induced lesion formation in LDLr^{-/-} mice. Female LDLr^{-/-} mice (n=6 per group) were placed on a Western type diet for 0-1.5-3-4.5 and 9 weeks. Total serum cholesterol was determined during the experiment and a significant increase is observed at 1.5 weeks of Western type diet feeding that is maintained throughout the experiment. Representative pictures of lesion formation in the aortic root at the indicated time points are visualized in the power panels. Oil red O staining is used to visualize lipids.

Results

Animal and tissue phenotype

We designed this experiment to identify genes and genetic pathways that are specific for very early atherosclerotic lesion formation. We used LDLr^{-/-} mice as a model for atherosclerosis as in this animal, the onset of lesion formation can be specifically timed by feeding a Western type diet. Female LDLr^{-/-} mice (n=6/group) were placed on a Western type diet for 1.5; 3; 4.5; and 9 weeks. Chow fed animals were used as control. Western type diet feeding resulted in a significant upregulation of serum cholesterol levels already after 1.5 weeks from 260 ± 23 mg/dl to 862 ± 46 mg/dl. This increase in cholesterol level was constant throughout the experiment (Figure 1, upper panel). To determine the degree of atherosclerotic lesion formation, we stained the aortic valve leaflet area for lipid deposition using Oil-red-O. Representative photomicrographs are shown in figure 1 lower panels.

In general, the onset of lesion formation in the aortic valve leaflet area precedes plaque formation in the aortic arch. To provide insight in the degree of lesion formation in the aortic arch, we used real time PCR to check for the presence of macrophages and T cells.

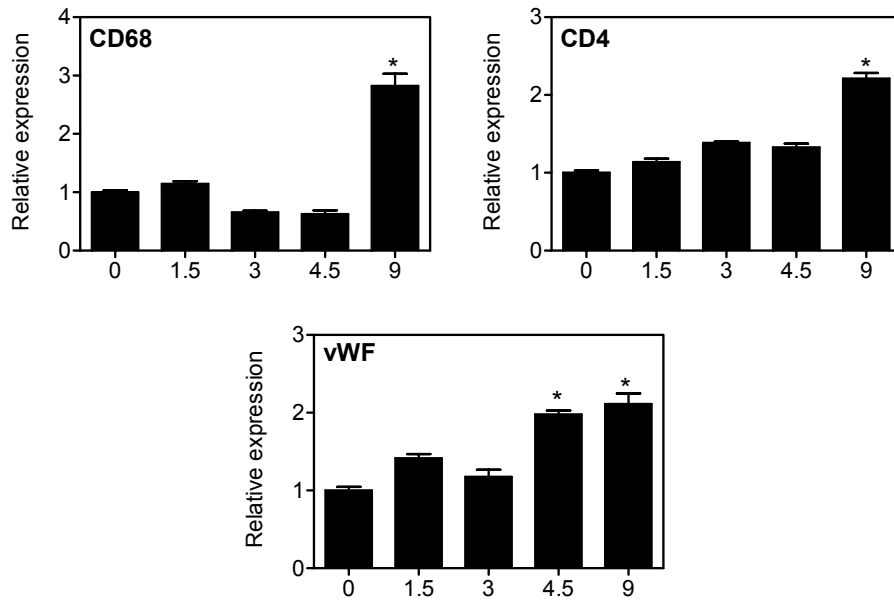


Figure 2: Leukocyte influx and endothelial activation in the aortic arch. We determined the extent of leukocyte influx within the aortic arch in time by RT-PCR on CD68 (macrophage specific) and CD4 (T cell specific) mRNA expression. Endothelial activation was assessed by vWF expression. mRNA was isolated from the aortic arch of LDLr^{-/-} mice using the GTC method and expression of genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student *t* test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (**p* < 0.05, n= 6 per time point).

Figure 2 shows that only after 9 weeks of Western type diet feeding, a significant upregulation of both CD68 mRNA (macrophage specific, 2.8 fold, $p=0.023$) and CD4 (T cell specific, 2.2 fold, $p=0.02$) was observed. This indicates that these leukocytes have entered the aortic wall and atherosclerotic plaque formation has been initiated. The activation of the endothelium that precedes the leukocyte influx is already visible after 4.5 weeks of Western type diet. This is shown by the significant upregulation of von Willebrand factor at 4.5 and 9 weeks of diet (2.0 fold, $p=0.01$, and 2.1 fold, $p=0.03$ respectively).

Transcriptional regulation in the aortic arch during Western type diet feeding

Transcriptional differences between the aortic arch of control mice and diet fed mice were determined by micro-array analysis. mRNA, isolated from every aortic arch was amplified one round, labeled with Cy5 and subsequently hybridized against a common reference (Cy3). Genes significantly regulated in time were identified by using Bayesian one-way ANOVA with Benjamini-Hochberg correction for multiple testing. Genes were considered differentially expressed when expression was observed in all the arrays, and the False Discovery Rate (FDR) < 25%. The resulting significant genes (N=354) were clustered using hierarchical clustering software. The time points were grouped in control tissue on chow diet resulting in low cholesterol levels (LC, 0 weeks), high serum cholesterol and no lesion formation (HC, 1.5 and 3 weeks,) and high cholesterol and initial lesion formation (HCIL, 4.5 and 9 weeks) and regulation profiles over these 3 situations were analyzed. The resulting clusters and gene lists are visualized in figure 3. Clustering resulted in 5 unique clusters as visualized in the following order; A: Induced HC and HCIL, N=76 genes; B: reduced HCIL only, N=19; C: induced HCIL only, N=16; D: reduced HC only, N=239; E: reduced HC and HCIL, N=10.

Transcription factor binding sites overrepresented within the clusters

Once we identified the above described differentially regulated gene clusters, we searched for coordinate regulation of these genes via possible common transcription factor binding sites in upstream regions of the genes present in each cluster by using rVISTA. We used the human-mouse alignment of May 2004 and searched within a 500 base pair upstream region of ATG. Distinct transcription factor binding sites were identified for each cluster and these are visualized in figure 3 right panel. In the genes that are present in the upper cluster, with induced expression in both high cholesterol situations, we found overrepresentation of transcription factor binding sites for FOXO1, GATA2, MEIS1, and c-myb. FOXO1 is involved several metabolic signaling pathways; overexpression of FOXO1 leads to hyperglycemia and hypertriglyceridemia, and exercise leads to downregulation of FOXO1 regulated genes¹⁷⁻²⁰. GATA2 regulation is described for vascular endothelial and/or smooth muscle cells and Seo *et al.* have shown that GATA2 expression is one of the most predictive genes for human aortic sites with high lesion burden⁵.

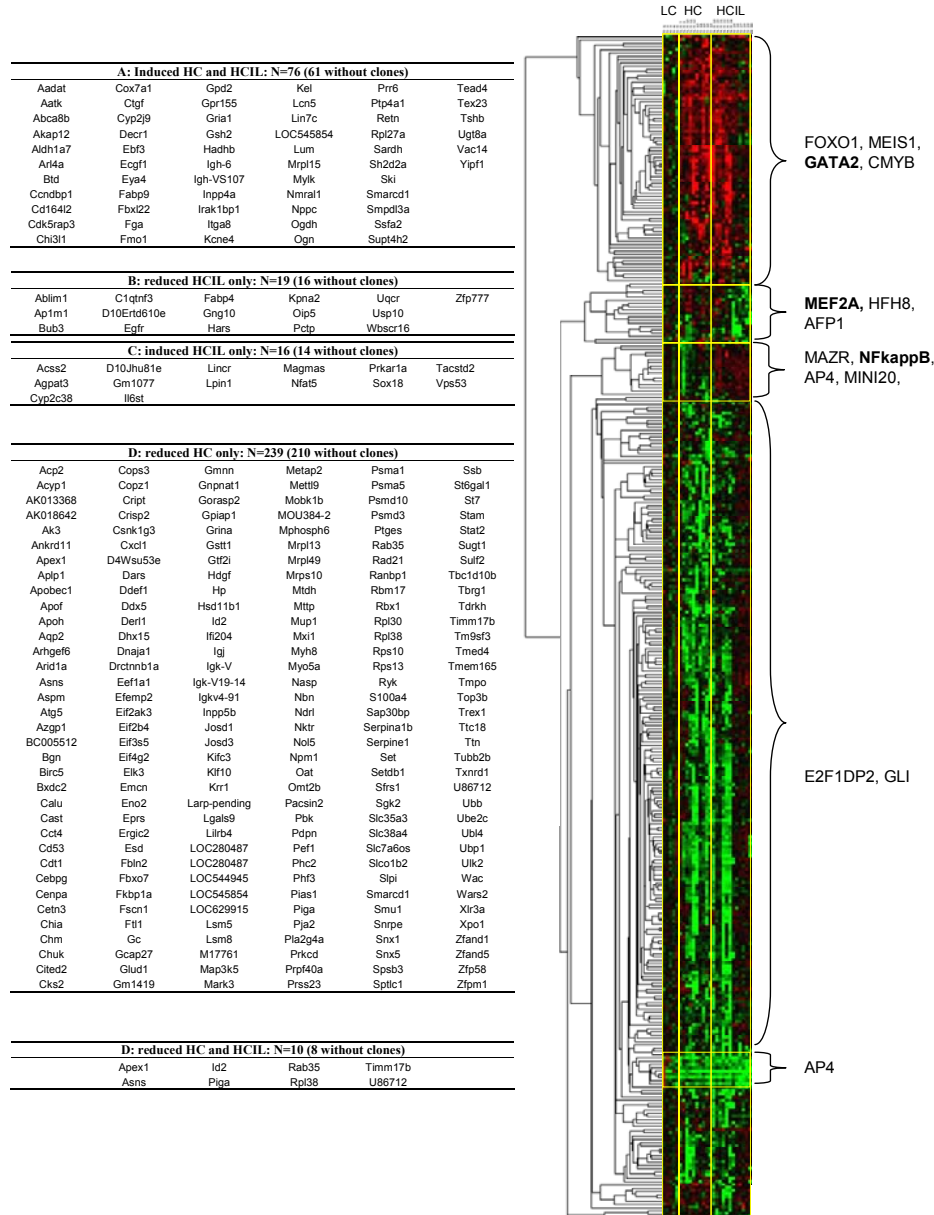


Figure 3: Differentially regulated genes during diet induced lesion formation. Differential regulation due to Western type diet feeding in the aortic arch was assessed by Bayesian one-way ANOVA with BHB correction and 354 genes were found to be significantly differentially expressed in time. Hierarchical clustering of these significant genes resulted in 5 distinctly regulated clusters as shown in by }. Presence of overrepresented TFBS in the promoter region of the genes in each cluster was identified by comparison with the 2004 mouse/human conserved genome database by using rVista. For each cluster these transcription factors are shown. Transcription factors that have been previously associated with vascular biology are shown in bold. **LC**: aortic arch from mice on chow; **HC**: aortic arch from mice on high cholesterol diet, no plaque formation; **HCIL**: aortic arch from mice on high cholesterol diet, initial lesion formation.

Next to this finding, 5 polymorphisms in the GATA2 gene were shown to be correlated to coronary artery disease^{5,21}. MEIS1 is mainly associated with leukemogenesis and lymphocyte cell differentiation during early life²². C-myb was initially described as proto-oncogene, but some evidence exists that it functions as a mediator of vascular smooth muscle cell proliferation and neointima formation²³⁻²⁶.

In initial lesion formation, when initial leukocyte infiltration has started, we observed an expected upregulation of genes regulated by the inflammatory regulator NFkappaB. This transcription factor has been extensively researched in the context of atherogenesis, and induces the expression of adhesion molecules, chemokines and matrix-metalloproteinases^{26,27}. Other transcription factor binding sites that are enriched within this time point are MAZR and AP4, both with no direct described function in vascular cells until now.

Interestingly, during initial lesion formation, genes with a MEF2 and HFH8 (FOXF1) transcription factor binding site are downregulated. MEF2 regulation is associated with both endothelial and smooth muscle cell function^{28,29}. Furthermore, a mutation in MEF2a is linked to coronary artery disease³⁰. HFH8 is less well described, but has been linked to vascular endothelial growth factor (VEGF) expression and LKLF mediated regulation during lung development³⁰.

The largest gene cluster contains genes that were reduced in both high cholesterol no lesions and high cholesterol with initial lesions. The overrepresented transcription factor binding sites are E2F1DP2 and GLI. E2F1DP2 has no downstream genes that are functionally described. GLI however, has been studied in more detail and is reported as a mediator of hedgehog signaling in oncogenic pathways leading to a variety of lethal tumors³¹. The last cluster contains genes that are downregulated during Western type diet feeding, both in initial and early atherogenesis, and shows enrichment for AP-4 binding sites. This transcription factor was also associated with an upregulation of genes in early atherogenesis.

The expression of HMG-CoA reductase is upregulated during Western type diet feeding

Recent reports link the expression of 3-hydroxy 3-methylglutaryl coenzyme A (HMGCoA) reductase directly to the level of active FOXO1¹⁷. In order to functionally validate the observed enrichment of FOXO1 induced genes after Western type diet feeding, we analyzed the expression of HMGCoA reductase in our samples. We observed a clear and significant upregulation of this gene due to Western type diet administration and this regulation profile correlates with the cluster profile (Fig 4). This indicates that FOXO1 downstream gene expression is enhanced in the aortic wall exposed to elevated cholesterol levels.

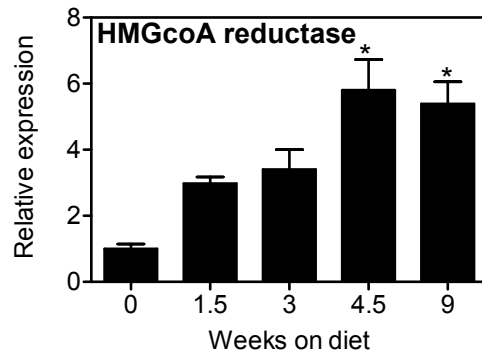


Figure 4: Expression of HMG Co-A reductase in the aortic arch on Western type diet feeding. mRNA was isolated from the aortic arch of LDLr^{-/-} mice using the GTC method and expression of genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student *t* test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (**p* < 0.05, *n* = 6 per time point).

GSEA

Next to the gene based pattern analysis, we performed pathway based Gene Set Enrichment Analysis to study transcriptome differences in the aortic arch of initial and early lesion formation. This method enables the interpretation of genome-wide expression profiles based on biological function. Next to the functional output, this analysis tool identifies a top 100 of signature genes of one situation compared to another¹⁵. An enrichment score and relevant *p* value is calculated based on the ranked difference in expression of genes between two situations. In this way, gene sets that are overrepresented at the extremes of the ranked gene list will result in high enrichment scores. Tables 1 A and B show the enriched pathways for both initial and early lesion formation, and the nominal *P* values calculated by weighted Kolmogorov-Smirnov-like statistics as described earlier¹⁵.

Immediately after Western type diet feeding (1.5 and 3 weeks of diet) a clear enrichment of metabolism associated genes is observed compared to early atherosclerosis (upper table). However, none of these sets were significantly enriched. In initial atherosclerosis (4.5 and 9 weeks of diet), the number of significant (*p* < 0.05) enriched gene sets is 24. Next to metabolism associated sets, cell adhesion and map kinase activation pathways are observed.

Figure 5 shows the top 100 signature genes (50 top, 50 bottom) according to the intensity differences between initial and early atherosclerosis.

We used real-time PCR to validate genes that were present in the gene list generated by GSEA. ABCB10 expression is one of the top enriched genes in the high cholesterol, no lesion time point, indicating a diet induced effect on its regulation. ABCB10 is a relatively new member of the ABC binding cassette transporter family and has not been linked to atherogenesis yet. A significant upregulation of ABCB10 expression is observed after 3 weeks of Western type diet.

CCL8 (formerly known as MCP-2) is one of the top ranked genes in early atherosclerosis. CCL8 functions as a chemotactic factor for monocytes via CCR1 and CCR2b³²⁻³⁵. Real-time PCR showed that CCL8 expression indeed increased markedly by 2.4 fold (*p* = 0.02) in aortic tissue with early lesions.

High Cholesterol	NOM p-val
MAP00330_Arginine_and_proline_metabolism	0.067
MAP03020_RNA_polymerase	0.070
GO_ROS	0.166
MAP00240_Pyrimidine_metabolism	0.288
MAP00220_Urea_cycle_and_metabolism_of_amino_groups	0.322
no1Pathway	0.176
MAP00562_Inositol_phosphate_metabolism	0.278
MAP00910_Nitrogen_metabolism	0.333
hdacPathway	0.321
INSULIN_2F_DOWN	0.308

Initial lesion	NOM p-val
fatty_acid_metabolism	<0.001
Eicosanoid_Synthesis	<0.001
MAP00590_Prostaglandin_and_leukotriene_metabolism	<0.001
SIG_Regulation_of_the_actin_cytoskeleton_by_Rho_GTPases	0.018
Wnt_Signaling	0.018
cell_adhesion_molecule_activity	0.019
tnfr2Pathway	0.019
GLUCO	0.020
MAP00052_Galactose_metabolism	0.021
electron_transporter_activity	0.025
MAP00710_Carbon_fixation	0.027
ST_GRANULE_CELL_SURVIVAL_PATHWAY	0.030
GLYCOL	0.037
KRAS_TOP100_KNOCKDOWN	0.041
GLUT_UP	0.042
mRNA_splicing	0.043
mprPathway	0.048
ST_Wnt_Ca2_cyclic_GMP_Pathway	0.056
SIG_PIP3SIGINCARDIACMYOCTES	0.057
wntPathway	0.058
ca_nf_at_signalling	0.062
CR_PROTEIN_MOD	0.063
cell_adhesion_receptor_activity	0.064
ST_Tumor_Necrosis_Factor_Pathway	0.068

Table 1: enriched gene sets identified using GSEA. GSEA was used on our data set to identify differential pathways in week 1.5-3 (high cholesterol and initial lesion formation (week 4.5-9) in the aortic arch. GSEA identified no significant pathways in the initial stage, but 24 pathways were significantly enriched in the initial lesion formation stage. Next to pathways associated with metabolism, 4 immunological pathways are identified (indicated in gray).

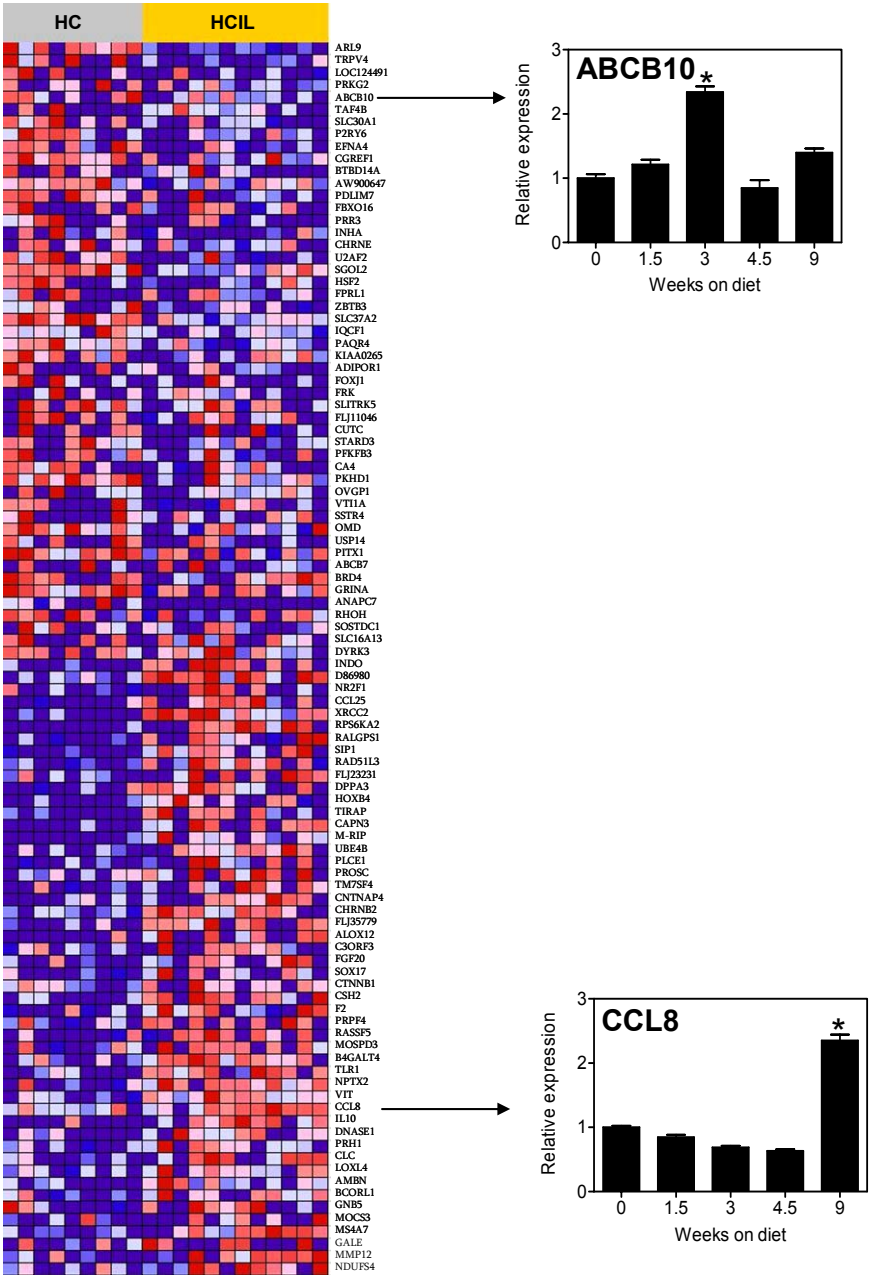


Figure 5: Ranked gene list of top 100 most differential genes between initial and early lesion formation. GSEA analysis uses a ranked gene list of expression differences between initial and early lesion formation to identify the above described pathways. A heat map of the 100 most differentially regulated genes, 50 from initial, and 50 from early lesion formation, is shown. Verification of some of these candidates was performed by RT-PCR and the resulting expression profiles are shown in the right panels.

Discussion

In the present study we compared gene expression profiles from vascular tissue of LDLr^{-/-} mice on different times of diet. We used whole mount material of the aortic arch, consisting of endothelial cells, smooth muscle cells and when plaque formation has started, leukocytes. This leukocyte influx was clearly demonstrated after 4.5 weeks and was significantly present at 9 weeks of diet feeding, as the mRNA levels of CD68 and CD4 were elevated at week 9. Furthermore, we showed that endothelial activation was already present at 4.5 weeks of Western type diet by increased expression of vWF³⁶. Based on these observations we decided to group our arrays in control (chow fed animals), high cholesterol and no lesions (1.5 and 3 weeks of diet) and initial lesion formation (4.5 and 9 weeks). In this way, we enlarged the statistical power of our analysis and created the opportunity to compare aortic tissue from mice on diet without any lesion with very early lesions. We used two statistical approaches to find relevant transcriptome differences between the time points, because no straight forward analysis pipeline exists for the analysis of time dependent microarray data. These two methods, one-way ANOVA followed by transcription factor binding site scan and GSEA pathway analysis are discussed separately below.

Transcription regulation during initial and early lesion formation

From the transcription factor binding site analysis, we identified FOXO-1 as a new possible regulator of diet induced vascular transcription. Genes regulated by this transcription factor are specifically overrepresented in the cluster with genes that are upregulated both in initial as well as in early lesion formation. This transcription factor expression inside the nucleus is regulated by phosphorylation by Akt and AMPK, as phosphorylation results in rapid degradation. Fisslaler *et al.* show that shear stress resulted in rapid phosphorylation FOXO1 and that its downstream target HMGCo-A reductase is subsequently decreased¹⁷. Next to this, FOXO1 has been shown to mediate responses in diabetes, glucose metabolism and exercise¹⁸⁻²⁰. We show in our study, that diet induced genes are regulated by FOXO1 and that subsequently, its downstream target HCR is significantly upregulated in the aortic arch during Western type diet feeding. This is an interesting observation and indicates that FOXO1 is a mediator of both shear stress, as shown by Fisslaler *et al.*, and diet induced alterations in vascular transcription. Pro-atherogenic conditions such as low shear stress, high glucose or lipid levels and little exercise all increase FOXO1 mediated transcription¹⁷⁻²⁰. Therefore FOXO1 provides an interesting switch mechanism uniting the pro- atherogenic effects of several identified risk factors in atherogenesis.

Furthermore, we identified specific transcription factors for initial lesion formation. At this point, a moderate influx of leukocytes was confirmed by increased mRNA expression of both CD68 and CD4. Genes with a TFBS for

MEF2 were significantly overrepresented in the gene set that was downregulated at this time point. In contrast, genes with a TFBS for the inflammation associated transcription factor NF κ B were overrepresented in the cluster with upregulated genes. MEF2 is a transcription factor that has been mostly associated with muscle development but is currently investigated in endothelial cell biology^{28,37}. A mutation of the MEF2A gene is associated with increased risk for coronary artery disease³⁰. Interestingly, MEF2 activity is inversely correlated with NF κ B in our aortic material with early lesions. Kumar *et al.* have described a similar activity regulation profile in TNF α stimulated endothelium. The pro-inflammatory mediator TNF α downregulates the expression of KLF2, and this decrease is mediated by inhibition of MEF2 by NF κ B³⁸. We propose that a similar regulatory mechanism is activated in vascular tissue in a high fat environment leading to atherosclerotic plaque formation.

Pathways and genes identified by GSEA

In the current study we have found some interesting new target genes that were overexpressed in aortic tissue with initial or early atherosclerosis. In the top 50 most differentially regulated genes in initial lesion formation we identified 2 ABC transporters namely ABCB7 and ABCB10. ABCB10 is a member of the MDR/TAP subfamily, which members are associated with multi-drug resistance. Until now, the function of this mitochondrial protein is unknown, but a role in antigen presentation is suggested^{39,40}. We confirmed the upregulation of ABCB10 by RT-PCR and identified this molecule as initially elevated due to Western type diet feeding.

CCL8, belonging to the small inducible cytokine family, was identified by GSEA as one of the top most differentially expressed within initial atherosclerosis. Real time PCR confirmed these findings by GSEA. Several chemokines, such as CCL5 and CCL2 have been shown to be upregulated in atherogenesis, and specific blockade or downregulation of these chemokines has been shown to be protective in atherogenesis^{8, 41-44}. CCL8 functions as an attractor for monocytes via the CCR2 receptor. Unlike the receptor CCR2, the ligand CCL8 has never been associated with (early) atheroma so far. Functional blockade of this (possibly endothelial derived) molecule in animal models for atherosclerosis is necessary to address its now theoretical role in lesion formation.

We conclude that leukocyte influx into the vessel wall to initiate plaque formation is preceded by regulation of genes which are transcriptionally regulated by FOXO1. When the first leukocytes enter the vessel wall, NF κ B regulation is enhanced, combined with a decrease in MEF2 associated transcription. Our study thus identifies new transcriptional pathways and genes that underlie the early changes in the vessel wall, ultimately leading to atherosclerotic plaque formation.

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Chapter 3

Transcriptional Profiling of CD4 T Cells During Diet Induced Atherogenesis

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Abstract

Atherosclerosis is an inflammatory disease of the larger arteries that is accompanied by an immune response in the context of oxidized lipids and lipoproteins within the vessel wall. We quantified the mRNA expression of interleukins, chemokines and T cell activation markers in the spleen of LDLr^{-/-} mice fed a Western type diet for 3, 6, 9, and 12 weeks and compared this to the expression on chow diet. We observed that week 6 of diet feeding is the most differentially regulated time point, with a vast upregulation of many pro-inflammatory mediators.

We then focussed on the dominant regulators of atherosclerosis associated immune responses, the CD4 positive T cell. We used magnetic beads to isolate CD4⁺ cells from the spleen of LDLr^{-/-} after 0-6-12 weeks of diet and determined the transcriptional differences in mRNA regulation at these time point by using micro-arrays. Interestingly, after 6 weeks of diet, the enriched gene sets identified are mostly associated with cholesterol homeostasis, and the single immunologically relevant gene set is the CCR5 pathway. After 12 weeks of diet, the cholesterol homeostasis genes are still significantly enriched, but are now accompanied by gene sets involved in leukocyte adhesion, Th1/Th2 activation and TNF receptor activation. The CCR5 gene set is also significantly enriched after 12 weeks of Western type diet.

We conclude that a pro-atherogenic diet results in the regulation of cholesterol homeostasis associated genes in CD4 cells in the spleen, and that this precedes the massive upregulation of the common T cell activation associated molecules such as TNF receptor member or adhesion molecules.

Introduction

Atherosclerosis is a disease of the larger vessels characterized by deposition of lipids and fibrous tissue and it has been recently recognized as an inflammatory disease. Next to lipid deposition within the vessel wall, an ongoing adaptive immune reaction against lipids or modified lipoprotein antigens is observed¹⁻⁵. CD4 positive T cells are the predominantly identified T cells subset within atherosclerotic plaques of humans⁶, ApoE deficient (ApoE^{-/-}) and LDL receptor deficient (LDLR^{-/-}) mice⁷⁻⁸ and a pathogenic role for these CD4 T cells is indicated by the reconstitution of immune-deficient SCID/SCID/ mice with CD4 cells which leads to accelerated atherogenesis⁹. This information is complemented by studies with depleting antibodies against CD4 that reduced plaque formation in C57/Bl6 mice on high fat diet and attenuated plaque formation in CD4 deficient mice¹⁰. However, these effects are not observed in ApoE^{-/-} mice¹¹⁻¹². Not only the removal of the total CD4 population affects atherogenesis, the specific prevention of T cell activation by blockade of T cell co-stimulatory molecules such as CD40, OX40 B7 and ICOS have shown to be involved in atherosclerosis¹³⁻¹⁸. The spleen plays an important role in orchestrating the immune response during lesion formation. Removal of the spleen leads to increased atherogenesis in ApoE^{-/-} mice and to reduced levels of antibodies against oxidized lipid antigens¹⁵.

In this study we evaluated the expression of an array of genes associated with the regulation of immune responses, such as chemokines, interleukins and T cell activation in the spleen of LDLR^{-/-} mice on a Western type diet. Diet dependent differential regulation was observed and several regulatory patterns are described. Based on these initial results we performed full gene expression analysis in isolated splenic CD4 cells during diet feeding and identified genes and pathways that are involved in the systemic ongoing immune response during atherosclerotic lesion formation.

Material and methods

Animal studies

LDLr^{-/-} mice (n=6 per time point) were fed a Western type diet for 0-3-6-9-12 weeks. After the indicated time points, mice were anesthetized and flushed with PBS. The spleen was snap frozen in liquid nitrogen for RNA isolation. Cryostat sections of the aortic root (10 µm) were collected and stained with Oil-red-O. Lesion size was determined in 10 sections of the aortic valve leaflet area. For the microarray experiments, spleen single cell suspensions were prepared and CD4 T cells were isolated using CD4 specific IMag beads (Becton Dickinson) according to the manufacturers' protocol.

Serum lipid levels

Blood samples were collected by tail bleeding from non-fasted animals. Concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

Real Time PCR assays

Total RNA was isolated from spleen using the guanidium isothiocyanate (GTC) method. Purified RNA was DNase treated (DNase I, 10 units/µg of total RNA) and reverse transcribed (RevertAid M-MuLV reverse transcriptase) according to manufacturers protocol. Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (Appendix I) were designed using Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and validated for identical efficiencies (slope = -3.3 for a plot of the threshold cycle number (*C_t*) versus log ng cDNA). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Microarray analysis

RNA was isolated from CD4 T cells using Trizol. RNA was linearly amplified for 1 round (starting material 1 µg total RNA) synthesizing anti-sense cRNAs with an average base length of 500 nucleotides using the Message-Amp kit from Ambion. Aminoallyl-UTP was incorporated with a molar ration of 1:1 to rUTP. Cy3 or Cy5 mono-reactive dyes were coupled according to the manufacturers' instructions (Amersham Bioscience, Piscataway, New Jersey). Labeled cRNA was purified using the RNeasy purification kit (Qiagen, Germany). All RNA samples were hybridized against a common reference containing (in equal amounts and amplified one round); liver, spleen and aorta of LDLr^{-/-} mice 6 weeks on Western type diet and LDLr^{-/-} mice treated with LPS, RAW cells treated with LPS, H5V cells treated with TNFα and Strategene universal mouse reference RNA.

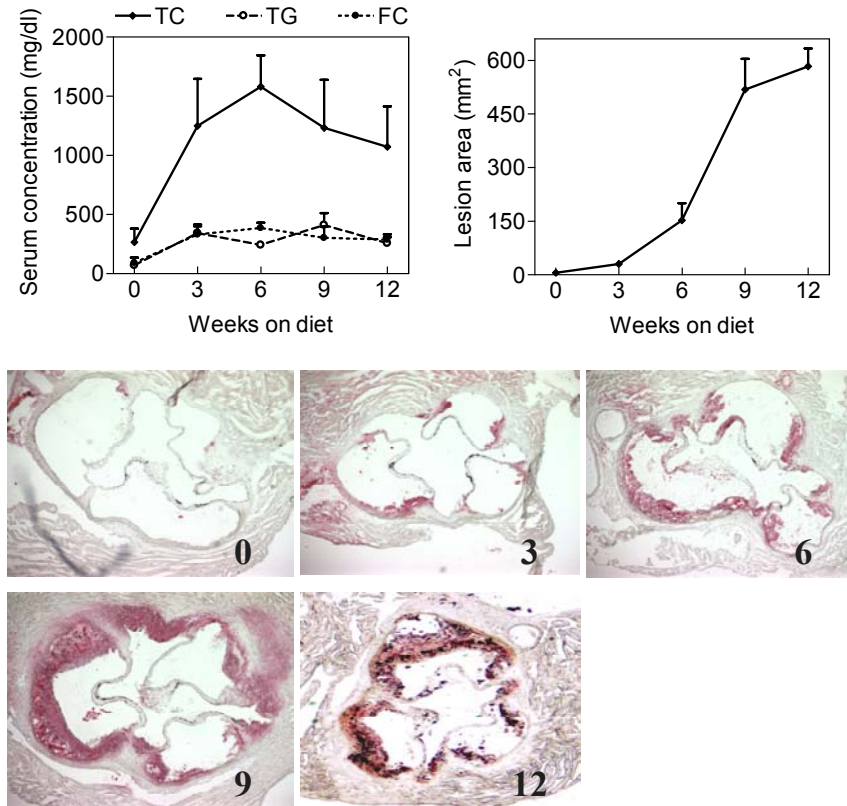


Figure 1: Diet induced atherosclerosis in LDL receptor deficient mice Female LDLr^{-/-} mice (n=6 per group) were placed on a Western type diet for 0-3-6-9-12 weeks. Serum free and total cholesterol and triglycerides were determined during the experiment and a significant increase is observed at 3 weeks of Western type diet feeding that is maintained throughout the experiment. Lesion formation in the aortic root was quantified at the indicated time points after Oil red o staining and average lesion size is indicated in upper right panel. Representative slides are shown for every time point.

Hybridization was performed on glass based micro-arrays representing 22,056 unique murine oligonucleotide sequences (Micro Array Department, University of Amsterdam, The Netherlands (<http://www.microarray.nl/libraries.html>)). Microarray data were acquired and imported in Rosetta Resolver database and Loess normalized (limma package, Bioconductor). Genome wide analysis at group level was performed using GSEA after conversion of mouse to human signature¹⁶. The molecular signature database that was used was a modified version of the original named c2.symbols.gmt (available on-line at http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html) set and earlier described by Volger *et al*¹⁶. The dataset contained 594 gene sets. Analysis was performed with the following settings: 100 permutations on phenotype, gene sets with more than 10 genes were included in the analysis.

