

Crosstalk between apoptosis and inflammation in atherosclerosis

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Crosstalk between Apoptosis and Inflammation in Atherosclerosis

Marijke M. Westra

Crosstalk between Apoptosis and Inflammation in Atherosclerosis

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Chapter 1

Introduction

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7 Thesis outline

1 Atherosclerosis and cardiovascular disease

Atherosclerosis can be defined as a multifactorial, progressive disease of medium and large sized arteries which sets off already in childhood[1] and is characterized by accumulation of lipid material and fibrous components in the artery wall[2]. Atherosclerosis is the pathophysiological cause of the majority of cardiovascular disease including myocardial infarction, angina pectoris and stroke. Most clinical complications are caused by plaque disruption and subsequent thrombus formation[3,4]. Its onset and progression was seen to associate with both environmental risk factors like smoking, high-fat diet and lack of exercise and factors with a strong genetic component like hypertension, hyperlipidemia, diabetes and male gender[5-8]. Therapies are mostly based on reducing these risk factors, such as lowering serum lipid levels using statins, lowering blood pressure and life style changes or consist of surgical intervention such as bypass surgery, percutaneous transluminal coronary angioplasty (PTCA) and stenting although the effectiveness of the latter interventions is often impaired by the recurrent narrowing of the vessel, a process referred to as restenosis[9]. Despite the available treatments, atherosclerosis continues to be one of the main causes of death in the world.

2 Pathogenesis of atherosclerosis

2.1 Leukocyte adhesion and migration

In the normal, healthy arterial wall the endothelium covers a layer of smooth muscle cells and produces various factors controlling vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, inflammation of the vessel wall and vascular remodeling[10]. Atherosclerotic plaques start as fatty streaks at specific predilection sites within the arterial tree, such as bifurcations and branches[1,2]. The first step herein lies in dysfunction of the endothelium due to increased turbulence or decreased shear stress often combined with aspects of the above mentioned risk factors[1,2]. As a result the expression by endothelial cells of adhesion and inflammatory molecules, essential in the recruitment of leukocytes, is increased[11]. The initial tethering and rolling of circulating leukocytes (monocytes and lymphocytes) is mediated by selectins, L-selectin expressed on circulating leukocytes and P-selectin and E-selectin on the activated endothelium, resulting in further leukocyte activation[12,13]. Subsequently firm adhesion of leukocytes requires the engagement of β_1 and β_2 integrins, e.g. VLA4 and CD18/CD11, which interact with upregulated intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expressed by endothelial cells[14,15]. Functional roles for ICAM-1 and both E-selectin and P-selectin in atherogenesis have been confirmed by gene deletion studies in mouse models for atherosclerosis, the ApoE and LDLr deficient mouse[16,17]. Transmigration of leukocytes into the subendothelial space is the final step in plaque initiation, a process also known as diapedesis. Various endothelial cell expressed molecules facilitate transmigration, such as platelet/endothelial-cell

adhesion molecule 1 (PECAM1), junctional adhesion molecule A (JAM-A), endothelial cell-selective adhesion molecule (ESAM), ICAM2 and CD99[18-22]. In addition to adhesion molecules chemokines are critically involved in the adhesion and migration of leukocytes[23]. Regarding lesion initiation chemokine receptor CCR2 and its ligand monocyte chemoattractant protein 1 (MCP1) are considered the most important. Deletion of MCP1 in LDLr$^{-/-}$ mice and (leukocyte) CCR2 in ApoE$^{-/-}$ or ApoE3 Leiden mice all resulted in significantly reduced atherosclerosis development[24-26]. Once migrated into the intima, monocytes differentiate into macrophages in response to macrophage-colony stimulation factor (M-CSF) secreted by endothelial cells and vascular smooth muscle cells (vSMC) and contribute to plaque progression[2]. Figure 1 shows a schematic overview of the processes described above.

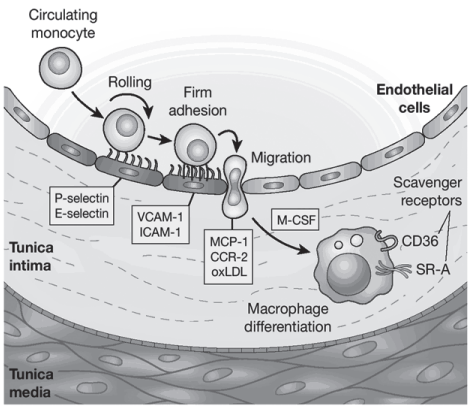


Figure 1. Atherosclerotic plaque initiation. Selectins mediate the first cell-cell interactions enabling capture, tethering and rolling of circulating monocytes. Once captured, integrins (interacting with ICAM-1 and VCAM-1) mediate the firm adhesion of monocytes to the endothelium after which they migrate into the subendothelial space along a chemokine gradient. Here they differentiate into macrophage under the influence of M-CSF and increase the expression of scavenger receptors. Adapted from Li and Glass[175].

2.2 Plaque progression and instability

Fatty streaks do not cause clinical symptoms but may progress to more complex plaques. They are characterized by continuous influx of inflammatory cells (macrophages and lymphocytes) and lipids into the vessel wall. Low-density-lipoprotein (LDL) within the intima can be modified by oxidation and aggregation[27-29]. In turn, these modified LDL particles and entrapped cholesteryl esters can be taken up by macrophages which have increased expression of scavenger receptors due to M-CSF stimulation[30]. As a result of this progressive accumulation of lipids, macrophages will convert into foam cells. Differentiated macrophages and infiltrated T lymphocytes will augment the inflammatory response by secreting growth factors and cytokines[31]. Formation of a more complex fibroatheromathous lesion involves the migration of vSMC from the vessel wall into the intima and vSMC proliferation under the influence of growth factors secreted by endothelial cells and macrophages. VSMC synthesize the bulk of the extracellular matrix such as collagen, elastin and proteoglycans within the plaque in response to transforming growth factor (TGF) β and platelet derived growth factor (PDGF). VSMC and extracellular matrix proteins form a fibrous cap overlying the lipid core[32]. Augmentation of the inflammatory response, vSMC migration and formation of a fibrous cap cause the initial fatty streak to develop into an advanced atherosclerotic lesion narrowing the

vessel lumen.

As the atherosclerotic plaque progresses a necrotic core is formed consisting of accumulated lipids and cell debris derived from apoptotic or necrotic cells. Whereas stable advanced lesions have a dense fibrous cap overlying this necrotic core, the potentially dangerous plaques, responsible for the majority of clinical manifestations, are unstable as a result of cap thinning which makes a plaque vulnerable to rupture and thrombus formation[33]. Several factors contribute to the progressive destabilization and thrombogenicity of atherosclerotic plaques. A large lipid core[34], accumulation of inflammatory cells[35], extracellular matrix degradation[36,37] and plaque cell death[38,39] comprise the most important contributors. In addition intraplaque hemorrhage has been proposed to be a critical factor in plaque destabilization[35]. Fibrous cap thinning and plaque inflammation in regard to lesion progression and destabilization will be discussed in more detail in the following sections.

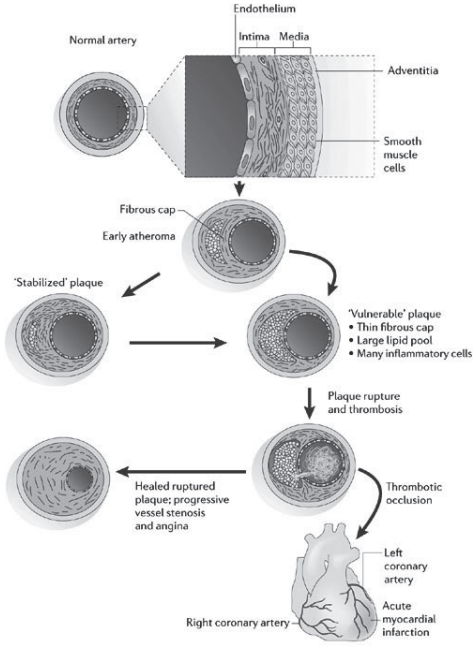


Figure 2.Atherosclerotic plaque progression from early atheroma to myocardial infarction. Early atheroma can progress into a stable fibrous plaque characterized by a small core and thick fibrous cap. Alternatively a vulnerable plaque develops with a large core containing lipids and cell debris, a high inflammatory cell content and a thin fibrous cap. Vulnerable plaques may rupture resulting in the formation of a thrombus. Ruptured plaques can either heal following vSMC migration and extracellular matrix production or result in myocardial infarction. Adapted from Watkins and Farrall[176].

3 The role of vascular smooth muscle cells in atherosclerosis

Vascular smooth muscle cells (vSMC) are one of the major cellular constituents of the atherosclerotic plaque. Evidence shows that intimal vSMC differ from medial vSMC in many aspects. Medial vSMC are predominantly of the contractile phenotype while most intimal vSMC have characteristics of the synthetic, migratory phenotype. This phenotypic switch can be induced by a variety of atherogenic stimuli like cytokines, shear stress, reactive oxygen species (ROS) and lipids. Synthetic vSMC migrate and proliferate better than contractile vSMC and synthesize more collagen[41]. VSMC migration can be triggered by various growth factors and chemokines secreted by

macrophages and T cells like platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor (TGF) β, monocyte chemoattractant protein (MCP) 1 and stromal cell-derived factor (SDF) 1α[1,42,43].

VSMC, like macrophage, are able to ingest lipids and form foam cells. They express several receptors involved in (modified) lipoprotein uptake including the LDL receptor, CD36, type I and type II scavenger receptors and SR-PSOX[44-47]. Furthermore, adhesion molecules like vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) have been demonstrated to be expressed by vSMC, these may enable them to increase monocyte adherence and infiltration into the atherosclerotic lesion[48.] The mechanisms and consequences of adhesion of leukocytes to vSMC *in vivo* however are not well characterized. Furthermore, intimal vSMC have been reported to produce a wide variety of growth factors and cytokines, including PDGF, TGFβ, MIF and MCP-1, contributing to the pro-inflammatory environment of the atherosclerotic lesion[41].

VSMC play a crucial role in fibrous cap formation and preserving plaque stability. Unstable plaques prone to rupture contain a higher macrophage and lipid content and a thinned fibrous cap due to loss of vSMC and extracellular matrix. The strength of the fibrous cap seems to depend on a balance between collagen synthesis and breakdown and on the type of collagen. Expression of genes promoting collagen synthesis by vSMC and of matrix metalloproteinases (MMPs), important in the breakdown of extracellular matrix, can be influenced by inflammatory cytokines[49]. For instance, TGFβ enhances the ability of vSMC to produce collagen, while TNFα, IL1 and IFNγ suppress collagen content either directly or by inducing MMPs[50-52]. In addition MMP expression was shown to be elevated in atherosclerotic plaque in comparison to normal vessels, a result of both inflammatory cytokine production and oxidative stress[33]. MMP activity is balanced by tissue inhibitors of metalloproteinases (TIMPs), MMP specific inhibitors expressed by vSMC. Expression of TIMPs can be either constitutive or upregulated by TGFβ and PDGF[53].

Apart from MMPs, cathepsins which are cysteine proteases, can degrade the extracellular matrix[54]. Cathepsins are secreted by macrophages and their expression is increased in atherosclerotic lesions compared to healthy arteries[55]. Comparable with MMPs, cathepsin activity can be inhibited by a family of proteins, the cystatins of which cystatin C is best described. As opposed to cathepsins, expression of cystatin C is decreased in atherosclerotic lesions[55,56].

Another role for vSMC may lay in the healing of fibrous cap breaks that remain subclinical. Mediators released at sites of thrombosis, for example PDGF and TGFβ released by platelets, can stimulate vSMC migration, mitogenesis and production of collagen, thus promoting a fibrous lesion morphology[49]. A thrombus caused by plaque rupture that doesn't occlude the vessel is reorganized and incorporated into the plaque. Recurring incidents of plaque rupture and healing can be visible in plaques[57,58].

4 Inflammation in atherosclerosis

Monocyte infiltration contributes largely to plaque initiation. Stimulation with M-CSF secreted by endothelial cells and vSMC, causes the infiltrated monocytes to differentiate into macrophages and induces expression of scavenger receptors and cytokine production[59-60]. Macrophages are able to take up cell-activating modified LDL, mainly oxidized LDL (Ox-LDL) via several scavenger receptors including type 1 and 2 scavenger receptor A (SRA), CD36, CD86, MARCO (macrophage receptor with a collagenous structure), SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein) and lectin-like oxidized low density lipoprotein receptor 1 (LOX-1)[61-65]. Uptake of modified lipoproteins by scavenger receptors not only leads to the formation of foam cells but also results in macrophage activation. Subsequently, activated macrophages produce inflammatory cytokines, growth factors, proteases and reactive oxygen species influencing endothelial cell activation, vSMC migration, proliferation and collagen production and T cell activation[35]. Expression of scavenger receptors can be influenced by various cytokines present in the plaque including TNFα, IFNγ, IL4 and TGFβ[66-68]. TGFβ was shown to inhibit foam cell formation[68].
Uptake of modified lipoproteins via macrophage scavenger receptors can result in MHC restricted antigen presentation to T cells[69]. T cells are recruited into the lesion by mechanisms similar to the recruitment of monocytes. The majority of lesional T cells are CD4+ effector cells although CD8+ cells are present as well[70]. The role of lymphocytes in atherosclerosis has been studied using $RAG^{-/-}$ mice lacking T and B cells. In $ApoE^{-/-}$ mice lymphocyte deficiency results in the development of smaller lesions[71,72] while transfer of CD4+ T cells into immunodeficient (scid/scid) $ApoE^{-/-}$ mice aggravated atherosclerosis[73]. Several antigens have been associated with atherosclerosis. An important group of antigens consists of altered self molecules. T cells within the atherosclerotic lesions have been shown to respond to *Chlamydia pneumoniae* related antigens and stress-induced heat shock protein (HSP) 60[70]. Apart from Ox-LDL which is recognized by T cells present in human plaques[74] peptides derived from modified LDL components, for example apolipoprotein B and phospholipids can serve as antigens in atherosclerotic plaques[70]. CD4+ T cells can be subdivided in several T helper (Th) cell subsets based on their cytokine secretion profile, e.g. Th1 cells (which produce IFNγ and TNFα), Th2 cells (producing IL4, IL5 and IL13) and regulatory T cells (IL-10 and TGFbeta)[70]. Mouse and human studies have demonstrated a predominant pro-inflammatory Th1 cytokine pattern in atherosclerotic plaques[75,76]. IL2 and IFNγ were shown to be abundantly present whereas only small amounts of Th2 cytokines IL4 and IL5 have been found in plaques. Mouse studies have demonstrated that IL12 and IL18, both Th1 inducing cytokines, have pro-atherogenic properties[77-81] as do Th1 cytokines IFNγ[82,83] and TNFα [84,85], while the role of Th2 cytokines is less clear. IL4 was demonstrated to be atheroprotective[78,86] but deficiency of IL5 increased atherosclerosis[87].
Production of cytokines by macrophages and lymphocytes in the plaques does not only influence inflammatory processes but also modulates smooth muscle

cell activity. IFNγ inhibits smooth muscle cell proliferation[88] and the production of collagen, whereas TGFβ stimulates collagen production[89]. In addition TGFβ downregulates the expression of MMPs, collagen degrading proteins[90], while macrophages are stimulated to produce MMPs by TNFα and IL1[91]. Finally TNFα and IFNγ can promote the uptake of modified lipoproteins by smooth muscle cells leading to smooth muscle cell derived foam cells[92].

In addition to macrophages and T cells other inflammatory cell types have been demonstrated to be involved in atherosclerosis, including B cells, dendritic cells, mast cells and neutrophils. Although few B cells are present in the plaque the majority is located in the adventitia[70]. B cell associated immunity was shown to be protective in atherosclerosis as splenectomy increased plaque development in $ApoE^{-/-}$ mice while transfer of spleen derived B cells counteracted this effect[93]. Dendritic cells are the most potent antigen presenting cells. They are present in healthy vessels but accumulate during atherogenesis, being mainly localized in the rupture prone shoulder areas[94]. Skin dendritic cells have been shown to be activated by dislipidaemia with surprising inhibition of migration into lymph nodes suggesting that they contribute to local inflammation[95]. However a recent study by Packard *et al.*[96] found opposing results. Here, dendritic cells were demonstrated to maintain their antigen presenting function and ability to prime $CD4^+$ T cells in vitro under hypercholesterolemic conditions[96]. Mast cells are present in the atherosclerotic plaque and were shown to accumulate in the shoulder region[97]. Activated mast cells secrete cytokines and proteases and mast cell derived TNFα and IL6 were shown to promote atherosclerosis[98]. In addition mast cells have been demonstrated to be involved in intraplaque hemorrhage, macrophage apoptosis and vascular leakage, promoting plaque instability[99]. Neutrophils are thought to be pro-atherogenic as well. They are mainly present in the adventitia and the luminal area of mouse plaques[100] and in ruptured human coronary artery plaques[101]. Depletion of circulating neutrophils resulted in reduced plaque formation in $ApoE^{-/-}$ mice[100].

5 Apoptotic cell death

5.1 Signal transduction pathways

Removal of defective, damaged or dangerous cells is critical for normal development and tissue homeostasis of all organisms[102]. Death of these cells takes place via a process called apoptosis or programmed cell death[103]. Apoptosis is characterized by morphological changes like cell shrinkage, DNA fragmentation, condensation of chromatin and membrane blebbing. In contrast, features of passive, traumatic cell death or necrosis are cell swelling and loss of membrane integrity[104].

The executers of apoptotic cell death are a family of cysteine proteases known as caspases. Caspases proteolytically cleave proteins necessary for maintaining cellular structure like lamins[105] and focal adhesions kinase (FAK)[106] but also proteins that protect from cell death such as DFF45 (a nuclease inhibitor)[107] and Bcl-2 family members[108]. A cascade of caspases in which a pro-apoptotic signal activates initiator

caspases (e.g. caspases 1, 8, 9 and 10) which in turn activate effector caspases (caspases 3, 6 and 7) results in cellular breakdown[109]. There are two signaling pathways regulating apoptosis that share the same effector caspases. The extrinsic or death receptor mediated pathway is activated in response to ligation of death receptors (fig. 3). Binding of specific ligands to the cognate death receptor causes formation of a death-inducing signaling complex (DISC) in which various adaptor proteins like FADD and TRADD interact with death domains (DD) of the receptors[110]. Initiator caspase 8 is essential for death receptor induced apoptosis[111]. Death receptors belong to the tumor necrosis factor (TNF) receptor family and include TNF receptor 1 (TNFR1), FAS, death receptor (DR) 3, DR4 and DR5. Their ligands are TNF family members, including Fas ligand, TNFα, TWEAK (TNF-like weak inducer of apoptosis) and TRAIL (TNF related apoptosis inducing ligand)[110].
The intrinsic apoptosis signaling pathway requires the involvement of members of the Bcl-2 (B cell lymphoma 2) family of apoptosis regulators and mitochondria. Apoptotic stimuli activating this pathway include DNA damage, UV radiation, hypoxia and growth factor withdrawal[112]. Apoptosis signaling via the intrinsic pathway depends on the release of cytochrome c and other apoptosis regulating proteins like Smac/Diablo and apoptosis inducing factor (AIF) from the mitochondria (fig. 3). Once in the cytosol cytochrome c associates with an adaptor molecule called apoptotic protease-activating factor-1 (APAF-1) and pro-caspase 9 forming the so-called apoptosome. The subsequently activated caspase 9 is then able to activate effector caspases[113].

5.2 Bcl-2 family of apoptosis regulators

The intrinsic apoptosis pathway is mainly regulated by proteins of the Bcl-2 family. This family consists of both pro- and anti-apoptotic proteins sharing one or more Bcl-2 homology (BH) domains[114]. Anti-apoptotic proteins contain three or four BH domains and include Bcl-2, Bcl-w, Bcl-x_L, Bfl-1 and Mcl-1. There are two classes of pro-apoptotic Bcl-2 family proteins: proteins of the multidomain group comprising Bax, Bak and Bok which contain BH domains 1-3 and Bcl-2 proteins which carry only the BH-3 domain. The latter BH-3 only proteins include Bid, Bad, Bik, Bim, Noxa, Puma, Bmf, Blk and Hrk[114]. BH-3 only proteins initiate the apoptotic cascade[115], whereas Bax and Bak function downstream of BH-3 only proteins[116]. Bcl-2 family proteins Bak and Bax are thought to form pores in the outer mitochondrial membrane or change pore size thereby affecting of the mitochondrial permeability for cytochrome c[113]. Cytochrome c release from mitochondria takes place through these pores. Under non-apoptotic circumstances activity of BH3-only proteins is inhibited by Bcl-2 and other anti-apoptotic Bcl-2 proteins[112]. Following an apoptotic stimulus, BH-3 only proteins can either directly activate multidomain pro-apoptotic proteins (Bid and Bim) or interact with anti-apoptotic Bcl-2 proteins and prevent their binding to other pro-apoptotic proteins (Bim). Activity of BH3-only proteins can be regulated by phosphorylation (for example Bad and Bim[117,118]), transcriptional control (Puma and Noxa which are p53 targets[119,120]) or cleavage (Bid[121]). The pro-apoptotic protein

Bid, which functions in the intrinsic pathway, can also be activated by caspase-8 after stimulation of the extrinsic apoptosis pathway, thereby connecting both pathways[112].

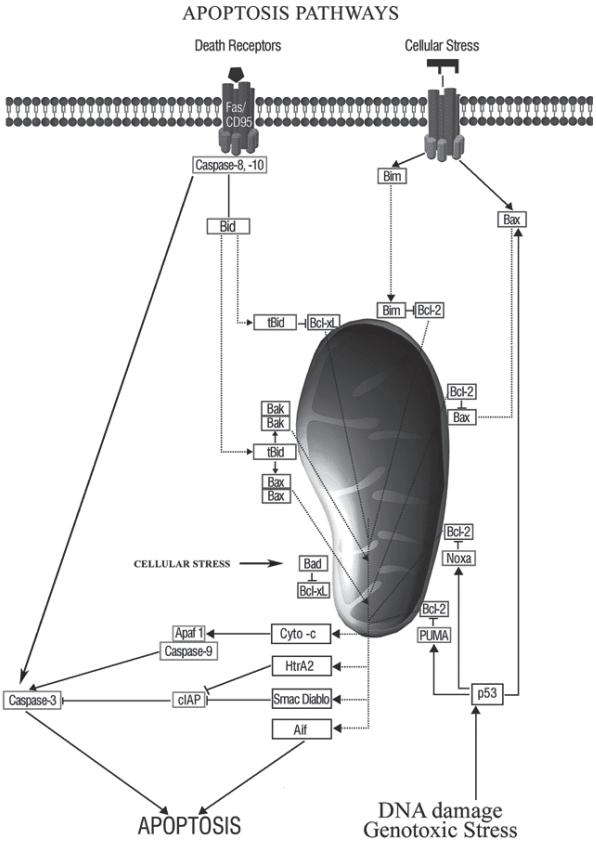


Figure 3. Apoptosis pathways. The death receptor (extrinsic) pathway is activated by ligation of death receptors. Subsequently initiator caspases activate effector caspases resulting in cell death. BH3-only proteins (e.g. Bim) initiate the mitochondrial or intrinsic pathway after apoptotic stimuli like DNA damage and oxidative stress, followed by activation of multidomain pro-apoptotic proteins (Bak and Bax) which form pores in the mitochondrial membrane. Apoptotic signaling is regulated by anti-apoptotic bcl-2 proteins (Bcl-2, Bcl-x_L, Mcl-1 etc). Cell death results from effector caspase activation and subsequent release of cytochrome c and other regulatory proteins from the mitochondria. Adapted from Kutuk and Basaga[112].

5.3 Apoptotic cell clearance

Apoptosis is followed by uptake of cellular remnants by professional phagocytes, macrophages, dendritic cells and granulocytes[122]. A wide range of receptors, ligands and adaptor molecules on both apoptotic cells and phagocytes are involved in the removal of apoptotic cells. One of the best described molecules in the recognition of apoptotic cells is phosphatidylserine (PS), which is translocated from the inner to the outer leaflet of the cell membrane early in the apoptotic process[123]. Other molecules implicated in the recognition and engulfment of apoptotic cells include scavenger receptors CD36, CD68 and SRA, Mer kinase, CD14 and integrins on the phagocyte membrane and bridging molecules such as milk fat globule epidermal growth factor 8 (Mfge8) and complement component C1q[122,124-127]. When removal of apoptotic cells is insufficient apoptotic cells may undergo secondary necrosis with leakage of cellular content. This may have pathological consequences since secondary necrotic cells and their debris can be taken up by antigen presenting cells and result in inflammation and autoimmunity[128].

6 Apoptosis and phagocytosis in the atherosclerotic plaque

Apoptosis occurs in atherosclerotic lesions affecting all major cell types, endothelial cells, macrophages, T cells and vSMC[129]. However, apoptosis increases with plaque progression, being virtually absent in initial lesions and increasingly present in advanced lesions[130]. Inducers of apoptotic cell death are abundant and include modified LDL, reactive oxygen species, cytokines with pro-apoptotic activity, hypoxia and death receptor ligation (Fas, TNFR1 and 2, DR4 and DR5)[131-137].

6.1 Endothelial cell apoptosis

Endothelial injury and apoptosis are late events in atherosclerosis[138]. Endothelial cells in lesion-prone regions in the vasculature have increased turnover due to increased apoptosis[139]. In endothelial cells in regions predisposed to atherosclerotic lesion development NF-κB signal transduction pathway was shown to be primed for activation[140] and NF-κB activation by various stimuli like hypoxia, IL18 and TNFα has been demonstrated to trigger apoptosis in endothelial cells[141-143]. Apoptosis is stimulated by exposure to oxidized LDL and oxidative stress among other factors. Nitric oxide (NO) may play a role in endothelial cell apoptosis in atherosclerosis as well. In healthy arteries NO derived from endothelial NO synthase (eNOS) acts protective against apoptosis[144]. In atherosclerotic lesion prone regions eNOS expression is decreased[145]. In addition, atherosclerotic plaque macrophages produce high amounts of inducible NOS (iNOS) which can generate peroxynitrite contributing to oxidative stress[146] which in turn can induce DNA damage and subsequent apoptosis in endothelial cells[138]. EC injury and apoptosis can have various consequences. Induction of EC apoptosis may promote thrombus formation followed by plaque erosion and leukocyte infiltration[147,148].

6.2 Vascular smooth muscle cell apoptosis

Apoptosis of vSMC has been shown to occur after injury in a rabbit balloon angioplasty model[149], in human abdominal aortic aneurisms[150] and in atherosclerotic lesions[21]. Surprisingly apoptosis of vSMC in atherosclerotic plaques can induce inflammation as shown *in vivo* in rat carotid arteries[151] where it triggered IL8 and MCP-1 expression together with massive macrophage infiltration after vSMC death. In $ApoE^{-/-}$ mice in which apoptosis was specifically induced in vSMC by diphtheria toxin (SM22α-hDTR / $ApoE^{-/-}$ mice) increased inflammation was observed after vSMC apoptosis as well[152]. Furthermore, vSMC apoptosis has been shown to lead to thrombin generation[153] and calcification[154] *in vitro*. In human atherosclerotic lesions apoptosis of both vSMC and macrophages was demonstrated to be elevated only in advanced lesions while in early lesions apoptosis was minimal[130]. In addition, human vSMC derived from coronary atherosclerotic plaques were shown to be more susceptible to cell death than vSMC from healthy coronary arteries *in vitro*[155] and vSMC may exhibit increased oxidative stress induced senescence[156]. VSMC senescence following ROS induced

DNA damage was shown to be mediated by p53 activation[156]. Abovementioned studies seem to support the general concept that apoptosis of vSMC promotes plaque vulnerability by thinning of the fibrous cap and also various studies in mice are in agreement with this concept. Induction of apoptosis by targeted overexpression of p53 into cap smooth muscle cells in advanced collar induced carotid artery plaques in ApoE$^{-/-}$ mice resulted in increased apoptosis of cap cells, reduced cap thickness, and in general a vulnerable plaque phenotype which was prone to phenylephrine induced rupture[157]. A comparable, vulnerable plaque phenotype was found after adenovirus mediated overexpression of the pro-apoptotic TNF family member Fas ligand in cap cells of ApoE deficient mice[158]. Plaques contained hemorrhage, buried caps and iron deposits, also indicating increased vulnerability. Recently, the above mentioned SM22α-hDTR / ApoE$^{-/-}$ mice were used to examine the impact of vSMC apoptosis on plaque phenotype and disease progression[152,159]. Induction of apoptosis in established atherosclerotic plaques resulted in plaque vulnerability as indicated by fibrous cap thinning, loss of collagen, accumulation of cell debris and increased inflammation[152]. In addition, persistent vSMC apoptosis throughout plaque development was seen to accelerate atherogenesis[159].

6.3 Macrophage apoptosis

Macrophage apoptosis occurs in both early and late stages of atherosclerosis and can be induced by a variety of stimuli including oxidized LDL, oxysterols, free cholesterol and hypoxia but also TNFα[160]. Apoptosis of macrophages has been demonstrated to be beneficial in early atherogenesis in several *in vivo* studies[161-164]. Inhibition of macrophage apoptosis due to leukocyte p53 deletion in ApoE3 Leiden transgenic mice[161] or LDLr$^{-/-}$ mice[162] and leukocyte Bax deletion in LDLr$^{-/-}$ mice[163], both pro-apoptotic factors, resulted in increased atherosclerotic lesion size. In addition deletion of pro-survival factor AIM (apoptosis inhibitor expressed by macrophages) in LDLr$^{-/-}$ mice led to increased macrophage apoptosis and decreased lesion area[164]. The consequences of macrophage apoptosis in advanced lesions are less clear. In advanced human lesions clearance of apoptotic cells was shown to be defective[165], suggesting that macrophage apoptosis will lead to secondary necrosis and accumulation of cell and lipid debris. This will translate in necrotic core expansion and elicit a pro-inflammatory response which could result in promotion of plaque instability[160]. However, others did not find such pronounced effects of macrophage apoptosis in advanced atherosclerotic plaques. For instance, Stoneman *et al.*[166] developed a model in which in ApoE$^{-/-}$ mice apoptosis could be induced specifically in macrophages with diphtheria toxin (DT), the CD11b-hDTR / ApoE$^{-/-}$ mouse[166]. Induction of apoptosis during early atherogenesis resulted in decreased plaque development together with reduced collagen content and necrotic core formation, confirming the atheroprotective effects of macrophage apoptosis in aforementioned studies regarding early atherogenesis. However in established plaques DT treatment induced macrophage apoptosis but this did not result in alterations in plaque size, cell composition or inflammation. In another

study macrophage apoptosis was achieved by LysM cre induced deletion of Bcl-2 in ApoE$^{-/-}$ mice[167]. Increased macrophage apoptosis was observed after 10 weeks of western type diet feeding but this resulted in a slight increase of 25% in necrotic core size only in female mice. No other characteristics of enhanced plaque instability were observed.

6.4 Phagocytosis of apoptotic cells

Phagocytosis of apoptotic cells in the atherosclerotic plaque limits plaque progression, inflammation and plaque instability as has been demonstrated by several gene deletion studies. Deficiency of leukocyte transglutaminase 2 (TG2) in LDLr$^{-/-}$ mice was seen to increase aortic valve lesion size and intimal macrophage infiltration[168]. LDLr$^{-/-}$ mice deficient in milk fat globule-EGF factor 8 (Mfge8) show accelerated atherosclerosis with increased necrotic core size and an elevated inflammatory status[169]. Finally, deletion of leukocyte Mer kinase in LDLr$^{-/-}$ mice led to increased accumulation of apoptotic cells, increased macrophage area and lymphocyte infiltration resulting in accelerated lesion development[170].

As mentioned in the previous section, phagocytic clearance of apoptotic cells is impaired at later stages of plaque progression[165]. Several mechanisms for defective phagocytosis have been proposed. First, Ox-LDL shares molecules involved in recognition by macrophages with apoptotic cells and as a result may compete with apoptotic cells for ingestion[171,172]. In addition auto-antibodies directed against Ox-LDL have been demonstrated to bind to apoptotic cells and inhibit their phagocytosis by macrophages[173]. Finally oxidative stress may inhibit the phagocytosis of apoptotic cells by macrophages as has been demonstrated in vitro for the oxidative stress mediators hydrogen peroxide (H2O2)[174] and peroxynitrite[165].

7 Thesis outline

In this thesis the role of several apoptosis regulating proteins in the development of atherosclerosis and atherosclerotic plaque stability is investigated. As many of these proteins also display immune-modulating features, we have particularly investigated effects of modulation of apoptosis regulating proteins on plaque and systemic inflammation. In chapter 2 current knowledge on pro- or anti-apoptotic proteins and their effects on inflammation in both murine and human atherosclerosis as well as the influence of pro- or anti-inflammatory mediators on apoptotic processes are reviewed.

Chapter 3 describes a study in which gene expression profiles of thin cap fibroatheroma are compared to those of thick cap fibroatheroma by micro-array technology in order to identify genes or pathways that are associated with plaque vulnerability. Two different mouse models for thin cap fibroatheroma are used to increase the significance of the findings.

In chapter 4 the relevance of Bim (Bcl-2 interacting mediator of cell death), a pro-apoptotic member of the Bcl-2 family identified as upregulated in both models in

the previous chapter, for atherosclerosis is investigated in LDLr$^{-/-}$ mice. Bim has been previously demonstrated to be an important regulator of B and T cell homeostasis. Therefore, apart from apoptotic processes relevant for atherosclerosis, we also assessed the role in disease associated innate and adaptive immunity. The pro-apoptotic activity of Bim is partly regulated by Mcl-1 (myeloid cell leukemia 1), an anti-apoptotic member of the Bcl-2 family. Mcl-1 is amongst others involved in proliferation and differentiation of monocytes and neutrophils and has been implicated in lipid accumulation by macrophages. In chapter 5 we therefore studied the impact of Mcl-1 deletion on cell death, lipid accumulation and inflammatory status of LDLr$^{-/-}$ mice.

Chapter 6 describes a study addressing the role of focal adhesion kinase (FAK), a kinase not only involved in cell death and proliferation, but particularly important in cell adhesion and migration, in atherosclerosis development and progression in ApoE$^{-/-}$ mice. Recently, FAK was shown to be involved in oxidized LDL mediated CD36 signaling. Thus, in chapter 6 the role of FAK in plaque apoptosis, inflammatory status and lipid metabolism in Western type diet fed ApoE$^{-/-}$ mice was investigated.

To conclude, in chapter 7 the main findings of the studies described in this thesis are summarized and discussed in relation to possible therapeutic approaches.

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Chapter 2

Apoptosis Associated Inflammation in Atherosclerotic Plaque Progression and Stability

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Abstract
Inflammation and apoptosis are regarded key processes in the development, progression and instability of atherosclerotic plaques. Although originally considered anti-inflammatory, recent insights promote the notion that apoptosis may under conditions prevalent in atheromathous tissue promote inflammation. Vice versa several pro-inflammatory mediators that contribute to atherogenesis can have pro- or anti-apoptotic effects. In this review we shall describe recent advances in our understanding of the crosstalk between apoptotic and inflammatory signaling pathways in the context of atherosclerosis.

