General Introduction

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Chapter 1

General Introduction

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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 world. 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 fibrosis. 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 sex. 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 layer. Normally functioning healthy endothelium produces anti-inflammatory and anti-thrombotic agents that prevent large scale infiltration of leukocytes and thrombus formation. So-called “injury” of the endothelium due to risk factors discussed above, results in an increased permeability and adhesiveness of the vessel wall. Endothelial cells enhance the expression of adhesion molecules and produce pro-inflammatory mediators including chemokines and interleukins.

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. 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-selectin. The expression of these molecules is induced after vascular stress, and is the result of NFkB induced activation. Firm adhesion of the now slowly rolling leukocyte is facilitated by intercellular adhesion molecule (ICAM)-1 and -2 and vascular adhesion molecule (VECAM)-1. 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-1\textsuperscript{19-21}. Additional molecules with adhesive properties on leukocytes are L-selectin, CD14 and P-selectin glycoprotein ligand (PSGL)-1\textsuperscript{22}. 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 CD99\textsuperscript{23-27}. 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.

\textbf{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-intima\textsuperscript{28}. 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)\textsuperscript{29-31}. Scavenger receptor mediated uptake of modified lipoproteins is however responsible for the uncontrolled uptake of
cholesterol within a high cholesterol environment. This excessive accumulation of cholesterol esters (CE) eventually leads to foam cell formation.

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 core. 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 destabilization. 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 wall. 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.

Laser capture micro-dissection (LCM) can be used to isolated 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 plaques.
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 1986 and certainly during the last decade it has become clear that atherosclerosis is an inflammatory disease. 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, leading 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. 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 cystein 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 cascades. 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. 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 cells. Studies with CCR2 and MCP-1 deficient and transgenic animals have shown the pivotal role of this interaction in the formation of atherosclerotic plaques. 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 formation.

A second well documented CC receptor/chemokine signalling axis in atherogenesis consists of CCR5 and its T cell derived ligands CCL3 (MIP-1α), CCL-4 (MIP-1β) and CCL-5 (RANTES). Expression of CCL3, -4 and -5 was shown in atherosclerotic plaques and antagonizing CCL-5 results in decreased plaque formation. 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 colleagues. CCR3 expression is associated with macrophage rich lesions and its main ligand, eotaxin (CCL11) is produced by vascular smooth muscle cells. 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 disease. 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 regression.

A theoretical role for CCR4 and CCR8 signalling has been proposed in literature. Expression of the CCR4 ligands CCL17 (TARK) and CCL22 (MDC) has been established in atheroma and is associated with macrophage rich areas and sites of neo-vascularization. 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 cells.
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 progression. 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 LDL. 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 plaque. A complicating factor is the rather large number of other CXCL molecules with affinity for CXCR2. These include CXCL1 (GROα), -2(GROβ), -3(GROγ), -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. 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). 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 ligands. Targeted deletion of CXCR3 in ApoE deficient (ApoE−/−) mice results in decreased lesion formation in the abdominal aorta. 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 plaque.

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. 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 plaque. In turn, reduced CXCL12 plasma levels have been associated with unstable coronary artery disease, suggesting anti-inflammatory or plaque-stabilizing properties for CXCL12 in atherosclerosis. 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 expression. Neither CXCR5 nor its ligand CXCL13 is reported to be expressed or differentially regulated during atherosclerotic lesion formation. CXCR16, also known as SR-PSOX, is a rather peculiar member of the chemokine family. It is membrane bound, and available for its receptor, CXCR6 upon cleavage by ADAM-10. Interestingly, the transmembrane form functions as a scavenger receptor and mediates the uptake of oxLDL by macrophages. It is expressed in human atherosclerotic plaques by all vascular cells, and not in healthy tissue. Furthermore, it mediates the adhesion of CXCR6 expressing cells. Recently, it was shown that a polymorphism of CXCL16 is associated with aggravated coronary artery stenosis.

CX3C family
Only one member of the CX3C family is described, CX3CL1, and this molecule was only identified in 1997. Like CXCL16, it is expressed as a membrane bound chemokine, and is accessible for its receptor CX3CR1 only after cleavage by ADAM-17. 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 adhesion. 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 root. 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 plaques.

Interleukins
Interleukins are messenger molecules that are used in the communication between 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-6. The profound effects in patients are attributable to the pro-inflammatory effects of IL-6 on monocytes, smooth muscle cell proliferation and endothelial cells. IL-1β has pro-inflammatory effects on endothelial cells, vascular smooth muscle cells and macrophages, all detrimental in the formation of atherosclerotic lesions. Increased levels of IL-1 lead to an upregulation of adhesion molecules and induce leukocyte migration to the growing lesion. Numerous studies in IL-1 or IL-1 receptor knock outs and transgenic mice underlined these findings. 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 IFNγ, IL-6 and vascular adhesion molecules. 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 plaque. Interestingly, combined stimulation of T and NK cells with IL-18 and IL-12 leads to activation of Th1 cells and subsequent IFNγ production. The absence or selective blockade of this differentiation route by inhibition of IL-12 or IFNγ profoundly inhibits atherosclerosis. IFNγ is the predominant cytokine produced by T helper type 1 (Th1) cells and the pro-atherogenic role of IFNγ and IL-12/IL-18 indicates that this cell type induces and accelerates atheromatous lesion formation. Buono et al. underlined this hypothesis by showing severe attenuation of atherogenesis in mice deficient in the Th1 specific transcription factor T-bet.