Statistical analysis

Values are expressed as mean $\pm$ SEM. Two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was used to not normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

Results

Atherosclerotic lesion formation in LDLr^{-/-} mice during Western type diet feeding

We used LDLr^{-/-} mice as a model for atherosclerosis and these animals were fed a Western type diet for 0-3-6-9-12 weeks. This diet does not contain cholate and only 0.25 % cholesterol. Diet feeding results in elevated cholesterol and triglyceride levels already after 3 weeks, and these elevated levels were maintained throughout the experiment (figure 1 upper left). Basal free cholesterol levels were 91 ± 44 mg/dl vs 331 ± 83 mg/dl at week 3. Total cholesterol level at chow feeding were 265 ± 115 mg/dl and these rose to 1249 ± 398 mg/dl after 3 weeks of Western type diet. An induction was also observed in triglyceride levels, from 109 ± 57 mg/dl to 229 ± 44 in week 3. The observed induction in cholesterol and triglyceride levels was accompanied by the formation of atherosclerotic plaques. Figure 1 clearly shows the induction in lesion formation in the aortic valve leaflet area in time. After 12 weeks, lesion size averaged 583 ± 50 mm² and a sharp increase in lesion size is observed between 3 and 9 weeks of diet feeding (from 31 ± 9.2 to 518 ± 86 mm²). Representative pictures of the aortic valves are shown for every time point.

Gene expression in the spleen during Western type diet feeding in LDLr^{-/-} mice

To investigate the systemic immunological changes within the spleen in relation to the observed induction of atherosclerosis, we isolated mRNA from spleens of LDLr^{-/-} mice at the indicated time points (0-3-6-9-12 weeks of diet). We determined the gene expression of a panel of genes associated with immune responses, including interleukins, chemokines and T cell activation molecules. The results are shown in figure 2.

We observed 3 distinct regulatory patterns within the array of interleukins we scanned. Panel 2A shows the regulation of, IL-9, IL-10 and TNF α , all clearly being downregulated during Western type diet feeding from week 6. Interestingly, IL-4 was significantly upregulated after 6 weeks of diet feeding, only being downregulated after 12 weeks. IL-1 β , IL-2 and IL-17A expression is already significantly upregulated at 3 weeks of diet feeding, showing a peak expression at week 6 and returning to basal level at 12 weeks (Panel 2B). This peak at week 6 was also observed when we determined the expression of IFN γ , IL-6, and not for the T cell activator IL-12 and the growth factor TGF β . (Panel 2C). This is highly interesting as normally IL-12 regulates the secretion of IFN γ

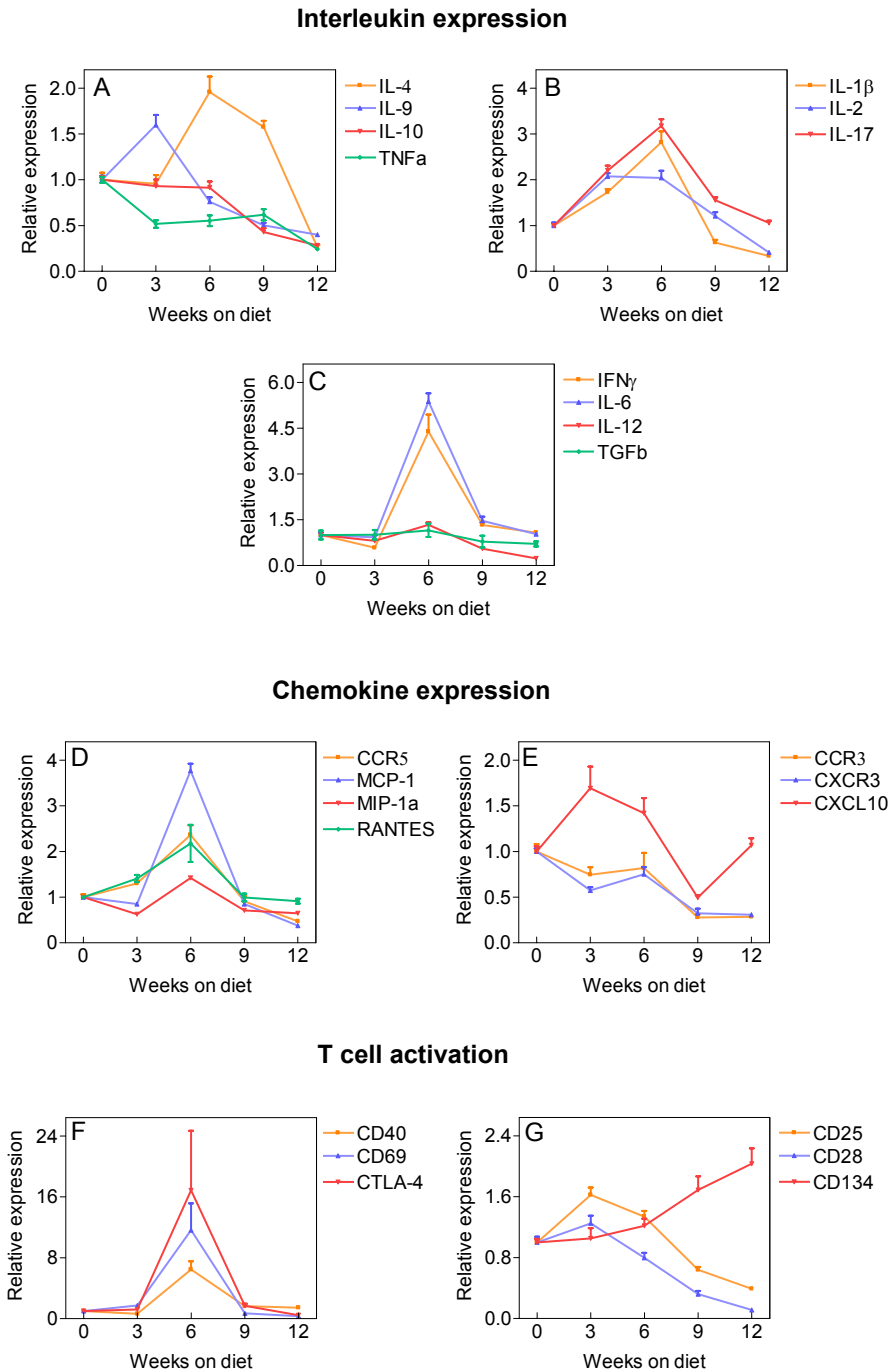


Figure 2: Transcriptional regulation of interleukins (A-C), chemokines (D-E) and T cell activation markers (F-G) in the spleen during atherogenesis

mRNA was isolated from the spleen of LDLr^{-/-} using the GTC method and expression of different genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression of mice on chow diet. An unpaired Student *t* test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (**p* < 0.05, n=6 per time point).

by T cells. The expression of TGF β was extremely high in all time points (as compared to house hold gene expression) and not differentially regulated. Not only interleukins, but chemokines are involved in immune activation and migration during atherogenesis. We therefore checked the expression profile of several chemokines and chemokine ligands during Western type diet feeding. Interestingly, the expression profile of CCR5, MCP-1, MIP-1 α and RANTES is similar to the regulation of IL-6 and IFN γ (panel 2C), showing a sharp increase in expression after 6 weeks of Western type diet feeding (Panel 2D). Diet feeding however resulted in a decreased expression of CCR3 and CXCR3 as shown in panel 2E. One of the ligands for CXCR3, CXCL10 was only downregulated after 9 weeks of diet feeding but was significantly upregulated after 3 weeks.

The differential expression of interleukins and chemokines may suggest an effect on the T cell population present in the spleen. We therefore determined the expression of the T cell activation markers CD40, CD69 and CTLA-4. Surprisingly, for these molecules a very vast and significant increase in expression was observed only after 6 weeks of Western type diet feeding (Panel 2F). A different profile is observed for CD25 (IL-2 receptor expressed on regulatory T cells) and CD28, a co-activation marker. The expression of these molecules is effectively downregulated by Western type diet feeding after week 6 (Panel 2G). A distinct pattern is observed for CD134 (OX40), a T cell activation marker from the TNF receptor superfamily used for co-stimulation. Its expression is nicely correlated with the induction of atherosclerosis, increasing steadily with the time of diet feeding.

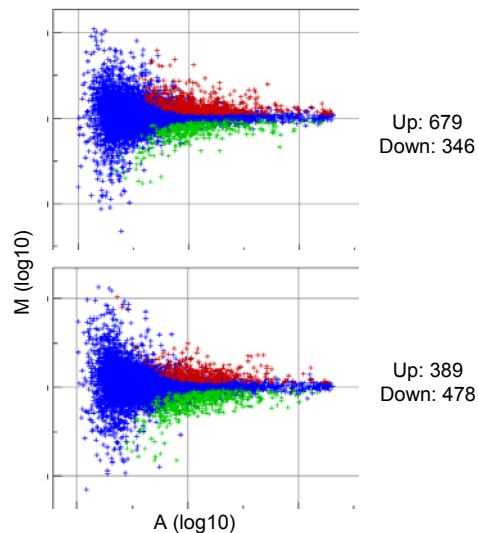


Figure 3: M versus A plots of re-ratio profiles from CD4 T cells isolated from the spleen after 6 and 12 weeks of diet compared to chow. CD4⁺ T cells were isolated from the spleen of LDLr^{-/-} mice after 0-6-12 weeks of Western type diet feeding by using anti-CD4 magnetic bead separation. RNA was isolated and amplified one round and hybridized against a common reference. Rosetta Resolver was used to generate M vs. A plots and the significantly regulated genes compared to chow fed animals are shown in green (down) and up (red).

Transcriptional regulation of CD4 T cells in the spleen during diet induced atherogenesis

The regulation patterns observed above indicate a differential regulation of genes associated with immune activation and regulation during diet induced lesion formation. Especially week 6, the time point that correlates with extensive plaque growth, and week 12 seem to show distinct regulatory patterns. The spleen however, consists of many cell types, including macrophages, T and B lymphocytes. As CD4⁺ effector T cells are the predominant T cell subclass linked to atherosclerotic lesion formation, we decided to investigate the transcriptional regulation of this specific cell type during atherogenesis. We isolated CD4⁺ T cells from the spleen after 0, 6 and 12 weeks of diet using magnetic beads. Homogeneity of the isolated population was tested using FACS and bead isolation resulted in >95% CD4⁺ T cells (data not shown). Transcriptional differences in this population during diet induced lesion formation (0, 6 and 12 weeks) were determined by micro-array. Isolated mRNA from every CD4 sample (n=4 per time point) was amplified one round and hybridized against a common reference. M versus A plots that show relative expression to week 0 versus intensity are shown in figure 3.

Gene set enrichment analysis

Pathway based Gene Set Enrichment Analysis (GSEA) was used to study transcriptome differences in CD4⁺ T cells in the spleen during Western type diet feeding. This method enables the interpretation of genome-wide expression profiles based on biological function. Next to the functional output, this analysis tool identifies a top 100 of signature genes of one situation compared to another¹⁶. In this way, gene sets that contain genes that are overrepresented at the extremes of the ranked gene list will result in high enrichment scores. Tables 1 A and B show the enriched pathways for both 6 weeks of diet feeding and 12 weeks of diet feeding, and the nominal P values calculated by weighted Kolmogorov-Smirnov-like statistic as described earlier¹⁶.

The gene sets enriched in CD4⁺ cells after 6 weeks of diet (compared to chow fed, early atherosclerosis) are visualized in table 1A. Interestingly, metabolic pathways such as PPAR α , fatty acid metabolism and glucose metabolism associated pathways are present at this time point. This indicates that the CD4⁺ T cells in the spleen respond to the increase in cholesterol and triglyceride levels upon Western type diet feeding. Next to these metabolic pathways, genes associated to the CCR5 pathway are enriched after 6 weeks of diet corresponding to extensive plaque growth.

After 12 weeks of diet, the number of gene sets significantly enriched compared to chow fed animals is 23, and a list of these gene sets is shown in table 1B. Again, several gene sets associated with metabolism, some similar to week 6, are observed. These include PPAR α and fatty acid metabolism. The number of gene sets associated with inflammation was clearly increased. Again, the gene set CCR5 is significantly enriched compared to week 0. Additional immunological

Week 6 (A)	NOM p-val
HOXA9_DOWN	<0.001
CR_DEATH	<0.001
CR_CYTOSKELETON	<0.001
extrinsicPathway	<0.001
par1Pathway	<0.001
achPathway	<0.001
ccr5Pathway	<0.001
pparaPathway	<0.001
fatty_acid_metabolism	<0.001
GLUCOSE_DOWN	0.032
Week 12 (B)	NOM p-val
cell_adhesion_molecule_activity	<0.001
CR_PROTEIN_MOD	<0.001
HUMAN_CD34_ENRICHED_TF_JP	<0.001
cell_adhesion	<0.001
fatty_acid_metabolism	<0.001
p53_signalling	<0.001
hivnefPathway	<0.001
GLUCOSE_UP	<0.001
ccr5Pathway	<0.001
MAP00240_Pyrimidine_metabolism	<0.001
41bbPathway	<0.001
ctlPathway	<0.001
calcineurinPathway	<0.001
cdmacPathway	<0.001
GNF_FEMALE_GENES	<0.001
pmlPathway	<0.001
pparaPathway	<0.001
HTERT_DOWN	<0.001
lairPathway	<0.001
tnfr1Pathway	<0.001
th1th2Pathway	<0.001
tnf_and_fas_network	0.025
stressPathway	0.029

Table 1 A + B: enriched gene sets identified using GSEA. GSEA was used on our data set from CD4⁺ T cells to identify differential pathways in week 6 and 12 compared to week 0. Gene set and nominal p value are shown for week 6 (table A) and week 12 (table B).

gene sets include 41bb, which consists of T cell co-activation genes, TNF pathways, which include T cell activation and cell death receptors, and Th1/Th2 genes that regulate the differentiation of T cells. The most significantly enriched pathway is a cell adhesion pathway, indicating increased adherence capacity of the CD4⁺ T cell population in the spleen.

Lipid metabolism associated genes

Western type diet feeding has a profound effect on cholesterol metabolism in CD4⁺ T cells. This is remarkable, as no specific lipid uptake or scavenging systems have been described in T cells. We looked in our array database for lipid associated genes with a minimum fold in- or decrease of 2 and a (Rosetta) p value < 0.05 in week 6 and 12 compared to the control mice and the results are shown in table 2.

When we compare the gene list of weeks 6 and 12, 4 genes are significantly induced in both time points; CD36, fatty acid binding protein (FABP) 1, ABCA8, and lipocalin 5 (shown in gray).

Week 6 (A)	Fold change	NOM p-val
CD36 antigen	4.57	0.0192
Fatty acid binding protein 1, liver	4.55	0.0000
Cytochrome P450, family 3, subfamily a, polypeptide 44	3.90	0.0018
Stearoyl-Coenzyme A desaturase 2	3.84	0.0000
ATP-binding cassette, sub-family A (ABC1), member 8b	3.50	0.0216
Lipocalin 5	3.07	0.0000
Tocopherol (alpha) transfer protein	2.89	0.0346
Cytochrome P450, family 4, subfamily a, polypeptide 14	2.87	0.0000
3-oxoacid CoA transferase 2A	2.77	0.0454
Apolipoprotein A-V	2.51	0.0138
Cytochrome P450, family 8, subfamily b, polypeptide 1	2.28	0.0321
ATP-binding cassette, sub-family C (CFTR/MRP), member 5	2.06	0.0025

Week 12 (B)	Fold change	NOM p-val
Fatty acid binding protein 1, liver	5.05	0.0000
ATP-binding cassette, sub-family A (ABC1), member 8b	4.81	0.0475
CD36 antigen	3.18	0.0700
Lipocalin 5	2.63	0.0017
Cytochrome P450, family 2, subfamily c, polypeptide 29	2.41	0.0001
Acyl-Coenzyme A dehydrogenase, very long chain	2.12	0.0013
Cytochrome P450, family 7, subfamily b, polypeptide 1	-2.44	0.0440
Bile acid-Coenzyme A: amino acid N-acyltransferase	-2.57	0.0077
Cytochrome P450, family 3, subfamily a, polypeptide 44	-3.99	0.0005
ATP-binding cassette, sub-family C (CFTR/MRP), member 2	-18.27	0.0172

Table 2 A + B: Significantly regulated genes involved in lipid metabolism. Genes with a role in lipid metabolism that were significantly regulated in week 6 (table A) and week 12 (B). Fold change and nominal p value are indicated.

CD36 is a member of the scavenger receptor family and has an important role in the uptake of modified LDL and is used in the clearance of pathogens. FABP1 binds long chain fatty acids and is associated with fatty acid metabolism. ABCA8 is identified as an ATP dependent lipophilic drug transporter. Lipocalin 5 belongs to the lipocalin family and shares functional homology with the FABP family. This family is involved in binding to hydrophobic molecules and has been suggested to play a role in cell homeostasis and regulation of the immune response, inflammation and in detoxification processes.

Discussion

The hypothesis that atherosclerosis is a lipid driven chronic inflammatory disease is now widely accepted. Several important publications have indicated the pivotal role of CD4 cells in the initiation and maintenance of this immunological response within the atherosclerotic plaque^{5, 11}.

Next to a local inflammatory response at the site of the atherosclerotic lesion, systemic upregulation of interleukins and elevated levels of antibodies against oxidized lipoproteins and heat shock proteins have been identified in humans with atherosclerotic lesions^{17,18}. This study shows that next to a local inflammation of the arterial wall, systemic immune-regulation takes place in the spleen during diet-induced atherosclerotic lesion formation. This is not unexpected, as the spleen is continuously exposed to the elevated circulating

lipoproteins. Both direct activation via the diet and indirect activation of the spleen cells due to migrated dendritic cells that have taken up antigens in the vascular wall can result in systemic immune-activation.

We used female LDLr^{-/-} mice on a pro-atherogenic Western type diet as a model for atherosclerosis. This diet induces lesion formation in the large vessels of the animal, and we examined the expression of known immunologically active genes in the spleen after 0-3-6-9-12 weeks of diet. We show that between 3 and 9 weeks of diet, lesion area is rapidly increased. One of the most interesting observations is the combined upregulation of known pro-inflammatory factors after 6 weeks of diet feeding, correlating to the point of extensive lesion growth. Upregulation at this time point was observed for interleukins (IFN γ , IL-6, IL-1 β , IL-17), chemokines (CCR5, MCP-1, MIP-1 α , RANTES) and T cell specific activation markers (CD40, CD69, CTLA-4, OX40). A pro-atherogenic role was established for the vast majority of these factors¹⁹. Next to this interesting peak profile observed at week 6, the severe downregulation of a number of genes at week 12 deserved attention. Downregulation is seen for identified anti-atherogenic factors such as IL-9 and IL-10 that are associated with Th2 and regulatory T cells²⁰⁻²². This decrease is accompanied by the reduced expression of CD25, expressed on regulatory T cells²³. Next to the expected downregulation of anti-atherogenic markers, the expression of CD28, a co-stimulatory molecule for T cells is also decreased at this time point²⁴. Furthermore, the expression of CXCL10 and CXCR3, both identified as being pro-atherogenic, decreased as well²⁵⁻²⁸. Due to the multi-cellular composition of the spleen, the observed regulatory profiles are hard to address. Macrophages, T cells and B cells all can contribute to the observed transcriptional differences especially for interleukins such as IL-6 and IL-10, which can be produced by many cell types.

During recent years, it was shown that CD4⁺ T cells are an important immunoregulatory cell associated with atherosclerosis⁶⁻¹⁰. We therefore focused our attention on this specific subclass of T cells, and determined the transcriptional regulation induced during atherosclerosis induction upon feeding atherosclerosis prone LDLr^{-/-} mice a Western type diet.

We isolated CD4⁺ T cells from mice that received 6 and 12 weeks of diet, and compared the transcriptome of these cells with CD4⁺ T cells isolated from mice on chow. We performed Gene Set Enrichment analysis on the expression profile datasets to identify gene sets that were significantly enriched at these time points. It appears that the gene set for CCR5 was enriched after both 6 and 12 weeks of diet feeding. This is interesting, as the expression of CCR5, RANTES and CCR2 in the whole spleen is only elevated after 6 weeks of diet feeding. After 12 weeks of atherosclerosis induction, more inflammatory pathways are enriched, including TNF receptor super family members, Th1/Th2 differentiation genes and cell adhesion markers. This clearly indicates the immunological activation of the isolated cell subset at this time point.

One intriguing issue is the difference in regulatory profile between the entire spleen and the isolated CD4 population. When analyzing the profiles of most pro-inflammatory genes such as IL-1 β , IL-6, IFN- γ , a peak is observed after 6 weeks of Western type diet feeding. When we compare this to the gene sets in the specific CD4⁺ population at week 6, only CCR5 is clearly associated with immunological activation as it is predominantly expressed on activated Th1 cells. Most of the gene sets identified at this time point are however metabolism related. Only after 12 weeks we observed gene sets that indicate T cell differentiation and activation, including Th1/Th2 differentiation and TNF receptor superfamilies. The CCR5 gene set was significantly enriched at this time point as well.

During inflammation, the innate immune response precedes the adaptive T cell mediated reaction. The innate response is mediated by macrophages and other antigen presenting cells and the interleukins IL-1 β , IFN γ and IL-6 are known mediators of this process. The observed upregulation of these molecules in the spleen already after 3-6 weeks most likely results from this early defence system, and possibly results from activation by elevated levels of circulating lipids that are possibly minimally modified. The adaptive, T cell mediated reaction is taking over between 6 and 12 weeks of atherosclerosis. This is shown by the upregulation of T cell activation pathways in the isolated CD4 subset. Possibly, at this time point, presentation of antigens derived from the atherosclerotic plaque is facilitated by dendritic cells that specifically induce the activation of these T cells.

An interesting and rather unexpected finding in our study was the profound representation of lipid homeostasis genes that are normally associated with hepatic lipid metabolism. Especially after 6 weeks of diet, these gene sets clearly outnumber the immune response associated gene sets (except for CCR5). When we looked for genes associated with lipid homeostasis in the single gene list we found several significantly differentially regulated genes. Most of these genes are involved in the removal or degradation of (potentially) harmful lipids and free fatty acids. These include the cytochrome P450 enzymes and ATP binding cassette transporters that mediate transport of cholesterol in and out of cells. Also the fatty acids binding proteins and lipocalins are involved in the neutralization, removal or degradation of molecules such as fatty acids.

A possible link between the observed regulation in the areas of lipid metabolism and immunological activation is provided by 2 transcription factors, PPAR α and NFAT.

PPAR α is a nuclear receptor that has been shown to modulate both lipid homeostasis and inflammation^{29,30}. PPAR α agonist such as fenofibrates and benzofibrates reduce triglycerides and induce HDL levels and attenuate atherosclerotic lesion formation in animal models^{31,32}. Interestingly, GSEA

analysis after diet feeding resulted in the enrichment of the PPAR α gene set in CD4⁺ T cells after both 6 and 12 weeks of Western type diet feeding, indicating a role for this nuclear receptor in T cell activation during atherosclerotic lesion formation. Possibly, in a high fat environment the expression of PPAR α in T cells is induced to prevent large scale cholesterol uptake by these cells. PPAR α is able to induce the removal of cholesterol from macrophages in a high fat environment via stimulation of efflux transporters such as ABCA1³³. However, not only the upregulation of efflux transporters is the effect of increased PPAR α activity. This also results in suppression of NF- κ B activity and decreased IFN γ production by T cells. This suggests a negative feedback loop on T cell activation, so further research will be necessary to address these effects. However, when looking at the general expression of inflammatory markers in the spleen during diet feeding for 12 weeks, downregulation of genes activated by NF- κ B such as IL-1 β and IL-12 is observed.

The nuclear factor of activated T cells (NFAT), is a transcriptional factor that is expressed in T cells³⁴. It regulates the transcription of (cytokine) genes that are critical during an immune response such as IL-2, IFN- γ , TNF- α and CD40 ligand³⁵. Immune-suppression using cyclosporine A and KF506 have been shown to be protective in atherosclerosis^{36,37} due to reduced calcineurin mediated phosphorylation of NFAT and its subsequent translocation to the nucleus³⁸. One of the most differentially regulated genes in CD4⁺ T cells during induction of atherosclerosis after Western type diet feeding is Lipocalin-5. The expression of this gene is regulated by the transcription factor FOXA1 that in turn is activated by NFAT^{34,39}. Mazière *et al.* provide evidence that oxidized LDL activates NFAT in a T cell line³⁴. Upregulation of NFAT signaling can therefore be caused by the increased levels of circulating (oxidized) lipoproteins and subsequently induce a systemic immune response.

The data shown in this study indicate that T cells are heavily influenced by high levels of circulating (oxidized) lipids. Further research is necessary to determine the specific effects of high serum lipids on T cell activation and its role in diet induced atherogenesis.

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Chapter 4

The HIV Entry Inhibitor TAK-779 Attenuates Atherogenesis in LDL Receptor Deficient mice

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Abstract

HIV combination therapy using protease-inhibitors is associated with elevated plasma levels of atherogenic lipoproteins and increased risk for atherosclerosis. We investigated whether the HIV entry inhibitor TAK-779, affects lipoprotein levels and atherogenesis in LDL receptor deficient mice.

TAK-779 is an antagonist for the chemokine receptors CCR5 and CXCR3, that are expressed on leukocytes, especially Th1 cells, and these receptors may be involved in recruitment of these cells to atherosclerotic plaques.

TAK-779 treatment of LDLr^{-/-} mice did not elevate the levels of atherogenic lipoproteins, whereas it dramatically reduced atherosclerosis in the aortic root and in the carotid arteries. The number of T cells in the plaque was reduced by 95%, concurrently with a 98% reduction in relative IFN- γ area. TAK-779 treated animals showed a decreased percentage of CD4⁺ and CD8⁺ T cells in peripheral blood and in mediastinal lymph nodes compared to control treated animals.

TAK-779 not only suppresses HIV entry via blockade of CCR5 but also attenuates atherosclerotic lesion formation by blocking the influx of Th1 cells into the plaque. TAK-779 treatment may be especially beneficial for young HIV patients as they face lifelong treatment and this drug impairs atherogenesis.

Introduction

Leukocyte recruitment into the vessel wall is a key step in atherosclerotic lesion formation and chemokines are known to regulate this process. Chemokines can be divided into four families including the CC receptors that bind CC chemokines, and the CXC receptors which bind CXC chemokines¹. CCR5 and CXCR3 are two chemokine receptors that are implicated in the migration of activated Th1 cells to the site of inflammation, and both receptors have been suggested as potential targets for the treatment of auto-immune like diseases^{2,3}.

A 32 base pair deletion in the CCR5 gene (CCR5 Δ 32) results in a non-functional receptor and individuals that are homozygous for this deletion are resistant to infection with HIV^{4,5}. Interestingly, it is also shown that this natural deficiency in CCR5 protects individuals from early myocardial infarction and severe coronary artery disease. The ligands for CCR5, RANTES and MIP-1 α , have been detected in atherosclerotic plaques of both humans and mice⁶⁻⁹. Furthermore, inhibition of CCR5/CCR1 using the receptor antagonist met-RANTES attenuates atherosclerosis in LDLr deficient mice¹⁰. Antibody mediated blockade of CXCR3 results in a decreased recruitment of Th1 cells to the site of inflammation³.

TAK-779 (N,N-dimethyl-N-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclopten-8-yl]carbonyl]amino]benzyl]-tetrahydro-2H-pyran-4-aminiumchloride) is a non-peptide CCR5/CXCR3 antagonist, that was developed for the treatment of HIV infection by inhibiting HIV cell entry via CCR5¹¹⁻¹³. TAK-779 however has also some anti-immunogenic effects. It has been shown to block the influx of CCR5 and CXCR3 positive T cells into inflamed joints in an experimental model for arthritis¹⁴.

Both CCR5 and CXCR3 are predominantly expressed on Th1 cells¹⁵. As atherosclerosis is considered to be a Th1 mediated disease^{16,17}, treatment with TAK-779 could possibly attenuate atherogenesis by blocking the influx of Th1 cells into the atherosclerotic lesion. Both studies in humans and mice demonstrate an increase in atherosclerotic lesion formation and myocardial infarction in relation to the protease inhibitor treatment in HIV positive patients¹⁸⁻²². TAK-779 could therefore have a dual function in these patients, as it not only blocks virus entry, but at the same time inhibits the severe side effects of their therapy.

In the present study we show that treatment of LDLr^{-/-} mice with TAK-779 attenuates atherosclerotic lesion formation. TAK-779 treatment may therefore serve as a new convenient treatment of HIV infection and at the same time attenuate atherosclerotic lesion formation, in contrast to the now available combination therapy with protease inhibitors.

Materials and methods

Animal experiments

Female LDLr deficient (LDLr^{-/-}) mice, 15 weeks old (n=10/group), were put on a Western-type diet containing 0.25 % cholesterol and 15% cocoa butter two weeks before collar placement. Silastic collars (0.3 mm inside diameter, Dow Corning, Midland, USA) were placed around the carotid artery to induce atherosclerosis as described previously²³.

Mice were treated immediately after collar placement with an injection of 150 µg TAK-779 in 100 µl of 5% mannitol(w/v) subcutaneously every other day during 6 weeks after which mice were anesthetized and subsequently sacrificed during tissue harvesting (5% mannitol was used as a control).

In a second experiment female LDLr^{-/-} (mice n=10/group) were fed a Western type diet and TAK-779 treatment was started simultaneously. Treatment was performed for 6 weeks and hearts were excised and stored in Zinc Formal-Fixx and the aortic root was taken out for analysis.

Histological analysis

Cryosections were stained with hematoxylin (Sigma Diagnostics) and eosin (Merck diagnostics). Corresponding sections were stained with antibodies directed against a macrophage specific antigen (MOMA-2, polyclonal rat IgG2b,; Research diagnostics); α-smooth muscle cell actin (monoclonal mouse IgG2a, clone 1A4, Sigma Diagnostics); IFN-γ (rat IgG1, clone XMG1.2, BD Pharmingen) or CD3 (molecular complex 17A2, BD Pharmingen) for 2 hours. As secondary antibodies (1 hour incubation) goat anti-rat IgG alkaline phosphatase conjugate (Nordic) with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates, or biotinylated goat anti-rat polyclonal Ig (BD Pharmingen) was used in combination with streptABC complex (DAKO), with Nova Red as enzyme substrate (Vector Laboratories). Collagen was visualized by picosirius red (Direct red 80) and lipids by Oil red O staining.

Serum lipid levels

Concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath was used as a standard. Cholesterol distribution over the different lipoproteins was determined by fractionation of the serum using a FPLC system and subsequent cholesterol assay.

Real time PCR assays

Total RNA was isolated from spleens of the mice using GTC method. Purified RNA was DNase treated (DNase I, 5 units/µg of total RNA) and reverse transcribed (RevertAid M-MuLV reverse transcriptase) according to manufacturers protocol. Quantitative gene expression analysis (Primers,

appendix I) was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) using SYBR Green technology.

Flow cytometry

Lympholite (Cedarlane Laboratories, Hornby, Ontario, Canada) was used to separate lymphocytes from whole blood and spleen. Cell suspensions from mediastinal lymph nodes, spleen and blood were incubated with 1% normal mouse serum in PBS and stained for surface markers (0.5 μ g Ab/200,000 cells, BD Bioscience). All data were acquired on a FACSCalibur and 10000 lymphocyte events were analyzed with CELLQuest software (BD Biosciences).

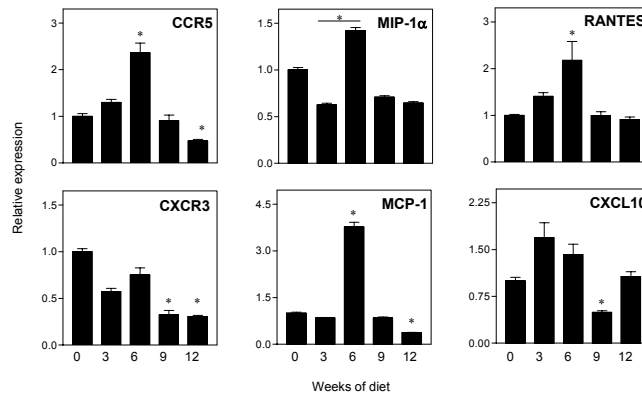


Figure 1. mRNA expression of inflammatory markers in the spleen in response to a Western type diet. mRNA was isolated from spleen using the GTC method and expression of different genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression of mice on chow diet. An unpaired Student *t* test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (**p* < 0.05, *n*=6 per time point).

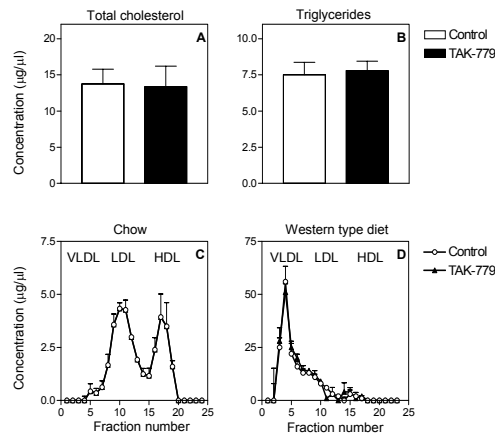


Figure 2. Effect of TAK-779 treatment on serum lipid levels. LDL^{-/-} mice (*n*=6 per group) were put on a Western type diet and treated with 150 μ g of TAK-779 *s.c.* per mouse every other day or control. At the indicated time points blood samples were taken and serum cholesterol (2A) and triglyceride (2B) levels were determined. Distribution of cholesterol over lipoproteins was determined using FPLC. 30 μ l aliquots of serum of individual mice were loaded on a Pharmacia SMART column and 24 fractions were collected. Cholesterol concentrations were determined at chow (2C) and Western type diet (2D) for TAK-779 treated and control mice.

Results

mRNA expression in spleen of CCR5, CXCR3 and their ligands

Atherosclerosis is considered to be an inflammatory disease, primarily characterized by a Th1 mediated inflammatory reaction^{16,17}. CCR5 and CXCR3 are chemokine receptors that are mainly expressed on Th1 cells^{25,26}. The spleen plays a central role in the immune system and is exposed to circulating antigens associated with atherosclerosis such as oxidized LDL. We examined the mRNA expression of CCR5 and CXCR3 and their ligands in the spleen during the process of atherosclerosis in LDLr^{-/-} mice on Western type diet (Figure 1).

After 6 weeks of diet, CCR5 relative mRNA expression in the spleen is significantly upregulated 2.4 fold (1.0 ± 0.06 vs. 2.4 ± 0.21 , $p=0.01$). The expression of CXCR3 is not differentially regulated during the first 6 weeks of feeding a Western type diet and atherosclerotic lesion formation. Both CCR5 and CXCR3 are downregulated in the spleen in later stages of atherosclerotic lesion formation in the used model (> 9 weeks of Western Type diet). The expression of established ligands for CCR5 was also monitored. RANTES and MIP-1 α are both significantly upregulated after 6 weeks of Western type diet. This coincides with the regulation pattern of CCR5 and MCP-1. CXCL10, a ligand specific for CXCR3 is downregulated after 9 weeks of Western type diet and showed no regulatory similarity with the other ligands.

TAK-779 reduces atherogenesis in LDLr^{-/-} mice without altering cholesterol levels

Two different experiments were performed to study the effect of TAK-779 on atherogenesis.

After two weeks of Western type diet LDLr^{-/-} mice were equipped with collars around both carotid arteries. Subsequently, the mice were treated with TAK-779 or control treated for 6 weeks. Yang *et al.* showed that administration of 150 μ g per mouse every other day was sufficient to induce the maximum blocking effect¹⁴. No difference in cholesterol levels and lipoprotein concentration could be observed between the control and TAK-779 treated group (Figure 2). Figure 3 shows representative sections of TAK-779 treated (figure 3D) and control treated (figure 3E) mice. Lesion size significantly decreased by 68% upon treatment with TAK-779 (18384 ± 3370 μ m² vs. 5926 ± 1842 μ m², $p=0.004$) (figure 3A), intima media ratio decreased by 49% (0.46 ± 0.07 vs. 0.26 ± 0.06) (figure 3B), and also the relevant clinical parameter, intima lumen ratio, decreased significantly by 56% (0.26 ± 0.04 vs. 0.11 ± 0.03 , $p=0.01$) (figure 3C).