1 Introduction

Immunoregulatory effects of cell death

Cells can die through two major processes, traumatic cell death ("necrosis") and or programmed cell death ("apoptosis"). Features of necrotic cell death are cell swelling and loss of membrane integrity, whereas apoptosis is characterized by a completely different repertoire of morphological changes including cell shrinkage, DNA fragmentation and membrane blebbing[1]. Necrosis will elicit a pro-inflammatory response. In contrast, apoptotic cells maintain their membrane integrity and in addition are readily taken up by phagocytes including macrophages and dendritic cells. Both measures will prevent the release of cell contents and as a result, apoptotic death generally does not cause an inflammatory response. In fact, apoptotic cells and phagocytes ingesting apoptotic remnants both can act anti-inflammatory by secreting transforming growth factor β1 (TGFβ1), interleukin (IL) 10 and prostaglandin E2 (PGE2) and by decreasing the secretion of pro-inflammatory cytokines such as TNF-α, IL1 and IL12[2,3,4,5]. However, recent studies have shown that apoptosis not always acts inflammation-neutral or anti-inflammatory (for reviews see 6 and 7). For example, various inducers of apoptosis, among which Fas, can influence cytokine secretion or immune cell behaviour[8]. Adequate elimination of apoptotic remnants to prevent secondary necrosis and the ensuing pro-inflammatory response is therefore of great importance.

Inflammation and apoptosis in atherogenesis

Atherosclerosis, a chronic inflammatory disease, is initiated by endothelial dysfunction and accumulation of lipoprotein and low-density lipoprotein (LDL) derived lipids in the vessel wall. Expression of adhesion molecules in the endothelium results in adherence and migration of T lymphocytes and monocytes into the intima, after which monocytes differentiate into macrophages[9]. Intimal leukocytes release a whole range of pro- and anti-inflammatory cytokines, chemokines and growth factors[10]. Moreover reactive oxygen species (ROS) are generated in the intima, which act pro-apoptotic on the one hand and modify (phospho)lipids and LDL promoting foam cell formation on the other hand[11]. Modified LDL can also cause further endothelial activation and dysfunction[10]. Furthermore lesional vSMC are also able to take up lipids to form foam cells and secrete adhesion molecules and cytokines, thereby contributing to inflammation[12].

The relevance of apoptosis to plaque progression and instability has been established in numerous studies. Clearly, both rate and consequences of apoptotic cell death are dependent on the actual cell type and on lesion stage. VSMC apoptosis increases with plaque progression. Differences in expression patterns of pro- or anti-apoptotic proteins and survival cytokines like decreased insulin-like growth factor 1 (IGF-1) and increased p53 levels and alterations in expression of Bcl-2 family members may cause increased sensitivity of intimal vSMC to apoptosis compared to medial vSMC[13]. In murine atherogenesis persistent apoptosis of vSMC was seen to result in accelerated lesion development[14] and both persistent vSMC apoptosis and induction

of vSMC apoptosis in established lesions leads to plaque vulnerability as indicated by increased necrotic core and fibrous cap thinning[14,15]. In addition, in established lesions increased intimal inflammation was detected[15].
Macrophage death occurs at all stages of atherosclerotic lesion development[16], although in human lesions macrophage death was elevated mainly in advanced lesions compared to healthy vessels[17]. Apoptosis of macrophages can be induced by various stimuli present in the plaque including reactive oxygen species (ROS), oxidized LDL (ox-LDL), free cholesterol, TNF-α and Fas ligand[16]. Experimental studies in mice revealed that macrophage apoptosis may be beneficial in early atherosclerosis, limiting lesion size and cellularity. On the contrary in late stage atherosclerosis macrophage death leads to increased lesion size and necrotic core expansion. The latter is possibly attributable to insufficient removal of apoptotic cells and resulting secondary necrosis. Apoptosis of the various plaque cell types in atherosclerosis and its consequences for lesion progression and stability has been extensively reviewed elsewhere[13,16,18]. Recent studies, which be will reviewed here, suggest that several apoptotic proteins are involved in inflammatory processes while inflammatory mediators can influence apoptosis and that the association of apoptosis and inflammation might be of great significance in atherosclerotic lesion progression and stability. This review will discuss this link between apoptosis and inflammation in the context of atherosclerosis elaborating on pro-inflammatory effects of plaque cell apoptosis on the one hand and on pro- or anti-apoptotic effects of the foremost inflammatory mediators in atherosclerotic lesions on the other hand.

2 Immunomodulatory effects of intraplaque apoptosis

2.1 Inflammatory consequences of apoptotic protein activity

2.1.1 Apoptosis inducing TNF family proteins

Fas/Fas ligand

Fas (CD95) is a death receptor of the TNF receptor super family that stimulates cells to undergo apoptosis after ligation of Fas ligand. The Fas /Fas ligand system is key to the elimination of immune cells (T cells) and thus in the regulation of adaptive immune responses. In fact *lpr* en *gld* mice, which have inactivating mutations in Fas and Fas ligand respectively, both develop autoimmune disease very reminiscent of lupus erythematosis with overt lymphadenopathy and splenomegaly[19,20].
In several studies Fas triggered apoptosis has been implicated in neointima formation and atherogenesis. Adenoviral Fas ligand gene delivery inhibit neointima formation in balloon-injured rat carotid arteries[21], while in advanced atherosclerotic lesions it reduced the number of vSMC in the cap inducing intraplaque hemorrhage and buried cap formation. Moreover overexpression in carotid artery plaques was seen to promote leukocyte adhesion, endothelial leakage and intraplaque bleeding[22]. Several other studies have explored the impact of Fas function in atherosclerosis on inflammatory status. Yang *et al*[23] describe that Fas induced apoptosis of endothelial cells has anti-inflammatory properties. Transgenic mice overexpressing Fas ligand

on vascular endothelial cells showed decreased plaque macrophage and CD8+ T cell content and overall decreased aorta lesion size[23]. Conversely Fas signaling in vSMC had rather pro-inflammatory effects. Overexpression of inducible Fas-associated death domain (FADD), a downstream signal transducer of Fas, in a rat vSMC line increased apoptosis[23]. Seeding of these FADD overexpressing vSMC on rat carotid arteries led to Fas ligand induced intimal vSMC apoptosis resulting in MCP-1 and IL-8 secretion and monocyte recruitment to adventitia and neointima. The link between auto-immune disease and atherosclerosis has been addressed in Fas ligand defective *gld* mice, backcrossed to ApoE$^{-/-}$ mice. *gld.*ApoE$^{-/-}$ double transgenics not only had increased atherosclerotic lesion size and apoptotic cell content compared to ApoE$^{-/-}$ mice, but also increased lymphadenopathy, splenomegaly and formation of autoantibodies compared to *gld* mice[25], implying that autoimmune effects associated with Fas ligand deficiency is exacerbated by the proinflammatory milieu in ApoE$^{-/-}$ mice. Finally, Fas has been implicated in oxidative stress and ox-LDL induced cell death[26]. Ox-LDL induced apoptosis of T lymphocytes has been demonstrated to be preceded by an increased expression of Fas and membrane associated and soluble Fas ligand, and apoptosis could be inhibited by blocking of the Fas/Fas ligand dyad[26]. The relevance of Fas signaling for oxidative apoptosis in atherosclerosis is illustrated by colocalisation of TUNEL positive apoptotic cells in human carotid plaques with Fas expression on T cells and that of Fas ligand on macrophages. Interestingly both cell types showed increased iNOS expression, implicating Fas in iNOS induced cell death[27].

The above mentioned studies identify Fas as an important factor in atherosclerotic lesion apoptosis, including Ox-LDL and ROS induced cell death, of several cell types while both pro- and anti-inflammatory effects have been reported.

TRAIL

TNF related apoptosis inducing ligand (TRAIL), another member of the TNF super family, was originally described as a protein inducing apoptosis exclusively in transformed and infected cells. TRAIL acts by binding to signaling (i.e. death receptors or DR's) and non-signaling receptors (osteoprotegerin and decoy receptors) and displays both pro-apoptotic and immunoregulatory capacity[28]. TRAIL expression on immune cells is upregulated in response to type I interferons IFNα and IFNβ[29]. IFNα induced TRAIL upregulation on T cells has also been shown in atherosclerotic lesions via colocalization. The enhanced TRAIL expression enabled these T cells to induce apoptosis in coronary SMC[30]. TRAIL colocalizes with CD3 and ox-LDL in human stable lesions with increased expression in vulnerable plaques[31]. Secchiero *et al*[32] showed that systemic adenoviral TRAIL delivery reduces atherosclerotic lesion size in diabetic ApoE$^{-/-}$ mice, while increasing apoptosis of infiltrating macrophages within the lesion and increasing plaque vSMC content.

CD40

CD40 and CD40 ligand (CD154) are expressed by all cell types of human atherosclerotic

lesions. CD40 activation acts proliferative and pro-apoptotic and stimulates the secretion of pro-inflammatory cytokines and chemokines[33]. Genetic or antibody based CD40 signaling blockage decreased atherosclerotic lesion size in ApoE$^{-/-}$ and LDLr$^{-/-}$ mice[34,35] and led to a more stable phenotype[35]. Leukocyte CD40 ligand deletion however did not affect lesion size at all[36] suggesting that pro-atherogenic activity of CD40 ligand can be attributed to non-hematopoietic cells. This is supported by the finding that human umbilical vein endothelial cells (HUVECs) stimulated with CD40 ligand undergo massive apoptosis along with VCAM-1 and ICAM-1 release[37], suggesting that stimulation of CD40 on endothelial cells may promote monocytes/macrophages recruitment and thereby lesion development.

TWEAK

TNF-like weak inducer of apoptosis (TWEAK) has various biological functions including induction of inflammation, activation of cell growth, and stimulation of apoptosis upon binding its receptor Fn14[38]. Fn14 and TWEAK are expressed by macrophages, foam cells and smooth muscle cells in human carotid atherosclerotic plaques and in human aortic SMC[39,40]. Fn14 expression is upregulated after stimulation with IL1β and IFNγ[39], and TWEAK induced pro-inflammatory cytokines IL-6, MCP-1 and IL-8 in THP-1 human macrophages[40], suggesting that this pro-apoptotic TNF family member may function in apoptosis and inflammation in atherosclerotic plaques. Experimental proof to support this is lacking to date.

2.1.2 Bcl-2 family

Bcl-2 family members, which can be either pro- or anti-apoptotic, are the major regulators of both extrinsic and intrinsic apoptosis signaling pathways. In the vasculature these proteins are expressed in all major cell types and regulate apoptosis in response to oxidation and inflammation. Expression of Bcl-2 family members has been studied in human carotid endarterectomy samples showing increased expression of pro-apoptotic Bax and Bak in lipid laden macrophages, vSMC and apoptotic cells, absence of anti-apoptotic Bcl-2 and Bcl-XL in apoptotic cells and expression of the latter in non-apoptotic vSMC. Expression patterns of Bcl-2 family members in atherosclerotic lesions have been reviewed in detail by Kutuk and Basaga[41].

Inhibition of Bcl-XL by antisense oligonucleotides was shown to induce apoptosis in intimal cells of rabbit vascular lesions, an effect that was absent in medial SMC and in control vessels. Increased apoptosis led to a reduction in intimal lesion size[42].

Bax is another Bcl-2 family member which has been studied in an atherosclerotic mouse model. Liu *et al* showed reduced macrophage apoptosis in hematopoietic LDLr$^{-/-}$ chimeras with Bax deficiency leading to an increase in atherosclerotic lesion size in the aorta[43].

Increasing evidence indicates that Bcl-2 family members are involved in apoptosis induced by ROS, Ox-LDL, and inflammation. Ox-LDL induced apoptosis in U937 human monocytes and in human differentiated endothelial progenitor cells was

shown to involve ROS generation with concomitant mitochondrial Bax translocation and activation[44,45]. Translocation of Bax from cytoplasm to mitochondria in these cells could be inhibited by Bcl-2 overexpression, which did not prevent ROS generation suggesting that Bax acts downstream of ROS[44]. In endothelial cells Bax activation and translocation was mediated by an increase in p53[45]. Similarly minimally oxidized LDL induced apoptosis in human coronary endothelial and vSMC was mediated by Fas and TNF receptor domains and, in endothelial cells, accompanied by an increase in pro-apoptotic Bad and a decrease in anti-apoptotic Bcl-2[46]. In addition, activation of Bim and Bad and downregulation of Bcl-XL was observed in oxysterol (25-hydroxycholesterol and 7-ketocholesterol) induced cell death in P388D1 murine macrophages, probably by promoting AKT degradation[47]. Finally Badrichani *et al* showed that Bcl-2 and Bcl-XL overexpression not only protects bovine aortic EC's from TNFα induced apoptosis but also inhibits TNFα dependent upregulation of pro-inflammatory genes like E-selectin and IL-8 by inhibiting NF-κB[48].
Bcl-2 family members appear to be involved in apoptosis signaling pathways induced by both pro-apoptotic proteins and inflammatory mediators Ox-LDL and ROS and in addition are able to affect inflammatory responses in vascular cells.

2.1.3 P53 and p21

Tumor suppressor p53 is a key regulator of cellular homeostasis exerting various functions such as cell-cycle arrest, senescence, differentiation and apoptosis. Downstream regulators of p53 in apoptosis and survival include Bax, NOXA, PUMA, PTEN, Fas, DR5 and p21[49]. Vascular p53 expression was reported to be increased in advanced atherosclerosis. Various studies have been undertaken to elucidate the role of p53 in atherosclerosis investigating the effect of (cell type specific) p53 deficiency and p53 overexpression on lesion development in animal models (reviewed in 50). From these data p53 function in atherosclerotic lesion appears to be complex and stage-, context- and cell-dependent. For instance p53 protected from apoptosis and stimulated proliferation in atherosclerotic lesions in one study[51], whereas its overexpression in advanced atherosclerosis was seen to promote vSMC apoptosis and destabilize plaques[52]. In macrophages p53 induced apoptosis. Generally, deficiency of p53 increased atherosclerotic lesion size[50]. Recently p53 was implicated in Ox-LDL induced apoptosis in endothelial cells via the pro-apoptotic Bcl-2 member Bax[45]. Ox-LDL led to activation and mitochondrial translocation of Bax and subsequent apoptosis, processes that could be prevented by siRNA induced p53 knockdown. Earlier, induction of apoptosis by mildly oxidized LDL in human coronary endothelial and vSMC has also been shown to increase p53 expression together with that of multiple other pro-apoptotic proteins[53]. In a human colorectal cancer cell line (DLD-1) p53 induced apoptosis was preceded by increased expression of several ROS generating genes[54], identifying p53 as a pro-oxidant factor. In comparison, relatively low levels of p53 were able to protect DNA from oxidation and damage by ROS[55].
An important p53 target, p21 cyclin-dependent kinase inhibitor, also known as WAF1

and CIP1, can upon activation inhibit apoptosis[49]. Recombinant p21 was reported to inhibit lymphocyte proliferation and expression of pro-inflammatory cytokines IL2, TNFα and IFNγ[56] as well. Adenoviral gene delivery of p21 into balloon injured porcine arteries inhibited the development of intimal hyperplasia[57]. In addition, overexpression of a p21 mutant with increased biological activity, reduced restenosis in $ApoE^{-/-}$ mice[58]. This was attributed to attenuated vSMC proliferation and macrophage infiltration and increased apoptosis of vascular cells. In contrast, both systemic and leukocyte p21 deficiency in $ApoE^{-/-}$ mice protected against atherosclerosis and led to increased apoptosis rates. In these mice systemic p21 deficiency reduced VCAM-1 expression[59]. Furthermore p21 deficient peritoneal macrophages expressed lower levels of pro-inflammatory cytokines such as macrophage inflammatory proteins (MIP) 1 and 2 and displayed increased phagocytosis of apoptotic cells.
The role of p53 in atherosclerotic lesions is stage-, context- and cell-dependent. P53 is implicated in apoptosis induced by various factors like Ox-LDL and ROS involving different Bcl-2 family members as well. Several studies indicate that p53 responsive protein p21, like p53, has a complex role in atherogenesis.

2.2 Phagocytosis of apoptotic cells

Apoptotic cell death was originally considered to be immunologically neutral or anti-inflammatory[6]. In general apoptotic cells are rapidly removed by phagocytes such as macrophages and dendritic cells, which is mediated by dedicated eat-me signals including phosphatidyl serine (PS) exposure on the surface of apoptotic cells.
Various molecules participate in recognition and engulfment of apoptotic cells including CD14, TG2, calreticulin, Mer receptor tyrosine kinase, lactadherin, complement components (C1q, CR3 and CR4), integrins and scavenger receptors CD36, CD68 and SRA[60-64]. Although considerable redundancy in uptake and signaling mechanisms exists, these mechanisms may differ between the various phagocytic cells. Moreover inflammatory responses after apoptotic cell recognition and uptake may depend on specific receptors and proteins implicated as demonstrated in knockout mouse models[60,61,65-67].
Anti-inflammatory effects are attributable to the apoptotic cells themselves as well as to the remnant ingesting phagocytes[61]. For example phagocytic DC's exhibit decreased secretion of the pro-inflammatory cytokine TNFα after exposure to apoptotic cells[68] but secretion of anti-inflammatory mediators like TGFβ, IL10, and IL1 receptor antagonist is promoted[61]. In addition apoptotic lymphocytes were shown to release TGFβ[61]. Apoptotic cell death can also be pro-inflammatory. For example Fas induced apoptosis *in vivo* resulted in hepatic inflammation and neutrophil recruitment[8]. Furthermore, apoptotic cells may undergo secondary necrosis when not readily cleared by phagocytes, to subsequently evoke an inflammatory response. Figure 1 shows potential immunomodulatory effects of apoptotic cell death.
Defective phagocytosis by plaque macrophages has been demonstrated by Schrijvers *et al*[69]. Analysis of human endarterectomy samples revealed a higher ratio of free

to phagocytised apoptotic cells when compared with human tonsils[69]. Tabas has postulated the hypothesis that in early lesions phagocytotic capacity is sufficient to clear cellular debris implying that macrophage apoptosis will result in decreased lesion progression. In more advanced lesions phagocytosis is impaired, which not only dampens immunoregulatory feedback normally observed after phagocytosis but also favors proinflammatory secondary necrosis promoting lesion progression and instability[16].

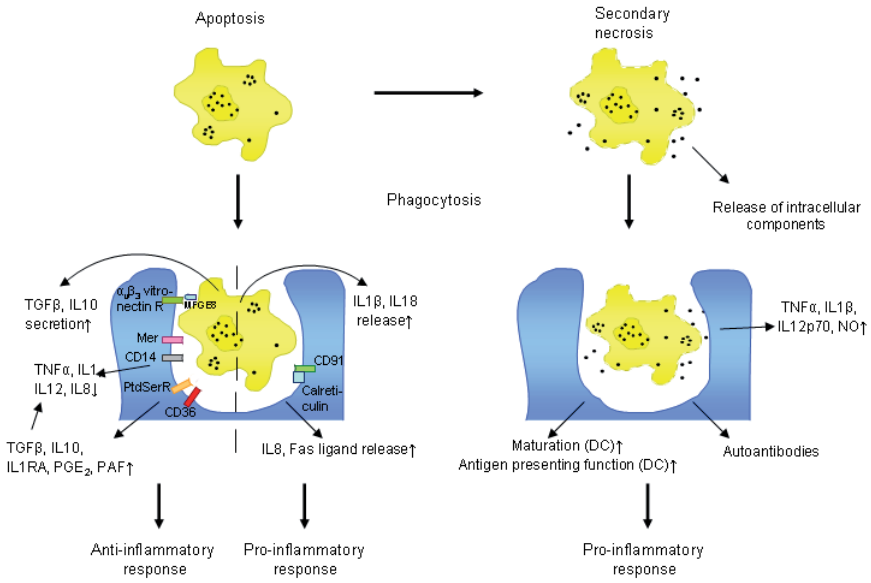


Figure 1. Modulation of inflammatory response through apoptotic cells and phagocytosis. Phagocytosis of apoptotic cells generally results in an anti-inflammatory response via increased release of anti-inflammatory molecules and inhibition of that of pro-inflammatory molecules by both apoptotic cells and phagocytes. However, apoptosis and subsequent phagocytosis can also be pro-inflammatory. A number of receptors and factors involved in apoptotic cell recognition and engulfment were shown to be associated with activation of pro- as well as with anti-inflammatory pathways, suggesting that a shifted pattern of apoptotic cell handling will translate in an altered inflammatory profile. Defective engulfment of apoptotic cells may lead to secondary necrosis. In contrast to apoptotic cells necrotic cells lose membrane integrity and their cellular content is released resulting in a pro-inflammatory response. TGFβ, transforming growth factor β; IL, interleukin; TNFα, tumor necrosis factor α; IL1RA, interleukin 1 receptor antagonist; PGE2, prostaglandin E2; PAF, platelet activating factor; NO, nitric oxide; DC, dendritic cell.

The relevance of phagocytosis to atherosclerosis was subject to various recent studies. Aprahamian *et al* [25] observed that the induced atherogenic response in Fas ligand deficient *gld*.ApoE$^{-/-}$ mice was associated with decreased apoptotic cell clearance and increased macrophage and lymphocyte infiltration. Even deficiency of ApoE itself may already affect phagocytosis[70]. *In vitro* ApoE deficient macrophages ingest less apoptotic thymocytes and in ApoE$^{-/-}$ mice apoptotic macrophage content is increased in various tissues compared with wild-type mice resulting in a pro-

inflammatory state as witness the elevated plasma TNFα levels in the former mice. One of the first macrophage receptors to be implicated in phagocytosis of apoptotic remnants is CD36, a class B scavenger receptor[61]. Ox-LDL, an established substrate for this receptor, was suggested to compete with apoptotic cells for ingestion by macrophages[71,72]. In keeping Khan *et al* [73] reported impaired uptake but not binding of apoptotic vSMC and fibroblast by thioglycolate elicited mouse peritoneal macrophages in the presence of excess Ox-LDL. In addition, release of IL6 and MCP-1, inhibited by phagocytosis without Ox-LDL, was no longer suppressed[72]. A similar apoptotic cell engulfment reducing effect of Ox-LDL was noted in peritoneal macrophages by Schrijvers *et al* [71]. Interestingly, minimally modified LDL, although not recognized by scavenger receptors, also can inhibit phagocytosis of apoptotic cells by binding to CD14, while increasing uptake of Ox-LDL[73]. Altogether these studies suggest partially shared but non-identical recognition sites for Ox-LDL and apoptotic cells. A second mechanism underlying the impaired apoptotic cell handling in atherosclerosis may involve Ox-LDL targeted auto-antibodies, which are present in both human and murine atherosclerotic lesions. These auto-antibodies were shown to bind to apoptotic cells and inhibit their phagocytosis by macrophages[74].

The role of several key genes in apoptotic cell removal on vascular disease progression has been recently addressed. Deficiency of leukocyte transglutaminase 2 (TG2), which mediates apoptotic cell uptake and TGFβ secretion by macrophages[62], in $LDLr^{-/-}$ mice was seen to increase aortic valve lesion size and intimal macrophage penetration[65]. In another study the effect of leukocyte milk fat globule-EGF factor 8 (also known as Mfge8 or lactadherin) deficiency was assessed on atherogenesis in $LDLr^{-/-}$ mice[66]. Mfge8 binds to PS exposed by apoptotic cells and enhances their uptake by macrophages[63]. Atherosclerosis was accelerated in $Mfge8^{-/-}LDLr^{-/-}$ mice and, importantly, lesions showed larger necrotic cores. In addition, Mfge8 deficiency appeared to favor proatherogenic Th1 type immune responses, with reduced IL10 production by spleen and increased IFNγ levels in both spleen and atherosclerotic arteries[66]. Another critical factor involved in phagocytosis of apoptotic cells is Mer receptor tyrosine kinase (Mer). Macrophages with dysfunctional Mer had defective phagocytosis of apoptotic thymocytes[64]. Moreover, Mer was shown to be necessary for apoptotic cell induced inhibition of NF-kB activation and pro-inflammatory cytokine secretion in dendritic cells[68]. Mer has been implicated in phagocytosis in atherosclerosis as well. Apoptosis induced in macrophages by free cholesterol loading elicited an inflammatory response in phagocytes, consisting of TNFα and IL1β production. This inflammatory response was constrained by Mer as shown by the use of Mer deficient phagocytes[67]. Similar to TG2 and Mfge8, deletion of leukocyte Mer in $LDLr^{-/-}$ mice also led to increased accumulation of apoptotic cells, increased macrophage area and lymphocyte infiltration resulting in accelerated lesion development[75].

3 Pro- and anti-apoptotic effects of immunomodulators in atherogenesis

3.1 Pro-inflammatory cytokines

IFNγ

Interferon gamma (IFNγ) is a pro-inflammatory cytokine produced by T and NK cells and macrophages. It is highly expressed in atherosclerotic lesions[76]. IFNγ has been shown to induce apoptosis in endothelial cells, SMC and macrophages in culture. Several pro-apoptotic proteins were suggested to be responsible for IFNγ induced cell death. In microvessel endothelial cells (EC) IFNγ synergizes with TRAIL to induce apoptosis, but not in large-vessel EC[77]. In agreement with the last finding, IFNγ lowers expression of death receptors DR4 and DR5 in HUVECs which is accompanied by decreased apoptosis[78]. In contrast, Li *et al* proposed a key role for Fas rather than TRAIL in IFNγ induced apoptosis in HUVECs[79]. Anti-Fas antibodies were able to completely block the pro-apoptotic effects of IFNγ. Likewise pro-apoptotic effects of IFNγ in vSMC were attributed to Fas[80] as well as to the pro-apoptotic X-linked inhibitor of apoptosis associated factor-1 (XAF1) and Bcl-2 family member Noxa[81] while in THP-1 macrophages upregulation of TNFR1 appeared to be implicated in IFNγ induced apoptosis[82].

Animal studies do support a pro-atherogenic role of IFNγ, but leave unanswered whether IFNγ dependent apoptosis could in part be held accountable for this effect. In IFNγ deficient ApoE$^{-/-}$ mice atherosclerotic lesion size and T lymphocyte content was shown to be reduced[83,84] while intraperitoneally administered recombinant IFNγ increases lesion size accompanied by an increase in lesional T lymphocytes[85]. A similar, reduced lesion size was observed in LDLr$^{-/-}$ mice lacking IFNγ[86].

Tumor Necrosis Factor alpha

Tumor Necrosis Factor alpha (TNFα) is highly expressed by activated macrophages and other immune cells in murine as well as human lesions. It exerts its, mainly pro-inflammatory, effects through two receptors, TNFR1 (p55) and TNFR1 (p75). For instance TNFα promotes monocyte/macrophage recruitment by inducing expression of E-selectin, VCAM-1 and ICAM-1 in endothelial cells via TNFR2 activation[87]. TNFα can induce apoptosis via TNFR1, which in contrast to TNFR2 contains a death domain, although TNFR1 is also capable of transducing survival signals[88-90]. TNFα induces apoptosis in endothelial cells and SMC, both on its own account and in response to various other pro-inflammatory cytokines like IFNγ and IL1β which are able to enhance expression of TNFα[91-93]. Boyle *et al* showed that in coculture macrophage derived TNFα acts proapoptotic on SMC via TNFR1[92]. Possibly, p73β, a protein closely related to p53, may mediate the pro-apoptotic effects of TNFα on vSMC as shown for rat aorta SMC by Tang *et al* [93]. The role of TNFα and TNF receptors in atherogenesis has been studied in various mouse models. The results are rather contradictory, but most data are supportive of a proatherogenic effect[76]. Two of the studies examining the effect of TNFα deletion assessed apoptotic cell content in atherosclerotic lesions. In ApoE*3 Leiden transgenic mice TNFα deletion resulted in decreased necrosis area but paradoxically an increased number of apoptotic cells[94]. In contrast, in ApoE$^{-/-}$ mice, TNFα deletion led to decreased apoptotic cell content[95].

Interleukins

Pro- and anti-inflammatory interleukins are regarded key mediators in vascular inflammation and atherogenesis[76,96]. More recently several interleukins such as IL1β, IL4 and IL10 have been shown to affect apoptotic processes as well, while others like IL6 and IL12 and IL18 promote proliferation[97,98]. IL1β can promote apoptosis of endothelial cells and SMC, an effect which could in human (HUVEC) and mouse lung EC be abrogated by overexpression of the naturally occurring interleukin 1 receptor agonist IL1-RA[99]. IL1β induced vSMC apoptosis however was shown to be TNFα dependent[91]. IL1β expression appears to regulate, and in turn is regulated, by various apoptotic proteins, including TWEAK receptor Fn14 and bcl-2 family members Bcl-2 and Bcl-XL[39,100]. The role of caspase-1 (Interleukin 1 converting enzyme, ICE) in pro-IL1β and pro-IL18 cleavage to their active form[101,102] further underlines the intertwined relationship between apoptosis and inflammation. Apart from IL1β, IL2[103] and IL4[104] have been implicated in apoptosis of vascular cells, at which the former acts in an indirect, TNFα and NO dependent manner.

Anti-apoptotic properties have been attributed to the anti-inflammatory IL10. In THP-1 macrophage IL10 was shown to decrease Ox-LDL induced apoptosis by upregulating anti-apoptotic Bcl-2 family members Bfl-1 and Mcl-1[105]. Interestingly plaque targeted and systemic overexpression of IL10 in LDLr$^{-/-}$ mice both inhibited atherosclerosis and resulted in decreased apoptosis of lesional macrophages[106]. An anti-apoptotic activity of this cytokine was supported by the finding that in advanced human atherosclerotic plaques high IL10 expression was seen to associate with decreased macrophage death[107].

Macrophage inhibitory factor

The pro-inflammatory macrophage inhibitory factor (MIF), which is increasingly expressed during atherogenesis, has recently been identified as an important contributor to inflammation in atherosclerotic lesions. MIF deletion in LDLr$^{-/-}$ mice resulted in reduced aortic atherosclerosis[108] while in ApoE$^{-/-}$ mice MIF blockade by neutralizing antibodies reduced macrophage infiltration and expression of adhesion and inflammatory molecules ICAM-1, MMP-2, TNF, IL-12, and CD40L[109]. Antibody blockade of MIF in LDLr$^{-/-}$ mice led to increased apoptosis in carotid arteries after endothelial denudation, pointing to a regulatory role of MIF in apoptosis[110]. In another study the anti-apoptotic properties of MIF in macrophages in culture were shown to be caused by inhibition of p53 expression[111].

3.2 Oxidative stress

During atherogenesis reactive oxygen species (ROS) and oxidized LDL (Ox-LDL) will accumulate in the vessel wall which leads to progressively increased oxidative stress. Ox-LDL, generated by *in situ* oxidation of extravasated LDL, can be taken up by macrophages leading to foam cell formation and promotion of inflammatory responses. Moreover, as discussed above, Ox-LDL can induce apoptosis in endothelial cells, vSMC and macrophages[112] and interfere with apoptotic cell handling, often

favoring secondary necrosis. ROS are thought to be pro-atherogenic by, amongst others, inducing cell proliferation and apoptosis, recruitment of leukocytes and expression of pro-inflammatory cytokines like TNFα, IL1β and IFNγ (reviewed in 113 and 114). ROS can also cause degradation of cellular components including (mitochondrial) DNA, proteins and lipids. Sources of ROS in the vasculature include cyclooxygenases, lipoxygenases, NO synthase and NADPH oxidase. Most of the atherosclerotic risk factors (hypercholesterolemia, diabetes, hypertension, smoking and aging) were seen to increase ROS production[114]. In inflammatory cells and in particular in phagocytes, NADPH oxidase, which is activated by phagocytic neutrophils and macrophages[114], is the major source of ROS. The importance of NADPH oxidase has been established in animal models. ApoE$^{-/-}$ that lack p47phox, an essential subunit of NADPH oxidase, showed reduced levels of O_2^- production and smaller atherosclerotic lesions in the aorta[115]. A similar protective effect was seen after pharmacological inhibition of NADPH oxidase in balloon injured rat carotid arteries[116]. Lipoxygenases (LO) are another class of ROS producing enzymes expressed in leukocytes and vSMC. Inhibition of 12/15–LO in vSMC reduces growth factor induced migration, proliferation and matrix production[117] and 5-lipoxygenase pathway products leukotriens are potent vascular inflammation inducing mediators[118]. In ApoE$^{-/-}$ mice 12/15-LO gene disruption led to substantially smaller aortic artery lesions together with decreased circulating levels of auto-antibodies to Ox-LDL[119] while pharmacological inhibition of the receptor for Leukotriene B4, a product of the 5-lipoxygenase pathway of arachidonic acid metabolism, in both ApoE$^{-/-}$ and LDLr$^{-/-}$ mice reduced monocyte infiltration and foam cells in atherosclerotic lesions[120].

In the vasculature nitric oxide (NO), produced by endothelial NO synthase (eNOS) and inducible NO synthase (iNOS), is instrumental in controlling vascular tone. At physiological concentrations it is suggested to be anti-apoptotic and atheroprotective. Under inflammatory conditions as apparent during atherogenesis, catalytically active iNOS will be upregulated and substrate as well as cofactors may become rate limiting favoring the production of the pro-oxidant nitroperoxide at the expense of a reduced NO production[114]. This translates in augmented synthesis of pro-inflammatory cytokines TNFα, MCP-1 and IFNγ and adhesion molecules VCAM-1 and ICAM-1, promoting recruitment, infiltration and activation of monocytes and macrophages. INOS has been shown to protect EC against apoptosis[121]. The critical importance of a proper NO/ROS balance for endothelial cell function was illustrated by a study in which antisense oligonucleotide induced downregulation of iNOS expression in EC was seen to lead to endothelial dysfunction and ROS mediated cell death[118]. Furthermore, foam cells show increased iNOS expression resulting in toxic levels of NO. Fas associated cell death may be involved in this toxicity[122].

Ox-LDL promotes generation of ROS in vascular and atherosclerotic lesion cells, which can in turn induce oxidation of LDL. In endothelial cells Ox-LDL stimulates expression of adhesion molecules and growth factors leading to monocyte recruitment[112]. Both mildly and extensively oxidized LDL can induce apoptosis of endothelial cells,

vSMC and macrophages including foam cells, although exposure of macrophages to low concentrations of oxidized LDL also was reported to stimulate proliferation[123]. Mildly oxidized LDL results not only in activation of Fas and TNF receptor signaling but also in an increased pro-apoptotic and decreased anti-apoptotic Bcl-2 family member expression in human coronary endothelial and SMC[53]. Furthermore Ox-LDL induces various transcription factors involved in oxidative stress pathways including p53 and NF-κB. Another mechanism by which oxidative stress influences Ox-LDL induced apoptosis and inflammation involves the endothelial oxidized LDL receptor LOX-1. Lectin-like oxidized low density lipoprotein receptor (LOX-1) activation by Ox-LDL stimulates NF-κB and subsequent adhesion molecule and pro-inflammatory gene expression. In endothelial cells Ox-LDL has been shown to reduce eNOS and increase LOX-1 expression thereby inhibiting endothelial cell proliferation and function[124]. In keeping, a recent study showed that LOX-1 disruption in LDLr$^{-/-}$ mice had reduced aortic atherosclerotic lesion size and pro-inflammatory markers within the lesions[125]. Furthermore LOX-1 expression has been linked to apoptosis in human endothelial cells[126]. Figure 2 gives a summary of the effects of ROS on apoptosis and inflammation.