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 subunit. 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 cell. IL-17 has clearly defined possible pro-atherogenic effects on various atheroma-associated cell types. 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. A separate group of interleukins that share a common subunit (in this case of their receptor) is the γc 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. 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.

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-15. 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 pectoris. However, lower levels of IL-7 mRNA were shown in unstable coronary atherosclerotic plaques. 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 TGFβ.
Antiartherogenic 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. It induces a shift towards a protective Th2 phenotype as it inhibits Th1 proliferation of T cells. A number of papers provided direct evidence for a link between a decrease in serum IL-10 levels and unstable angina and CAD and have addressed the protective effects of IL-10 on atherosclerotic lesion formation. 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 effect. A similar function is postulated for the Th2 associated cytokine IL-9. This molecule is able to reduce TNFα, IFNγ and IL-12 secretion and induce IL-10 expression after LPS injection.

As discussed, Th1 associated interleukins such as IL-12 and IFNγ 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 colleagues. They however found that its protective effects were mainly due to increased production of oxidized low density lipoprotein-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 attenuated.

The potent anti-inflammatory molecule TGFβ was linked to atherogenesis in a study that showed reduced serum levels of active TGFβ in patients with advanced atherosclerosis. TGFβ 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. Interestingly, naturally occurring regulatory T cells identified as CD4+CD25+FOXP3+ positive, exert their protective effects via the release of IL-10 and TGFβ. This is also observed for the Th3 cells that mediate tolerance and subsequent suppression of inflammation after oral administration of antigens.

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-27p28. Effects on both Th1 and Th2 subsets have been described for IL-27. In addition, IL-27 is a suppressor of pro-atherogenic Th17 cells and in this way can provide protection against atherosclerotic lesion formation.
**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 centers\(^{175}\). 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 deceased levels of MHCII and IFN\(\gamma\) in the lesion\(^{176}\). 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 cells\(^{164}\).

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 processes\(^{177-181}\). 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 atherogenesis\(^{182}\).

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 cells\(^{183}\). The combined exposure of CD40 to CD40L and MHCII to the T cell
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receptor (TCR) leads to further activation of the APC and secretion of pro-inflammatory cytokines such as IL-12\textsuperscript{184,185}. As expected, blockade of CD40-CD40L signaling markedly decreased atherosclerotic lesion formation and enhanced plaque stability as shown by numerous authors\textsuperscript{186–189}.

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\textsuperscript{190,191}. The ligand for OX40 (OX40L, TNFSF4) is expressed by APC’s and by vascular endothelium\textsuperscript{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\textsuperscript{194,195}. In the present thesis, chapter 8 describes the atheroprotective 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 plaques\textsuperscript{196}.

GITR (TNFRSF18) in mice, and AITR in humans is a surface receptor molecule that is upregulated upon T cell stimulation via CD28\textsuperscript{197}. It has been shown to be constitutively expressed in T-regulatory cells and extending the survival of T-effector cells\textsuperscript{198}. Recently, Kim et al demonstrated that GITR and its ligand are expressed mainly in lipid-rich macrophages within atherosclerotic plaques\textsuperscript{199}. Furthermore they showed that GITR stimulation increased TNFα 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 (LTβR), 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\textsuperscript{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 expected\textsuperscript{202}. 
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 of 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 atherogenesis. 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 injection. 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 protection.

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 immunization\textsuperscript{210}. 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 \textit{Salmonella Typhimurium} that has been described for vaccination purposes\textsuperscript{211}. An advantage of \textit{Salmonella} is that it egresses from the gut lumen via the M cells to Peyers patches, and in this way the vector (via the pathogen) is efficiently transported to the immune system for translation and processing\textsuperscript{212-214}. DNA vaccination by using oral administration of \textit{Salmonella} can lead to different immune responses, based on the T cell subset that is activated by APC’s present in the Peyers patch\textsuperscript{215}. By activation of CD8\textsuperscript{+} cells, a cellular immune response is initiated. This leads to the specific killing of cells that express and present the antigen via MHCI. 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\textsuperscript{+} T cells to antigen specific B cells. The following two paragraphs will discuss the mechanisms leading to cellular or humoral immunity.

\textbf{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 \textit{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\textsuperscript{216, 217}. Not only bacterial strains can be the target of CTL mediated apoptosis, also eukaryotic cells expressing high levels of the protein via MHCI 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 cancer\textsuperscript{218-221}. 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\textsuperscript{+} VEGF specific CTL’s that remove activated endothelial cells from the damaged vascular wall\textsuperscript{222}. 

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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 vaccination\textsuperscript{215}. This indicates that a moiety has to be inserted that drives activation of the T helper subset, instead of the activation of the CD8\textsuperscript{+} 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 Lysosome (HEL), PanDRepitope (PADRE) and ovalbumine (OVA)\textsuperscript{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 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.
References


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Chapter 1


General Introduction