The general composition of the carotid lesions of TAK-779 treated mice was not altered compared to control treated mice (figure 4). The relative MOMA-2 stained area was comparable in control (4B) and TAK-779 treated animals (4C), (0.265 ± 0.05 vs. 0.252 ± 0.06 , $p=0.88$). We visualized the collagen content of the plaque by picosirius red staining. No difference was observed between control (4E) and treated mice (4F) (0.095 ± 0.03 vs. 0.101 ± 0.03 , $p=0.69$). Staining for α -

actin, specific for smooth muscle cells (ASMA) is shown in on line figure 4H (control) and 4I (treated). TAK-779 treatment did not affect relative ASMA area (0.084 ± 0.016 vs. 0.078 ± 0.011).

In a second experimental setup, Western type diet and TAK-779 treatment were started simultaneously and 8 weeks later LDLr^{-/-} mice were sacrificed. Atherosclerosis in the aortic valves was decreased by 40% in TAK-779 treated mice compared to control mice without altering relative macrophage content (Figure 5).

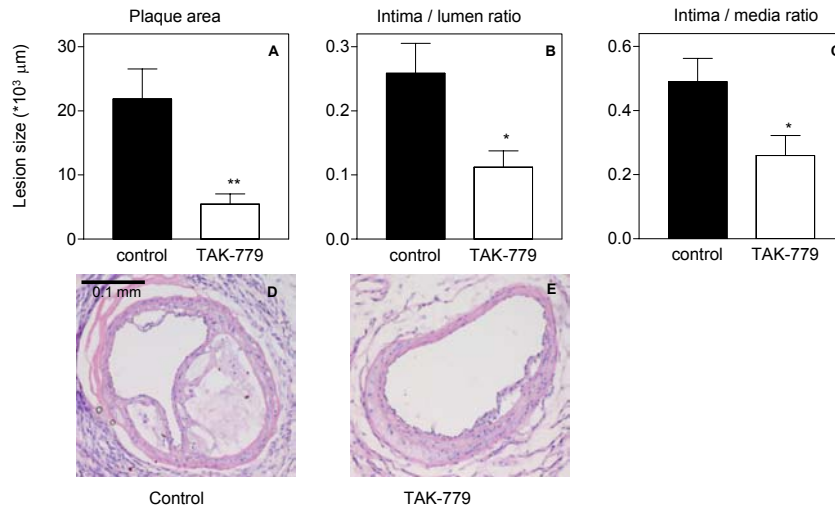


Figure 3. Effect of TAK-779 treatment on plaque formation in a collar induced carotid artery atherosclerosis model. Cross-sectional carotid plaque area (3A), intima/ lumen ratio (3B), and intima / media ratio (3C) following a treatment with 150 μg of TAK-779 in 100 μl of 5% mannitol injection fluid *s.c.* every other day during 6 weeks. A marked and significant decrease was seen for all three parameters. Lower panel gives representative pictures of the carotid artery of control (3D) and TAK-779 treated (3E) LDLr^{-/-} mice. Error bars represent SEM, n = 10 per group, (**= p<0.005, *=p<0.05, Mann Whitney test).

TAK-779 treatment impairs the influx of Th1 T cells to the atherosclerotic plaque

Both CCR5 and CXCR3 are expressed predominantly on Th1 cells and both receptors are implicated in the migration of these cells to their site of action and treatment with TAK-779 may block the migration of Th1 cells into the atherosclerotic plaque. We performed a staining for CD3 (figure 6A), a general T cell marker and scored for positive cells in both the plaque and the adventitia. A vast and significant decrease is observed in the number of T cells in the plaque and adventitia of TAK-779 treated mice compared to control (plaque 0.53 ± 0.31 vs. 0.03 ± 0.03 , p=0.04, adventitia 4.98 ± 0.63 vs. 2.01 ± 0.38 , p=0.0007). In order to investigate whether the reduction of T cells resulted in a decreased expression of Th1 specific markers we stained sections of control (6D) and TAK-779 treated

mice (6E) for IFN- γ . A significant reduction of 98% was observed in the IFN- γ positive area in TAK-779 treated animals compared to control ($0.55\% \pm 0.21\%$ vs. $0.01\% \pm 0.0004\%$, $p=0.013$).

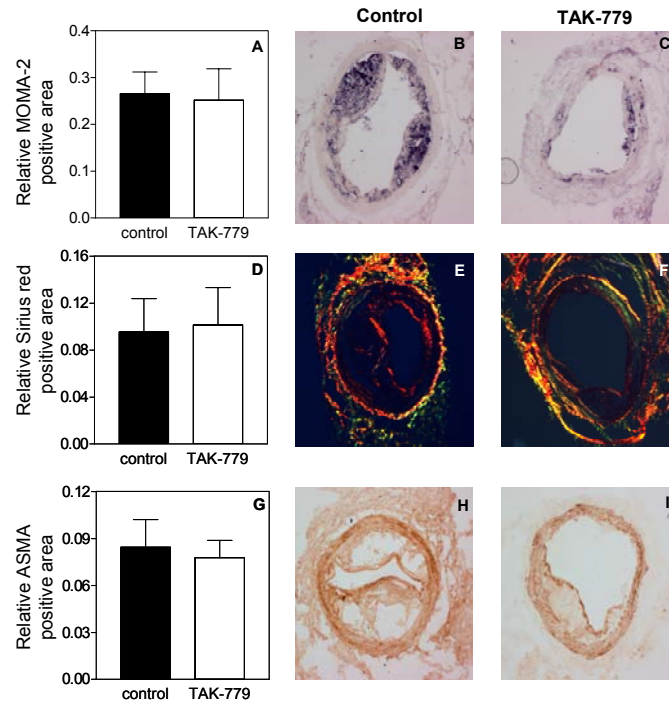


Figure 4. General plaque composition is not altered by TAK-779 treatment. Plaque composition of TAK-779 treated and control treated mice was determined using a macrophage specific antibody (MOMA-2) (figure 4A-C), picosirius red staining to visualize collagen (figure 4D-F) and smooth muscle cell specific staining (α -SM acin) (figure 4G-I). Representative sections are shown for each group. No significant effects could be observed between control and treated animals ($n=10$ per group).

TAK-779 reduces T cell counts in LDLr^{-/-} mice

Both CCR5 and CXCR3 are reported to be involved in T cell migration to inflamed tissue^{3,14}. To investigate whether T cell numbers were affected during atherogenesis in TAK-779 treated mice versus control treated mice, lymphocytes were harvested from blood, from mediastinal lymph nodes, which drain from the aortic arch, and from spleen 6 weeks after the start of Western type diet. Cells were stained for CD4 and CD8, gated for lymphocytes in the FSC/SSC plot, and the percentages of CD4⁺ and CD8⁺ T cells were determined by flow cytometric analysis. Figure 7A shows representative dot plots for control treated mice (upper three panels) and TAK-779 treated mice (lower three panels) on a Western type diet. A vast decrease in the percentage of CD4⁺ T cells in peripheral blood ($21.6 \pm 3.11\%$ vs. $10.46 \pm 1.57\%$, $p=0.02$) and a modest decrease in lymph nodes ($35.3 \pm 2.5\%$ vs. $26.9 \pm 4\%$, $p=0.14$) was observed for the TAK-779 treated group.

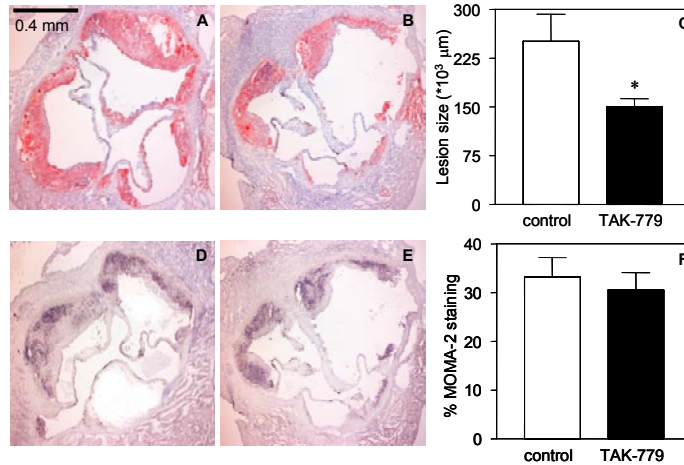


Figure 5. TAK-779 treatment reduces plaque formation in the aortic leaflet area.

Representative photomicrographs of oil red O stained cross sections of the aortic root of control (5A) and TAK-779 treated mice (5B) are shown. A significant reduction in plaque size was found as compared to control ($p=0.001$, $n=8$ per group) (5C). Relative macrophage staining of control (5D) and treated mice (5E) is visualized using a monoclonal antibody specific for macrophages (MOMA-2). No difference in the percentage MOMA positive area is observed between control and treated mice (5F).

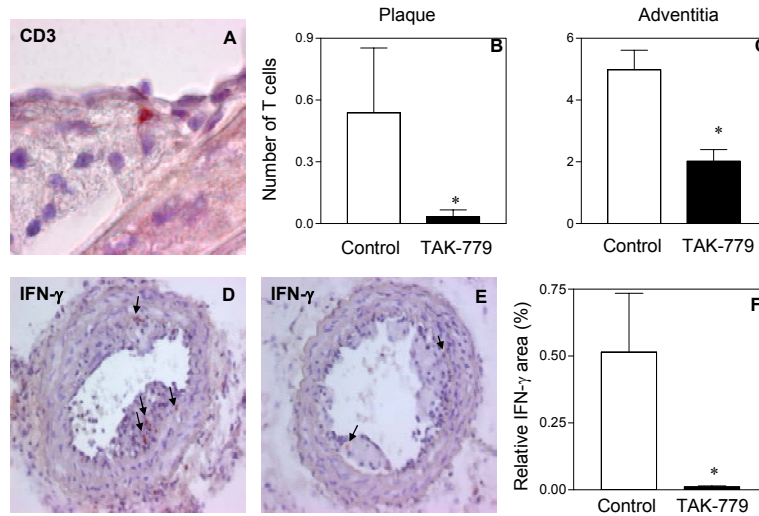


Figure 6. Migration of Th1 cells in TAK-779 treated animals is reduced compared to control treated animals.

Sections of collar induced lesions were stained for T cells using an anti-CD3 antibody (6A). The number of positive cells in both plaque and adventitia was quantified. A significant reduction of the number of CD3 positive cells was observed both in the plaque (6B) as in the adventitia (6C) of TAK-779 treated animals compared to control ($n=10$ per group). Representative sections with IFN- γ staining are shown for control (6D) and treated mice (6E). A significant reduction in the relative IFN- γ area is observed after TAK-779 treatment compared to control (6F).

In contrast, the CD4⁺ population was significantly increased in the spleen of the TAK-779 treated mice (9.6 ± 1.22 % vs. 13.5 ± 0.9 %, $p=0.03$) (figure 7B). An even more pronounced decrease was observed in the CD8⁺ T cell population. In the circulation the percentage CD8⁺ T cells decreased from 10.2 ± 1.8 % in the control group to 3.8 ± 0.7 % in the treated group ($p=0.01$). In the mediastinal lymph nodes draining from the aortic arch, a reduction of 34% in the number of CD8⁺ cells was observed (21.4 ± 0.52 % vs. 14.6 ± 2.0 %, $p=0.02$) (figure 7C).

No significant differences in total white blood cell counts were observed between control and treated mice.

Inflammatory status in the spleen after TAK-779 treatment

After 6 weeks of simultaneous Western type diet feeding and TAK-779 treatment, the mRNA expression of CCR5 and CXCR3 was determined to evaluate the expression of the two chemokine receptors that were specifically blocked by TAK-779 (figure 8). CCR5 expression was 1.9-fold increased in TAK-779 treated mice in comparison with control mice ($p=0.02$), whereas the expression of CXCR3 remained unchanged. The expression of CCR2, a chemokine receptor involved in the migration of macrophages and T cells and also implicated in atherosclerosis, is highly elevated in the spleen upon TAK-779 treatment. The expression of endogenous ligands for these receptors was also evaluated. The splenic mRNA levels for MIP-1 α , RANTES and CXCL10, which bind to CCR5 and CXCR3 respectively, were not affected by TAK-779 treatment, while the expression of monocyte chemoattractant protein-1 (MCP-1), a ligand for CCR2, was significantly upregulated 8.1-fold. We also determined the expression of some cytokines that are important in the Th1/Th2 balance. IL-12, a general Th1 stimulatory interleukin is 2.8-fold upregulated in the spleen of TAK-779 treated mice as compared to control treated mice, while the Th2 interleukin IL-4 is 3.2-fold upregulated. This results in an unaffected Th1/Th2 balance upon TAK-779 treatment, and the observed increase in splenic cytokine expression might be explained by the increased number of CD4⁺ T cells in spleen (figure 8).

Discussion

Infiltration of mononuclear cells into the vessel wall is an important hallmark of atherosclerosis. It has become clear that next to macrophages, T lymphocytes are present in the atherosclerotic plaque and exert a significant role in plaque progression. The majority of these T cells is CD4 positive. Activation of CD4⁺ T cells via MHC class II on antigen presenting cells results in the release of cytokines and the expression of several surface markers. Activated CD4⁺ T cells can be divided in 2 distinct categories based on their phenotype. Th1 cells secrete IFN- γ , IL-2 and TNF- α . Th2 cells produce IL-4, IL-5 and IL-13 leading to antibody production and the elimination of extracellular pathogens.

The HIV entry inhibitor TAK-779 is a CCR5 and CXCR3 antagonist in mice and several studies have shown that TAK-779 reduces autoimmune responses by interfering with the migration of Th1 cells to the inflamed tissue¹⁴. We argue that TAK-779 may therefore also affect atherogenesis in a similar way. As the current treatment for HIV patients is associated with increased atherogenic lipoprotein concentration and accelerated atherosclerosis, an anti-HIV drug that reduces atherosclerosis would be of great importance.

As atherosclerosis is considered to be a Th1 mediated disease^{16,17}, modulation of the activation and migration of Th1 T cells provides an attractive therapeutic possibility.

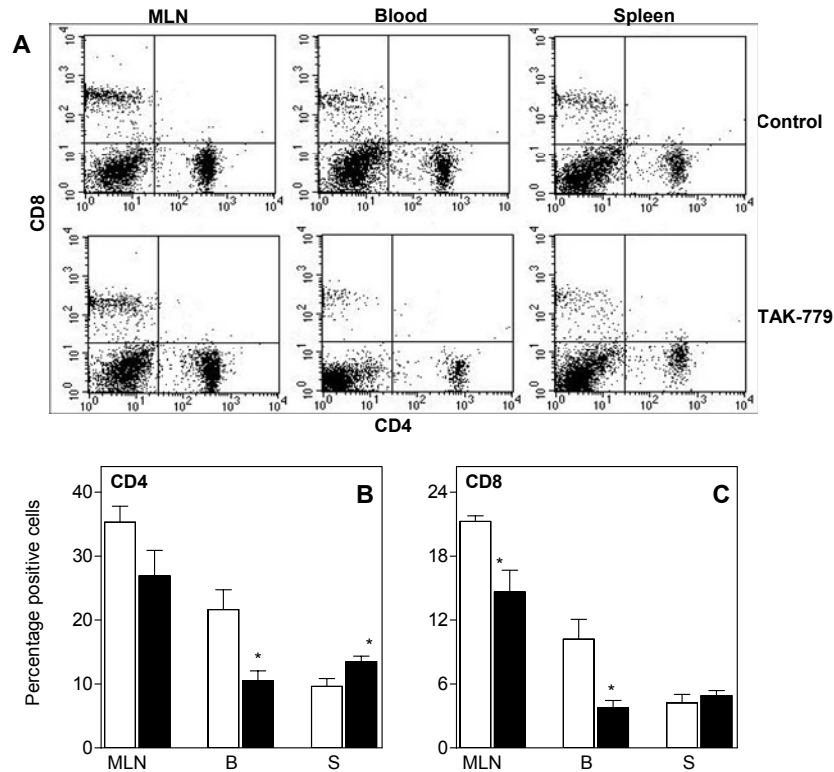


Figure 7. Percentages of CD4 and CD8 positive T cells in mediastinal lymph nodes (MLN), blood and spleen. Mononuclear cell suspensions of draining lymph nodes, spleen and blood were isolated from control mice and mice following a treatment with 150 μ g of TAK-779 in 100 μ l of 5% mannitol injection fluid *s.c.* every other day during 6 weeks. Representative dot plots are shown in figure 7A. Cells were stained for CD4 (7B), and CD8 (7C). Results represent the mean percentage of positive cells $\pm$ SEM from 5 individual mice (**= $P < 0.005$, *= $P < 0.05$, Students T test). A decrease is seen in the percentage CD4 and CD8 positive T cells in DLN and blood in the TAK-779 treated group (White bars) compared to control (Black bars). In the spleen, the percentage of CD4 positive cells is increased in the treated group.

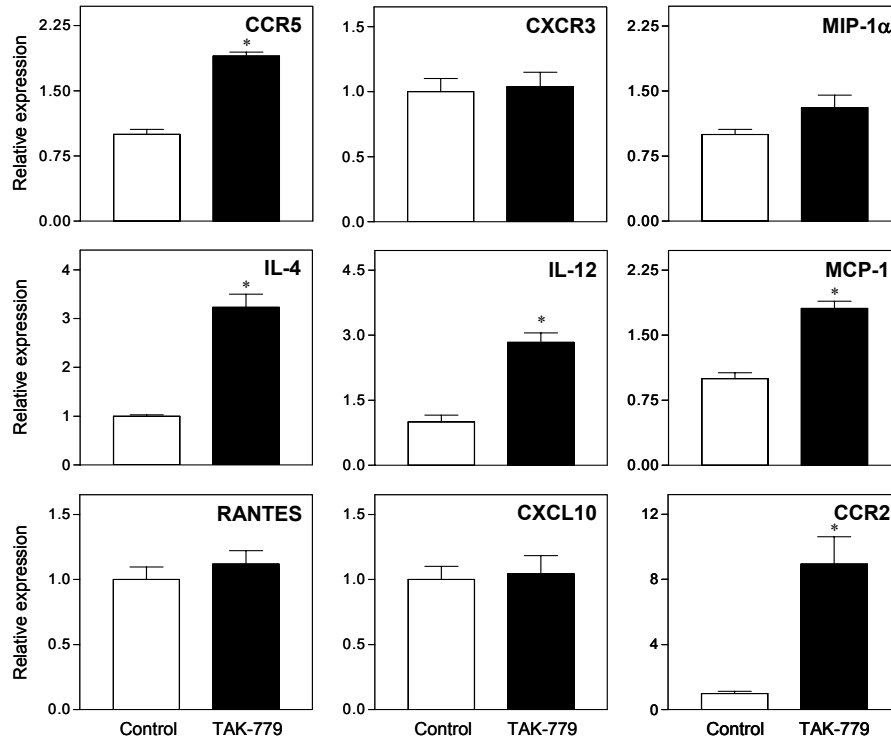


Figure 8. Spleen mRNA expression of inflammatory markers after TAK-779 treatment. mRNA expression of different genes in the spleen of TAK-779 treated mice (8 weeks) is expressed relative to 36B4 and HPRT, and subsequently related to the expression in control mice. White bars represent control mice, black bars represent TAK-779 treated mice. An unpaired Student *t* test was applied to test if mRNA levels were significantly different from the mRNA levels in chow fed animals (n=10 per group) (**P*<0.05).

CCR5 and CXCR3 are chemokine receptors that are both primarily expressed on Th1 T cells¹⁵. A putative role for CCR5 and CXCR3 in atherogenesis is implicated by studies in which a mutation in the CCR5 sequence ($\Delta 32$ mutation) was found to be associated with a reduced incidence in myocardial infarction and severe coronary artery disease^{6,7}. Furthermore, antagonizing CCR5 using met-RANTES also resulted in reduced atherosclerotic lesion formation¹⁰.

In the present study we firstly examined the expression of CCR5 and CXCR3 in the spleen of LDLr^{-/-} mice during Western type diet feeding. It appeared that CXCR3 was not differentially regulated in the spleen during the first 6 weeks of atherogenic diet, whereas CCR5 showed a significant upregulation in this period. The known ligands of CCR5, RANTES and MIP-1 α have been detected in atherosclerotic plaques, and these molecules were also upregulated at 6 weeks of Western type diet feeding in the spleen⁸. No distinct regulatory pattern could be observed for the ligands of CXCR3, interferon gamma inducible protein-10 (IP-10) and CXCL10. These findings suggest that CCR5 may have a more

prominent role in the response of the immune system towards elevated cholesterol levels and atherogenesis.

In our studies the synthetic low molecular weight CCR5/CXCR3 antagonist TAK-779 was used to assess the role of these receptors in the process of atherosclerotic lesion formation. Treatment with this potential HIV entry inhibitor appeared to reduce plaque formation in the carotid artery with 68% and lesion formation in the aortic root with 40%. These findings are in line with a study by Veillard *et al.*, who used met-RANTES to antagonize CCR5 and CCR-1, and observed a decrease in lesion formation as a result of reduced infiltration of mononuclear cells into the lesion¹⁰. In contrast with these observations, Kuziel *et al.* have published that disruption of the mouse CCR5 gene has no effect on early lesion formation in apoE deficient mice²⁸. As these mice lack CCR5 throughout their development, it may be that impaired T cell migration due to CCR5 disruption is counterbalanced by a compensatory chemokine receptor mediated mechanism such as CCR-2/MCP-1.

The expression of CCR5 and CXCR3 has been well characterized on CD4⁺ T cells and both chemokine receptors are preferentially expressed on the Th1 cell subset^{25,26}. Limited expression of CCR5 is also reported on CD8⁺ cells, human vascular smooth muscle cells and macrophages^{30,31}. Our data show that the relative macrophage content of the atherosclerotic plaques did not differ between TAK-779 treated and control treated mice. This implies that migration of macrophages into the intima is not impaired by CCR5/CXCR3 blockade by TAK-779. Therefore the decreased lesion formation is more likely to be explained by the diminished migration of T cells from the spleen towards the sites of atherogenesis. This is further supported by the finding that the number of CD3 positive T cells in the plaque as well as in the adventitia is significantly decreased in TAK-779 treated animals. We also observed that the relative T cell counts in the blood and draining lymph nodes for both CD4⁺ and CD8⁺ T cells are significantly decreased in TAK-779 treated animals. Simultaneously, the percentage of CD4⁺ T cells in the spleen is increased, suggesting that these T cells are retained in the spleen upon TAK-779 treatment.

Veillard *et al.* described that antagonizing CCR1 and CCR5 with met-RANTES results in decreased macrophage and T cell influx into the atherosclerotic lesion, in contrast to antagonizing CCR5/CXCR3 with TAK-779 which only affects T cell influx. This difference may be explained by the fact that met-RANTES antagonizes CCR1 which is expressed at high levels on macrophages, in contrast to CCR5 and CXCR3. TAK-779 is therefore more selective in antagonizing Th1 T cells, leaving macrophage recruitment mostly intact. We furthermore used an *in vitro* setup to test whether TAK-779 had any effect on macrophage adhesion to LPS stimulated endothelial cells. No dose-effect relationship for TAK-779 incubation of macrophages on endothelial adhesion was observed (data not shown). This further strengthens our findings that there is no major effect on macrophage adhesion and infiltration in TAK-779 treated animals.

It has been shown that local T cells secrete cytokines, which in turn activate and attract other inflammatory cells and therefore stimulate the inflammatory process. As CCR5 and CXCR3 are most strongly expressed on Th1 cells it is expected that mice treated with TAK-779 show smaller amounts of Th1 cytokines in the atherosclerotic plaque. We stained atherosclerotic plaque sections of control and treated mice for IFN- γ , a Th1 marker. In plaques of treated animals, the relative IFN- γ area was decreased by 98%, indicating that IFN- γ production by Th1 cells was almost completely absent upon TAK-779 treatment.

Our data show that TAK-779 blockade resulted in a marked upregulation of CCR2/MCP-1 in the spleen, which coincides with an upregulation of CCR5 in the spleen in comparison to control mice. This may be explained by the fact that CCR2 and CCR5 coexist in the same lipid raft domain and both receptors share a migratory function²⁹. Inhibition of CCR5 is then compensated by an upregulation of CCR2 to sustain migratory capacity.

Baba *et al.* have shown that TAK-779 has some affinity for CCR2b in humans, but this is 100 fold lower in comparison with CCR5¹¹. In addition, TAK-779 is not able to bind human CXCR3. Therefore it is likely that TAK-779 exerts its action by inhibiting CCR5 function in humans. We observed similar effects in our study in mice, as the expression of CXCR3 in spleen of mice treated with TAK-779 is not increased, in contrast to the expression of CCR5, thus implying a dominant role for CCR5 as target of TAK-779.

The increase in individual expression of the different chemokine receptors, combined with the unaltered Th1/Th2 ratio supports the theory that TAK-779 targets CCR5 and impairs T cell migration to the site of action, the atherosclerotic plaque, and does not alter the general inflammatory status of the animals.

Combination therapy which is commonly used to treat HIV patients has been shown to lead to elevated serum levels of the atherogenic lipoproteins LDL and VLDL, which result in a higher incidence in cardiovascular disease. We now show that TAK-779, a novel HIV entry inhibitor, does not elevate serum total cholesterol levels or triglyceride levels. More important, no increase in atherogenic lipoprotein levels was observed in the blood of TAK-779 treated animals, and a decrease was observed in atherosclerotic lesion formation. This is in contrast with patients and animal models treated with protease inhibitors.

In summary, the use of HIV entry inhibitors in the long-term treatment of HIV would be preferential over the now used combination therapy. It achieves long-term suppression of the virus and as shown in this study, retained atherosclerotic lesion formation by blocking the influx of Th1 cells in the atherosclerotic plaque. This finding is primarily beneficial for young HIV patients, as they face a lifelong treatment and are currently confronted with the severe side effect of heart disease.

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Chapter 5

The CXCR3 Antagonist NBI-74330 Attenuates Atherosclerotic Plaque Formation In LDL Receptor Deficient Mice

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Abstract

The chemokine receptor CXCR3 is implicated in migration of leukocytes to sites of inflammation. Antagonizing CXCR3 could therefore be a potential strategy to inhibit inflammation induced leukocyte migration, and subsequently reduce atherosclerotic lesion formation. In this study we used the CXCR3 specific antagonist NBI-74330 to block CXCR3 mediated signaling in thioglycolate induced peritonitis and diet induced atherosclerosis. Antagonizing CXCR3 with NBI-74330 resulted in a significant reduction in CD4⁺ T cell and macrophage migration to the peritoneal cavity. Ex-vivo migration studies with cells isolated from the peritoneal cavity after NBI-74330 treatment showed that this reduction in migration was totally CXCR3 dependent. Atherosclerotic lesion formation in both the aortic valve leaflet area as well as the total aorta was significantly inhibited in NBI-74330 treated mice. Lymph nodes draining from the aortic arch were significantly smaller in treated mice and showed enrichment in regulatory- and less activated T cells. This study shows for the first time that treatment with a CXCR3 antagonist results in attenuating atherosclerotic lesion formation, not only by blocking direct migration of effector cells from the circulation to the atherosclerotic plaque, but also by beneficially modulating the inflammatory response in lymph nodes draining from the atherosclerotic lesion.

Introduction

Atherosclerosis is a progressive multi-factorial disease of the larger arteries characterized by cholesterol deposition, leukocyte influx, cell death and fibrosis. In recent years, it has become increasingly clear that next to a lipid storage disorder, atherosclerosis can be considered as an ongoing inflammatory process within the vasculature^{1,2}. Migration of leukocytes into the vessel wall is an essential step in atherosclerotic lesion formation and progression and chemokines are defined as key regulators of this process^{3,4}.

Chemokine receptors are trans-membrane spanning, G-protein-coupled receptors which are classified by the position of the N-terminal cysteins (CC, CXC, C, CXXXC). They play an important role in the recruitment, migration and trafficking of immune cells to sites of inflammation. An increasing amount of evidence underscores the relevance of chemokines in the pathogenesis of atherosclerosis⁵⁻¹⁰.

The chemokine receptor CXCR3 is expressed on different types of leukocytes, including T cells, B cells, natural killer (T) cells and monocytes^{11, 12, 13, 14}. Its expression is highly induced upon CD4 T cell activation and is preferentially expressed on activated auto-reactive T cells¹⁵. Antibody-mediated blockade of CXCR3 results in a decreased recruitment of Th1 cells to sites of inflammation¹⁶. CXCR3 has 3 known ligands; MIG (monokine induced by IFN- γ (CXCL9)), IP-10 (IFN- γ -inducible protein, (CXCL10)) and ITAC (IFN- γ -inducible T-cell α -chemoattractant (CXCL11)). The expression of these ligands is highly inducible by interferon- γ (IFN- γ) and associated with several inflammatory disorders.

Recent publications point towards a prominent role for CXCR3 mediated migration of inflammatory cells in atherosclerosis. Human atherosclerotic lesions express high amounts of all three CXCR3 ligands¹⁷. Targeted deletion of CXCR3 in ApoE deficient (ApoE^{-/-}) mice resulted in decreased lesion formation in the abdominal aorta⁵. Furthermore, deletion of the CXCR3 ligand CXCL10 in ApoE^{-/-} mice resulted in decreased lesion formation by reducing the migration of CD4 effector T cells to the atherosclerotic plaque¹⁸.

Blockade of CXCR3 mediated migration could therefore provide a potential strategy to reduce leukocyte migration to sites of inflammation and in this way attenuate atherosclerotic lesion formation. In this study, we describe the highly specific CXCR3 antagonist NBI-74330 and its inhibitory effects on cell migration and diet induced atherosclerosis in LDL receptor deficient (LDLr^{-/-}) mice.

Material and methods

Determination of appropriate in vivo dosage of NBI-74330

The quinazolinone-derived CXCR3 antagonist NBI-74330 was synthesized as described by Medina *et al* (WO02083143, 2002 oct 24). Mice were treated with NBI-74330 in 0.1% Na Docusate in 0.5% 400Cp Methylcellulose and serum concentration at indicated time points were determined using LC-MS-MS. Serum was subjected to protein precipitation prior to analysis. HPLC mobile phase consisted of H₂O with 0.1% (v/v) formic acid and Acetonitrile with 0.1% (v/v) formic acid using a gradient profile.

Peritonitis induced migration and mobility assay

LDLr^{-/-} mice were treated with a subcutaneous injection of 100mg/kg CXCR3 antagonist NBI-74330 in 100 µl 0.1% Na Doc in 0.5% 400Cp Methylcellulose (n=6) or vehicle (n=5) for 6 days. At day 2, all mice were injected intra-peritoneally with sterile 3% (w/v) Brewers thioglycolate solution. Peritoneal cells were isolated by peritoneal cavity lavage with PBS and counted and phenotyped by flow cytometry at day 6. Migration capacity of the isolated peritoneal cells in response to CXCL10 (100ng/ml), and the chemotactic peptide FMLP (1µM) was quantified using a chemokinesis assay¹⁹.

Atherosclerosis experiments

Female LDLr^{-/-} mice, 10 weeks old (n=8-12 per group), were fed a Western-type diet containing 0.25 % cholesterol and 15% cocoa butter two weeks before collar placement²⁰. Mice were treated with a subcutaneous injection of 100mg/kg CXCR3 antagonist NBI-74330 in 100 µl 0.1% Na Doc in 0.5% 400Cp Methylcellulose every day during the entire experiment. After 8 weeks of Western-type diet and treatment, the mice were sacrificed and organs were harvested for histology, FACS and RNA isolation. Blood samples were collected by tail bleeding from non-fasted animals and concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures.

Histological analysis

Cryostat sections of the aortic root (10 µm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:50). Goat anti-rat IgG-AP (dilution 1:100) was used as secondary antibody and NBT-BCIP as enzyme substrates. Masson trichrome staining (Sigma Diagnostics) was used to visualize collagen (blue staining). TGFβ was stained with a polyclonal rabbit antibody (Santa Cruz, USA) and biotinylated goat anti rabbit (Dako cytomatics, The Netherlands) was used

as a secondary antibody with Nova Red as enzyme substrate (Vector Laboratories).

Real time PCR assays

Total RNA was isolated from aortic arch and collar induced atherosclerotic plaques and was DNase treated. Quantitative gene expression analysis was performed on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (Online table I) were designed using Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Flow cytometry

Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada). Cell suspensions from spleen, blood, lymph nodes draining from the aortic arch and peritoneal cavity were stained for surface markers (0.2 μ g Ab/300,000 cells) and subsequently subjected to flow cytometric analysis (FACS). Antibodies were purchased from Immunosource (Belgium). All data were acquired on a FACSCalibur and were analyzed with CELLQuest software (BD Biosciences).

Statistical analysis

Values are expressed as mean $\pm$ SEM unless indicated otherwise. Two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was used to compare not normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

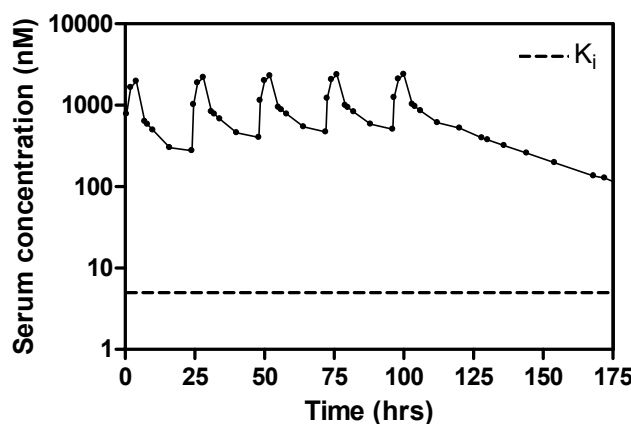


Figure 1: Daily *s.c.* injections of 100 mg/kg NBI-74330 result in serum levels sufficient to fully antagonize CXCR3 *in vivo*. A formulation of NBI-74330 was constructed using 1% Na Doc in 0.5% 400Cp Methylcellulose and the mice ($n=3$) were treated with 100 mg/kg compound every day for 5 days. Serum levels were determined at indicated time points using LC-MS-MS. Dotted line indicates $\sim K_i$.