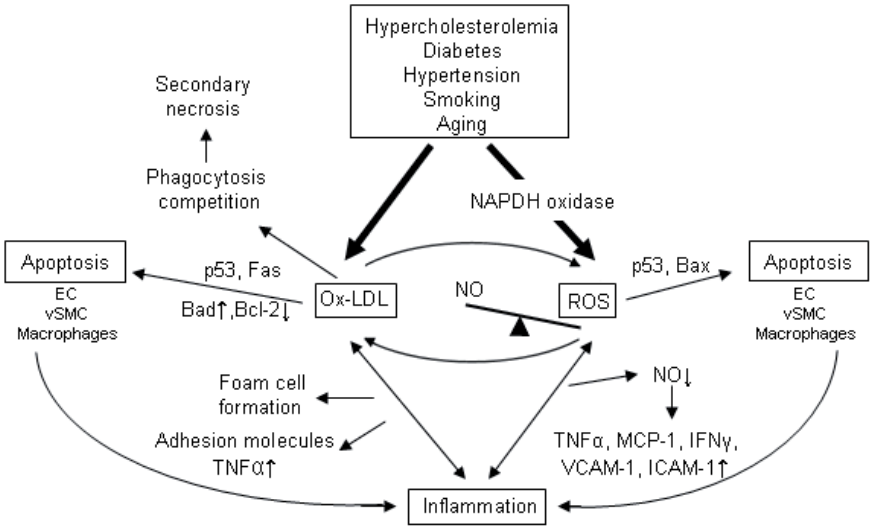


Figure 2. Generation of reactive oxygen species and its effects on inflammation, apoptosis and atherogenesis. During early atherogenesis ROS are generated in the vascular wall, reducing NO production and resulting in an unfavorable NO-ROS balance. ROS promotes endothelial activation, oxidation of LDL, apoptosis and inflammation. Ox-LDL in turn promotes generation of ROS and exerts pro-apoptotic and pro-inflammatory effects as well. Ox-LDL, oxidized low-density lipoprotein; NO, nitric oxide; ROS, reactive oxygen species; EC, endothelial cell; vSMC, vascular smooth muscle cell, TNFα, tumor necrosis factor α; MCP-1, monocyte chemoattractant protein 1; IFNγ, interferon γ; VCAM-1; vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1.

4 Summary and conclusions

Inflammatory and apoptotic processes in atherogenesis are closely intertwined. This notion has been conclusively demonstrated in various recent *in vitro* studies in cell types relevant to atherosclerosis as well as in *in vivo* studies in atherosclerosis prone animal models. First many of the proapoptotic members of the TNF super family, Fas, CD40 and Fn14, can promote secretion of inflammatory cytokines like MCP-1 and IL8 and/or adhesion molecules accompanying induction of apoptosis. Reactive oxygen species and Ox-LDL, accumulating in the vessel wall during plaque progression, are able to induce cell death in endothelial cells, vSMC and macrophages by upregulating several apoptotic proteins including Fas, TNFα, p53 and Bcl-2 family proteins. In addition, several key cytokines present in atherosclerotic lesions, apart from being induced in apoptosis, are able to induce apoptosis themselves. Vice versa, apoptotic cells were seen to modulate inflammatory responses either on their own account by elaborating amongst others cytokines and proteases or after uptake and processing by phagocytes. Impaired and altered phagocytosis, as apparent during progressive atherosclerosis, will, by eliciting a proinflammatory response and by promoting secondary necrosis, further aggravate inflammation leading to lesion progression and instability. Although several pro- or anti-apoptotic proteins and inflammatory mediators are attractive targets for therapeutic strategies, crosstalk between and the complex consequences of inflammatory, apoptotic and phagocytic processes have to be accounted for.

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Chapter 3

Gene Expression Profiling in Atherosclerotic Plaque Vulnerability Identifies Neuropeptide Y as a Marker of Plaque Vulnerability

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Abstract

While the significance of mouse models for atherosclerotic plaque rupture still is subject to discussion, various models have been claimed to display features of enhanced plaque vulnerability. Conceivably, models for vulnerable plaque formation could share pathways or factors that predispose to plaque rupture. Here we have pursued a genomics approach to identify pathways or factors that are operative early on in the plaque destabilization process. Total RNA was obtained from collar induced carotid artery plaques of Western type diet fed ApoE$^{-/-}$ mice 7 days after transduction with adenoviral P53 to generate thin cap fibroatheroma (ThCFA) or with AdLacZ (thick cap fibroatheroma control; TkCFA). Brachiocephalic artery plaques of the same ApoE$^{-/-}$ mice isolated after 9 weeks of Western type diet feeding served as second model for plaque vulnerability. DNA micro array analysis on oligonucleotide arrays (22,000 genes) revealed a total of 57 genes that were significantly upregulated in Adp53 (ThCFA) versus AdLacZ overexpressing carotid artery plaques (TkCFA). 58 Genes were significantly upregulated in brachiocephalic (ThCFA) versus non-transduced carotid artery plaques (TkCFA), whereas 87 and 66 genes were downregulated in the respective models with an overrepresentation of gene clusters relevant to cell death. Five of these genes were found to be upregulated while nine were downregulated in both types of ThCFA. Differential expression of several of these genes was confirmed by real-time PCR. In particular neuropeptide Y showed increasing gene expression not only with disease progression in both mouse models but also in human plaques. In conclusion, mouse models of vulnerable plaque formation share various dysregulated genes and gene sets that may potentially be relevant to the destabilization process in the human situation.

Introduction

Rupture of an atherosclerotic plaque generally underlies coronary thrombosis and subsequent myocardial infarction and cardiac death[1]. Although plaque rupture rarely occurs in mouse models of atherosclerosis, some studies have demonstrated that ApoE and LDL receptor deficient mice do develop complex thin cap fibroatheroma[2,3,4]. At age 42 weeks brachiocephalic artery plaques of chow diet fed ApoE$^{-/-}$ mice show overt features of enhanced vulnerability, as amongst others reflected by fibrous cap break and intraplaque hemorrhage[2]. When fed a Western type diet for only 8 weeks, ApoE$^{-/-}$ mice were even claimed to form complex fibroatheromathous lesions with ruptured caps in 62% of cases[5]. In addition to cap breaks and intraplaque hemorrhages, plaques frequently contained buried fibrous caps, possibly remnants of a previous plaque rupture, although some debate exists on the significance of this finding[6-8].

In 2002 we have reported that targeted overexpression of p53 into cap smooth muscle cells in advanced collar induced carotid artery plaques resulted in increased apoptosis of cap cells, in reduced cap thickness, and in general in a vulnerable plaque phenotype which proved prone to phenylephrine induced rupture[9].

Several genomics studies have been performed to identify disease related gene expression patterns in atherosclerosis in humans and mice[10-14] leading to the identification of differentially expressed genes or functional groups of genes like growth factors, chemokines and other inflammatory and non-inflammatory genes resulting from differences in strain, site (atherosclerosis-prone or -resistant), age and diet. However transcriptional profiling in established mouse models of vulnerable plaque formation, particularly of great importance inducing most clinical complications, has not been addressed to date.

In this study, genome wide profiling is used to identify gene expression patterns characteristic of vulnerable thin cap fibroatheroma (ThcFA) compared to more stable thick cap fibroatheroma (TkCFA) in ApoE$^{-/-}$ mice. To minimize the impact of patterns intrinsic to the models used and thus to increase the significance of our findings we have studied two different mouse models at an early stage of the destabilization process before vulnerable plaque formation had become manifest. We hypothesize that at this stage these models may share pathways or factors that predispose to plaque vulnerability.

Methods

Animals and surgery

All animal work was approved by regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. ApoE$^{-/-}$ mice were obtained from the local animal breeding facility. Starting at 15 weeks of age 18 male ApoE$^{-/-}$ mice were fed a Western type diet containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK) three weeks before surgery and throughout the experiment. Carotid atherosclerotic plaque development was induced by bilateral perivascular collar placement as described by Von der Thüsen *et al.*[15] Five weeks after collar placement

right carotid arteries were treated with adenovirus containing a CMV promoter and p53 (AdP53, n=9) or β-galactosidase transgene (AdLacZ, n=9) as described by Von der Thüsen *et al.*[9] Contralateral carotid arteries were left untreated.

Cholesterol levels
Blood samples were taken by tail cut after four weeks and seven weeks of Western type diet feeding, and at the time of sacrifice. Total serum cholesterol levels were measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, The Netherlands).

Tissue harvesting and plaque morphometry
One week after transduction with either AdP53 or AdLacZ the mice were anesthetized and perfused with PBS. AdP53 or AdLacZ transduced carotid arteries and half of the non-transduced carotid and brachiocephalic arteries were isolated and snap frozen in liquid nitrogen. Mice were subsequently fixated by 4% formaldehyde perfusion (4.5 times diluted Zinc Formal-Fixx, Thermo Electron Corporation, Breda, The Netherlands). The remaining carotid and brachiocephalic arteries were isolated and stored in 4% formaldehyde solution. Cryosections were prepared of carotid and brachiocephalic arteries and stained with hematoxylin and eosin and analyzed using Leica Qwin image analysis software.

Micro-array hybridization and analysis
Total RNA was isolated from snap frozen transduced carotid arteries, their non-transduced contralateral counterparts and brachiocephalic arteries using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and quality controlled with RNA 6000 Nano Lab-on-Chip kit (Agilent Technologies, Amstelveen, The Netherlands). RNA of brachiocephalic artery and transduced or non-transduced carotid arteries from RNA samples of three different mice was pooled, giving three samples per artery, and used for cDNA synthesis followed by cRNA amplification with aminoallyl modified UTP's (MessageAmp aRNA Amplification kit, Ambion, Nieuwerker a/d IJssel, The Netherlands) and coupling to Cy3 or Cy5 monoreactive dyes (GE Healthcare Europe GmbH, Diegem, Belgium). cRNA of carotid artery samples transduced with AdP53 and AdLacZ were hybridized together on oligonucleotide arrays containing 22.000 genes as were cRNA of brachiocephalic artery and non-transduced carotid artery in a dyeswap manner. Genepix v6.0 (Axon instruments, Sunnyvale, CA, USA) was used for feature extraction. Analysis was performed using Rosetta Resolver software (Rosetta Biosoftware, Seattle, WA, USA) and applying the standard Rosetta Resolver error model for GenePix. Only spots with significantly higher signal ($p\text{-value} < 10^{-6}$) than the surrounding background were considered in the analysis. Differences in gene expression between AdP53 transduced carotid artery plaques and AdLacZ transduced carotid artery plaques or brachiocephalic artery plaques and non-transduced carotid artery plaques were considered significant when $p<0.001$, as determined by a Student's t-test. The obtained lists of differentially

expressed genes were further analyzed using Ingenuity Pathway Analysis (http://www.ingenuity.com/products/pathways_analysis.html) to determine the biological function of differentially expressed genes.

Verification of gene expression by realtime PCR

cDNA was prepared from total RNA samples of mouse brachiocephalic and carotid arteries (used for cRNA synthesis). Gene expression was analyzed by realtime PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems) with SYBR-Green technology. The different primers and sequences are listed in table 1. HPRT, 18S and β-actin were used as standard housekeeping genes.

Table 1. Primer sequences for realtime PCR.

Gene	Species	Forward Primer (5'-3')	Reverse Primer (5'-3')
HPRT	Mouse	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
18S	Mouse	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG
β-actin	Mouse	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
Cd5l	Mouse	GGAGTTGGGACCTCGTTAGAAGA	AGGTGGTCAAGCTGTGGACAA
GC	Mouse	GCAGAACGGCTAAGGACAAAA	AGTCCGAGTGTTTCTCCACCAT
Mup3	Mouse	CTCGAGGCCCGAGAATGAA	TGACGACCAACCTCCTCCTT
Npy	Mouse	TTTTCCAAGTTTCCACCCTCAT	AGTGGTGGCATGCATTGGT
Fabp5	Mouse	GGAAGGAGAGCACGATAACAAGA	GGTGGCATTGTTCATGACACA
HPRT	Human	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
Cyclophilin	Human	CCCACCGTGTTCTTCGACAT	CCAGTGCTCAGAGCACGAAA
Npy	Human	TGGTGATGGGAAATGAGACTTG	TGGATTCTCGTTTTACACGATGA

Expression profiling in human atherosclerotic carotid artery plaques

For gene expression profiling in human stable and unstable plaques RNA was obtained from the AtheroExpress Biobank[16]. Gene expression was analyzed as described for verification of gene expression with mouse RNA with primers listed in table 1. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and cyclophilin were used as standard housekeeping genes. For immunohistochemical analysis of neuropeptide Y expression paraffin embedded sections of plaques corresponding with the RNA samples used for gene expression analysis were obtained from the AtheroExpress Biobank. Antibodies used were rabbit anti-NPY (Abcam, Cambridge, UK) and goat anti-rabbit HRP conjugated secondary antibody (Dako, Heverlee, Belgium).

Neuropeptide Y gene expression during plaque development

In a separate experiment 20 male LDLr$^{-/-}$ mice, obtained from the local animal breeding facility, were fed a Western type diet two weeks prior to surgery and throughout the experiment. Atherosclerotic carotid artery plaques were induced by perivascular collar placement as described before. From 0 to 8 weeks after collar placement every two weeks a subset of 4 mice was sacrificed. The mice were anaesthetized and perfused with PBS after which both carotid arteries were isolated, snap-frozen in liquid nitrogen and stored at -80ºC. Two or three carotid arteries were pooled

per sample and total RNA was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands). Gene expression was analyzed as described before using the (mouse) PCR primers listed in table 1.

Statistics expression analysis

Values are expressed as mean ± SEM or presented as mean + upper limit of the SEM. For analysis of relative mRNA expression data t-test was performed on ΔCt values. For these experiments statistical significance was set at $p<0.05$.

Results

Animal and atherosclerotic plaque characteristics

In the present study we have pursued a genomics approach to identify pathways or factors involved in atherosclerotic plaques vulnerable to rupture early on in the plaque destabilization process. Collar induced carotid artery plaques of ApoE$^{-/-}$ mice fed a Western type diet for eight weeks were transduced with either AdP53 or AdLacZ and carotid and brachiocephalic arteries were isolated one week later. Serum total cholesterol levels did not differ significantly between the two groups of mice transduced with either AdP53 or AdLacZ at the time of sacrifice, 1468 ± 229 mg/dl and 1226 ± 193 mg/dl respectively. Total body weight was comparable between both groups as well (26.8 ± 4.8 g for AdP53 transduced mice and 27.5 ± 2.7 g for AdLacZ transduced mice). Carotid and brachiocephalic arteries of ApoE$^{-/-}$ mice isolated after nine weeks of Western type diet feeding and six weeks after collar placement were sectioned and stained with hematoxylin and eosin. The average size of carotid artery plaques was 2.97 ± 1.27 x 10^4 µm^2 and the average size of brachiocephalic artery plaques was 1.03 ± 0.27 x 10^5 µm^2. Representative micrographs of non-transduced carotid and brachiocephalic artery plaques are shown in figure 1. At the time of isolation, brachiocephalic artery plaques had progressed to cap fibroatheromas; cap breaks however were not evident at this stage. Morphological characteristics are depicted in table 2.

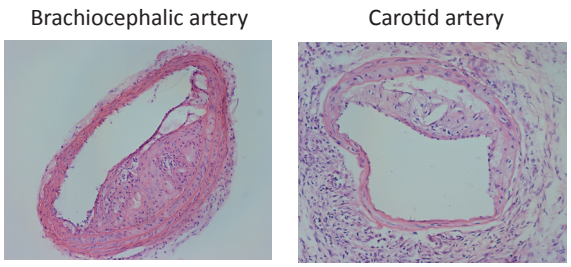


Figure 1. Representative pictures of brachiocephalic (left) and carotid (right) artery plaques stained with hematoxylin and eosin.

Table 2. Morphological characteristics of carotid and brachiocephalic arteries.

	Carotid artery	Brachiocephalic artery
Plaque size (µm^2)	2.97 ± 1.27 x 10^4	1.03 ± 0.27 x 10^5
Media size (µm^2 *10^4)	3.97 ± 0.99	8.36 ± 1.24
Intima/Media ratio	0.79 ± 0.42	1.25 ± 0.36
Intima/Lumen ratio	0.34 ± 0.16	0.55 ± 0.11

Microarray analysis

To identify pathways or factors shared between the two different mouse models of ThCFA and potentially predisposing to plaque rupture we performed genome wide analysis using microarray technique. Carotid artery plaques transduced with AdP53 (ThCFA), were compared with control TkCFA (AdLacZ transduced) carotid artery plaques and brachiocephalic artery plaques (ThCFA) were compared with non-transduced carotid artery plaques (TkCFA). For differential gene expression analysis we applied a student's t-test using a p-value < 0.001 as treshold. In this analysis 87 genes appeared to be significantly down- and 57 genes upregulated in AdP53 vs LacZ transduced carotid artery plaques. Comparison of brachiocephalic artery plaques to non-transduced carotid artery plaques showed 66 genes to be down- and 58 genes to be upregulated. Comparison of both microarray experiments revealed that 10 (out of 143) downregulated and 5 (out of 110) upregulated genes, listed in table 3, were dysregulated in both models of unstable plaque formation. Importantly, of the five genes upregulated in both microarrays, three appeared to be involved in apoptosis, *Cd5l, Plagl1* and *Bim*.

Table 3. Genes differentially expressed in two models of unstable atherosclerotic plaque formation as identified by micro-array analysis and/or genes verified by realtime PCR.

		Fold change	
GenBank Accession nr.	Gene	AdP53 vs AdLacZ transduced Carotid Artery	Brachiocephalic vs carotid artery
NM009538	Pleiomorphic adenoma gene-like 1 (Plagl1)	2.86 ↑	9.77 ↑
NM009690	CD5 antigen-like (CD5l)	3.18 ↑	7.70 ↑
NM009754	BCL2-like 11 (Bim)	2.08 ↑	3.43 ↑
AK002310	RIKEN cDNA 0610008A10 gene	1.67 ↑	1.58 ↑
AK019647	RIKEN cDNA 4930478K11 gene	1.49 ↑	1.95 ↑
AF327059	Apolipoprotein A-V (ApoA5)	2.81 ↓	41.25 ↓
U89889	Hemopexin (Hpxn)	2.70 ↓	5.69 ↓
NM010168	Coagulation factor (F2)	2.42 ↓	16.06 ↓
AK004470	RIKEN cDNA 1190003J15 gene	2.38 ↓	10.98 ↓
AB039380	Cytochrome P450, family 3, subfamily a, polypeptide 44 (Cyp3a16)	2.34 ↓	39.34 ↓
L04150	Apolipoprotein C-III (ApoC3)	2.33 ↓	2.70 ↓
M55413	Group specific component (GC)	2.16 ↓	24.01 ↓
M27608	Major urinary protein 3 (Mup3)	2.07 ↓	16.08 ↓
L13622	Methionine adenosyltransferase I, alpha (Mat1a)	2.04 ↓	10.70 ↓
AJ011413	Albumin 1 (Alb1)	2.02 ↓	30.40 ↓
NM010634	Fatty acid binding protein 5, epidermal (Fabp5)		4.20 ↑
AF273768	Neuropeptide Y (Npy)		2.28 ↑

In order to rule out the possibility that differential gene expression is caused by differences in plaque macrophage, smooth muscle cell, endothelial cell or T lymphocyte content, several cell type specific markers were verified for gene expression. The majority of these genes were not differentially regulated (table 4).

Table 4. Expression of cell type specific markers.

			Innominate vs. carotid		AdP53 vs. AdLacZ	
Cell type	Accession nr.	Gene name	Fold change	P-value	Fold change	P-value
Smooth muscle cells	AK019969	Myh10	1.31 ↑	0.0620	1.14 ↑	0.1262
	AK020381	Myh10	1.03 ↓	0.3179	1.19 ↑	0.7684
	NM_009922	Cnn1	1.32 ↑	0.0564	1.05 ↑	0.8895
	NM_007725	Cnn2	1.71 ↑	0.2912	1.35 ↓	1
Macrophages	NM_009853	Cd68	1.99 ↑	0.4650	1.35 ↑	0.0593
	NM_032465	Cd96	1.09 ↑	0.9240	1.40 ↓	0.0059
Endothelial cells	NM_008816	Pecam1	1.03 ↓	0.3463	1.33 ↓	1
	NM_011345	E-selectin	1.17 ↓	0.8508	1.99 ↓	0.8278
T-lymphocytes	NM_007648	Cd3e	1.41 ↓	0.0035	1.22 ↓	0.3214
	NM_013487	Cd3d	1.13 ↑	0.4174	1.07 ↓	0.5757
	NM_031162	Cd3z	1.14 ↑	0.4439	1.09 ↑	0.5043
	NM_009850	Cd3g	1.14 ↑	0.8235	1.29 ↓	0.0962
	NM_009858	Cd8b1	1.11 ↑	0.0619	1.05 ↑	0.1087
	AJ131778	Cd8a	1.17 ↓	0.3207	1.17 ↑	0.1979
	M12052	Cd8a	1.22 ↑	0.7407	1.30 ↑	0.5904
Dendritic cells	AF065893	Cd80	1.53 ↓	0.1476	1.04 ↑	0.8488
	NM_019388	Cd86	1.07 ↓	0.7801	1.21 ↑	0.3581

Involvement of differentially expressed genes in functional groups

Ingenuity Pathway Analysis was performed to identify the underlying pathways and processes dysregulated in both models of ThCFA. Processes with particular overrepresentation of dysregulated genes, shared by both models included cell death (43 and 30 genes differentially regulated in AdP53 versus AdLacZ treated carotid artery plaques (p=0.01) and brachiocephalic versus carotid artery plaques (p=0.01) respectively, lipid metabolism (21 [p=0.02] and 22 [p=0.01] genes), small molecule biochemistry (28 [p=0.01] and 26 [p=0.01] genes), metabolic disease (21 [p=0.008] and 16 [p=0.01] genes), cellular growth and proliferation (9 [p=0.01] and 27 [p=0.01] genes) and cell-to-cell signaling and interaction (19 [p=0.02] and 8 [p=0.01] genes). These functional groups remain significantly affected when only differentially expressed genes are analyzed that are shared between both models of ThCFA.

Verification of differentially expressed genes by realtime PCR

Genes differentially expressed in advanced plaques of both models of ThCFA compared to TkCFA control plaques and genes differentially expressed in advanced plaques of one model with previously reported involvement in atherosclerotic

plaque development were selected for verification by quantitative realtime PCR using the same cRNA samples as originally used for microarray analysis. Differential regulation of Cd5 antigen like (*Cd5l*), Pleiomorphic adenoma gene-like-1 (*Plagl1*), Group specific component (*Gc*) and Major urinary protein 3 (*Mup3*) in both microarray experiments and Neuropeptide Y (*Npy*) and Fatty acid binding protein 5 (*Fabp5*) in brachiocephalic artery plaques compared to carotid artery plaques could be confirmed by realtime PCR (fig. 2). Bcl-2 like interacting mediator of cell death (*Bim*) showed a tendency towards increased expression in brachiocephalic compared to carotid artery plaques (p=0.056). Several other selected genes failed to reach statistical significance.

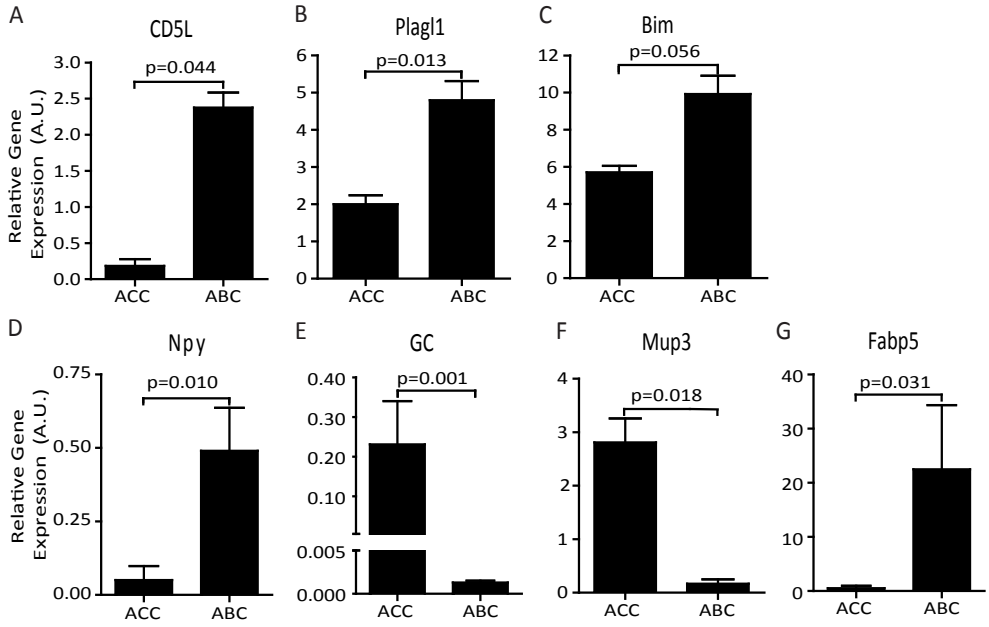


Figure 2. Quantitative PCR assisted validation of genes differentially expressed in mouse stable (ACC, common carotid artery) versus unstable plaques (ABC, brachiocephalic artery) as identified by microarray analysis.

Expression of Neuropeptide Y in human carotid artery atherosclerotic plaques

Expression of the genes that were significantly altered in mouse ThCFA compared to TkCFA was assessed in human stable and unstable carotid artery plaques. Plaque RNA was obtained from the AtheroExpress Biobank of endartorectomy specimen of patients who had undergone endarterectomy of the carotid artery (n=9/12)[16]. Plaques from all patients in this study were previously phenotyped[16]. Fibrous plaques low in inflammatory cell and fat content with strong staining for collagen and smooth muscle cells were considered stable, while lesions were categorized unstable atheromathous plaques if they had strong staining for macrophages and no or minor staining for collagen and smooth muscle cells[16]. Neuropeptide Y (*Npy*) gene expression appeared to be increased more than twofold (p=0.036) in unstable compared to stable human carotid artery plaques (fig. 3a). Immunohistochemical

analysis was performed on paraffin embedded sections from the same carotid artery plaques that served as source for gene expression analysis. Npy was demonstrated to be strongly present in de medial layer of carotid arteries in both stable and unstable plaques and was virtually absent in the intima (fig. 3b). Immunological analysis did not allow establishing quantitative differences in Npy protein expression between stable and unstable plaques.

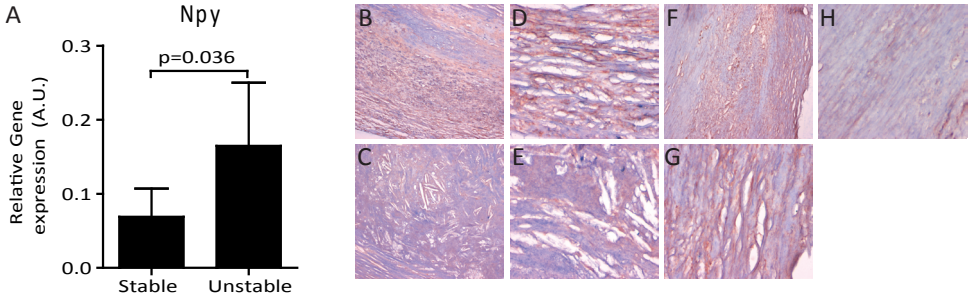


Figure 3. A. Neuropeptide Y expression is increased in unstable versus stable human carotid artery atherosclerotic plaques, specimen from the AtheroExpress Biobank. B-H. Representative sections of Npy immunohistochemical staining in human unstable (B-E) and stable (F-H) carotid artery atherosclerotic plaques. Magnification 100x (B,C and F) and 400x (D,E,G, H).

Neuropeptide Y gene expression during atherogenesis

Next we have monitored *Npy* expression during atherogenesis. Carotid arteries of western type diet fed LDLr$^{-/-}$ mice equipped with semi-constrictive collars to accelerate low shear stress induced plaque formation. *Npy* expression steadily increased with plaque progression from 0 (no plaques) to six weeks after initiation of plaque development (t=6, fig. 4). After six weeks of plaque development *Npy* expression leveled off to maintain a high level at least until week eight.

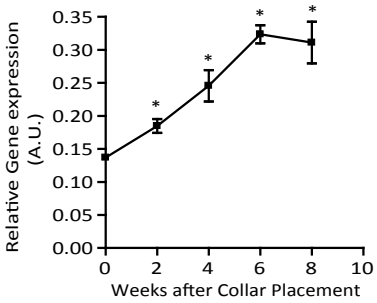


Figure 4. Neuropeptide Y expression increases with atherosclerotic plaque progression in carotid arteries of LDLr$^{-/-}$ mice. Mice underwent surgery to place perivascular collars around the carotid arteries and were fed a Western type diet. (*p<0.05)

Discussion

In the current study we analyzed gene expression profiles of thick cap fibroatheroma and thin cap fibroatheroma in mice in order to identify genes or functional groups of genes that predispose the vascular wall to plaque rupture. To increase the power of genomic analysis and facilitate the identification of actual stability- rather than model-associated pathways we included two different models of ThCFA in our

genomics approach and analyzed them for genes and pathways shared by both mouse models. As a first model we considered spontaneous vulnerable plaque development in the brachiocephalic artery of ApoE$^{-/-}$ mice fed a Western type diet for 9 weeks[5]. As a second model we have used low shear stress induced carotid artery plaques in ApoE$^{-/-}$ mice that were further destabilized by adenoviral overexpression of P53 in the cap zone[9]. The impact of factors intrinsic to the models or changes in cellular plaque composition was minimized by assessing the early stage vulnerable plaque formation in both models. Using this strategy we could identify 87 genes that were downregulated and 57 genes that were upregulated in ThCFA AdP53 versus AdLacZ transduced carotid artery plaques (TkCFA controls) and 66 downregulated and 58 upregulated genes when brachiocephalic artery ThCFA were compared to TkCFA. Of these differentially regulated genes 10 downregulated and 5 upregulated genes were shared between the two models of ThCFA. In addition, Ingenuity Pathway Analysis identified several functional clusters consisting of differentially regulated genes that were overrepresented in both models. Cell death, lipid metabolism, small molecule biochemistry, metabolic disease, cellular growth and proliferation and cell to cell signaling and interaction comprise the functional processes that were most significantly affected in ThCFA versus TkCFA. With 43 and 30 regulated genes in brachiocephalic and AdP53 transduced carotid artery plaques respectively, cell death was the most prominently dysregulated process in our analysis. This finding is in line with the observed increase in plaque apoptotic cell content after adenoviral p53 overexpression[9], and with the reported enhanced rate of apoptosis of both macrophages and vascular smooth muscle cells (vSMC) in human type IV to VI plaques[17]. Direct induction of vSMC apoptosis in mouse aortic atherosclerotic plaques was recently shown to promote features of plaque vulnerability including fibrous cap thinning, loss of collagen, intimal inflammation and increased necrotic core size[18], and in carotid artery atherosclerotic plaques reduced cap cellularity and cap/intima ratio with induction of plaque rupture[9]. Enhanced macrophage apoptosis in mouse plaques results in an increased necrotic core size after 10 weeks of Western type diet feeding[19]. This, together with the fact that phagocytosis of apoptotic cells is impaired in advanced human atherosclerotic plaques[20], also point to a destabilizing effect of increased macrophage apoptosis at later stages of plaque development, although more recent studies could not support this notion[21].

In this study, of the five genes that are upregulated in both ThCFA models compared to their respective TkCFA counterparts three are regulating cell death, while the other two have unknown functions, which confirms the important role of apoptosis in plaque vulnerability. Pleiomorphic adenoma gene-like-1 (Plagl1, also called Zac-1) and Bcl-2 like interacting mediator of cell death (Bim or Bcl2-like-11) are both pro-apoptotic proteins. Plagl1 induces apoptosis and cell cycle arrest[22] and, importantly, is a cofactor for p53[23] the expression of which was seen to be increased in advanced human plaques[24], as well as in mouse plaques after adenoviral p53 overexpression. Although contradictory results have been reported on its role in atherosclerotic plaque development and progression depending on the cell type

affected, generally deficiency of p53 increased atherosclerotic lesion size[24]. Bim is a pro-apoptotic BH3 only member of the Bcl-2 family of apoptosis regulators. Bim was found to be involved in negative selection of T lymphocytes and its deficiency resulted in expansion of lymphocytes and autoimmunity in mice[25-26]. To date, an involvement of Bim in atherosclerotic plaque development or progression has not been reported. CD5 ligand (CD5l, also known as AIM or api6) has been demonstrated to possess both pro- and antiapoptotic properties[27-29]. It inhibits apoptosis of NKT and T cells[27,28], but induces apoptosis in resting B cells[29]. CD5l has been implicated in atherosclerosis development. First it promoted macrophage survival in response to pro-apoptotic stimuli such as oxidized LDL[30]. Second, plaque macrophages have been shown to express CD5l and deletion of CD5l in LDLr$^{-/-}$ mice resulted in a marked inhibition of atherosclerotic plaque development[30]. A possible role for CD5l in plaque instability has however not been assessed. We propose that these three genes, *Cd5l*, *Bim* and *Plagl1*, may play a role in the transition of TkCFA into a more vulnerable plaque phenotype.

The differential regulation of one of the genes upregulated in brachiocephalic artery ThCFA versus perivascular collar induced carotid artery TkCFA in ApoE$^{-/-}$ mice, neuropeptide Y, could be verified by realtime PCR. In addition we show that in LDLr$^{-/-}$ mice *Npy* RNA expression gradually increases with plaque progression. Interestingly in human unstable carotid atherosclerotic plaques Npy gene expression was upregulated as well. Moreover Npy protein was abundantly expressed in atherosclerotic plaques, but immunohistochemical detection unfortunately did not allow to detect significant differences in protein content between unstable and stable plaques probably due to the higher sensitivity of RNA expression profiling over immunohistochemistry. Npy is a neurotransmitter involved in regulation of both central and peripheral processes mediating its effect through G coupled receptors Y1-Y6[31]. In the vasculature Npy proved to be a potent vasoconstrictor[32,33] and to exert pro-angiogenic[34,35] and pro-atherogenic[36] activities via different receptors. In rat ischemic tissue Npy release is stimulated and exogenous Npy induces angiogenesis[34]. This effect of Npy was shown to be mediated by receptors Y2 and/or Y5. Furthermore, Npy was shown to act mitogenic on endothelial[37] and smooth muscle cells[38]. In a rat model of carotid artery balloon angioplasty vascular expression of Npy was seen to be upregulated in response to injury and exogenous Npy in the same model aggravated intimal hyperplasia at which atherosclerotic-like lesions are formed with a high macrophage, matrix and lipid content, an effect that was likely mediated by receptors Y1/Y5[36]. Our data suggest that apart from its documented pro-angiogenic and pro-atherogenic properties Npy colocalizes with vSMC and may be involved in induction of ThCFA.