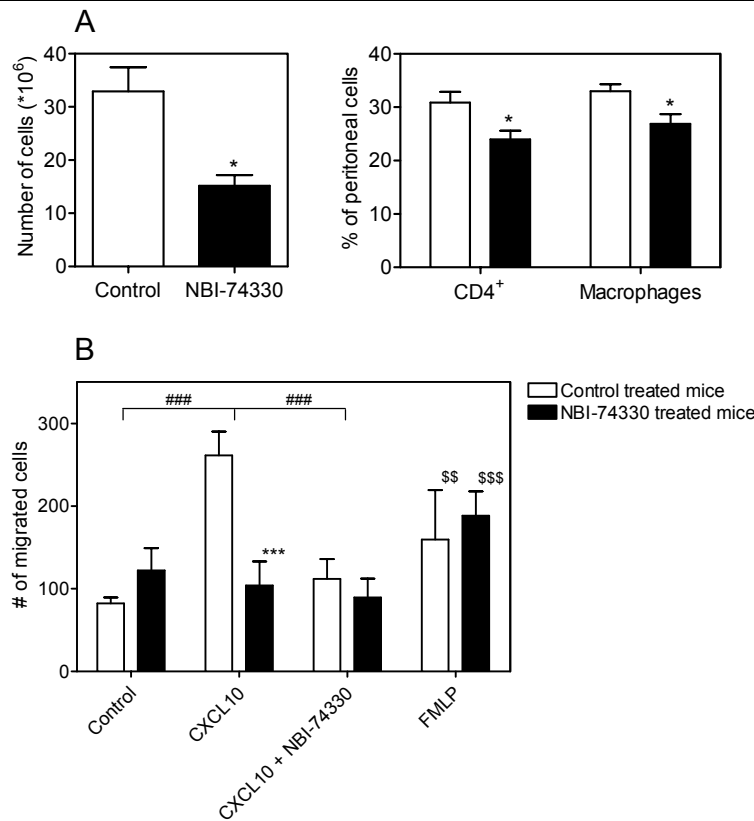


Figure 2: Macrophage and T cell migration to thioglycollate induced peritonitis is inhibited by NBI-74330 treatment. **A:** Female LDLr^{-/-} mice were treated with a subcutaneous injection of 100mg/kg CXCR3 antagonist NBI-74330 in 100 μ l 1% Na Doc in 0.5% 400Cp Methylcellulose (n=6) or vehicle (n=5) for 6 days. At day 2, all mice were injected intra-peritoneally with sterile 3% (w/v) Brewers thioglycollate solution. Peritoneal cells were isolated by peritoneal cavity lavage with PBS and counted and phenotyped by FACS at day 6. **A:** recruitment of cells to the peritoneal cavity was reduced by 56% after NBI-74330 treatment. The percentage of CD4⁺ T cells and macrophages (F4/80⁺, Gr-1^{low}) present in the peritoneal cavity was significantly decreased in mice treated with NBI-74330 compared to control treated mice. (Error bars represent SEM, *: p< 0.05, **: p<0.01)

B: 5000 isolated peritoneal cells (triplicate per mouse/ situation) were seeded on fibrinogen coated 96-wells and left to adhere at a nearly upright position. Migration in response to CXCL10 and FMLP (positive control) was then assessed at a 15° tilted position by counting the number of cells that migrated across a defined threshold. (Error bars represent SD, ***: p<0.001 compared to cells from control mice cultured with X100ng/ml CXCL10, ###: p<0.001 compared to cells from control mice cultured with 100 ng/ml CXCL10, \$\$: p<0.01, \$\$\$p<0.001 FMLP versus control stimulated cells).

Results

In vivo use of NBI-74330

NBI-74330 is a small molecular high affinity CXCR3 antagonist that is a potent inhibitor of CXCR3 ligand binding with a K_i in the low nanomolar range (~8nM). *In vitro* data have shown that it inhibits CXCL10 and ITAC induced calcium mobilization at concentrations below 10 nM²¹. A formulation of NBI-74330 was constructed using 1% Na Docusate in 0.5% 400Cp Methylcellulose.

We tested the optimal dosing of this formulation *in vivo* and found that daily dosage of 100mg/kg via subcutaneous injections resulted in serum concentrations of approximately 1 μ M (figure 1). This concentration is sufficient to fully block the CXCR3 receptor *in vivo*.

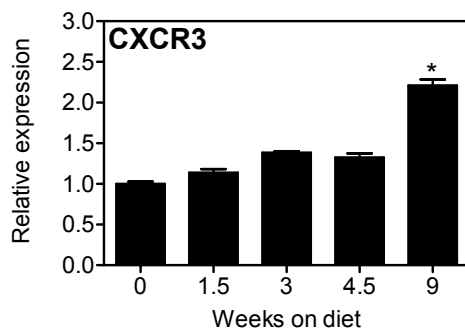


Figure 3: mRNA expression of CXCR3 is significantly upregulated in the aortic arch of LDLr^{-/-} mice on Western typed diet feeding. mRNA was isolated from the aortic arch of LDLr^{-/-} mice using the GTC method and expression of CXCR3 is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. (*p < 0.05, n= 6 per time point).

The CXCR3 antagonist NBI-74330 inhibits CD4 T cell and macrophage migration during thioglycolate induced peritonitis

To show the *in vivo* capacity of NBI-74330 to antagonize CXCR3 mediated cell migration, we used a peritonitis model. In this model, leukocytes migrate to the peritoneal cavity in response to a single intraperitoneal thioglycolate challenge. Female LDLr^{-/-} mice were treated with NBI-74330 or control for 6 days. At day 2, all mice were injected intra-peritoneally with sterile 3% (w/v) Brewers thioglycolate solution and 5 days later the number of cells present in the peritoneum was quantified. A significant 56% reduction in leukocyte recruitment could be observed after 5 days in NBI-74330 treated mice compared to control treated mice (Fig. 2A, p=0.01). FACS analysis of the isolated peritoneal cells showed that this reduction was mainly due to reduced migration of CD4 T cells and macrophages (Fig 2A). To investigate the capacity of the isolated peritoneal cells from control and NBI-74330 treated mice to migrate in response to the CXCR3 ligand CXCL10 we performed an *ex vivo* mobility study. Isolated peritoneal cells from control and treated mice were allowed to accumulate at the lower end of a 96-well culture plate and migration in response to 100ng/ml CXCL10 and FMLP (positive control) was then assessed. Results are shown in figure 2B.

Peritoneal cells isolated from mice treated with vehicle for 6 days had a clear migratory response when exposed to 100 ng/ml CXCL10, and this effect could be completely reversed by the *in vitro* addition of the CXCR3 antagonist NBI-74330 to the culture medium. Cells isolated from mice treated with CXCR3 antagonist *in vivo* during the thioglycolate challenge were not able to respond to CXCL10. Their capability to migrate while exposed to the general chemotactic peptide FMLP was not different compared to control treated mice.

These findings clearly show that the reduced migration towards thioglycollate induced peritonitis by NBI-74330 treatment is the result of an effective *in vivo* blockade of CXCR3.

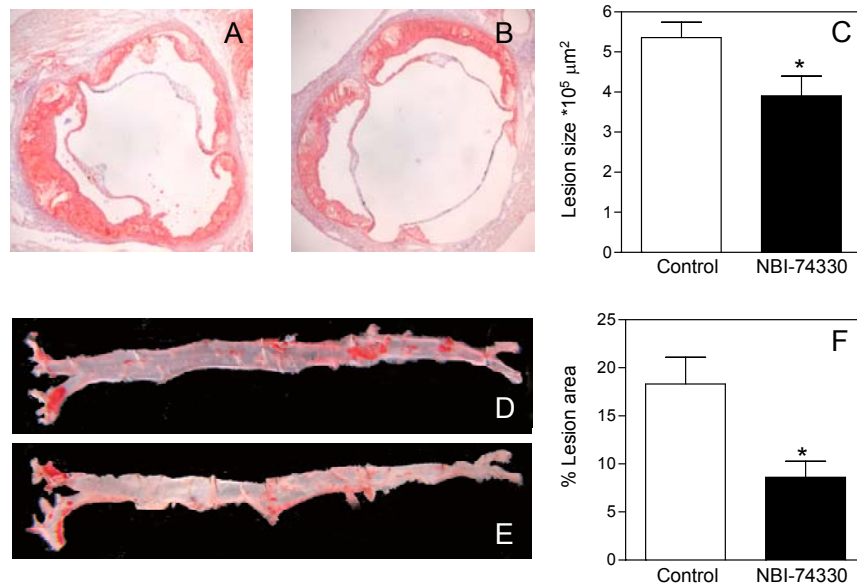


Figure 4: Atherosclerotic lesion formation is significantly inhibited in mice treated with NBI-74330

Representative photomicrographs of oil red O stained cross sections of the aortic root of control treated mice (A) and NBI-74330 treated mice (B) are shown. A significant reduction in plaque size was found as compared to control ($p=0.03$, $n \geq 8$ per group) (C).

Relative plaque area was quantified in *en face* pinned aortas. Representative pictures are shown for control (D) and NBI-74330 treated mice (E). Lesion formation is significantly inhibited by NBI-74330 treatment by 53% ($p=0.01$).

Atherosclerotic lesion formation in LDLr^{-/-} mice is attenuated by antagonizing CXCR3

We use LDL receptor deficient mice on a Western type diet as a model for atherosclerosis and the mRNA expression of CXCR3 during lesion formation was monitored in the aortic arch at different stages of western type diet feeding. A significant increase was observed in CXCR3 mRNA expression after 9 weeks of diet, indicating initial influx of CXCR3 expressing leukocytes (Figure 3). This showed that CXCR3 could be involved in the atherosclerotic process in our model. We then assessed the effect of NBI-74330 treatment on atherosclerotic lesion formation.

Female LDLr^{-/-} mice were fed a Western-type diet and received daily subcutaneous injections of 100mg/kg NBI-74330 or vehicle. No difference was observed in serum cholesterol and triglyceride levels between control and treated animals (data not shown). Figure 4 shows representative sections of control treated (figure 4A) and NBI-74330 treated (figure 4B) mice.

Atherosclerotic lesion formation in the aortic valve leaflet area was significantly inhibited in mice treated with NBI-74330 ($536 \times 10^3 \mu\text{m}^2$ vs. $391 \times 10^3 \mu\text{m}^2$, $p < 0.05$). Relative macrophage staining (MOMA-2) was comparable in plaques from control and treated animals, as well as the relative collagen content as determined by Masson trichrome staining (Figure 5).

Next to lesion formation in the aortic valve leaflets, we quantified the percentage of lesion area in the aorta of control and NBI-74330 treated mice by en face pinned out aortas stained with oil red O. Representative pictures of control (4D) and NBI-74330 treated aortas (4E) are shown. NBI-74330 treatment resulted in a 53 % reduction in lesion formation compared to control treated mice ($18 \pm 2\%$ vs. $8 \pm 2\%$, $p = 0.01$).

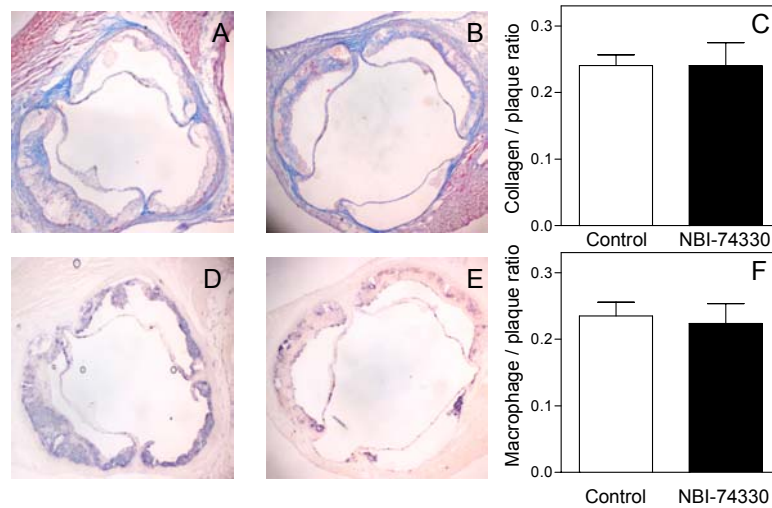


Figure 5: NBI-74330 treatment has no effect on relative macrophage and collagen content of atherosclerotic plaques. Plaque composition of NBI-74330 treated and control treated mice was determined using a macrophage specific antibody (MOMA-2) (figure 4A-C), and Masson Trichrome staining to visualize collagen (figure 4D-F). Representative sections are shown for each group. No significant effects could be observed between control and treated animals ($n \geq 8$ per group).

CXCR3 antagonist NBI-74330 treated mice have smaller lymph nodes draining from the aortic arch and a beneficial regulatory/effector T cell balance during atherogenesis

Recent publications suggest a role for CXCR3 in the migration of effector cells towards the site of inflammation^{18,22,23}. We isolated the lymph nodes draining from the aortic arch of $\text{LDLr}^{-/-}$ mice after 8 weeks of Western type diet and subsequent treatment with either control or the CXCR3 antagonist NBI-74330. The size of the isolated lymph nodes was assessed by quantifying the number of cells. Treatment with NBI-74330 resulted in a 64% reduction of cell numbers in lymph nodes draining from the aortic arch (Fig. 6). Characterization of the isolated cell population was performed using FACS.

A significant increase was observed in the percentage CD4⁺CD25⁺ regulatory T cells in NBI-74330 treated mice ($p<0.05$). This increase in regulatory T cells was accompanied by an increase in the expression of CD62L on CD4 cells ($p<0.001$), suggesting a reduction in the activation state of effector T cells. These effects were restricted to the lymph nodes draining from the aortic arch, as no such effects were observed in spleen or the circulating white blood cell population.

Plaques from mice treated with NBI-74330 express more genes associated with regulatory T cells

To determine whether the local reduction in effector cells and the increase in regulatory T cell phenotype resulted in a resembling cytokine profile inside the atherosclerotic plaque we stained for the regulatory T cell cytokine TGF β . Representative slides are shown for control (7A) and NBI-74330 treated (7B) mice. A significant increase was observed in the relative expression of TGF β in the plaque of mice treated with NBI-74330 compared to control.

We performed QPCR on atherosclerotic plaques isolated from the carotid artery after collar induced atherosclerosis from both control and treated mice and determined the expression of the regulatory T cell associated genes FOXP3, CD25 and CTLA-4. The expression of FOXP3 was 2.5 fold upregulated ($p<0.05$), and the expression of CTLA-4 was 6 fold upregulated ($p<0.05$) compared to expression in control treated mice. The expression of CD25 showed a trend towards an increased expression, but this was not significant.

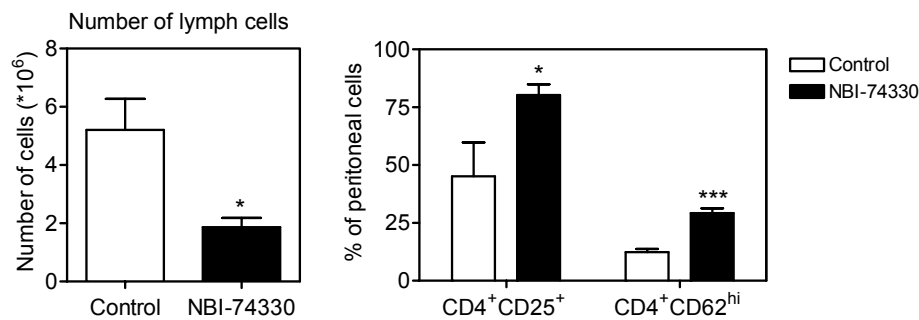


Figure 6: Draining lymph nodes from the aortic arch of NBI-74330 treated mice are smaller in size and contain less activated and more regulatory T cells

Draining lymph nodes were isolated from control and NBI-74330 treated mice after 8 weeks of western type diet feeding. Single cell suspensions were prepared and total cell number was quantified. NBI-74330 treatment resulted in a 64% decrease in cell number compared to control. (Error bars represent SEM, *: $p<0.05$). FACS was used to assess the relative amount of CD4⁺CD25⁺ regulatory cells, and the % CD4⁺CD62L^{hi} effector cells. (Error bars represent SEM, n=6 per group, *: $p<0.05$, ***: $p<0.001$)

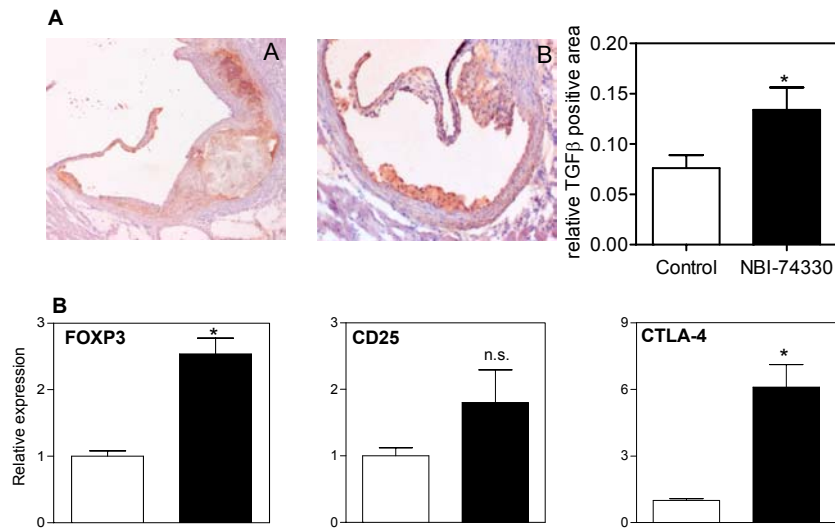


Figure 7: Atherosclerotic plaques from NBI-74330 treated mice express increased levels of regulatory T cell associated molecules

A: Sections of the aortic leaflet area were stained for TGFβ using anti TGFβ antibodies. We then quantified the TGFβ positive area relative to total plaque area. Treatment with NBI-74330 resulted in a significant increase in relative TGFβ positive area (red staining).

B: mRNA expression of different genes isolated from collar induced atherosclerotic plaques (8 weeks) is expressed relative to 36B4 and HPRT, and subsequently related to the expression in control mice. White bars represent control mice; black bars represent NBI-74330 treated mice. An unpaired Student *t* test was applied to test if mRNA levels were significantly different from the mRNA levels in chow fed animals (n=5-10 per group) (**P*<0.05).

Discussion

Evidence is building that CXCR3 mediated cell migration plays an important role in several (auto-) immune diseases²⁴⁻²⁷. CXCR3 positive cells enter the site of inflammation followed by a local upregulation of CXCR3 expression. These CXCR3 positive cells are attracted by the 3 known ligands, CXCL9, 10 and 11, and it was shown that these ligands are highly expressed in atherosclerotic lesions¹⁷.

In this study we used the highly specific CXCR3 antagonist NBI-74330 to block CXCR3 mediated signalling and migration. Firstly, we showed the *in vivo* capacity of this compound to reduce cell migration to a site of inflammation using a thioglycolate induced peritonitis model. Total leukocyte migration to the peritoneal cavity was reduced by 56% upon daily treatment with NBI-74330. The reduction in total cell migration was the consequence of a reduction in the number of CD4⁺ T cells and macrophages to the peritoneal cavity. The observed decrease in migration was the result of selective blockage of CXCR3, as shown by a subsequent *ex vivo* mobility assay. Peritoneal cells isolated from control mice were able to migrate in response to both CXCL10 and FMLP. Cells isolated

from mice that received NBI-74330 during the induction of peritonitis were not responsive to CXCL10, but were still capable of migrating in response to an FMLP stimulus.

After we established the *in vivo* efficacy of the compound, we used LDL receptor deficient mice to test the effect of antagonizing CXCR3 on atherosclerotic lesion formation.

We show that the expression of CXCR3 is upregulated in the aortic arch of LDLr^{-/-} mice fed a western type diet for 9 weeks. This indicates that CXCR3 positive leukocytes migrate to the developing atherosclerotic plaque. It was shown that CXCR3 deficient mice on an ApoE background show reduced lesion burden compared to control ApoE mice. In addition, mice lacking CXCL10, the main ligand for CXCR3, show decreased atherogenesis compared to control ApoE mice. These are interesting observations, clearly suggesting that CXCR3 is involved in the disease initiation and progression of atherosclerosis in ApoE deficient mice. However, these mice lack the expression of these proteins throughout their development and compensating mechanisms to overcome this deficiency may have taken place.

In this study, we use the compound NBI-74330, a highly specific low molecular weight CXCR3 antagonist, to investigate the effect of antagonizing CXCR3 in a diet induced model for atherosclerosis. Treatment with NBI-74330 resulted in a significant decrease in atherosclerotic lesion formation at the aortic valve leaflet area as well as the total aorta. We observed a more pronounced decrease in lesion size in the aorta compared to the valve leaflet area. In their study with CXCR3/apoE double deficient animals, Veillard *et al.* also showed a difference in lesion formation between these two sites, since they observed a significant decrease in the degree of lesion formation in the descending aorta was observed, but no effect in the aortic sinus. They suggest a more prominent role for CXCR3 in the initial stages of lesion formation. Our findings strengthen this idea as we had quite large and advanced lesions in the valves, while the lesion burden in the aorta was more moderate.

As shown in literature, CXCR3 mediates the migration of effector cells to the site of inflammation. Absence of CXCR3 or its main ligand CXCL10 in mice on an ApoE background resulted in an induction of regulatory T cells markers within the atherosclerotic plaque but not in lymph nodes or in the circulation.

Our experiments however clearly show that administration of a pharmacologically active CXCR3 antagonist results in smaller lymph nodes draining from the aortic arch compared to control treated mice. Next to this, the activation state of T cells is decreased and regulatory phenotype is enhanced in these lymph nodes of NBI-74330 treated mice.

When LDLr^{-/-} mice are fed a Western type diet, the lymph nodes draining from the aortic arch increase in size due to locally induced inflammatory signals. Administering a CXCR3 antagonist clearly reduces the migration of leukocytes to the lymphatic sites draining to the site of inflammation. This indicates that not only direct migration of effector cells from the circulation to the atherosclerotic plaque is inhibited, but that also migration to the draining lymph system is beneficially modulated. Possibly CXCL9 and 11 are involved in this process, because no increase in regulatory T cells was observed in lymph cells isolated from CXCL10 deficient mice. The amount of TGFβ in plaques from mice treated with NBI-74330 is significantly increased. Several mechanisms could lead to this finding. We observed that NBI-74330 treated mice have an increased number of CD4⁺CD25⁺ positive regulatory T cells in the lymph nodes draining directly from the aortic arch²⁸. Next to this treated mice have an increased expression of the regulatory T cell markers FOXP3, CTLA-4 and CD25 within the atherosclerotic plaque. This indicates that regulatory T cells are the likely source of the observed increase in (surface bound) TGFβ expression within the atherosclerotic plaque²⁹⁻³⁴.

In general, we hypothesize that the observed reduction in lesion formation and the accompanying induction in regulatory T cell phenotype is a result of a reduction in migration of effector cells both from the circulation to the atherosclerotic plaque and to the locally draining lymph nodes. This will in turn lead to a relative induction of anti-inflammatory and regulatory cells. Eventually, due to the continuing exposure to antigens such as oxLDL or heat shock proteins, the ongoing attraction of leukocytes via other pathways than CXCR3 will facilitate the process of lesion formation. The blockade of CXCR3 thus provides a “lag-time” in this response.

It is clear that selective blockade of CXCR3 migration *in vivo* using NBI-74330 provides an attractive way to beneficially balance the immune response in an auto-inflammatory situation such as atherosclerosis. A possible drawback of interfering with CXCR3 mediated migration could be a potentially hampered immune response against invading pathogens. For example, lung infection with *B. Bronchiseptica* results in strong upregulation of CXCR3 ligands and influx of CXCR3⁺ cells. However, CXCR3 deficient mice do not show increased mortality to this pathogen. Furthermore, Chackavarty *et al.* have shown that CXCR3 deficient mice are more resistant to *Mycobacterium tuberculosis* infection³⁵. CXCR3 is also associated with cellular influx into CMV infected liver. Infection of the liver with CMV attracts CXCR3⁺ CD8⁺ cells that contribute to the protective response to the virus but these cells are not exclusive required for its clearance³⁶. Based on these observations treatment with a selective CXCR3 antagonist is unlikely to result in severe infections with pathogens.

Small molecular antagonists are the preferential and most widely used drugs and have clear advantages over protein or antibody formulations. We conclude that

this study shows for the first time that a small molecular CXCR3 antagonist inhibits lesion formation in an animal model for atherosclerosis. This study therefore provides evidence that CXCR3 antagonists can be a possible new therapeutic strategy to counteract the development of atherosclerosis or other (auto)-immune diseases.

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Chapter 6

Vaccination Against CD99 Inhibits Atherogenesis in LDL Receptor Deficient Mice

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Abstract

Murine CD99 was recently found to be expressed on leukocytes and endothelial cells where it is concentrated at inter-endothelial contacts. Blockade of CD99 by specific antibodies inhibits leukocyte extravasation to inflamed sites in vivo.

We constructed a DNA vaccine against CD99 by cloning the extracellular domain of murine CD99 into pcDNA3. Vaccination was performed by oral administration of attenuated *Salmonella typhimurium* transformed with pcDNA3-CD99. This vaccination results in a CD8 mediated cytotoxic response targeting cells transfected with CD99 and the subsequent reduction of CD99 expressing cells. We showed that CD99 is expressed on vascular endothelium overlying atherosclerotic plaques and found that CD99 expression is up-regulated during Western type diet feeding. CD99 vaccination induced the formation of CD8 positive T cells that were cytotoxic against cells transfected with pcDNA3-CD99. Activation of CD8⁺ T cells was demonstrated by a 30% increase in CD8⁺CD69⁺ double positive T cells in spleen and mediastinal lymph nodes. Furthermore, lymphocytes isolated from CD99 vaccinated mice specifically lysed CD99 expressing cells. More importantly, vaccination against CD99 attenuated atherosclerotic lesion formation in the aortic valve leaflets by 38% and in the carotid artery by 69% as compared to mice that were vaccinated with a control vector. Furthermore, a lower number of cells were found in atherosclerotic lesions implying that fewer leukocytes were recruited to these sites. These observations were accompanied by a decrease in CD99 expression on leukocytes. We conclude that vaccination against CD99 decreases atherogenesis by the selective removal of CD99 expressing cells, which could reduce leukocyte recruitment into atherosclerotic lesions and attenuate atherogenesis.

Introduction

Atherosclerosis is a chronic inflammatory disorder of the large arteries and the prominent cause of death in the western world¹. Recruitment of leukocytes into the vessel wall is driving the initiation and progression of atherosclerotic plaque formation^{2,3}. The atherosclerotic process is orchestrated by several groups of molecules on both the activated endothelium as well as on the infiltrating leukocytes⁴. Activation of the endothelium due to low shear stress and/or local damage results in the upregulation of leukocyte adhesion molecules and secretion of chemoattractants⁵.

Recruitment of blood leukocytes to sites of inflammation is initiated by interaction of P- and E-selectins that are upregulated on activated endothelium with their sialylated ligands on leukocytes⁶⁻¹⁰. This transient interaction slows down leukocytes from the flowing blood resulting in rolling behavior on the endothelial surface. After activation, leukocytes come to a firm arrest by binding to endothelial cell adhesion molecules. In experimental animals, endothelial cells in the arteries express in particular vascular cell-adhesion molecule-1 (VCAM-1) resulting in the predominant recruitment of monocytes and lymphocytes to atherosclerotic regions¹¹. Blocking this interaction results in attenuation atherosclerosis and other inflammatory disorders¹²⁻¹⁴. The last step in leukocyte transendothelial migration that is also known as diapedesis occurs largely through junctions between adjacent endothelial cells. Indeed a number of cell adhesion molecules located at endothelial cell junction have been implicated in this process. These molecules include platelet-endothelial cell adhesion molecule (PECAM-1)¹⁵, members of the junctional adhesion molecule (JAM) family¹⁶, CD99¹⁷⁻¹⁹, ESAM²⁰ and ICAM-2²¹.

CD99, a long known leukocyte membrane protein that was initially described to function in T cell activation and lymphocyte aggregation²²⁻²⁴, was only recently reported to participate in the transmigration of human monocytes through cultured endothelial cells¹⁹. Schenkel *et al.* showed that CD99 is expressed at endothelial cell contacts and that a monoclonal antibody against CD99 inhibits diapedesis of human monocytes across a monolayer of cultured endothelial cells by 90%¹⁹. The mouse counterpart was identified and cloned few years later by Bixel *et al.*¹⁷⁻¹⁸. Antibodies against mouse CD99 efficiently block migration of lymphocytes and neutrophils through a monolayer of cultured endothelioma cells¹⁷⁻¹⁸. More importantly, these antibodies block recruitment of in vivo-activated T cells into inflamed skin¹⁷ and inhibit neutrophil extravasation to inflamed sites in two inflammatory mouse models¹⁸. As the recruitment of both monocytes and T cells contribute to the initiation and progression of the atherosclerotic plaque, blockade of their transmigration may provide protection against atherosclerosis³.

In the present study we assessed the role of CD99 in the process of atherosclerosis by vaccinating atherosclerosis prone mice against CD99. Oral administration of a DNA vaccine encoding the extracellular domain of murine

CD99 by attenuated *Salmonella typhimurium* evoked a T-cell mediated immune response against cells expressing CD99 in mice. We demonstrate that vaccination of mice against CD99 generates antigen-specific CD8⁺ T cells that target 3T3 fibroblasts transfected with CD99. Vaccination of LDL receptor deficient mice against CD99 significantly reduced the formation of atherosclerotic lesions in the aortic valve leaflet and the carotid artery as compared to mice vaccinated with the vector alone.

Material and methods

Construction of the vaccine

The cDNA encoding the extracellular part of murine CD99 (BC019482), obtained by HindIII/EcoRI digestion of the Fc fusion plasmid earlier described by Bixel et al¹⁷ was cloned into pcDNA3 plasmid (Invitrogen California). This plasmid was electroporated into *S. typhimurium* Aro/A (strain SL7207) bacteria as previously described²⁵. Female LDL receptor deficient mice, aged 10-12 weeks were immunized by oral administration of 1*10⁸ cfu *S.typhimurium* transformed with either pcDNA3-CD99 or pcDNA3 empty (control) 3 times with 2 week intervals.

Induction of CD8⁺ specific cytotoxic T cells

Spleens were isolated from control and CD99 vaccinated mice and their capacity to lyse CD99 expressing cells was determined in the following assay. Murine fibroblasts (3T3) were cultured in a 24 wells plate and incubated with DMEM containing 10% Fetal bovine serum, 2mmol/L L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere (5% CO₂) at 37 °C. Cells were co-transfected with pcDNA3-CD99 (encoding for the extracellular part of murine CD99) and pEGFP-N1 vector using ExGen500 (Fermentas, Germany) as transfection reagent according to the manufacturers protocol. After 24 hours the transfected cells were incubated with splenocytes derived from control or CD99 vaccinated mice (2 weeks after last vaccination) for 24 hours. Non-adherent cells were washed away with PBS and specific lysis of the cells expressing CD99 was analyzed on a FACScalibur (BD Biosciences, The Netherlands) by quantifying the percentage of eGFP positive cells. The percentage of specifically lysed of cells transfected with CD99 was plotted as percentage of eGFP positive cells in absence of splenocytes.

Atherosclerosis experiments and histology

Female LDLr deficient (LDLr^{-/-}) mice (n=12 per group), 8 weeks old, were vaccinated with pcDNA3 empty or pcDNA3-CD99 3 times in two week intervals. After vaccination, mice were placed on a Western type diet containing 0.25 % cholesterol and 15% cocoa butter two weeks before collar placement. Silastic collars (0.3 mm inside diameter, Dow Corning, Midland, USA) were

placed around the carotid artery to induce atherosclerosis. After 8 weeks of western type diet, mice were sacrificed and organs were harvested.

Cryosections from carotid artery (5 μm) were stained with hematoxylin and eosin. Site of maximal stenosis was used for morphometric assessment using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK). Cryostat sections of the aortic root (10 μm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Expression of CD99 was visualized using an affinity-purified anti-mouse CD99 polyclonal antibody¹⁷. As secondary antibody, biotinylated goat anti-rabbit (DAKO, the Netherlands) was used in combination with streptABC complex (DAKO), with Nova Red as enzyme substrate (Vector Laboratories).

Real time PCR assays

Total RNA was isolated from aortic arch and collar induced atherosclerotic plaques using the guanidium isothiocyanate (GTC) method. Purified RNA was DNase treated (DNase I, 10 units/ μg of total RNA) and reverse transcribed (RevertAid M-MuLV reverse transcriptase) according to manufacturers protocol. Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using SYBR Green technology. (PCR primers see appendix 1)

Flow cytometry

Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) according to manufacturer's protocol. Cell suspensions from spleen, blood, lymph nodes draining from the aortic arch and peritoneal cavity were stained for surface markers (0.20 μg Ab/300.000 cells) and subsequently subjected to flow cytometric analysis. Antibodies were purchased from eBiosciences (Immunosource, Belgium). All data were acquired on a FACS-Calibur and were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Statistical analysis

Values are expressed as mean $\pm$ SEM unless indicated otherwise. Two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was applied to analyze not-normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

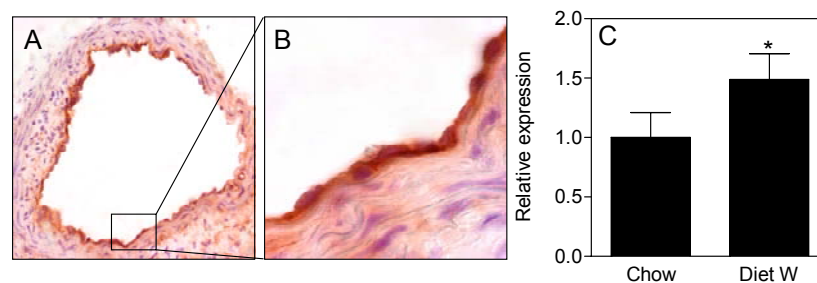


Figure 1: CD99 is expressed by vascular endothelial cells in an atherosclerotic vessel and is upregulated after Western type diet feeding. Expression of CD99 was visualized using an affinity purified polyclonal antibody against CD99. Representative pictures are shown in figure 1A and at higher magnification in 1B. mRNA was isolated from the aortic arch of LDLr^{-/-} mice using the GTC method. mRNA levels for CD99 were determined for mice receiving Chow or Western type diet and levels were related to mRNA levels of 36B4 and HPRT. Statistical analysis was done by an unpaired Student *t* test (week 0) (**P* < 0.05, n= 6 per time point).

Results

CD99 expression is upregulated on vascular endothelium overlying atherosclerotic plaques

Several molecules expressed at endothelial cell junctions such as JAM-A have been associated with atherosclerosis, and are upregulated in atherosclerosis prone sites of the vasculature²⁶. We stained cryosections of collar induced atherosclerotic plaques in LDLr^{-/-} mice and observed a profound expression of CD99 by vascular endothelial cells covering the plaque. A representative picture is shown in figure 1A-B. To further investigate the regulation of this expression, we isolated the aortic arch of LDLr^{-/-} mice after 2 weeks of western type diet and compared CD99 mRNA levels of mice that received a chow diet. We observed a 1.5-fold increase in CD99 expression upon western type diet feeding (Fig. 1C). This increase was not accompanied by elevated expression of T cell or macrophage markers, indicating that the cellular composition of the aorta was not changed due to an influx of leukocytes.

Vaccination against CD99 induces T cell-mediated lysis of cells expressing CD99

We developed a vaccination strategy based on the induction of CD99 specific cytotoxic T cells that specifically lyse cells that express high levels of CD99 as recently shown for FLK-1²⁷. To test the functionality and specificity of this vaccination protocol we determined the antigen-specific cytotoxicity of splenic CD8⁺ T cells derived from mice vaccinated against CD99. Therefore 3T3 fibroblasts were co-transfected with pcDNA-CD99 and EGFP-N1, which allows the identification of CD99 expressing cells by their simultaneous expression of GFP using flow cytometry. Splenocytes were isolated from control and CD99 vaccinated mice and were co-cultured for 24 hours with 3T3 fibroblasts that

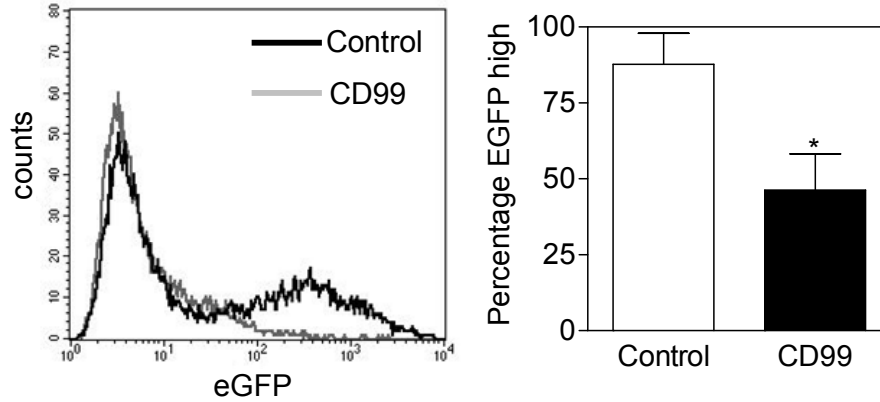


Figure 2: Vaccination against CD99 generates cytotoxic cells that specifically lyse cells expressing CD99. Murine fibroblast CD99 transfected with pcDNA-CD99 and eGFP were exposed to spleen cells isolated from control and CD99 vaccinated mice. After 24 hours, the percentage of eGFP positive cells was quantified using flow cytometry. Representative histograms are shown for spleen cells isolated from mice vaccinated with control vector (black line) or with pcDNA-CD99 (gray line). Specific lysis of 3T3 fibroblasts that present CD99 peptides on MHC class I was determined by quantifying the percentage of GFP positive cells compared to controls where no splenocytes were added. The percentage of GFP positive cells was significantly decreased when splenocytes were used derived from animals that were vaccinated against CD99 (right panel, $p=0.01$, $n=7$ per group).

were transfected with CD99. Specific lysis of CD99 expressing cells was determined by quantifying the percentage of eGFP positive cells compared to controls where no splenocytes were added. The percentage of CD99 expressing cells was significantly decreased after incubation with splenocytes from CD99 vaccinated mice (Fig. 2, $86.6 \pm 10.2\%$ vs. $46.3 \pm 11.8\%$), whereas no decrease was observed when splenocytes from control vaccinated mice were added (Fig. 2). This observation indicates that CD99 vaccination induces the formation of T cells that specifically lyse CD99 expressing cells.