In conclusion, comparative transcriptome analysis of two ThCFA models and respective TkCFA controls by microarray technique shows that several differentially expressed genes and functional gene clusters are shared by both models of ThCFA. From our study genes involved in cell death signaling are most prominent, confirming the importance of apoptosis in plaque vulnerability. In addition, we have

identified neuropeptide Y as a gene potentially involved in plaque vulnerability. Further studies are necessary to determine the exact contribution of Npy to plaque destabilization.

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Chapter 4

Leukocyte Bim Deficiency Induces Anti-Ox-LDL Autoantibody Formation and T Cell and Immunoglobulin Accumulation in Atherosclerotic Lesions of LDLr$^{-/-}$ Mice

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Abstract

Proapoptotic Bcl-2 family member Bim is an important regulator of leukocyte apoptosis. It is particularly relevant for the deletion of autoreactive and activated T and B cells implicating Bim in autoimmunity. As atherosclerosis is regarded as a chronic inflammatory process with features of an autoimmune disease, we investigated the impact of hematopoietic Bim deficiency on plaque formation in Western type diet fed LDLr$^{-/-}$ mice.

Bim$^{-/-}$ bone marrow transplanted LDLr$^{-/-}$ mice displayed marked splenomegaly. The pro-inflammatory status of Bim$^{-/-}$ chimeras was also reflected by enhanced T cell activation and proliferation, which could be partly attributed to an increased capacity of matured Bim$^{-/-}$ DCs to induce T cell proliferation. Moreover, circulating and mediastinal lymph node lymphocyte levels were increased while atherosclerotic lesions were seen to be enriched in T cells as well. Leukocyte Bim deficiency was accompanied by elevated levels of Ox-LDL specific autoantibodies of the IgG1 but not IgG2a isotype, suggestive of a Th2 biased T-cell response. In addition, plaques of Bim$^{-/-}$ but not WT transplanted mice contained massive immunoglobulin deposits. Although circulating monocyte levels were increased in Bim$^{-/-}$ BM recipients and bone marrow derived macrophages proved to be less sensitive for Ox-LDL induced apoptosis, Bim deficiency did not translate in altered lesion macrophage content. Surprisingly, despite these profound effects and unexpected 30-50% reductions in serum lipid levels, atherosclerotic lesion burden in aortic root and descending aorta as well as lesion stability were unaffected in Bim$^{-/-}$ BM transplanted mice.

In conclusion, leukocyte Bim deficiency in LDLr$^{-/-}$ mice results in general T cell activation, increased lesion T cell but not macrophage content, enhanced anti-Ox-LDL autoantibody formation and lesional immunoglobulin deposition. Apparently, pro-inflammatory effects counteract the atheroprotective effect of Bim deficiency on serum lipid levels leading to an overall unchanged atherogenic response.

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by lipid accumulation[1]. With disease progression vascular apoptosis gradually increases[2]. In early atherogenesis apoptosis is regarded a beneficial factor as it dampens plaque inflammation and limits lesion expansion[3,4]. At later stages of atherosclerosis however clearance of apoptotic cells has been demonstrated to be insufficient[5], and apoptotic cell death might here promote necrotic core formation and inflammation.

The Bcl-2 family of pro- and antiapoptotic proteins regulates apoptosis induced by cellular stressors such as DNA damage, UV radiation and oxidative stress[6]. Proteins of this family share one to four Bcl-2 homology (BH) domains[6]. *In vivo* studies in atherosclerotic mouse models have established a role for various Bcl-2 family members in apoptosis of atherosclerotic lesion macrophages[7,8]. For instance lesions of lethally irradiated LDLr$^{-/-}$ mice transplanted with proapoptotic Bax deficient bone marrow were seen to contain 53% less apoptotic cells and demonstrated to be 50% larger than Bax$^{+/+}$ transplanted mice[7]. However in ApoE$^{-/-}$ mice macrophage specific deletion of antiapoptotic Bcl-2 did not result in altered lesion size, despite an increase in apoptotic cell content[8]. Bim (Bcl-2 interacting mediator of cell death) is a BH3-only proapoptotic protein of the Bcl-2 family[9,10] and, like other BH3-only proteins, binds to antiapoptotic Bcl-2 family members thereby initiating apoptosis[11]. Studies in Bim deficient mice have revealed crucial functions in leukocyte homeostasis. Bim deficient granulocytes and lymphocytes are less sensitive to apoptosis induced by cytokine deprivation or various apoptotic stimuli[12,13]. Moreover, Bim deficient mice have markedly increased numbers of B and T cells in circulation, spleen and thymus, and of circulating monocytes and granulocytes[12]. Bim deficiency results in auto-immunity and lymphadenopathy due to defective removal of autoreactive T and B cells[12,14,15]. Bim was also demonstrated to be necessary for appropriate termination of immune responses[16].

Atherosclerotic lesions contain a large number of inflammatory cell types including lymphocytes. Lymphocyte deficiency in mice has previously been demonstrated to result in reduced atherosclerotic lesion size[17,18] while transfer of CD4^{+} T cells in immunodeficient mice was seen to aggravate atherosclerosis[19]. Various mediators of both innate and adaptive immunity are involved in the pathogenesis of atherosclerosis. In fact atherosclerosis exhibits several features of an autoimmune disease[20-22], at which Ox-LDL derived epitopes and heat shock proteins (HSP) were shown to act as autoantigen[22]. T-cell autoreactivity is generally suppressed by regulatory T cells (Tregs), and Treg deficiency can result in autoimmune disease[23]. Concordant with various autoimmune diseases, also in atherosclerosis Treg function is impaired[23]. Likewise functional studies demonstrated that depletion or functional impairment of Tregs exacerbated atherosclerosis in ApoE$^{-/-}$ mice[24,25]. As Bim is instrumental in preventing autoimmunity a role for this protein in the pathogenesis of atherosclerosis is anticipated. Therefore, in the present study we have investigated the role of Bim regulated apoptosis in atherosclerosis-prone LDLr$^{-/-}$ mice. Our study shows that leukocyte Bim deficiency in LDLr$^{-/-}$ mice results in increased atherosclerotic

lesion T cell content and massive immunoglobulin deposition as well as increased circulating anti-oxidized LDL autoantibodies. Furthermore, we demonstrate that loss of leukocyte Bim interferes with lipid metabolism.

Methods

Bone marrow transplantation

All animal work was approved by regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. LDLr$^{-/-}$ mice were obtained from the local animal breeding facility and Bim$^{-/-}$ mice from the Department of Biochemistry, Biosciences Research Institute from the University College Cork, Ireland. Male LDLr$^{-/-}$ mice (n=12) were housed in sterile individual ventilated cages with food and water ad libitum. The drinking water was supplied with antibiotics (83 mg/l ciprofloxacin and 67 mg/l Polymixin B) and 5 g/l sugar. The mice were exposed to a single dose of 9 Gy total body irradiation (0.19Gy/min, 200 kV, 4 mA) using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) one day before transplantation. Bone marrow was extracted from femurs and tibia of male Bim$^{-/-}$ and wild-type (WT) littermates. Irradiated LDLr$^{-/-}$ mice received either 5 x 10^6 Bim$^{-/-}$ bone marrow cells or 5 x 10^6 WT bone marrow cells via tail vein injection. After a recovery period of eight weeks the diet was changed from normal chow (RM3, Special Diet Services, Witham, Essex, UK) to Western type diet (WTD) containing 0.25% cholesterol and 15% cacao butter (Diet W, Special Diet Services, Witham, Essex, UK) for an additional ten weeks.

Cholesterol and triglyceride levels

Blood samples were taken by tail bleeding before bone marrow transplantation (BMT) and before and five weeks after start of Western type diet feeding and at the time of sacrifice. Hepatic lipids were extracted as described by Bligh and Dyer[26]. Total cholesterol, triglyceride and phospholipid content was measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, The Netherlands) in serum and hepatic extracts.

Tissue harvesting and analysis

Sixteen weeks after BMT mice were anesthetized and perfused with PBS after which heart, aorta, spleen, thymus and mediastinal lymph nodes were isolated and stored in 4% formaldehyde solution or snap-frozen in liquid nitrogen and stored at -80ºC. Cryosections were prepared of aortic valves, spleen and liver and stained with hematoxylin and eosin (HE) and/or Oil Red 'O. The descending aorta was cut open longitudinally for *en face* analysis after staining with Oil Red 'O. Immunohistochemical stainings were performed for macrophage (MOMA-2, Sigma, Zwijndrecht, The Netherlands) and T cell (CD3, Immunologic, Duiven, The Netherlands) content in aortic root lesion, liver and/or spleen sections. Apoptotic cell content was quantified using terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics) and presence of immunoglubulins using FITC

labeled rabbit anti-mouse Ig (DakoCytomation, Heverlee, Belgium). Lesion size and tissue morphology was analyzed using Leica image analysis system, consisting of a Leica DMRE microscope with camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Fluorescent immunohistochemistry was analyzed on a Nikon Eclipse E600 using ImagePro 4.5 software.

Blood cell analysis and flow cytometry

Blood samples were taken by tail bleeding before BMT (week 0), before (week 6) and five weeks after start of Western type diet feeding (week 11) and at the time of sacrifice (week 16). Peritoneal leukocytes were isolated at the time of sacrifice by peritoneal lavage with 10 ml cold PBS. Whole blood and peritoneal lavage samples were analyzed on a Sysmex blood cell analyzer (XT-2000i, Sysmex Europe GmbH, Norderstedt, Germany). White blood cells (WBC) were isolated after erythrocyte lysis of whole blood samples obtained by orbital puncture. For flow cytometry, WBC and peritoneal leukocytes or single cell suspensions of spleen and lymph nodes were stained with fluorescently labelled antibodies from eBioscience (Halle-Zoersel, Belgium; CD3, CD4, CD8, CD11c, CD69, CD71, CD62L, CCR7 and MCH-II) and BD Pharmingen (Breda, The Netherlands; CD4, CD8 and CD45). Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCanto with FACSDiva software (BD Biosciences).

Macrophage apoptosis

Femurs and tibia were flushed with phosphate buffered saline (PBS) to isolate bone marrow. A single cell suspension was obtained by passing the bone marrow through a 70 µm nylon cell strainer (BD Falcon, Breda, The Netherlands). Bone marrow cells were differentiated into macrophages by culturing in 70% RPMI, supplemented with 20% FCS, glutamine, pyruvate, penicillin/streptomycin and non essential amino acids, and 30% M-CSF conditioned DMEM (obtained from L929 cells) for 7 days. Bone marrow derived macrophages (BMDM) were stimulated with 40 ug/ml Ox-LDL, cultured without growth factors (30% M-CSF conditioned DMEM) or in control medium for 24 hours. The macrophages were detached with accutase (PAA Laboratories GmbH, Cölbe, Germany), stained with Annexin V (ImmunoTools, Friesoythe, Germany) and propidium iodide (Sigma, Zwijndrecht, The Netherlands) and subsequently analyzed by flow cytometry using a FACSCalibur with CellQuest software (BD Biosciences).

Proliferation assay

Single cell suspensions from spleens of $Bim^{-/-}$ and WT BM transplanted mice were obtained by passing spleen tissue through a 70 µm nylon cell strainer (BD Falcon), after which splenocytes were washed with PBS. When indicated T cells were isolated from spleen and lymph nodes of $LDLr^{-/-}$ mice fed a Western type diet for 2 weeks using a Mouse T Lymphocyte Enrichment Set – DM ("purified T-cells"; BD Pharmingen, Breda, The Netherlands). Bone marrow cells of $Bim^{-/-}$ and

WT BM transplanted mice were differentiated into dendritic cells (DCs) by culturing in 70% IMDM, supplemented with 8% FCS, 2 mM glutamine, 100 U/ml penicillin/ streptomycin and 20 μM beta-mercaptoethanol, and 30% GM-CSF conditioned IMDM (obtained from R1 cells) for ten days. DCs were then stimulated with LPS (1 μg/ml), Ox-LDL (7.5 μg/ml) or left untreated for 24 h.
Splenocytes (2 x 10^5) or purified T-cells (6 x 10^5) were plated in triplicate in a round-bottom 96-well plate in RPMI containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol and incubated for 24 hours in the presence or absence of 2 μg/ml concanavalin A (splenocytes) or in the presence of stimulated or nonstimulated DCs (purified T-cells; cell ratio 1:10) at 37ºC. [^{3}H]-thymidine (0.5 μCi/well, GE Healthcare, Diegem, Belgium) was added and after incubation for another 24 hours samples were washed with PBS and cells were lysed in 0.1 M NaOH. [3H]-thymidine incorporation was measured by a liquid scintillation analyzer (Tri-Carb 2900R, Packard).

Detection of anti-Ox-LDL antibodies
An EIA/RIA high binding 96-well plate (Corning, Schiphol, The Netherlands) was coated with Ox-LDL (5 μg/ml) in a 50 mM Na_2CO_3/$NaHCO_3$ coating buffer (pH 9.6). IgM, IgG1 and IgG2a antibodies against Ox-LDL in serum were measured using an enzyme-linked immunosorbent assay (ELISA) Ig detection kit (Zymed Laboratories, San Francisco, CA, USA) according to the manufacturer's protocol.

Statistical analysis
Values are expressed as mean ± SEM or presented as mean + upper limit of the SEM. Analysis to compare two groups was performed by two-tailed Student's t-test. P-values less than 0.05 are considered significant.

Results
Serum lipid levels are reduced in Bim$^{-/-}$ BM transplanted mice
The role of leukocyte Bim in atherogenesis was studied in chimeras generated by transplanting bone marrow (BM) from Bim$^{-/-}$ and WT littermates to irradiated LDLr$^{-/-}$ recipients. First we analyzed whether leukocyte Bim deletion in western type diet fed LDLr$^{-/-}$ mice influenced serum total cholesterol (TC) and triglyceride (TG) levels. To our surprise TC and TG levels of Bim$^{-/-}$ BM transplanted mice were reduced by a very significant 30-48% compared to controls both after 5 and after 10 weeks of western type diet feeding ($p<0.05$, fig. 1A and B). The reduction in TC was clearly induced by Western type diet since there was no difference between groups when mice were fed a chow diet, eight weeks after BMT. However, at this time point serum TG levels had already dropped by 35% ($p<0.05$). Body weight was comparable between the two groups of mice (39.4 ± 1.7 g vs. 38.9 ± 1.8 g for WT and Bim$^{-/-}$ BM transplanted mice respectively), suggesting that the decreased TC and TG levels were not caused by differences in the general metabolic state.
Reduction of serum lipid levels neither did translate in nor was caused by perturbed

hepatic lipid handling as we did not observe differences in hepatic lipid content (fig. 1C and D). Livers of Bim$^{-/-}$ BM transplanted mice did however show an increased leukocyte content, consisting for a substantial part of T cells, suggestive of increased hepatic inflammation (fig 1E and F).

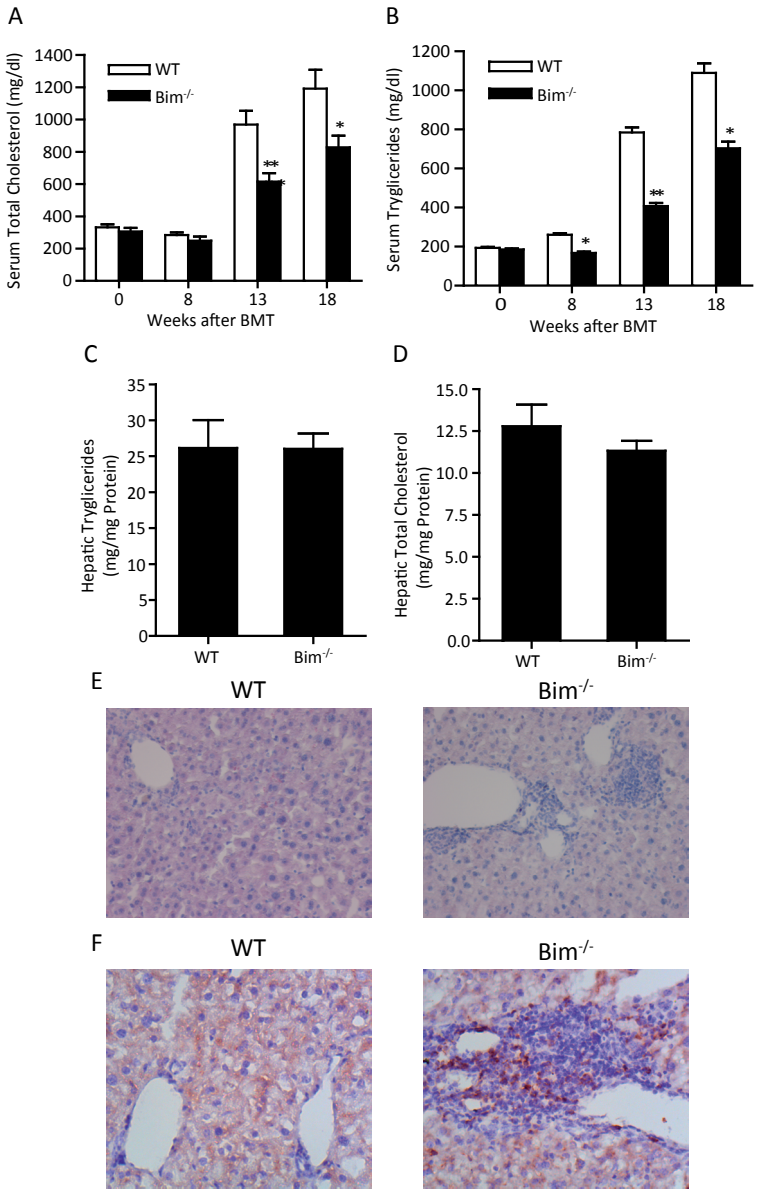


Figure 1. Serum lipid levels are reduced. Circulating total cholesterol (TC) levels are reduced after Western type diet feeding of Bim$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice (A), circulating triglyceride (TG) levels are reduced independent of diet (B). The reductions in lipid levels are not caused by changes in hepatic TC and TG content (C and D). Livers of Bim$^{-/-}$ chimeras show increased cell influx, consisting partly of T cells. Representative picture of a HE (E) and CD3 stained (brown) cryosections (F). (*p<0.05, **p<0.01)

Loss of leukocyte Bim in LDLr$^{-/-}$ mice affects T cell levels and characteristics

As Bim is instrumental in lymphocyte development and T cell response, we assessed these characteristics in Bim$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice. The spleen of Bim$^{-/-}$ BM recipients was enlarged (+81% in relative weight; p<0.001, fig. 2A) compared to WT BM recipients. Thymus weight and mediastinal lymph node (MLN) total cell number were unaltered (fig 2B and C). Total white blood cells in Bim$^{-/-}$ BM transplanted mice were increased by almost two-fold, which was mainly attributable to a 90% increase in circulating lymphocyte numbers (p<0.01, fig. 3A). Next, T cell content and activation in spleen and MLN was analyzed. Compared to controls MLN from Bim$^{-/-}$ BM recipients were slightly enriched in CD4^{+} and CD8^{+} T cells (p<0.01, fig. 3B). Relative spleen T cell content was unchanged (fig. 3C), indicating that the different leukocyte subsets were enhanced proportionally. However both CD4^{+} and CD8^{+} T cell subsets were found to be more activated in Bim$^{-/-}$ BM transplanted mice in comparison with WT transplanted mice as judged from the enhanced expression of activation markers CD69 and CD71 (p<0.05, fig. 3D and E).

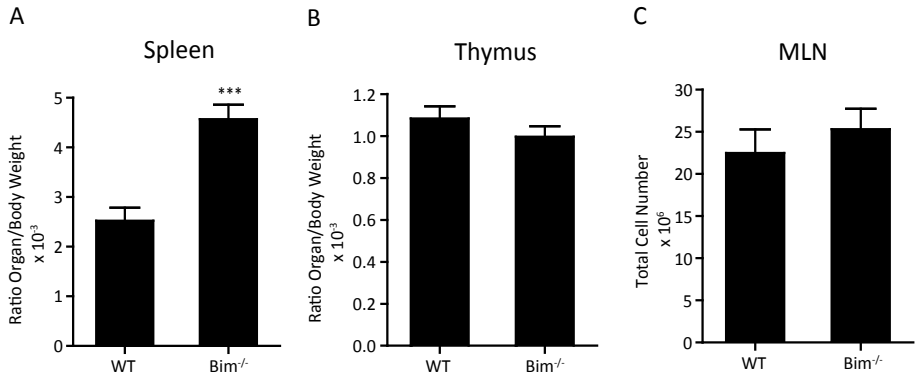


Figure 2. Organ weights in Bim$^{-/-}$ versus Bim$^{+/+}$ bone marrow transplanted LDLr$^{-/-}$ mice. Spleen weight was increased in Bim$^{-/-}$ BM recipients (A), while thymus weight was unaltered (B). Values are expressed as organ weight relative to total body weight. (***p<0.001)

In keeping with this finding, basal proliferation of unstimulated splenocytes derived from Bim$^{-/-}$ BM recipients, as measured by [^{3}H]-thymidine incorporation, was increased by 38% (p<0.05, fig. 4A). Concanavalin A induced proliferation was however not affected in Bim$^{-/-}$ splenocytes (fig. 4B), indicating that Bim deficiency does not alter the splenocyte proliferation capacity. Next, we examined the ability of WT and Bim$^{-/-}$ dendritic cells to stimulate T cell proliferation. Bone marrow cells from Bim$^{-/-}$ and WT transplanted mice were differentiated into dendritic cells (DCs) by growth factor stimulation. DCs were subsequently matured in the presence of lipopolysaccharide (LPS) or Ox-LDL to stimulate antigen presentation. Proliferation of T cells isolated from western type diet fed LDLr$^{-/-}$ mice was measured after co-incubation for 24h with matured DCs. Proliferation of T cells incubated with immature WT or Bim$^{-/-}$ DCs was essentially similar as was proliferation of T cells incubated with Ox-LDL pulsed DCs (fig. 4C). However Bim$^{-/-}$ DCs stimulated with LPS were able to stimulate T cell proliferation to a slightly higher extent than unstimulated Bim$^{-/-}$ DCs

while for WT DCs no significant increase in T cell proliferation was observed after LPS stimulation ($p<0.05$, fig. 4C).

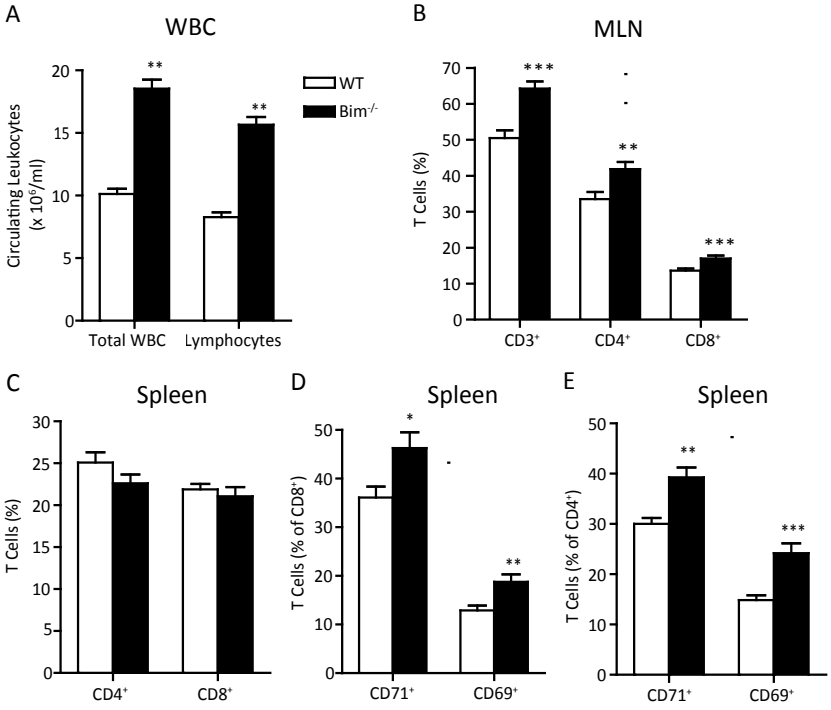


Figure 3. Effect of hematopoietic Bim$^{-/-}$ deficiency on lymphoid organ T cell content and activation. Total white blood cell numbers were largely increased in Bim$^{-/-}$ chimeras, mainly due to the large increase in lymphocyte counts (A). Both CD4$^+$ and CD8$^+$ T cell levels were elevated in mediastinal lymph nodes (MLN; B), but not in spleen (C). However in spleen a larger portion of CD4$^+$ and CD8$^+$ T cells were activated as shown by increased CD69 and CD71 expression (D and E). (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

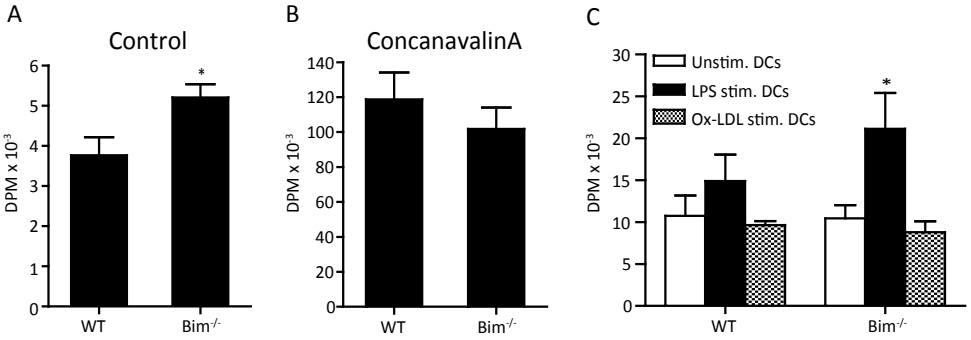


Figure 4. Proliferative capacity of Bim deficient splenocytes and T cells. Basal proliferation of splenocytes is enhanced after leukocyte Bim deletion (A), while that of concanavalin A stimulated splenocytes is unchanged (B). Proliferation of T cells isolated from LDLr$^{-/-}$ mice fed a Western type diet for two weeks was increased after incubation with Bim$^{-/-}$ dendritic cells (DCs) stimulated with LPS, but not after incubation with nonstimulated Bim$^{-/-}$ DCs or DCs stimulated with Ox-LDL as compared to T cells incubated with stimulated or nonstimulated WT DCs (C). (*$p<0.05$)

Leukocyte Bim deficiency affects circulating myeloid cell and lymph node DCs

In addition to elevated circulating lymphocyte numbers, monocyte/macrophage and neutrophil levels in blood were elevated by 87% and 42%, respectively ($p<0.05$, fig 5A). Mediastinal lymph nodes surprisingly showed a reduced total dendritic cell (DC) content (10.0 ± 0.8 % in $Bim^{-/-}$ BM transplanted mice vs. 7.6 ± 0.7% in WT BM transplanted mice; $p<0.05$, fig. 5B). $MHCII^{+}$ DCs were reduced to the same extent as total DCs, suggesting that Bim deficiency does not influence activation of DCs in $LDLr^{-/-}$ mice. Relative DC content in spleen remained unchanged (fig. 5C).

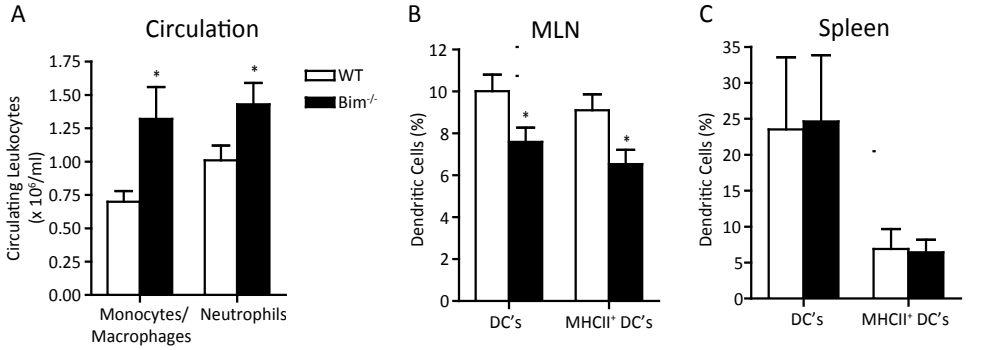


Figure 5. Consequences of Bim deletion on circulating, splenic and MLN myeloid cell content. Circulating monocytes/macrophages and neutrophils are increased in Bim deficient $LDLr^{-/-}$ mice fed a Western type diet for ten weeks (A). Total DC and $MHCII^{+}$ DC content is reduced after Bim deletion in mediastinal lymph nodes (MLN; B) but not in spleen (C). (*$p<0.05$)

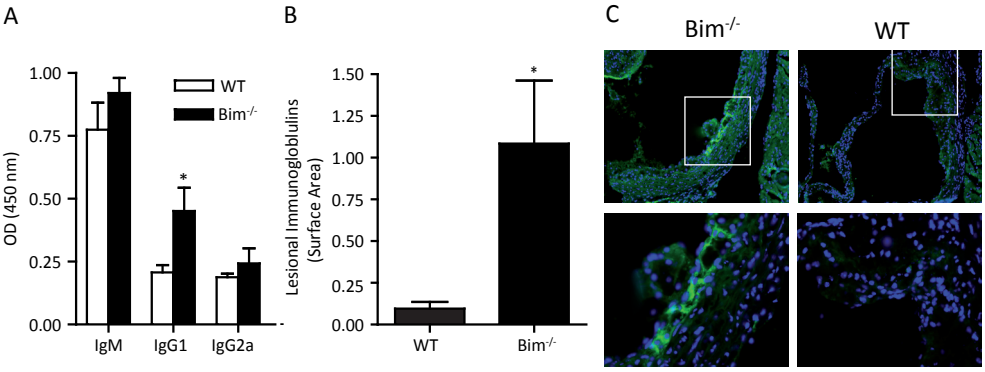


Figure 6. Effect of leukocyte Bim deficiency on humoral immune response. Ox-LDL specific antibody titers in serum after 10 weeks of Western type diet feeding were measured by ELISA. IgG1 antibody levels were increased while IgM and IgG2a antibody levels were unchanged suggestive of a Th2 shifted T-cell response (A). Total immunoglobulin (Ig) deposition in aortic root lesions was massively increased in $Bim^{-/-}$ transplanted mice (B). Representative cryosections showing overt staining for total Ig (green); nuclei are stained with DAPI (blue) (C). (*$p<0.05$)

Leukocyte Bim deficiency affects the humoral immune response in $LDLr^{-/-}$ mice

Apart from affecting T cell homeostasis, loss of Bim was seen to be associated with impaired deletion of autoreactive B cells. The resulting accumulation of these cells and derived autoreactive antibodies *in vivo*[15] can promote autoimmunity[12]. We therefore measured Ox-LDL directed autoantibody titers in serum. IgG1 anti-Ox-

LDL antibodies were increased by more than two-fold ($p<0.05$, fig. 6A) in Bim$^{-/-}$ BM transplanted mice compared to controls, but no differences in IgM and IgG2a anti-Ox-LDL auto-antibodies were detected. Consequently the IgG1/IgG2a ratio, a measure of Th2/Th1 polarized T-cell response, was sharply increased in Bim$^{-/-}$ chimeras from 1.19 ± 0.22 for WT BM recipients to 2.84 ± 0.71 for Bim$^{-/-}$ BM recipients ($p=0.06$). Importantly, staining for deposits of total immunoglobulins (Ig) in lesions revealed the striking presence of Ig complexes in lesions of Bim$^{-/-}$ chimeras but not littermate controls (0.10 ± 0.04 % versus 1.08 ± 0.04%; $p<0.05$, fig. 6B and C).

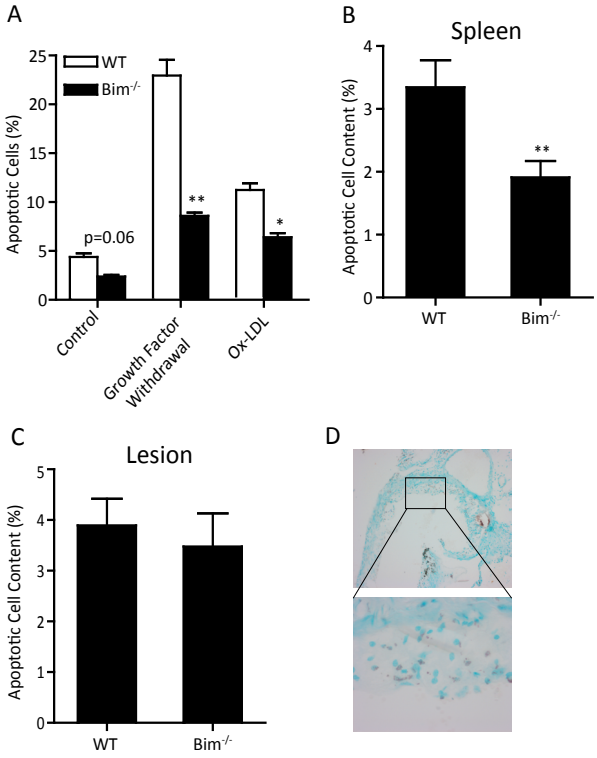


Figure 7. Apoptotic cell death in leukocyte Bim deficient LDLr$^{-/-}$ mice. Apoptosis of bone marrow derived macrophages is reduced in Bim$^{-/-}$ transplanted mice at basal level, after growth factor withdrawal, and after stimulation with Ox-LDL (40 µg/ml) (A). Spleen apoptotic cell content is decreased by 45% in Bim$^{-/-}$ chimeras (B), but is unaffected in atherosclerotic lesions (C). Representative pictures of TUNEL stained aortic root cryosection (D). (*$p<0.05$, **$p<0.01$)

Apoptotic cell death is affected by loss of leukocyte Bim

Loss of Bim in bone marrow derived macrophages (BMDM) resulted in decreased sensitivity to apoptotic cell death in response to a number of stimuli. Basal apoptosis levels were already reduced in Bim$^{-/-}$ compared to WT BMDM (45%, $p=0.06$, fig. 7A). While in WT BMDM apoptosis increased from 4.39 ± 0.81% to 22.94 ± 3.61% upon growth factor withdrawal, in Bim$^{-/-}$ BMDM apoptosis increased from 2.39 ± 0.37 to only 8.59 ± 0.70% ($p<0.01$, fig. 7A). OxLDL induced apoptotic cell death was 43% less in Bim$^{-/-}$ compared to WT BMDM ($p<0.05$), but interestingly the relative increase over non-stimulated cells was similar in WT and Bim$^{-/-}$ BMDM (fig. 7A). Since we observed profound splenomegaly in Bim$^{-/-}$ BM recipients we measured apoptotic cell content in spleens, which appeared to be significantly lower in Bim$^{-/-}$ BM compared to WT BM transplanted mice (3.34 ± 0.43 vs. 1.84 ± 0.29 % of total splenocytes for WT and Bim$^{-/-}$ BM transplanted mice respectively, $p<0.01$, fig. 7B).