Vaccination against CD99 inhibits atherosclerotic lesion formation in LDLr^{-/-} mice

After showing the effectiveness of our vaccination strategy, we investigated the effect of CD99 vaccination on atherosclerotic lesion formation. Female LDLr^{-/-} mice were vaccinated with pcDNA3-empty (control) or pcDNA-CD99 3 times at a 2 week time interval. Mice were subsequently placed on a Western type diet (0.25% cholesterol) and two weeks later mice were equipped with peri-carotid collars. Six weeks later, mice were sacrificed and the atherosclerotic lesion burden was determined in the carotid artery and aortic root. Representative photomicrographs of cross sections of the aortic root of control and treated mice are shown in figure 3A and B respectively. The mean lesion area in mice vaccinated against CD99 was decreased by 38% compared to control animals (Fig. 3C, $5.25 \pm 0.68 \times 10^5 \mu\text{m}^2$ vs. $3.37 \pm 0.57 \times 10^5 \mu\text{m}^2$).

Middle panels show representative sections of the carotid artery of control (Fig. 3D) and CD99 vaccinated (Fig. 3E) mice. Lesion size was significantly decreased by 69% after CD99 vaccination (Fig. 3F. $24.15 \pm 7.7 \times 10^3 \mu\text{m}^2$ vs. $7.71 \pm 4.0 \times 10^3 \mu\text{m}^2$, in Fig. 3F $2.4 \pm 0.7 \times 10^4 \mu\text{m}^2$ is shown, which is accompanied by an 83% decrease in intima/media ratio (Fig. 3G) and a 78% diminished degree of lumen stenosis (Fig. 3H).

In addition, the number of cells that had infiltrated the carotid plaques of control and CD99 vaccinated mice was determined by quantifying the number of nuclei per square micrometer (Fig. 3I). Vaccination against CD99 resulted in a 35% decrease in number of nuclei per area in atherosclerotic plaques from CD99 vaccinated mice compared to controls, indicating that a lower number of cells have infiltrated the vessel wall.

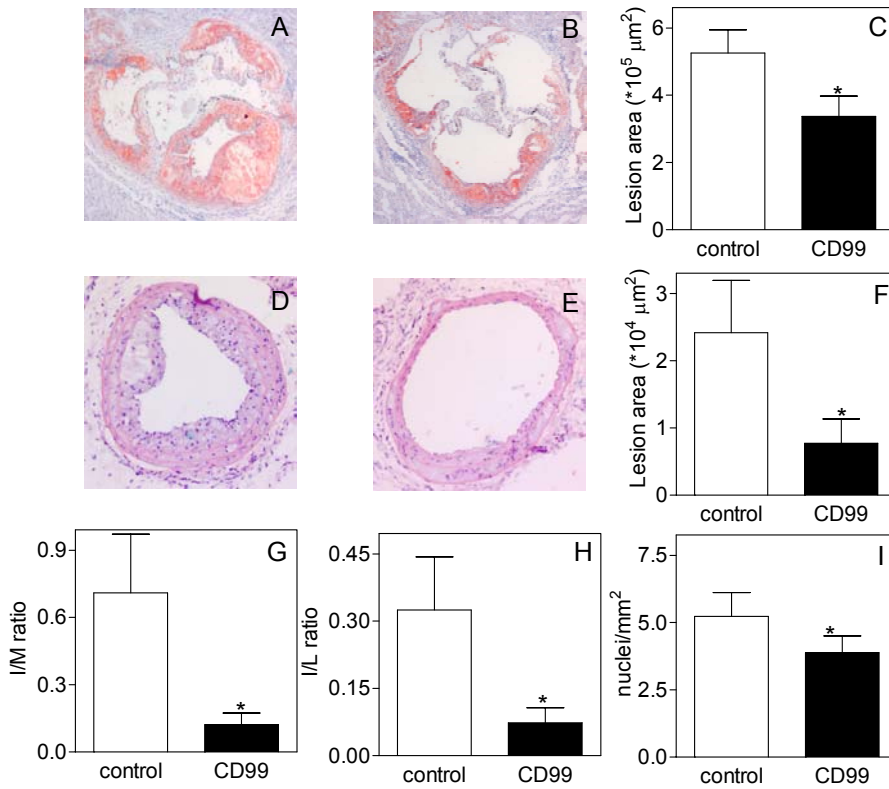


Figure 3: Atherosclerotic lesion formation is significantly inhibited in mice vaccinated against CD99. Representative photomicrographs of oil red O stained cross sections of the aortic root of control treated mice (A) and vaccinated mice (B) are shown. A significant reduction in plaque size was found in CD99 vaccinated mice compared to control mice (C). Lower panels demonstrate representative pictures of the carotid artery of control (D) and CD99 vaccinated animals (E). A significant decrease ($p=0.02$) was observed in carotid plaque area after vaccination against CD99 (F). This decrease in lesion formation is accompanied by a significant decrease in lumen stenosis (G) and intima/lumen ratio (H). Number of cells that infiltrated into the plaque area was decreased in mice vaccinated against CD99 compared to controls (I). Error bars represent SEM, $n = 12$ per group. Statistical analysis was done by a Mann Whitney test, $*=p<0.05$.

Vaccination activates CD8⁺ T cell in LDLr^{-/-} mice

The vaccination strategy is based on the induction of specific CD8⁺ T cells that induce apoptosis of cells that express high levels of CD99 via MHC class I. To test whether our vaccine had an effect on the activation state of the CD8⁺ T cell population during atherogenesis, we isolated lymphocytes from spleen and lymph nodes of control and CD99 vaccinated mice at the end of the atherosclerosis experiment. The activation state of the T cell population was determined by flow cytometry using CD69 as an early marker for T cell activation. CD8⁺ cells were gated and the percentage of CD8⁺ T cells expressing CD69 was determined (Fig. 4, upper panels). A significant increase of CD8⁺CD69⁺ double positive cells within the CD8⁺ T cell population was observed in spleen ($43.4 \pm 3.9\%$ vs. $59.3 \pm 4.5\%$) and in lymph nodes draining the aortic arch ($4.8 \pm 0.2\%$ vs. $5.9 \pm 0.1\%$, Fig. 4, lower panels). This observation was restricted to the CD8⁺ T cell population, since the activation state of CD4⁺ T cells was not changed as shown by measuring the percentage of CD4⁺/CD69⁺ double positive cells among the CD4⁺ T cell population (data not shown). These findings indicate that vaccination against CD99 specifically activates CD8⁺ T cell subsets, but not CD4⁺ T cells.

Vaccination against CD99 decreases expression of CD99 on CD4⁺ T cells and F4/80⁺ macrophages

CD99 is expressed on most leukocytes, including lymphocytes and monocytes derived from peripheral blood¹⁷⁻¹⁸. To determine whether vaccination against CD99 would affect CD99 expression levels on leukocytes, we stained CD4⁺ T cells and F4/80⁺ macrophages with anti-CD99 antibodies and determined CD99 expression on these cells by flow cytometry. The percentage CD99 positive cells within the CD4⁺ and F4/80⁺ cell population is shown in Figure 5.

The expression of CD99 on CD4⁺ T cells was significantly decreased upon vaccination against CD99 (Fig. 5, left panel; $37.6 \pm 2.7\%$ vs. $28.5 \pm 1.6\%$). In addition, the percentage of CD99 expressing cells within the macrophage population was also decreased by 31% after vaccination (Fig. 5, right panel; 24.5 ± 1.7 vs. 16.9 ± 2.7). The percentage of macrophages and CD4⁺ T cells was not changed compared to control vaccinated mice as determined by FACS (data not shown).

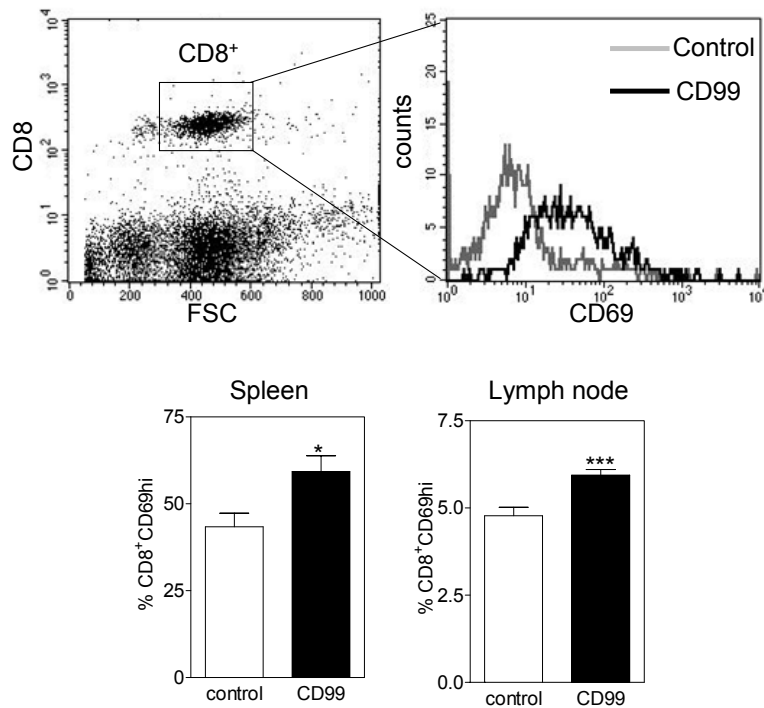


Figure 4: Vaccination against CD99 activates CD8⁺ T cells. Activation of CD8⁺ cells was analyzed by flow cytometry. CD8⁺ cells were gated and expression of the early T cell activation antigen CD69 was determined (upper panels). The percentage of CD8⁺CD69⁺ double positive cells was quantified within the CD8⁺ cell population. In CD8⁺ T cells derived from spleen and lymph nodes, vaccination against CD99 resulted in a significant up-regulation of CD69. White bars represent controls, black bars represent CD99 vaccinated mice, error bars display SEM, n=6 per group, *:p<0.05)

Discussion

Human CD99 was recently reported to be involved in migration of human monocytes through a monolayer of cultured endothelial cells¹⁹. Cloning of mouse CD99 by Bixel *et al.* opened the possibility to examine the physiological relevance and role of CD99 in mouse models of inflammatory diseases¹⁷.

Recruitment of leukocytes into the vessel wall is a hallmark of atherosclerosis and several facilitators of this process have been identified. A prominent role for cell adhesion molecules that are expressed at cell contacts of endothelial cells was reported, i.e. increased expression of PECAM-1 was found to be associated with atherosclerosis-prone regions of the vessel wall and blocking anti-PECAM-1 antibodies reduced leukocyte migration into atherosclerotic plaques^{15,28,29}. Mice deficient in JAM-A, another junctional cell adhesion molecule that participates in leukocyte extravasation, were shown to have reduced neointima formation on apoE background³⁰. We hypothesized that CD99 may also be involved in the recruitment of leukocytes into atherosclerotic plaques. A lower amount of CD99 expressing cells both endothelial and leukocyte could possibly slow down

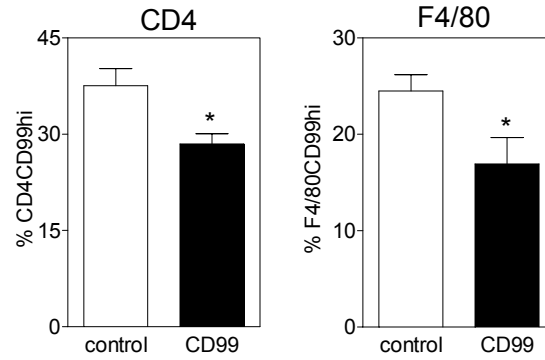


Figure 5: Expression of CD99 on CD4⁺ T cells and F4/80⁺ macrophages is decreased after vaccination against CD99. Single cell suspensions of spleens from control and CD99 vaccinated mice were prepared and analyzed by flow cytometry to determine CD99 expression on CD4⁺ T cells and F4/80⁺ macrophages. Expression of CD99 was quantified by assessing the relative amount of CD99 positive cells among the CD4⁺ and the F4/80⁺ positive cell population. Expression of CD99 in CD4⁺ T cells and F4/80⁺ macrophages was significantly decreased after CD99 vaccination compared to controls. White bars represent control, black bars represent CD99 vaccinated mice, error bars display SEM, n=6 per group, *:p<0.05.

leukocyte recruitment to inflamed areas of the artery and could attenuate the formation of atherosclerotic plaques.

First we demonstrate that CD99 is expressed on vascular endothelium overlying vessels with atherosclerotic lesions. Second, Western type diet feeding of atherosclerosis prone LDLr^{-/-} mice induced a 1.5-fold upregulation of CD99 mRNA levels in the aortic arch after 2 weeks of diet. This upregulation was not due to CD99 expressing leukocytes infiltrating the vessel wall, as CD99 expression was already increased before an increase in CD68⁺ or CD4⁺ cells was observed. This indicates that no additional CD99 expressing leukocytes were recruited and that the cellular composition of the examined vessels after Western type diet or chow diet feeding was not changed (data not shown).

To test the hypothesis that CD99 is involved in leukocyte recruitment to sites in the vessel wall where atherosclerotic lesions are formed we developed a DNA vaccination strategy to immunize LDL receptor deficient mice against CD99. An attenuated *S. typhimurium* strain was transformed with a pcDNA3 vector encoding the extracellular domain of murine CD99. The *S. typhimurium* carrier is taken up by M cells and processed in the Peyer's patches of the gastrointestinal tract. The bacteria are taken up by antigen presenting cells; the vector is transcribed and translated into protein. Processing the protein by the antigen presenting cells leads to presentation of peptide fragments in a complex with MHC class I. These MHC class I presented peptides activate specific cytotoxic natural killer cells and CD8⁺ T cells that clonally expand and break peripheral tolerance against CD99. This vaccination protocol therefore generates T cells that specifically target cells that express high levels of CD99 via MHC-I. The

DNA vaccination strategy has been successfully validated for other proteins in animal models for tumor growth and cancer therapy and atherosclerosis^{25,27,31}.

Here we show that vaccination against murine CD99 of LDLr^{-/-} mice specifically activates CD8⁺ T cells during atherogenesis that are able to target CD99 expressing cells. Based on these observations we conclude that our vaccination strategy generates cytotoxic CD8⁺ T cells that break through immune tolerance against a self antigen and kill cells that express high levels of CD99.

Once we had validated our vaccination protocol, we induced atherogenesis in LDLr^{-/-} mice that were vaccinated with control (pcDNA3-empty) or CD99 (pcDNA3-CD99). This approach has been shown to reduce plaque formation in mice that were vaccinated against vascular endothelial growth factor (VEGF) receptor 2 (flk-1), which is expressed by activated endothelial cells that cover the atherosclerotic plaque²⁵.

The importance of CD99 for leukocyte diapedesis was first shown by Schenkel et al.¹⁹ for human CD99 and later by Bixel for mouse CD99¹⁸. For human monocytes and neutrophils and for mouse lymphocytes it has been shown that the separate blockade of both endothelial or leukocyte CD99 by specific antibodies is sufficient to inhibit leukocyte transmigration¹⁷. Lower CD99 expression levels or a reduced functionality of CD99 on leukocytes could possibly interfere with leukocyte extravasation and reduce their migration through the vascular endothelium. We have shown that vaccination against CD99 significantly decreases CD99 expression on CD4⁺ T cells and macrophages. It was suggested for human CD99 that homotypic interaction of CD99 on leukocytes with CD99 on endothelial cells is important for leukocyte diapedesis through endothelial contacts. A lower expression level of CD99 on leukocytes and/or endothelial cells could possibly attenuate plaque formation due to a reduced transmigration rate. We clearly show that vaccination against CD99 indeed strongly reduced the formation of atherosclerotic lesions and attenuated atherogenesis at two different sites within the vasculature. In addition to smaller size of atherosclerotic plaques they contained fewer cells when mice were vaccinated against CD99. This indicates that fewer cells that could contribute to plaque initiation and growth have migrated into the activated vessel wall. The observed reduction in lesion formation is not the result of differences in cholesterol and triglyceride levels between control and CD99 vaccinated mice (data not shown).

In summary, we generated an attenuated *Salmonella* vaccine against mouse CD99 that induced the generation of a CD8⁺ T cell population that specifically targets CD99 expressing cells. Vaccination against CD99 of LDL receptor deficient mice significantly reduced the formation of atherosclerotic plaques and attenuated clinical symptoms of atherogenesis. This flexible, easy to prepare, and non invasive method provides a rationale for the future development of DNA based *Salmonella* vaccines to prevent the initiation or progression of atherosclerosis.

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Chapter 7

Vaccination Against IL-16 Accelerates atherogenesis in LDL Receptor Deficient Mice

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Abstract

Atherosclerosis is an auto-immune disease characterized by the influx of leukocytes into the vessel wall and the deposition of lipids. Interruption of the migration of specific subsets of leukocytes into the lesion can exert protective effects on lesion formation. IL-16 is a chemotactic factor that specifically induces the migration of CD4⁺ cells to sites of inflammation. It has been shown to have pro-inflammatory effects in several auto-immune diseases and we therefore proposed that blockade of IL-16 would be protective in atherogenesis. We constructed an orally administrated DNA IL-16 vaccine and confirmed the induction of anti-IL16 specific antibodies. LDL receptor deficient mice were vaccinated against IL-16 and we determined the effect on lesion formation in the aortic leaflet area and in collar induced atherogenesis after 8 weeks of diet feeding. No effects were observed in the aortic leaflets, but lesion formation was significantly induced in the carotid artery. At this time point, the number of circulating T cells, both CD4⁺ and CD8⁺, was significantly increased in IL-16 vaccinated mice compared to control. Vaccinated mice on a diet for 20 weeks showed an even more profound 2-fold induction of lesion formation in the total aorta. Vaccination did not affect serum cholesterol and triglyceride levels. We propose that IL-16 has a protective role, especially in late stage atherogenesis. More research will be necessary to address the mechanism behind the observed anti-atherogenic effect.

Introduction

Accumulation of leukocytes in the vessel wall is a hallmark of atherosclerosis. Both macrophages and CD4⁺ T cells are attracted to the activated endothelium and contribute to the growing atherosclerotic plaque. IL-16 is one of the first described proteins that displays chemoattractant properties for lymphocytes and IL-16 was therefore originally designated as Lymphocyte Chemoattractant Factor (LCF)^{1,2}. It selectively mediates the migration of CD4⁺ T cells to sites of inflammation and has been extensively studied since it was cloned in 1994³. IL-16 is produced by a variety of cells, including leukocytes, fibroblasts and epithelial cells and is synthesized as a precursor that has to be cleaved by caspase-3 to become biologically active^{4,5}. Secreted IL-16 forms homo-tetramers that are able to induce cross linking of its receptor CD4. CD4 is expressed by a subset of T cells and to a lesser extent on most monocytes/macrophages. IL-16 induces cell activation and migration, and therefore a prominent role for this molecule can be suggested in (auto-)immune diseases. IL-16 seems to exert a pro-inflammatory stimulus in animal models for experimental autoimmune encephalomyelitis (EAE)^{6,7}, rheumatoid arthritis (RA)^{8,9} and asthma¹⁰⁻¹².

Leukocyte accumulation in the arterial wall initiates the formation of atherosclerotic plaques and inhibition of this migration, for example by selective blockade of chemokines or pro-inflammatory mediators, has resulted in the successful attenuation of lesion formation. It can be speculated that blockade of IL-16 will result in decreased migration of CD4 positive cells, and thus will result in attenuated plaque formation. This hypothesis was further underlined by the finding that CD4 deficient mice show reduced lesion formation when backcrossed to several atherosclerosis prone animal models and adoptive transfer of CD4⁺ cells led to accelerated atherosclerosis¹³⁻¹⁵.

Lynch et al have identified CCR5 as a co-receptor for IL-16¹⁶. As blockade of CCR5 results in significant attenuation of atherosclerotic lesion formation^{17, 18}, this could point to a pro-atherogenic effect of IL-16 as well.

In this study, we used oral DNA vaccination to induce IL-16 specific antibodies that selectively block IL-16. To break IL-16 tolerance, we used a PAN-DR epitope that enables a MHC-II mediated antibody response against the self-peptide IL-16. We show that vaccination against IL-16 results in increased lesion formation in the carotid artery in LDL receptor deficient (LDLR^{-/-}) mice. This effect was more prominent in later stages of plaque formation in the total aorta. We therefore propose an anti-inflammatory role for IL-16 in atherosclerotic lesion formation.

Material and methods

Vaccination strategy

The sequence of murine IL-16 was fused with the PADRE sequence and cloned into pcDNA3.1 plasmid (Invitrogen, CA) and electroporated into attenuated *Salmonella typhimurium* (Aro/A (strain SL7207). HEK 293-T cells were transfected with the plasmids and IL-16 protein expression was determined by Western blotting. Vaccination was performed by 3 times oral administration of 1×10^8 cfu of *S. typhimurium* transformed with pcDNA3.1-PADRE-IL-16 or pcDNA3.1-PADRE (control) in 100 μ l of PBS at 2 week intervals¹⁹. All animal experiments were in accordance with guidelines issued by the Dutch law and approved by the ethics committee for animal Experiments of Leiden University (DEC).

Antibody titer determination

96-wells plates were coated with 250 ng/mL IL-16 in PBS and incubated overnight at 4°C. After washing, the plates were blocked with FCS for 1 hour at room temperature. Plasma of IL-15 or control vaccinated mice was added in serial dilutions with PBS 5% Triton-X100 and incubated for 1 hour at room temperature. Plates were washed with PBS + 0.1% Tween-20, and horseradish peroxidase conjugated Goat-anti Mouse antibody (Dako) was used as detection antibody. After washing, the anti-IL-16 antibody titer was determined using a TMB kit (Pierce).

Atherosclerosis experiments

Female LDLr^{-/-} mice were vaccinated with control (pcDNA3.1-PADRE) or pcDNA3.1-PADRE-IL-16 as described (n=12/13 per group). After the last vaccination, mice were fed an atherogenic diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Service, UK). After 2 weeks of diet, atherosclerosis was induced by placing silastic collars around both common carotid arteries as previously described²⁰. After 8 and 20 weeks of Western type diet, mice were sacrificed and lesion size (8 weeks aortic root, 20 weeks en face aorta) was analyzed using a Leica-DM-RE microscope and Leica Qwin software. Concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

Histological analysis

Cryostat sections of the aortic root (10 μ m) and carotid artery (5 μ m) were collected and stained with Oil-red-O and H/E respectively. Lesion size was determined in 5 sections of the aortic valve leaflet area and at the site of maximal stenosis in the carotid artery. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:50).

Goat anti-rat IgG-AP (dilution 1:100) was used as secondary antibody and NBT-BCIP as enzyme substrates.

Real time PCR assays

Total RNA was isolated from aortic arch and was DNase treated. Quantitative gene expression analysis was performed on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (Appendix I) were designed using Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Flow cytometry

Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada). Cell suspensions from spleen, blood, lymph nodes draining from the aortic arch and peritoneal cavity were stained for surface markers (0.2 µg Ab/300,000 cells) and subsequently subjected to flow cytometric analysis (FACS). Antibodies were purchased from Immunosource (Belgium). All data were acquired on a FACSCalibur and were analyzed with CELLQuest software (BD Biosciences).

Statistical analysis

Values are expressed as mean ± SEM unless indicated otherwise. Two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was used to compare not normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

Results

IL-16 mRNA is increased during initial lesion formation

IL-16 functions as a chemotactic factor for CD4⁺ cells and we speculated that IL-16 could be involved in the attraction of leukocytes during atherosclerotic plaque formation.

We used LDL receptor deficient mice on a Western type diet as a model for atherosclerosis and the mRNA expression of IL-16 during lesion formation was monitored in the aortic arch at different stages of western type diet feeding. A vast and significant increase was observed in IL-16 mRNA expression after 9 weeks of diet (20-fold, $p = 0.001$), indicating a role for IL-16 in diet-induced atherosclerotic lesion formation (Figure 1). At this time point a moderate influx of CD4⁺ and CD68⁺ cells was observed in the vessel wall (data not shown), indicating that lesion formation has started.

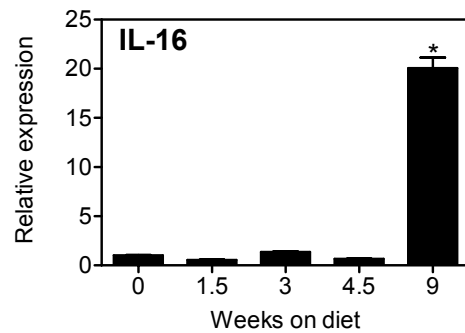


Figure 1: mRNA expression of IL-16 is significantly upregulated in the aortic arch of LDLr^{-/-} mice on Western typed diet feeding. mRNA was isolated from the aortic arch of LDLr^{-/-} mice and expression of IL-16 is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student *t* test was applied to test whether IL-16 mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (**p* < 0.05, *n* = 6 per time point).

PADRE- IL-16 vaccination

Once we found a diet induced regulation of IL-16, we constructed a DNA vaccine against IL-16 to address the functional role of this interleukin during lesion formation. The vaccination strategy is based on the generation of IL-16 specific antibodies that will functionally block IL-16 signaling. We used a PAN-DR epitope (PADRE) to overcome tolerance against the self peptide IL-16 by facilitating MHC-II mediated T cell activation. We cloned a fusion construct of PADRE-IL-16 into pcDNA 3.1 and *in vitro* experiments were used to determine the expression of the used construct in eukaryotic cells. HEK-293T cells were transfected with the PADRE-IL-16 plasmid and the presence of pro-IL-16 protein was confirmed by Western blot (Figure 2A). Lane 4 and 5 clearly show a band of approximately 70 kD that indicated pro-IL-16 protein (68 kD). Non-transfected control cells and transfection with pcDNA 3.1 empty did not show the expression of pro-IL-16 (Lane 2 and 3).

To test the induction of anti-IL-16 antibodies after vaccination, we titrated serum from control and IL-16 vaccinated mice to determine the concentration of anti-IL-16 antibodies by ELISA. Serial dilutions of serum from IL-16 vaccinated mice resulted in a sigmoidal decrease in the binding of antibodies to the IL-16 coated wells as shown in figure 2B. Binding of antibodies in serum of control vaccinated mice to IL-16 was not observed in control vaccinated mice (open dots).

Vaccination against IL-16 aggravates atherogenesis in LDLr^{-/-} mice

Subsequently, we used the plasmid in a vaccination protocol to determine the effect of the induction of anti-IL-16 antibodies on lesion formation. Mice were vaccinated 3 times by oral administration of attenuated *S. typhimurium* transformed with pcDNA 3.1PADRE-IL16 or control (pcDNA 3.1PADRE). After vaccination, mice were fed a Western type diet for 2 weeks after which collars were placed around the carotid artery to induce atherosclerosis in the carotid arteries. Six weeks after collar placement, animals were sacrificed and initial atherosclerotic lesion formation was assessed. Vaccination did not affect cholesterol (2753 ± 432 vs. 2733 ± 544 mg/dl) and triglyceride levels (475 ± 134 vs. 533 ± 205 mg/dl) during the experiment.

Representative sections of the aortic valve leaflet area of control (A) and IL-16 vaccinated mice (B) are shown in figure 3. Lesion formation at this site was not affected by IL-16 vaccination ($2.85 \pm 0.26 \times 10^5 \mu\text{m}^2$ vs. $2.56 \pm 0.56 \times 10^5 \mu\text{m}^2$, $p=0.55$).

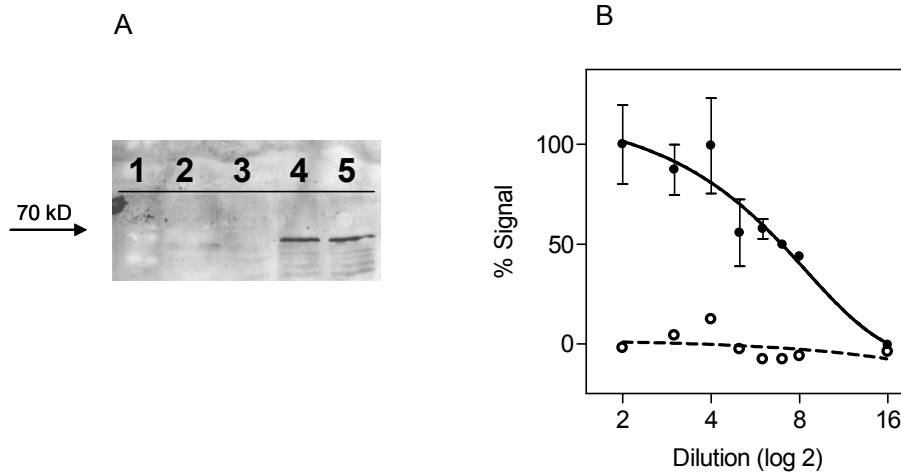


Figure 2: IL-16 vaccination results in protein expression in eukaryotic cells and specific antibody titers. A: HEK 293T cells were transfected with pcDNA3.1 (lane 3) and pcDNA containing PADRE-IL-16 (lane 4+5) and incubated for 24 hours. Cells were lysed and subjected to Western blotting. Pro-IL16 protein (68kD) was detected with a IL-16 specific antibody. Control cells (lane 1) and cells transfected with pcDNA3.1 showed no expression of IL-16, in contrast to PADRE-IL16 transfected cells (lanes 4+5).

B: Serum from control and vaccinated mice was obtained at the termination of the atherosclerosis experiments (8 weeks) and anti-IL-16 titer was determined by ELISA. Serial dilutions are plotted against the relative signal with average control serum stated as 0%. A clear sigmoidal decrease in ELISA signal is appreciated in mice vaccinated against IL-16 (closed dots). This was not visible in mice that were vaccinated with pcDNA3.1 PADRE only (open dots). N=12 per dilution, at all points the amount of antibody as determined against a calibration curve was significantly increased in IL-16 vaccinated mice compared to control vaccinated mice.

We also assessed the degree of atherosclerosis in a model for flow-induced lesion formation. Representative slides of collar induced atherosclerosis in the carotid artery are shown for control (A) and PADRE-IL-16 (B) vaccinated mice in figure 4. Lesion formation at this site was significantly increased by 60% upon IL-16 vaccination. ($44,850 \pm 7,036 \mu\text{m}^2$ vs. $71,739 \pm 10,383 \mu\text{m}^2$, $p=0.04$) (figure 4C). This was reflected in a 73% increase in intima media ratio (0.86 ± 0.12 vs. 1.50 ± 0.20 , $p=0.01$, 4E) and also the relevant clinical parameter, intima lumen ratio, increased by 41% (0.46 ± 0.06 vs. 0.65 ± 0.06 , $p=0.05$, 4D).

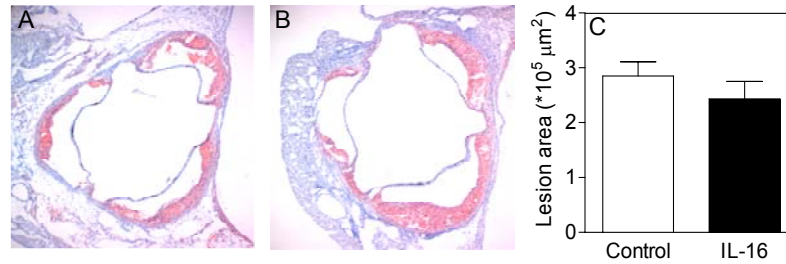


Figure 3: Initial atherosclerotic lesion formation after IL-16 vaccination. Representative photomicrographs of oil red O stained cross sections of the aortic root of control vaccinated mice (A) and IL-16 vaccinated mice (B) are shown. No difference in lesion area was observed after IL-16 vaccination.

In addition to the effect of IL-16 vaccination on initial lesion formation after 8 weeks of Western type diet, we assessed the result of IL-16 vaccination on advanced lesion formation. Again, animals were vaccinated with control or PADRE- IL-16 3 times at a two-week interval and were fed a Western type diet for 20 weeks. The degree of plaque formation was determined along the aorta by en face staining of the lipid rich areas using Oil red O. Representative examples of control (A) and IL-16 vaccinated (B) aortas are shown in figure 5. The lesion burden in IL-16 vaccinated mice is 1.9-fold higher in comparison to control vaccinated mice (13.8 ± 1.3 % vs. 26.2 ± 2.3 %, $p=0.0004$).

Circulating T cell numbers are increased after IL-16 vaccination

IL-16 functions as a chemotactic factor for CD4 positive cells and we were interested in the effect of IL-16 vaccination on the leukocyte population during atherogenesis. To investigate this we harvested leukocytes from blood, spleen and lymph nodes, which drain from the aortic arch from control and IL-16 vaccinated mice after 6 weeks of Western type diet. We stained the cell suspensions for CD4, CD8 and F4/80 and the percentage of positive cells was determined by FACS.

Figure 6A shows the relative percentage of CD4+ T cells in blood, spleen and lymph nodes. A significant 50 % increase is observed in the percentage of these cells in the circulation of IL-16 vaccinated mice compared to PADRE vaccinated mice (17.2 ± 1.5 vs. 24.9 ± 2.1 , $p=0.01$). No differential effects of IL-16 vaccination were observed on the CD4 population in spleen and lymph nodes. A similar pattern, however more modest, can be observed in the CD8+ population (6B). The percentage CD8+ cells is significantly increased in blood after IL-16 vaccination (10.8 ± 1.6 vs. 12.8 ± 0.8 , $p=0.03$), and no such effects were observed in spleen or lymph nodes. As macrophages and monocytes can express (low amounts of) CD4, we also analyzed the F4/80 positive population. No differential effects were observed on this cell type after vaccination against IL-16 (figure 6C). No significant differences in total white blood cell counts were observed between control and treated mice.

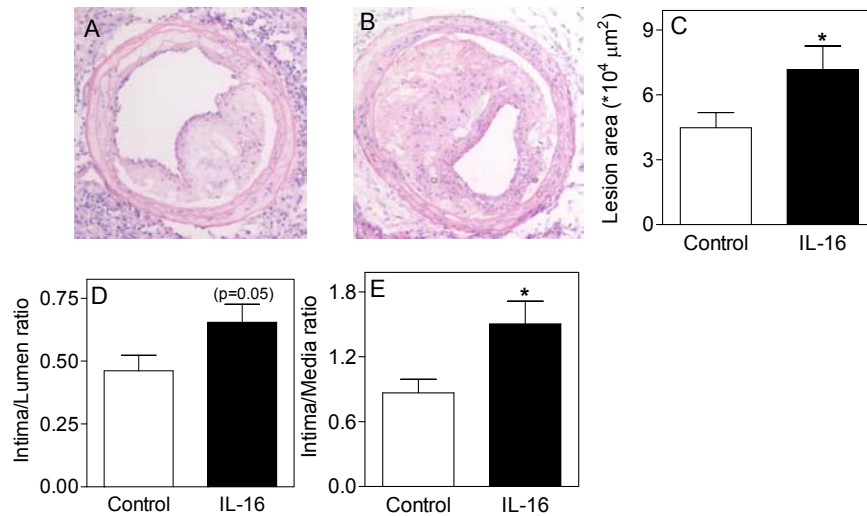


Figure 4: Initial atherosclerotic lesion formation after IL-16 vaccination. Collar induced plaques from control (A) and IL-16 vaccinated (B) mice were stained with H/E and lesion area was assessed. Lesion size significantly increased by 60% upon treatment vaccination against IL-16 (n=12 per group, p=0.04). This translated into a 73 % increase in intima media ratio (E) and also the relevant clinical parameter, intima lumen ratio, increased by 41% (D).