In striking contrast, apoptotic cell content of atherosclerotic lesions remained unchanged (fig. 7C and D).

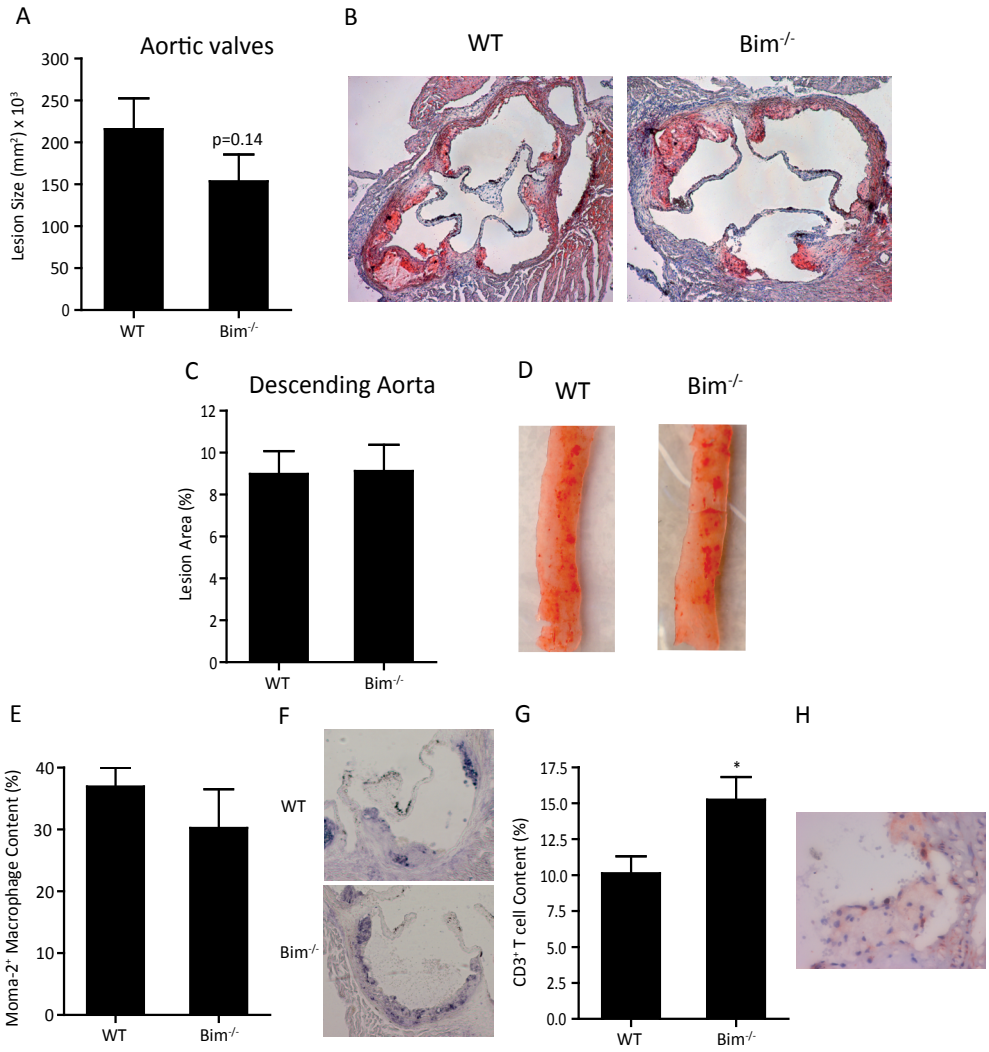


Figure 8. Atherosclerotic lesion size and composition. Lesion size in aortic root and descending aorta were unchanged in LDLr$^{-/-}$ mice with hematopoietic Bim deficiency (A and C respectively, with representative pictures [B and D]) as was Moma-2^{+} macrophage content (E, with representative pictures [F]). CD3^{+} T cell content was increased in Bim$^{-/-}$ transplanted mice (G). Representative picture of a CD3 stained (brown) cryosection (H). (*p<0.05)

Atherosclerotic lesion size and stability are not changed after loss of leukocyte Bim

After ten weeks of Western type diet feeding mice were sacrificed and the aortic root and descending aorta were isolated and analyzed for size and composition. In keeping with elevated T cell levels in circulation and lymphoid organs T cell content in the atherosclerotic lesions (intima and adventitia) was increased by 51% from 10.1 ± 1.2 per section in WT BM recipients to 15.3 ± 1.6 per section in Bim$^{-/-}$ BM

transplanted recipients ($p<0.05$, fig. 8G and H). We did not observe any significant differences in lesion size in aortic root between groups (fig. 8A and B). In agreement *en face* analysis of the descending aorta showed no differences in atherosclerotic lesion area as well (fig. 8C and D). Regarding composition, lesional moma-2 positive macrophage content in $Bim^{-/-}$ did not differ from that in WT BM transplanted mice (fig. 8E and F). Collagen and necrotic core were virtually absent in the early lesions of WT and $Bim^{-/-}$ BM transplanted mice.

Discussion

Bim was demonstrated to be essential for apoptosis of various leukocyte subsets, including T and B cells, dendritic cells, macrophages and granulocytes[12,13,27]. As these cell types are all shown to be present in atherosclerotic lesions and to contribute to disease development, a role for Bim in lesional leukocyte apoptosis and atherogenesis may be anticipated. Therefore we generated $LDLr^{-/-}$ mice with Bim deficiency in hematopoietic cells by bone marrow transplantation and addressed effects of Bim deficiency on lesion development. The most profound consequences of Bim deficiency following BMT were observed on T cell homeostasis. Lymphocytes, although in atherosclerotic lesions less abundantly present than macrophages, are importantly involved in regulating immune responses in lesions[20,28]. Lymphocyte deficiency in mice has previously been demonstrated to result in reduced atherosclerotic lesion size[17,18] and transfer of $CD4^+$ T cells in immunodeficient mice was seen to aggravate atherosclerosis[19]. In keeping with previous studies[12] we show markedly elevated circulating lymphocyte levels in $Bim^{-/-}$ BM transplanted $LDLr^{-/-}$mice. Furthermore, mediastinal lymph node $CD4^+$ and $CD8^+$ T cell content is slightly increased and $LDLr^{-/-}$ mice suffer from considerable splenomegaly after leukocyte Bim deletion. Our results are consistent with previous observations that systemic Bim deficient mice have enlarged spleens and highly elevated lymphocyte content in circulation and lymphoid organs[12], although as expected in systemic Bim deficiency the above-mentioned differences were more pronounced. As suggested by Bouillet and Hildeman and colleagues this overt lymphocytosis may be caused by impaired apoptosis of autoreactive thymocytes and activated T cells for which Bim has been demonstrated to be essential[14,16]. In agreement with the latter, both $CD4^+$ and $CD8^+$ splenic T cells are more activated in $Bim^{-/-}$ BM recipients than in control mice, both at an absolute (% of total splenocytes) and at a relative level (% of the respective T cell subset). The observed elevated T cell numbers are reflected in increased T cell content in atherosclerotic lesions indicating an enhanced infiltration of T cells from circulation into the lesions. Decreased apoptotic cell death in spleen was accompanied by a slightly increased basal splenocyte proliferation in $Bim^{-/-}$ BM transplanted mice. The proliferation capacity of $Bim^{-/-}$ splenocytes was not affected. Although increased proliferation of $Bim^{-/-}$ splenocytes has not been described before, Bim interacts with anti-apoptotic Bcl-2 family members, which in turn were seen to influence cell cycle entry[29,30]. Interestingly, T-cell proliferation was increased after stimulation with LPS but not Ox-LDL pulsed $Bim^{-/-}$ DCs compared

to immature DCs. This effect was absent in WT DCs. Previously loss of Bim in DCs was seen to decrease cell death and to promote proliferation of antigen-specific T cells[27]. We extend these findings by showing that pulsed Bim$^{-/-}$ DCs are able to enhance proliferation of nonspecific T cells as well.

In addition to T cell homeostasis and activation Bim was previously reported to be necessary for apoptosis of autoreactive B cells[15] and to prevent autoimmunity[12]. Atherosclerosis is currently recognized as a lipid driven inflammatory process with features of autoimmune disease[20-22], implicating T cell responses to auto-antigens, such as Ox-LDL and heat shock proteins (HSP)[22]. In fact, Ox-LDL specific antibodies have been detected in atherosclerosis-prone ApoE$^{-/-}$ mice[31], in human and rabbit serum and in atherosclerotic lesions[32]. Moreover, a subset of T cells present in human lesions was demonstrated to be Ox-LDL specific[33]. In the present study we observed markedly elevated Ox-LDL antibody levels in serum of Bim$^{-/-}$ BM transplanted mice. In addition, total immunoglobulin deposition in atherosclerotic lesions of Bim$^{-/-}$ BM recipients was dramatically increased. Generally, these auto-antibodies directed against Ox-LDL are thought to be atheroprotective as several studies have demonstrated decreased lesion formation after immunization with modified LDL[34-37]. Furthermore IgG antibodies against modified LDL and oxidized phospholipids were increased in immunized LDLr$^{-/-}$ mice and correlated with decreased lesion formation[37]. Furthermore B cell associated immunity was shown to be protective in atherosclerosis as splenectomy increased lesion size of ApoE$^{-/-}$ mice which could be counteracted by transfer of spleen derived B cells[38]. The observed increase in anti-Ox-LDL antibodies in serum of Bim$^{-/-}$ BM recipients is mainly due to elevated anti-Ox-LDL IgG1 levels, whereas anti-Ox-LDL IgM and IgG2a isotypes were not affected. This may point to a shift towards Th2 type immune responses, which may by itself influence the progression of atherosclerosis[20]. Both mouse and human studies have demonstrated a predominant Th1 response in atherosclerotic plaques, with Th1 cytokines such as IFNγ, IL2 and TNFα abundantly present within the lesion[39,40]. Th2 cells, in contrast, produce IL4, IL5, IL10 and IL13 which promote antibody formation and inhibit pro-inflammatory cytokine expression and are thought to protect against atherosclerosis[20,21].

In agreement with previous studies[12], not only circulating lymphocytes numbers but also that of monocyte/macrophages and neutrophils were increased in Bim$^{-/-}$ BM transplanted mice. Surprisingly, this does not translate into increased plaque macrophage content or elevated relative macrophage and DC content in lymphoid organs, except for a slight relative decrease in mediastinal lymph node DCs. Since loss of Bim has been demonstrated to increase half life and proliferation of DCs[27] our result may indicate that either trafficking of antigen presenting DCs into lymph nodes is impaired or that uptake of antigen by DCs and subsequent DC maturation is reduced in Bim$^{-/-}$ BM recipients compared to WT BM recipients.

Absence of Bim decreased spontaneous apoptosis of bone marrow derived macrophages as well as apoptosis induced by growth factor withdrawal. These results are consistent with previous studies showing decreased apoptosis in Bim deficient

T cells[12], DCs[27] and granulocytes[13] in response to various stimuli. In addition we identify Bim as a regulator of Ox-LDL induced apoptosis of macrophages. However while spleen apoptotic cell content was decreased in Bim$^{-/-}$ BM recipients as expected, we did not observe any differences in atherosclerotic lesion apoptotic cell content, predominantly representing macrophages and foam cells. Bim dependent pro-apoptotic effects on T cells, which are present at much lower numbers in atherosclerotic lesions[20], are probably not detectable.

Despite marked effects on T cell levels and characteristics, humoral immune response and myeloid subset levels, leukocyte Bim deficiency in LDLr$^{-/-}$ mice did not alter atherosclerotic lesion size or stability as assessed at two different vascular sites, early atherogenesis in the descending aorta and intermediate stage atherosclerosis in the aortic root valves. Apart from an increased lesional T cell content no differences were observed in aortic root lesion composition. Surprisingly leukocyte Bim deficiency following BMT led to a striking reduction in serum lipids, e.g. total cholesterol and triglycerides, in LDLr$^{-/-}$ mice. For triglycerides this effects was independent of the diet. Decreased serum lipid levels were not caused by differences in hepatic lipid content. To what extent hepatic inflammation represented by T-cells which were elevated in livers of Bim$^{-/-}$ BM transplanted mice had contributed to the hypolipidemia remains to be determined. Nevertheless, such involvement of Bim in hepatic lipid metabolism has not yet been described thus far and warrants further study..

In conclusion, we show here that leukocyte Bim deficiency in LDLr$^{-/-}$ mice results in increased activated T-cell content in circulation, lymphoid organs and atherosclerotic lesions, in increased levels of autoreactive antibodies directed against oxidized LDL in circulation and in substantial immunoglobulin deposition in atherosclerotic lesions. Collectively these pro- and anti-atherogenic effects of leukocyte Bim deficiency, summarized in table 1, together with the surprising reduction in serum lipid levels likely counterbalance so that atherosclerosis development remains unaffected.

Table 1. Pro- and anti-atherogenic consequences of leukocyte Bim deficiency in LDLr$^{-/-}$ mice.

Pro-atherogenic		**Anti-atherogenic**	
Circulating and lymph node T cell levels	↑	Serum cholesterol	↓
T cell activation	↑	Serum triglycerides	↓
Circulating Monocyte/macrophage and neutrophil levels	↑	Ox-LDL antibodies	↑
Apoptosis (depending on cell type)	↓	Apoptosis (depending on cell type)	↓

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Chapter 5

Increased Foam Cell Formation and Atherosclerotic Plaque Apoptosis and Markedly Decreased Neutrophil Recruitment in LDLr$^{-/-}$ Mice Lacking Macrophage Mcl-1

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Abstract

The anti-apoptotic Bcl-1 family member myeloid cell leukemia 1 (Mcl-1) plays an important, in neutrophils essential, role in survival and differentiation of leukocyte subsets. Here, we investigated the impact of Mcl-1 deletion in neutrophils and macrophages on atherosclerotic plaque development and stability in Western type diet fed LDLr$^{-/-}$ mice by a bone marrow (BM) transplantation strategy.

First, Mcl-1 deficient peritoneal macrophages had an increased sensitivity to Ox-LDL induced cell death and showed altered expression of several pro-apoptotic Bcl-2 family members as compared to WT macrophages. In keeping apoptotic cell content in aortic root lesions of Mcl-1$^{-/-}$ chimeras was elevated by 77% compared to WT controls. Second, lipid accumulation by peritoneal Mcl-1$^{-/-}$ macrophages, induced by Ox-LDL and VLDL, was enhanced in vitro as well as in vivo. Third, Mcl-1$^{-/-}$ macrophages showed a shift towards a pro-inflammatory M1 phenotype as apparent from their cytokine expression pattern and reduced phagocytic capacity. Despite these profound pro-atherogenic effects of Mcl-1 deficiency, the atherosclerotic plaque development and progression in LDLr$^{-/-}$ mice on a Western-type diet did not differ be-tween Mcl-1$^{-/-}$ and WT BM recipients. This seeming paradox can be explained by the markedly lower neutrophil numbers and migratory capacity in Mcl-1 deficiency, which translates in impaired atherosclerotic lesional neutrophil infiltration thus corroborating the significance of neutrophils for atherogenesis.

In conclusion, myeloid Mcl-1 deletion enhances Ox-LDL induced foam cell formation and cell death, favors a pro-inflammatory macrophage phenotype and markedly lowers neutrophil recruitment. The unaffected plaque initiation and growth indicates that neutrophil recruitment is essential to translate the pro-inflammatory phenotype into increased plaque formation.

Introduction

Macrophages are critical in the onset and progression of atherosclerotic lesion development. Monocyte infiltration with subsequent differentiation into macrophages, accumulation of lipids and secretion of various cytokines and growth factors all contribute to lesion progression[1]. Plaque macrophages have been shown to undergo apoptosis at all stages[2], although apoptosis tends to increase with plaque progression[3]. Macrophages are also the predominant lesional phagocytes[4]. Defective macrophage apoptosis in early lesions has been shown to promote lesion development in different atherosclerotic mouse models[5-7]. In advanced lesions, macrophage apoptosis contributes to the formation of large necrotic cores consisting mostly of lipids and apoptotic cell debris[2]. However, studies addressing effects of CD11b monocyte/macrophage apoptosis in advanced lesions did not reveal any differences in lesion size despite increased apoptotic cell content[7,8]. In human atherosclerotic lesions apoptosis of macrophages appeared to be enhanced only in more advanced stages[9]. This increase may at least in part be due to defective apoptotic cell phagocytosis in advanced lesions[10].

Anti-apoptotic Mcl-1 is a member of the apoptosis regulating Bcl-2 family[11]. It directly interacts with proapoptotic BH3-only proteins Bim and Bid and multidomain proapoptotic Bak[12-14], thereby inhibiting apoptosis. Mcl-1 is expressed in various tissues including hematopoietic cells[15], in which overexpression delays cell death in response to various stimuli[16]. Deletion of Mcl-1 in mice results in embryonic lethality[17]. A recent *in vivo* study in conditional knockout mice lacking Mcl-1 in neutrophils and macrophages demonstrated that Mcl-1 is necessary for neutrophil but not macrophage survival[18,19]. Mcl-1 induced neutropenia could be prevented by combined deletion of pro-apoptotic Bak and Bax[19]. In synovial fluid macrophages of rheumatoid arthritis patients Mcl-1 expression is enhanced and demonstrated to be essential for survival since Mcl-1 inhibition largely increased apoptosis[20]. Mcl-1 has been implicated in macrophage lipid accumulation. Mcl-1 RNA expression was found to be increased in THP-1 macrophages following Il-10 exposure, which also augmented Ox-LDL induced foam cell formation [21]. Furthermore, silencing Mcl-1 and Bfl-1, another anti-apoptotic Bcl-2 family member, in these macrophages prevented IL-10 induced lipid accumulation[21]. The critical importance of macrophage apoptosis and foam cell formation in atherosclerosis points to a potential role of this anti-apoptotic factor in atherosclerosis.

In the present study we have investigated the role of myeloid Mcl-1 in atherosclerotic plaque development and progression using bone marrow transplantation of LDLr$^{-/-}$ mice with Mcl-1$^{fl/fl}$ LysMcre or control bone marrow, with a specific emphasis on neutrophil recruitment as pro-atherogenic factor.

Materials and Methods

Animals

All animal work was approved by regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. LDLr$^{-/-}$ mice were obtained from

the local animal breeding facility. Mcl-$1^{fl/fl}$ LysMcre mice were obtained from the Department of Immunology, Duke University Medical Center, Durham, NC, USA.

Mcl-1 gene expression during atherogenesis

To assess Mcl-1 gene expression during atherosclerotic lesion progression 20 male $LDLr^{-/-}$ mice were fed a Western type diet (WTD) two weeks prior to surgery and throughout the experiment. Atherosclerotic carotid artery lesions were induced by perivascular collar placement as described by Von der Thüsen *et al.*[22] and subsets of 4 mice were sacrificed at 0, 2, 4, 6 and 8 weeks after collar placement for expression analysis. The mice were anaesthetized and perfused with phosphate buffered saline (PBS, 150 mM NaCl, 1.5 mM NaH_2PO_4, 8.6 mM Na_2HPO_4, pH 7.4) after which both common carotid arteries were isolated, snap-frozen in liquid nitrogen and stored at -80ºC. Two to three carotid arteries were pooled per sample and total RNA was isolated using Trizol reagent (Invitrogen, Breda, The Netherlands). Gene expression was analyzed by real time PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems) with SYBR-Green technology. The primers used for expression of Mcl-1 and standard housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) are listed in table 1.

Bone marrow transplantation and atherosclerosis induction

Male $LDLr^{-/-}$ mice (n=10) were housed in sterile ventilated cages with food (RM3, Special Diet Services, Witham, Essex, UK) and water ad libitum. The drinking water was supplied with antibiotics (83 mg/l ciprofloxacin and 67 mg/l Polymixin B) and 5 g/l sugar. The mice were exposed to a single dose of 9 Gy total body irradiation (0.19Gy/min, 200 kV, 4 mA,) using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) one day before transplantation. Bone marrow was extracted from femurs and tibia of male Mcl-$1^{fl/fl}$ LysMcre (hereafter Mcl-$1^{-/-}$) and wild type (WT) littermates. Irradiated $LDLr^{-/-}$ mice received either 2.5 x 10^6 Mcl-$1^{-/-}$ bone marrow cells (n=10) or 2.5 x 10^6 WT bone marrow cells (n=10) via tail vein injection. After a recovery period of eight weeks mice were put on a WTD containing 0.25% cholesterol and 15% cacao butter (Diet W, Special Diet Services, Witham, Essex, UK) for an additional five (plaque initiation) or ten weeks (advanced plaque formation).

Cholesterol and triglyceride assay

Blood samples were taken by tail bleeding before bone marrow transplantation (BMT), before start of (week 0), after two and six weeks of WTD feeding (week 2 and week 6) and at the time of sacrifice (week 5 or week 10). Total cholesterol and triglyceride levels in serum were measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, The Netherlands).

Blood cell analysis and flow cytometry

Blood samples were taken by tail bleeding immediately before BMT, before start of

(week 0) and after four weeks of WTD feeding (week 4) and at the time of sacrifice (week 5 or week 10). Peritoneal leukocytes were isolated at the time of sacrifice by peritoneal lavage with 10 ml PBS. Whole blood and peritoneal lavage samples were analyzed using a Sysmex blood cell analyzer (XT-2000i). White blood cells (WBC) were isolated by erythrocyte lysis of whole blood samples obtained by bleeding through the orbital sinus. For flow cytometry, WBC and peritoneal leukocytes were stained with fluorescently labeled antibodies against F4/80, CD19, CD4, CD71 and CD11b (eBioscience, Halle Zoersel, Belgium) and Gr1, CD8 and CXCR4 (BD Pharmingen, Breda, The Netherlands). Fluorescence-activated cell sorting (FACS) analysis was performed on FACSCalibur with CellQuest software (BD Biosciences).

Tissue harvesting and analysis

Two hours before sacrifice five mice from each group, Mcl-$1^{-/-}$ or WT, received intraperitoneal injections of the chemokine KC (200 ng/ml in 1 ml PBS) or PBS control. The mice were anesthetized and perfused with PBS. Heart, spleen and liver were harvested and stored in 4% formaldehyde solution. Cryosections were prepared of the aortic root and of spleen tissue and stained with hematoxylin and eosin (HE) or Oil Red O. Lesion size was quantified using Leica image analysis system, consisting of a Leica DMRE microscope with camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Immunohistochemical stainings were performed for macrophage (MOMA-2, Sigma, Zwijndrecht, The Netherlands) and vSMC (α-smooth muscle actin, Sigma) content. Apoptotic cell content was quantified using terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics).

LDL and VLDL isolation

LDL and VLDL were obtained from human plasma by density gradient ultracentrifugation for 20h at 4ºC[23]. Oxidation of LDL was performed by incubation with $CuSO_4$ for 24 hours at 37ºC after which the oxidation reaction was terminated by addition of EDTA (final concentration 200 µM).

Lipid loading of peritoneal macrophages

Peritoneal leukocytes were isolated from mice that received an i.p. injection of PBS before sacrifice and subsequently combined resulting in 3 pools of Mcl-$1^{-/-}$ and of WT peritoneal leukocytes. Cells were plated at 0.25 x 10^6 cells/well in 8 chamber culture slides (BD Falcon, Breda, The Netherlands). Non-adherent cells were removed and adherent macrophages were stimulated with 20 µg/ml oxidized LDL (Ox-LDL) or 50 ug/ml VLDL for 24 hours after which slides were washed with PBS and stained with Oil Red O. Lipid loading was quantified as the ratio between the Oil Red O stained cell area and total cell surface.

Apoptosis of peritoneal macrophages

Mcl-$1^{-/-}$ and WT peritoneal leukocyte pools (n=3 each) were plated at a density of 0.5 x 10^6 cells/well in a 24 well plate. Non-adherent cells were removed and adherent

macrophages were stimulated with 40 ug/ml Ox-LDL for 24 hours. The macrophages were detached with Accutase (PAA Laboratories GmbH, Cölbe, Germany), stained with FITC labeled Annexin V (ImmunoTools, Friesoythe, Germany) and propidium iodide (Sigma) and subsequently analyzed by flow cytometry (FACSCalibur, BD Biosciences, Breda, The Netherlands).

Isolation and differentiation of bone marrow derived macrophages

Bone marrow cells were isolated by flushing femurs and tibia with PBS and single cell suspensions were obtained by passing the suspension through a 70 µm nylon cell strainer (BD Falcon). Bone marrow cells were differentiated into macrophages by culturing in 70% RPMI, supplemented with 20% FCS, glutamine (2 mM), sodium pyruvate (1%), penicillin (100 U/ml), streptomycin (100 µg/ml) and non-essential amino acids (1%), and 30% L929 conditioned DMEM for 7 days. Bone marrow derived macrophages (BMDM) were used for apoptosis assay. BMDM experiments were performed in RPMI containing FCS (10%), glutamine (2 mM) and penicillin (100 U/ml) and streptomycin (100 µg/ml).

Phagocytosis assay

Jurkat cells were labeled with CellTracker Orange CMTMR fluorescent dye (Molecular Probes, Merelbeke, Belgium). Apoptosis was induced by incubation with 1 µM staurosporine (Sigma) for 2 hours. Apoptosis was assessed by flow cytometry after FITC labbeled Annexin V and propidium iodide staining of the cells. 70% of Jurkat cells were found to undergo apoptosis after incubation with staurosporine. BMDM were incubated for 1 hour with apoptotic Jurkat cells or red fluorescent beads (2 µm Fluospheres, Molecular Probes) at 37ºC. After thorough washing with PBS (5 times) phagocytosis was analyzed on a FACSCanto with FACSDiva software (BD Biosciences).

Table 1. Primer sequences

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Mcl-1	TCATTCTGGTAGAGCACCTAACACTT	AGCAGGTCAGCAAAGAACTTATAG
Bim	GGACTCTAAGAAAATACCCATCAAACC	TCCCCATCTGCTGCTAATACTTC
PUMA	CCCCATCCATCTCATTGCAT	GTGGCTTCCGCCAATATCTC
NOXA	GGTTGATGGAAATGCCTGGTAT	GAAACTCATTCCTATCTCCTTCATCAT
IL10	TCTTACTGACTGGCATGAGGATCA	GTCCGCAGCTCTAGGAGCAT
Arg1	GGTTCTGGGAGGCCTATCTTACA	TCTTCACCTCCTCTGCTGTCTTC
TNFα	GCCAGCCGATGGGTTGTA	AGGTTGACTTTCTCCTGGTATGAGA
p35	CCAAACCAGCACATTGAAGA	CTACCAAGGCACAGGGTCAT
iNOS	CCTGGTACGGGCATTGCT	GCTCATGCGGCCTCCTTT

RNA isolation and realtime PCR

Murine RAW 264.7 cells or BMDM were incubated for 24 h with or without Ox-

LDL (20 or 40 µg/ml). Total RNA was extracted using guanidine thiocyanate (GTC, Sigma, according to Chomczynski[24]). After RT-PCR, gene expression was analyzed by quantitative real-time PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR-Green technology and the primers listed in table 1. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and acidic ribosomal phosphoprotein P0 (36B4) were used as standard housekeeping genes.

Statistic analysis

Values are expressed as mean ± SEM or presented as mean + upper limit of the SEM. Analysis to compare two groups was performed by two-tailed Student's t-test. For analysis of relative mRNA expression data t-test was performed on ΔCt values. Statistical significance was set at $p<0.05$.

Results

Expression of Mcl-1 in murine macrophages and during atherogenesis

We first examined whether Mcl-1 has a potential role in atherosclerotic lesion development by assessing Mcl-1 gene expression in murine RAW 264.7 macrophages and the influence of oxidized LDL (Ox-LDL) on Mcl-1 expression. Mcl-1 is expressed in RAW 264.7 cells (fig. 1A). RAW 264.7 derived foam cells, generated by exposure to 20 or 40 µg/ml OxLDL for 24h displayed decreased mcl-1 expression ($p\leq0.05$, fig. 1A).

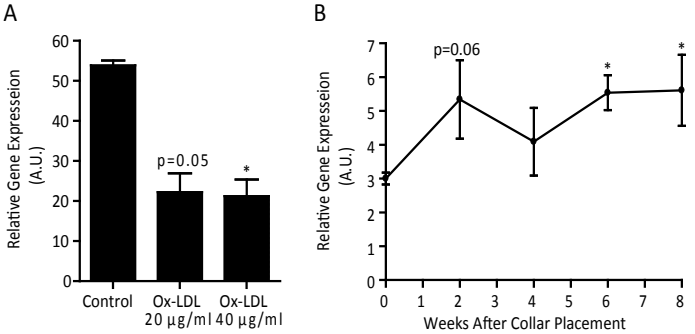


Figure 1. Regulation of Mcl-1 expression in vitro in RAW 264.7 macrophages and in vivo during atherogenesis. A. Mcl-1 was abundantly expressed in non-stimulated RAW 264.7 macrophages but downregulated upon exposure to 20 µg/ml or 40 µg/ml Ox-LDL for 24h. B. Vascular Mcl-1 expression gradually increased during lesion progression in a model of collar induced carotid artery atherogenesis in $LDLr^{-/-}$ mice (*$p<0.05$).

Next, we monitored Mcl-1 gene expression in $LDLr^{-/-}$ mice during atherogenesis. Mcl-1 gene expression during atherogenesis was measured in carotid artery lesions of western type diet fed $LDLr^{-/-}$ mice equipped with semi-constrictive collars to accelerate atherosclerotic lesion formation[22]. Mcl-1 gene expression was gradually increased during lesion development and in particular advanced plaques (> six weeks after collar induction of atherosclerotic lesion development) displayed a significant upregulation of increased Mcl-1 ($p<0.05$, fig. 1B). These results suggest that Mcl-1 is regulated during lesion progression which could contribute to atherosclerotic lesion

development or lesion stability.

Total cholesterol levels and body weight

Next we generated LDLr$^{-/-}$ mice with myeloid Mcl-1 deletion by reconstituting lethally irradiated LDLr$^{-/-}$ mice with Mcl-1$^{fl/fl}$ LysMCre (hereafter Mcl-1$^{-/-}$) or wild type (WT) bone marrow cells. After a recovery of eight weeks mice were put on a Western type diet (WTD) and effects of Mcl1$^{-/-}$ deficiency on lipid levels were monitored by measuring serum total cholesterol (TC) and triglyceride (TG) levels throughout the experiment. Average TC and TG levels did not differ between Mcl-1$^{-/-}$ and WT transplanted animals. Mcl-1 deletion did not affect total body weight.

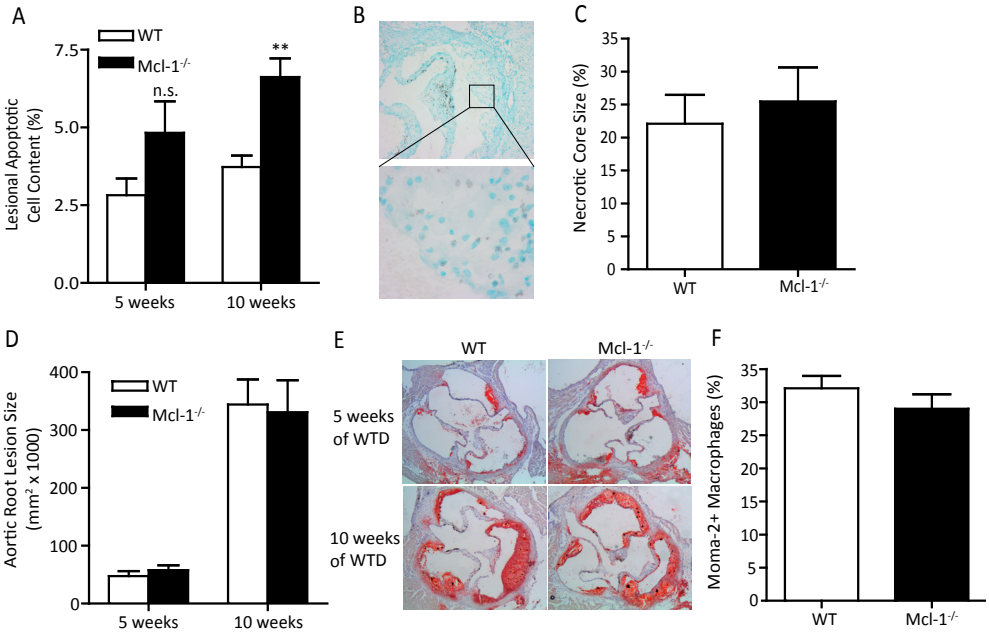


Figure 2. Effect of myeloid Mcl-1 deficiency on atherosclerotic lesion size and composition. A. Plaque apoptotic cell content is increased in Mcl-1$^{-/-}$ BM transplanted mice after 10 weeks of WTD feeding. B. Representative micrographs of TUNEL-stained aortic root sections (100x and 650x magnification (insert)). C. Plaque necrotic core size however is unchanged in Mcl-1 deficiency. D. Atherosclerotic lesion size is unaffected by myeloid Mcl-1 deficiency both at early and at late stages of disease progression. E. representative micrographs of Oil Red O stained aortic root sections. F. Moma-2 positive macrophage content of plaques was unaffected by myeloid Mcl-1 deficiency as well. (*p<0.05)

Myeloid Mcl-1deletion increases lesional apoptotic cell content but does not affect atherosclerotic lesion size

Five (early plaque formation) or ten weeks (advanced plaque formation) after WTD feeding aortic roots were isolated, sectioned and analyzed. Apoptotic cells content appeared to be increased by 71% (NS) and 77% (p=0.002) in atherosclerotic lesions of Mcl-1$^{-/-}$ fed a WTD for 5 and 10 weeks, respectively, compared to WT transplanted mice (Fig. 2A,B). However, despite its profound impact on apoptosis, myeloid Mcl-1 deletion did neither alter early atherogenesis (5.97 ± 2.62 *10^4 µm^2 and 4.73 ±

2.29 *10^4 µm² for Mcl-$1^{-/-}$ and WT reconstituted mice respectively, 5 weeks of WTD) nor advanced lesion formation (3.31 ± 1.66 *10^5 µm² and 3.44 ± 1.38 *10^5 µm², 10 weeks of WTD) (Fig. 2D) Necrotic core size and lesion macrophage (Moma-2) and collagen content were unaffected by myeloid Mcl-1 deletion as well (fig. 2C,E,F).