Discussion

In this study we showed anti-atherogenic effects of IL-16 in atherosclerosis. We used a DNA vaccination strategy to block IL-16 function *in vivo*. IL-16 is an endogenous protein and antibody formation against a endogenous proteins is inhibited due to the lack of T cell help resulting in tolerance²¹. A vaccination strategy that will result in antibodies against a self protein is therefore depending on overcoming this T cell unresponsiveness²². Vaccination against IL-12 by using the fusion protein of IL-12 and the T helper cell restricted epitope PADRE has been shown to be successful in preventing both experimental autoimmune encephalomyelitis (EAE) and atherosclerosis by induction of IL-12 autoantibodies^{23, 24}. In this study, we use a DNA vaccination strategy where we overcome tolerance by linking the PADRE sequence to murine pro-IL-16 and cloned the fusion construct into pcDNA 3.1.

As protein expression of the construct is essential for further immunological processing, we show that transformation of eukaryotic cells with this plasmid resulted in protein production of the fusion construct. We then transformed *Salmonella typhimurium* with the plasmid and vaccinated mice by multiple oral administrations of the transformed bacteria. Niethammer *et al.* have nicely shown that oral vaccination results in the uptake of the transformed *Salmonella* by M cells and subsequent processing in the Peyer's patch leads to translation of the proteins encoded by the plasmid¹⁹. In our case, by using the PADRE epitope, the fusion protein will be recognized by IL-16 specific B cells.

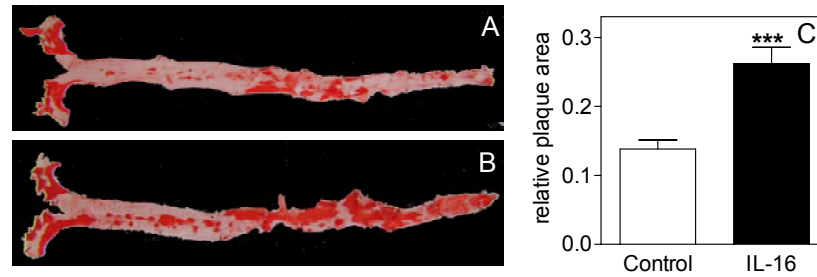


Figure 5: Lesion formation after 20 weeks of diet is significantly induced after IL-16 vaccination. Relative plaque area was quantified in *en face* pinned aortas after vaccination and 20 weeks of diet feeding. Representative pictures are shown for control (A) and IL-16 vaccinated mice (B) and lesion formation is significantly induced by 100% (n=8 per group, p=0.0004).

T cell help will be facilitated via the specific MHC class II presentation of PADRE by the antigen presenting cells (ie the B cell), which results in T cell help for the IL-16 specific B cell. We confirmed the resulting IL-16 specific antibody titers by ELISA.

Once we successfully validated the vaccination protocol, we vaccinated LDLr^{-/-} mice against IL-16 to determine the role of this molecule in atherogenesis. No effect of IL-16 was observed on serum cholesterol and triglyceride levels and IL-16 vaccination did not affect initial lesion formation in the aortic valves. However, an increase in lesion formation was determined in the carotid artery and after 20 weeks of diet feeding, lesion formation in the total aorta was almost 100 % increased compared to control vaccinated mice. This indicated that there is a discrepancy in the effects of IL-16 vaccination between different sites and time points, and this phenomenon is observed by more authors^{25,26}. For example Frederickson et al. observed a significant reduction in lesion size in the descending aorta and not in the aortic valve leaflets in apoE deficient mice after immunization with apoB-100²⁵. Furthermore, Hauer et al. showed that DNA vaccination against vascular endothelial growth factor receptor 2 very significantly reduced lesion formation in the carotid artery and in contrast, no effect was observed in the valve leaflet²⁶.

The observed profound effects of IL-16 vaccination on advanced lesion formation and to a lesser extend on initial lesion formation may be due to the processing route of IL-16. It is synthesized as a pro-form and only actively secreted after cleavage by caspase-3^{5, 27}. This enzyme is highly expressed in lipid rich plaques and associated with macrophage apoptosis due to oxLDL loading²⁸. It was also shown that IL-16 is spontaneously released from monocytes cells that undergo apoptosis²⁹. In general, during plaque growth and differentiation the lipid rich core associated with necrosis and apoptosis will increase. This indicates that the amount of caspase-3 will increase with plaque progression, and therefore the active form of IL-16 will probably be more present in advanced atherosclerosis. Blocking the function of this molecule will thus have a more

profound effect in advanced plaque progression than in the development of (initial) fatty streaks. This mechanism is not in contrast with the observed elevation of IL-16 mRNA in the vessel wall already after 9 weeks of diet feeding. Pro-IL-16 mRNA is constitutively expressed in leukocytes, and cellular activation or apoptosis induction is required to release the active form of IL-16.

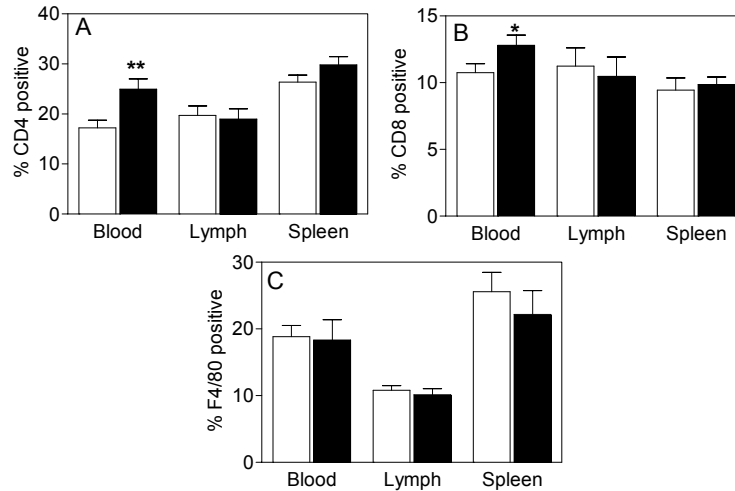


Figure 6: Relative number of circulating T cells is increased upon IL-16 vaccination. Mononuclear cell suspensions of draining lymph nodes, spleen and blood were isolated from control mice and mice following control or IL-16 vaccination. Cells were stained for CD4 (A), and CD8 (B) and F4/80 (C). Results represent the mean percentage of positive cells $\pm$ SEM from 6 individual mice (*= $P < 0.05$, Students T test). An increase is seen in the percentage CD4 and CD8 positive T cells in blood in the IL-16 vaccinated group (White bars) compared to control (Black bars).

Although IL-16 is mainly presented in literature as a pro-inflammatory mediator, we propose that IL-16 has a protective effect during diet induced lesion formation. This rather unexpected finding can have several reasons. IL-16 is able to induce migration of CD4⁺ cells and vaccination against IL-16 will thus result in the reduction of IL-16 mediated migration. Not all attracted cells during the formation of an atherosclerotic plaque contribute to its growth. Specific attraction of cell subsets such as naturally occurring regulatory T cells or other suppressive subsets have profound protective effects on lesion formation. It was shown that IL-16 can induce expression of CD25, and is a possible stimulator of regulatory T cells in this way³⁰. Blocking this signalling route can locally modify the balance between regulatory and effector T cells and lead to accelerated atherosclerosis^{31,32}. The effects of IL-16 are not restricted to the T cell population. Macrophages and dendritic cells are known to express low to moderate amounts of CD4. When these cells are exposed to IL-16, the production of several pro-inflammatory and pro-atherogenic mediators is increased³³. However, IL-16 is also able to induce the secretion of IL-10 in these

cells, indicating a possible protective role via the induction of this potent anti-atherogenic molecule.

In conclusion we propose that IL-16 has protective effects in advanced lesion formation, possibly by the attenuation of anti-atherogenic T cell subsets or by reducing the amount of protective cytokine production. Further research will be necessary to determine the specific mechanism by which IL-16 exerts these beneficial effects on plaque formation.

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Chapter 8

Interruption of the OX40/OX40L Pathway Attenuates Atherogenesis

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Abstract

Atherosclerosis is a chronic (auto-) inflammatory disease and T cell activation is an important factor in this process. Tnfrsf4 (OX40) and Tnfsf4 (OX40 ligand) are members of the TNF and TNF receptor family and OX40/OX40L mediated signaling is important in co-activation of T cells and facilitates B-T cell interaction. In this study we assessed the role of the OX40/OX40L pathway in atherosclerosis and the effect of interruption of the OX40/OX40L pathway on lesion development.

We treated LDLr^{-/-} mice with an anti-OX40L antibody which lead to a 53% decrease in atherosclerotic lesion formation. Treatment resulted in inhibition of Th2 mediated isotype switching by decreasing IL-4 secretion and subsequent low IgG1 serum levels against oxLDL, while protective anti-oxLDL specific IgM titers were increased in treated mice compared to control.

We conclude that blocking the OX-40/OX40L interaction reduced atherogenesis by inhibition of IL-4 mediated Th2 induced isotype switching and subsequent increased levels of anti-oxLDL IgM.

Introduction

Atherosclerosis is considered to be a chronic (auto-) inflammatory disease in the context of high plasma cholesterol levels, in which both innate and adaptive immune responses play a role^{1, 2}. It is characterized by the infiltration of the arterial vasculature by leukocytes in response to both endothelial injury and elevated levels of oxidized lipoproteins^{3, 4}. These elevated levels of oxidized lipids induce a strong humoral response, resulting in the production of specific antibodies against oxidized LDL (oxLDL) and malondialdehyde LDL (MDA-LDL). T cell activation is an important step in this process and is initiated by antigen (possibly oxidized lipoproteins or heat shock proteins) recognition in combination with co-stimulatory signals provided by antigen presenting cells (APCs). The TNF receptor pathway can provide co-stimulatory signals and has been implicated in the onset and progression of atherosclerosis⁵⁻⁷.

Tnfrsf4 (OX40, CD134) is a less well-explored member of the TNF receptor family and is primarily expressed on activated CD4-positive and CD8-positive T cells in mice and humans⁸⁻¹⁰. OX40L (Tnfsf4, CD134L) is expressed on a wide variety of cells including dendritic cells, B-lymphocytes, microglia, and vascular endothelial cells^{11,12}.

OX40 and OX40L have been implicated in T cell activation and migration into inflamed tissue, as well as in the activation of B cells and macrophages¹³. Activated OX40 positive T cells can provide help to B cells via OX40L expressed on the B cell surface, and can subsequently induce isotype switching dependent on their cytokine production¹⁴.

Interruption of OX40-OX40L interaction has been shown to ameliorate several autoimmune like diseases^{15,16, 17}. The expression of OX40 by T cells during EAE¹⁸ and graft versus host disease (GVHD)¹⁹ is correlated with the severity of disease, increasing during disease onset, and decreasing when the clinical parameters decline. Interestingly, the OX40L gene is located within the Ath-1 QTL on chromosome 1, a region associated with aggravated atherosclerosis, indicating a role for this molecule in atherogenesis^{20, 21}.

In the present study we examined the expression of OX40 during the formation of atherosclerotic lesions in LDLr^{-/-} on a Western type diet and determined the effect of an OX40L blocking monoclonal antibody (RM134) on atherogenesis.

Material and methods

Animal experiments

Female LDLr deficient (LDLr^{-/-}) mice, 8 weeks old, were put on a Western type diet containing 0.25 % cholesterol and 15% cocoa butter two weeks before collar placement. Silastic collars (0.3 mm inside diameter, Dow Corning, Midland, USA) were placed around the carotid artery to induce atherosclerosis as described previously²². After collar placement mice were treated with 300 µg of anti-OX40L antibody (RM134) in 100 µl of sterile PBS *i.p.* twice a week during 6 weeks. As a control rat IgG (Sigma) in sterile PBS was used. Cryosections from the carotid artery (5µm) were stained with hematoxylin and eosin. Site of maximal stenosis was used for morphometric assessment using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK). Cryostat sections of the aortic root (10 µm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:50). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100) was used as secondary antibody (1h, room temperature) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. Lesion collagen content was visualized by a 90-minute incubation of slides in 0.1% Sirius Red (Direct Red 80, Sigma) in saturated picric acid and subsequent rinsing in 0.01M HCl.

Serum lipid levels

Blood samples were collected by tail bleeding from non-fasted animals. Concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

Real Time PCR assays

Total RNA was isolated from spleen using the guanidium isothiocyanate (GTC) method. Purified RNA was DNase treated (DNase I, 10 units/µg of total RNA) and reverse transcribed (RevertAid M-MuLV reverse transcriptase) according to manufacturers protocol. Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (Table I) were designed using Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and validated for identical efficiencies (slope = -3.3 for a plot of the threshold cycle number (C_t) versus log ng cDNA). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Flow cytometry

Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) according to manufacturers protocol. Cell suspensions from spleen and blood were incubated with 1% normal mouse serum in PBS and stained for surface markers (0.25 μg Ab/200,000 cells) and subsequently subjected to flow cytometric analysis (FACS). Antibodies were purchased from eBiosciences. All data were acquired on a FACSCalibur and were analyzed with CELLQuest software (BD Biosciences).

Serum antibody and cytokine level determination

IgG1, IgG2A and IgM levels against oxLDL were detected in serum using the Mouse MonoAb ID kit (Zymed Laboratories Inc., South San Francisco, USA). OxLDL (5 $\mu\text{g}/\text{ml}$) dissolved in a $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer (pH 9.0) was coated o/n onto a flat-bottom 96-wells high binding plate (Corning, NY). Serum samples were 1:1 diluted in PBS and absorbance was detected at 405 nm. IL-5 concentration was determined using an IL-5 specific ELSIA (Immunosource). Spleen and peritoneal cells from mice which received 2 weeks of Western type diet and were treated with control IgG or RM134 were cultured at 2×10^6 cells/ml and stimulated with anti-CD3 and CD28 coated antibodies. Supernatants were collected after 24 hours and IFN- γ , IL-5 and IL-4 concentrations were determined by ELISA (eBioscience, Belgium).

Statistical analysis

Values are expressed as mean $\pm$ SEM. Two-tailed student's T-test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was used to not normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

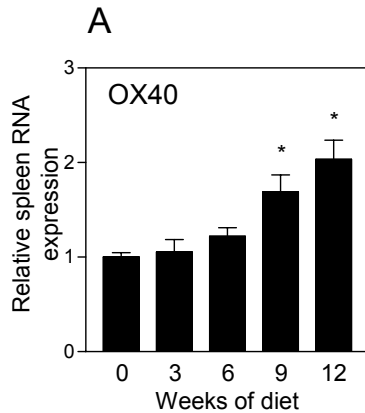


Figure 1A. mRNA expression of OX40 in response to a Western type diet. mRNA was isolated from spleen using the GTC method and expression of the indicated genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student *t* test was applied to test whether OX40 mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (* $P < 0.05$, $n = 6$ per time point).

Results

Expression of OX40 during Western type diet feeding

Atherosclerosis is characterized by both locally presented (within the vessel wall) and circulating antigens such as modified LDL. The spleen is therefore continuously exposed to atherogenic factors such as oxLDL, and the reaction of the spleen in response to a high cholesterol diet used in our model can provide insight into the systemic immunological response. We examined the mRNA expression of OX40 in the spleen of LDLr^{-/-} mice fed a Western type diet for 0, 3, 6, 9 and 12 weeks. A significant increase in the relative expression of OX40 was determined in the spleen of LDLr^{-/-} mice, showing a strong positive correlation with time of diet administration (figure 1A).

To correlate the elevated mRNA expression of OX40 in spleen to protein expression, we determined cell surface expression of OX40 during Western type diet feeding. The expression of CD4, CD8 and OX40 in spleen and blood was determined in female LDLr^{-/-} mice using FACS analysis at 0, 2 and 4 weeks of Western type diet feeding.

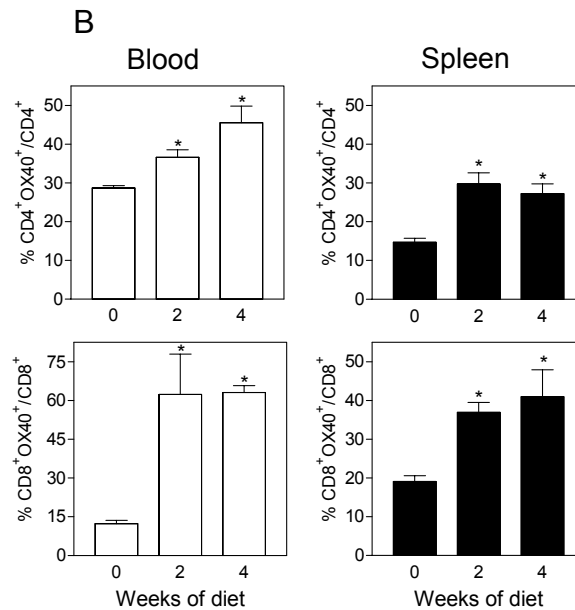


Figure 1B. Protein expression of OX40 in response to a Western type diet. Mononuclear cell suspensions from spleen and blood were isolated from control mice and mice following 0, 2 or 4 weeks of Western type diet feeding. Results in panel 1B represent the mean percentage of positive cells $\pm$ SEM from 6 individual mice (*= $P < 0.05$, student's T-test). A significant increase is seen in the percentage CD4 (upper panels) and CD8 (lower panels) OX40 double positive T cells in blood (white bars) and spleen (black bars).

The expression is plotted as the percentage OX40⁺ cells in the CD4⁺ or CD8⁺ positive population. In the spleen, a 2-fold increase was found in both CD4/OX40 double positive and CD8/OX40 double positive T cells after 2 and 4 weeks of Western type diet as compared to control. The induction of OX40 expression in the spleen is associated with an increase in the expression of OX40 in the circulation. Within the CD4⁺ population, a significant increase in the OX40 positive population is observed in blood after 2 and 4 weeks of Western type diet feeding as compared to chow fed mice. The percentage OX40 positive cells in the CD8⁺ population shows a 5-fold increase as compared to chow fed mice on both 2 and 4 weeks of Western type diet (figure 1B).

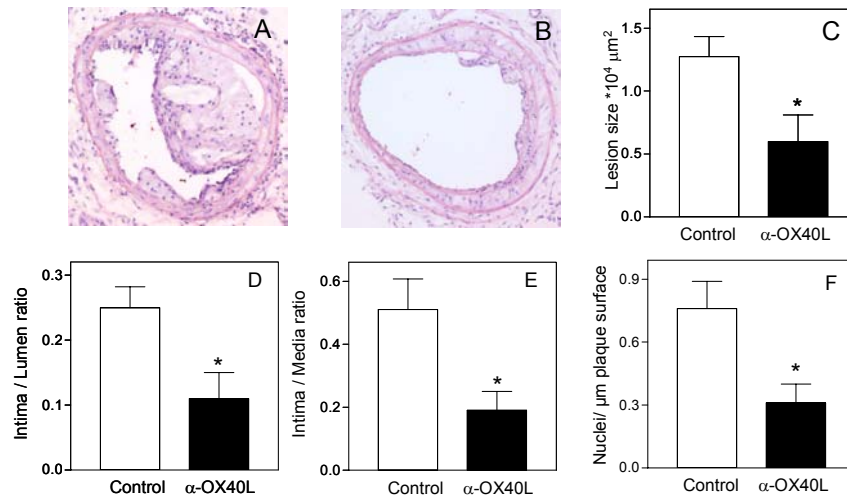


Figure 2. Anti OX40L antibody treatment reduces collar induced atherosclerotic plaque formation.

A significant decrease was observed in carotid plaque area after treatment with anti- OX40L antibody. Representative pictures of the carotid artery of control IgG (A) and anti- OX40L (B) treated mice are shown. A marked and significant decrease was seen for intima/ lumen ratio (D), and intima / media ratio (E). Nuclei density of the plaque significantly decreased after treatment (F). Error bars represent SEM, n =10 per group (*= $P < 0.05$, Mann Whitney test).

Attenuation of atherogenesis by anti-OX40L antibody administration

OX40 positive T cells require OX40L on the vascular endothelium to enter inflamed tissue from the circulation, and this interaction can be inhibited by the anti-OX40L antibody RM134. Furthermore, the anti-OX40L antibody can inhibit T cell activation, and subsequently reduce their help to B cells.

In order to investigate the effect of specific blockade of OX40L on atherogenesis, we used the anti-OX40L antibody in LDLr^{-/-} mice. After two weeks of Western type diet, female LDLr^{-/-} mice were equipped with pericarotid collars and subsequently treated twice a week with 300 μg of anti-OX40L for a period of 6 weeks. Figure 2 shows representative sections of the carotid artery of control IgG treated (2A) and anti-OX40L treated mice (2B) stained with hematoxylin and eosin. Lesion size was significantly decreased by 53% after blockade of

OX40L ($1.27 \pm 0.16 \times 10^4 \mu\text{m}^2$ vs. $5.98 \pm 0.21 \times 10^4 \mu\text{m}^2$, $P=0.02$), which translated into a diminished degree of lumen stenosis (2D) and a marked decrease in intima/media ratio (2E). Anti-OX40L antibody treatment reduced the cellularity of the plaques by 60% ($P=0.01$) compared to control treated mice (2F). No significant effects of antibody treatment were seen on body weight, serum cholesterol and triglyceride levels (data not shown).

In addition, we quantified the lesion size in the aortic valve leaflet of the same mice. Representative photomicrographs of cross sections of the aortic root of control and treated mice are shown in figure 3A and B respectively.

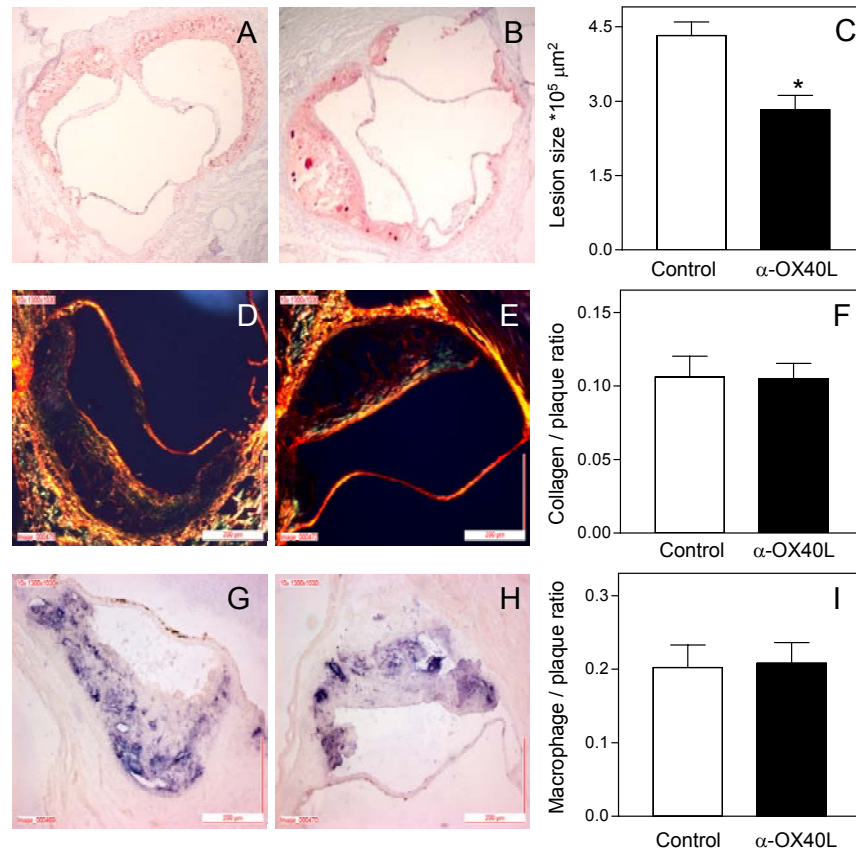


Figure 3. Anti OX40L antibody treatment reduces atherosclerotic plaque formation in the aortic valve leaflet. Representative photomicrographs of oil red O stained cross sections of the aortic root of control IgG treated mice (A) and anti-OX40L treated mice (B) are shown. A significant reduction in plaque size was found as compared to control ($p=0.01$, $n=10$ per group) (C). Plaque collagen content is stained with Pico Sirius Red in control (D) and treated (E) mice. Relative collagen area does not differ between groups (F). Relative macrophage staining of control (G) and treated mice (H) is visualized using a monoclonal antibody specific for macrophages (MOMA-2). No difference in the percentage MOMA positive area is observed between control and treated mice (I).

Mean lesion area was decreased by 35% upon anti-OX40L antibody treatment compared to control mice ($P=0.004$, figure 3C). No significant difference in relative collagen (figures 3D-F) or macrophage content (figures 3G-I) was observed.

Expression of OX40 after anti-OX40L treatment

After sacrificing the animals we determined the mRNA expression of OX40. A significant 45% decrease in the spleen after OX40L antibody treatment compared to control treated mice ($P<0.05$) was observed (figure 4A). In addition, we also quantified the expression of OX40 on T cells in spleen, mediastinal lymph nodes and blood by FACS analysis (Figure 4B). A clear and significant reduction can be seen in the percentage CD4 and CD8/OX40 double positive cells in blood after anti-OX40L treatment. Strikingly, no decrease in OX40 double positive cells was observed in spleen and lymph nodes. In addition no difference in the expression of IL-12 and IFN- γ in spleen was observed after anti-OX40L treatment (data not shown).

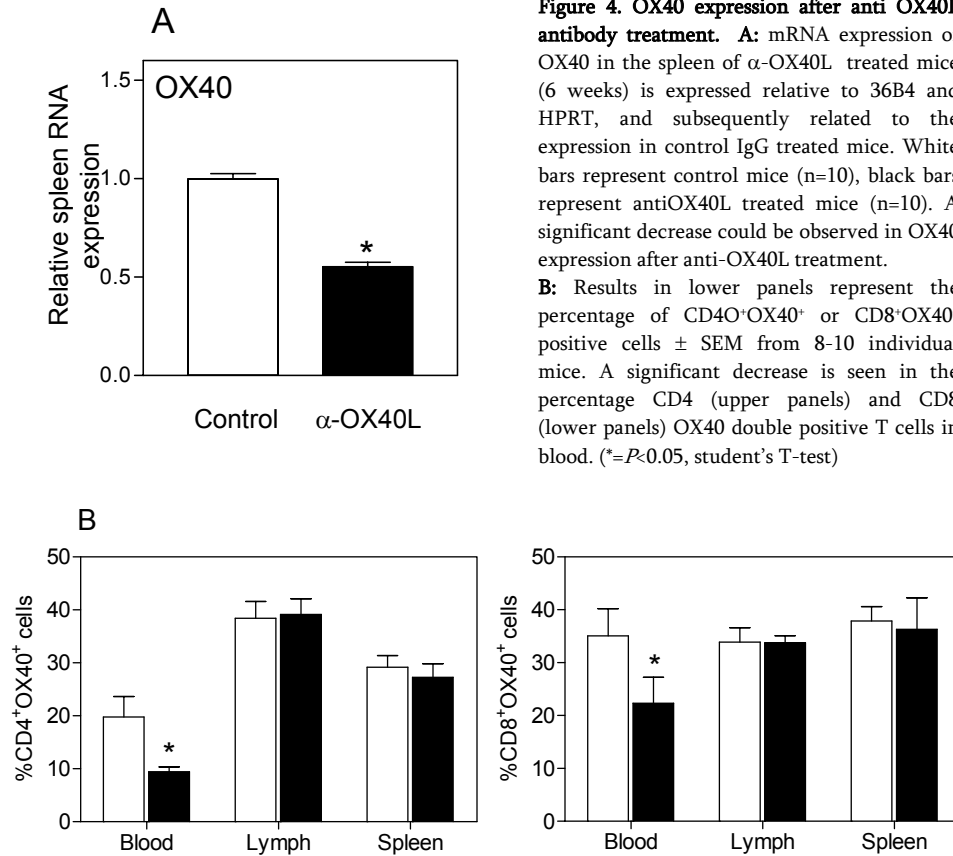


Figure 4. OX40 expression after anti OX40L antibody treatment. **A:** mRNA expression of OX40 in the spleen of α -OX40L treated mice (6 weeks) is expressed relative to 36B4 and HPRT, and subsequently related to the expression in control IgG treated mice. White bars represent control mice (n=10), black bars represent antiOX40L treated mice (n=10). A significant decrease could be observed in OX40 expression after anti-OX40L treatment.

B: Results in lower panels represent the percentage of CD4⁺OX40⁺ or CD8⁺OX40⁺ positive cells $\pm$ SEM from 8-10 individual mice. A significant decrease is seen in the percentage CD4 (upper panels) and CD8 (lower panels) OX40 double positive T cells in blood. (*= $P<0.05$, student's T-test)

Antibody response against oxLDL

OX40-OX40L interaction plays an important role in T cell dependent humoral responses. B cells differentiate into IgG producing cells after help from OX40 positive, activated T cells¹⁴. In this way, OX40 positive T cells induce B cell isotype switching. We determined the isotype specific antibody response to oxLDL in control and anti-OX40L treated mice after 6 weeks of treatment and 8 weeks of Western type diet. Figure 5 shows a significant decrease in IgG1 antibodies against oxLDL, while IgG2a levels remained constant in treated mice compared to control. This resulted in a very significantly increase in the IgG2a/IgG1 ratio ($P < 0.001$).

Antigen-specific IgG production is preceded by a more general IgM antibody response. Anti-OxLDL specific IgM titers increased significantly in anti-OX40L treated mice compared to control. Even after 2 weeks of treatment (and 4 weeks of Western type diet) significantly higher IgM levels were observed in treated mice. In addition to an enhanced IgM levels due to an inhibition of isotype switching after OX40L blockade, the increase in IgM may result from the production of IgM by B1 cells, which is under control of IL-5 (Binder *et al.*²³). The concentration of IL-5 in serum of control and treated mice was determined after 6 weeks of treatment and a significant increase in IL-5 levels was determined in anti-OX40L treated mice (figure 5).

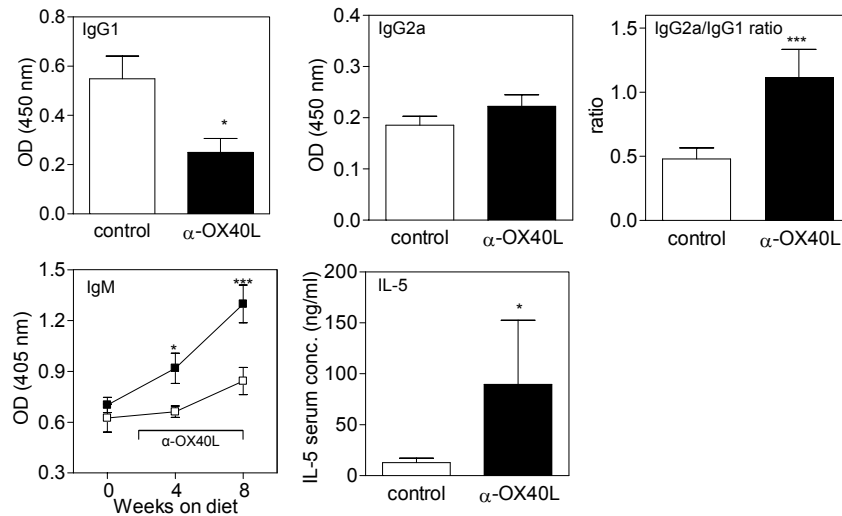


Figure 5. OxLDL specific antibody production

Specific isotypes of antibodies against oxLDL were determined by ELISA in mice after 8 weeks of Western type diet. Relative concentrations of control and antiOX40L treated mice are shown. A significant reduction in IgG1 serum level was observed. Circulating IgM levels increased during treatment, as well as IL-5 serum levels. White bars represent control mice (n=8), black bars represent anti-OX40L treated mice (n=10). Error bars represent SEM, *: $p < 0.05$, ***: $p < 0.001$.

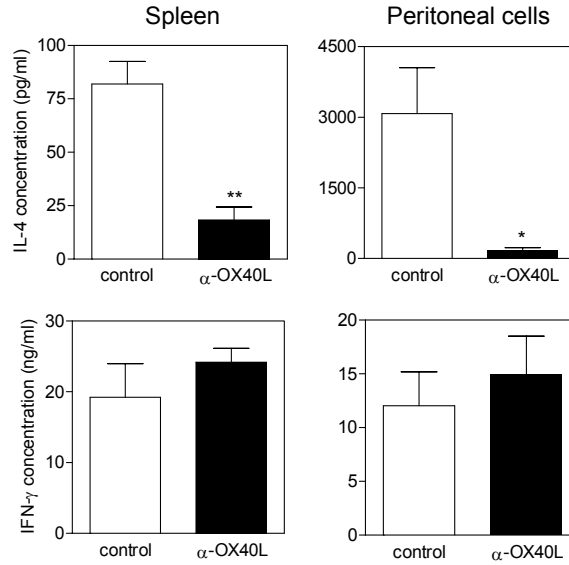


Figure 6. IFN- γ , IL-5 and IL-4 production by spleen and peritoneal cells Spleen and peritoneal cells isolated from mice that received two weeks of western type diet feeding and treatment with control or anti-OX40L antibody. A significant reduction in IL-4 production is observed in both spleen and peritoneal cells after anti CD3-CD28 stimulation for 24 hours. IL-5 production is significantly decreased by peritoneal cells treated with anti-OX40L antibody, but is significantly decreased in spleen cell cultures from these mice. IFN- γ production is not affected by anti-OX40L treatment. White bars represent control mice (n=5), black bars represent anti-OX40L treated mice (n=5). Error bars represent SEM, *: $p < 0.05$, **: $p < 0.01$.

Th2 activation is inhibited by anti-OX40L treatment

Isotype switching of B cells to IgG1 or IgG2a producing cells is dependent on interleukin secretion by T cells. IL-4 secretion can induce an IgG1 isotype switch, while IFN- γ can induce an IgG2a switch. As shown above, anti-OX40L antibody treatment resulted in reduced IgG1 levels against OxLDL. We determined the capacity of spleen and peritoneal cells to secrete IFN- γ , IL-4 and IL-5 after CD3/CD28 stimulation for 24 hours (Figure 6). Cells were isolated from mice which received 2 weeks of Western type diet feeding and anti-OX40L treatment. Two weeks should be sufficient to induce isotype switching, as a normal IgM responses are switched to specific IgG production within 14 days. IL-4 production was significantly decreased after anti-OX40L treatment, while IFN- γ production remained unchanged in both spleen and peritoneal leukocytes. IL-5 production by peritoneal cells was reduced, which correlated with the decreased IL-4 production by these cells. In contrast, spleen cells isolated from the same mice secreted increased levels of IL-5 after activation for 24 hours.

Discussion

A recent publication of Wang *et al.* describes the identification of *Tnfsf4* (OX40L) as the underlying gene in the *Ath-1* QTL^{20 21 24}, which renders C57Bl/6J mice susceptible to atherosclerosis in contrast to C3H/HeJ and BALB/cJ mice. Mice overexpressing *Tnfsf4* showed significantly larger atherosclerotic lesions compared to control, and mice with targeted deletion of this gene had significantly smaller lesions compared to control. Furthermore, the same study showed that humans with a SNP in the OX40L gene have a higher incidence of myocardial infarction.

As Calderhead *et al.* have shown, OX40 expression is very low on naïve T cells, and upregulated after activation²⁵, we monitored OX40 protein expression on T cells in spleen and blood of *LDLr^{-/-}* mice fed a Western type diet. In spleen a significant increase is shown in the number of CD4⁺OX40⁺ and CD8⁺OX40⁺ cells during 2 and 4 weeks of Western type diet feeding. This increase coincides with an increase of these populations in the circulation. OX40 mRNA levels significantly increased at a later time point than the increase in the percentage of OX40 double positive cells. The percentage of positive cells however, does not reflect the expression level of OX40 per cell since it only discriminates between OX40 positive and negative cells. The difference between mRNA and protein expression of OX40 may reflect the dynamic structure of the spleen and cells expressing high levels of OX40 may be more prone to migration.

The correlation between OX40 positive CD4 and CD8 cells and autoimmune diseases has also been shown for EAE and GVHD¹⁸. We show that Western type diet feeding leads to T cell activation based on OX40 expression and OX40 may be an ideal candidate for modulating T cell responses in atherogenesis.

The effect of anti-OX40L antibody treatment on atherosclerosis was investigated in *LDLr^{-/-}* mice. Treatment led to a significant reduction (53%) in de novo lesion formation. The underlying mechanism of the observed plaque reduction by anti-OX40L treatment may be complex. Not only systemic or local T cell activation, but also B cell function may be involved. Furthermore, very recently Ito *et al.* showed that OX40L strongly inhibited the generation of IL-10 producing type 1 regulatory T cells. These T cells are important in the maintenance of peripheral tolerance and were shown to be protective in the development of atherosclerosis^{26 27}. Future experiments must indicate whether the blockade of OX40L by the anti-OX40L antibodies also results in increased numbers of type 1 regulatory T cells.

As atherosclerosis is classically considered to be a Th1 mediated disease we quantified the mRNA levels of IFN- γ and IL-12 in spleens of anti-OX40L treated mice. No significant difference could be observed between the two groups, despite the significant reduction in plaque size and general T cell activation determined by expression of CD62L and CD69 was not altered between groups (data not shown). We did however observe a significant reduction in OX40

mRNA expression in spleen and a decrease in circulating OX40 positive T cells, indicating reduced secondary T cell activation.

OX40 activation has been linked to both Th1 and Th2 activation in several disease models²⁸⁻³⁴. We determined the capability of spleen and peritoneal cells to produce Th1 (IFN- γ) and Th2 (IL-4, IL-5) cytokines and found a vast and significant decrease in IL-4 secretion after anti-OX40L treatment in these cells, while the production of IFN- γ was unaffected. We determined the percentages of T cells in these cell suspensions and no significant differences were observed between the two groups.

Since knocking out IL-4 has previously been shown to be protective in atherosclerosis^{35, 36}, our data further undermine the hypothesis that Th2 cytokine IL-4 provides protection in atherosclerosis and supports the pro-atherogenic effects of IL-4.

IL-4 is an inducer of IgG1 isotype switching in B cells. In accordance with the reduced IL-4 production by lymphocytes, OxLDL specific IgG1 levels in mice treated with anti-OX40L were significantly decreased. IFN- γ production was not different in anti-OX40 treated mice compared to control, and correspondingly, IgG2a antibodies against OxLDL were not altered.

An isotype switch towards IgG production is preceded by the production of IgM. The reduced isotype switching to IgG1 as observed in anti-OX40L treated mice may explain the observed enhanced anti-OxLDL IgM levels. Anti-OxLDL IgM has been shown to provide protection against atherosclerosis by contributing to the elimination of oxidized lipoproteins and cellular debris³⁷⁻³⁹. In addition to reduced isotype switching, B-1 cells may form another source for anti-OxLDL IgM production. These B-1 cells depend on IL-5 for differentiation and maturation⁴⁰⁻⁴². We observed an induction of serum IL-5 in mice treated with anti-OX40L antibodies. IL-5 production by peritoneal cells from anti-OX40L treated mice was significantly decreased, and this is in correlation with the decreased production of IL-4 by these cells after anti-CD3/CD28 activation. In spleen, anti-OX40L treatment leads to increased secretion of IL-5. IL-5 is produced by Th2 cells but since the anti-OX40L treated mice showed a decrease in Th2 response (lower IL-4 and IgG1), we conclude that other cell types such as mast cells and NK2 cells may be responsible for the increased IL-5 production, and that these cells are (partially) located in the spleen. Since the anti-OX40L treatment resulted not only in a significant increase in IL-5, but also in a trend towards an increased number of CD5 IgM expressing B-1 cells in the peritoneal cavity (Figure 7), we conclude that the increased level of atheroprotective anti-OxLDL IgM upon OX40L blockade may also result from B-1 cells in addition to a reduction in isotype switching. Based on the data it is unlikely that the increased OxLDL specific IgM is derived from the peritoneum, however, migration of activated B-1 cells from peritoneum to germinal centers in the spleen has been reported⁴³.

Based on this study we conclude blockade of the OX40/OX40L pathway by an anti-OX40L antibody results in protection against atherosclerosis due to elevated protective IgM titers and a decreased IL-4 production.

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Chapter 9

CD127 is Required for Macrophage and Regulatory T Cell Function and Has a Protective Role in Atherosclerosis

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Abstract

CD127 is the receptor for interleukin 7 (IL-7) and is expressed on immature B cells, activated T cells and macrophages. IL-7 induces T cell activation via CD127, and is implicated in the survival of regulatory T cells. Furthermore, premature B cells need IL-7 to proliferate into mature B cells. In LDLr^{-/-} mice CD127 mRNA was upregulated 5-fold ($p < 0.001$) in the aortic arch after 9 weeks of Western type diet, indicating a correlation between expression of CD127 and atherosclerotic lesion formation. Macrophages showed elevated the expression of CD127 when exposed to oxidized LDL in vitro. In order to elucidate the function of CD127 in the process of atherosclerosis we constructed an anti-CD127 DNA vaccine by cloning murine CD127 into pcDNA3.1. Vaccination was performed by repeated oral administration of attenuated *Salmonella typhimurium* transformed with pcDNA3.1-CD127. Vaccination of LDLr^{-/-} mice against CD127 aggravated collar induced lesion formation by 3-fold without an effect on serum cholesterol levels. Lesions from vaccinated mice showed reduced cellularity and increased apoptotic area compared to control vaccinated animals. Macrophage numbers in the spleen from vaccinated mice were reduced by 37%, CD4 and CD8 T cell percentages in spleen, lymph nodes and blood were not altered. Strikingly, the regulatory T cell population (CD4⁺CD25⁺) in draining lymph nodes from the aortic arch was significantly decreased by 30% ($p=0.02$). We conclude from these results that CD127 is an essential factor in the function and/or survival of regulatory T cells and macrophages, which may contribute to its protective role in the process of atherosclerotic lesion formation.

Introduction

IL-7 plays an essential role in the development of the immune system and deficiencies in the IL-7 pathway lead to severe lymphopenia^{1, 2}. It is secreted by fetal liver cells, stromal cells in bone marrow and thymus, and other epithelial cells³⁻⁵. Alongside its prominent role in thymic development, IL-7 is recently been identified as essential for the induction of CD4⁺ and CD8⁺ T cells with a memory phenotype during adult life^{6, 7}. Next to these predominant effects on T cells, evidence exists that IL-7 may induce activation and expansion of monocytes and natural killer cells^{8, 9}. IL-7 signals through the IL-7 receptor that includes two polypeptides, a common gamma chain (CD132) and an alpha chain (CD127). The alpha chain is specific for the IL-7 receptor (and together with TSLPR for TSLP^{10, 11}), while the gamma chain is part of several other interleukin receptors (IL-2,4,9,15 and 21). CD127 is expressed by B cell precursors, most T cells and monocytes¹².

Expression of CD127 is down-regulated when the cell is exposed to stimulatory factors such as OX40L, CD40L or activating cytokines such as IL-2 or IL-4¹³. In contrast, it was shown that memory T cells are derived from a population of activated T cells that do not reduce the expression of this receptor¹⁴. IL-7 is identified as a survival factor for CD4⁺CD25⁺ regulatory T cells, but recent publications put forward CD127 as a negative marker for regulatory T cells. It was shown that the expression of the transcription factor FOXP-3 is inversely correlated with the expression of CD127 on CD4⁺ T cells¹⁵⁻¹⁷. IL-7 mediated activation and expansion of T cells is associated with several (auto-) immune diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and graft versus host disease (GVHD)¹⁸⁻²⁰. Next to these clear pro-inflammatory effects of IL-7 in auto-immune disease, Ponchel *et al.* describe that serum levels of IL-7 are decreased in patients with RA, suggesting a negative association of this molecule with disease²¹. Atherosclerotic plaques are characterized by the infiltration of both monocytes and T cells. IL-7 may have a potential role in the initiation or progression of lesion formation by modulating the activation state of local or systemic T cells and the involvement in regulatory T cell homeostasis. No direct evidence exists on the function of IL-7/CD127 signaling in atherosclerosis. Raised serum levels of IL-7 were reported for patients with angina pectoris and correlated with the severity of disease^{22,23}. Randi *et al.* however found that unstable coronary artery plaques show decreased levels of IL-7.

In this study we show that macrophages exposed to different concentrations of OxLDL induce their expression of CD127, which correlates with the finding that expression of CD127 in the aorta is upregulated during lesion formation. We designed a vaccination strategy that induced CD8⁺ T cells that specifically removed cells that express high levels of CD127. Vaccination of atherosclerosis prone LDL receptor deficient (LDLr^{-/-}) mice against CD127, resulted in a vast and

significant induction of lesion formation as compared to control vaccinated mice. This is accompanied by a reduction in the local number of regulatory T cells. These findings may indicate a protective effect of IL-7/CD127 signaling in atherosclerosis.

Material and methods

Cell culture

Bone marrow derived macrophages were grown from bone marrow from C57/Bl6 mice using complete DMEM medium supplemented with 1% non essential amino acids, 1% pyruvate, 20% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamin (all from BioWhittaker) and 25% L-929 medium (containing MCSF) for one week in a humidified atmosphere with 5% CO₂ in non tissue culture plates. Subsequently, cells were incubated with oxLDL and/or LPS at various concentrations for 16 hours and CD127 expression was determined by FACS.

Vaccination strategy

Murine CD127 was cloned into pcDNA3.1 plasmid (Invitrogen, CA) and electroporated into attenuated *Salmonella typhimurium* (Aro/A (strain SL7207). Vaccination was performed by 3 times oral administration of 1x10⁸ cfu of *S. typhimurium* transformed with pcDNA3.1-CD127 or pcDNA3.1 empty (control) in 100 µl of PBS at 2 week intervals²⁴. All animal experiments were in accordance with guidelines issued by the Dutch law and approved by the ethics committee for animal Experiments of Leiden University (DEC).

Atherosclerosis experiments

Female LDLr^{-/-} mice were vaccinated with control or pcDNA3.1-CD127 as described (n=12 per group). After the last vaccination, mice were fed an atherogenic diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Service, UK). After 2 weeks of diet, atherosclerosis was induced by placing silastic collars around both common carotid arteries as previously described²⁵. After 8 weeks of Western type diet all mice were sacrificed and lesion size was analyzed using a Leica-DM-RE microscope and Leica Qwin software. To assess intimal apoptosis, corresponding sections were subjected to TUNEL staining using the IN Situ Cell Death detection kit (Roche Diagnostics). Concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

FACS analysis

Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) was used to separate lymphocytes from whole blood and spleen by density gradient centrifugation according to manufacturer's protocol. Cell suspensions from

spleen and blood were incubated with 1% normal mouse serum in PBS and stained for surface markers (0.25 μ g Ab/200,000 cells) and subsequently subjected to flow cytometric analysis (FACS). Antibodies were purchased from eBiosciences. All data were acquired on a FACSCalibur and 10000 lymphocyte events were analyzed with CELLQuest software (BD Biosciences).

Gene expression

Total RNA was isolated from the aortic arch of the mice using the guanidium isothiocyanate (GTC) method. Purified RNA was DNase treated (DNase I, 10 units/ μ g of total RNA) and reverse transcribed (RevertAid M-MuLV reverse transcriptase) according to manufacturers protocol. Quantitative gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) using SYBR Green technology. PCR primers (appendix I) were designed using Primer Express software with the manufacturer's default settings (Applied Biosystems). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Statistical analysis

Values are expressed as mean $\pm$ SEM. Two-tailed student's T-test was used to compare normally distributed data. Mann-Whitney test was used to compare lesion area data and other not normally distributed data. A probability value of $P < 0.05$ was considered to be significant for both tests.

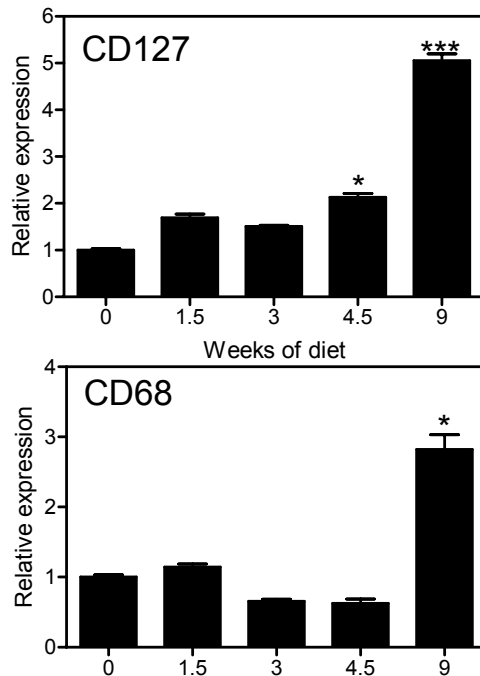


Figure 1: CD127 mRNA expression is upregulated in the aortic arch during atherosclerotic lesion formation. mRNA was isolated from the aortic arch of LDLr^{-/-} mice using the GTC method and expression of genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student *t* test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (week 0) (* $P < 0.05$, $n = 6$ per time point).

Results

Expression of CD127 is upregulated in the aortic arch of LDLr^{-/-} mice after western type diet feeding

CD127 is expressed on several types of (activated) T cells, bone marrow derived macrophages and immature B cells. As the influx of leukocytes into the vessel wall is considered to be essential in the formation of atherosclerotic lesions, CD127 may also be expressed in the atherosclerotic plaque. In order to investigate CD127 expression during atherosclerosis, we isolated the aortic arch of female LDLr^{-/-} mice at several time points of western type diet feeding. Figure 1 shows a vast and significant 2.2 ($p < 0.03$) and 5.1-fold ($p < 0.0002$) increase in CD127 mRNA expression after 4.5 and 9 weeks of western type diet feeding, respectively. The increase in CD127 expression coincides with the increased expression of the macrophage marker CD68 in the vessel wall (lower panel). This suggests a possible role for CD127 expression on macrophages that are present in the atherosclerotic plaque.

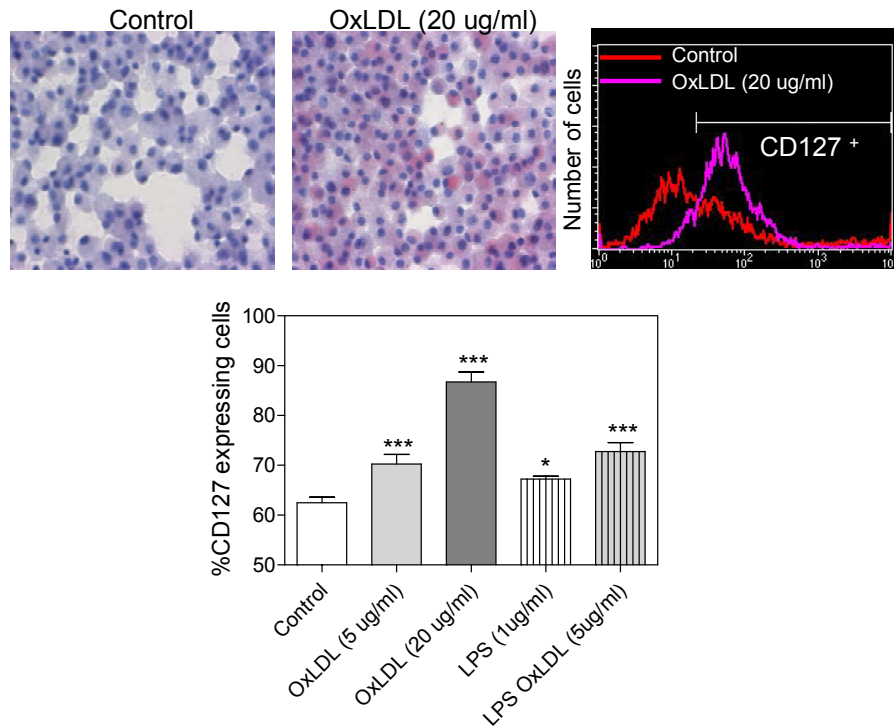


Figure 2: Bone marrow derived macrophages upregulate the expression of CD127 when exposed to oxidized LDL. Bone marrow derived macrophages were cultured using L929 conditioned medium and subsequently exposed to different concentrations of OxLDL and/or LPS. After 16 hours of incubation, the expression of CD127 on the cells was monitored using FACS. Upper panels give representative examples of control cells and cells exposed to 20 μ g/ml OxLDL stained with Oil red O and in the right panel representative histograms are shown for CD127 expression of these cells. Lower panel, *, $p < 0.05$, ***, $p < 0.001$ compared to control situation)

Macrophage expression of CD127 is upregulated after exposure to oxLDL

To investigate the expression and regulation of CD127 on macrophages during atherosclerotic plaque formation we cultured bone marrow derived macrophages from C57/Bl6 mice by using M-CSF. We exposed these bone-marrow derived macrophages to increasing concentrations of oxLDL and/or LPS to induce foam cell formation or cellular activation. Figure 2 shows representative pictures of control and oxLDL treated (20µg/ml) cells (upper panels). After 16 hours we assessed the expression of CD127 by FACS as shown in upper right panel. OxLDL exposure very significantly induced the expression of CD127 during foam cell formation already at low concentrations (5µg/ml), and the effect is more pronounced at an oxLDL concentration of 20 µg/ml. TLR4 stimulation of the macrophages with LPS did only result in modest upregulation of CD127 and LPS had no additional effect on the expression of CD127 when administered simultaneously with 5µg/ml oxLDL.

Lesion formation is severely aggravated after vaccination against CD127 in LDL receptor deficient mice

To investigate the role of IL-7/CD127 signaling during atherogenesis we constructed a DNA vaccine against CD127 using *S. typhimurium* as a carrier. Vaccination will result in the generation of CD8⁺ cytotoxic cells (as shown previously by our group²⁶), and these cells selectively remove cells that express high levels of CD127 and present epitopes thereof via MHC-I.

We vaccinated female LDLr^{-/-} mice by oral administration of *S. typhimurium* transformed with pcDNA3.1-CD127 or pcDNA3.1 empty (control) in 100 µl of PBS at two-week time intervals (n=12-13/group). Following the last vaccination, mice were placed on a Western type diet and two weeks later collars were placed around the common carotid arteries to generate flow-induced atherosclerosis. Six weeks later the mice were sacrificed and atherosclerotic plaque formation was analyzed. Representative pictures of control (A) and CD127 vaccinated mice (B) are shown in figure 3. Vaccination against CD127 resulted in a significant 2.7-fold induction of atherosclerotic lesion formation ($2.13 \pm 0.73 \cdot 10^4 \mu\text{m}^2$ (control) vs. $5.67 \pm 1.06 \cdot 10^4 \mu\text{m}^2$ p=0.004). This translated into a significant increase in both intima/media (D) as well as intima/lumen (E) ratio, showing a significant narrowing of the lumen.

Vaccination against CD127 affected the composition of the plaque and reduced the cellularity of plaques compared to control vaccinated mice (Figure 3F). Nuclei density per mm² was decreased by 32% (4.10 ± 0.67 (control) vs. 2.76 ± 0.22 nuclei/mm², p=0.03). This decrease in cellular density was accompanied by a significant increase in apoptosis identified by relative TUNEL positive area ($5.2 \pm 1.8 \cdot 10^{-3}$ (control) vs. $11.2 \pm 2.3 \cdot 10^{-3}$, p=0.04, Figure 4). Representative pictures of control (A) and CD127 vaccinated (B) mice are shown in figure 4. No significant effects on cholesterol and triglyceride levels were observed.

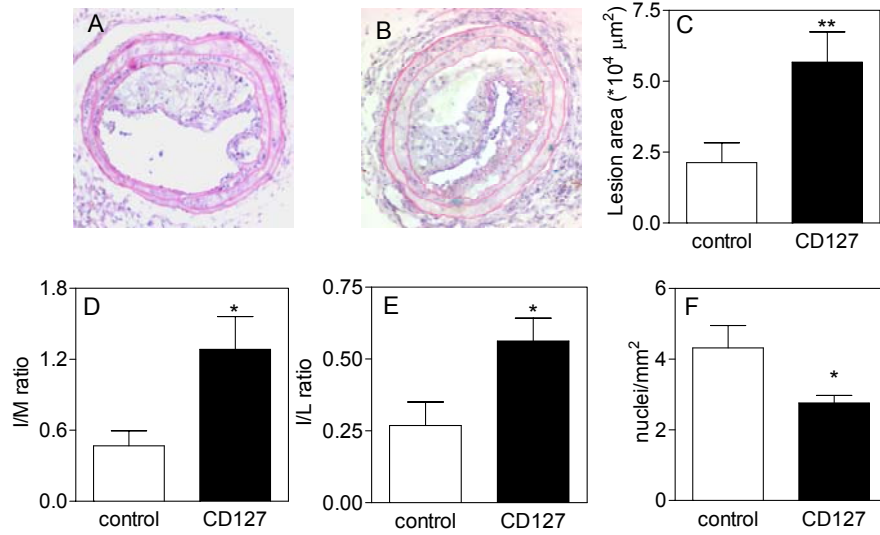


Figure 3: Vaccination against CD127 significantly induced atherosclerotic plaque formation in LDL receptor deficient mice. Upper panels demonstrate representative pictures of the carotid artery of control (A) and CD127 vaccinated animals (B). A significant increase ($p=0.004$) was observed in carotid plaque area after vaccination against CD127. This decrease in lesion formation is accompanied by a significant increase in lumen stenosis (D) and intima/lumen ratio (E). Plaque cellularity is decreased in mice vaccinated against CD127 (F). Error bars represent SEM, $n = 12/13$ per group. (*= $p<0.05$, Mann Whitney test).

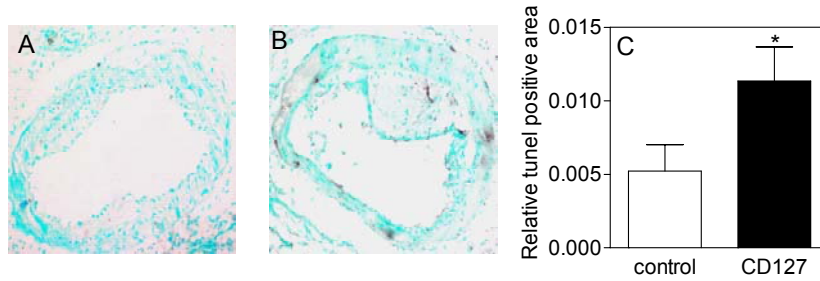


Figure 4: Apoptosis is increased in atherosclerotic plaques of mice vaccinated against CD127. Corresponding sections of carotid artery atherosclerotic plaques were stained for apoptosis using a TUNEL stain. Apoptosis is indicated by brown staining. Representative slides are shown for control (A) and CD127 vaccinated (B) animals. We then quantified the TUNEL positive area relative to total plaque area. Vaccination against CD127 led to a significant increase in TUNEL positive area compared to control vaccinated mice ($n=12/13$, $p=0.04$).

Vaccination against CD127 leads to a relative reduction in macrophages and an increase in B cells in the spleen

Vaccination against CD127 will result in the removal of activated cells that express high levels of CD127 and present epitopes thereof via their MHC-I molecules. As T cells, B cells and macrophages express CD127, we analyzed the effect of CD127 vaccination on the cellular phenotypes in the spleen by FACS (Figure 5). Spleen cells were isolated and macrophages were identified as F4/80

positive. A significant reduction was seen in the relative number of macrophages after vaccination against CD127 ($22.16 \pm 1.20\%$ (control) vs. $16.15 \pm 1.03\%$, $p=0.003$). A relative increase was shown for B cells, identified by their expression of CD19 ($32.90 \pm 1.78\%$ vs. $40.04 \pm 0.41\%$, $p=0.004$). In order to determine whether the capacity of the mature B cells to produce antibodies against antigens was changed, we determined isotope specific antibodies against oxLDL in control and CD127 vaccinated mice. No significant effects were seen on the circulating IgG1, IgG2a or IgM antibodies against oxLDL after vaccination (Figure 6). This indicated that CD127 vaccination had no apparent effects on B cell function. No effect was observed on the relative numbers of CD4 and CD8 positive T cells in the spleen after vaccination against CD127 (Figure 5, lower panels).

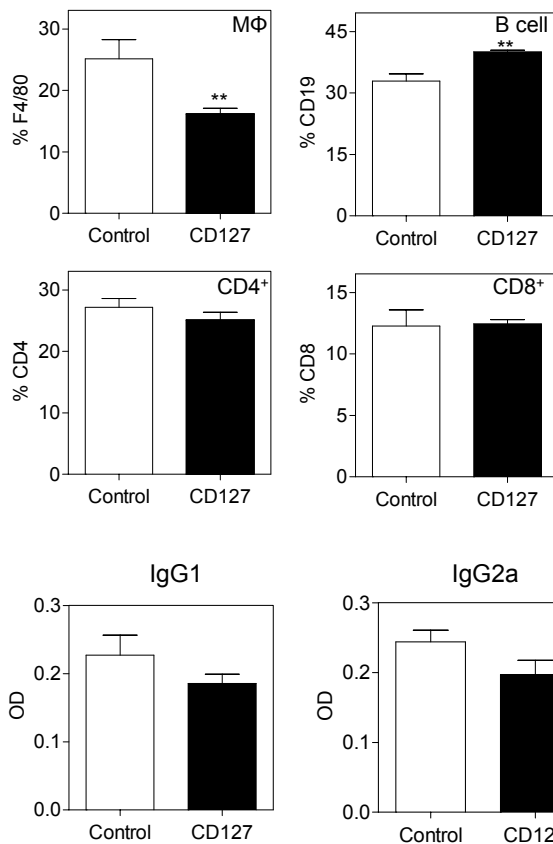


Figure 5: The effect of CD127 vaccination on different cell types of the spleen. Single cell suspensions of spleens of control and CD127 vaccinated mice were prepared and FACS was used to determine the percentage of CD4 and CD8 positive T cells, macrophages (F4/80) and B cells (CD19). Vaccination against CD127 led to a significant decrease in macrophages and to a significant increase in the percentage of B cells. (White bars represent control, black bars represent CD127 vaccinated mice, error bars display SEM, $n=6$ per group, *: $p<0.05$)

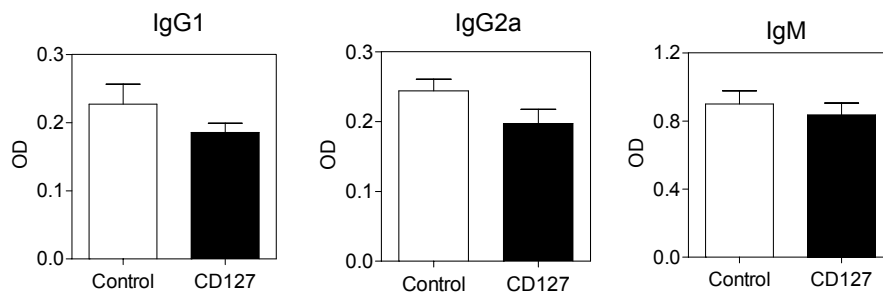


Figure 6: Antibody production against oxLDL is not changed after vaccination against CD127. Specific isotypes of antibodies against oxLDL were determined by ELISA in mice after 8 weeks of Western type diet. Relative concentrations of control and CD127 vaccinated mice are shown. No significant effects could be observed. (White bars represent control mice ($n=12$), black bars represent CD127 vaccinated mice ($n=13$). Error bars represent SEM).

Vaccination against CD127 reduced the number of CD4⁺CD25⁺ regulatory T cells

Recent publications suggest a role for CD127 regulation in the survival and activation of CD4⁺CD25⁺ regulatory T cells¹⁵. We isolated lymph nodes draining from the aortic arch of control and CD127 vaccinated mice after 8 weeks of Western type diet. A significant reduction was observed in the percentage of CD4⁺CD25⁺ regulatory T cells in the draining lymph nodes from the aortic arch after vaccination against CD127 (6.00 ± 0.53 vs. 4.17 ± 0.46 %, $p=0.02$, Figure 7). The relative number of CD4 and CD8 single positive T cells was not altered in the lymph nodes.

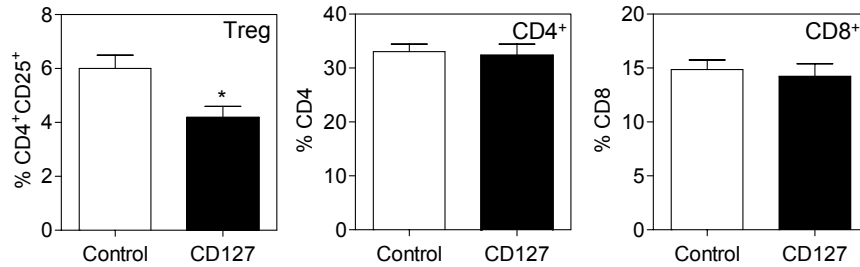


Figure 7: Vaccination against CD127 decreases the number of regulatory T cells in lymph nodes draining from the aortic arch without altering general T cell numbers. Draining lymph nodes were isolated from control and CD127 vaccinated mice after 8 weeks of western type diet feeding. Single cell suspensions were prepared and FACS was used to assess the relative amount of CD4⁺CD25⁺ regulatory cells present. Vaccination resulted in a significant decrease in the number of regulatory T cells defined as CD4⁺CD25⁺ double positive. No effects were seen on the general T cell percentages. (White bars represent control, black bars represent CD127 vaccinated mice, error bars display SEM, $n=6$ per group, *: $p<0.05$)

Discussion

The regulation and activation of the immune system during atherogenesis has received considerable attention over the last few years²⁷⁻²⁹. The function of T cells and their interaction with other leukocytes has received special interest. IL-7 has primarily been described as a factor in the development of the immune system, but recent evidence suggests that it also has a prominent role after the embryonic phase, in the regulation and activation of regulatory and memory T cells. Next to these described effects on T cells, a limited number of publications indicate a role for the IL-7/IL-7 receptor pathway in myelopoiesis and erythropoiesis⁹.

The receptor for IL-7 is composed of two subunits, the γ subunit, constitutively expressed at low levels on lymphocytes, and CD127 (the α subunit) that is restricted to specific stages of differentiation to mediate cellular responses^{12,30}.

To investigate the role of IL-7/CD127 signaling in atherosclerosis, we analyzed the expression of CD127 in the aortic arch of mice on a western type diet.

Expression of CD127 increased over time, which largely paralleled the increase in macrophage influx in the aorta as determined by expression of CD68. This led us to determine the regulation of CD127 expression on macrophages in an athero-prone environment with high concentrations of oxidized LDL. Interestingly, a significant upregulation of the IL-7 receptor was observed in a concentration dependent mechanism after 24 hours of oxLDL treatment of isolated bone-marrow derived macrophages. This is not the effect of general macrophage activation, as the exposure to LPS did only result in modest upregulation of the IL-7 receptor. Furthermore, no additional effects of LPS were observed when it was added in combination with oxLDL. This indicated the CD127 expression on the surface of macrophages is induced in a pro-atherogenic environment where high concentrations of oxidized lipid are present. This is a very interesting finding that indicated a functional role for IL-7/CD127 signaling in macrophages during atherogenesis, next to the suggested function in T cell homeostasis.

We then used a vaccination strategy to study the functional role of CD127 in a diet induced model for atherosclerosis. In this vaccination strategy, *S. typhimurium* is transformed with a vector encoding murine CD127. The *S. typhimurium* carrier is taken up by M cells and processed in the Peyer's patches in the gastrointestinal tract where antigen presenting cells become activated by the live attenuated bacteria. The bacteria are processed by the antigen presenting cell and in this way the vector is transcribed and presented. These MHC class I presented peptides activate specific cytotoxic T cells that will clonally expand and break peripheral tolerance against CD127. This vaccination protocol results in the induction of CD8⁺ cytotoxic T cells that specifically target cells that express high levels of CD127 and has been successfully validated for other proteins in animal models for tumor growth and atherosclerosis^{24, 26, 31}.

Vaccination against CD127 will lead to the removal of cells that express high levels of CD127 and the observed upregulation of CD127 on macrophages is therefore likely to lead to removal of these cells via CD8 mediated cytotoxicity. We clearly show that vaccination against CD127 indeed leads to a significant reduction in macrophage numbers in the spleen of mice on a western type diet compared to control vaccinated mice. Next to this systemic effect, the cellularity of atherosclerotic plaques from CD127 vaccinated mice was reduced after vaccination indicating a reduction in the number of viable macrophages within the plaque.

We therefore conclude that CD127 is induced by macrophages in a pro-atherogenic situation. This upregulation can have several functions. IL-7 signaling in T cells is a potent inducer of anti-apoptotic survival factors such as bcl-2 and its family members³². It may be speculated that macrophages need survival signals to cope with the pro-apoptotic and toxic environment of the atherosclerotic plaque with high levels of oxidized lipoproteins and reactive oxygen species. The resulting upregulation of CD127 will result in the removal

of these cells via CD8⁺ cells in mice vaccinated against CD127. To further investigate this hypothesis we stained plaques from control and CD127 vaccinated mice for apoptosis using an anti-TUNEL antibody. As expected, plaques from CD127 vaccinated mice showed increased apoptosis, even when corrected for the increased plaque area.

Induction of macrophage apoptosis in the atherosclerotic plaque due to the vaccination possibly has a direct impact on plaque growth. In literature, it is however shown by several authors that specific induction of macrophage apoptosis during lesion formation does not induce, but reduces or does not affect lesion formation. Among others this was shown by selective induction of macrophage apoptosis by using Diphtheria toxin receptor (DTr) transgenic bone marrow transplantation which resulted in decreased lesion formation³³. Bone marrow transplantation using Bax deficient donors resulted in increased lesion formation³⁴. This is in concordance with the DTr experiment, as Bax is a pro-apoptotic factor and deletion of this factor will reduce macrophage apoptosis. No effect was observed on plaque growth in apoE mice overexpressing the pro-apoptotic factor p53³⁵. These studies indicate that in our experiments, an additional effect is most likely responsible for the vast induction on lesion formation.

Next to this surprising effect on the macrophage population we evaluated the number and phenotype of the lymphocyte population after vaccination in our model for atherosclerosis. Extensive studies have shown the importance of IL-7 for the maintenance of the lymphocyte population in both humans and mice. Most of this research however, concentrates on the role of this molecule in early life. We show that the relative number of both CD4 and CD8 positive cells in spleen and lymph nodes is not altered after vaccination against CD127. This is in line with studies that show IL-7 acts predominantly as a thymopoietic molecule in the development of the immune system³⁶.

Farrar et al. however have proposed that the activation STAT-5 via IL-7/CD127 signaling is important in the development of CD4⁺CD25⁺ T cells³⁷. Next to this, IL-7 is essential for the survival of these regulatory T cells¹⁵. Our data clearly show that vaccination against CD127 led to a significant reduction in CD4⁺CD25⁺ regulatory T cells in the draining lymph nodes of the aorta. Several recent publications have shown the protective effect of these CD4⁺CD25⁺ regulatory T cells in atherosclerosis^{38,39}. The decrease we observe in this important T cell population could possibly explain the vast increase in plaque formation in the carotid artery of mice vaccinated against CD127.