Macrophage sensitivity to Ox-LDL induced apoptosis is increased

Incubation of peritoneal macrophages with 40 µg/ml Ox-LDL for 24 hours showed that Mcl-$1^{-/-}$ macrophages have an increased sensitivity towards Ox-LDL induced cell death compared to WT macrophages (Fig. 3A). Mcl-1 deletion also appeared to decrease cell survival in non-stimulated macrophages. Gene expression of several BH3-only proapoptotic Bcl-2 family proteins was assessed in Mcl-$1^{-/-}$ and WT bone marrow derived macrophages (BMDM). The expression of Bim, PUMA ($p<0.01$) and NOXA (n.s.), proapoptotic proteins that all were seen to interact with Mcl-1[25-27], appeared to be decreased in non-stimulated Mcl-$1^{-/-}$ macrophages (fig. 3B-D). Exposure to Ox-LDL decreased gene expression of PUMA and NOXA but did not change that of Bim in WT macrophages. Conversely, in Mcl-$1^{-/-}$ macrophages Ox-LDL incubation resulted in elevated Bim and PUMA expression.

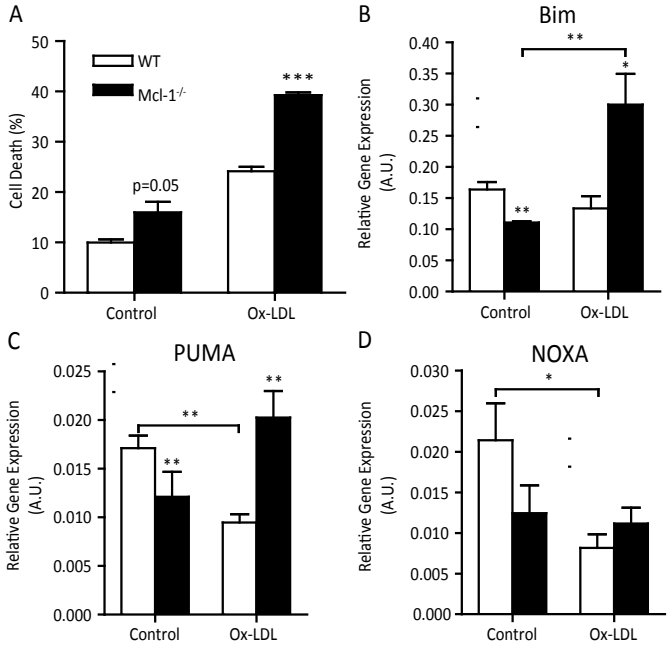


Figure 3. Cell death and ex-pression of Bcl-2 family members in peritoneal and bone marrow derived macrophages. A. Mcl-1 deficiency leads to enhanced cell death of unstimulated and Ox-LDL (40 µg/ml) stimulated peritoneal macrophages. B-D. Bim, PUMA and NOXA gene expression was affected by Mcl-1 deletion (black bars) in both unstimulated and Ox-LDL (40 µg/ml) stimulated BMDM. (*$p<0.05$, **$p<0.01$)

Macrophage lipid loading is enhanced

Peritoneal foam cell numbers were increased by 2.5 fold ($p<0.05$) in Mcl-$1^{-/-}$ compared

to WT transplanted mice (Fig. 4A). Total peritoneal macrophage numbers were unchanged. This finding led us to examine the lipid loading capacity of peritoneal macrophages *in vitro*. Macrophages were incubated for 24h with 20 µg/ml Ox-LDL or 50 µg/ml VLDL and stained with Oil Red O. In agreement with the elevated peritoneal foam cell counts *in vivo*, lipid accumulation in non-stimulated Mcl-1$^{-/-}$ macrophages was markedly increased (fig. 4B,C). Lipid loading in WT macrophages remained unchanged after incubation with Ox-LDL for 24h, but was substantially enhanced in Mcl-1$^{-/-}$ macrophages ($p<0.01$). While incubation with VLDL increased lipid content in both WT and Mcl-1$^{-/-}$ macrophages, this increase was considerably (3,6 fold) higher in the latter cells ($p=0.01$).

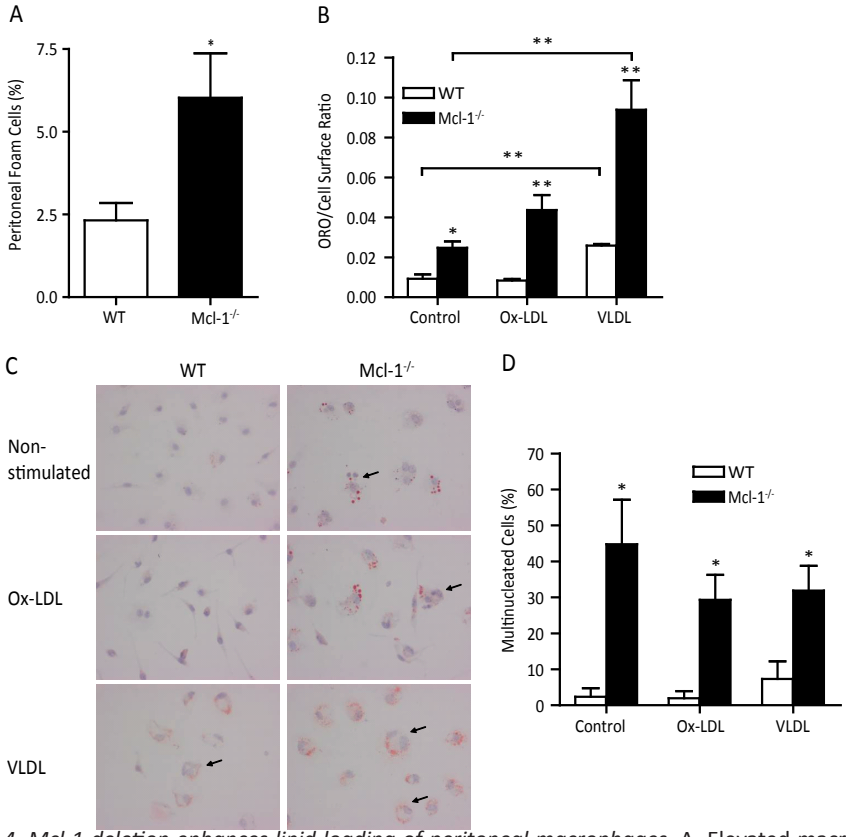


Figure 4. Mcl-1 deletion enhances lipid loading of peritoneal macrophages. A. Elevated macrophage derived foam cell numbers in peritoneum of Mcl-1$^{-/-}$ BM (black bars) compared to WT BM transplanted mice (white bars). B. Lipid loading of Mcl-1$^{-/-}$ peritoneal macrophages (black bars) was dramatically increased after incubation with Ox-LDL (20 µg/ml) or VLDL (20 µg/ml), whereas WT macrophages (white bars) only showed a moderate increase in lipid loading after incubation with VLDL. C. Representative micrographs of Oil Red O stained unstimulated and Ox-LDL or VLDL stimulated peritoneal macrophages. Multinucleated giant cells are indicated by an arrow. (*$p<0.05$, **$p<0.01$) D. Multinucleated giant cell levels were largely increased among Mcl-1$^{-/-}$ peritoneal macrophages compared to WT macrophages.

In addition to enhancing the lipid loading capacity, Mcl-1 deficiency resulted in an increased presence of multinucleated giant cells (MGC) within the peritoneal

macrophage population both at baseline and after incubation with Ox-LDL or VLDL ($p<0.05$, Fig. 4C and 4D, MGC are indicated by arrows).

Mcl-1$^{-/-}$ transplanted LDLr$^{-/-}$ mice display splenomegaly and altered macrophage phenotype.

Relative spleen weight was increased by nearly two-fold ($p=0.006$) in Mcl-1$^{-/-}$ compared to WT transplanted mice (fig. 5B), which in Mcl-1 LysMCre deleted mice has been shown to result from increased myeloid cell content in splenic red pulp[19]. BM derived macrophages with MCL1 deletion showed features of a pro-inflammatory M1 phenotype (fig 5D-H). IL10 expression in OxLDL stimulated Mcl-1$^{-/-}$ macrophages was reduced while that of the M2 marker arginase I was unaltered. Conversely, expression of proinflammatory M1 cytokines TNFα, and IL12 subunit p35 was upregulated after Ox-LDL stimulation, an effect absent in WT macrophages. In agreement with the M1 shift, macrophages exhibited reduced phagocytosis of early apoptotic Jurkat cells (fig. 5C), a phenomenon that was reported to be more pronounced in M2 than in M1 macrophages[28].

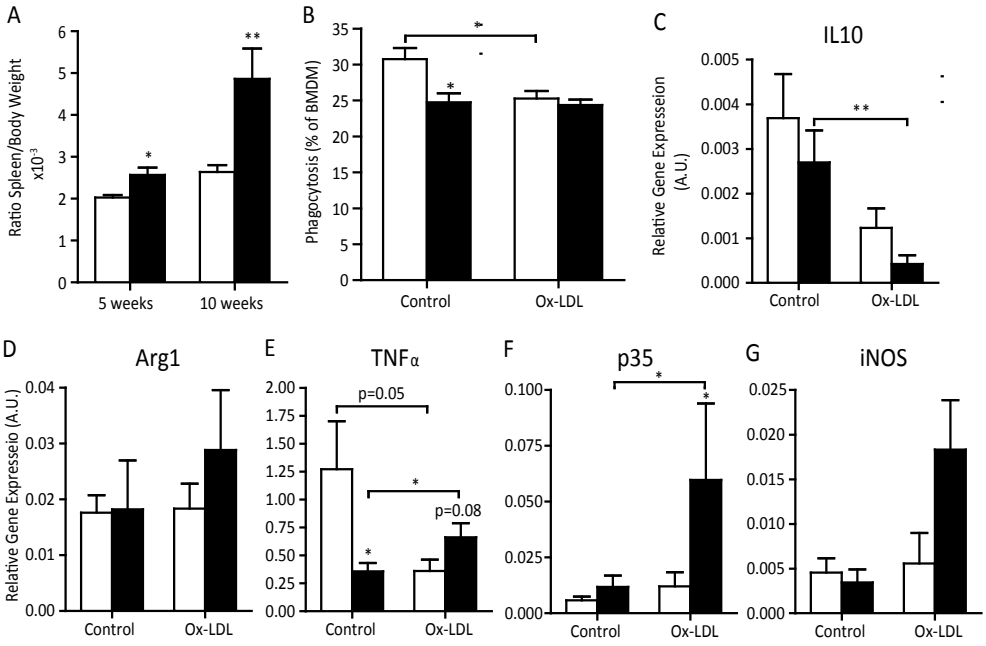


Figure 5. Myeloid Mcl-1 deficiency affects inflammatory status of LDLr$^{-/-}$ mice. A. Mcl-1$^{-/-}$ BM recipients showed more pronounced and progressive splenomegaly after Western type diet feeding as compared to WT recipients. B. Baseline phagocytosis of apoptotic Jurkat cells by Mcl-1$^{-/-}$ BMDM was slightly decreased. Phagocytosis by Ox-LDL stimulated Mcl1$^{-/-}$ BMDM was however comparable to that by WT BMDM, however phagocytosis was decreased compared to unstimulated WT BMDM. C-G. Ox-LDL incubation led to a decrease in IL10 gene expression and unaltered expression of arginase-I in Mcl-1$^{-/-}$ BMDM (black bars). Gene expression of TNFα, and IL12 subunit p35 were increased after Ox-LDL stimulation (black bars), an effect absent in WT BMDM (white bars), pointing towards a more pro-inflammatory M1 phenotype of Mcl-1$^{-/-}$ macrophages. (*$p<0.05$, **$p<0.01$)

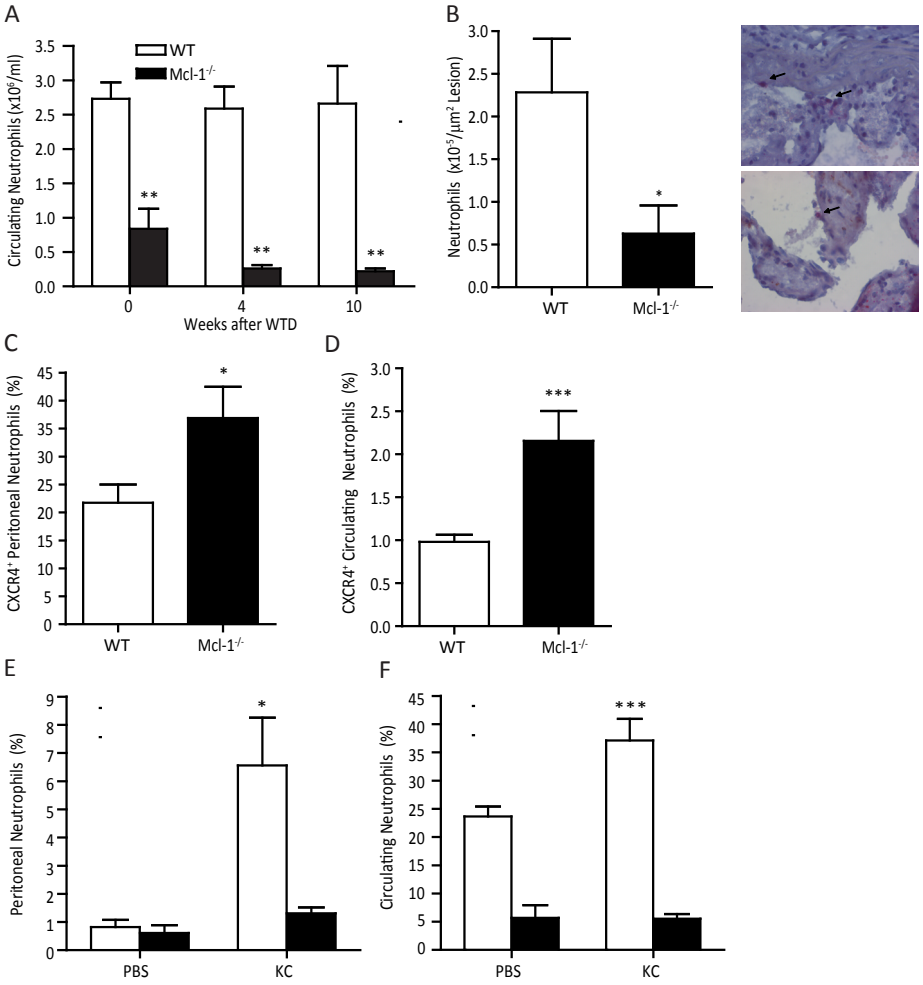


Figure 6. Mcl-1 deletion affects neutrophil levels and phenotype. A. Circulating neutrophil levels were profoundly reduced in Mcl-1$^{-/-}$ compared to WT transplanted mice and even more so after Western type diet feeding. B. Aortic root atherosclerotic lesions of Mcl-1$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice contained fewer neutrophils as can also be seen in representative micrographs (right panel, arrows indicate neutrophils). C,D. CXCR4 expression on Mcl-1$^{-/-}$ neutrophils is elevated compared to WT neutrophils. E. WT BM transplanted mice displayed a massive neutrophil influx into the peritoneal cavity in response to i.p. KC injection (white bars) which is almost completely prevented in Mcl-1$^{-/-}$ BM transplanted mice (black bars). F. The KC induced increase in circulating neutrophil numbers observed in WT chimeras (white bars) was absent in Mcl-1$^{-/-}$ BM transplanted mice as well (black bars). (*p<0.05, **p<0.01, ***p<0.001)

Mcl-1 deletion alters neutrophil levels and characteristics

Mcl-1 was shown to be essential for neutrophil survival and as a result circulating and splenic neutrophils numbers were reduced by 80 and 86%, respectively, in Mcl-1$^{fl/fl}$ LysMcre mice[18]. Indeed Mcl1$^{-/-}$ chimeras were almost depleted in circulating neutrophils both before WTD feeding and under hyperlipidemic conditions (four and ten weeks after WTD feeding) (-82% to 91%, p<0.001, Fig. 6A). Neutrophil

content in Mcl-$1^{-/-}$ atherosclerotic lesions was decreased by 72% (0.63 ± 0.31 *10^{-5} per μm^2 lesion) compared to WT lesions (2.28 ± 0.63 *10^{-5} neutrophils per μm^2 lesion, $p<0.05$, Fig. 6B), which might point to an enhanced adhesive capacity of residual neutrophils in circulation.

We therefore examined neutrophil phenotype and function in both groups of animals. CXCR4 expression was increased on circulating (2.2 fold, $p<0.001$) as well as on peritoneal (1.7 fold, $p<0.05$) Mcl-$1^{-/-}$ neutrophils (Fig. 6C,D), suggesting altered migratory capacity. Remarkably, Mcl-$1^{-/-}$ neutrophils showed a decreased responsiveness to KC, a potent neutrophil chemoattractant. Neutrophil content in the peritoneal cavity and circulation was measured 2 hours after i.p. injection of KC or PBS. Peritoneal neutrophil influx in response to KC was prominent in WT transplanted mice whereas Mcl-$1^{-/-}$ transplanted mice only showed a minor, non-significant, increase in peritoneal neutrophils (Fig. 6E,F). Of note, neutrophil recruitment was paralleled by a moderate increase in circulating neutrophils in WT but not Mcl$1^{-/-}$ mice.

Discussion

In this study we determined the role of Mcl-1 in atherosclerotic plaque initiation and progression. Mcl-1 is an anti-apoptotic Bcl-2 family member[19] and although several members of this family have been investigated in the context of atherosclerosis[6,8,29], the role of Mcl-1 in disease progression has not been assessed thus far. Here, we studied effects of specific deletion of Mcl-1 in lysozyme M expressing myeloid subsets such as neutrophils and macrophages on early and advanced atherosclerosis.

First we established Mcl-1 expression in RAW 264.7 macrophages and showed that Mcl-1 expression is decreased in response to Ox-LDL. In addition vascular Mcl-1 expression steadily increases with lesion progression in LDLr$^{-/-}$ mice. Given that macrophage content and apoptosis gradually increases with plaque progression[3] and that Mcl-1 was seen to affect macrophage lipid accumulation[21] these findings suggest that Mcl-1 could be a critical player in atherosclerosis. Therefore we investigated the role of Mcl-1 atherosclerotic lesion development and stability, in LDLr$^{-/-}$ chimeras with Mcl-1 deficiency in macrophages and neutrophils generated by transplantation of Mcl-$1^{fl/fl}$ LysMcre or wildtype (WT) bone marrow. The use Mcl-1 deficiency in lysozyme M expressing cells enabled us to specifically study effects of Mcl-1 deficiency in macrophage and neutrophil differentiation and apoptosis and their contribution to disease. Mcl-1 was previously demonstrated to be involved in cell death of various leukocyte subsets[16,18,30-33]. Overexpression of Mcl-1 in hematopoietic cells protected from cell death in response to various stimuli[16] and inhibition of Mcl-1 expression in human neutrophils and macrophages resulted in increased apoptosis[31,32]. *In vivo* overexpression of Mcl-1 promoted survival of lymphocytes and myeloid cells[18,30,33]. Our data largely correspond with these findings in that Mcl-$1^{-/-}$ macrophages displayed decreased survival compared to WT macrophages. Additionally, we show that Mcl-1 participates in Ox-LDL induced cell death as Mcl-$1^{-/-}$ macrophages were more susceptible to cell death

after Ox-LDL incubation. Mcl-1 deficiency was seen to influence the expression of other apoptosis regulating Bcl-2 family members, such as Bim, PUMA and NOXA, which all were decreased in unstimulated Mcl-$1^{-/-}$ compared to WT macrophages. Whereas Ox-LDL stimulation resulted in decreased PUMA and NOXA expression in WT macrophages, in Mcl-$1^{-/-}$ macrophages this led to an increase in PUMA expression together with increased Bim. Bim, PUMA and NOXA are BH3-only pro-apoptotic Bcl-2 family members involved in the initiation of apoptosis[34], which exhibit reduced pro-apoptotic activity upon interaction with Mcl1 and all other pro-survival Bcl-2 family members (Bim and PUMA) or Mcl-1 and A1 only (NOXA)[12,25-27]. The dysregulated expression of BH3-only proteins in Mcl-$1^{-/-}$ macrophages suggests that apart from inhibiting apoptosis by interaction with BH3-only proteins, Mcl-1 might also exert anti-apoptotic effects at the transcriptional level in response to an apoptotic stimulus.

In line with the *in vitro* findings apoptotic cell content in advanced aortic root lesions (10 weeks of WTD) was increased in mice with myeloid Mcl-1 deficiency, indicating that Mcl-1 is a major survival protein in atherosclerotic lesions. However despite a 77% increase in apoptosis, atherosclerotic lesion burden was unaltered in Mcl-$1^{-/-}$ BM recipients, as were necrotic core size and macrophage and collagen content. Similar findings were obtained when studying plaque initiation (five weeks of WTD feeding). Our results correspond with those from Thorp *et al.*[8], who showed increased macrophage apoptosis but unchanged lesion burden in Bcl-2_{flox}-LysMCre ApoE$^{-/-}$ mice that are deficient in macrophage and neutrophil Bcl-2[8]. However macrophage apoptosis in early atherogenesis was demonstrated to be beneficial in several studies in LDLr$^{-/-}$ and ApoE$^{-/-}$ mice with deleted expression of pro-apoptotic or survival factors[5,6,35,36]. Two studies assessing apoptosis in advanced atherosclerosis presented contradictory results. Stoneman *et al.*[7] observed in CD11b-hDTR / ApoE$^{-/-}$ mouse that diphtheria toxin induced ablation of CD11b^{+} monocytes and macrophages did not result in any effects on lesion initiation or progression although lesion apoptotic cell content was largely increased. Conversely, in a recent study by Gautier *et al.*[37] apoptosis in advanced lesions in ApoE$^{-/-}$ mice was seen to aggravate disease progression. Here, we found that macrophage and neutrophil specific deletion of Mcl-1 in LDLr$^{-/-}$ mice had additional consequences, both at a systemic level and within the lesion itself, which may have masked effects of the observed increased lesion apoptosis.

First, apart from an increased sensitivity to Ox-LDL induced cell death Mcl-$1^{-/-}$ macrophages showed augmented lipid accumulation after incubation with Ox-LDL and VLDL. In keeping we observed elevated foam cell levels *in vivo* in the peritoneal cavity of Mcl-$1^{-/-}$ BM compared to WT BM recipients. These findings seems to contrast with those of Halvorsen *et al.*[21], who reported reduced IL-10 induced OxLDL loading by THP-1 macrophages *in vitro* after siRNA mediated silencing of Mcl-1 and Bfl-1 expression. The authors did not assess effects of Mcl-1 inhibition alone, without IL10 stimulation. Based on our data we hypothesize that the apoptosis-prone phenotype of Mcl-$1^{-/-}$ macrophages facilitates uptake of lipids.

Another remarkable characteristic was the high propensity of Mcl-1$^{-/-}$ cells to form multinucleated giant cells (MGC). MGC originate from monocyte-macrophage lineage and result from aberrant cell fusion[38]. Although the actual mechanism of MGC formation is hitherto unclear, their presence is thought to reflect an elevated inflammatory status with a crucial role for the cytokine environment in which they are formed[38]. The latter notion is corroborated by the splenomegaly in Mcl-1$^{-/-}$ chimeras and a shifted polarization of Mcl-1$^{-/-}$ macrophages towards a pro-inflammatory M1 macrophage phenotype[39] as judged from their cytokine expression pattern and reduced phagocytic capacity[28].

Previously Mcl-1 has been proven to be essential for appropriate differentiation of hematopoietic stem cells[40] and development of lymphocytes[12] and neutrophils[18], whereas monocytes and macrophages display normal development in the absence of Mcl-1[19]. Lately, neutrophils are increasingly perceived as important players in atherogenesis. Increased neutrophil count is positively correlated with coronary artery disease[41,42] and in mouse models neutrophils have been demonstrated to be present in atherosclerotic lesions and adventitia[43,44]. Depletion of neutrophils in ApoE$^{-/-}$ mice results in reduced atherosclerotic lesion formation[43]. In our study, Mcl-1$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice were displaying overt neutropenia, consistent with previous findings that Mcl-1 is essential for neutrophil survival[18]. Impaired neutrophil survival translated in a reduced atherosclerotic lesion neutrophil content although the decrease was less pronounced than in circulation. The latter finding may be explained by an altered migratory capacity of residual neutrophils. Dzhagalov *et al*.[18] showed that residual neutrophils in plain Mcl-1$^{fl/fl}$ LysMcre mice were shown to express Mcl-1 and apparently had escaped Cre-mediated Mcl-1 deletion[18]. In our study, both in circulation and in the peritoneal cavity CXCR4^{+} neutrophil numbers were increased in Mcl-1 deficiency, indicative of increased stromal retention and decreased release of neutrophils from the bone marrow. Administration of CXCR4 blocking antibody to mice was reported to reduce retention of circulating neutrophils in while increasing their release from bone marrow[45]. In addition it was shown that CXCR2 ligand KC induces desensitization to effects of CXCR4 ligand SDF-1α[45]. Vice versa, SDF-1α is able to attenuate neutrophil response to KC[46]. In our study, circulating neutrophils in Mcl-1$^{-/-}$ BM recipients had a reduced migratory response to chemoattractant KC *in vivo*. This reduced response might be partly attributable to enhanced SDF-1α /CXCR4 signaling and partly be a result of the substantially reduced neutrophil pool.

In summary, myeloid Mcl-1 deficiency enhanced Ox-LDL induced cell death of macrophages *ex vivo* as well as atherosclerotic lesion apoptosis in BM transplanted LDLr$^{-/-}$ mice. Furthermore, mcl-1 deficiency was seen to reduce phagocytic capacity and to promote macrophage lipid uptake and macrophage polarization towards a pro-inflammatory M1 phenotype. Despite these supposedly pro-atherogenic effects of Mcl-1 deficiency, it did not aggravate lesion development or progression. This apparent paradox indicates that the markedly lower neutrophil numbers in circulation and plaque, contravene the pro-atherogenic capacity of Mcl-1 deficiency,

illustrating that neutrophil recruitment is essential to translate a pro-inflammatory phenotype into increased atherosclerotic plaque formation.

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Chapter 6

Compromised Focal Adhesion Kinase Function Does Not Alter Atherogenesis in ApoE$^{-/-}$ Mice despite Profound Effects on Lipid Metabolism and Inflammatory Status

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Abstract

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase involved in cell survival, cell-matrix adhesion, migration and cell proliferation and apoptosis of all major cell types involved in atherosclerotic plaque development. While suggestive of a role in cellular homeostasis in atherosclerotic plaques, experimental evidence to support this hypothesis is lacking to date. The aim of this study was to address the role of impaired FAK function on inflammatory status and atherosclerotic lesion development in Western type diet fed $ApoE^{-/-}$ mice.

$FAK^{+/-}ApoE^{-/-}$ mice had significantly lower plasma total cholesterol levels, which was attributable to decreased hepatic VLDL production rather than an altered intestinal cholesterol absorption. Hepatic lipid content was unchanged in $FAK^{+/-}ApoE^{-/-}$mice. Furthermore, $FAK^{+/-}ApoE^{-/-}$ mice displayed splenomegaly (31% increase in weight) with a decreased number of white pulp nodules in the spleen and enlarged germinal centres, suggesting perturbed immune function. Macrophage content in spleen was found to be decreased whereas the number of lymphocytes was increased. In contrast, the peritoneal cavity and the circulation showed increased monocytes numbers accompanied by a reduced $CD4^+$ and $CD8^+$ T cell content. Surprisingly, despite these marked effects on lipid homeostasis and immune status, impaired FAK expression neither affected size and composition of collar induced carotid artery plaques nor that of spontaneous brachiocephalic artery and aortic root plaques.

We conclude that compromised FAK function beneficially affects cholesterol metabolism in Western type diet fed $ApoE^{-/-}$ mice and alters their inflammatory status. Despite these marked peripheral effects impaired focal adhesion does not translate in an altered atherogenic response.

Introduction

Focal adhesion kinase (FAK) is a ubiquitously expressed non-receptor protein tyrosine kinase and is activated by (auto)phosphorylation upon integrin binding. Known targets of FAK include paxillin, Cas and the Src family of kinases[1]. In addition to activation by phosphorylation following integrin binding, FAK phosphorylation has been shown to occur in response to growth factor stimulation (VEGF and HGF)[2,3]. Several studies have established a role for FAK in cell-matrix adhesion, cell migration, proliferation and apoptosis[4]. FAK-deficient fibroblast-like cells were shown to have an increased number of focal adhesions, most likely to be caused by a decreased turnover, and to spread more slowly on extracellular matrix proteins[5]. Furthermore FAK deficient hematopoietic precursor cells display an impaired migratory response to chemotactic signals[6]. FAK is essential in embryonic development in particular of the vasculature, since FAK knockout embryo's die at 8.5 days of age due to perturbed vasculogenesis[5].

In the vasculature FAK was found to affect adherence, migration and proliferation of vascular smooth muscle cells (vSMC's)[7,8] and endothelial cells[9-11]. Moreover, study of endothelial cells from FAK knockout embryo's showed that endothelial FAK is required for tubulogenesis[9]. In addition, targeted FAK deletion in embryonic endothelial cells resulted in impaired vascular network stability and cell survival[10]. Consequently, FAK knockout embryo's exhibit severe defects in vascular development. The non-catalytic carboxyl-terminal protein binding domain of FAK called FRNK (FAK related non kinase) is independently and selectively expressed in vSMC's, acting as an endogenous inhibitor of FAK signaling. Following balloon-induced carotid artery injury FRNK is strongly upregulated in vSMC's indicating that following injury vSMC migration and adhesion complex turnover may be negatively regulated[7].

These findings combined with the recent observation that FAK is overexpressed in VSMC's in human intimal hyperplasia[12] might suggest a role for FAK in cellular homeostasis in atherosclerotic plaques. However to date the precise role of FAK in atherogenesis has not been addressed. In this study we therefore assessed the effect of compromised FAK function in ApoE$^{-/-}$ mice using perivascular collar placement around the common carotid arteries to induce atherosclerotic lesion development. Although our results surprisingly demonstrated no overt effect on atherosclerotic lesion size or composition, lipid metabolism and inflammatory status of these mice appeared to be seriously modified in the heterozygote status of FAK expression.

Materials and Methods

Animals

All animal work was approved by regulatory authority of Leiden and performed in compliance with the Dutch government guidelines. FAK$^{+/-}$ mice were obtained from the Department of Toxicology (LACDR, Leiden, The Netherlands) and were crossbred with ApoE$^{-/-}$ mice, obtained from the local animal breeding facility.

Carotid collar placement

At 16-18 weeks of age carotid atherosclerotic lesion development was induced by bilateral perivascular collar placement in female $FAK^{+/-}ApoE^{-/-}$ mice (n=10) and $FAK^{+/+}ApoE^{-/-}$ littermates (n=9) as described by Von der Thüsen *et al*.[13] Starting 4 weeks before collar placement and throughout the whole experiment mice were fed a Western type diet containing 0.25% cholesterol and 15% cacao butter (Diet W, Special Diet Services, Witham, Essex, UK). In a separate experiment bilateral perivascular collar were placed in female $FAK^{+/-}ApoE^{-/-}$ mice (n=11) and $ApoE^{-/-}$ littermates (n=11) at the age of 12-16 weeks. These mice were fed a regular chow diet throughout the experiment.

Cholesterol, triglyceride and phospholipid assay

Blood samples were taken by tail cut before start of (week 0), after four weeks of the Western type diet (week 4), and at the time of sacrifice (week 10). Hepatic lipids were extracted as described by Bligh and Dyer[14]. Total cholesterol, triglyceride and phospholipid content was measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, The Netherlands) in serum and hepatic extracts. Serum samples were fractionated using Superose 6 column (SMART System, Pharmacia Biotech, Uppsula, Sweden) to obtain total cholesterol distribution patterns.

Cholesterol absorption

To analyze cholesterol absorption from the intestine, $FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates (n=5) were intravenously injected with 500 mg Triton WR1339/Tyloxapol per kg body weight (Sigma, Zwijndrecht, The Netherlands) to block lipoprotein lipase mediated lipolysis after overnight fasting. Subsequently the mice were gavaged with 1.2 µCi ^{14}C-cholesterol (GE Healthcare, Diegem, Belgium) and 200 µl unlabeled cholesterol (Sigma) in olive oil. Blood samples were taken by tail cut before and 1, 2, 3 and 4 hours after oral cholesterol administration. After 4 hours the animals were anesthetized and gal bladder, small intestine and liver were harvested. ^{14}C content was determined in serum, gall bladder, small intestine (20 cm from pylorus) and liver sections using a liquid scintillation analyzer (Tri-Carb 2900R, Packard).

VLDL production rate

For analysis of Very Low-Density Lipoprotein (VLDL) production rate, mice were intravenously injected with Triton WR1339/Tyloxapol (500 mg per kg body weight, Sigma). Blood samples were taken via tail vein before and 1 and 2 hours after injection. Serum triglyceride accumulation was measured as described above.

FAK gene and protein expression

$FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates (n=4) were anesthetized and blood was obtained by bleeding through the orbital sinus. Erythrocytes in whole blood were lysed to obtain white blood cells (WBC's). Various organs including liver, heart

and aorta were harvested in liquid nitrogen after perfusion with PBS. Total RNA was extracted using guanidine thiocyanate (GTC, Sigma). After RT-PCR, FAK gene expression in various organs and expression of genes involved in lipid homeostasis in liver were analyzed by quantitative real-time PCR using ABI PRISM 7700 Sequence Detector (Applied Biosystems) and SYBR-Green technology. The primer sequences are listed in table 1. HPRT, β-actin and/or acidic ribosomal phosphoprotein P0 (36B4) were used as standard housekeeping genes.

Protein expression was analyzed by Western blotting using mouse anti-FAK, clone 4.47 (Upstate Biotech, Charlottesville, VA, USA) primary antibody and rabbit anti-mouse-HRP (DakoCytomation, Heverlee, Belgium) secondary antibody. Signal intensity was analyzed using ImageJ software (http://rsb.info.nih.gov/ij/).

Table 1. Primer sequences for realtime PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
18S	CCATTCGAACGTCTGCCC	GTCACCCGTGGTCACCATG
β-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
FAK	GAGAATCCAGCTTTGGCTGTT	GGCTTCTTGAAGGAACTTCT
CD36	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
FAS	GGCATCATTGGGCACTCCTT	GTCGCAAGCACAGCCTCTCT
SCD1	TACTACAAGCCCGGCCTCC	CAGCAGTACCAGGGCACCA
SREBP-1	GACCTGGTGGTGGGCACTGA	AAGCGGATGTAGTCGATGGC
SREBP-2	TGAAGCTGGCCAATCAGAAAA	ACATCACTGTCCACCAGACTGC

Tissue harvesting and analysis

Six (for Western type diet fed mice) or ten weeks (for normal chow diet fed mice) after collar placement the animals were anesthetized and perfused with PBS followed by fixation with 4% formaldehyde (4.5 times diluted Zinc Formal-Fixx, Thermo Electron Corporation, Breda, The Netherlands). Carotid arteries, brachiocephalic artery, heart, spleen and liver were harvested and stored in 4% formaldehyde solution. Cryosections were prepared of carotid and brachiocephalic arteries, aortic valves, spleen and liver and stained with hematoxylin and eosin (HE) and/or Oil Red O. Lesion size was quantified using Leica image analysis system, consisting of a Leica DMRE microscope with camera and Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Immunohistochemical stainings were performed for macrophage (MOMA-2, Sigma), VSMC (α-smooth muscle actin, Sigma), T cell (CD3, Serotec) and B cell (Cd45R/B220, Pharmingen) content. Apoptotic cell content was quantified using terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics, Almere, The Netherlands).

Blood cell analysis and flow cytometry

Peripheral blood mononuclear cells, splenocytes and peritoneal leukocytes were isolated from FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates fed normal chow or Western type diet for 4 weeks and analyzed using a Sysmex blood cell analyzer (XT-2000i), or stained for cell surface markers and analyzed by flow cytometry. White blood cells

were isolated after erythrocyte lysis of whole blood samples obtained by bleeding through the orbital sinus, peritoneal leukocytes by peritoneal lavage with 10 ml PBS and splenocytes by passing (part of) the spleen through a 70 µm nylon cell strainer (BD Falcon). White blood cells were stained with fluorescently labeled antibodies against CD4, CD19, CD23, CD69, CD71, CD86, F4/80 and MHCII (eBioscience, San Diego, California) and CD8 and CD11b (BD Pharmingen, Breda, The Netherlands). Fluorescence-activated cell sorting (FACS) analysis was performed on a FACS-Calibur and analyzed with CellQuest software (BD Biosciences).

Statistical analysis

Values are expressed as mean ± SEM. Analysis to compare two groups was performed by two-tailed Student's t-test. For analysis of relative mRNA expression data t-test was performed on ΔCt values. Statistical significance was set at $p<0.05$.

Results

FAK expression

To determine the level of FAK deletion in $FAK^{+/-}ApoE^{-/-}$ mice, gene and protein expression was analyzed in heart, aorta, liver and/or spleen of these mice and $ApoE^{-/-}$ littermates. FAK gene expression appeared to be reduced by 34-45 % in $FAK^{+/-}ApoE^{-/-}$ mice compared to $ApoE^{-/-}$ littermates as determined by quantitative real-time PCR (Fig. 1A). In keeping total FAK protein expression was reduced by a similar 35-50% in these organs as shown by Western blotting and Image J analysis software (Fig. 1B and C).

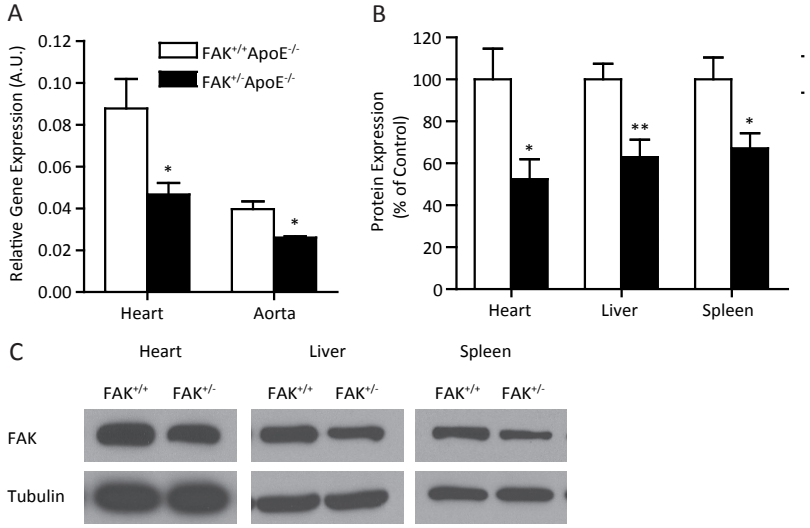


Figure 1. FAK expression in $FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates. Total RNA was isolated from different organs with GTC and used for real-time PCR. Proteins were isolated with protein lysis buffer and used for Western Blotting. FAK gene expression in heart and aorta (A) and FAK protein expression in heart, liver and spleen (B) are reduced in $FAK^{+/-}ApoE^{-/-}$ mice compared to $ApoE^{-/-}$ littermates. (*$p<0.05$, **$p<0.01$)

Lipid metabolism

Throughout the study we have monitored lipid levels in FAK$^{+/-}$ApoE$^{-/-}$ and ApoE$^{-/-}$ mice by measuring serum total cholesterol (TC) and triglyceride (TG) levels before and after four or ten weeks of Western type feeding. Surprisingly, while total cholesterol levels were increased in both groups after Western type feeding, the diet induced increase in TC levels in FAK$^{+/-}$ApoE$^{-/-}$ mice was 18% lower than in ApoE$^{-/-}$ mice ($p<0.01$, Fig. 2A). Triglyceride levels were comparable in both groups as were total cholesterol levels when fed a regular chow diet. The reduced hypercholesterolemic response to Western type diet was not caused by apparent differences in health status or food intake, since total body weight remained unchanged, 30.3 ± 2.1 g for FAK$^{+/-}$ApoE$^{-/-}$ mice and 29.8 ± 2.1 g for ApoE$^{-/-}$ mice. As shown in fig. 2B, when serum was fractionated by Superose 6 column to analyze the TC distribution over the different lipoproteins, the total VLDL cholesterol levels were found to be responsible for the decrease in total cholesterol.

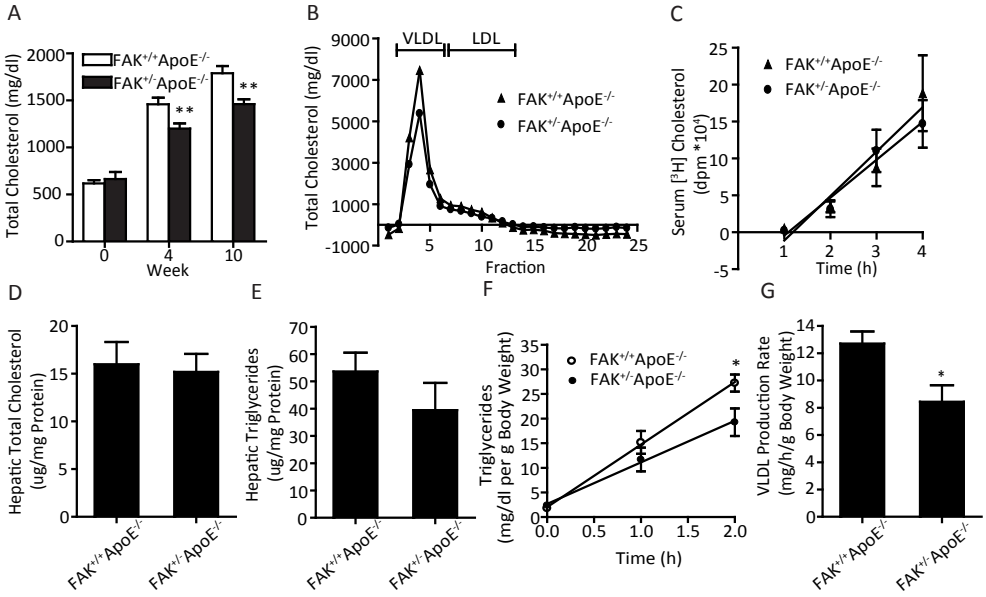


Figure 2. Lipid metabolism is altered in FAK$^{+/-}$ApoE$^{-/-}$ mice. Blood samples were taken by tail vein puncture before starting the Western type diet (WTD), after four and ten weeks of WTD feeding and at the time of sacrifice. Serum total cholesterol levels after high fat diet feeding are reduced (A), mainly due to reduced VLDL associated TC. Fractions 2-6 represent VLDL associated TC, fractions 7-13 represent LDL associated TC (B). This reduction was not caused by altered absorption of cholesterol from the intestine (C) or changes in hepatic lipid content (D,E) but is likely due to decreased VLDL production rate in FAK$^{+/-}$ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$ littermates (F,G).

Liver morphology of FAK$^{+/-}$ApoE$^{-/-}$ mice did not reveal pathological changes or features of inflammation. Moreover the reduction in TC levels was not caused by changes in hepatic lipid content or cholesterol absorption rate from the intestine (fig. 2C-E). However hepatic VLDL production rate appeared to be decreased in FAK$^{+/-}$ApoE$^{-/-}$ mice from 12.71 ± 0.89 mg/dl/h/g body weight in ApoE$^{-/-}$ mice to 8.44

± 1.21 mg/dl/h/g body weight (p=0.02, fig. 2F-G), suggesting that decreased total cholesterol levels are caused by reduced VLDL production. Remarkably, assessment of hepatic gene expression of several genes involved in lipid metabolism by realtime PCR revealed an upregulation of key genes in lipogenesis in $FAK^{+/-}ApoE^{-/-}$ mice. Figures 3A-E show that expression of stearoyl-coenzyme A desaturase 1 (SCD1, 94% p=0.04) and sterol regulatory element-binding proteins (SREBP) 1 and 2 is increased (56%, p=0.01 and 32%, p=0.02 respectively) while fatty acid synthase (FAS) shows a tendency towards increased gene expression as well (59%, p=0.06). In addition CD36 expression was increased by 25% (p=0.003). Expression of several other genes involved in lipoprotein metabolism such as acetyl CoA carboxylase α (ACACA), ATP citrate lyase (ACLY), microsomal triglyceride transfer protein (MTP), lipoprotein lipase (LPL) and its cofactor apolipoprotein C2 (ApoC2) or transcription regulators like peroxisome proliferator-activated receptor α (PPARα) and PPARγ coactivator (PGC) 1a remained unchanged.

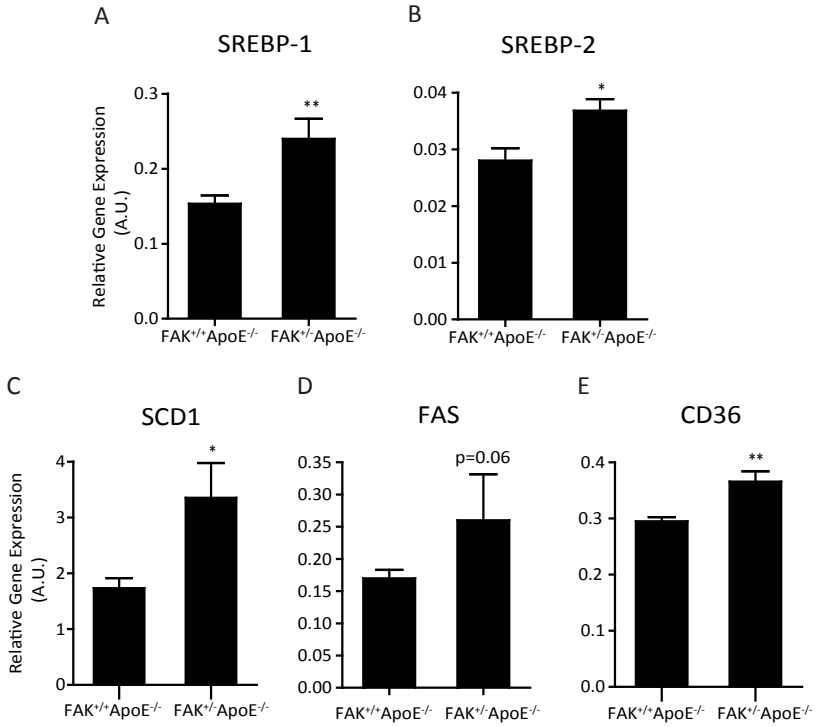


Figure 3. Hepatic expression of various key genes in lipogenesis is upregulated with reduced FAK levels. Relative gene expression of SREBP1 (A) and 2 (B), SCD1 (C), FAS (D) and CD36 (E). FAS, fatty acid synthase; SCD1, stearoyl-coenzyme A desaturase 1; SREBP, sterol regulatory element-binding protein. (*p<0.05, **p<0.01)

Spleen morphology and leukocyte composition

$FAK^{+/-}ApoE^{-/-}$ mice displayed moderate splenomegaly with a 31% increase in organ weight after Western type feeding for 10 weeks compared to $ApoE^{-/-}$ littermates (p=0.04, Fig. 4A). The spleen/body weight ratio was increased as well. The increase

in spleen weight appeared to be at least partly diet dependent, in that spleen weight of mice fed a regular chow diet was increased by only 12% (p=0.06, Fig. 4A). Further pathophysiological analysis revealed a decreased number of white pulp nodules in spleen while the germinal centers were enlarged, resulting in an increased germinal centre area. The germinal centre expansion was accompanied by an altered germinal center organization. As shown in Fig. 4B, germinal centers appear to be fused together exhibiting irregular arrangement.

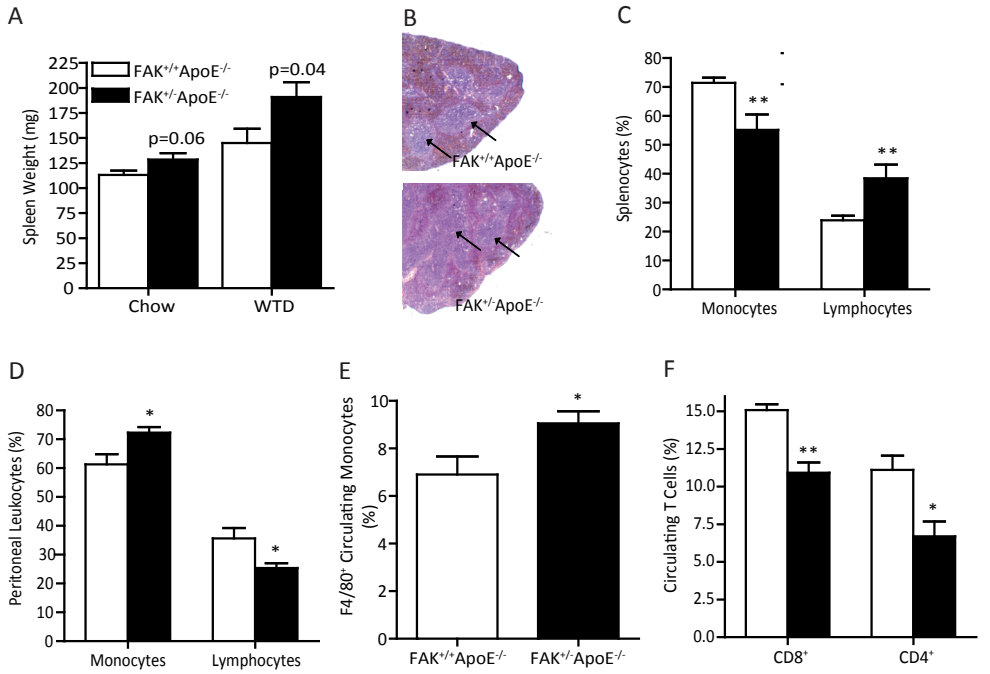


Figure 4. Reduced FAK levels lead to splenomegaly, altered spleen morphology and altered monocyte/macrophage and (T)-lymphocyte content in spleen, circulation and peritoneal cavity. Spleen size of approximately 5 month old FAK$^{+/-}$ApoE$^{-/-}$ mice (black bars) and ApoE$^{-/-}$ littermates (white bars) after normal chow or 10 weeks of Western type diet feeding (A). Cryosections of spleen were prepared and stained with hematoxylin and eosin. Spleens of FAK$^{+/-}$ApoE$^{-/-}$ mice show a decreased number of white pulp nodules in the spleen while the germinal centres (arrows) all seem to be enlarged (B). Peripheral blood mononuclear cells, splenocytes and peritoneal leukocytes were isolated from FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates fed normal chow or Western type diet for 4 weeks and analyzed by Sysmex blood cell analyzer (C,D) or flow cytometry (E,F). In spleen, reduced FAK expression resulted in decreased number of monocytes and elevated lymphocyte numbers (C) while the monocyte content in the peritoneal lavage was increased in FAK$^{+/-}$ApoE$^{-/-}$ mice accompanied by a decrease of peritoneal lymphocyte numbers (D). Also, reduced FAK expression led to an increase in circulating F4/80^{+} monocytes (E), and a decrease in circulating CD4^{+} as well as CD8^{+} T cells (F). (*p<0.05, **p<0.01)

To assess whether the increase in spleen weight was proportional between the different cell subsets, splenocytes were isolated and analyzed. In spleen, reduced FAK expression resulted in a 23% decreased relative number of monocytes (p=0.01, Fig. 4C) while the number of lymphocytes was elevated by 61% (p=0.01, Fig. 4C). In contrast, the leukocyte distribution profile in the peritoneal cavity showed the inverse pattern with increased monocyte numbers (+18%, p=0.02) and a concomitant

29% decrease in lymphocyte numbers (p=0.02) (fig. 4D) The changes in peritoneal leukocyte profile corresponded with those in circulation, where F4/80+ monocytes were up (+31%, p=0.04) and and CD4+ and CD8+ T cells were down by 40% (p=0.04) and 28% (p=0.005) respectively (fig. 4E-F). Reduced FAK expression did not affect macrophage phenotype and activation, T cell activation, circulating B cell and total white blood cell numbers or total peritoneal leukocyte numbers.

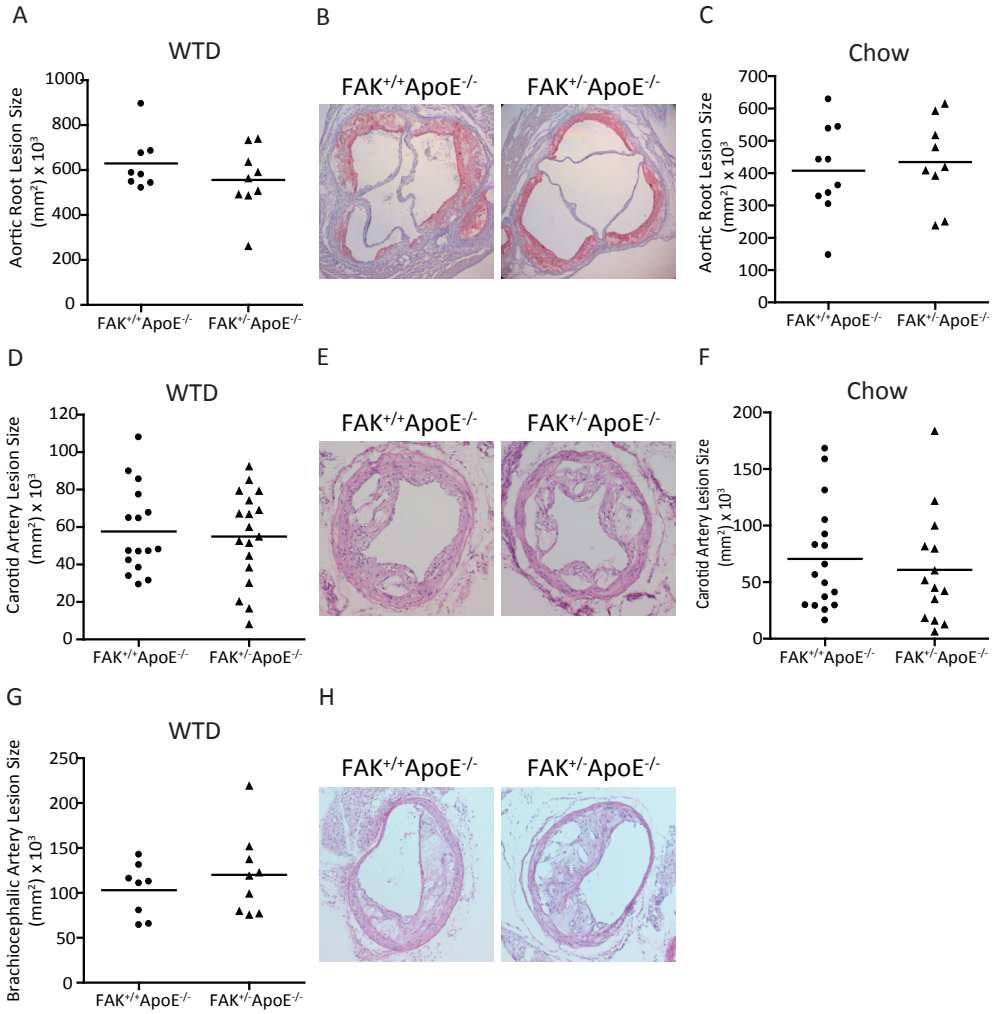


Figure 5. Reduced FAK expression does not affect atherogenesis. Cryosections were prepared of formaldehyde fixed aortic roots, carotid and brachiocephalic arteries and stained Oil Red O or HE. Aortic root (A,C), collar aided carotid artery (D,F) and brachiocephalic artery (G) atherosclerotic lesion size did not differ between FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates fed a Western type or chow diet. B, E and H show representative pictures of the lesions.

Atherosclerotic plaque size and composition

After ten weeks of high-cholesterol diet feeding and eight weeks after collar placement carotid and brachiocephalic arteries and aortic roots were isolated,

sectioned and stained with hematoxylin and eosin or Oil Red O. Remarkably, reduced FAK expression did not alter the atherosclerotic plaque size in collar aided carotid artery, brachiocephalic artery or aortic root (Fig. 5). Plaque composition as determined by immunohistochemical staining for monocytes/macrophages (MoMa-2) and vSMC's (alfa-SM-actin), for collagen (Sirius Red staining) and apoptotic cell content (TUNEL staining) at the three different assessed sites was essentially similar in FAK$^{+/-}$ApoE$^{-/-}$ versus ApoE$^{-/-}$ littermates. Moreover, lesion cellularity and necrotic core size were unaffected as well (fig. 6).

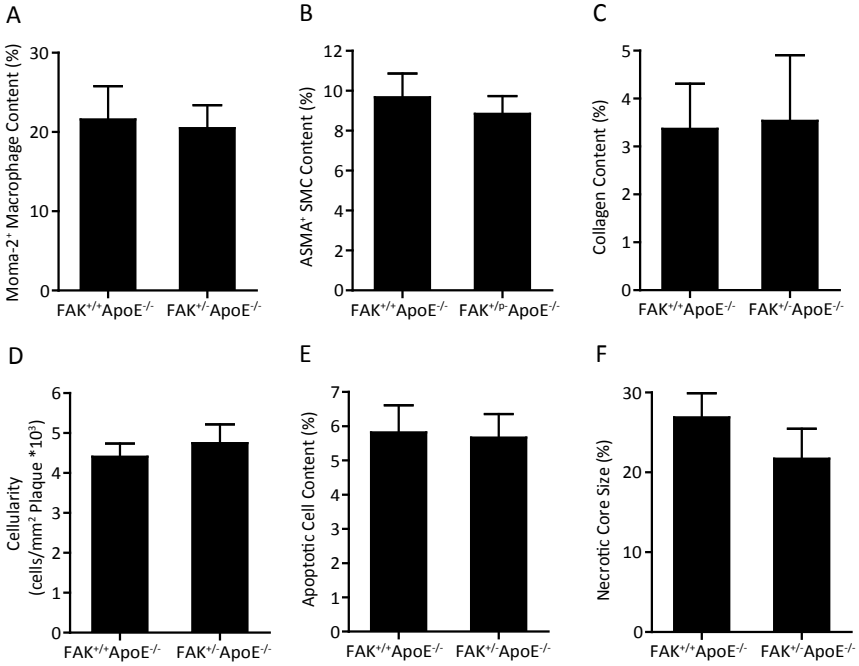


Figure 6. Atherosclerotic lesion composition is unchanged in FAK$^{+/-}$ApoE$^{-/-}$ mice. Moma-2+ macrophage (A), ASMA+ smooth muscle cell (B) and collagen (C) content, cellularity (D), apoptosis (E) and necrotic core size (F) did not differ between FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ controls. ASMA, α-smooth muscle-actin.

Because serum total cholesterol levels were reduced in FAK$^{+/-}$ApoE$^{-/-}$ mice after Western type feeding only, we have assessed whether this may have indirectly counteracted any pro-atherogenic responses of reduced FAK activity. However collar induced carotid artery formation as well as spontaneous brachiocephalic artery and aortic root plaque sizes of chow diet fed FAK$^{+/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ littermates were completely comparable (Fig. 5). Apparently differences in cholesterol load did not mask pro-atherogenic effects of FAK heterozygousity on leukocyte homeostasis.

Discussion

In this study we show that reduction of FAK expression in ApoE$^{-/-}$ mice, as achieved by cross breeding FAK$^{+/-}$ with ApoE$^{-/-}$ mice, leads to a reduction of total cholesterol (TC)

levels in response to Western type diet feeding. This hypocholesterolemic response was high fat diet feeding dependent, since it was not observed when mice were fed a regular chow diet and suggests an until now unknown involvement of FAK in cholesterol metabolism. Closer analysis of the cholesterol distribution over different lipoproteins, showed that mainly VLDL associated cholesterol was decreased. The absorption rate of cholesterol after oral administration of cholesterol did not differ between $FAK^{+/-}ApoE^{-/-}$ mice and $ApoE^{-/-}$ littermates. Hepatic total cholesterol, tryglyceride and phospholipid content, was comparable between the two groups as well. Thus the decreased TC levels are most likely caused by the observed lower hepatic VLDL production rate in $FAK^{+/-}ApoE^{-/-}$ mice. Of note, the hepatic expression of critical genes in lipogenesis, SREBP1 and 2, SCD1 and FAS, was increased in $FAK^{+/-}ApoE^{-/-}$ mice. SREBPs are strongly regulated by a feedback mechanism in cholesterol and fatty acid metabolism[15] and contribute to the regulation of various effector genes in lipogenesis, such as SCD1 and FAS[16,17]. Hepatic CD36 expression was slightly increased in response to FAK reduction as well. In a recent study oxidized LDL mediated CD36 signaling was shown to inhibit macrophage migration and stimulate cell spreading by continued FAK phosphorylation and activation[18], suggesting a role for FAK in trapping macrophages in the arterial wall and promoting atherogenesis. Thus the elevated hepatic expression of these genes may reflect a compensatory mechanism in response to lowered VLDL and TC levels in the case of lipogenesis genes and decreased FAK levels in the case of CD36.

In addition to cholesterol lowering, we found that reduced FAK expression in $ApoE^{-/-}$ mice leads to splenomegaly accompanied by altered spleen morphology. The increase in total spleen weight and in particular germinal centre area suggest an aberrant splenocyte composition, with a relative increase in germinal centre B and T lymphocytes content and a decrease in monocytes/macrophages numbers. This notion was confirmed by further analysis of splenocyte composition. Although initially FAK was reported to be absent in monocytes/macrophages[19,20], recent publications have conclusively proven this to be incorrect[21,22]. GM-CSF and M-CSF, key regulators of myeloid differentiation, were found to induce FAK phosphorylation in monocytes/macrophages while inhibition of FAK activity by FRNK led to impaired macrophage spreading and adhesion[21,22]. Deletion of FAK in monocytes/macrophages was seen to attenuate their migratory capacity, random motility as well as M-CSF, SDF-1α and MCP-1 directed migration[23]. The observed decrease in macrophage content in spleen in $FAK^{+/-}ApoE^{-/-}$ mice may likely be attributable to impaired ingress from circulation, even despite enhanced circulating monocyte numbers, whereas the increased peritoneal macrophage levels may be due to impaired migration from the peritoneal cavity. However, no reduction in plaque macrophages was observed.

In contrast to the change in splenocyte composition reduced FAK expression led to decreased circulating $CD4^+$ and $CD8^+$ T lymphocyte numbers, however activation of T cells or T cell subset numbers were unchanged. FAK is expressed in both human T and B lymphocytes[24]. In the present study we observed an increased spleen lymphocyte content in FAK heterozygous mice along with a seemingly paradoxical decrease in

relative circulating and peritoneal lymphocyte numbers. In vitro, in T-lymphocytes FAK has been shown to have an essential role in β_1 integrin dependent migration[25] and lymphocyte function-associated antigen-1 (LFA-1 or $\alpha_L\beta_2$ integrin) mediated T lymphocyte adhesion. FAK dephosphorylation and thus inhibited activity was shown to impair LFA-1 mediated adhesion[26,27]. Furthermore, FAK was indicated in chemokine mediated lymphocyte trafficking as FAK phosphorylation and activation in T cells is induced by RANTES[28]. Similarly SDF-1 induced FAK phosphorylation in T cells, hematopoietic progenitor cells and pro-B cells appeared to be essential for its chemotactic and adhesive responses[6,29,30]. In agreement with these findings, our study seems to point to enhanced retention of lymphocytes in spleen in the case of compromised FAK function.

Despite the previously reported involvement of FAK in migration and proliferation of vSMC's, macrophages and endothelial cells in the vasculature and the impaired hypercholesterolemic response to Western type diet and altered inflammatory cell distribution pattern in circulation, peritoneal cavity and spleen of FAK$^{+/-}$ApoE$^{-/-}$ mice observed in this study, to our surprise we did not observe any effects of impaired FAK function on size or composition of collar aided carotid, brachiocephalic artery or aortic root atherosclerotic plaques. The lack of effect of FAK heterozygousity on atherogenesis was not due to the observed reduced hypercholesterolemic response to high fat diet feeding, which potentially could have masked FAK mediated adverse effects on leukocyte trafficking. This suggests that the marked immune effects of compromised FAK function per se do not influence atherogenesis. Apparently the complex interaction of immune cells in the arterial wall leads to a new equilibrium in the FAK$^{+/-}$ApoE$^{-/-}$ mice culminating into an unchanged development of atherosclerotic lesions. It might be concluded that therapeutical approaches to diminish atherosclerotic lesions must be focused upon targets which play a more unidirectional role in lesion formation.

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Chapter 7

Summary and Discussion

Despite available treatments, be it lipid lowering or blood pressure lowering therapy, surgical intervention or life style changes, cardiovascular diseases (CVD) continue to be one of the main causes of death in the world[1]. Most clinical complications of CVD can be attributed to atherosclerotic plaque disruption and subsequent thrombus formation[2,3]. Atherosclerosis is a progressive disease of medium and large sized arteries characterized by accumulation of lipids in the artery wall[4]. It is a multifactorial process featuring in addition to subendothelial lipid deposition, excessive tissue remodeling, inflammation, and oxidative stress[5].

An important process generally considered to contribute to plaque progression and instability is apoptotic cell death. Apoptosis, programmed cell death characterized by morphological changes like cell shrinkage, DNA fragmentation, condensation of chromatin and membrane blebbing[6], occurs in atherosclerotic lesions and affects all major cell types relevant to the disease process such as endothelial cells, macrophages, T cells and vSMC[7]. Apoptosis increases with plaque progression, being virtually absent in initial lesions and overtly present in advanced lesions[8], as a result of enhanced apoptosis rates or impaired phagocytosis. Although originally considered anti-inflammatory or inflammation-neutral, recent insights support the notion that apoptosis may under conditions present in atheromathous tissue, promote inflammation[9].The consequences of apoptosis also depend on the cell type. Apoptosis of vSMC was seen to promote plaque vulnerability[10] whereas the impact of macrophage apoptosis is less well defined. At early stages macrophage apoptosis has been reported to be beneficial[11,12], whereas in advanced plaques phagocytosis is impaired[13] and apoptotic macrophages may undergo secondary necrosis, potentially leading to lipid core expansion, plaque inflammation and thus progression. On the contrary, others did not observe any effects of macrophage apoptosis on plaque size or inflammation in advanced stages of atherosclerosis[14].

In chapter 2 we review the inflammatory consequences of altered apoptosis and effects of plaque inflammation on cell death. Several pro-inflammatory mediators that contribute to atherogenesis can have pro- or anti-apoptotic effects. For example, proapoptotic members of the TNF super family, Fas, CD40 and Fn14, were also seen to promote secretion of inflammatory cytokines like MCP-1 and IL8 and/or adhesion molecules[15-17]. Apoptotic cells modulate inflammatory responses either on their own account by inducing amongst others cytokines[18] or after uptake and processing by phagocytes[19]. Furthermore, impaired and altered phagocytosis, as apparent during progressive atherosclerosis[20], elicits a proinflammatory response as a result of secondary necrosis thereby further aggravating inflammation leading to lesion progression and instability[9]. Reactive oxygen species and Ox-LDL, accumulating in the vessel wall during plaque progression, are able to induce cell death in endothelial cells, vSMC and macrophages[21-23] by upregulating several apoptotic proteins including Fas, TNFα, p53 and Bcl-2 family proteins[24-26]. In addition, several cytokines present in atherosclerotic lesions, including IFNγ[27,28], TNFα[29,30], IL1β[31], IL2[32], IL4[33] and MIF[34,35], are able to induce apoptosis themselves.

Since most clinical complications of cardiovascular disease are caused by plaque rupture in this thesis we first aimed to identify genes or pathways promoting thin cap fibroatheroma formation. Thin cap fibroatheroma (ThCFA) can be defined as plaques containing a large lipid core with an overlying thin fibrous cap, as opposed to thick cap fibroatheroma (TkCFA) which are more fibrous in nature and have as indicated by their name a thick cap. ThCFA and TCFA are categorized as type IV and V lesions, respectively, according to the classification system of the American Heart Association (AHA). ThCFA are considered as the most vulnerable to rupture[36]. In chapter 3 of this thesis we describe a microarray analysis on RNA obtained from mouse TkCFA and ThCFA early on in the plaque destabilization process in two different models of thin cap fibroatheroma formation. As a first model we chose spontaneous vulnerable plaque development in the brachiocephalic artery of ApoE$^{-/-}$ mice, after 9 weeks of Western type diet feeding, when plaques were reported to display the first features of enhanced vulnerability[37]. As a second model we considered low shear stress induced plaque formation in the carotid artery of ApoE$^{-/-}$ mice that were further destabilized by cap overexpression of the tumor suppressor protein p53[38]. We were able to identify several functional clusters consisting of differentially regulated genes that were overrepresented in both models of thin cap fibroatheroma. These functional gene clusters include lipid metabolism, small molecule biochemistry, metabolic disease, cellular growth and proliferation and cell-to-cell signaling and interaction. Genes regulating cell death were most significantly dysregulated in thin cap fibroatheroma versus thick cap fibroatheroma. Within this functional cluster, five upregulated genes were shared between both thin cap fibroatheroma models (Cd5l, Plagl1, Bim and two genes of unknown function). This indicates that CD5l, Plagl1 and Bim, the differential expression of which was confirmed by realtime PCR, may play a role in the transition of thick cap fibroatheroma into a more vulnerable

plaque phenotype.
In addition to the identification of cell death regulating genes possibly involved in thin cap fibroatheroma development, one particular gene, Npy, a neurotransmitter with both pro- and anti-atherogenic properties[39-41], was evident. Apart from an upregulation in thin cap fibroatheroma in the brachiocephalic artery compared to thick cap fibroatheroma in the carotid artery, Npy expression was shown to increase with disease progression not only in murine but also in human plaques.

Having identified cell death associated genes and gene clusters which were differentially regulated in thin cap fibroatheroma versus thick cap fibroatheroma, we set out to assess the role of several apoptosis regulating proteins in atherosclerotic plaque development and stability in different mouse models. One of the first genes of interest was Bim, which as microarray analysis indicated is one the cell death regulating factors differentially expressed in thin cap fibroatheroma compared to thick cap fibroatheroma. In chapter 4 we have investigated effects of leukocyte Bim deficiency on plaque development in LDLr$^{-/-}$ mice. Previous studies in Bim deficient mice have already revealed that Bim is a key regulator of leukocyte apoptosis[42,43], and particularly important in the deletion of autoreactive and activated T and B cells[42,44-46]. T and B cell levels, including autoreactive T and B cell numbers, are markedly increased in circulation, spleen and thymus of Bim deficient mice[42] facilitating the development of auto-immunity and lymphadenopathy[42,44,45]. As lymphocytes are important regulators of immune responses in atherogenesis[47,48] and atherosclerosis is considered to have features of an autoimmune disease[49,50] Bim may be of particular relevance in atherosclerosis.
Deletion of leukocyte Bim in Western type diet fed LDLr$^{-/-}$ mice resulted in an increased pro-inflammatory status as demonstrated by marked splenomegaly and enhanced T cell activation and proliferation resulting in increased circulating and mediastinal lymph node T cell levels. Leukocyte Bim deficiency led to increased lesion T cells as well. Leukocyte Bim deficient LDLr$^{-/-}$ mice had increased Ox-LDL antibody levels of the IgG1 but not IgG2a isotype in serum and in addition we found dramatically increased total immunoglobulin levels in atherosclerotic lesions of Bim$^{-/-}$ BM transplanted mice. In atherosclerotic lesions of ApoE$^{-/-}$ mice, human and rabbit as well as in serum Ox-LDL specific antibodies have been detected[24-26]. Studies in which mice were immunized with modified LDL resulted in decreased lesion formation[54-57], suggesting that the Bim deficiency related increase in Ox-LDL specific auto-antibodies are atheroprotective. Although Bim$^{-/-}$ bone marrow derived macrophages were less sensitive to cell death after culturing and growth factor withdrawal as well as after stimulation with oxidized LDL, lesion apoptotic cell content was not altered in leukocyte Bim deficient LDLr$^{-/-}$ mice. Since apoptotic cells in spleen were clearly elevated, the observed lack of effect on apoptosis in lesions may be a result of increased resistance of plaque macrophages against Bim deficiency associated cell death or of the low T cell content in atherosclerotic lesions. Despite these profound effects on lymphocyte homeostasis, inflammatory

status and unexpectedly, the reduction in lipid serum levels, atherosclerotic lesion burden and stability in both aortic root and descending aorta was unchanged in Bim$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice. We hypothesize that the observed pro- and anti-atherogenic effects of Bim deletion on inflammatory response, together with decreased lipid levels counterbalance, resulting in unaltered atherogenesis. Possibly cell-type specific modulation of Bim expression will clarify the contribution of Bim functioning in specific cells to atherogenesis.

Bim activity is in part regulated by the Bcl-2 family member myeloid cell leukemia 1 (Mcl-1) which directly interacts with Bim, Bid, another pro-apoptotic BH3-only protein, and multidomain pro-apoptotic Bak[39-41], to inhibit apoptosis. Mcl-1 plays an important role in promoting survival and differentiation of leukocyte subsets[58,59] being essential for neutrophil survival[60]. In chapter 5 we investigated the consequences of modulating macrophage apoptosis on atherosclerotic plaque development and stability by deleting Mcl-1 in lysozyme M (lysM) expressing, myeloid cells. Lethally irradiated LDLr$^{-/-}$ mice were transplanted with Mcl-1$^{fl/fl}$ LysMcre or wildtype bone marrow, followed by Western-type diet feeding to induce atherosclerotic lesion development.
We show that Mcl-1 participates in Ox-LDL induced cell death, and regulates gene expression of other apoptosis regulating Bcl-2 family members Bim, PUMA and NOXA after Ox-LDL exposure. In atherosclerotic aortic root lesions of Mcl-1$^{-/-}$ bone marrow transplanted mice apoptotic cell content was elevated by 77% compared to WT controls. However, atherosclerotic plaque size did not differ between Mcl-1$^{-/-}$ and WT bone marrow recipients as a result of additional effects of myeloid Mcl-1 deficiency. Mcl-1$^{-/-}$ macrophages exhibited increased lipid uptake and increased peritoneal foam cell counts, marked neutropenia and altered neutrophil phenotype in Mcl-1$^{-/-}$ bone marrow transplanted mice. Mcl-1$^{fl/fl}$ LysMcre transplanted mice not only carry an Mcl-1 deletion in macrophages but also in neutrophils[61]. In keeping with the notion that Mcl-1 is essential for neutrophil survival, neutrophil levels in circulation and lesions were sharply decreased in Mcl-1$^{-/-}$ bone marrow transplanted mice. The remaining neutrophils, that escaped Cre mediated deletion, displayed a decreased response to chemoattractant KC, partly due to reduced responsiveness and partly a result of the substantially reduced neutrophil pool Furthermore, CXCR4 expressing neutrophil levels in Mcl-1$^{-/-}$ BM transplanted mice were increased compared to WT BM recipients, indicative of increased release of neutrophils from stroma.
Finally, myeloid Mcl-1 deficiency resulted in alteration of the inflammatory properties of LDLr$^{-/-}$ mice. Isolated peritoneal macrophages from Mcl-1$^{-/-}$ BM recipients contained a high amount of multinucleated giant cells as compared to WT macrophages, suggestive of an altered apoptotic response and macrophage polarization. Another indication for an elevated inflammatory status of Mcl-1$^{-/-}$ BM transplanted mice was found in the apparent shift towards a pro-inflammatory M1 macrophage phenotype as judged from their cytokine expression patterns and reduced capacity for apoptotic

cell clearance. Thus analogous to leukocyte Bim deletion, myeloid Mcl-1 deletion in LDLr$^{-/-}$ mice results in several cell death and inflammation related effects but, surprisingly, without affecting atherosclerotic plaque size or stability, emphasizing the importance of pro-atherosclerotic neutrophil recruitment into plaques.

In chapter 6 we studied the atherogenic role of focal adhesion kinase (FAK), a kinase not only involved in cell death and proliferation, but also in adhesion and migration of various cell types[62]. Plaque initiation starts with adhesion of leukocytes to the luminal endothelial cell layer followed by migration into the subendothelial space[63]. After initial tethering and rolling of circulating leukocytes, mediated by selectins, adhesion involves the engagement of β-integrins which interact with ICAM-1 and VCAM-1[64,65]. FAK is activated by (auto)phosphorylation upon integrin binding, resulting in increased turnover of focal adhesions thereby stimulating migration[66]. We generated ApoE$^{-/-}$ mice with heterozygous FAK expression, since FAK deletion is embryonically lethal[67]. Atherosclerosis was induced by Western type diet feeding.
Reduced FAK levels resulted in an increased inflammatory status in ApoE$^{-/-}$ mice as witnessed by splenomegaly and enlarged splenic germinal centers. Furthermore, leukocyte composition in spleen, peritoneal cavity and circulation was affected by FAK reduction. Macrophage content in spleen was found to be decreased, whereas that of lymphocytes was increased. In contrast, peritoneal cavity and circulation showed increased monocyte numbers accompanied by reduced CD4^{+} and CD8^{+} T cell content. These differential leukocyte composition was likely caused by impaired monocyte ingress from circulation to spleen even despite enhanced blood monocyte counts and enhanced retention of lymphocytes in spleen. FAK$^{+/-}$ApoE$^{-/-}$ mice had significantly lower plasma total cholesterol levels, which was shown to be specifically evident upon a Western-type diet and attributable to a decreased hepatic VLDL production rather than to an altered intestinal cholesterol absorption. Hepatic lipid content was unchanged in FAK$^{+/-}$ApoE$^{-/-}$ mice. However, hepatic expression of critical genes in lipogenesis, SREBP1 and 2, SCD1 and FAS, was increased in FAK$^{+/-}$ApoE$^{-/-}$ mice in addition to a slight increase in CD36 expression. OxLDL mediated CD36 signaling was shown to inhibit macrophage migration and stimulate cell spreading by continued FAK phosphorylation and activation[68], suggesting a role for FAK in trapping macrophages in the arterial wall and promoting atherogenesis.
Surprisingly, despite these marked effects on immune status, impaired FAK expression neither affected size and composition of semi-constrictive perivascular collar induced carotid artery plaques nor that of spontaneous brachiocephalic artery and aortic root plaques. We therefore assessed atherosclerotic lesion size in chow fed ApoE$^{-/-}$ mice with reduced FAK expression in order to eliminate the impact of reduced lipid levels on atherosclerosis development. However, also here atherosclerotic lesion size of FAK$^{+/+}$ and FAK$^{+/-}$ mice was essentially comparable. Thus, FAK reduction beneficially affects lipid metabolism in Western type diet fed ApoE$^{-/-}$ mice and alters their inflammatory status without resulting in an altered atherogenic response.

In chapter 6 we show that systemic reduction of FAK activity alters various inflammatory parameters. In addition we are the first to show an unexpected effect of FAK on hepatic lipid metabolism in mice. The results obtained from the studies described in chapter 4 and 5 provide new insights on the role of apoptosis regulating Bcl-2 family members in atherosclerosis. We show that both pro-apoptotic Bim and anti-apoptotic Mcl-1 have several functions during atherogenesis including intraplaque cell death, foam cell formation and lipid metabolism. Moreover, modulation of Bim and Mcl-1 expression resulted in major effects on inflammatory processes. Apoptosis, and in particular that mediated by Bcl-2 family members, is essential in homeostasis and functioning of the immune system[69]. Deletion of autoreactive T and B cells, termination of the immune response and survival of activated T and B cells are all regulated by Bcl-2 family member and death receptor dependent apoptosis. Dysregulated apoptosis of immune cells may result in various pathological conditions including immunodeficiency, autoimmunity, tumor growth and infection[69]. The Bim and Mcl-1 associated modulation of inflammatory parameters observed in our studies correspond with the previously reported role of these proteins in immune system homeostasis. We further show that both Bim and Mcl-1 regulate specific cell death and inflammatory processes relevant to atherosclerosis. However, the key role in systemic immunity and the multifaceted mode of action of these proteins involving apoptotic, phagocytic and inflammatory processes and lipid metabolism should be taken into account when considering therapeutic approaches targeting these apoptosis regulating proteins. In the studies described in chapter 4 and 5 we specifically deleted Bcl-2 family proteins in total hematopoietic (Bim) or myeloid cells (Mcl-1), whereas in the study described in chapter 6 FAK expression was reduced at a systemic level. Local modulation of gene expression, for example by targeted gene or drug delivery, or in the case of FAK, cell type specific modulation, might be a valuable approach to restrict therapeutic effects to the atherosclerotic lesions without affecting the systemic immune response. In particular, local intervention in Mcl-1 signaling, to prevent pathological consequences of altered systemic immunity, to modify macrophage lipid accumulation and plaque apoptosis could potentially be a valuable approach to modulate atherogenesis. Further studies are necessary to confirm this hypothesis.

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Nederlandse Samenvatting

Hart- en vaatziekten, zoals hartinfarct of beroerte, hebben meestal atherosclerose, ook wel aderverkalking genoemd, als oorzaak. Atherosclerose is een vaak progressieve verdikking van de vaatwand in grote en middelgrote slagaders, waarvan het ontstaan een proces is dat tientallen jaren in beslag neemt en dat vaak al begint in de tienerjaren. De belangrijkste risicofactoren voor de ontwikkeling van atherosclerose zijn een vet-rijk dieet, roken, gebrek aan lichaamsbeweging en hoge bloeddruk, geslacht en leeftijd. Daarnaast is er een erfelijke component die de ontwikkeling van atherosclerose kan beïnvloeden.

Bij het onstaan van atherosclerose spelen zowel de ophoping van vetten in de wand van het bloedvat als het immuunsysteem een belangrijke rol. Een gezonde vaatwand bestaat uit endotheelcellen die een gladde spiercellaag bedekken. In het eerste stadium van plaque ontwikkeling raakt het endotheel lokaal geirriteerd door de chronische blootstelling aan cholesterol in het bloed en hoge bloeddruk, en vindt lekkage door de vaatwand en ophoping van lipidemateriaal zoals cholesterol in de vaatwand plaats als gevolg waarvan endotheelcellen geactiveerd kunnen raken. Deze brengen vervolgens verschillende eiwitten tot expressie die de rekrutering van ontstekingscellen zoals monocyten en T cellen naar de vaatwand kunnen bevorderen. In de vaatwand differentieren de monocyten tot macrofagen die de opgehoopte lipiden kunnen opnemen leidend tot de vorming van zogenaamde schuimcellen. Wanneer de plaque zich verder ontwikkelt migreren gladde spiercellen vanuit de vaatwand naar de plaque en vormen een beschermend kapsel. In verder gevorderde stadia van atherosclerose heeft de plaque zich ontwikkeld tot een forse verdikking met een kern van vetmateriaal, schuimcellen en dood celmateriaal. Naarmate het ziekteproces vordert kunnen deze groter wordende plaques het bloedvat vernauwen of zelfs afsluiten waardoor de bloedtoevoer naar stroomafwaarts gelegen weefsel beperkt wordt. Daarnaast

kan een atherosclerotische plaque scheuren waarbij plaquemateriaal vrijkomt in de bloedbaan waardoor een omvangrijke ontstekingsreactie en vorming van een bloedstolsel kan ontstaan. Dit laatste proces kan gepaard gaan met de klinische symptomen als een hart- of herseninfarct.
Behandeling van hart- en vaatziekten is gericht op het verminderen van risicofactoren, zoals een verhoogd cholesterolgehalte in het bloed (statines), en chirurgische ingrepen zoals dotteren. In een groot aantal patienten hebben deze interventies helaas onvoldoende effect en dit legitimeert verder onderzoek naar de ontwikkeling van nieuwe, effectievere behandelmethoden. De in dit proefschrift beschreven studies zijn uitgevoerd met als uiteindelijke doel om nieuwe aangrijpingspunten voor een effectievere therapie te identificeren.

De studies beschreven in dit proefschrift hebben voornamelijk betrekking op apoptose oftewel geprogrammeerde celdood. Wanneer een cel stress ondervindt als gevolg van bijvoorbeeld DNA schade, infectie door een pathogeen, of binding van bepaalde eiwitten aan de cel en reparatie van de schade niet meer haalbaar lijkt zal er een programma in werking treden met als uiteindelijke uitkomst celdood. Dit proces wordt apoptose genoemd. Apoptose leidt tot afbraak van de cel en opname van het restmateriaal door naburige cellen zonder dat de inhoud van de cel vrijkomt. In dit laatste aspect onderscheidt apoptose zich van een andere vorm van celdood, necrose genoemd, dat een veel willekeuriger verloop heeft en waarbij de celinhoud wel vrijkomt, wat een reactie van het immuunsysteem in gang kan zetten.
Aangetoond is dat alle cellen in de atherosclerotische plaque apoptose kunnen ondergaan en dat apoptose toeneemt naarmate een plaque verder ontwikkelt. De gevolgen van apoptose voor de ontwikkeling van een plaque hangen af van het celtype en het stadium van de plaque. Algemeen wordt aangenomen dat apoptose van gladde spiercellen in het kapsel dat de plaque bedekt schadelijk is omdat deze daardoor zwakker wordt en de plaque kan gaan scheuren. Apoptose van macrofagen in vroege stadia van atherosclerose remt de plaqueontwikkeling doordat macrofagen en schuimcellen vroegtijdig worden afgebroken. In late stadia wordt macrofaag apoptose echter vaak als pro-atherosclerotisch gezien. Opname van apoptotische macrofagen door naburig cellen via een proces dat fagocytose wordt genoemd, is dan onvoldoende waardoor de cel necrose ondergaat en de celinhoud alsnog vrijkomt. De daarop volgende immuunreactie kan bijdragen aan plaque-ontsteking.

Omdat hart- en vaatziekten vaak het gevolg zijn van scheuren van een atherosclerotische plaque hebben wij in hoofdstuk 3 genen geidentificeerd, die mogelijk betrokken zijn bij de overgang van stabiele plaques naar meer kwetsbare plaques. Hierbij hebben we gebruik gemaakt van de zogenaamde micro-array techniek, waarbij de mate van aanwezigheid (de expressie) van een grote hoeveelheid genen in één meting kan worden bepaald, en van twee muismodellen voor stabiele en meer kwetsbare plaques. In onze studie is de expressie van 22.000 genen

gemeten. Apolipoproteine E (ApoE) deficiënte muizen, die spontaan atherosclerose ontwikkelen, werden na een aantal weken van vetrijk dieet langs operatieve weg voorzien van een licht constrictief bandje rond de halsslagaders. Na de vorming van de atheroslerotische plaques in de halsslagader is p53 tot overexpressie gebracht in het kapsel van de plaque. Dit eiwit induceert apoptose van de gladde spiercellen in het kapsel, als gevolg waarvan deze laag zwakker wordt en de kwetsbaarheid van de plaque toeneemt. Als controle werd het enzyme beta-galactosidase gebruikt, dat geen invloed op de plaque heeft maar goed detecteerbaar is. Het tweede model voor kwetsbare plaques is de *arteria bracchiocephalica*. Atherosclerotische plaques die zich in deze slagader vormen in ApoE deficiënte muizen na een 9 weken durend vetrijk dieet hebben van nature een extra dun kapsel en zijn daarmee extra gevoelig voor ruptuur. Als controle hiervoor werden stabiele plaques die zich iets verder stroomafwaarts in de halsslagader vormen gebruikt.
Wanneer per model de genexpressie in voor ruptuur gevoelige of kwetsbare plaques, (ook wel ThCFA voor thin cap fibro-atheroma genoemd) en meer stabiele plaques (TkCFA voor thick cap fibro-atheroma genoemd) wordt vergeleken kan worden bepaald welke genen anders gereguleerd zijn in de meer kwetsbare plaques. Deze analyse liet zien dat 57 en 58 genen een verhoogde expressie en 87 en 66 genen een verlaagde expressie hadden in de twee respectievelijke modellen voor kwetsbare plaque vorming ten opzichte van hun stabiele tegenhangers. Van deze genen hebben 5 een verhoogde expressie in beide modellen en 10 een verlaagde expressie in beide modellen. Van de vijf genen met verhoogde expressie in kwetsbare plaques bleken er drie een functie te hebben in de regulering van apoptose, nl. *Cd5l, Plagl1* en *Bim.*
Vervolgens hebben we gekeken of er nog andere processen waren waarin meerdere betrokken genen verhoogd of verlaagd waren in de meer kwetsbare plaque. Hieruit bleek dat verschillende processen zoals vetstofwisseling, celgroei en -proliferatie en cel-cel communicatie significant beïnvloed waren in de meer kwetsbare plaque. Het proces dat in de kwetsbare plaque het meest verschilde van de meer stabiele plaque bleek celdood te zijn.
Verder hebben we in hoofdstuk 3 een ander gen met toegenomen expressie aangetoond, nl. het neuropeptide Y (Npy), dat ook in weefsel verkregen uit humane plaques een verhoogde productie bleek te hebben in kwetsbare vergeleken met stabiele plaques. Npy is een eiwit dat voornamelijk een functie heeft als neurotransmitter in het zenuwstelsel. Daarnaast is in diermodellen aangetoond dat Npy zowel een schadelijke als een beschermende werking kan hebben in het ontstaan van atherosclerose.
Met deze studie hebben we aangetoond dat in de diverse modellen voor kwetsbare plaquevorming een aantal genen waarvan de productie verhoogd of verlaagd is in vergelijking met de meer stabiele plaque en die mogelijk bijdragen tot de vorming van kwetsbare plaques gemeenschappelijk zijn. Daarnaast hebben wij Npy geidentificeerd als mogelijke indicator voor een toegenomen kwetsbaarheid van atherosclerotische plaques.

Na de identificatie van processen en genen die karakteristiek zijn voor de ruptuurgevoelige plaque hebben we in de daaropvolgende hoofdstukken de functionele rol van enkele bij celdood betrokken eiwitten in de ontwikkeling van atherosclerose onderzocht met behulp van dierexperimenteel onderzoek in muismodellen.

In hoofdstuk 4 is het effect van Bim onderzocht, een pro-apoptotisch eiwit en lid van de Bcl-2 familie van apoptose regulerende eiwitten, waarvan in hoofdstuk 3 al was aangetoond dat het verhoogd aanwezig is in de kwetsbare plaque.
Eerder is aangetoond dat Bim cruciaal is voor een goede werking van het immuunsysteem doordat het mede verantwoordelijk is voor de eliminatie van ongewenste T en B cel klonen. In hoofdstuk 3 werden muizen met een defecte LDL (lage dichtheids lipoproteine) receptor ($LDLr^{-/-}$), en die als gevolg daarvan spontaan atherosclerose ontwikkelen, bestraald om uit beenmerg afkomstige cellen te depleteren. Hierna werd het beenmerg gerepopuleerd door injectie van beenmerg uit Bim deficiënte ($Bim^{-/-}$) of controle donormuizen. Na een herstelperiode van acht weken werd de muizen een vetrijk dieet gegeven om de ontwikkeling van atherosclerotische plaques te initieren. De $LDLr^{-/-}$ muizen waarin Bim ontbrak in de witte bloedcellen bleken een vergrote milt en niet alleen verhoogde aantallen maar ook meer geactiveerde T cellen in bloed en lymfeknopen te hebben, hetgeen wijst op een verhoogde ontstekingsgraad van deze muizen. Daarnaast bleken ook de atherosclerotische plaques van muizen waarin Bim ontbrak in witte bloedcellen verhoogde aantallen T cellen te bevatten. Bim zorgt, naast het induceren van apoptose in T cellen, ook voor eliminatie van B cellen. Deze B cellen kunnen antilichamen of antistoffen produceren die lichaamsvreemde stoffen kunnen herkennen om vervolgens andere cellen van het immuunsysteem te stimuleren deze stoffen of cellen af te breken. Indien B cellen antilichamen maken tegen lichaamseigen stoffen wordt gesproken van auto-immuniteit. Recent onderzoek heeft een verhoogde spiegel van antilichamen tegen lichaamseigen stoffen laten zien in het bloed van muizen en patienten met hart- en vaatziekten, hetgeen suggereert dat atherosclerose deels een auto-immuunziekte is. In onze Bim studie zagen we dat het aantal antilichamen in het bloed dat geoxideerd LDL herkent aanmerkelijk verhoogd was in muizen waarin Bim ontbrak in witte bloedcellen. Van deze antilichamen is aangetoond dat ze een beschermende werking hebben in atherosclerose. Verder analyseerden we dat het totaal aantal antilichamen in de atherosclerotische plaques in deze muizen sterk was toegenomen. Zoals verwacht waren Bim deficiënte macrofagen minder gevoelig voor apoptose dan controle macrofagen. Als eerste hebben we nu ook laten zien dat stimulatie van macrofagen met geoxideerd LDL, dat in hoge concentraties celdood kan induceren, resulteert in minder apoptose in deze macrofagen.
Ondanks de bovenbeschreven effecten bleken de atherosclerotische plaques in Bim deficiënte muizen vergelijkbaar met die in controle muizen wat betreft grootte en stabiliteit. Mogelijk is dit het gevolg van het grote aantal verschillende effecten veroorzaakt door Bim deficiëntie die elkaar opheffen.

Mcl-1 behoort, net als Bim, tot de Bcl-2 familie, maar heeft in tegenstelling tot Bim een celdood remmende werking. Mcl-1 gaat een interactie aan met pro-apoptotische eiwitten, waaronder Bim, waardoor hun apoptose bevorderende werking geremd wordt. In hoofdstuk 5 hebben we een vergelijkbare strategie gevolgd als voor Bim, om het effect van Mcl-1 deficiëntie op de ontwikkeling van plaques te bestuderen. Omdat Mcl-1 vooral een belangrijke rol speelt in de regulatie van apoptose in macrofagen en neutrofielen, beide immuuncellen die celresten, bacterien en dergelijke kunnen opnemen, hebben we in deze studie gebruik gemaakt van muizen waarin Mcl-1 specifiek ontbreekt in deze celtypen.
Mcl-1 deficiënte macrofagen waren gevoeliger voor de celdood die optreedt na incubatie met geoxideerd LDL. Daarnaast bleek de opname van vetmateriaal door en de daaropvolgende vorming van schuimcellen uit macrofagen na incubatie met geoxideerd LDL of VLDL, sterk verhoogd. In overeenstemming hiermee bleek ook het aantal macrofaag afgeleide schuimcellen toegenomen in Mcl-1 deficiënte vergeleken met controle muizen. Daarnaast bleek ook dat Mcl-1 deficiënte macrofagen meer ontstekingsbevorderend zijn dan controle macrofagen. Ten slotte bleek zowel in vroege als in meer geavanceerde atherosclerotische plaques het aantal apoptotische cellen verhoogd in Mcl-1 deficiënte muizen.
Ondanks deze atherosclerose bevorderende effecten van macrofaag Mcl-1 deficiëntie was de plaquegrootte in Mcl-1 deficiënte en controle muizen in beide stadia vergelijkbaar. Dit is mogelijk te wijten aan de sterke afname in het aantal neutrofielen, zowel in bloed als in de plaque, en het door ons waargenomen veranderde migratiepatroon van dit celtype. Van neutrofielen is aangetoond dat ze bijdragen aan de ontwikkeling van atherosclerose, dus door de verlaagde aantallen neutrofielen in deze studie is waarschijnlijk die ontwikkeling geremd.

In hoofdstuk 6 is ten slotte de rol van FAK, een eiwit dat de adhesie en migratie van cellen reguleert en daarnaast celdood beïnvloedt, in atherosclerose bestudeerd. Adhesie en hechting van cellen aan andere cellen of de vaatwand, en inbedding van deze cellen in de weefselmatrix zijn essentiele processen bij het ontstaan van plaques. FAK reguleert deze sleutelprocessen in alle in de plaque aanwezige cellen, waaronder macrofagen, gladde spiercellen, endotheelcellen en T cellen. Daarom is het aannemelijk dat FAK een belangrijke factor is in plaque-ontwikkeling. Een volledige verlies van FAK functie is helaas lethaal en daarom hebben we voor deze studie transgene muizen die heterozygoot waren voor FAK ($FAK^{+/-}$) gebruikt en deze gekruist met ApoE deficiënte ($ApoE^{-/-}$) muizen, die spontaan atherosclerose ontwikkelen. Op deze manier werden ApoE deficiënte muizen met verminderde FAK functie verkregen. Tot onze verrassing hadden $FAK^{+/-}ApoE^{-/-}$ muizen een verlaagd cholesterolniveau in het bloed. Dit werd veroorzaakt door een verminderde productie van VLDL deeltjes in de lever. In $ApoE^{-/-}$ deficiënte muizen is cholesterol voornamelijk verbonden met VLDL dat in de lever wordt gemaakt en uitgescheiden in het bloed. De verlaagde VLDL productie was waarschijnlijk het gevolg van de

verminderde expressie van een aantal sleutelgenen in de VLDL productie in de lever.
Daarnaast leidde vermindering van FAK tot een veranderde inflammatoire status in ApoE deficiënte muizen. Zo was de milt vergroot en bevatte het minder macrofagen maar meer T cellen, terwijl in bloed en in de buikholte meer macrofagen en minder T cellen aanwezig waren. Deze verschillen werden waarschijnlijk veroorzaakt door een verstoorde migratie van monocyten/macrofagen vanuit het bloed naar de milt en een verhoogde retentie van T cellen in de milt.
Ook in deze studie leidt de optelsom van pro- (ontstekings-bevorderend effect) en anti-atherogene (verlaagde cholesterol) effecten van verminderde FAK functie tot een gelijkblijvende grootte en samenstelling van atherosclerotische plaques in verschillende vaten.

Samenvattend kan gesteld worden dat de studies beschreven in dit proefschrift laten zien dat celdood-regulerende genen mogelijk bijdragen tot de overgang van stabiele naar meer kwetsbare plaques (hoofdstuk 3) en dat uitschakeling van enkele van sleutelgenen in de regulatie van apoptose, Bim en Mcl-1, diverse effecten heeft op vetmetabolisme en ontsteking (hoofdstuk 4 en 5). Verder blijkt uit hoofdstuk 6 dat FAK aan de ene kant betrokken is bij VLDL productie in de lever en daaraan gerelateerd het cholesterolniveau in het bloed, en aan de andere kant de migratie en adhesie van immuuncellen beïnvloedt in ApoE deficiënte muizen. Wanneer dus wordt overwogen de hier bestudeerde genen te moduleren zal een celtype specifieke of plaque-gerichte aanpak moeten worden gebruikt om gewenste effecten te isoleren van ongewenste effecten. Lokale beïnvloeding van de werking van bijvoorbeeld Mcl-1 zou bijvoorbeeld de lipide-ophoping in en apoptose van macrofagen in de plaque gunstig kunnen beinvoeden hetgeen de ontwikkeling van atherosclerose zou kunnen afremmen. Via deze aanpak worden systemische effecten op onder andere neutrofielen vermeden. Om deze inzichten te bevestigen zullen aanvullende studies nodig zijn.

Abbreviations

36B4	Acidic ribosomal phosphoprotein P0
ACC	Carotid artery
Ad	Adenoviral
AIF	Apoptosis inducing factor
AIM	Apoptosis inhibitor expressed by macrophages
APAF	Apoptotic protease-activating factor
ApoE	Apolipoprotein E
Arg1	Arginase 1
ASMA	α-smooth muscle actin
Bad	Bcl-2-associated agonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
BCA	Brachiocephalic artery
Bcl-2	B cell lymphoma 2
Bfl-1	Bcl-2-related gene expressed in fetal liver 1
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 like interacting mediator of cell death
Blk	Bik-like killer protein
BM	Bone marrow
Bmf	Bcl-2 modifying factor
BMT	Bone marrow transplantation
BMDM	Bone marrow derived macrophages
CCR	CC chemokine receptor
CD	Cluster of differentiation
CMV	Cytomegalovirus
Ct	Threshold cycle
CXCR	CXC chemokine receptor
DC	Dendritic cell
DD	Death domain
DFF	DNA fragmentation factor
DISC	Death inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DR	Death receptor
DT(R)	Dyphtheria toxin (receptor)
EC	Endothelial cell
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESAM	Endothelial cell-selective adhesion molecule

Fabp	Fatty acid binding protein
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FAS	Fatty acid synthase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FRNK	FAK related non kinase
GC	Group specific component
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTC	Guanidine thiocyanate
HE	Hematoxylin and eosin
HGF	Hepatocyt growth factor
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
Hrk	Harakiri, Bcl-2 interacting protein
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor
IL	Interleukin
IL1-RA	Interleukin 1 receptor antagonist
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible nitric oxide synthase
LDL(r)	Low density lipoprotein (receptor)
LO	Lipoxygenase
LOX-1	Lectin-like oxidized low density lipoprotein receptor
LPS	Lipopolysaccharide
MARCO	Macrophage receptor with a collagenous structure
Mcl-1	Myeloid cell leukemia 1
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony stimulating factor
Mfge8	Milk fat globule epidermal growth factor 8
MHC	Major Histocompatibility complex
MIF	Macrophage migration inhibitory factor
MLN	Mediastinal lymph node
MMP	Matrix metalloproteinases
Moma-2	Monocyte/macrophage antibody 2
Mup	Major urinary protein
NF-κB	Nuclear factor κB
NK	Natural killer
NO	Nitric oxide
Npy	Neuropeptide y

n.s.	Non significant
Ox-LDL	Oxidized LDL
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PECAM	Platelet/endothelial-cell adhesion molecule
PGE2	Prostaglandin E2
Plagl1	Pleiomorphic adenoma gene-like-1
PTCA	Percutaneous transluminal coronary angioplasty
Puma	P53-up-regulated modulator of apoptosis
RAG	Recombination activating gene
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR
SCD1	Stearoyl-coenzyme A desaturase 1
SDF-1	Stromal cell derived factor 1
SEM	Standard error of the mean
SRA	Scavenger receptor 1
SREBP	Sterol regulatory element-binding protein
SR-PSOX	Scavenger receptor that binds phosphatidylserine and oxidized lipoprotein
TC	Total cholesterol
TG	Triglyceride
TG2	Transglutaminase 2
TGFβ	Transforming growth factor β
Th	T helper
TNF(R)	Tumor necrosis factor (receptor)
TRAIL	TNF related apoptosis inducing ligand
TUNEL	Terminal deoxytransferase dUTP nick-end labeling
TWEAK	TNF-like weak inducer of apoptosis
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA-4	Very late activation antigen 4
VLDL	Very low-density lipoprotein
vSMC	Vascular smooth muscle cells
WBC	White blood cells
WT	Wild-type
WTD	Western type diet
XAF1	X-linked inhibitor of apoptosis associated factor-1

Publications

Full papers

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Curriculum vitae

Marijke Westra werd geboren op 14 juli 1979 te Haskerland. In juni 1997 behaalde zij haar VWO diploma aan scholengemeenschap 'OSG Sevenwolden' in Heerenveen, en in juni 1998 aanvullende VWO certificaten aan het Friesland College in Leeuwarden. In september 1998 werd begonnen met de studie Farmacie aan de Univeriteit Utrecht. Het doctoraal examen van deze studie met als afstudeerrichting Farmaceutische Biologie werd in oktober 2004 behaald aan de Rijksuniversiteit Groningen. Tijdens deze opleiding werden twee onderzoeksstages gedaan. De eerste stage vond plaats bij de Basiseenheid Farmaceutische Biologie aan de Rijksuniversiteit Groningen en betrof de ontwikke-ling van RANK antagonisten en een trimeerconstructie voor antagonisten en agonisten voor receptoren van de TNF-familie onder begeleiding van Dr. M.M. Mullally en Prof. Dr. W.J. Quax. De tweede stage was getiteld 'Identificatie van genen betrokken bij, en mechanismen van sensitisatie voor TRAIL geïnduceerde apoptose in kanker cellijnen' en werd uitgevoerd bij het Department of Biochemistry, Cell Stress and Apoptosis Research Group aan de National University of Galway in Ierland, onder begeleiding van Dr. E. Szegezdi en Dr. A. Samali. Van januari 2005 tot april 2009 werkte zij als assistent in opleiding bij de afdeling Biofarmacie van het Leiden/Amsterdam Center for Drug Research aan de Universiteit Leiden. Hier werd het onderzoek beschreven in dit proefschrift, gesubsidieerd door NWO/ZonMW (project 912-02-037), uitgevoerd onder begeleiding van Prof. Dr. E.A.L Biessen en Prof. Dr. Th.J.C. van Berkel. Sinds mei 2009 is zij werkzaam als octrooigemachtigde in opleiding bij Vereenigde te Amersfoort.