CD127 is shared by the TSLP receptor, so besides modulating IL-7 signaling, it is possible that the signaling of TSLP is influenced as well. TSLP is an IL-7 like cytokine that supports the activation of immature dendritic cells (DC's) and it might be an early trigger for DC-mediated allergic inflammation⁴⁰. TSLP is also

produced in the thymus where it instructs thymic DC's to induce auto-reactive T cells to become CD4⁺CD25⁺FOXP3⁺ regulatory T cells⁴¹. Furthermore, TSLP is associated with Th2 induction via OX40L⁴². These findings indicate that TSLP is associated with an athero-protective function via the induction of regulatory T cell function and the production of Th2 cytokines with anti-atherogenic properties⁴². Vaccination against CD127 would lead to decreased TSLP function, and, based on its athero-protective properties described above, to an increased atherogenesis. We show that vaccination against CD127 indeed leads to accelerated lesion formation, including TSLP as a possible target cytokine of our vaccination.

In general we conclude that macrophages upregulate CD127 in response to oxLDL and that the expression of CD127 is increased in atherosclerotic plaques compared to healthy vascular tissue. Vaccination against CD127 leads to severely increased atherosclerotic plaque formation, by reducing the number of regulatory T cells in draining lymph nodes and by increasing macrophage apoptosis. Based on the results presented here, CD127 signaling is providing an anti-atherogenic stimulus to both macrophages and regulatory T cells and further research is necessary to fully understand the regulation of this multi-functional and pleiotropic signaling route in vascular biology.

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Chapter 10

Summary and Perspectives

EJA van Wanrooij

Summary

Despite impressive progress in the treatment of cardiovascular disease by widespread statin administration and reduction of other risk factors, atherosclerosis derived clinical events are still the major cause of death in the Western hemisphere. As established in the last decade, atherosclerosis is a lipid associated inflammatory disease of the larger and medium sized arteries¹⁻⁵. Although the beneficial effects of statins are partially due to their pleiotropic anti-inflammatory effects, no therapy is available that specifically reduces the inflammation associated with atherogenesis. In this thesis, several approaches are presented that offer therapeutic immuno-modulatory possibilities for atherosclerosis. At present, therapy is provided to patients that already show clinical cardiovascular symptoms or have elevated cholesterol levels. This implies that lesion formation in these patients can only be targeted at an already advanced stage. Since atherosclerosis is a disease that starts at a very young age, identification of new targets during the initiation of the atherosclerotic process will be highly relevant. There is however, little knowledge on the mechanisms of the initial changes in the vessel wall that precede the formation of an atherosclerotic plaque.

Differential gene regulation during initial atherogenesis

To get insight in the initial vascular changes preceding lesion formation, the transcriptional regulation within the vessel wall was monitored in an animal model for atherosclerosis, the LDL receptor deficient mouse. In this model, atherosclerosis develops upon Western type diet administration as a consequence of an increase in serum VLDL/LDL resembling a more “human” like pro-atherogenic lipoprotein profile⁶. As gene regulation is mostly exerted group-wise by means of transcription factors, we searched for transcription factors that potentially are involved in the regulation of genes associated with very early changes in atherogenesis. It appears that FOXO1 mediated transcription is enhanced already after 1.5 weeks during Western type diet feeding, and that this is maintained throughout the administration of the diet. This was further substantiated by the significant upregulation of the FOXO1 downstream gene HMG-CoA reductase. Interestingly, HMGCoA reductase is the target enzyme of statins that specifically inhibit this enzyme and the subsequent synthesis of cholesterol.

Infiltration of leukocytes into the vessel wall is one of the hallmarks of atherosclerosis. Attraction of leukocytes is mediated not only by the upregulation of adhesion molecules and chemokines by the vessel wall, but by the activation of leukocytes as well. Therefore, the reaction of the spleen in response to the diet was monitored during atherosclerotic lesion formation. High fat diet administration resulted in profound upregulation of established pro-inflammatory cytokines such as IFN γ , IL-1 β , CD40 and CCL5. Interestingly, this response showed a sharp peak at 6 weeks of diet administration. A number of T cell activation markers from the TNF receptor superfamily were vastly upregulated at this time point as well. As upregulation of these molecules reflected a specific activation of T cells, the response of CD4⁺ cells was investigated on mRNA level by using micro-arrays. This cell type is identified as the predominant T cell type in atherosclerosis^{7, 8}. The transcription of the CD4⁺ cells revealed a different time dependent regulation pattern. At 6 weeks of diet feeding no upregulation of immune response related activation markers was observed, but after 12 weeks the expression of TNFRSF members, adhesion molecules and Th1/Th2 differentiation was increased. The only upregulated gene set associated with immune responses that was present at both 6 and 12 weeks was the CCR5 pathway. This indicates that the observed pattern in the spleen is possible a primary (innate) response to the high fat diet feeding. Only after a longer period of time (possibly 9 weeks), the activation of the adaptive T helper cells is initiated.

After the identification of several genes and pathways that are clearly differentially regulated during atherogenesis, some of these targets were verified in a model for atherosclerotic plaque formation.

Inhibition of leukocyte migration in atherogenesis

Our microarray studies indicated that the CCR5 pathway was associated with plaque formation as upregulation of CCR5 in the vessel wall coincided with an enrichment of the CCR5 pathway on T cells during initial lesion formation. This chemokine receptor has a prominent role in the attraction of leukocytes, predominantly Th1 cells, to sites of inflammation⁹. As atherosclerotic plaque formation is enhanced by the activation and subsequent release of Th1 cytokines, it could be speculated that inhibition of CCR5 signaling would lead to attenuated plaque formation. To specifically target the migration of these Th1 cells we used the HIV entry inhibitor TAK-779. This molecule is a potent antagonist for both CCR5 and CXCR3, a chemokine receptor that is also predominantly expressed on Th1 cells¹⁰. Treatment with TAK-779 resulted in a vast decrease in lesion formation. This decrease was due to reduced migration of Th1 cells which led to a 98% reduction in the expression of the pro-atherogenic Th1 cytokine IFN γ . TAK-779 is an HIV entry inhibitor and can be used in HIV-positive patients to repress HIV replication. Current treatment of these patients with protease inhibitors has been associated with increased evidence of cardiovascular events and increased levels of atherogenic lipoproteins such as LDL and VLDL¹¹. Our study shows that TAK-779, besides blocking HIV entry, also reduces atherosclerotic lesion formation and in this way has potential benefits for the growing population of young HIV positive patients.

After we established the protective effects of a CCR5/CXCR3 antagonist, we focused our attention on CXCR3 itself. CXCR3 is a chemokine receptor that is mostly expressed on Th1 effector cells¹². CXCR3 positive T cells have been shown to aggravate several immune diseases and we anticipated that the specific inhibition of CXCR3 by the CXCR3 antagonist NBI-74330 would lead to a reduction of plaque formation. We indeed observed a significant reduction in lesion formation upon treatment with NBI-74330. CXCR3 is expressed on effector T cells that drive the inflammation. We assessed the balance between activated effector cells and regulatory cells and found that treatment with a CXCR3 antagonist beneficially increased the number of regulatory T cells.

The trans-endothelial migration of leukocytes to the atherosclerotic plaque is facilitated via adhesion molecules expressed in the endothelial junction and this process is named diapedesis. Blockade of this transmigration route provides an attractive way to inhibit atherosclerotic plaque formation. Previously it was shown that mice deficient in PECAM-1 or JAM-1, two proteins that facilitate diapedesis, develop less atherosclerosis than control mice^{13, 14}. When an additional member of the junctional adhesion molecules was identified, CD99, a pro-atherogenic role for this molecule could be suggested. In order to elucidate the function of CD99 during atherosclerotic lesion formation, a DNA vaccine that encoded the extracellular part of CD99 was constructed and attenuated

Salmonella transformed with the CD99 plasmid was used to induce cellular immunity against cells that express CD99. This vaccination strategy induces a CD8⁺ mediated cytotoxic response against cells that express CD99. Vaccination against CD99 resulted in a vast decrease in lesion formation and this decrease was accompanied by decreased expression of CD99 on leukocytes. By using a fast and flexible vaccination strategy, we identified CD99 as a pro-atherogenic molecule. However, more research is needed to delineate the exact mechanism by which CD99 induces lesion formation.

Not only chemokines and adhesion molecules contribute to the migration of leukocytes to the plaque, but some interleukins also display chemotactic properties. IL-16 is one of these signaling molecules, and based on this property it was originally named Leukocyte Chemotactic Factor (LCF)¹⁵⁻¹⁷. It attracts CD4⁺ cells and was implicated as pro-inflammatory in several auto-immune diseases. Based on the pro-inflammatory properties described in literature, we anticipated that IL-16 would attract leukocytes to the atherosclerotic plaque, and that by blocking IL-16 signaling would inhibit atherosclerotic lesion formation. Surprisingly, in chapter 7 it is described that IL-16 has anti-atherogenic properties. Vaccination against this interleukin results in accelerated lesion formation, especially after prolonged periods of diet feeding. IL-16 is synthesized as a precursor that needs to be cleaved by caspase 3 in order to be secreted. Caspase 3 is a rate limiting enzyme in the apoptosis cascade, and it was shown that apoptotic cells spontaneously released IL-16. Apoptosis is a phenomena predominantly associated with more advanced stages of atherosclerosis, indicating why IL-16 vaccination would be more detrimental in late stage atherogenesis. Possibly IL-16 mediates a migration signal for CD4⁺ T cells with regulatory phenotype, as IL-16 induces CD25 expression on T cells. When IL-16 signaling is blocked, lesion formation is accelerated by the reduced attraction of regulatory T cells.

Inhibition of leukocyte activation in atherogenesis

Next to the migration of leukocytes, the activation of immune cells provides another interesting signaling cascade associated with atherosclerotic lesion formation. It was shown that the activation of Th1 cells and NKT cells leads to aggravation of atherogenesis¹⁸⁻²⁰. As shown in chapter 3, T cell co-activation markers from the TNF receptor superfamily are upregulated after Western type diet feeding and subsequent atherosclerotic lesion formation. For OX40, a member of this superfamily, a relation was found between the expression of this molecule on T cells, and the severity of several auto-immune diseases such as RA and EAE^{21, 22}. We therefore monitored the expression of OX40 on T cells during lesion formation and found a significant upregulation of this molecule during atherogenesis providing a rationale for therapeutic blocking of this pathway

during atherogenesis. The antibody mediated blockade of the ligand of OX40 (OX40L) that is expressed by APC's is described in chapter 8. Blockade of OX40/OX40 ligand signaling results in reduced lesion formation, however not by modulating the expression of Th1 cytokines such as IFN γ or the migration of effector cells to the atherosclerotic plaque. This was rather puzzling, as the reduction in lesion formation was quite prominent. OX40 was however not only described as a potent inducer of Th1 responses, but could induce Th2 activation as well. We therefore determined the concentration of oxLDL specific antibody isotypes IgG1, IgG2a and IgM. The production of antigen specific IgG is mediated by the activation of B cells by specific subsets of T helper cells. Th1 cells induce isotype switching to IgG2a, and Th2 cells induce production of IgG1 antibodies. It appears that Th2 mediated isotype switching was reduced due to decreased production of IL-4, and this led to decreased levels of IgG1 oxLDL specific antibodies. Because isotype switching was inhibited, B cells continued to produce the more aspecific IgM antibodies. Binder et al however showed that IgM antibodies are produced in response to IL-5, which is a Th2 associated interleukin. We showed that IL-5 levels indeed were increased upon OX40L antibody administration. The discrepancy between decreased IL-4 levels and increased IL-5 levels was rather unexpected, as these interleukins are mainly regulated in a similar way. More research is necessary to determine the exact role of these Th2 cytokines in atherogenesis.

As described in the introduction, T cell homeostasis is essential in the immune response during atherogenesis. IL-7 is an interleukin that has major functions in the regulation of T cell survival and differentiation²³. We therefore were interested in the function of IL-7 during atherosclerotic lesion formation. A vector that contained the coding region for IL-7 receptor (CD127) was designed and used to create a DNA vaccine. Vaccinated LDL receptor deficient mice was performed and the effect of the vaccination on lesion formation was determined. Vaccination against this receptor for IL-7 severely increases the lesion burden in LDLr^{-/-} mice by 300%. Lesions from vaccinated mice showed increased macrophage apoptosis and reduced numbers of regulatory T cells were found in the lymph nodes draining from the aortic arch. The increase in macrophage apoptosis was rather unexpected, as CD127 expression was mostly reported on T cells. We however showed that exposure of macrophages to oxLDL greatly increases the expression of CD127. As our vaccination strategy selectively targets cells that express high levels of this molecule, removal of these cells will be expected.

Concluding remarks and future perspectives

Upon review of the various aspects of this thesis, the question raises which therapeutic field will eventually become most promising for use in a clinical setting. The research in this thesis provides tools for inhibition of leukocyte migration, and interference with leukocyte activation. Both fields have their own pros and cons and this paragraph will discuss some of these considerations.

Inhibition of leukocyte migration has been shown to be very effective in the attenuation of atherosclerosis. It must however be stated that leukocyte egress is essential in almost all inflammatory processes. Non selective inhibition of leukocyte migration will most likely interfere with essential defense mechanisms such as the clearance of pathogens. This especially is a problem when the treatment can not be reversed, which is evident with vaccinations. Vaccination against CD99 non selectively impairs leukocyte migration, and therefore its application may lead to clinical inflammatory complications. Ewing sarcoma's however highly express CD99, have a very bad clinical outcome and are predominantly seen in very young children. In this patient population, vaccination against CD99 would be very beneficial and probably life saving.

Inhibition of migration of a leukocyte subset, instead of the total population would erase some of the above stated risks. The inhibition of migration of CXCR3 and/or CCR5 positive cells potentially decreased the formation of atherosclerotic plaques. These receptors are predominantly expressed on a small subset of T cells that have a clear pro-atherogenic effect. It was shown in literature that bacterial infections and viral infections in mice deficient for both receptors did not result in increased lethality²⁴⁻²⁷. This indicates that no large scale systemic immune suppression is reached during treatment with CCR5/CXCR3 antagonists. It was already mentioned that an additional property of CCR5/CXCR3 antagonists is their inhibitory effects on HIV replication. Treating HIV positive patients with these antagonists will therefore be hitting two birds with one stone²⁸. The replication of the virus will be attenuated, but at the same time, severe cardiovascular side effects that are the result of the currently used protease inhibitors will be counteracted.

This thesis has clearly shown that inhibition of the migratory properties of a specific subset of T cells can also have adverse effects. Vaccination against the CD4⁺ T cell migration factor IL-16 severely increased the lesion burden in later stages of atherosclerosis. However, no effects were visible during initial lesion formation. This shows that intervention in a signaling cascade can have differential effects at different time points of lesion formation. It implies that a drug or vaccination with significant beneficial effects on initial lesions can lead to destabilization of advanced plaques or the other way around. Evidently, plaque formation in humans is not a linear process, and plaques of different stages are present at the same time. It is therefore of the utmost importance to identify the exact role of a molecule in initial as well as advanced lesions.

Not only minimizing migration of pro-atherogenic cells can provide protection against atherogenesis, inhibition of activated T cell signaling and co-stimulation can lead to attenuated lesion formation as well. This was shown by antibody treatment against OX40 ligand. OX40L is expressed on antigen presenting cells, and binds to OX40 on activated T cells. Targeting OX40 positive T cells has a clear advantage above general immune-suppression since these cells are mainly associated with auto-inflammatory disorders. The selective suppression of the activation and proliferation of this subset has clinically relevant properties for atherosclerosis and other auto-immune diseases. Repression of co-stimulation may be preferred above stimulation of suppressive T cell subsets, as was shown by the administration of a CD28 antibody designed to boost T cell activation and the expansion of regulatory T cells. Due to the lack of well designed pro-clinical experiments, 6 healthy volunteers that received this antibody developed severe clinical symptoms associated with major release of T cell cytokines and subsequent multiple organ failure²⁹.

Next to the specific de-activation of T cells, we determined the role of IL-7 signaling in atherosclerosis. For this purpose, we developed a vaccination strategy to remove cells that expressed high levels of IL-7 receptor (CD127). We designed this study to remove activated T cells and presumably to decrease lesion formation in this way. However, we found that macrophages show increased expression of this receptor as well, especially in the context of high levels of oxidized LDL. This was not documented yet, and the increase in lesion formation after vaccination against CD127 was at least partially due to increased macrophage apoptosis. This shows that a signaling pathway can be very well documented in a certain subset of cells, but completely ignored in the analysis of other relevant cell types.

In conclusion, modulation of leukocyte activation and migration provides an attractive possibility for future drug design in the field of atherosclerosis. However, due to the complexity of immunological signaling cascades, extensive pre-clinical characterization is needed before these potential new drug candidates can be applied in the clinic.

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Nederlandse Samenvatting

EJA van Wanrooij

Hart en vaatziekten zijn de meest voorkomende doodsoorzaak in de westerse wereld. De onderliggende oorzaak van infarcten en beroertes is voornamelijk het dichtslippen van de grote slagaders door middel van de vorming van een atherosclerotische plak, ook wel aderverkalking genoemd. De klinische manifestaties van atherosclerose zijn het gevolg van volledige blokkade van de bloedsomloop van het vat. Dit kan gebeuren doordat de plaque het volledige vat afsluit, maar ook na een eventuele scheuring van de plaque waardoor een blokkerend bloedstolsel ontstaat. In beide gevallen blijft achterliggende weefsel verstoken van voedingsstoffen en het meest desastreus, zuurstofrijk bloed. Op deze manier kan atherosclerose in de vaten in het hart leiden tot een myocard infarct, en een vernauwing van de vaten in de hersenen zorgen voor een beroerte.

In de laatste 20 jaar is in kaart gebracht welke risicofactoren bijdragen aan het ontstaan van atherosclerose, en de meest belangrijke daarvan zijn verhoogde cholesterol waarden in het bloed, roken, hoge bloeddruk en weinig lichaamsbeweging. De huidige therapieën spitsen zich dan ook vooral toe op het verlagen van deze risicofactoren. Zo wordt een te hoog cholesterol gehalte in het bloed behandeld met statines, en wordt mensen aangeraden minimaal 30 minuten per dag te bewegen. Dankzij grootschalige behandeling is de incidentie van hart en vaatziekten sterk gedaald, maar nog steeds vormen zij de belangrijkste doodsoorzaak in Nederland en in andere Westerse landen.

In de afgelopen tien jaar is het duidelijk geworden dat niet alleen hoge cholesterol waarden en roken een rol spelen in atherosclerose. Ook het immuun systeem blijkt betrokken te zijn bij de vorming van atherosclerotische plaques. De algemeen geaccepteerde theorie op dit moment is dat de binnenwand van het vat geactiveerd raakt door bijvoorbeeld roken, een hoge bloeddruk, genetische aanleg of andere risicofactoren. De initiële activatie van de vaatwand zorgt ervoor dat de cholesterolvervoerende deeltjes in het lichaam, de lipoproteïnen, door de vaatwand heen kunnen migreren. Eenmaal binnenin de vaatwand aangekomen, worden deze deeltjes gemodificeerd. Daarnaast zorgt het geactiveerde bloedvat ervoor dat er immuuncellen worden aangetrokken om de beschadiging te verhelpen. Deze immuuncellen, in het bijzonder macrofagen zijn bijzonder geschikt voor het opruimen van allerlei ongewenste deeltjes, zoals bacteriën en dode cellen, maar ook de gemodificeerde lipoproteïnen. Naast macrofagen zijn ook T cellen betrokken in deze fase van atherosclerose. Deze cellen scheiden moleculen uit die het immunologische proces kunnen beïnvloeden. Er bestaan T cellen die remmend werken, maar ook T cellen die ervoor zorgen dat er meer ontstekingscellen worden aangetrokken, die vervolgens bijdragen tot de groei van de atherosclerotische plaque.

In dit proefschrift worden verschillende experimenten beschreven waarin het ontstekingsproces dat plaatsvindt tijdens atherosclerose beïnvloed wordt. In **hoofdstuk 1** worden de componenten van het immuunsysteem beschreven die een rol spelen bij atherosclerose. Er wordt eerst aandacht besteed aan moleculen die de aantrekking van immuuncellen (leukocyten) reguleren, de zogenaamde chemokines. Daarnaast worden moleculen besproken die een immunologische reactie kunnen versterken of afremmen, de zogenaamde interleukines. Als laatste wordt aandacht besteed aan de oppervlakte markers op leukocyten die gebruikt kunnen worden bij de activatie van de deze cellen.

Omdat het proces dat leidt tot de vorming van atherosclerotische plaques nog lang niet volledig bekend is, hebben wij in **hoofdstuk 2** dit (gedeeltelijk) in kaart gebracht en eventuele nieuwe eiwitten geïdentificeerd die als target kunnen dienen voor de toekomstige behandeling. Hiervoor werd een genetisch gemodificeerd muismodel gebruikt dat door middel van een Westers dieet dat hoog is aan vetten en cholesterol atherosclerose ontwikkeld. In deze muis is de receptor voor LDL, een pro-atherogeen lipoproteïne weggehaald, en op dit dieet ontwikkelt deze muis een menselijk lipoproteïnen profiel. De genregulatie in de vaatwand van deze muis is geanalyseerd op verschillende tijdstippen gedurende het vette dieet en vergeleken dit vervolgens met de genexpressie in de vaatwand van gezonde muizen.

Niet alleen de vaatwand speelt een rol bij het ontstaan van atherosclerose maar ook de activatie en migratie van immuuncellen is hierbij betrokken. Naast de regulatie van de genexpressie in de vaatwand, wordt in **hoofdstuk 3** de

genregulatie van T cellen gedurende een vetrijk dieet bestudeerd. Hierbij worden nieuwe moleculen en receptoren ontdekt die een eventuele rol spelen in de immunologische activatie gedurende het ontstaan van atherosclerose.

Nadat in hoofdstuk 2 en 3 een inventarisatie is gemaakt van de mechanismen die betrokken zijn bij dieet geïnduceerde laesie vorming, hebben we in de volgende hoofdstukken verschillende therapeutische benaderingen geëxploreerd om de immunologische reactie te beïnvloeden.

In **hoofdstuk 4** wordt het effect van de toediening van een antagonist voor de chemokine receptoren CCR5 en CXCR3 beschreven. Deze receptoren bevinden zich hoofdzakelijk op de atherosclerose bevorderende T helper 1 cellen en zijn betrokken bij de migratie van deze cellen in de richting van een ontsteking. Het blokkeren van deze Th1 migratie leidde tot een vermindering in plaquevorming in LDL receptor deficiënte muizen. Deze vermindering wordt veroorzaakt doordat de migratie van de Th1 cellen naar de plaque volledig geblokkeerd is. Dit is onder andere aangetoond door een bijna totale afwezigheid van het pro-atherogene Th1 cytokine interferon gamma in de plaques van de behandelde muizen.

Een vergelijkbaar experiment is uitgevoerd met een specifieke antagonist van CXCR3. De resultaten van dit onderzoek zijn beschreven in **hoofdstuk 5**. Ook hier was duidelijk te zien dat een blokkade van CXCR3 het ontstaan van atherosclerotische plaques remt. Deze vermindering in plaque formatie blijkt het gevolg van een toename in lokaal aanwezige regulatoire T cellen. Deze T cellen zijn bijzonder efficiënt in het onderdrukken van een immuunrespons door het uitscheiden van anti-inflammatoire cytokines. De aanwezigheid van één van deze cytokines, TGF β , was significant verhoogd in plaques van therapeutisch behandelde muizen.

In **hoofdstuk 6** beschrijven we voor de eerste keer de pro-atherogene rol van het onlangs geïdentificeerde molecuul CD99. CD99 is een receptor die zowel tot expressie komt op leukocyten als op het endotheel, de cellaag die de binnenkant van een bloedvat bekleedt. Interactie tussen CD99 op de immuun-cel met CD99 op het endotheel is nodig voor de migratie van leukocyten naar de plaque. Een vetrijk dieet activeert het endotheel waardoor de expressie van CD99 verhoogd. De rol van CD99 in atherosclerose is vervolgens bestudeerd door een vaccin te genereren dat specifiek cellen met een hoge expressie van CD99 wegvangt. Wanneer LDLr deficiënte muizen gevaccineerd worden tegen CD99 ontwikkelen ze 70 procent minder atherosclerose en daarnaast is de celdichtheid van deze plaques ook lager.

In dit proefschrift wordt ook een vaccinatie beschreven tegen interleukine-16 (IL-16) waarvan de resultaten zijn weergegeven in **hoofdstuk 7**. Dit molecuul induceert de migratie van een specifiek type T cel, de CD4 positieve T helper

cel. Omdat er in verschillende ziektemodellen een pro-inflammatoire werking voor IL-16 werd aangetoond, is een vaccinatieprotocol ontwikkeld om de functie van IL-16 te blokkeren in de LDL receptor deficiënte muis. Verrassend genoeg resulteert vaccinatie tegen IL-16 in een vermeerdering van atherosclerose, vooral in de verder gevorderde stadia van atherosclerose. De onverwachte anti-atherogene rol voor IL-16 is mogelijk het gevolg van de late IL-16 gemedieerde aantrekking van regulatoire T cellen. Actief IL-16 kan namelijk alleen vrij komen nadat de pro-vorm door caspase-3 wordt geknipt. Dit enzym is essentieel in het aanzetten tot geprogrammeerde celdood, ook wel apoptose genoemd. Dit proces vindt hoofdzakelijk plaats in meer ontwikkelde atherosclerotische plaques.

Niet alleen de migratie van leukocyten speelt een belangrijke rol in de vorming atherosclerotische plaques. Ook de activatie van deze cellen heeft invloed op de lokale immunologische respons. In dit proefschrift worden twee studies beschreven die de hypothese testen dat de activatie van pro-atherogene T cellen bijdraagt aan het atherosclerotische proces.

Een van de moleculen die betrokken is bij de activatie van T cellen is OX40. Dit molecuul wordt tot expressie gebracht ongeveer 3 dagen na de initiële activatie van T cellen, en heeft een co-stimulatoire functie. In **hoofdstuk 8** wordt de blokkade van de interactie van OX40 met haar ligand OX40L dat voorkomt op antigen-presenterende cellen zoals B cellen en macrofagen beschreven. Toepassing van een selectief antilichaam tegen OX40 ligand leidt tot een reductie in atherosclerose in LDL receptor deficiënte muizen. Deze reductie wordt veroorzaakt doordat de interactie tussen geactiveerde Th2 cellen en B cellen niet meer functioneert. Deze B cellen blijven daardoor beschermende IgM antilichamen tegen geoxideerd LDL aanmaken wat resulteert in verminderde atherosclerose.

IL-7 is een interleukine dat bijdraagt aan de activatie en differentiatie van T cellen. Omdat de rol van IL-7 in het proces van atherosclerose nog niet onderzocht is, hebben wij een vaccinatie ontwikkeld tegen de receptor van IL-7, CD127 (IL7R). De resultaten van deze experimenten staan beschreven in **hoofdstuk 9**. Expressie van de IL-7 receptor was beschreven op T cellen, en de vaccinatie was zo ontworpen dat deze zou moeten leiden tot de verwijdering van T cellen met een hoge expressie van IL-7R. Uit onze experimenten bleek dat niet alleen T cellen, maar ook macrofagen een zeer hoge expressie hebben van CD127, vooral nadat ze worden blootgesteld aan geoxideerd LDL. Deze blootstelling aan geoxideerd LDL zal in het bijzonder het geval kunnen zijn in de atherosclerotische plaque. Vaccinatie tegen IL-7R resulteerde dan ook in verhoogde apoptose van macrofagen in de plaque, en een toegenomen plaquegroei in LDL receptor deficiënte muizen. Daarnaast was ook het aantal regulatoire T cellen in de buurt van de plaque verlaagd, wat nog verder bijdraagt aan de inductie van laesievorming.

Het onderzoek dat beschreven staat in dit proefschrift heeft verschillende nieuwe targets opgeleverd die een aanknopingspunt kunnen vormen voor de ontwikkeling van nieuwe medicijnen tegen atherosclerose. Naast deze nieuwe targets is ook aangetoond dat door middel van vaccinaties, eenvoudig en snel de rol van een nieuw molecuul of receptor in het proces van atherosclerose onderzocht kan worden. De resultaten dragen bij aan een beter inzicht in de gecompliceerde processen die ten grondslag liggen aan de vorming van een atherosclerotische plaque, en met name in de immunologische component hiervan. Het toegenomen inzicht in de interactie tussen verschillende immunologisch actieve cellen in atherosclerose zoals beschreven in dit proefschrift, zal leiden tot een meer doelgerichte therapeutische utilisatie van het immuunsysteem bij de bestrijding van hart en vaatziekten.

Curriculum Vitae

Eva van Wanrooij was born on the 29th of March 1979 in Weert, The Netherlands. After her secondary education at the Philips van Horne SG, she obtained a masters degree in Bio-Pharmaceutical sciences at Leiden University in 2002. For her master thesis entitled “Differential gene expression during atherogenesis” she received the Susanne Hovinga award for best bio-medical master thesis. In the same year she started the PhD project described in this thesis under supervision of Dr. Johan Kuiper and Prof. Theo van Berkel. Vascular genomics, focusing on activation and infiltration of leukocytes in the vessel wall was the main area of research. Various aspects of these studies have been published and presented at international conferences. Next to her scientific research, she obtained a Bachelor of Law degree at Leiden University Law School in 2006, and will finish a Master of Corporate Law at Leiden University in September 2007. In October 2007 she will start as Patent Attorney Trainee at Philips Intellectual Properties & Standards in Eindhoven.

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Van Wanrooij EJA, Volger OL, De Vos,P, Michon I, Horrevoets AJ, Van Berkel TJC, Kuiper J: Transcriptional profiling of initial atherogenesis in LDL receptor deficient mice identifies diet induced upregulation of FOXO1 controlled genes. *Manuscript in preparation.*

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Van Wanrooij EJA, Segers FME, Willemse ME, de Vos P, Peterse N, van Berkel TJC, Biessen EAL, Kuiper J: Vaccination against IL-16 accelerates atherogenesis in LDL receptor deficient mice. *Manuscript in preparation.*

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Appendix 1: List of PCR primers

GENE	REVERSE PRIMER	FORWARD PRIMER
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
ABCB10	CAGAAACGTGCACTTCACATACC	GATGGAAAGACTGAAATCCTGGA
CCL8	AGCTACGAGAGAATCAACAATATCCA	CAGAGAGACATACCCTGCTTGGT
CCR2	CCTTGGGAATGAGTAACTGTGTGA	TGGAGAGATACCTTCGGAACCTCT
CCR3	TGCAGGTGACTGAGGTGATTG	CGGAACCTCTCACCAACAAAG
CCR5	GACTGTCAGCAGGAAGTGAGCAT	CTTGACGCCAGCTGAGCAA
CD 4	GTGAGCTGGAGAACAGGAAAGAG	GGCTGGTACCCGGACTGA
CD127	TACCGTGAGCGACAAAGATG	GCTGAATCATTGGGTACCT
CD134	GCCTTCAAAGAACTACCTAGGGACTATG	GCTCTCCTGGTTTTCTTTGCA
CD25	GGAGCCGCAAGCTAAAAGC	TGCTTCGTGCCACTGT
CD28	AGACTCCTTCAAGTGACTACCATGAAC	GGGCTGGTAAGGCTTTCGA
CD40	GGTAAGATGGCTTTTGTGGTAAA	GAAGTCCCAGGGATCAAAATCA
CD68	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTGA
CD69	AAAGCACGAGCGATCCAGTTA	AGAAAAAT AATTCGTTCTCACCAACTA
CD99	GCCGCTGCATCAGTGATG	TTGTGAGTGGAGGTTATGATGTCA
CTLA-4	GGACCACAAAGGCTTGATC	CGAGGTCTGCACCAACTG
CXCL10	GGATGGCTGTCCTAGCTCTGTAC	CCTGGCATGGCACATG
CXCR3	GCTGCTGTCCAGTGGGTTTT	AGTTGATGTTGAACAAGGCGC
FOXP3	GGAGCCGCAAGCTAAAAGC	TGCTTCGTGCCACTGT
GITR	GCCCTATGGCTGCCTTCTC	GCTAAACGTGGTGCTCTTGGT
HMGeoAred.	TCTGGCAGTCAGTGGAACTATT	CCTCGTCCTTCGATCCAATTT
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAAGCTATAG
IFN γ	ATAACTATTTAACTCAAGTGGCATAGATG T	TCT GGC TCT GCA GGA TTT TCA
IL-10	TCTTACTGACTGGCATGAGGATCA	GTCCGCAGCTCTAGGAGCAT
IL-12	GACCAAAGGGACTATGAGAAGTATTCA	CTGCTGCCGTGCTTCCA
IL-16	AGGCAACGAGGTCTTTCCA	CCGAGCTTGGCGAAGGATA
IL-17	CCAGGGAGAGCTTCATCTGTGT	AAGTCCTTGGCCTCAGTGTTTG
IL-1 β	TGG TGT GTG ACG TTC CCA TTA	AGGTGGAGAGCTTTCAGCTCATAT
IL-2	TGCCAATTCGATGATGAGTCA	GGCTTGTTGAGATGATGCTTTG
IL-4	ACTTGAGAGAGATCATCGGCATTT	AGCACCTTGAAGCCCTACAG
IL-6	GAAGAATTTCTAAAAGTCACTTTGAGATCTA	CAC AGT GAG GAA TGT CCA CAA AC
IL-9	CCGTGCTACAGGGAGGGA	TGGAAAACAGGCAAGAGTCTTG
MCP-1	GCATCTGCCCTAAGGTCTTCA	TTCACTGTCACACTGGTCACTCCTA
MIP-1 α	CCAGGGTTCTCAGCACC AAT	CTTCTCTGGGTTGGCACACA
RANTES	GCAAGTGCTCCAATCTTGCA	CTTCTCTGGGTTGGCACACA
TGF β	AGGGCTACCATGCCAACTTCT	GCAAGGACCTTGCTGTACTGTGT
TNF α	GCCAGCCGATGGGTTGTA	AGGTTGACTTTCCTGGTATGAG A
vWF	AACTTCAATAAGAGCAAGGAGTTCGT	ACTGAGATGCGCGTTGCAT

Appendix 2: Abbreviations

36B4	acidic ribosomal phosphoprotein PO
Ab	antibody
ABC	ATP binding cassette
APC	antigen presenting cell
apoE/apoB	apolipoprotein E/apolipoprotein B
CCR/CXCR	CC/CXC chemokine receptor
CCL/CXCL	chemokine ligand
CD	cluster of differentiation
CMV	Cytomegalovirus
CTLA	cytotoxic T-lymphocyte antigen
CVD	cardiovascular disease
DC	dendritic cell
EAE	experimental acquired encephalomyelitis
FABP	fatty acid binding protein
FACS	fluorescent-activated cell sorting
FMLP	formyl-met-leu-phe
Foxp3	forkhead box p3
GFP	green-fluorescent protein
GSEA	gene set enrichment analysis
HDL	high-density lipoprotein
HMG-coA	3-hydroxy-3-methylglutaryl coenzyme A
HPRT	hypoxanthine-guanine phosphoribosyltransferase
ICE	interleukin-1 β converting enzyme
i.p.	intraperitoneal
i.v.	intravenous
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon γ inducible protein
ITAC	interferon γ inducible T cell α -chemoattractant
(ox)LDL	(oxidized) low-density lipoprotein
LDLr	low-density lipoprotein receptor
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein
MHC	major histocompatibility complex
MIG	monokine induced by interferon γ
MIP-1	macrophage inflammatory protein-1
MMP	matrix metalloproteinase
NFAT	nuclear factor of activated T cells
NF- κ B	nuclear factor κ B
NK	natural killer

NKT cell	natural killer T cell
oxLDL	oxidized low-density lipoprotein
PBS	phosphate buffered saline
PPAR	peroxisome proliferators-activated receptor
RANTES	regulated on activation, normal T cell expressed and secreted
SMC	smooth muscle cell
SR	scavenger receptor
TCR	T cell receptor
TGF β	transforming growth factor β
Th1/Th2	T helper 1/T helper 2
TNF	tumor necrosis factor
Tr1	regulatory T cell type 1
Treg	regulatory T cell
TUNEL	terminal deoxynucleotidyl transferase end-labeling
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein