Adaptive immunity in atherosclerosis:
an *in vivo* evaluation

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**Adaptive immunity in atherosclerosis:
an in vivo evaluation**

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List of abbreviations

AHA	American Heart Association
APC	Antigen-presenting cell
APOE	Apolipoprotein E
BCR	B-cell receptor
bFGF	Basic-fibroblast growth factor
β 2-GPI	β 2-Glycoprotein I
BM	Bone marrow
CDR3	Complementarity-determining region 3
CRP	C-reactive protein
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
HDL	High-density-lipoprotein
HFD	High fat diet
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IFN γ	Interferon- γ
Ig	Immunoglobulin
IL	Interleukin
LDL	Low-density-lipoprotein
LDLR	Low-density-lipoprotein receptor
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex

MMP	Matrix metalloproteinase
NC	Normal chow
NK cell	Natural killer cell
OVA	Ovalbumin
oxLDL	Oxidised low-density-lipoprotein
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PC	Phosphorylcholine
p-Smad2/3	Phospho-Smad2/3
PTX	Pentoxifylline
RAG	Recombination activating gene
SCID	Severe combined immune deficiency
T _C -lymphocyte	Cytotoxic T-lymphocyte
TCR	T-cell receptor
TGF- β	Transforming growth factor- β
TGF β R	TGF- β receptor
TGF β RII:Fc	Recombinant soluble TGF- β receptor II
T _H -lymphocyte	Helper T-lymphocyte
TMX	Tamoxifen
TNF α	Tumour necrosis factor- α
VALT	Vascular-associated lymphoid tissue
VCAM	Vascular cell adhesion molecule
VLA-1	Very late antigen-1

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Chapter 1

General introduction

Most inhabitants of developed countries of the world suffer from atherosclerosis. Atherosclerosis is a chronic inflammatory disease of the large and medium sized arteries, which progresses during a person's lifetime. It is the main cause of cardiovascular events such as myocardial infarction, peripheral arterial disease and stroke.¹ In the Netherlands, one in three deaths in 2004 were the result of cardiovascular disease.² The aetiology of atherosclerosis is clearly multifactorial and epidemiologic studies have identified many risk factors for atherosclerosis including smoking, lack of exercise, a high fat diet, hyperlipidemia and hypertension.^{3,4} Although reducing these risk factors will decrease cardiovascular events, it will not prevent atherosclerosis completely.

1.1 Pathogenesis of atherosclerosis

Over the past decades, many researchers have investigated the initiation and progression of atherosclerosis and the prerequisites needed for atherosclerosis to cause cardiovascular events. Our current knowledge on the pathogenesis of atherosclerosis is the result of combining the findings of clinical investigations, population studies, *in vitro* experiments and studies in animal models. Russell Ross gave in his famous review of 1999⁵ a clear overview of the current insights in the pathogenesis of atherosclerosis and especially of the role of the immune system in this disease. Based on this review, supplemented with the latest concepts, the key stages in the initiation and progression of atherosclerosis are described.

1.1.1 Initiation of atherosclerosis

Atherosclerosis develops in large and medium sized arteries at predisposed sites where the pulsatile blood flow is causing changes in local hemodynamic forces, like shear stress and oscillatory or turbulent flow.^{6,7} In Figure 1.1 the distribution of these high-susceptible sites of the vasculature is illustrated. The initiation of atherosclerosis can be mainly attributed to hypercholesterolemia, the endothelium overlying the arterial wall and the inflammatory response of innate immune cells, primarily monocytes/ macrophages. However, in humans, T-lymphocytes are already found in the adventitia of atherosclerosis-prone areas prior to the presence of atherosclerotic lesions. These regions are called 'vascular-associated lymphoid tissue' (VALT).^{8,9} Recently, it was shown that these adventitial lymphocyte

infiltrates are also present in atherosclerosis-prone mice.^{10,11} Nevertheless, the contribution of this VALT to early atherogenesis is not yet understood.

Figure 1.1 Distribution of the high-susceptible sites (gray) of the aorta (right): 1, aortic root; 2, aortic arch; 3, carotid artery; 4, thoracic aorta; 5, abdominal aorta and 6, iliac artery. LV, left ventricle. Schematic representation of atherosclerotic lesions in the aortic root (lower middle) and hematoxylin and eosin-stained section of an aortic root containing atherosclerotic lesions (left). AS, aortic sinus. See page 194 for full colour figures.

At present, there are three hypotheses describing the early events in the pathogenesis of atherosclerosis: the response-to-injury hypothesis, the response-to-retention hypothesis and the oxidative modification hypothesis. The response-to-injury hypothesis attributes endothelial dysfunction or endothelial injury at atherosclerosis-prone sites as the primary step in atherogenesis. Endothelial dysfunction results in enhanced endothelial permeability to atherogenic lipoproteins.⁵ The response-to-retention hypothesis and the oxidative modification hypothesis emphasise on the retention of LDL as the critical first event in early atherogenesis.¹² LDL is retained by binding to extracellular glycoproteins of the

extracellular matrix in the subendothelial space as a result of hypercholesterolemia^{13,14} or the subsequent modification of LDL. A combination of all three hypotheses describes the early events of atherogenesis, however, the retention and oxidation of LDL must precede endothelial dysfunction or endothelial injury, since it was found in animal models that although the rate of LDL entry in the vascular wall is quite uniform the retention of atherogenic lipoproteins is enhanced at lesion-prone sites.^{12,13} The retained and modified LDL will activate the endothelium and attract monocytes and T-lymphocytes, which is described in the response-to-injury hypothesis.¹² The ultimate outcome of these initiating events is inflammation resulting in an early atherosclerotic lesion or fatty streak consisting mainly of lipid-laden monocyte-derived macrophages (foam cells) and T-lymphocytes (Figure 1.2).

Figure 1.2 Schematic representation of an early atherosclerotic lesion (upper panel) and an advanced atherosclerotic lesion (lower panel). NC, necrotic core. Hematoxylin and eosin-stained section of an aortic sinus containing an early atherosclerotic lesion (upper panel) and an advanced atherosclerotic lesion (lower panel). See page 195 for full colour figures.

1.1.2 Progression of atherosclerosis

As early lesions grow larger, cells die and form a necrotic core. To shield the lesion from the blood and stabilise it, a fibrous cap is formed consisting of extracellular matrix proteins,

like collagen and glycoproteins produced by fibroblasts and smooth muscle cells. This stage is classified as an advanced lesion (Figure 1.2) and it can be subdivided into a stable, vulnerable or ruptured atheroma. Stable lesions have a thick fibrous cap and a relative low level of inflammation. Continuous chronic inflammation or enhanced inflammation results in constant trafficking of leukocytes, like monocytes and T-lymphocytes into and throughout the atherosclerotic lesion. To migrate, leukocytes need to degrade the extracellular matrix proteins of the atherosclerotic lesion with their secreted matrix metalloproteinases (MMPs) thereby thinning the fibrous cap. A lesion where the fibrous cap starts to thin at the shoulder region and the amount of inflammatory cells increases is classified as vulnerable. At the ultimate stage of atherosclerosis, the thin fibrous cap is so thin that it ruptures resulting in the formation of a thrombus. At the site of rupture, infiltrates of macrophages and T-lymphocytes are often found. This final stage is called a ruptured atheroma and is frequently correlated with acute cardiovascular events.^{5,15-17}

Hence, in all stages of atherosclerosis, inflammatory cells or mediators of the immune system are involved. In the upcoming part of the introduction, the murine models used in atherosclerosis research are introduced, followed by an overview of the components of the immune system and their processes that contribute to the pathogenesis of atherosclerosis.

1.2 Murine models of atherosclerosis

Unravelling the contribution of individual components and their interactions to the pathogenesis of atherosclerosis can only be done in animal models, in particular murine models. Research on atherosclerosis was first performed in the most susceptible mouse strain, C57Bl/6.¹⁸ These mice were given a high fat diet containing a pro-inflammatory ingredient, cholic acid, to induce atherosclerosis. However, they developed only small early lesions and hardly any advanced lesions.^{18,19} As genetic engineering techniques evolved, better murine models for atherosclerosis, such as apolipoprotein E-deficient (APOE^{-/-}) mice^{20,21} and low-density lipoprotein receptor-deficient (LDLR^{-/-}) mice,²² were developed. These mice are nowadays the most commonly used murine models for human atherosclerosis. LDLR^{-/-} mice have only slightly elevated total plasma cholesterol levels, but when given a high fat diet their cholesterol levels markedly increase. Depending on the type of diet the total plasma cholesterol level ranges from 300 to >1500 mg/dl.^{23,24} On the

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other hand, APOE^{-/-} mice have a five times higher total plasma cholesterol level (400-600 mg/dl) than wild-type mice and spontaneously develop atherosclerosis, which progresses with age.^{19,21,25}

The first site containing atherosclerotic lesions is the aortic sinus. Subsequently, atherosclerosis develops at the branch points of the aortic arch and finally at the branch points of the abdominal aorta.²⁶ From 8 weeks of age, APOE^{-/-} mice have atherosclerotic lesions in the aortic sinus that resemble human fatty streaks, which are rich in foam cells. Over another 3 months they progress into more advanced lesions containing multiple layers of foam cells, a necrotic core, smooth muscle cells and a fibrous cap.^{26,27} In LDLR^{-/-} mice, the development of atherosclerosis is similar to that of APOE^{-/-} mice. To study the role of adaptive immunity in atherosclerosis, T-lymphocytes need to be present in these murine models as they are in human disease. T-lymphocytes are present in atherosclerotic lesions of the aortic root in both APOE^{-/-} and LDLR^{-/-} mice, although the density of T-lymphocytes is greater in LDLR^{-/-} mice than in APOE^{-/-} mice.²⁸ A disadvantage of the APOE^{-/-} mouse model is that the APOE gene has been reported to inhibit the proliferation of monocytes and T-lymphocytes.^{28,29} Additionally, APOE^{-/-} mice have an elevated humoral immune response and the cell-mediated immunity is impaired.²⁹ Therefore, we consider the LDLR^{-/-} mice to be the best model for investigating the involvement of the immune system in atherosclerosis.

1.3 The immune system: an introduction

The main function of the immune system is to defend the host against foreign invaders by a collective and coordinated inflammatory response, thereby enabling the healing processes of injured tissues. The immune system consists of cells and inflammatory mediators, which can be divided into either innate or adaptive immunity. In vertebrates, innate immunity provides the first rapid lines of defence against foreign invaders and consists of the following components: (1) physical and chemical barriers, like (mucosal) epithelia, (2) blood proteins, including components of the complement system and other mediators of inflammation, (3) immune cells, such as phagocytes (neutrophils and macrophages) and other leukocytes (NK cells and mast cells). The inflammatory response by innate immune cells is blunt because it focuses mainly on highly conserved motifs in pathogens, which are

recognized by germline-encoded receptors. In contrast, the immune response by adaptive immune cells is highly specific for distinct pathogenic substances (antigens) by using a great variety of antigen-recognising receptors (BCR and TCR) and it is specialised with multiple diverse ways of responses to pathogens. Another feature of adaptive immunity is that memory cells can develop, thereby enabling a more vigorous response when repeatedly exposed to the same pathogen. Adaptive immunity comprises of T-lymphocytes, B-lymphocytes and their products (antibodies and cytokines). Although innate and adaptive immunity can be separated, together they form an integrated system of host defence in which various cells and inflammatory mediators function in cooperation.³⁰

In order to defend the host most efficiently against invading pathogens, immune cells need to circulate and travel among blood, lymph and tissues throughout the host to recognise and eliminate pathogens. Especially for the induction of adaptive immunity centralisation of antigens from invading pathogens is required. This is accomplished by the gathering of antigens from the blood and lymph, which drain all tissues of the body, into lymphoid organs, like spleen, lymphoid tissues and lymph nodes.³⁰

1.4 Immune mechanisms in atherosclerosis

Atherosclerosis is an example of a chronic inflammatory disease. Cells and mediators of the immune system dominate in all stages of atherosclerosis. Macrophages and T-lymphocytes are involved in early atherosclerosis and contribute profoundly to the progression of the atherosclerotic lesion. Especially in vulnerable and ruptured plaques, an enhanced intraplaque inflammation is found. Inflammatory cells accumulate at the sites of plaque rupture and their numbers correlates with the severity of angina pectoris.³¹⁻³³ In addition, plaque vulnerability is related to general markers of inflammation, like C-reactive protein (CRP), IL-6, soluble CD40L and neopterin, but also more specific ones, like antibodies against oxLDL and heat shock protein (HSP). In particular, CRP was shown to be an independent risk factor for cardiovascular events and its levels are increased in patients suffering from unstable angina or cardiovascular events.^{34,35} Therefore, the clinical outcome of atherosclerosis seems to be the result of a balance between inflammation with continued recruitment of leukocytes leading to weakening of the lesion on the one hand and tissue healing with generation of connective tissue matrix resulting in strengthening of the lesion

on the other hand. Cells and mediators of innate and adaptive immunity involved in the inflammatory response in atherosclerosis are described below.

1.4.1 Innate immunity and atherosclerosis

Macrophages

In the arterial wall, modified LDL induces secretion of chemokines, like MCP-1, by endothelial cells and smooth muscle cells to attract leukocytes (Figure 1.3). Leukocytes are recruited to the subendothelial space by the activated endothelium, which expresses adhesion molecules (VCAM, ICAM, P- and E-selectin). To induce differentiation of monocytes into macrophages and proliferation of smooth muscle cells, the activated endothelial cells produce growth factors, like M-CSF.^{5,12,36,37}

Figure 1.3 Schematic overview of effects of oxLDL on atherosclerosis: 1, secretion of chemokines to attract leukocytes; 2, foam cell formation. ScR, scavenger receptor. See page 196 for full colour figures.

Macrophages contribute to the innate immune response in both the initiation and progression of atherosclerosis. Monocytes migrate into the subendothelial space by digesting the extracellular matrix using direct phagocytosis or by secreting several

proteolytic enzymes such as plasminogen activator, $\text{TNF}\alpha$ and a variety of MMPs. In the subendothelial space monocytes differentiate into macrophages, thereby up-regulating their pattern-recognition receptors, including scavenger receptors and toll-like receptors. The scavenger receptors are used to internalise the trapped oxLDL particles from the intima (Figure 1.3). Endocytosis of oxLDL particles by scavenger receptors is tightly regulated by cytokines such as $\text{IFN}\gamma$, $\text{TNF}\alpha$ and $\text{TGF-}\beta$ through adjustment of the expression of scavenger receptors on macrophages.^{36,38} After degrading the oxLDL particles, free cholesterol may not be sufficiently effluxed to HDL or adequately enzymatically modified and is thus stored in cytosolic droplets. Macrophages containing these cytosolic droplets are called foam cells and they are the main hallmark of atherosclerosis.^{3,5,38-40}

Activation of macrophages is the result of oxLDL particles binding to toll-like receptors (Figure 1.4). Activated macrophages produce inflammatory cytokines, chemokines, growth factors and hydrolytic enzymes, which results in generation of more oxLDL particles and eventually in attraction of more monocytes.⁴⁰ Furthermore, they are better capable of processing and presenting the internalised oxLDL particles in MHC molecules to adaptive immune cells, which become activated. Activated adaptive immune cells produce $\text{IFN}\gamma$, the classic macrophage activation factor, thereby synergistically amplifying the inflammatory response.^{39,41} Besides pattern-recognition receptors macrophages also express Fc receptors by which they can take up immune complexes containing oxLDL particles and anti-oxLDL antibodies (Figure 1.4). This internalisation can be enhanced by complement binding to the immune complex. Binding of immune complexes to the Fc receptors results in activation of the macrophage.⁴¹

Figure 1.4 Schematic overview of the generation of activated macrophages and their effects on atherosclerosis: 1, secretion of inflammatory products; 2, antigen presentation. Ab, antibody; TLR, toll-like receptor; FcR, Fc receptor. See page 197 for full colour figures.

Innate B-lymphocytes

A minority of B-lymphocytes, called B1 cells or innate B-lymphocytes, is mainly found in the peritoneal cavity and to a lesser extent in the spleen. They arise during fetal and neonatal development and do not mature in the bone marrow (BM) like B-lymphocytes do. They remain as a self-replenishing population and do not require help from T-lymphocytes for their activation.³⁰ Natural antibodies produced by innate B-lymphocytes are capable of blocking the uptake of oxLDL particles by scavenger receptors on macrophages. These

natural antibodies recognise microbial determinants and a variety of self-determinants including cell membrane components such as phosphorylcholine (PC), which is also present in oxLDL. In experimental atherosclerosis, it was shown that an increase in these natural antibodies by pneumococcal immunisation or immunisation with oxLDL resulted in reduced atherosclerosis. Whether these natural antibodies can also have such an atheroprotective effect on human atherosclerosis is unknown.⁴²

NK cells

NK cells are found in low numbers in human and experimental atherosclerosis. They are scattered throughout the lesion, but are more prominent in the shoulder region than in the necrotic core. NK cells and their mediators were correlated with severe atherosclerotic disease. Activated NK cells have two effector functions: cell-mediated cytolytic activity by perforin or granzymes and cytokine production, among which is IFN γ . The activity of NK cells is influenced by other cytokines such as IL-2, IL-15 and IL-12, which are all present in atherosclerotic lesions. Until now, only a small number of studies have investigated the involvement of NK cells in experimental atherosclerosis. On the one hand, the cytolytic activity of NK cells was hindered, which gave no effect on atherosclerosis or the extent of atherosclerosis increased. On the other hand, depletion of NK cells reduced the development of atherosclerosis. Therefore, the involvement of NK cells in atherosclerosis is still inconclusive.^{41,43}

Mast cells

Mast cells are present in human atherosclerotic lesions in relatively low densities during plaque development. They accumulate especially in the shoulder region of rupture-prone lesions, but are also found in the core region and fibrous cap susceptible to erosion. Mast cells can be activated by a variety of stimuli: IgE-mediated cross-linking of Fc ϵ receptor I through antigen binding, histamine-releasing factors secreted by neighbouring T-lymphocytes or macrophages, components of the complement cascade, direct neural stimulation or excessive cholesterol incorporation into lipid rafts. When activated, mast cells release vasoactive substances (histamine and leukotrienes), proteolytic enzymes (tryptase and chymase), inflammatory cytokines (TNF α , TGF- β , MCP-1 and various ILs) and growth factors (PAF and bFGF), which are stored in large cytoplasmic granules. These

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components can contribute to the destabilisation of the atherosclerotic lesion by degrading matrix proteins of the fibrous cap, by inhibiting vascular smooth muscle cell proliferation and collagen synthesis or by inducing endothelial and smooth muscle cell death.^{39,41,43,44}

Neutrophils

Neutrophils are rarely found during the development of human atherosclerosis, but they are prevalent in eroded or ruptured lesions of patients having acute coronary syndromes. Another illustration of the possible involvement of neutrophils in plaque rupture is that an increased level of neutrophils in the circulation is an independent risk factor for coronary heart disease. Activated neutrophils contribute to acute inflammation by taking up damaged tissue and secreting proteolytic enzymes (elastases and MMPs). Their contribution to atherosclerosis remains unclear in whether the recruitment of neutrophils leads to plaque erosion or rupture or whether they enter shortly after disruption.^{39,43}

Dendritic cells

Dendritic cells (DCs) are a major component of the proposed VALT, present in the healthy human intima at atherosclerosis-prone sites.^{8,9} DCs are identified in all stages of human atherosclerosis and their numbers increase with the severity of atherosclerosis. In experimental atherosclerosis, DCs are also found in atherosclerotic lesions.^{8,9,43,45} DCs express C1q to facilitate the capture of immune complexes in the atherosclerotic lesion. There are several DC subclasses known, which all have distinctive cytokine profiles and therefore have different functions. Especially myeloid or classical DCs are highly efficient antigen-presenting cells (APCs).⁴¹ To present antigen it is necessary that DCs mature and increase their expression of MHC and co-stimulatory molecules, which is all promoted by oxLDL. These DCs are extremely important for the initial activation of naive T-lymphocytes in lymphoid tissue or lymphoid organs, thereby regulating the adaptive immune response to foreign and self-antigens.^{36,43}

In conclusion, innate immunity is triggered first in an immune response as in atherosclerosis. If the innate immune response cannot eradicate the inflammation in the vascular wall, adaptive immune mechanisms are needed to complete the inflammatory response. Activation of adaptive immune cells is accomplished through antigen-dependent

cell-cell interactions with macrophages and DCs or indirectly by secreted substances, like inflammatory cytokines and chemokines, thereby attracting and activating adaptive immune cells.

1.4.2 Adaptive immunity and atherogenesis

As already mentioned, cells and mediators of adaptive immunity are found in atherosclerotic lesions and are suggested to contribute to atherosclerosis. The participation of adaptive immune cells in the development of experimental atherosclerosis was only conclusively shown since the last decade. This was achieved by the analysis of atherosclerosis in immunodeficient mice on a C57Bl/6 background or crossed with the atherosclerosis-prone APOE^{-/-} or LDLR^{-/-} mice (Table 1.1). The first results were obtained in the spontaneous murine models for immunodeficiency: the SCID (scid/scid) mouse and the nude (nu/nu) mouse. The scid/scid mouse lacks functional T- and B-lymphocytes and the nu/nu mouse does not have a thymus, therefore it cannot generate mature T-lymphocytes and is deficient in cellular immunity. Analysis of the atherosclerotic lesions in these mice demonstrated that adaptive immunity is not essential for the initiation of atherosclerosis,⁴⁶⁻⁴⁸ but that the progression of atherosclerosis into advanced lesions is hampered in the absence of adaptive immune cells.^{47,48} This observation was confirmed in APOE^{-/-} or LDLR^{-/-} mice crossed with RAG^{-/-} mice, which lack a gene needed for the TCR or BCR rearrangement resulting in deficiency in mature T- and B-lymphocytes. In these mice the progression of atherosclerosis was delayed when compared to immunocompetent mice,^{9,49-53} although in the study of Song *et al.* the delay gradually subsided.⁵² The experiments done with immunodeficient mice revealed that adaptive immunity contributes mainly to the progression of experimental atherosclerosis. The results of further exploration of individual components of adaptive immunity are described next.

B-lymphocytes

B-lymphocytes express membrane bound antibodies (or immunoglobulin, Ig) as BCRs. Matured B-lymphocytes express IgM and IgD on their membrane and are antigen-responsive. Upon antigen binding to the BCR the B-lymphocyte becomes activated, which results in proliferation and differentiation into either memory B-lymphocytes or antibody secreting plasma cells.

Table 1.1 *The adaptive immune system and atherogenesis*

Defect or treatment	Effect on the immune system	Mouse model	Chow	Effect on atherosclerosis	References
scid/scid	no T- and B-lymphocytes	C57Bl/6	high fat diet	none	46
scid/scid	no T- and B-lymphocytes	APOE ^{-/-}	normal chow	↓ atherosclerosis	48
RAG1 ^{-/-}	no T- and B-lymphocytes	APOE ^{-/-}	normal chow	↓ atherosclerosis	9, 49
RAG2 ^{-/-}	no T- and B-lymphocytes	APOE ^{-/-}	normal chow	↓ atherosclerosis	51
RAG2 ^{-/-}	no T- and B-lymphocytes	APOE ^{-/-}	high fat diet	↓ atherosclerosis	9, 50
RAG1 ^{-/-}	no T- and B-lymphocytes	LDLR ^{-/-}	high fat diet	↓ atherosclerosis	9, 49, 52, 53
RAG2 ^{-/-}	no T- and B-lymphocytes	LDLR ^{-/-}	high fat diet	↓ atherosclerosis	53
nu/nu	no thymus (no T-lymphocytes)	C57Bl/6	high fat diet	none	46
nu/nu	no thymus (no T-lymphocytes)	C57Bl/6	high fat diet	↓ atherosclerosis	47

Activated B-lymphocytes can undergo isotype class switching generating an antibody (IgG, IgA or IgE) that can perform different effector functions and is better adjusted to eliminate particular pathogens. Isotype class switching can only occur when regulated by helping T-lymphocytes, which is mediated by the CD40-CD40L interaction.³⁰

The involvement of B-lymphocytes in atherosclerosis has been shown by the following observations (Table 1.2). First, B-lymphocytes and plasma cells were identified by immunostaining in experimental atherosclerosis.^{54,55} In human atherosclerosis, B-lymphocytes are present in adventitial infiltrates underneath the atherosclerotic lesion.⁵⁶ Second, in the absence of B-lymphocytes the size of atherosclerotic lesions is increased in LDLR^{-/-} mice.⁵⁷ Third, transfer of B-lymphocytes into APOE^{-/-} mice resulted in a reduced lesion area.⁵⁸ This protective effect was confirmed in a study in which transfer of B-lymphocytes could rescue the pro-atherogenic effect of a splenectomy.⁵⁸ These data show that B-lymphocytes are present in atherosclerotic lesions and that their overall effect on atherosclerosis is protective. However, it is not evident how this protective effect is accomplished; whether it is by cell-cell interactions with either T-lymphocytes or DCs or by the secretion of antibodies.

One of the first observations demonstrating antibody involvement in atherosclerosis was that antibodies and complement could be extracted from atherosclerotic lesions. In early lesions IgM is predominant, while IgG and IgA are more prominent in advanced lesions.⁵⁹ Antibodies extracted from human and experimental atherosclerotic lesions were shown to be directed against oxLDL.⁶⁰ Since these observations suggest a humoral response in atherosclerosis that is directed against lesion constituents, circulating antibodies directed against oxLDL were analysed in relation to the extent of human or experimental atherosclerosis. In humans, it was found that circulating anti-oxLDL antibodies are present in healthy individuals and in patients suffering from atherosclerosis.⁶⁰⁻⁶² Furthermore, the level of circulating anti-oxLDL antibodies correlated with the severity of atherosclerosis,^{63,64} although this was not consistently found.^{65,66} Circulating anti-oxLDL antibodies were also detected in experimental models for atherosclerosis^{60,61,67,68} and they correlated with increased atherosclerosis.^{69,70} Apart from oxLDL, more antigens were found to induce an immune response in atherosclerosis, among which are HSP and β 2-GPI.⁹

Table 1.2 *B-lymphocytes and atherogenesis*

Defect or treatment	Effect on the immune system	Mouse model	Chow	Effect on atherosclerosis	References
$\mu\text{MT}^{-/-}$ BMT	no B-lymphocytes	$\text{LDLR}^{-/-}$	high fat diet	$\uparrow$ atherosclerosis	57
Transfer of B-lymphocytes		$\text{APOE}^{-/-}$	normal chow	$\downarrow$ atherosclerosis	58
Splenectomy & transfer of B-lymphocytes		$\text{APOE}^{-/-}$	normal chow	$\downarrow$ atherosclerosis	58
Early immunisation with oxLDL		$\text{LDLR}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	78
Early immunisation with oxLDL		$\text{LDLR}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	86
Early immunisation with oxLDL		$\text{APOE}^{-/-}$	normal chow	$\downarrow$ atherosclerosis	79
Early immunisation with oxLDL		$\text{APOE}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	85
Early immunisation with oxLDL		$\text{APOE}^{-/-}$	normal chow	$\downarrow$ atherosclerosis	83
Early immunisation with LDL		$\text{LDLR}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	78
Early immunisation with LDL		$\text{APOE}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	84
Late immunisation with LDL		$\text{APOE}^{-/-}$	high fat diet	none	84
Early immunisation with CFA		$\text{APOE}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	81
Early immunisation with CFA		$\text{LDLR}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	82
Early immunisation with CFA		$\text{APOE}^{-/-}$	normal chow	$\downarrow$ atherosclerosis	83
Treatment with human IVIg		$\text{APOE}^{-/-}$	high fat diet	$\downarrow$ atherosclerosis	87, 88

The next important question is how anti-oxLDL antibodies play a role in the development of atherosclerosis. First, circulating anti-oxLDL antibodies may contribute to maintain the plasma level of oxLDL low.⁷¹ Second, in the atherosclerotic lesion anti-oxLDL antibodies can block the uptake of oxLDL by macrophages, thereby preventing the accumulation of oxLDL and foam cell formation.^{72,73} On the other hand, anti-oxLDL antibodies might have a pro-atherogenic effect by complexing with oxLDL and binding to Fc receptors present on macrophages, thereby providing an inflammatory signal.⁷⁴⁻⁷⁶ Another pro-atherogenic effect of anti-oxLDL antibodies might be that the uptake of apoptotic cells by macrophages is prevented.⁷⁷ Thus, the overall effect on atherogenesis of the anti-oxLDL antibodies is dependent on the contribution of their individual effects.

Further research focused on interfering with B-lymphocytes or their antibodies to reduce atherosclerosis. The first attempted treatment strategy was to immunise atherosclerosis-prone mice with oxLDL prior to the induction of atherosclerosis (Table 1.2). These studies reported that immunisation with oxLDL increased the level of anti-oxLDL antibodies and reduced the extent of experimental atherosclerosis.^{78,79} However, this effect was not mediated through the induced anti-oxLDL antibodies, because immunisation with LDL also resulted in less atherosclerosis, but with no or moderately enhanced anti-oxLDL antibodies.^{78,80} Probably the protective effect of oxLDL immunisation was the result of the used adjuvant, since Freund's adjuvant alone was shown to reduce atherosclerosis extensively.⁸¹⁻⁸³ Intervention studies in mice showed that early immunisation with LDL did result in smaller atherosclerotic lesions, but late immunisation did not.⁸⁴ Normally, immunisation does not only boost antigen-specific antibodies but can also activate T cells⁸⁵ and stimulate innate B-lymphocytes.⁸⁶ This might have added to the beneficial effect of immunisation on atherosclerosis. Another treatment strategy that was used to interfere with the B-lymphocyte response in atherosclerosis was administering a polyclonal Ig preparation. The polyclonal human Ig preparation was given at the onset or after the initiation of atherosclerosis. These treatments both reduced the extent of atherosclerosis and the inflammatory response in atherosclerosis-prone mice.⁸⁷ The effectiveness of the treatment was dependent on the Fc portion of the Ig, since F(ab')₂ fragments did not have any effect on the development of atherosclerosis.⁸⁸ The effectiveness of this therapy can be explained through multiple ways of action: first, Ig can block the uptake of immune

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complexes by Fc receptors on phagocytes, thereby preventing activation of the phagocytes. Second, Ig can inhibit antibody synthesis by B-lymphocytes and modulate the T-lymphocyte function. Third, Ig can attenuate the complement-mediated tissue damage.^{87,88}

These studies show that the humoral response is involved in atherosclerosis. Moreover, immunisation therapy resulted in elevation of the athero-protective antibodies and polyclonal Ig therapy dampened the pro-atherogenic inflammatory response. Depending on the therapy used, also other inflammatory cells, like macrophages and T-lymphocytes, might be additionally affected, thereby reversing or enhancing the effects of the applied therapy.

T-lymphocytes

T-lymphocytes surviving maturation in the thymus start to survey the body. They express a TCR, which can only bind to small peptides of antigens present in a unique structure called the MHC. There are two classes, MHC I and MHC II, which have distinct functions. MHC I molecules bind only to peptides of endogenous antigens, produced by cells of the host (self-antigens) or by internal pathogens and they are expressed on all cells. Peptides present in the MHC I molecule are recognised by a subset of T-lymphocytes, the CD8 or cytotoxic T (T_C)-lymphocyte (Figure 1.5). On the other hand, MHC II molecules contain only peptides of extracellular pathogens or internalised cells, which were taken up by specialised cells (mononuclear phagocytes, B-lymphocytes and DCs). Peptides bound to MHC II molecules are recognised by CD4 or helper T (T_H)-lymphocytes (Figure 1.5). The initial activation of naive T-lymphocytes can only be properly done in lymphoid tissues by APCs, which are DCs. Antigen recognition does only result in activation of T-lymphocytes if they are provided with a second signal: costimulation. Costimulation is accomplished by an interaction of certain surface molecules (*e.g.* CD80, CD86 or CD40) on the APC with specific receptors (*e.g.* CD28 or CD40L) on T-lymphocytes (Figure 1.5). If T-lymphocytes are not provided with both signals, they die or become anergic.³⁰

Figure 1.5 Schematic representation of molecules involved in antigen recognition by T-lymphocytes: the MHC I-TCR interaction (left panel); the MHC II-TCR interaction (right panel). See page 198 for full colour figures.

In the last decade, another important regulatory mechanism of T-lymphocyte activation was discovered. This mechanism is provided by regulatory T cells (Tregs), which can dampen the immune response and maintain immune homeostasis by direct cell-cell interactions or by the production of immunosuppressive cytokines, like IL-10 and TGF- β .^{89,90} Activation of naive T-lymphocytes by APCs in the lymphoid tissue leads to proliferation and differentiation into effector cells. T_H-lymphocytes can differentiate into multiple effector T-lymphocyte subsets (Figure 1.6). In the presence of IL-12, naive T_H-lymphocytes differentiate into T_H1-lymphocytes, but in the presence of IL-4 they differentiate into T_H2-lymphocytes. All effector T-lymphocytes have different functions: T_C-lymphocytes re-enter the circulation to go to the site of inflammation where they lyse cells presenting pathogenic peptides in MHC I. T_H1-lymphocytes produce IFN γ and lymphotoxin to stimulate phagocyte-dependent immune reactions (macrophages and neutrophils). In addition, they contribute to the antigen recognition by naive CD8 T-lymphocytes and their differentiation into effector T_C-lymphocytes and they help B-lymphocytes to switch the isotype of their antibodies towards complement-binding and opsonising antibodies. Finally, the IL-2 production stimulates the suppressive effects of Tregs. T_H2-lymphocytes produce IL-4, IL-5 and IL-10, which is important for the phagocyte-independent reactions (humoral immunity). These cytokines suppress macrophage activation, but enhance eosinophil activation. Furthermore, T_H2-lymphocytes help B-lymphocytes to switch the isotype of

their antibodies towards neutralising antibodies and IgE, which stimulate the degranulation of mast cells.³⁰

Figure 1.6 Schematic overview of CD4 T-lymphocyte differentiation into effector T-lymphocyte subsets. Treg, regulatory T cell; APC, antigen-presenting cell. See page 199 for full colour figures.

T-lymphocytes have multiple functions in an immune response, they secrete cytokines and chemotactic factors, which can attract mast cells, macrophages and smooth muscle cells. They also determine the differentiation and function of B-lymphocytes and monocytes and they can actively contribute to the elimination of pathogens by their cytolytic capacities. Therefore, the discovery of the involvement of T-lymphocytes in atherosclerosis was crucial for understanding the pathogenesis of atherosclerosis. The first observation that T-

lymphocytes might contribute to the pathogenesis of atherosclerosis was their presence in human and experimental atherosclerotic lesions. Both T_H-lymphocytes and T_C-lymphocytes are found in all stages of human and experimental atherosclerosis.⁹¹⁻⁹³ In advanced lesions, T-lymphocytes are mainly located in the shoulder of the fibrous cap^{93,94} or at the site of a plaque rupture.⁹⁵ In atherosclerotic lesions, some T-lymphocytes are in an activated state as shown by their expression of HLA-DR, VLA-1 and CD45 and only a minority also expresses the IL-2 receptor.^{91-94,96,97} Activated T-lymphocytes are mainly found in interaction with macrophages or smooth muscle cells, which express MHCII molecules (HLA-DR)^{91-94,98,99} and several of those T-lymphocytes also express co-stimulatory molecules.¹⁰⁰ The T-lymphocytes found in atherosclerotic lesions mostly express the α/β TCR, though some T-lymphocytes have the γ/δ TCR on their surface.¹⁰¹ The importance of T-lymphocytes in atherosclerosis was further explored by analysing their specificity. In human atherosclerosis, the rearrangement patterns of the TCR β and TCR δ genes and the TCR-CDR3 sequences of cloned T-lymphocytes from advanced atherosclerotic lesions showed that the T-lymphocyte response is polyclonal and that there is no repetition of CDR3 nucleotide motifs.¹⁰²⁻¹⁰⁴ However, recently it was published that for a subpopulation of CD28^{null} CD4 T-lymphocytes the response is monoclonal.¹⁰⁵ In experimental atherosclerosis, the analysis of the TCR demonstrated that usage of the TCR V domain was limited and that the CDR3 variability was restricted, indicating an oligoclonal T-lymphocyte response.¹⁰⁶ Besides analysis of the TCR expression pattern, T-lymphocytes isolated from atherosclerotic lesions were also examined for their antigen recognition. T-lymphocytes extracted from human atherosclerotic lesions recognised oxLDL,¹⁰⁷ but also HSP¹⁰⁸⁻¹¹⁰ and pathogens such as Chlamydia¹¹¹⁻¹¹³ or P. gingivalis.^{110,114} Since the T-lymphocyte response in atherosclerotic lesions seems to be antigen-specific, the importance of antigen presentation for the development of atherosclerosis was investigated (Table 1.3A). One study showed that the extent of atherosclerosis was enhanced in MHCI^{-/-} mice when compared to wild-type mice, but no difference was found in MHCII^{-/-} mice.⁴⁶ However, another study found that the total plaque area in MHCII^{-/-} mice was reduced in comparison to wild-type mice.¹¹⁵ Based on these observations the involvement of antigen presentation by MHC is not fully established and needs to be investigated further.

Table 1.3A *T-lymphocytes and atherogenesis*

Defect or treatment	Effect on the immune system	Mouse model	Chow	Effect on atherosclerosis	References
MHCI ^{-/-}	no antigen presentation to T _C -lymphocytes	C57Bl/6	high fat diet	↑ atherosclerosis	46
MHCII ^{-/-}	no antigen presentation to T _H -lymphocytes	C57Bl/6	high fat diet	none	46
MHCII ^{-/-}	no antigen presentation to T _H -lymphocytes	C57Bl/6	high fat diet	↓ atherosclerosis	115
Treatment with anti-CD4 mAb & anti-CD8 mAb	no T-lymphocytes	C57Bl/6	high fat diet	↓ atherosclerosis	47
TCRβ ^{-/-}	no TCRαβ ⁺ T-lymphocytes	APOE ^{-/-}	normal chow	↓ atherosclerosis	116
TCRδ ^{-/-}	no TCRγδ ⁺ T-lymphocytes	APOE ^{-/-}	normal chow	none	116
Neonatal tolerance by treatment with oxLDL	Clonal deletion of oxLDL reactive T-lymphocytes	APOE ^{-/-}	high fat diet	↓ atherosclerosis	117
Treatment with anti-CD8 mAb	no T _C -lymphocytes	C57Bl/6	high fat diet	none	47
CD8 ^{-/-}	no T _C -lymphocytes	APOE ^{-/-}	normal chow	none	116

Table 1.3A (continued) *T-lymphocytes and atherogenesis*

Defect or treatment	Effect on the immune system	Mouse model	Chow	Effect on atherosclerosis	References
Treatment with anti-CD4 mAb	no T _H -lymphocytes	C57Bl/6	high fat diet	↓ atherosclerosis	47, 115
CD4 ^{-/-}	no T _H -lymphocytes	C57Bl/6	high fat diet	↓ atherosclerosis	115
CD4 ^{-/-}	no T _H -lymphocytes	APOE ^{-/-}	normal chow	↓ atherosclerosis	83
CD4 ^{-/-}	no T _H -lymphocytes	APOE ^{-/-}	normal chow	↑ atherosclerosis	116
Treatment with PTX	no T _H 1-lymphocyte differentiation	APOE ^{-/-}	normal chow	↓ atherosclerosis	118
T-bet ^{-/-}	no T _H 1-lymphocyte differentiation	LDLR ^{-/-}	high fat diet	↓ atherosclerosis	119
scid/scid & transfer of T _H -lymphocytes		APOE ^{-/-}	normal chow	↑ atherosclerosis	48
Transfer of β ₂ -glycoprotein I-reactive T-lymphocytes		LDLR ^{-/-}	high fat diet	↑ atherosclerosis	120

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The involvement of T-lymphocytes in atherogenesis was established in animal models (Table 1.3A). Depletion of CD4⁺ and CD8⁺ cells reduced atherosclerosis in mice.⁴⁷ In T-lymphocyte-deficient LDLR^{-/-} mice the extent of atherosclerosis was decreased when compared to LDLR^{-/-} mice.¹¹⁶ Clonal deletion of anti-oxLDL reactive T-lymphocytes by inducing neonatal tolerance in APOE^{-/-} mice also reduced the extent of atherosclerosis.¹¹⁷ Additionally, less atherosclerosis was observed in mice depleted for T_H-lymphocytes by treatment with anti-CD4 antibodies^{47,115} and T_H-lymphocyte-deficient mice¹¹⁵ or T_H-lymphocyte-deficient APOE^{-/-} mice.⁸³ It was shown that especially T_H-lymphocytes are important for atherogenesis, although one study with T_H-lymphocyte-deficient APOE^{-/-} mice could not confirm this.¹¹⁶ The importance of pro-inflammatory T_H1-lymphocytes in the progression of atherosclerosis was further established in APOE^{-/-} mice treated with pentoxifylline (PTX, an inhibitor of the T_H1 differentiation pathway)¹¹⁸ and T_H1-lymphocyte-deficient LDLR^{-/-} mice.¹¹⁹ Both studies reported that the atherosclerosis was reduced. To prove that especially T_H-lymphocytes aggravate atherosclerosis, T_H-lymphocytes harvested from 5-month-old APOE^{-/-} mice were transferred into scid/scid APOE^{-/-} mice and these mice displayed increased atherosclerosis.⁴⁸ In another study, transfer of T-lymphocytes isolated from LDLR^{-/-} mice immunised with β 2-GPI into LDLR^{-/-} mice enhanced the atherosclerotic lesion formation.¹²⁰ Thus, T_H-lymphocytes have a pro-atherogenic effect on the development of atherosclerosis.

The pro-inflammatory properties of T-lymphocytes in atherosclerosis might be due to the production of inflammatory cytokines (Table 1.3B). Cloned T-lymphocytes from human advanced lesions were found to primarily produce IFN γ ^{107,111,121} and IFN γ mRNA was mainly detected in T_H1-lymphocytes present in atherosclerotic lesions of APOE^{-/-} mice.¹²² In the absence of the IFN γ -receptor in APOE^{-/-} mice, the atherosclerotic lesion size significantly reduced accompanied by an increase of collagen content.¹²³ These results were confirmed in IFN γ -deficient LDLR^{-/-} and APOE^{-/-} mice, which developed less atherosclerosis and a slightly diminished cellular content,^{124,125} although this effect was gender-specific in the APOE^{-/-} mice.¹²⁵ These studies suggest a pro-atherogenic effect for IFN γ . This was further confirmed by administering IFN γ to atherosclerosis-prone mice, which enhanced the extent of atherosclerosis and the number of inflammatory cells within

the lesions.¹²⁶ To further explore which cells are responsible for the observed pro-atherogenic effect of IFN γ on atherosclerosis, the contribution of IFN γ producing leukocytes was investigated by transplanting LDLR^{-/-} mice with IFN γ ^{-/-} BM. These mice had more atherosclerosis, but the lesions were of a more stable plaque phenotype characterised by more collagen and unchanged cellular content.¹²⁷ Overall, these published results show that IFN γ acts pro-atherogenic on atherosclerosis by enhancing the inflammatory response.

The production of IFN γ by T-lymphocytes, NK cells and macrophages is regulated by other secreted cytokines, acting as stimulator, like IL-12 and IL-18 or as inhibitor, like IL-10 and TGF- β (Table 1.3B). Besides a direct action on the production of IFN γ , these cytokines also participate in the stimulation of IFN γ producing T-lymphocytes. That is, IL-12 is needed to induce the IFN γ producing T_H1-lymphocytes and IL-18 can enhance this action. However, if IL-10 is present, it down-regulates the T_H1-lymphocyte response by inhibiting the production of IL-12. In human and experimental atherosclerotic lesions, IL-12,^{128,129} IL-18,^{130,131} IL10^{128,132,133} and TGF- β ^{132,134} are present and they are mainly localised in macrophages. Therefore, the effect of these cytokines on experimental atherosclerosis was investigated. Inhibition or deletion of IL-12 or IL-18 resulted in diminished experimental atherosclerosis and a more stable phenotype,¹³⁵⁻¹³⁷ whereas administering IL-12 or IL-18 accelerated experimental atherosclerosis.^{129,138} On the other hand, IL-10 deficiency or reduction of TGF- β levels markedly enhanced the extent of atherosclerosis in atherosclerosis-prone mice,¹³⁹⁻¹⁴³ while overexpression of IL-10 reduced experimental atherosclerosis.¹³⁹ This protective effect of IL-10 and TGF- β on atherosclerosis was shown to be mediated by leukocytes. In atherosclerosis-prone mice, IL-10 deficiency in leukocytes increased the development of atherosclerotic lesions,¹⁴⁴ whereas overexpression of IL-10 in leukocytes decreased the extent of atherosclerosis.¹⁴⁵ For TGF- β it was reported that disruption of TGF- β signalling in T cells aggravated experimental atherosclerosis in one study.¹⁴⁶ Another study found that disruption of TGF- β signalling in T cells reduced the extent of atherosclerosis, although the lesions had a more vulnerable plaque phenotype.¹⁴⁷

Table 1.3B *T-lymphocytes and atherogenesis*

Defect or treatment	Effect on the immune system	Mouse model	Chow	Effect on atherosclerosis	References
IFN γ R ^{-/-}		APOE ^{-/-}	high fat diet	↓ atherosclerosis	123
IFN γ ^{-/-}	no IFN γ	APOE ^{-/-}	normal chow	female: none male: ↓ atherosclerosis	125
IFN γ ^{-/-}	no IFN γ	APOE ^{-/-}	high fat diet	female: none male: ↓ atherosclerosis	125
IFN γ ^{-/-}	no IFN γ	LDLR ^{-/-}	high fat diet	↓ atherosclerosis	124
Treatment with IFN γ		APOE ^{-/-}	normal chow	↑ atherosclerosis	126
IFN γ ^{-/-} BMT	no IFN γ producing leukocytes	LDLR ^{-/-}	high fat diet	↑ atherosclerosis	127
Electrotransfer with a murine IL-18BP expression plasmid	no IL-18	APOE ^{-/-}	normal chow	↓ atherosclerosis	136
IL-18 ^{-/-}	no IL-18	APOE ^{-/-}	normal chow	↓ atherosclerosis	137
IL-12 ^{-/-}	no IL-12	APOE ^{-/-}	normal chow	↓ atherosclerosis	135
Treatment with IL-18		APOE ^{-/-}	normal chow	↑ atherosclerosis	138
Treatment with IL-12		APOE ^{-/-}	normal chow	↑ atherosclerosis	129

Table 1.3B (continued) *T-lymphocytes and atherogenesis*

Defect or treatment	Effect on the immune system	Mouse model	Chow	Effect on atherosclerosis	References
IL-10 ^{-/-}	no IL-10	C57Bl/6	high fat diet	↑ atherosclerosis	139
IL-10 ^{-/-}	no IL-10	C57Bl/6	high fat diet	↑ atherosclerosis	140
IL-10 ^{-/-}	no IL-10	APOE ^{-/-}	normal chow	↑ atherosclerosis	141
TGFβ1 ^{+/-}	selective deficiency of the TGFβ1 isoform	C57Bl/6	high fat diet	↑ atherosclerosis	142
Treatment with anti-TGF-β mAb	systemic and local TGF-β neutralisation	APOE ^{-/-}	high fat diet	↑ atherosclerosis	143
IL-10 Tg	IL-10 overexpression in IL-2 producing cells	C57Bl/6	high fat diet	↓ atherosclerosis	139
IL-10 ^{-/-} BMT	no IL-10 producing leukocytes	LDLR ^{-/-}	high fat diet	↑ atherosclerosis	144
IL-10 Tg BMT	IL-10 overexpression in IL-2 producing leukocytes	LDLR ^{-/-}	high fat diet	↓ atherosclerosis	145
CD4-dnTGF-βRII Tg	No TGFβ signalling in all T-lymphocytes	APOE ^{-/-}	normal chow	↑ atherosclerosis	146
CD2-dnTGFβRII Tg BMT	No TGFβ signalling in all T-lymphocytes	LDLR ^{-/-}	high fat diet	↓ atherosclerosis	147

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In summary, IL-12 and IL-18 promote whereas IL-10 and TGF- β delay the development atherosclerosis by regulating the production of IFN γ .

In the last few years the recognition of Tregs as potent suppressors of the T-lymphocyte response has provided new insights into the pathogenesis of inflammatory diseases. Based on the phenotype or cytokine secretion, two specialised types of Tregs can be distinguished: the induced (or acquired) Tregs and the natural (or innate) Tregs. Induced Tregs are generated from naive T-lymphocytes upon activation by antigens not present in the thymus, which are presented in a tolerogenic manner. At present, two types of induced Tregs have been reported: the T_H3 and the T_R1 cells.^{89,148} T_H3 cells were discovered in mice, which were orally tolerised with foreign antigen and they were identified by their abundant secretion of TGF- β .^{89,148} On the other hand, *in vitro* repetitive antigen-stimulation of naive T-lymphocytes in the presence of IL-10 generates T_R1 cell clones, which are characterised by their secretion of high levels of IL-10.^{89,148} In mice and humans, these induced Tregs are most often found at sites of inflammation and at mucosal surfaces, where they locally regulate T-lymphocyte activity by their cytokine secretion.¹⁴⁹ Recently, induced Tregs were used as a therapeutic strategy in atherosclerosis. In this study, it was shown that transfer of OVA-specific T_R1 cells into APOE^{-/-} mice reduced the development of atherosclerosis and this was mainly due to an increased production of IL-10.¹⁵⁰

The other type of Tregs is the natural Treg, which was discovered when immunodeficient mice received T-lymphocytes depleted of CD25⁺ cells and spontaneously developed autoimmune disease in multiple organs. Natural Tregs originate from the thymus, they constitute 5-10% of the CD4 T-lymphocyte population in mice and are characterised by the constitutive expression of CD25 (the IL-2 receptor α chain).¹⁵¹ Furthermore, the natural Treg population presumably recognises a diversity of thymus-derived self-antigens similar to the specificity of circulating naive T-lymphocytes. Natural Tregs suppress the activation and proliferation of activated naive T-lymphocytes mainly by cell-cell interactions and inhibition of IL-2 production in primarily lymphoid organs.^{149,152} Natural Tregs have been shown to limit disease in several chronic inflammatory disorders like inflammatory bowel

disease, transplantation tolerance and thyroiditis.^{153,154} Therefore, natural Tregs might participate in the pathogenesis of atherosclerosis by regulating the immune response.

Overall, the published data clearly show that both B- and T-lymphocytes contribute to the pathogenesis of atherosclerosis. Especially T-lymphocytes are capable of enhancing and directing the effector mechanisms of the innate immune response in addition to modulating the inflammatory response by communicating with other cells involved in the atherosclerotic process and by producing cytokines.

1.4.3 CD40-CD40L interaction and atherogenesis

The CD40-CD40L interaction is a generally used interaction for the communication between inflammatory cells. CD40 is constitutively expressed on many cell types, among which are B-lymphocytes, DCs, activated macrophages and smooth muscle cells. CD40L, on the other hand, is inducible on a variety of cells such as T-lymphocytes, mast cells, NK cells, platelets, endothelial cells and smooth muscle cells. T-lymphocytes use the CD40-CD40L interaction to communicate with other cells, therefore signalling through this interaction has multiple effects. One effect is that co-stimulatory molecules are induced on antigen presenting B-lymphocytes, which enable full activation of T-lymphocytes.¹⁵⁵⁻¹⁵⁷ Another effect is that antigen presenting DCs or macrophages start producing IL-12, thereby stimulating a T_H1-lymphocyte response.¹⁵⁵⁻¹⁵⁷ Lastly, the CD40-CD40L interaction is essential for the T-lymphocyte dependent B-lymphocyte activation, differentiation and isotype class switching.¹⁵⁵⁻¹⁵⁷ In atherosclerotic lesions expression of both CD40 and CD40L is found, thereby implicating the involvement of the CD40-CD40L interaction in atherosclerosis.¹⁵⁶⁻¹⁵⁹ This role of the CD40-CD40L interaction in atherogenesis was further established by the determination of atherosclerosis in the absence of CD40L. Advanced atherosclerotic lesions of APOE^{-/-} mice deficient for CD40L were of a more stable plaque phenotype, characterised by increased collagen content and less inflammation.¹⁶⁰ Moreover, treatment of APOE^{-/-} or LDLR^{-/-} mice with anti-CD40L antibodies also resulted in the development of advanced atherosclerotic lesions with a more stable plaque phenotype.^{161,162} Even more important, treatment with anti-CD40L antibodies could transform atherosclerotic lesions, that were already present, into a more stable plaque phenotype.^{162,163}

Summarising, these experiments show that interruption of the CD40-CD40L interaction reduces inflammation and stimulates fibrosis in the atherosclerotic lesion, thereby inducing a more stable plaque phenotype.

1.5 This thesis

Nowadays, atherosclerosis is generally accepted as a chronic inflammatory disease. The key findings upon which this statement is based have been elucidated in the previous paragraphs. The development of atherosclerosis is initiated by innate immunity, mainly by macrophages, but the progression into the advanced stage of atherosclerosis is the result of a balance between inflammation and tissue healing. Both innate and adaptive immunity contribute to this inflammatory response. In particular, B-lymphocytes produce antibodies directed against lesion constituents that can accelerate or delay the uptake of oxLDL by macrophages in the atherosclerotic lesion. Additionally, T-lymphocytes are capable of modulating the inflammatory processes involved in atherogenesis by stimulating or inhibiting innate immune cells by cell-cell interactions or by secreting cytokines, like IFN γ , IL-10 or TGF- β .

1.5.1 Aim of this thesis

The overall aim of this thesis was to investigate how adaptive immunity is involved in advanced atherosclerosis. This aim was subdivided into three hypotheses. Anti-oxLDL antibody levels correlate with the severity of human atherosclerosis, therefore, anti-oxLDL antibodies might be a good marker for discriminating between stable and unstable or vulnerable atherosclerotic lesions. As described earlier, disruption of the CD40-CD40L interaction in APOE^{-/-} mice resulted in advanced lesions with a more stable plaque phenotype. Furthermore, the CD40-CD40L interaction is essential for isotype switching of B-lymphocytes and for the induction of T_H1-lymphocytes. Based on these findings, our first hypothesis is that in APOE^{-/-}CD40L^{-/-} mice the pro-atherogenic IgG anti-oxLDL antibody levels will be absent and the protective IgM anti-oxLDL antibody levels will be enhanced when compared to APOE^{-/-} mice. Our second hypothesis is that adaptive immune cells are responsible for the observed stable plaque phenotype in APOE^{-/-}CD40L^{-/-} mice.

In the second part of this thesis the T-lymphocyte response in atherosclerosis will be further assessed. T-lymphocytes are shown to contribute to the progression of atherosclerosis. However, it is still unknown whether the observed T-lymphocyte response in atherosclerosis is the result of defective suppression by Tregs. Additionally, it has not been proven yet that T-lymphocytes present in the atherosclerotic lesions are innocent bystanders or antigen driven. Therefore, our third hypothesis is that Tregs and/ or MHCII are essential components for the development of advanced atherosclerotic lesions.

1.5.2 Outline of this thesis

The first chapters of this thesis describe whether the observed stable plaque phenotype of advanced atherosclerotic lesions after disruption of the CD40-CD40L interaction in atherosclerosis-prone mice is the result of CD40L defective leukocytes. In *Chapter 2*, the levels of IgM and IgG antibodies directed against oxLDL as markers for the severity of atherosclerosis was investigated in APOE^{-/-}CD40L^{-/-} mice. In *Chapter 3*, the involvement of leukocytes in CD40L-deficient atherosclerosis-prone mice was studied by comparing atherosclerosis development in LDLR^{-/-} mice transplanted with BM isolated from CD40L^{-/-} or wild-type mice. Additionally, *Chapter 3* contains preliminary results of atherosclerosis developed in LDLR^{-/-} mice transplanted with CD40L^{-/-} BM, which were either treated with anti-CD40L antibodies or isotype control antibodies.

In the last chapters of this thesis, the focus is on the role of adaptive immunity in atherosclerosis. First, the effect of Tregs on the development of advanced atherosclerotic lesions was explored (*Chapter 4 and 5*). In *Chapter 4*, APOE^{-/-} mice were treated with recombinant soluble TGF- β receptor II thereby inhibiting TGF- β signalling, while in *Chapter 5* natural Tregs were depleted by treating LDLR^{-/-} mice with anti-CD25 antibodies. Finally, the involvement of antigen presentation in atherosclerosis was studied in *Chapter 6*. This was done by analysing the progression of atherosclerosis in LDLR^{-/-}MHCII^{-/-} mice. In *Chapter 7*, the results of these studies and implications for further research are discussed.

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Chapter 2

IgM anti-oxLDL antibodies, but not IgG anti-oxLDL antibodies, as plasma marker for progressive atherosclerosis

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In preparation

2.1 Abstract

In humans and animal models antibodies against oxLDL have been associated with atherosclerosis severity, although the exact relation is not clear. In the present study, we examined whether IgM and/or IgG anti-oxLDL antibody levels are reliable markers for lesion development in atherosclerotic mice. This was investigated in APOE^{-/-}CD40L^{-/-} mice, which are deficient for IgG and develop moderate advanced atherosclerosis. They were compared to mice developing severe (APOE^{-/-}) or no atherosclerosis (C57Bl/6). In addition, APOE^{-/-} mice were used as model for progressive atherosclerosis. Anti-oxLDL antibody levels were determined by an enzyme linked immunosorbent assay (ELISA). Results revealed that 24-week-old APOE^{-/-}CD40L^{-/-} mice had enhanced IgM anti-oxLDL antibody levels when compared to wild-type mice, but similar levels to those of APOE^{-/-} mice. As expected, the IgG anti-oxLDL antibody levels were almost absent in APOE^{-/-}CD40L^{-/-} mice. In APOE^{-/-} mice, the transition from early to advanced lesions was reflected by elevated IgM anti-oxLDL antibody levels, although they did not further increase during progression to more advanced lesions. In conclusion, the level of IgM anti-oxLDL antibodies does reflect the transition from early to advanced atherosclerosis. However, anti-oxLDL antibody levels are not a reliable marker for the severity of advanced atherosclerosis.

2.2 Introduction

The key initiating event in atherosclerosis is retention and oxidation of LDL in the vascular wall.¹⁻³ The LDL particle consists of an outer layer of phospholipids, apoB and free cholesterol and a core of cholesterol, which is bound into long esters by fatty acid chains.⁴ The LDL particle can diffuse into the arterial wall, where apoB can bind to the matrix proteoglycans, thereby facilitating the subendothelial retention of LDL. Trapped LDL undergoes aggregation or oxidation, thereby further enhancing its retention in the vascular wall.^{1,5} Oxidation of the fatty acid chains of the LDL particle results in oxidised lipids and the generation of aldehydes, like malondialdehyde. The generated malondialdehyde then forms adducts with the lysine residues of apoB, thereby generating oxLDL.^{2,3,5} The *in vivo* existence of oxLDL has been shown by the extraction of oxLDL from atherosclerotic lesions of both humans and animals.^{2,3} Considerable evidence indicates that oxLDL participates not only in the induction, but also in the progression of atherosclerosis. *In vitro*, oxLDL, but not LDL, is rapidly internalised by macrophages, which results in the generation of foam cells.^{2,4} Furthermore, oxLDL attracts monocytes and T-lymphocytes thereby amplifying the inflammatory response in atherosclerosis. Finally, it was demonstrated that oxLDL is immunogenic.^{2,4}

Antibodies directed against oxLDL can be extracted from human and experimental atherosclerotic lesions^{6,7} and circulating anti-oxLDL antibodies can be detected in human individuals^{6,8} and animal models of atherosclerosis.^{6,9} Both in humans and animals, it has been reported that the level of circulating IgG anti-oxLDL antibodies positively correlates with the severity of atherosclerosis,¹⁰⁻¹³ although this is not a consistent finding.^{14,15} Additionally, the level of anti-oxLDL antibodies can be further enhanced by immunisation with oxLDL and this results in reduced development of atherosclerosis in atherosclerosis-prone mice.¹⁶⁻¹⁸ However, this effect cannot only be attributed to the enhanced anti-oxLDL antibody levels, since mice immunised with LDL or CFA alone also developed less atherosclerosis.^{16,19-21} Recently, it was reported that pneumococcal vaccination or immunisation with oxLDL clearly enhanced the natural IgM anti-oxLDL antibody response and subsequently decreased the extent of atherosclerosis in atherosclerosis-prone mice.^{22,23}

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In the present study, we examined whether IgM and/or IgG anti-oxLDL antibody levels are reliable markers for lesion development in atherosclerotic mice. For this purpose, we used APOE^{-/-}CD40L^{-/-} mice, which are deficient for IgG and develop smaller advanced lesions with a more stable plaque phenotype than APOE^{-/-} mice.²⁴ APOE^{-/-}CD40L^{-/-} mice were compared with mice developing severe (APOE^{-/-})²⁵⁻²⁷ or no atherosclerosis (C57Bl/6).²⁸ In addition, the effect of progressive atherosclerosis on the level of anti-oxLDL antibodies was determined in 12- and 24-week-old APOE^{-/-} mice fed normal chow (NC) or a high fat diet (HFD).^{29,30}

2.3 Methods

Mice

APOE^{-/-}CD40L^{-/-} and APOE^{-/-} mice (all tenth generation C57Bl/6, originally obtained from Jackson's lab, Bar Harbor, Maine) were maintained at our animal facility (Maastricht, The Netherlands). Wild-type (C57Bl/6) mice were obtained from Iffa Credo (Lyon, France). Male littermates were given NC or at 5 weeks of age a HFD containing 16% fat and 0.15% cholesterol (Hope Farms, Woerden, the Netherlands) for 7 or 19 weeks (Table 2.1).

Table 2.1 Overview of collected plasma samples

	Wild-type (C57Bl/6)	APOE ^{-/-} CD40L ^{-/-}	APOE ^{-/-}
12 wks old – 7 wks NC			N = 11
24 wks old – 19 wks NC	N = 10	N = 10	N = 12
12 wks old – 7 wks HFD			N = 4
24 wks old – 19 wks HFD			N = 5

Prior to sacrifice, mice were anaesthetised by i.p. injection of 25 µg/g xylazine (Sedamun, Eurovet, Bladel, the Netherlands) and 125 µg/g ketamine (Nimatek, Eurovet). Subsequently, heparinised blood was obtained from the inferior vena cava, which was followed by perforation of the diaphragm. Plasma samples were collected from the blood samples. All procedures were approved by the animal care and use committee of the Maastricht University and were in accordance with national regulations on animal experiments.

Anti-oxLDL antibody ELISA

Native LDL ($d = 1.030\text{--}1.045$ g/ml) was isolated from human plasma by density-gradient ultracentrifugation.³¹ Salts were removed by rapid filtration through disposable desalting columns (Econo-Pac 10 DG, Bio-Rad, Hercules, California). Isolated LDL was modified by malondialdehyde (MDA, Merck, Hohenbrunn, Germany) in a final concentration of 169 mM.³² Native LDL and oxLDL were stored in PBS with 1 mg/ml EDTA as a preservative under a protective atmosphere of nitrogen at 4°C and used within 14 days. MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µg native LDL or oxLDL in 100 µl PBS under a protective N₂-atmosphere at 4°C. Plates were washed 5 times with a buffer containing 0.01 M Tris, 0.15 M NaCl and 0.05% Tween20 (pH 8.0). Mouse plasma was added in duplicate at a 1:50 dilution in incubation buffer (0.1 M Tris, 0.3 M NaCl and 0.05% Tween20 (pH 8.0)) overnight at 4°C. After washing the plates, they were incubated with either alkaline phosphatase-labeled anti-mouse IgM or IgG (Jackson Immuno-Research Laboratory, Westgrove, Pennsylvania) both at a 1:4000 dilution in incubation buffer for 1 hour at 37°C. Then the plates were washed again and developed by adding freshly made substrate containing 1 mg/ml disodium p-nitrophenyl phosphate (Sigma, St. Louis, Missouri) in diethanolamine buffer at pH 9.8. After 120 minutes on a shaking platform at room temperature, the absorbance of the wells was read at 405 nm in an ELISA reader. Anti-oxLDL antibody levels were calculated by subtracting the mean absorbance of native LDL from that of oxLDL.

Lipid parameters

Plasma cholesterol levels were determined using standard colorimetric assays (Sigma, St. Louis, Missouri) in all collected plasma samples.

Statistical analysis

Results are presented as mean±SEM. The data were analysed with the Kolmogorov-Smirnov (KS) test to assess normality and the F-test to compare variances. When results passed both tests ($p < 0.05$) they were analysed with the one-way ANOVA (two-sided) and Bonferroni posttest. A p -value of $p < 0.05$ was accepted as statistically significant.

2.4 Results

Anti-oxLDL antibody response as marker for the severity of advanced atherosclerosis

To test whether the anti-oxLDL antibody response reflects the severity of advanced atherosclerosis in mice, we measured the levels of IgM and IgG anti-oxLDL antibodies by ELISA. The anti-oxLDL antibody levels of 24-week-old $APOE^{-/-}CD40L^{-/-}$ mice with moderate atherosclerosis and impaired isotype switching were compared with those of 24-week-old wild-type (C57Bl/6) mice with no atherosclerosis or $APOE^{-/-}$ mice with severe atherosclerosis. All examined mice, including wild-type mice, had detectable levels of circulating IgM and IgG anti-oxLDL antibodies (Figure 2.1).

Figure 2.1 Analysis of the antibody response to oxLDL in $APOE^{-/-}CD40L^{-/-}$ mice. In plasma of $APOE^{-/-}CD40L^{-/-}$, $APOE^{-/-}$ and C57Bl/6 mice, binding of IgM (A) and IgG (B) antibodies directed against native LDL or oxLDL were determined by ELISA. The net absorbance was calculated by subtracting the mean absorbance of native LDL from that of oxLDL. Data represent individual mice and the mean values (horizontal lines).

In $APOE^{-/-}CD40L^{-/-}$ mice, the level of circulating IgM anti-oxLDL antibodies was significantly enhanced in comparison to that of wild-type mice (Figure 2.1A: 2.1 ± 0.1 , $APOE^{-/-}CD40L^{-/-}$ vs. 1.4 ± 0.2 , C57Bl/6, $p < 0.05$). Despite the difference in atherosclerosis severity, the IgM anti-oxLDL antibody level of $APOE^{-/-}CD40L^{-/-}$ and $APOE^{-/-}$ mice were comparable. IgM anti-oxLDL antibody levels of $APOE^{-/-}$ mice were also not significantly

higher than those in wild-type mice (Figure 2.1A). Since CD40L-deficient mice have impaired isotype switching, it was expected that the IgG anti-oxLDL antibody levels were almost absent in APOE^{-/-}CD40L^{-/-} mice when compared to either wild-type or APOE^{-/-} mice (Figure 2.1B: 0.04 ± 0.01 , APOE^{-/-}CD40L^{-/-} vs. 0.11 ± 0.02 , C57Bl/6, $p < 0.05$ and vs. 0.13 ± 0.02 , APOE^{-/-}, $p < 0.01$). There was no difference detected between the IgG anti-oxLDL antibody levels of APOE^{-/-} and wild-type mice (Figure 2.1B).

Anti-oxLDL antibody response as indicator for the progression of atherosclerosis

To further investigate whether the anti-oxLDL antibody response might correlate with the progression of atherosclerosis, we assessed the levels of IgM and IgG anti-oxLDL antibodies in APOE^{-/-} mice at various stages of atherosclerosis. In 24-week-old APOE^{-/-} mice with predominantly advanced atherosclerotic lesions the levels of IgM anti-oxLDL antibodies were markedly elevated in comparison to 12-week-old APOE^{-/-} mice with mostly early lesions (Figure 2.2A: 1.8 ± 0.2 , APOE^{-/-} 24 wk old vs. 1.1 ± 0.1 , APOE^{-/-} 12 wk old, $p < 0.05$). To determine whether this elevation of the IgM anti-oxLDL antibody response is the result of hypercholesterolemia, a correlation between cholesterol levels and IgM anti-oxLDL antibodies was determined. A clear correlation was found (Figure 2.2B: $R^2 = 0.3033$, $p < 0.01$). Further progression of atherosclerosis into more advanced atherosclerotic lesions induced by HFD feeding did not additionally enhance the IgM anti-oxLDL antibody levels in APOE^{-/-} mice (Figure 2.2A). The IgG anti-oxLDL antibody response was unaltered by the progression of atherosclerosis (Figure 2.2C).

Figure 2.2 Analysis of the antibody response to oxLDL in APOE^{-/-} mice. In plasma of APOE^{-/-} mice fed NC or HFD, binding of IgM (A) and IgG (C) antibodies directed against native LDL or oxLDL were determined by ELISA. The net absorbance was calculated by subtracting the mean absorbance of native LDL from that of oxLDL. Data represent individual mice and the mean values (horizontal lines). Plasma cholesterol levels were correlated with the IgM anti-oxLDL antibodies in 12 wk old (■) and 24 wk old (◆) APOE^{-/-} mice (B). Data represent individual mice.

2.5 Discussion

In the current study, we examined whether circulating anti-oxLDL antibody levels are reliable markers for atherosclerotic lesion development in mice. All mice had detectable levels of circulating IgM and IgG anti-oxLDL antibodies, regardless of the presence or absence of hypercholesterolemia and atherosclerosis. We found that the severity of advanced atherosclerosis was not reflected by IgM or IgG anti-oxLDL antibody levels in mice. However, the level of IgM anti-oxLDL antibodies did increase during the transition from early to advanced lesions in APOE^{-/-} mice, although it did not increase further upon progression to more advanced lesions.

In contrast to previously published results,^{9,33} 24-week-old wild-type mice had comparable basal levels of IgM and IgG anti-oxLDL antibodies as APOE^{-/-} mice. This discrepancy might be the result of variations between the studies. One source of variability is the used ELISA to detect anti-oxLDL antibodies. The origin of LDL and its oxidation may differ, but also the detection system and representation of the obtained results are not entirely the same. Unfortunately, the ELISA to detect anti-oxLDL antibodies is not yet standardised.³⁴ Another source of variability is the used C57Bl/6 strain, which might differ slightly from that examined by Palinski *et al.*⁹ or Zhou *et al.*,³³ because of different specified pathogen free conditions. On the other hand, in the splenectomy study of Caligiuri *et al.*,³⁵ it was found that spleen cells isolated from C57Bl/6 mice were capable of rescuing APOE^{-/-} mice from the pro-atherogenic effect of splenectomy. This rescuing effect of C57Bl/6 splenocytes was more pronounced than when spleen cells isolated from 6-week-old APOE^{-/-} mice were transferred. The protective effect of the splenocytes was shown to be established by B-lymphocytes³⁵ suggesting that this effect may have been due to anti-oxLDL antibodies. Also in healthy humans, IgG anti-oxLDL antibodies are detectable^{6,8} suggesting that these antibodies are part of the normal circulating reservoir of antibodies.

In this study, we examined whether the anti-oxLDL antibody levels reflect the development of advanced atherosclerotic lesions. Circulating IgM anti-oxLDL antibodies may contribute to maintain low plasma levels of oxLDL.³⁶ In addition, IgM anti-oxLDL antibodies can block the uptake of oxLDL by macrophages in the atherosclerotic lesion, thereby preventing the accumulation of oxLDL and foam cell formation.^{37,38} These data suggest that

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IgM anti-oxLDL antibodies have a mainly protective role in atherosclerosis. On the other hand IgG anti-oxLDL antibodies might have a pro-atherogenic effect, since they form immune complexes with oxLDL that can bind to Fc receptors present on macrophages, thereby generating an inflammatory signal.³⁹⁻⁴¹ Based on these findings, we hypothesised that moderate advanced atherosclerosis would be reflected by a relatively high level of IgM anti-oxLDL antibodies and a lower level of IgG anti-oxLDL antibodies, whereas severe advanced atherosclerosis would be associated with a significantly higher level of IgG anti-oxLDL antibodies and a lower level of IgM anti-oxLDL antibodies. As a model for moderate advanced atherosclerosis, we used 24-week-old APOE^{-/-}CD40L^{-/-} mice, which develop smaller advanced lesions with a more stable plaque phenotype characterised by less inflammatory cells and a thickened collagen cap.²⁴ 24-week-old APOE^{-/-} mice or APOE^{-/-} mice on a HFD were used as model for severe advanced atherosclerosis.^{29,30} In the current study, we did not find a significantly enhanced IgM anti-oxLDL antibody level in APOE^{-/-}CD40L^{-/-} mice when compared to APOE^{-/-} mice, nor did the progression of atherosclerosis in APOE^{-/-} mice result in an increased level of IgG anti-oxLDL antibodies. Therefore, our findings do not support this hypothesis. We did find, as was previously shown by Zhou *et al.*,³³ that the progression of atherosclerosis from early to advanced lesions was associated with an increased level of IgM anti-oxLDL antibodies in APOE^{-/-} mice. One possible explanation might be that the natural antibody response was enhanced in 24-week-old APOE^{-/-} mice, which can be investigated by determining the clonotype of the IgM anti-oxLDL antibodies. A more plausible explanation is that in early lesions, oxLDL is predominantly localised in macrophage-derived foam cells, while in advanced lesions oxLDL is mainly present extracellularly in the necrotic core.⁹ In growing early lesions, foam cells die and eventually form a necrotic core, a characteristic of advanced lesions. During this formation of the necrotic core oxLDL might become more freely available, which can then more easily be taken up by dendritic cells and presented to the immune cells in lymphoid organs to generate an anti-oxLDL antibody response. However, apparently the produced IgM anti-oxLDL antibodies are not sufficient to stop or delay the atherosclerotic process. Since the high fat feeding does only result in a more advanced lesion with more necrosis and less collagen, the oxLDL present in the necrotic core will not be able to additionally enhance the anti-oxLDL antibody response.

In conclusion, not only atherosclerotic mice, but also wild-type mice have circulating IgM and IgG anti-oxLDL antibodies. The transition of early to advanced atherosclerosis is accompanied by the induction of an increased IgM anti-oxLDL antibody response. However, measurement of the anti-oxLDL antibody levels is not a reliable marker for the extent of advanced atherosclerosis. Further research should clarify whether the level of IgM anti-oxLDL antibodies as marker for advanced atherosclerosis can be improved by discriminating between natural and induced IgM anti-oxLDL antibodies.

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Chapter 3

Leukocyte CD40L deficiency affects the CD25⁺ CD4 T cell population but does not affect atherosclerosis

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3.1 Abstract

Inhibition of CD40-CD40L interactions results in a reduction of natural Tregs in CD40^{-/-} mice and induces a stable plaque phenotype in atherosclerosis-prone mouse strains. Here we investigated the effects of leukocyte CD40L on the Treg population and on atherosclerosis. LDLR^{-/-} mice were reconstituted with wild-type or CD40L^{-/-} BM. These BM chimeras were analysed by flowcytometry for the presence of natural Tregs (CD45RB^{low} CD25⁺ CD4⁺) in lymphoid organs and peripheral blood. As in CD40^{-/-} mice, the CD45RB^{high}:CD45RB^{low} CD4 T cell ratio significantly increased and the CD25⁺ CD4⁺ subpopulation significantly decreased in LDLR^{-/-} mice receiving CD40L^{-/-} BM compared to LDLR^{-/-} mice receiving wild-type BM. However, atherosclerotic plaque progression and plaque phenotype did not change in LDLR^{-/-} mice reconstituted with CD40L^{-/-} BM. In conclusion, the present study shows that CD40-CD40L interactions on leukocytes are essential for the size of the CD45RB^{low} CD25⁺ CD4 Treg subpopulation. Nevertheless, CD40L deficiency on haemopoietic cells did not affect atherosclerosis, implying that CD40L expressing leukocytes alone are not responsible for the stable plaque phenotype observed after total CD40L blockade.

3.2 Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall. Atherosclerotic lesions contain large numbers of immune cells, particularly macrophages and T-lymphocytes, indicating a role for innate and adaptive immunity in the pathogenesis of atherosclerosis. The importance of adaptive immunity in atherogenesis has been established using animal models.^{1,2} Depleting adaptive immune cells, *i.e.* both T- and B-lymphocytes, does not prevent development of disease in atherosclerosis-prone mice, although lesion initiation and disease progression in early atherogenesis in RAG1/LDLR double knockout mice are delayed.³ Recently, the role of T-lymphocytes or B-lymphocytes in atherogenesis was further assessed. Depletion of B-lymphocytes in LDLR^{-/-} mice results in increased atherosclerosis⁴ and transfer of B-lymphocytes into APOE^{-/-} mice reduces atherosclerosis.⁵ On the other hand, transferring CD4⁺ T cells from APOE^{-/-} to immunodeficient scid/scid APOE^{-/-} mice aggravates atherosclerosis and is associated with the infiltration of transferred T-lymphocytes into the lesions.⁶ In addition, atherosclerosis is enhanced in LDLR^{-/-} mice after adoptive transfer with antigen-reactive T-lymphocytes.⁷

Recent work has documented the importance of the CD40-CD40L interaction in the pathogenesis of atherosclerosis.^{8,9} Interruption of the CD40-CD40L interaction not only diminishes the formation and progression of atheroma, but also results in the development of an atherosclerotic plaque phenotype, that is characterised by a reduced T cell and macrophage content and a thickened collagen cap.^{10,11} The interaction between CD40 and CD40L (CD154) was originally described to be important for the interaction between T- and B-lymphocytes mainly affecting B cell functions.¹² Further research showed that various other cells, including APCs, express CD40L and CD40 and that these CD40-CD40L interactions are critically involved in T-lymphocyte priming, activation and differentiation.¹³ Furthermore, CD40 signalling is important for the induction of Tregs by interacting with APCs.¹⁴ Tregs have originally been described in experimental animal models for autoimmune disease. Several subsets of Tregs can be distinguished by phenotype or function. One subset, the natural Tregs, has been phenotypically characterised as CD45RB^{low}, CD25⁺ T_H-lymphocytes that are able to maintain immunological self-tolerance and avoid autoimmunity mainly by direct cell contact. Another subset, the

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acquired Tregs, that include the T_{R1} and T_{H3} Tregs, produce cytokines like IL-10 and TGF β upon activation.¹⁵⁻¹⁸ Recently, it was shown that T_{R1} cells can alter the development of atherosclerosis by secreting IL-10.¹⁹ However, whether natural Tregs are implicated in the pathogenesis of atherosclerosis is unknown.

Given the importance of IFN- γ producing T-lymphocytes in plaque destabilisation²⁰ and the role of CD40-CD40L interactions on the size of the Treg population and in atherosclerosis,^{1,14,21} we examined the relative presence of CD40L dependent Tregs and investigated the role of leukocyte CD40L in atherosclerosis development. Therefore, BM chimeras were generated by reconstituting LDLR^{-/-} mice with CD40L^{-/-} or wild-type BM.

3.3 Methods

Mice

Male CD40L^{-/-} mice (provided by Dr R.A. Flavell, Yale Medical School, New Haven, Connecticut) were maintained at our animal facility (Maastricht, The Netherlands) and were backcrossed to C57Bl/6J for at least 10 generations. Male wild-type (C57Bl/6J) mice were obtained from Iffa Credo (Lyon, France). Female LDLR^{-/-} mice were maintained at our animal facility and backcrossed to C57Bl/6J for 4 generations. Mice were given regular chow unless stated otherwise. All procedures were approved by the ethical committee and in accordance with national regulations on animal experiments.

Bone marrow transplantation

Female LDLR^{-/-} mice were 9-13 weeks old and maintained in filter-top cages. They were given water containing polymyxin B sulphate (60,000 U/l) and neomycin (100 mg/l) from 1 week before transplantation until 4 weeks thereafter. Mice were lethally X-irradiated (10 Gy, 0.5 Gy/min, Philips MU15F/ 225 kV, Hamburg, Germany) and intravenously injected with 10^7 BM cells from either male wild-type (C57Bl/6J) or male CD40L^{-/-} mice on the next day. Four weeks after BM transplantation, mice were put on a HFD containing 16% fat and 0.15% cholesterol (Hope Farms, Woerden, the Netherlands) for 20 weeks. One week prior to sacrifice, DNA was isolated from blood cells by GFXTM Genomic Blood DNA purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and used for analysing the degree of chimerism by TaqMan analysis of the LDLR gene. Chimerism did

not significantly differ between LDLR^{-/-} mice transplanted with wild-type BM (95.3±1.2%) or CD40L^{-/-} BM (95.1±2.2%).

Flowcytometry

Heparinised blood obtained from the inferior vena cava, spleen and lymph nodes (superficial and deep cervical nodes, brachial nodes and mesenteric nodes) were isolated from the mice. Single-cell suspensions were made of spleen and lymph nodes.

Erythrocytes were removed by hypotonic lysis with NH₄Cl in peripheral blood and spleen.²² Cells (1×10⁶ per sample) were triple stained with CD3-FITC (17A2), CD8α-PE (53-6.7), CD4-biotin (RM4-5), or CD45RB-FITC (16A), CD25-PE (PC61) and CD4-biotin (RM4-5) (All antibodies were from BD-Pharmingen, San Diego, California). The biotin-labelled antibodies were detected in a second step with Streptavidin-CyChrome (BD-Pharmingen). Flowcytometry analysis was performed on a FACS Calibur and analysed with CellQuest software (BD-Bioscience, San Jose, California). Data were collected of 1×10⁴ CD3⁺ cells when stained with CD3-FITC, CD8α-PE and CD4-biotin or of 1×10⁴ CD4⁺ cells when stained with CD45RB-FITC, CD25-PE and CD4-biotin.

Lipid parameters

One week prior to sacrifice, a blood sample was taken from the LDLR^{-/-} mice after an overnight fast. Plasma cholesterol and plasma total triglyceride levels were determined using standard colorimetric assays (Sigma, St. Louis, Missouri).

Tissue processing

Mice were anaesthetised by i.p. injection of 25 µg/g xylazine (Sedamun, Eurovet, Bladel, the Netherlands) and 125 µg/g ketamine (Nimatek, Eurovet). Mice were perfused through the apex of the heart for 1 min with phosphate-buffered saline (PBS) containing sodium nitroprusside (Sigma) and for 3 min with 1% phosphate-buffered paraformaldehyde, pH 7.4. The heart was removed by cutting the aorta just above the atria and fixed overnight in 1% phosphate-buffered paraformaldehyde. Subsequently, the heart was divided in two by cutting perpendicular to the heart axis just below the atrial tips and processed and

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embedded in paraffin. Eighty serial sections of the aortic root, 4 μ m each, were cut and used for analysis.

Histomorphometry and immunohistometry

Eight sections of the aortic root, 40 μ m apart, were stained with either hematoxylin and eosin or Lawson and were used for determining lesion area and lipid core content. Tissue sections were stained for collagen content (Sirius red), smooth muscle cell content (monoclonal anti-smooth muscle actin-FITC, Sigma), macrophage content (Mac-3, BD-Pharmingen) and T cell content (CD3, DAKO, Glostrup, Denmark). All morphometric parameters were determined using a microscope coupled to a computerised morphometry system (Quantimet 570, Leica, Rijswijk, the Netherlands). Lesion area was determined as an average of at least 3 sections per mouse. In the central section of the aortic root lipid core content, collagen content, smooth muscle cell content and macrophage content were determined. The content was expressed as positive area relative to the total plaque area. The number of T cells was counted and expressed relative to the total amount of cells counted in the aortic root.

Statistical analysis

Results are presented as mean $\pm$ SEM. The data were analysed with the Kolmogorov-Smirnov (KS) test to assess normality and the F-test to compare variances. When results passed both tests ($p < 0.05$) they were analysed with the unpaired T-test (two-sided). A p -value of $p < 0.05$ was accepted as statistically significant.

3.4 Results

Regulatory T cell population is reduced after CD40L bone marrow transplantation

To determine whether both BM chimeras were comparable for their overall T cell compartment, total CD3, CD4 and CD8 T cells and the CD4:CD8 T cell ratio were measured in peripheral blood, spleen and lymph nodes by flowcytometry. Since the results for the three tissues were comparable, only the results of lymph nodes are shown. Table 3.1 shows that the CD3, CD4 and CD8 T cell compartment and the CD4:CD8 T cell ratio did not differ between LDLR^{-/-} mice transplanted with either wild-type BM or CD40L^{-/-} BM.

Table 3.1 *T cell values of LDLR^{-/-} mice receiving either wild-type or CD40L deficient BM*

	LDLR ^{-/-} with wild-type BM	LDLR ^{-/-} with CD40L ^{-/-} BM
	N = 6	N = 6
# CD3 ⁺ cells (×10 ⁶)	2.5 ± 0.2	3.3 ± 0.5
# CD4 ⁺ T cells (×10 ⁶)	1.3 ± 0.1	1.6 ± 0.2
# CD8 ⁺ T cells (×10 ⁶)	0.9 ± 0.1	1.1 ± 0.1
CD4:CD8	1.37 ± 0.04	1.46 ± 0.04

Values are mean±SEM. Presented results are derived from lymph nodes.

To assess the CD4 Treg population two generally used phenotypic determinants, the CD45RB^{high}:CD45RB^{low} T cell ratio and the percentage of CD25⁺ T cells within the CD4 T cell compartment, were measured in the BM chimeras as well as in wild-type and CD40L^{-/-} donor mice. Analysis of the CD45RB^{high}:CD45RB^{low} ratio revealed an increase due to a decrease of the CD45RB^{low} population in CD40L^{-/-} donor mice, but also in LDLR^{-/-} mice transplanted with CD40L^{-/-} BM. As shown in Figure 3.1, this ratio was 4.1±0.4 in CD40L^{-/-} donor mice and 3.3±0.4 in LDLR^{-/-} mice receiving CD40L^{-/-} BM compared to 2.2±0.1 in wild-type donor mice and 1.6±0.1 in LDLR^{-/-} mice receiving wild-type BM. In Figure 3.2, analysis of the percentage of CD25⁺ cells within the CD4 T cell population showed that the observed increases in the CD45RB^{high}:CD45RB^{low} ratio in CD40L-deficient mice were accompanied by significant decreases in the percentage of CD25⁺ CD4⁺ T cells (7.1±0.2%, CD40L^{-/-} vs. 13.1±0.4%, wild-type and 8.2±0.2%, CD40L^{-/-} BM vs. 14.7±0.4%, wild-type BM). Since the percentage of CD25⁺ CD4⁺ T cells of transplanted LDLR^{-/-} mice was similar to that of the donor mice, the size of the Treg compartment is intrinsic to the genotype of the BM stem cell. Characteristically, all CD25⁺ CD4⁺ T cells were included in the CD45RB^{low} population (Figure 3.2A). In summary, the Treg compartment was reduced in LDLR^{-/-} mice receiving CD40L^{-/-} BM compared to LDLR^{-/-} mice receiving wild-type BM as determined by the CD45RB^{high}:CD45RB^{low} ratio and by the percentage of CD25⁺ CD4⁺ T cells.

Figure 3.1 The $CD45RB^{high}:CD45RB^{low}$ ratio is increased in $LDLR^{-/-}$ mice receiving $CD40L^{-/-}$ BM. Isolated lymph node cells were incubated with anti-CD4 (T_H -lymphocytes) and anti-CD45RB mAb and analysed by flowcytometry. A, The histograms display the CD45RB expression of $CD4^+$ T cells of a representative mouse. B, Representation of the $CD45RB^{high}:CD45RB^{low}$ ratio of the mice groups (mean $\pm$ SEM).

Figure 3.2 The size of the $CD25^{+}$ $CD4$ T cell population is intrinsic to haemopoietic cells and results in a reduced $CD25^{+}$ $CD4$ T cell compartment in $LDLR^{-/-}$ mice receiving $CD40L^{-/-}$ BM. Isolated lymph node cells were incubated with anti- $CD4$ (T_H -lymphocytes) and anti- $CD25$ mAb and analysed by flowcytometry. A, The histograms display the $CD25$ expression of $CD4$ T cells of a representative mouse. All $CD25^{+}$ cells were included in the $CD45RB^{low}$ population as shown by the dotplots. B, Representation of the $CD25$ expression of $CD4$ T cells of the mice groups (mean $\pm$ SEM).

Transplantation of CD40L BM does not affect the atherosclerotic plaque phenotype.

LDLR^{-/-} mice transplanted with either wild-type BM or CD40L^{-/-} BM developed similar plasma cholesterol levels (1,659±175 mg/dl, CD40L^{-/-} BM vs. 1,682±149 mg/dl, wild-type BM) and triglyceride levels (177±23 mg/dl, CD40L^{-/-} BM vs. 150±10 mg/dl, wild-type BM) after 20 weeks of HFD. Histomorphometric analysis of the atherosclerotic lesions in the aortic root revealed that none of the tested parameters were significantly different between LDLR^{-/-} mice transplanted with wild-type BM or CD40L^{-/-} BM. Total plaque area (358,231±25,913 μm^2 , CD40L^{-/-} BM vs. 309,366±29,345 μm^2 , wild-type BM), total cell number (944±74, CD40L^{-/-} BM vs. 960±74, wild-type BM), lipid core content (39.9±3.5%, CD40L^{-/-} BM vs. 39.6±2.5%, wild-type BM) and collagen content (26.6±2.3%, CD40L^{-/-} BM vs. 26.4±1.9%, wild-type BM) did not differ between the transplantation groups (Figures 3.3 and 3.4).

Figure 3.3 Atherosclerotic plaques of LDLR^{-/-} receiving either wild-type BM or CD40L^{-/-} BM after a HFD of 20 weeks. Tissue sections of paraffin embedded mouse aortic roots were stained with A, hematoxylin and eosin or B, Sirius red (collagen). See page 200 for full colour figures.

Figure 3.4 Quantification of plaque characteristics in $LDLR^{-/-}$ receiving either wild-type BM or $CD40L^{-/-}$ BM after a HFD of 20 weeks. Tissue sections of paraffin embedded aortic roots were analysed by computer-assisted morphometric quantification. The total plaque area (A) was measured and the total number of cells (B) were counted in sections stained with hematoxylin and eosin. The lipid core content (C) was determined and expressed relative to the total plaque area. The percent positive area in sections stained with Sirius red (collagen, D) was determined and expressed relative to the total plaque area. Data represent individual mice and the mean values (horizontal lines).

There were also no differences in macrophage content ($23.2 \pm 3.9\%$, CD40L^{-/-} BM vs. $21.1 \pm 3.0\%$, wild-type BM), smooth muscle cell content ($6.2 \pm 0.9\%$, CD40L^{-/-} BM vs. $5.9 \pm 1.0\%$, wild-type BM) and T cell content ($2.8 \pm 0.6\%$, CD40L^{-/-} BM vs. 3.4 ± 0.4 , wild-type BM) as shown in Figure 3.5. Overall, these results indicate that the CD40L deficiency on BM derived cells does not alter atherosclerotic plaque phenotype.

Figure 3.5 Quantification of plaque characteristics in *LDLR*^{-/-} receiving either wild-type BM or CD40L^{-/-} BM after a HFD of 20 weeks. Tissue sections of paraffin embedded mouse aortic roots were analysed by computer-assisted morphometric quantification. The percent positive area in sections stained with anti-Mac-3 mAbs (macrophages, A) or anti-actin mAbs (SMCs, B) was determined and expressed relative to the total plaque area. T cells (C), stained with anti-CD3 mAbs, were counted and expressed relative to the total amount of cells present in the aortic root. Data represent individual mice and the mean values (horizontal lines).

In a pilot study the involvement of CD40L expression on non-haemopoietic cells in the induction of the reported stable plaque phenotype after CD40L deficiency was verified. LDLR^{-/-} mice receiving CD40L^{-/-} BM were given a HFD for 14 weeks and were treated with either anti-CD40L (n=5) or isotype (n=6) antibodies. Flowcytometry analysis showed no differences in leukocyte populations between the two mice groups and the observed reduction of the Treg population in LDLR^{-/-} mice receiving CD40L^{-/-} BM was confirmed (data not shown). Plasma cholesterol levels (804±43 mg/dl, anti-CD40L vs. 649±62 mg/dl, isotype) and triglyceride levels (79±15 mg/dl, anti-CD40L vs. 119±12 mg/dl, isotype) were comparable between the two mice groups. Unexpectedly, analysis of the atherosclerotic lesions in the aortic root did not show a more stable plaque phenotype after anti-CD40L antibody treatment. Total plaque area (575,100±27,540 μm^2 , anti-CD40L vs. 504,200±47,610 μm^2 , isotype), lipid core content (43.4±1.5%, anti-CD40L vs. 39.8±4.9%, isotype), collagen content (63.3±3.1%, anti-CD40L vs. 61.5±2.5%, isotype) and T cell content ($0.2\pm0.1 \times 10^{-4} \mu\text{m}^{-2}$, anti-CD40L vs. $0.5\pm0.2 \times 10^{-4} \mu\text{m}^{-2}$, isotype) did not differ between the mice groups (Figure 3.6 and 3.7). These preliminary results suggest that the role of leukocyte CD40L in atherosclerosis development is not easily examined in the BM transplantation model.

Figure 3.6 Atherosclerotic plaques of LDLR^{-/-} receiving CD40L^{-/-} BM and treated with either isotype or anti-CD40L antibodies after a HFD of 14 weeks. Tissue sections of aortic roots were stained with hematoxylin and eosin. See page 201 for full colour figures.

Figure 3.7 Quantification of plaque characteristics in $\text{LDLR}^{-/-}$ receiving $\text{CD40L}^{-/-}$ BM and treated with either isotype or anti-CD40L antibodies after a HFD of 14 weeks. Tissue sections of aortic roots were analysed by computer-assisted morphometric quantification. The total plaque area (A) was measured in sections stained with hematoxylin and eosin. The lipid core content (B) was determined and expressed relative to the total plaque area. The percent positive area in sections stained with Sirius red (collagen, C) was determined and expressed relative to the total plaque area. T cells (D), stained with anti-CD3 mAbs, were counted and expressed relative to the mean total plaque area. Data represent individual mice and the mean values (horizontal lines).

3.5 Discussion

The present study shows that the stable plaque phenotype observed after inhibition of CD40L in atherosclerosis⁸ is not dependent on leukocyte CD40L alone. Although our findings show that CD40L deficiency affects the Treg population and that the size of the Treg population is intrinsic to BM derived cells, atherosclerosis analysed in the BM transplanted LDLR^{-/-} mice showed no effect of leukocyte CD40L deficiency on plaque progression and plaque phenotype.

In the present study, the Treg population was identified by two commonly used phenotypic parameters: the CD45RB^{high}:CD45RB^{low} ratio and the CD25 (IL-2 α receptor) expression of CD4 T cells. The use of these phenotypic parameters is hampered because the CD45RB^{low} and CD25⁺ phenotype are not unique for Tregs. The CD45RB marker is expressed at high levels on peripheral naive T-lymphocytes and at low levels on three distinct subsets: activated T-lymphocytes, memory T-lymphocytes and Tregs. Regarding the expression of CD25, it is expressed on both activated T-lymphocytes and Tregs. High amounts of activated T-lymphocytes are not likely to be present in mice that were maintained at specified pathogen free conditions. Therefore, the phenotypic reduction observed in CD40L-deficient mice when compared to naive wild-type mice is most likely to be a reduction of Tregs. In several functional studies the regulatory function of the CD45RB^{low} CD25⁺ CD4 T cell population has been elucidated. In these studies, CD45RB^{high} cells or CD25⁻ CD4 T cells were transferred into recipient nu/nu or scid/scid mice, which resulted in the development of autoimmune disease. The disease can be prevented by co-transferring CD45RB^{low} cells or CD25⁺ cells proving that CD45RB^{low} or CD25⁺ cells contain Tregs.^{23,24} In these functional studies the CD45RB^{high}:CD45RB^{low} ratio is used as a parameter for the regulatory capacity, that is a high ratio results in disease and a low ratio does not. In rats, the CD45RC^{high}:CD45RC^{low} ratio, which is equivalent to the CD45RB^{high}:CD45RB^{low} ratio in mice, seems to be better associated with the susceptibility to autoimmune disease than the number of CD25⁺ CD4 T cells.²⁵ This can be explained by the recent finding that Treg activity was shown in the CD25⁻ CD4 T cell population in both mice and rats.^{26,27} Although in the current study the Treg population was only identified by phenotype and not by function, it is to be expected that the regulatory function in CD40L-deficient mice was also

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declined. In line with this hypothesis, Kumanogoh *et al.* showed in CD40-deficient mice a reduction in Tregs both by phenotype and function.¹⁴

To investigate whether the size of the Treg compartment is dependent on CD40L expressed by the haemopoietic stem cell, we performed a BM transplantation. Our data show that the reduction of the Treg compartment induced by the CD40L deficiency is transferable to LDLR^{-/-} mice similar to the level found in naive CD40L^{-/-} mice. These results are in accordance with observations in the rat with respect to the CD45RC^{high}:CD45RC^{low} ratio.²⁸ In addition, these results also fit the hypothesis postulated by Singh *et al.* that the CD45RB^{low} CD25⁺ CD4 T cells are a population of natural Tregs in contrast to the inducible T_H3 and T_R1 cells.¹⁵

The effect of CD40L expressing leukocytes on atherosclerosis was studied in LDLR^{-/-} mice transplanted with CD40L-deficient BM. These mice had no different plaque phenotype when compared to LDLR^{-/-} mice receiving wild-type BM. Also, the number of T-lymphocytes and macrophages present in the plaque was comparable, suggesting that the recruitment of leukocytes was not affected by the leukocyte CD40L deficiency. These data imply that although CD40L expression on leukocytes is important for the development of Tregs, the effect of CD40L deficiency on non-haemopoietic cells, like smooth muscle cells, fibroblasts and endothelial cells, might be more important for the generation of a stable plaque phenotype. A possible approach to study whether CD40L deficiency of non-haemopoietic cells results in the change of plaque phenotype is to reconstitute atherosclerosis-prone CD40L-deficient mice with wild-type BM. However, CD40L-deficient mice do not survive BM transplantation and die within two weeks after transplantation (data not shown). Indications for the importance of CD40L expressing non-haemopoietic cells in atherogenesis are that CD40 ligation on vascular endothelial cells, smooth muscle cells and macrophages results in the expression of tissue factor, leukocyte adhesion and migration and induction of pro-inflammatory cytokines, chemokines and matrix-degrading enzymes.^{8,9,29} Moreover, vascular endothelial cells, smooth muscle cells and macrophages express both CD40 and CD40L suggesting besides a paracrine activation, a potential strong autocrine activation of the CD40-CD40L system in these cells.^{29,30} In our BM transplantation model, plaque macrophages still express CD40. A possibility might be

that these macrophages can still be activated by CD40L released by platelets or other cell-types,^{9,31} thereby producing pro-atherogenic mediators. Besides the direct effects of the CD40-CD40L interaction on non-haemopoietic cells, the reduced inflammation reported after CD40L deletion or anti-CD40L treatment can be the result of reduced recruitment of CD40 expressing leukocytes, especially macrophages, by endothelial cells. The increased collagen cap observed after CD40L deletion or anti-CD40L treatment might be the result of manipulating the balance between synthesis and degradation of collagen by vascular smooth muscle cells³² towards synthesis of collagen rather than degradation. Further research should elucidate the effects of CD40L-deficient non-haemopoietic cells on atherosclerosis.

In a pilot study, we tried to confirm the conclusion of our first experiment and show that CD40L expression on non-haemopoietic cells contributes to the induction of the reported stable plaque phenotype after anti-CD40L antibody treatment. Therefore, LDLR^{-/-} mice receiving CD40L^{-/-} BM were treated with either anti-CD40L or isotype antibodies, during which atherosclerosis was induced by high fat feeding. Surprisingly, the developed plaque phenotype in the mouse group treated with anti-CD40L antibodies did not differ from that in the isotype treated mouse group. This unexpected result suggests that the efficacy of the antibody treatment may have been attenuated by the BM transplantation. Transplantation of WT BM into LDLR^{-/-} mice was reported to enhance the extent of atherosclerosis.^{33,34} This effect on atherosclerosis might have interfered with that induced by the CD40L^{-/-} BM transplantation or the anti-CD40L antibody treatment. To exclude this problem, another mouse group should have been included: LDLR^{-/-} mice receiving WT BM and treated with anti-CD40L antibodies. These preliminary results show that a role for CD40L expressing non-haemopoietic cells could not be proven and a role for leukocyte CD40L cannot be excluded either.

In conclusion, the development of the Treg population is dependent on CD40L expressing BM derived cells. On the basis of our first experiment the CD40L expression on leukocytes is not involved in the development of a stable plaque phenotype in mice deficient for CD40L. However, the preliminary data of the second study cannot confirm a role for the CD40L expressing non-haemopoietic cells either. Therefore, future research on the role of

the CD40:CD40L interaction in plaque stability should be focussed on clarifying this peculiarity.

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Chapter 4

Transforming growth factor- β mediates balance between inflammation and fibrosis during plaque progression

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4.1 Abstract

The transition from stable to rupture-prone and ruptured atherosclerotic plaques involves many processes, including an altered balance between inflammation and fibrosis. An important mediator of both is TGF- β and a pivotal role for TGF- β in atherogenesis has been postulated. Here, we determine the *in vivo* effects of TGF- β inhibition on plaque progression and phenotype in atherosclerosis. Recombinant soluble TGF- β receptor II (TGF β RII:Fc), which inhibits TGF- β signalling, was injected in APOE^{-/-} mice for 12 weeks (50 μ g, twice a week i.p.) as early treatment (treatment age 5 to 17 weeks) and delayed treatment (age 17 to 29 weeks). In the early treatment group, inhibition of TGF- β signalling treatment resulted in a prominent increase in CD3- and CD45-positive cells in atherosclerotic lesions. Most profound effects were found in the delayed treatment group. Plaque area decreased 37.5% after TGF β RII:Fc treatment. Moreover, plaque morphology changed into an inflammatory phenotype that was low in fibrosis: lipid cores were 64.6% larger and inflammatory cell content had increased 2.7-fold. The amount of fibrosis decreased 49.6% and intraplaque haemorrhages and iron and fibrin deposition were observed frequently. TGF β RII:Fc treatment did not result in systemic effects. These results reveal a pivotal role for TGF- β in the maintenance of the balance between inflammation and fibrosis in atherosclerotic plaques.

4.2 Introduction

TGF- β is a growth factor that exerts many regulatory actions. It is known for its role in development, proliferation, migration, differentiation and extracellular matrix biology, but it is also an important immunomodulator.^{1,2} These functions are best reflected in TGF- β 1-deficient mice, which die in utero or perinatally because of widespread inflammation.³ Moreover, mice expressing a dominant-negative TGF- β receptor II (TGF β RII) under a T-lymphocyte-specific promoter in which TGF- β signalling is specifically blocked in T-lymphocytes showed disruption of T-lymphocyte homeostasis and inflammation in many organs.⁴ On the other hand, mice with cardiovascular overexpression of TGF- β 1 are embryonically lethal because of abnormal yolk sac vasculogenesis,⁵ and adenoviral gene therapy of TGF- β 1 in the balloon-injured rat causes an increased extracellular matrix deposition in intimal lesions.⁶ Furthermore, mice overexpressing TGF- β 1 under a liver- or kidney-specific promoter show excessive fibrosis in the targeted organ.^{7,8}

Nowadays, atherosclerosis is considered to be a chronic inflammatory disease and the balance between inflammation and extracellular matrix deposition is thought to be important for the maintenance of plaque stability in humans.^{9,10} Because TGF- β is an immunomodulator and a fibrosis modulator, we postulate that TGF- β might play a key role in the maintenance of the phenotype of an atherosclerotic plaque.

The first evidence of an important role for TGF- β in vascular disease has been obtained from studies in balloon-injured rats. TGF- β levels were increased 6 to 24 hours after balloon injury,¹¹ and TGF- β overexpression⁶ or inhibition¹² influences the disease process by an alteration of neointima formation, extracellular matrix deposition, or smooth muscle cell proliferation. Consistent with these results is the observation that patients with severe restenosis also exhibit increased serum levels of TGF- β .¹³

In contrast to the large number of data that report a role for TGF- β on neointima formation, data regarding the effects of TGF- β on primary atherosclerosis are limited. The expression patterns of TGF- β 1 to - β 3 and of TGF β RI and TGF β RII in atherosclerotic plaques are well known, but functional *in vivo* studies in primary atherosclerosis are sparse.^{14,15} However,

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the limited *in vivo* data available suggest a protective role for TGF- β in atherosclerosis. Some research,¹⁴ but not all,¹⁶ has reported that advanced plaque cells contain mutations in the type II receptor that disable proper signalling and consequently decrease sensitivity of these cells for TGF- β .¹⁴ Furthermore, patients suffering from unstable angina exhibited decreased levels of TGF- β ,¹⁷ indicating a correlation of low TGF- β levels with advanced atherosclerosis. APOE^{-/-} mice that were treated with tamoxifen (TMX, an anti-estrogen) exhibited increased TGF- β levels that were associated with a decrease in early lesion formation.¹⁸ Furthermore, in mice heterozygous for the deletion of the TGF- β 1 gene (TGF β 1^{-/-} mice) that were fed a HFD exhibited an increased endothelial activation and lipid infiltration was observed in the vascular wall.¹⁹ Recently, Mallat *et al.*²⁰ treated APOE^{-/-} mice with a neutralizing antibody against TGF- β 1, - β 2 and - β 3 from weeks 6 to 15. They observed that atherosclerotic lesions contained an high inflammatory cell content and a decreased amount of collagen.

In the present study, we confirm and expand the data of Mallat *et al.*²⁰ with the use of a different approach. Inhibition of the TGF- β pathway with TGF β RII:Fc in APOE^{-/-} mice, in an early treatment (weeks 5 to 17) and delayed treatment (weeks 17 to 29) setting, resulted in increased inflammation and decreased plaque fibrosis and was associated with intraplaque haemorrhages and iron and fibrin deposition. No systemic effects of TGF β RII:Fc treatment were observed.

4.3 Methods

TGF β RII:Fc fusion gene

The recombinant mouse TGF β RII:Fc fusion gene, containing the extracellular domain of the mouse type II TGF- β receptor fused to the Fc portion of mouse IgG2a, was constructed as described previously.²¹ TGF β RII:Fc was purified and its activity was tested in mink lung epithelial cells (Mv1Lu).²¹ *In vivo* studies confirmed the effectiveness the TGF β RII:Fc.^{22,23}

Mice

APOE^{-/-} (male, n=36) mice on a C57Bl6 background were obtained from Iffa Credo (Lyon, France). Mice were injected with TGF β RII:Fc fusion protein or murine control IgG twice a

week for 12 weeks (100 μ g/wk i.p.). The dosing regimen used in the present study was based on earlier *in vivo* studies using the same compound.^{22,23}

Treatment started at the age of 5 weeks (early treatment, n=10 TGF β RII:Fc mice and n=10 control mice) or at the age of 17 weeks (delayed treatment, n=8 TGF β RII:Fc mice and n=8 control mice). After the experimental procedure, mice were euthanized after a 24-hour fast. Blood (0.5 to 1 ml) was obtained from the caval vein for lipoprotein analysis. The arterial tree was perfused and the aortic arch, including its main branch points (brachiocephalic trunk, left common carotid artery and left subclavian artery), was excised and fixed as described previously.^{24,25} The aortic arch including branch points was embedded longitudinally and cut into ~40 sections. A series of twenty 4 μ m sections, which represented the central area of the arch with an intact morphology of the complete arch and branch points, were analysed.^{24,25}

Lipid profile

For the assessment of lipid profiles, standard enzymatic techniques, automated on the Cobas Fara centrifugal analyzer (Hoffmann-La Roche, Basel, Switzerland), were used. Total plasma cholesterol and HDL were measured by using kit No. 07 3663 5 and kit No. 543004 (Hoffmann-La Roche); total glycerol was measured by using kit No. 337-40A/337-10B (Sigma, St. Louis, Missouri); and free glycerol was measured by using kit No. 0148270 (Hoffmann-La Roche). Standardized serum (Precipath) was used as an internal standard. LDL was calculated as follows: total cholesterol–[(total glycerol–free glycerol)/2.2]–HDL.

Evaluation of possible systemic effects

To evaluate possible systemic effects of TGF β RII:Fc treatment, flowcytometry analysis (FACS Calibur, BD-Bioscience, San Jose, California) with T-lymphocyte-specific antibodies was performed on spleen and lymph nodes of TGF β RII:Fc-treated (n=3) and control (n=3) mice of the delayed treatment group. Antibodies used for staining were CD3-FITC (17A2) and biotinylated CD4 (RM4-5), CD8 α -PE (53-6.7), CD45RB-FITC (16A) and CD25-PE (PC61) (all BD-Pharmingen, San Diego, California).

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In addition, >20 organs from each mouse (n=34 mice) were excised and analysed on 4 μ m sections stained with hematoxylin and eosin and with CD45.

Histology and morphometry

For histological analysis of atherosclerosis, 4 sections (20 μ m apart) were stained with hematoxylin and eosin. Atherosclerotic lesions were analyzed and classified according to American Heart Association (AHA) criteria.²⁶ To obtain precise insights of the effects of TGF β RII:Fc treatment on plaque progression, all analyses were performed on separate lesion types, representing all stages of atherogenesis, as defined by the AHA. Because the data on plaque burden and inflammatory and fibrotic parameters between the separate early plaque stages (AHA type I, II and III lesions) as well as between the separate advanced stages (type IV and V lesions) were similar, data were presented in 2 groups: early lesions (AHA type I/II/III) and advanced lesions (AHA type IV/V).^{26,27} Morphometric parameters were determined as described previously.²⁴

Immunohistochemistry

Sections were immunolabeled with the following: α -smooth muscle actin (monoclonal anti-smooth muscle actin-FITC, Sigma) as a marker for vascular smooth muscle cells and fibroblasts; Mac3 (BD-Pharmingen) to detect macrophages; CD3 polyclonal antibody (Dako, Glostrup, Denmark) to detect T-lymphocytes; CD45 (BD-Pharmingen) to detect inflammatory cells; CD40 (Santa Cruz, Santa Cruz, California), CD40L (Santa Cruz), TGF- β (R&D Systems, Minneapolis, Minnesota), factor VIII (Dako) and fibrin(ogen) (Nordic Immunologies, Tilburg, the Netherlands); and MMP-2 and MMP-9, as described.^{24,25} Perls' stain was used to detect iron. The relative amounts of cells were determined as described previously.²⁴

All measurements were performed by two independent investigators who were blinded regarding treatment group. Intraobserver as well as interobserver variation was <10%.

Signalling

To obtain more insight into the effects of TGF β RII:Fc treatment on downstream signalling effects, immunohistochemistry with antibodies against Smad2 (goat polyclonal antibody,

Santa Cruz), Smad3 (goat polyclonal antibody, Santa Cruz), phospho-Smad2/3 (rabbit polyclonal antibody, Santa Cruz), Smad4 (goat polyclonal antibody, Santa Cruz) and Smad7 (goat polyclonal antibody, Santa Cruz) was used.

Statistical analysis

Data are expressed as mean $\pm$ SEM. TGF β RII:Fc-treated APOE^{-/-} mice were compared with control APOE^{-/-} mice by a nonparametric Mann-Whitney U test. Data were considered statistically significant at P<0.05.

4.4 Results

Mouse study: histology

Survival rates were 100% in the early treatment study and 87.5% in both groups of the delayed treatment study. Body weight and heart weight did not differ between the treatment groups, nor did the levels of plasma cholesterol (early treatment, 13.1 $\pm$ 1.0 mM for TGF β RII:Fc vs. 12.2 $\pm$ 0.4 mM for control IgG; delayed treatment, 11.8 $\pm$ 0.9 mM for TGF β RII:Fc vs. 13.8 $\pm$ 1.5 mM for control IgG), triglyceride (early treatment, 1.6 $\pm$ 0.2 mM for TGF β RII:Fc vs. 1.5 $\pm$ 0.1 mM for control IgG; delayed treatment, 1.0 $\pm$ 0.1 mM for TGF β RII:Fc vs. 1.1 $\pm$ 0.2 mM for control IgG), LDL (12.6 $\pm$ 0.9 mM for TGF β RII:Fc vs. 11.7 $\pm$ 0.4 mM for control IgG; delayed treatment, 11.4 $\pm$ 0.9 mM for TGF β RII:Fc vs. 13.3 $\pm$ 1.5 mM for control IgG), or HDL (early treatment, 0.2 $\pm$ 0.1 mM for TGF β RII:Fc vs. 0.3 $\pm$ 0.1 mM for control IgG; delayed treatment, 0.3 $\pm$ 0.0 mM for TGF β RII:Fc vs. 0.3 $\pm$ 0.1 mM for control IgG). Autopsy (>20 organs) revealed no abnormalities on sections stained with hematoxylin and eosin or with CD45 (data not shown); especially, there was no generalized inflammation in organs (Figure 4.1), nor was there adventitial inflammation in normal arteries (Figure 4.2A and B) or atherosclerotic arteries (Figure 4.2C and D), indicating that the effects described below were plaque specific and not due to a systemic effect. Furthermore, no signs of ischemic end-organ damage were observed either macroscopically or microscopically.

Figure 4.1 *Histological characterisation of systemic effects of TGFβRII:Fc treatment.* Tissue sections of heart (A and B), spleen (C and D), liver (E and F) and kidney (G and H) were stained with hematoxylin and eosin. Treatment with TGFβRII:Fc or control IgG showed no systemic effects. See page 202 for full colour figures.

Figure 4.2 *Histological characterisation of effects of TGF β RII:Fc treatment on arteries.* Tissue sections of normal arteries (A and B) and atherosclerotic arteries (C and D) were stained with hematoxylin and eosin. Treatment with TGF β RII:Fc or control IgG showed no adventitial inflammation. A = adventitia, L = lumen. See page 203 for full colour figures.

Mouse study: flowcytometry

FACS analysis of lymph nodes and spleen revealed no differences in the amount of CD3-positive cells (T-lymphocytes) or in the activation status of the T-lymphocytes between the groups (CD4/CD8 ratio, CD25⁺ T cells), confirming the absence of systemic inflammatory effects of TGF β RII:Fc treatment (Figure 4.3).

Figure 4.3 Characterisation of effects of TGFβRII:Fc treatment on the T cell population. Spleen and peripheral lymph nodes were analysed with flowcytometry. Treatment with TGFβRII:Fc or control IgG showed no systemic inflammatory effects.

Signalling

In atherosclerotic plaques of the early and delayed TGFβRII:Fc treatment groups, immunoreactivity of Smad2 and phospho-Smad2/3 (p-Smad2/3) decreased (Figure 4.4), whereas immunoreactivity of Smad3, Smad4 and Smad7 did not change. The decrease in p-Smad2/3 and the decrease in Smad2 indicate the *in vivo* inhibition of TGF-β signalling.

Figure 4.4 *Immunoreactivity of p-Smad2/3.* Tissue sections of atherosclerotic plaques of the delayed TGF β RII:Fc treatment groups were stained with anti-p-Smad2/3. TGF β RII:Fc treatment significantly decreased p-Smad2/3 signalling. See page 204 for full colour figures.

Plaque burden

We analyzed 43 lesions in the aortic arches of TGF β RII:Fc-treated APOE^{-/-} mice (n=10) and 47 lesions of the control mice (n=10) in the early treatment study and 40 lesions in the aortic arches of TGF β RII:Fc-treated APOE^{-/-} mice (n=7) and 41 lesions of the control mice (n=7 mice) in the delayed treatment study. In the early treatment study, total plaque burden was not affected. However, in the delayed treatment study, total plaque area of the aortic arch decreased 37.5% after TGF β RII:Fc treatment (early treatment, 111,125 $\pm$ 24,924 μ m² for TGF β RII:Fc vs. 138,246 $\pm$ 23,574 μ m² for control IgG, P>0.05; delayed treatment, 547,359 $\pm$ 68,266 μ m² for TGF β RII:Fc vs. 876,869 $\pm$ 68,847 μ m² for control IgG, P<0.05). The number of plaques did not change after TGF β RII:Fc treatment (Figure 4.5A and 4.5C). Interestingly, TGF β RII:Fc treatment altered the distribution of AHA lesion types. In the early treatment group, the absolute and relative amounts of type Va lesions increased after TGF β RII:Fc treatment (72.7% for TGF β RII:Fc vs. 30% for control IgG, as percentage of all advanced lesions), whereas the absolute and relative amounts of type IV lesions (18.2% for TGF β RII:Fc vs. 70% for control IgG, as percentage of all advanced lesions) had decreased. Similar results were obtained in the delayed treatment group (Figure 4.5B and 4.5D). In that group, the number of Va lesions (fibrous cap and large lipid core) had increased (64.0% for TGF β RII:Fc vs. 39.1% for control IgG), whereas the number of Vc

lesions (fibrous lesions) was decreased (8% for TGF β RII:Fc vs. 30.4% for control IgG). This indicates that TGF β RII:Fc treatment induces a plaque phenotype with large lipid cores and thin fibrous caps. Individual plaque area per lesion type had significantly decreased in type IV lesions of the early treatment study and in type V lesions of the delayed treatment study (Figure 4.5B and 4.5D).

Figure 4.5 Characterisation of atherosclerosis after TGF β RII:Fc or control IgG treatment. Quantification of the distribution and number of the different lesion types (A and B) and plaque area of the different lesion types (C and D). *P<0.05.

Plaque composition

Besides the decrease in plaque area, the composition of the plaques significantly differed after TGF β RII:Fc treatment compared with control IgG treatment.

Early atherosclerotic plaques (fatty streaks)

Further analysis of early lesion development in the aortic arch revealed that the relative and absolute numbers of CD3-positive cells (T-lymphocytes) increased after TGF β RII:Fc treatment: relative numbers were $5.0 \pm 1.0\%$ for TGF β RII:Fc vs. $1.8 \pm 1.2\%$ for control IgG ($P < 0.05$) for early treatment and $4.9 \pm 0.6\%$ for TGF β RII:Fc vs. $2.0 \pm 1.0\%$ for control IgG ($P < 0.05$) for late treatment; absolute numbers were 10 ± 3 for TGF β RII:Fc vs. 3 ± 1 for control IgG for early treatment and 2 ± 1 for TGF β RII:Fc vs. 1 ± 0 for control IgG for late treatment). The relative and absolute numbers of CD45-positive cells also increased after TGF β RII:Fc treatment: relative numbers are shown in Figure 4.6A; absolute numbers were 3 ± 1 for TGF β RII:Fc vs. 1 ± 0 for control IgG for early treatment and 9 ± 4 for TGF β RII:Fc vs. 2 ± 1 for control IgG for late treatment. Furthermore, CD40 and CD40L immunoreactivity in early lesions of the early treatment and delayed treatment studies also increased after TGF β RII:Fc treatment. Macrophage content decreased in early lesions of the treated mice of the early treatment study ($82.1 \pm 2.2\%$ for TGF β RII:Fc vs. $73.5 \pm 2.0\%$ for control IgG, $P < 0.05$; delayed treatment, $74.4 \pm 5.6\%$ for TGF β RII:Fc vs. $70.8 \pm 3.4\%$ for control IgG, $P > 0.05$). Collagen content (Figure 4.6B), α -smooth muscle actin content (early treatment, $0.4 \pm 0.2\%$ for TGF β RII:Fc vs. $0.9 \pm 0.5\%$ for control IgG, $P > 0.05$; delayed treatment, $0.7 \pm 0.5\%$ for TGF β RII:Fc vs. $0.8 \pm 0.8\%$ for control IgG) and MMP-2 and MMP-9 immunoreactivity did not differ.

Advanced atherosclerotic plaques

In advanced lesions, the effects of inhibition of TGF- β signalling on plaque composition were even more pronounced. Lipid cores of the TGF β RII:Fc treatment group were 84.2% and 64.6% larger than in control IgG-treated mice, respectively (Figures 4.6C, 4.7A and 4.7B) and lipid core expansion was accelerated (148.2% vs. 50% increase in lipid core content from weeks 17 to 29, $P < 0.05$; Figure 4.6C).

Figure 4.6 Quantification of plaque characteristics in *TGFβRII:Fc*-treated and control *APOE*^{-/-} mice of the early and delayed treatment study. A, CD45-positive cell content. B, Collagen content. C, Lipid core content. *P<0.05.

Inflammatory cell content was significantly increased after *TGFβRII:Fc* treatment, as reflected by macrophage cell content (relative numbers for early treatment were 60.8±4.8% for *TGFβRII:Fc* vs. 64.2±5.7% for control IgG; relative numbers for delayed treatment were 60.9±2.3% for *TGFβRII:Fc* vs. 43.6±2.7% for control IgG, P<0.05), CD3 cell content (relative numbers for early treatment were 3.7±0.4% for *TGFβRII:Fc* vs. 2.0±0.7% for

control IgG; relative numbers for delayed treatment were $5.0 \pm 0.6\%$ for TGF β RII:Fc vs. $2.0 \pm 0.5\%$ for control IgG; absolute numbers for early treatment were 5 ± 2 for TGF β RII:Fc vs. 1 ± 1 for control IgG; and absolute numbers for delayed treatment were 8 ± 2 for TGF β RII:Fc vs. 4 ± 1 for control IgG) and CD45-positive cell content (Figures 4.6A, 4.7C and 4.7D; absolute numbers for early treatment were 6 ± 1 for TGF β RII:Fc vs. 2 ± 1 for control IgG; absolute numbers for delayed treatment were 12 ± 2 for TGF β RII:Fc vs. 6 ± 1 for control IgG). CD40 and CD40L (Figures 4.7E and 4.7F) immunoreactivity was also significantly increased after TGF β RII:Fc treatment. In addition, the amount of fibrosis, reflected by the collagen content, had decreased 50.3% in the delayed treatment study (Figures 4.6B, 4.7G and 4.7H). MMP-2 and MMP-9 immunoreactivity was increased. The amount of α -smooth muscle actin-positive cells did not differ (early treatment, $1.2 \pm 0.3\%$ for TGF β RII:Fc vs. $1.0 \pm 0.2\%$ for control IgG, $P > 0.05$; delayed treatment, $1.0 \pm 0.3\%$ for TGF β RII:Fc vs. $1.0 \pm 0.2\%$ for control IgG, $P > 0.05$). Interestingly, the increase in inflammation and decrease in fibrosis in plaques of mice treated with TGF β RII:Fc were associated with a significant increase in the frequencies of recent and older intraplaque bleedings and fibrin and iron deposition in the delayed treatment group (Figure 4.8). This was not associated with acute ischemic events. These data show that blockade of TGF- β signalling decreases the advanced plaque area and alters the balance between plaque inflammation and fibrosis. This results in an inflammatory plaque phenotype with a low extracellular matrix content. Systemic effects, as well as changes in lipid profile, were not observed.

Figure 4.7 *Histological characteristics of effects on atherosclerotic lesions after delayed TGFβRII:Fc treatment.* Hematoxylin and eosin stained section of advanced atherosclerotic lesions in the aortic arch, revealing an enlarged lipid core (arrow) after TGFβRII:Fc treatment (A) compared with control IgG treatment (B). CD45 staining of the shoulder region of advanced atherosclerotic lesions, revealing an increased amount of CD45-positive cells (arrows) after TGFβRII:Fc treatment (C) compared with control IgG treatment (D). CD40L staining of advanced atherosclerotic lesions. CD40L is most abundantly present after TGFβRII:Fc treatment (E) compared with control IgG treatment (F). Sirius red staining of advanced atherosclerotic lesions. The amount of collagen significantly decreased after TGFβRII:Fc treatment (G) compared with control IgG treatment (H). See page 205 for full colour figures.

Figure 4.8 *Histological characteristics of intraplaque haemorrhage in advanced atherosclerotic lesions after delayed treatment with TGF β RII:Fc in APOE^{-/-} mice. A, Overview of an advanced atherosclerotic lesion showing erythrocytes, iron deposition and fibrin deposition (arrows). (hematoxylin and eosin staining). B, Panel A in more detail. C, Perls' iron staining combined with factor VIII staining showing iron deposition (in blue) and endothelium (in red) in an advanced atherosclerotic lesion. D, Frequency of erythrocytes and fibrin and iron deposition. See page 206 for full colour figures.*

4.5 Discussion

In accordance with the present results, Mallat *et al.*²⁰ also reported that inhibition of TGF- β in primary atherosclerosis induces an increase in plaque inflammation and a decrease in plaque fibrosis. However, besides these similarities in outcome, there are also important differences between both studies. First of all, we used a different kind of TGF- β blockade.

Whereas Mallat *et al.* treated the mice with an inhibiting antibody against TGF- β 1, - β 2 and - β 3, we used a soluble recombinant TGF β RII. Second, besides the early time points that were studied by Mallat *et al.* (treatment weeks 6 to 15) and us (treatment weeks 5 to 17), we also studied the effects of TGF- β blockade after 29 weeks. In this delayed treatment group, we were able to show that the increase in plaque inflammation and decrease in plaque fibrosis were associated with intraplaque haemorrhages and iron and fibrin deposition. Last, in contrast to the study of Mallat *et al.*, we did not observe systemic effects after inhibition of TGF- β signalling. Besides the increase in plaque inflammation and decrease in plaque fibrosis, Mallat *et al.* also observed adventitial inflammation (vasculitis) as well as systemic inflammation in other organs.^{20,28} Because vasculitis and (systemic) inflammation are associated with an acceleration of atherosclerosis,^{29,30} the systemic effects of the anti-TGF- β antibody might have confounded the results of Mallat *et al.* (see Lutgens and Daemen³¹).

Our results are in agreement with the data earlier reported in *in vitro* studies. These studies show that TGF- β functions as an anti-inflammatory cytokine in cell types that are also present in atherosclerotic plaques.³²⁻³⁴ TGF- β is also known to be an important fibrotic cytokine that plays an important role in matrix remodelling and collagen synthesis.³⁵

The signalling cascade by which TGF- β exerts its actions is well known. In brief, TGF- β binds via receptor III to receptor II or directly to receptor II and this complex binds to receptor I. This induces the phosphorylation of Smad2 or Smad3. Subsequently, phospho-Smad-2 or -Smad-3 binds to Smad-4 and the resulting complex moves to the nucleus, where it interacts with various transcription factors to regulate the transcription of TGF- β -responsive genes and mediates the effects of TGF- β at the cellular level.² In the present study, it was shown that TGF β RII:Fc treatment suppresses phosphorylation of Smad2/3, thereby inhibiting the actions of TGF- β .

The phenomenon that inhibition of inflammation causes a plaque phenotype that contains a high extracellular matrix content has been described previously. Genetic disruption of interferon- γ in APOE^{-/-} mice has been reported to result in smaller atherosclerotic plaques

with higher collagen content.³⁶ Furthermore, we and others observed a collagen-rich phenotype after inhibition of CD40L in APOE^{-/-} and LDL receptor-deficient mice.^{24,25,37} Overexpression of the anti-inflammatory cytokine interleukin-10 increased collagen content in atherosclerotic lesions, whereas inhibition of interleukin-10 decreased collagen content.^{38,39}

Our earlier reports suggested the importance of TGF- β as a mediator of plaque fibrosis. Inhibition of CD40L signalling in atherosclerosis resulted in a lipid-poor collagen-rich plaque phenotype. Interestingly, we observed an increased immunoreactivity of TGF- β in these lesions.^{25,40} Additional analysis with a cDNA expression array revealed that anti-CD40L treatment induced a 2.3-fold upregulation of TGF- β mRNA. These results suggest that upregulation of TGF- β may be associated with the CD40L inhibition-induced fibrotic plaque phenotype. The observed balance between CD40L and TGF- β and the effects on plaque phenotype observed after CD40L (fibrosis-rich inflammatory-poor plaque phenotype) or TGF β RII inhibition (inflammatory-rich fibrosis-poor plaque phenotype) reflect the importance of the balance between inflammation and fibrosis on atherosclerotic plaque phenotype.

The results of the present study suggest that TGF- β may be used as a therapeutic target. However, whether TGF- β treatment would be beneficial or detrimental still needs to be determined. On the one hand, TGF- β would induce plaques with a high extracellular matrix content and a low inflammatory cell content, which may help to stabilize the plaque. On the other hand, the possible drawbacks of administering of TGF- β may be possible systemic fibrotic effects and an increase in plaque size. Targeted administering of TGF- β may solve the first problem. A role for TGF- β in atherosclerotic plaque growth has been postulated previously. Immunohistochemical analysis revealed that TGF- β and TGF β RII are most abundant in early lesions and lesions with pathological intimal thickening and may stimulate the production of lipid-trapping proteoglycans.⁴¹⁻⁴³ In other models of neointima formation, such as the balloon-injured rat, TGF- β also induced neointimal growth and inhibition of TGF- β -signalling was able to significantly reduce neointima formation.¹²

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A decrease in plaque size is beneficial in preventing clinical symptoms of atherosclerosis. However, because in humans, plaque composition is a more important predictor of the acute vascular symptoms than is plaque size,^{27,44} one may hypothesize that inhibition of TGF- β may increase plaque instability and evoke more clinical symptoms, even when plaque size decreases. Thus, local activation of TGF- β -signalling may provide a therapeutic target in atherosclerosis. Although it may not prevent the initiation of atherosclerosis, it may prevent the transition into an unstable plaque phenotype because of its anti-inflammatory and profibrotic effects.

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Chapter 5

Depletion of CD4⁺ CD25⁺ regulatory T cells enhances the inflammatory T-lymphocyte response in experimental atherosclerosis

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In preparation

5.1 Abstract

Recent experimental studies demonstrated the involvement of Tregs in atherosclerosis. Tregs (CD4⁺ CD25⁺ cells) are a subset of T-lymphocytes that control the activation and proliferation of pathogenic T-lymphocytes. In the present study, we investigated the role of CD4⁺ CD25⁺ cells in atherosclerosis. LDLR^{-/-} mice on a HFD were treated with anti-CD25 (PC61) antibodies or control IgG for 10 weeks. Analysis of blood, spleen and lymph nodes by flowcytometry confirmed the reduction of the CD4⁺ CD25⁺ cell population in anti-CD25-treated LDLR^{-/-} mice. Despite the markedly diminished CD4⁺ CD25⁺ cell population, the extent of atherosclerosis in LDLR^{-/-} mice treated with anti-CD25 antibodies was not affected. Also lipid core content, collagen content and macrophage content revealed no differences between the two experimental groups. Interestingly, the number of CD3 T-lymphocytes in the atherosclerotic lesions had increased by 2-fold in anti-CD25-treated LDLR^{-/-} mice and this increase was accompanied by enhanced accumulation of both CD4 T-lymphocytes and CD8 T-lymphocytes. Moreover, anti-CD25 antibody treatment resulted in a reduced accumulation of CD25⁺ cells in the atherosclerotic lesions confirming effective anti-CD25 antibody treatment. Thus, depletion of CD4⁺ CD25⁺ cells results in a more vigorous inflammatory T-lymphocyte response thereby inducing an inflammatory plaque phenotype.

5.2 Introduction

Atherosclerosis is a multi-factorial disease of the arterial wall with many characteristics of a chronic inflammation. Accumulating evidence shows that cells of both innate and adaptive immunity, especially macrophages and T-lymphocytes, contribute together to this chronic inflammatory process.¹⁻³ T-lymphocytes are found in atherosclerotic lesions at all stages of human and experimental atherosclerosis. Most of the T-lymphocytes isolated from atherosclerotic lesions are T_H-lymphocytes, which predominantly produce the T_H1 cytokine IFN γ and are thereby able to activate plaque macrophages.¹ Additionally, the T-lymphocytes are often located in the vicinity of MHCII-expressing macrophages, indicative for an active immune response.^{1,4} The importance of adaptive immunity in the development and progression of atherosclerosis has been shown in several mouse models. Absence of both T- and B-lymphocytes (scid/scid, nu/nu or RAG^{-/-} mice) in atherosclerosis-prone mice resulted in a hampered progression of atherosclerosis.⁵ Moreover, depletion of T-lymphocytes⁶ or CD4 T-lymphocytes⁷⁻⁹ delayed the development of atherosclerosis, while transfer of T-lymphocytes into atherosclerosis-prone mice substantially aggravated atherosclerosis.^{10,11}

A special subset of T-lymphocytes is the Treg population. Tregs are crucial in maintaining self-tolerance and in regulating immune responses. At present, at least two types of Tregs have been identified: the induced (or acquired) Tregs and the natural (or innate) Tregs. The induced Tregs include the T_H3 and the T_R1 cells, which are induced upon activation by antigen. They regulate T-lymphocyte activity mostly at the sites of inflammation by local secretion of the immunosuppressive cytokines, IL-10 and TGF- β .^{12,13} Transfer of ovalbumin (OVA)-specific T_R1 cells into APOE^{-/-} mice reduced the development of atherosclerosis, indicating a protective role for induced Tregs in atherosclerosis. This effect was mainly due to decreased levels of IFN γ and increased production of IL-10.¹⁴ The natural Tregs are characterised by the constitutive expression of CD25 (the IL-2 receptor α chain) and constitute 5-10% of the CD4 T-lymphocyte population in mice.¹⁵ The IL-2 receptor is essential for the generation of these Tregs in the thymus, but also for their maintenance and survival in the periphery and for their activation and functional integrity.¹⁶⁻¹⁸ Natural Tregs prevent the activation, proliferation and differentiation of

pathogenic T-lymphocytes primarily in lymphoid organs mainly by cell-cell interactions and inhibition of IL-2 production.^{12,13,17,19,20} Recently, it was shown that the natural (CD4⁺ CD25⁺) Tregs act also as powerful inhibitors of atherosclerosis.²¹

To confirm and extend the published data on the importance of the natural Tregs in atherosclerosis,²¹ we depleted CD4⁺ CD25⁺ cells in LDLR^{-/-} mice with anti-CD25 antibodies. The anti-CD25-treated mice were compared to mice treated with control IgG and analysed for their atherosclerotic lesion development and plaque phenotype.

5.3 Methods

Anti-CD25 antibody

For the depletion of the CD25⁺ cell population *in vivo*, rat anti-CD25 mAb (IgG1) produced by the PC61 hybridoma was used.²² The PC61 hybridoma was cultured in the presence of 1% FCS. The supernatant containing the anti-CD25 mAb was purified with protein G sepharose and the concentration was measured using the BCA protein assay kit (Pierce Chemical Company, Rockford, Illinois). As control treatment, rat IgG isolated from rat serum was used (Sigma, St. Louis, Missouri).

Mice

Female LDLR^{-/-} mice (tenth generation C57Bl/6, originally obtained from Jackson's Lab, Bar Harbor, Maine) were maintained at our animal facility (Maastricht, The Netherlands). Mice, 9-13-week-old, were treated with anti-CD25 antibodies (n=18) or control IgG (n=17). I.p. injections with 500 µg antibody were started one week prior to the high fat feeding (weekly injection; T = -1, 0 and 1) and the treatment was maintained once every two weeks for eight weeks. One week after the first anti-CD25 or control IgG injection, mice were given a HFD containing 16% fat and 0.15% cholesterol (Hope Farms, Woerden, the Netherlands). After 10 weeks of high fat feeding, mice were sacrificed. Prior to sacrifice, mice were anaesthetised by i.p. injection of 25 µg/g xylazine (Sedamun, Eurovet, Bladel, the Netherlands) and 125 µg/g ketamine (Nimatek, Eurovet). Subsequently, mice were bled by vena cava inferior puncture followed by perforation of the diaphragm. All procedures were approved by the animal care and use committee of the Maastricht University and were in accordance with national regulations on animal experiments.

Flowcytometry

To monitor the reduction of CD25⁺ cells, heparinised blood (50 µl per mouse) was obtained from the tail vein after an overnight fast before treatment and just before the injections at one and eight weeks of treatment. Erythrocytes were removed by hypotonic lysis with NH₄Cl.²³ Cells were double stained with CD25-PE (3C7, recognising a non-competing epitope distinct from that of PC61) and CD4-biotin (RM4-5) (all antibodies were from BD-Pharmingen, San Diego, California). The biotin-labelled antibody was detected in a second step with Streptavidin-CyChrome (BD-Pharmingen). Data were collected of 1×10⁴ CD4⁺ cells. Flowcytometry analysis was performed on a FACS Calibur and analysed with the CellQuest software (BD-Bioscience, San Jose, California).

Prior to sacrifice, heparinised blood was obtained from the inferior vena cava. Spleen and lymph nodes (superficial and deep cervical nodes, brachial nodes and mesenteric nodes) were collected from sacrificed mice. Single-cell suspensions from spleen and lymph nodes were prepared. In blood and spleen, erythrocytes were removed by hypotonic lysis with NH₄Cl²³. Blood cells (2×10⁵ per sample) were double stained with Gr1 (Ly-6G and Ly-6C)-FITC (RB6-8C5) and Mac1 (CD11b)-PE (M1/70) (all antibodies were from BD-Pharmingen). Data were collected of 0.5×10⁴ Gr1⁺ cells. In addition, cells (1×10⁶ per sample) were triple stained with CD3-FITC (17A2), CD8α-PE (53-6.7), CD4-biotin (RM4-5) or double stained with CD25-PE (3C7) and CD4-biotin (RM4-5) (all antibodies were from BD-Pharmingen). The biotin-labelled antibodies were detected in a second step with Streptavidin-CyChrome (BD-Pharmingen). Data were collected of 1×10⁴ CD3⁺ cells when stained with CD3-FITC, CD8α-PE and CD4-biotin or of 1×10⁴ CD4⁺ cells when stained with CD25-PE and CD4-biotin. Flowcytometry analysis was performed on a FACS Calibur and analysed with CellQuest software (BD-Bioscience).

Lipid parameters

Plasma cholesterol and plasma total triglyceride levels were determined using standard colorimetric assays (Roche Diagnostics, Mannheim, Germany). Pooled plasma was fractionised over a Superose 6PC 3.2/30 column in an AKTABasic chromatography system (Amersham Biosciences, Roosendaal, the Netherlands) to separate the lipoproteins. The

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amount of cholesterol in the lipoprotein fractions was measured using standard colorimetric assays (Roche Diagnostics).

Tissue processing

The heart was removed from sacrificed mice by cutting the aorta just cranial from the atria. The heart was divided in two by cutting perpendicular to the heart axis just below the atrial tips. The upper part of the heart containing the aorta was embedded in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) with the base facing downward, frozen in isopentane that was cooled by dry ice and stored at -80°C . Serial sections of the aortic root area of the heart were cut at $7\text{ }\mu\text{m}$. Sections showing the presence of the aortic valves were collected and used for analysis.

Histomorphometry and immunohistometry

Collected sections of the aortic root, $42\text{ }\mu\text{m}$ apart, were stained with hematoxylin and eosin, Sirius red (collagen content) or anti-CD68 (supernatant of clone FA-11, macrophage content). Morphometric analysis was performed using a microscope coupled to a computerised morphometry system (Quantimet Qwin version 3, Leica, Rijswijk, the Netherlands). Total plaque area was quantified as an average of at least 3 sections per mouse. Lipid core content, collagen content and macrophage content were determined in the central part of the aortic root. The content was expressed as positive area relative to the mean total plaque area.²³ All analyses were performed without prior knowledge of the type of administered antibodies.

To quantify the infiltrating inflammatory cells in the aortic lesions, aortic root sections were stained with anti-CD3 (supernatant of clone KT3, T-lymphocytes), anti-CD4 (RM4-5 (BD Pharmingen), T_H -lymphocytes), anti-CD8 (53-6.7 (BD Pharmingen), T_C -lymphocytes), or anti-CD25-biotin (purified from clone PC61, CD25^+ cells). As control staining, aortic root sections were stained with anti-rat Ig antibodies (DAKO, Glostrup, Denmark). The number of positive cells was counted in the same area of the aortic root, which was used for the total plaque area measurement and expressed relative to the mean total plaque area. All analyses were performed blinded to the type of administered antibodies.

Statistical analysis

Results are presented as mean $\pm$ SEM. The data were analysed with the Kolmogorov-Smirnov (KS) test to assess normality and the F-test to compare variances. When results passed both tests ($p < 0.05$) they were analysed with the unpaired T-test (two-sided, except the FACS analysis of the CD4⁺ CD25⁺ cells, which was tested one-sided). The monitoring of the CD4⁺ CD25⁺ cell population by FACS analysis was tested by two-way ANOVA and Bonferroni posttest. A p-value of $p < 0.05$ was accepted as statistically significant.

5.4 Results

Anti-CD25 antibody treatment reduces the CD4⁺ CD25⁺ cell population

Treatment of LDLR^{-/-} mice with anti-CD25 antibodies resulted, as expected, in a significantly sustained reduction of circulating CD4⁺ CD25⁺ cells during the experiment (Figure 5.1A; at 0 weeks: 2.2 $\pm$ 0.1%, anti-CD25 vs. 7.9 $\pm$ 0.2%, control IgG, $p < 0.001$ and at 7 weeks: 1.8 $\pm$ 0.2%, anti-CD25 vs. 5.3 $\pm$ 0.2%, control IgG, $p < 0.001$). After 10 weeks of anti-CD25 antibody treatment the CD4⁺ CD25⁺ cell population was still significantly reduced in blood, as well as in spleen and in lymph nodes (Figure 5.1B-D; blood: 3.2 $\pm$ 0.7%, anti-CD25 vs. 9.6 $\pm$ 0.8%, control IgG, $p < 0.001$; spleen: 9.3 $\pm$ 0.8%, anti-CD25 vs. 13.6 $\pm$ 1.1%, control IgG, $p = 0.004$ and lymph nodes: 9.1 $\pm$ 0.8%, anti-CD25 vs. 15.5 $\pm$ 0.9%, control IgG, $p < 0.001$).

Analysis of possible systemic effects of the anti-CD25 antibody treatment on the leukocyte subsets revealed that in blood the distribution of granulocytes, monocytes and lymphocytes as well as the CD4:CD8 T cell ratio were similar in both treatment groups (Table 5.1). In addition, analysis of spleen and lymph nodes by flowcytometry showed no alterations in the number of CD3⁺ T cells or CD4:CD8 T cell ratio between the treatment groups (data not shown). These data confirm that treatment of LDLR^{-/-} mice with anti-CD25 antibodies systemically reduced the CD4⁺ CD25⁺ cell population, but did not have any additional systemic effects on the distribution of leukocytes when compared to control LDLR^{-/-} mice.

Figure 5.1 Treatment with anti-CD25 antibodies reduces the CD4⁺ CD25⁺ cell population in LDLR^{-/-} mice. Blood, spleen or lymph node cells were incubated with anti-CD25 mAbs in combination with anti-CD4 mAbs and analysed by flowcytometry. A, Monitoring of the CD4⁺ CD25⁺ cells in blood of LDLR^{-/-} mice treated with anti-CD25 antibodies (---■---) or control IgG (—■—) during the experiment. The arrow at T=0 depicts the start of high fat feeding. *p<0.001 at T=0 and T=7. B, C, D, Representation of the CD25 expression on CD4 T-lymphocytes in blood (B), spleen (C) and lymph nodes (D) of the mice groups after sacrifice (mean ± SEM).

Table 5.1 Circulating leukocyte values of anti-CD25 or control IgG treated LDLR^{-/-} mice

	LDLR ^{-/-} , anti-CD25 N = 6	LDLR ^{-/-} , control IgG N = 6
Granulocytes, %	26.2 ± 3.5	29.4 ± 5.3
Monocytes, %	13.3 ± 2.4	10.5 ± 1.6
T cells, %	21.5 ± 1.4	19.5 ± 2.0
CD4:CD8	1.25 ± 0.07	1.18 ± 0.06

Values are mean±SEM.

Reduction of the CD4⁺ CD25⁺ cell population results in an enhanced accumulation of T-lymphocytes in the atherosclerotic lesion

After 10 weeks high fat feeding, LDLR^{-/-} mice treated with either anti-CD25 antibodies or control IgG had similar weights (19.2±0.3 g, anti-CD25 vs. 19.1±0.4 g, control IgG), cholesterol levels (948±74 mg/dl, anti-CD25 vs. 1,086±75 mg/dl, control IgG) and triglyceride levels (186±22 mg/dl, anti-CD25 vs. 202±22 mg/dl, control IgG). Lipid profiles were also comparable between both treatment groups (VLDL: 56.5%, anti-CD25 vs. 52.1%, control IgG; IDL/LDL: 28.8%, anti-CD25 vs. 33.2%, control IgG and HDL: 4.7%, anti-CD25 vs. 7.6%, control IgG).

Quantification of the extent of atherosclerosis in the aortic root revealed that total plaque area did not differ between both treatment groups (Figure 5.2A and 5.3A; 315,900±24,210 μm², anti-CD25 vs. 373,200±21,550 μm², control IgG). Analysis of the plaque phenotype revealed that lipid core content, collagen content and macrophage content were also not affected by the anti-CD25 antibody treatment (Figure 5.2B-D; lipid core content: 25.4±2.9%, anti-CD25 vs. 25.9±2.2%, control IgG; collagen content: 56.3±2.6%, anti-CD25 vs. 57.4±2.4%, control IgG and macrophage content: 47.1±4.7%, anti-CD25 vs. 41.9±2.7%, control IgG).

Figure 5.2 Quantification of plaque characteristics in $LDLR^{-/-}$ mice treated with anti-CD25 antibodies or control IgG after a HFD of 10 weeks. Tissue sections of aortic roots were analysed by computer-assisted morphometric quantification. The total plaque area was measured in sections stained with hematoxylin and eosin (A). The lipid core content (B) was determined and expressed relative to the mean total plaque area. The percent of positive area in sections stained with Sirius red (collagen, C) or anti-CD68 mAbs (macrophages, D) was determined and expressed relative to the mean total plaque area. Data represent individual mice and the mean values (horizontal lines).

Effect of anti-CD25 antibody treatment on atherosclerosis

In LDLR^{-/-} mice treated with anti-CD25 antibodies, the T-lymphocyte content had markedly increased (Figure 5.3B and 5.4A; $1.6 \pm 0.2 \times 10^{-4} \mu\text{m}^{-2}$, anti-CD25 vs. $0.9 \pm 0.1 \times 10^{-4} \mu\text{m}^{-2}$, control IgG, $p < 0.05$). This increase was due to an enhanced accumulation of both CD4 and CD8 T-lymphocytes (Figure 5.4B and C; CD4 T-lymphocytes: $1.0 \pm 0.2 \times 10^{-4} \mu\text{m}^{-2}$, anti-CD25 vs. $0.6 \pm 0.1 \times 10^{-4} \mu\text{m}^{-2}$, control IgG, $p < 0.05$ and CD8 T-lymphocytes: $0.4 \pm 0.1 \times 10^{-4} \mu\text{m}^{-2}$, anti-CD25 vs. $0.2 \pm 0.0 \times 10^{-4} \mu\text{m}^{-2}$, control IgG, $p < 0.05$). Quantification of the accumulation of CD25⁺ cells in atherosclerotic lesions showed that it had significantly decreased in anti-CD25-treated LDLR^{-/-} mice when compared to LDLR^{-/-} mice treated with control IgG (Figure 5.3C and 5.4D; $0.02 \pm 0.02 \times 10^{-4} \mu\text{m}^{-2}$, anti-CD25 vs. $0.08 \pm 0.02 \times 10^{-4} \mu\text{m}^{-2}$, control IgG, $p < 0.05$). Furthermore, staining with anti-rat Ig antibodies did not reveal any *in vivo* bound anti-CD25 on cells in the aortic root. Taken together, reduction of CD4⁺ CD25⁺ cells did not alter the extent of atherosclerosis, but did induce an inflammatory plaque phenotype that is rich in CD4 T-lymphocytes and CD8 T-lymphocytes.

Figure 5.3 Atherosclerotic plaques of *LDLR*^{-/-} treated with anti-CD25 antibodies or control IgG after a HFD of 10 weeks. Tissue sections of aortic roots were stained with hematoxylin and eosin (A), anti-CD3 mAbs (T-lymphocytes, B) or anti-CD25 mAbs (CD25⁺ cells, C). See page 207 for full colour figures.

Figure 5.4 Analysis of the atherosclerotic plaque phenotype in *LDLR*^{-/-} treated with anti-CD25 antibodies or control IgG after a HFD of 10 weeks. Tissue sections of aortic roots were stained with anti-CD3 mAbs (T-lymphocytes, A), anti-CD4 mAbs (T_H-lymphocytes, B), anti-CD8 mAbs (T_C-lymphocytes, C) or anti-CD25 mAbs (CD25⁺ cells, D). Positive cells were counted and expressed relative to the mean total plaque area. Data represent individual mice and the mean values (horizontal lines).

5.5 Discussion

In the present study, we provided evidence for a role of natural Tregs in atherosclerosis. Treatment of LDLR^{-/-} mice with anti-CD25 antibodies resulted in a depleted CD4⁺ CD25⁺ cell population. Analysis of atherosclerosis in these mice showed that in the absence of CD4⁺ CD25⁺ cells the extent of atherosclerosis was not reduced, but the plaque phenotype was a more inflammatory one, characterised by an increased number of plaque CD4 T-lymphocytes and CD8 T-lymphocytes.

The protective effect of Tregs on the development of atherosclerosis was previously shown. The group of Mallat *et al.* transferred *induced* Tregs, clones of OVA-specific T_R1 cells, into APOE^{-/-} mice and showed a reduction of atherosclerosis. Moreover, plaques of these mice had a reduced accumulation of T-lymphocytes and macrophages.¹⁴ In a second paper, this group proved an important role for *natural* Tregs in atherosclerosis as well.²¹ They used several mouse models deprived of CD4⁺ CD25⁺ Tregs by transplanting CD80^{-/-}CD86^{-/-} bone marrow (BM) into LDLR^{-/-} mice (which yields a reduction in CD4⁺ CD25⁺ splenocytes) or by transplanting Treg-poor CD28^{-/-} splenocytes into APOE^{-/-}RAG2^{-/-} mice. Analysis of atherosclerotic disease in these Treg-deficient mouse models showed that it was more severe than in LDLR^{-/-} or APOE^{-/-} mice with normal numbers of Treg cells.²¹ Additionally, Ait-Oufella *et al.* treated APOE^{-/-} mice twice, every 4 weeks with 100 µg anti-CD25 antibody (PC61 clone), reducing the Treg population by >75% in lymph nodes. This anti-CD25 antibody therapy did not only induce a 50% increase in lesion size, it also induced a decrease in collagen content and an increase in macrophage and T-lymphocyte content in their APOE^{-/-} mice.²¹ Although we used a higher dosage of anti-CD25 and administered the anti-CD25 antibody more frequently, we did not observe such a severe effect on atherosclerotic disease. However, in accordance with both Treg studies,^{14,21} we did find a profound increase in CD3 T-lymphocytes in the atherosclerotic plaque in anti-CD25-treated LDLR^{-/-} mice. One explanation for the difference in outcome of the atherosclerotic disease in the two depletion studies may be the efficiency of the anti-CD25 depletion. The anti-CD25 antibody treatment in the current study reduced the Treg cell population by >75% in blood, but only by 40% in lymph nodes, which is less than the >75% in lymph nodes in the study of Ait-Oufella *et al.*²¹ The incomplete depletion in the

lymphoid organs might explain why the atherosclerotic lesions of the anti-CD25-treated LDLR^{-/-} mice still contained some CD25⁺ cells. Another, more plausible explanation for the effect on atherosclerosis between the two studies might be that the two mouse models, LDLR^{-/-} and APOE^{-/-}, are distinct in atherosclerotic disease.²⁴⁻²⁶ In comparison to LDLR^{-/-} mice, APOE^{-/-} mice might be more susceptible for Treg-mediated regulation of the plaque phenotype of advanced atherosclerotic lesions than in LDLR^{-/-} mice. This would imply that a more stringent depletion of Tregs is required in LDLR^{-/-} mice to achieve the same effect on atherosclerosis as in APOE^{-/-} mice. Indeed, transplanting CD80^{-/-}CD86^{-/-} BM into LDLR^{-/-} mice did result in a far greater reduction of Treg cells than depletion obtained by anti-CD25 antibody treatment and aggravated advanced atherosclerosis in these LDLR^{-/-} mice.²¹ Altogether, depleting Tregs in APOE^{-/-} mice seems to have a much more detrimental effect on the atherosclerotic disease than in LDLR^{-/-} mice.

Although depletion with anti-CD25 antibodies in LDLR^{-/-} mice apparently did not affect the extent of atherosclerotic disease, there was a clear effect on T lymphocyte infiltration in the lesions. In healthy animals, depletion of the CD4⁺ CD25⁺ cell population is not sufficient to induce auto-immunity.²² A secondary stimulus, like immunisation with an auto-antigen, seems to be required.²² In case of LDLR^{-/-} mice, as well as APOE^{-/-} mice, we postulate that the atherosclerosis-prone background of these mouse strains serves as such a secondary stimulus, resulting in increased T-lymphocyte infiltration upon CD25 depletion. The observed inflammatory plaque phenotype therefore might reflect a more vigorous auto-immune reaction. Another mechanism by which the CD4⁺ CD25⁺ cell deficiency might have induced the observed inflammatory atherosclerotic plaque phenotype can be that the lack of natural Tregs has resulted in an enhanced immune response and/ or a decrease in anti-inflammatory cytokines IL-10 and TGF- β . Natural (CD4⁺ CD25⁺) Tregs have been shown to limit disease in several other chronic inflammatory disorders like inflammatory bowel disease, transplantation tolerance and thyroiditis.^{27,28} However, the mechanism by which these natural Tregs do that is not fully elucidated. In addition to controlling T-lymphocyte responses in lymphoid tissue by cell-cell contact, natural Tregs have also been identified at sites of inflammation.²⁹ Interestingly, it has been reported that some of the protective effects of natural Tregs is mediated by IL-10 and/or TGF- β ,¹⁹ indicating that natural Tregs not only act on a systemic level (lymphoid tissues), but also at the site of

inflammation itself. In our study, more CD4 T-lymphocytes and CD8 T-lymphocytes were present in the atherosclerotic lesion after anti-CD25 antibody treatment, reflecting activation of the immune system. The enhancing effect of a vigorous immune response on atherosclerotic disease has been extensively reviewed.³ Especially augmentation of the T_H1 response is associated with aggravation of atherosclerotic disease. IFN γ deficiency, TNF α and IL-6 deficiency all result in a reduction of atherosclerotic disease,³⁰ whereas transfer of IFN γ producing CD4 T-lymphocytes showed clear pro-atherogenic effects.^{10,11} Moreover, both IL-10 and TGF- β have been shown to play a major role in suppressing atherosclerotic plaque development and progression.^{3,30}

In conclusion, absence of natural Tregs enhanced the accumulation of both CD4 T-lymphocytes and CD8 T-lymphocytes in atherosclerotic plaques, thereby inducing an inflammatory plaque phenotype, which is most likely the result of an enhanced (auto-)immune response. This study confirms the importance of the natural Treg population in atherosclerosis and opens perspectives for novel therapeutic strategies to limit complications of atherosclerotic disease.

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Chapter 6

MHCII deficiency attenuates the development of atherosclerosis in LDLR-deficient mice

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Submitted

6.1 Abstract

Many cells among which are T-lymphocytes contribute to atherosclerosis. T-lymphocytes recognise antigen through binding of their TCR to MHC containing a peptide of the antigen. In the current study, we investigated the importance of the MHCII-TCR interaction in atherosclerosis. For this purpose, MHCII^{-/-} mice were backcrossed with LDLR^{-/-} mice and given a HFD for 9 weeks. Flowcytometry confirmed the MHCII deficiency and demonstrated a reduced CD4 T-lymphocyte population in LDLR^{-/-}MHCII^{-/-} mice. High fat feeding increased the cholesterol level in LDLR^{-/-}MHCII^{-/-} mice, but significantly less than in their LDLR^{-/-} littermates. Analysis of the aortic root revealed that lesion severity was markedly attenuated in LDLR^{-/-}MHCII^{-/-} mice and the total plaque area was reduced by 64% in comparison to their LDLR^{-/-} littermates. Interestingly, cholesterol levels explained only 55% of the variation in total plaque area. Therefore, further phenotypic analysis of the atherosclerotic lesions was performed. These analyses showed that the CD4 T-lymphocyte population was reduced in plaques of LDLR^{-/-}MHCII^{-/-} mice. Moreover, LDLR^{-/-}MHCII^{-/-} mice showed a reduced anti-MDA-LDL IgG antibody response. In conclusion, MHCII deficiency hampers the T_H-lymphocyte response and thereby reduces the severity and extent of atherosclerosis.

6.2 Introduction

Atherosclerosis, a chronic inflammatory disease of the large arteries, is the underlying cause of cardiovascular diseases, such as myocardial infarction, peripheral arterial disease and stroke. Atherosclerotic lesions contain mostly macrophages and T-lymphocytes. It has been shown that these components of innate and adaptive immunity are the main players of this chronic inflammatory process.¹⁻³ Several lines of evidence suggest that the progression of atherosclerosis is the result of an antigen-driven T-lymphocyte mediated immune response. Atherosclerotic lesions contain activated T-lymphocytes, which are mainly found in the vicinity of MHCII expressing macrophages or smooth muscle cells.^{4,5} Most T-lymphocytes present in atherosclerotic lesions are T_H-lymphocytes, which produce mainly IFN γ .^{6,7} Deficiency of adaptive immunity, like in RAG^{-/-} or scid/scid mice, does not prevent the initiation of atherosclerosis, but hampers its progression.⁸ Atherosclerosis initiation and progression can also be delayed by depletion of T-lymphocytes⁹ or T_H-lymphocytes.¹⁰⁻¹² Moreover, T-lymphocytes aggravate atherosclerosis if they are transferred into atherosclerosis-prone mice.^{13,14}

T-lymphocytes express TCRs, which are specific for each individual T-lymphocyte. TCRs are essential for the recognition of antigen by T-lymphocytes, because they bind to MHC, which contains a peptide of the antigen. The MHC-TCR interaction is needed for the initiation and effector phase of a T-lymphocyte response. Therefore, analysis of the TCRs expressed by T-lymphocytes in atherosclerotic lesions can provide evidence about whether the T-lymphocyte response is antigen-driven. The T-lymphocyte response in human atherosclerosis was shown to be polyclonal¹⁵⁻¹⁷ and monoclonal for a subpopulation of CD28^{null} CD4 T-lymphocytes¹⁸ and in mice the T-lymphocyte response was oligoclonal,¹⁹ suggesting that the response is not directed against a small range of antigens. On the other hand, T-lymphocytes isolated from atherosclerotic lesions specifically recognised oxLDL.⁶ These data do not give a conclusive answer to the question whether T-lymphocytes participate to atherogenesis by an antigen-driven response or as innocent bystanders. To elucidate the importance of the MHCII-TCR interaction in atherosclerosis, LDLR^{-/-}MHCII^{-/-} mice were generated. These mice were analysed for the extent and phenotype of atherosclerosis, as well as plasma lipid profiles and T_H-lymphocyte function.

6.3 Methods

Mice

LDLR^{-/-}MHCII^{-/-} mice were generated by backcrossing LDLR^{-/-} mice (fourth generation C57Bl/6) with MHCII^{-/-} mice. MHCII^{-/-} mice were generated in C57Bl/6 mice by disrupting the Aa gene²⁰ (a generous gift from H. Bluethmann, Hoffmann-La Roche, Basel, Switzerland). LDLR deficiency and MHCII deficiency were determined by PCR analysis. The primer sequences of the LDLR gene were: 1) 5'-GCAGTGCTCCTCATCTGACTTG-3'; 2) 5'-CATATGCATCCCCAGTCTTTG-3' and 3) 5'-ATAGATTGCCCTTGTGTC-C-3' and of the MHCII gene were: 4) 5'-CACGTACCATTGGTAGCTGG-3'; 5) 5'-GGAGACTGTCTGGATGCTTC-3' and 6) 5'-ATATTGCTGAAGAGCTTGGCGG-C-3'. The LDLR gene was amplified by using primers 1 and 2, resulting in a 223 base pair fragment and the amplification of the mutated gene with primers 2 and 3 generated a 175 base pair fragment. The MHCII gene was amplified by using primers 4 and 5, resulting in a 144 base pair fragment and the amplification of the mutated gene with primers 5 and 6 generated a fragment of approximately 650 base pairs. All mouse strains were maintained at our animal facility (Maastricht, The Netherlands). 8-12-weeks-old Female littermates were given a HFD containing 16% fat and 0.15% cholesterol (Hope Farms, Woerden, the Netherlands) for 9 weeks. Prior to sacrifice, mice were anaesthetised by i.p. injection of 25 µg/g xylazine (Sedamun, Eurovet, Bladel, the Netherlands) and 125 µg/g ketamine (Nimatek, Eurovet). Subsequently, mice were bled by vena cava inferior puncture followed by perforation of the diaphragm. All procedures were approved by the animal care and use committee of the Maastricht University and were in accordance with national regulations on animal experiments.

Flowcytometry

Prior to sacrifice, heparinised blood was obtained from the inferior vena cava. Spleen and lymph nodes (superficial and deep cervical nodes, brachial nodes and mesenteric nodes) were collected from sacrificed mice. Single-cell suspensions from spleen and lymph nodes were prepared. In blood and spleen, erythrocytes were removed by hypotonic lysis with NH₄Cl.²¹ Blood cells (2×10⁵ per sample) were double stained with Gr1 (Ly-6G and Ly-6C)-FITC (RB6-8C5) and Mac1 (CD11b)-PE (M1/70) (BD-Pharmingen, San Diego,

California). Data were collected of 0.5×10^4 Gr1⁺ cells. In addition, cells (1×10^6 per sample) were stained with MHCII (NIMR-4) (Southern Biotechnology Associates, Birmingham, Alabama), which was detected in a second step with anti-rat Ig-FITC cells. Data were collected of 2.5×10^4 cells MHCII⁺ cells. Finally, cells (1×10^6 per sample) were triple stained with CD3-FITC (17A2), CD8 α -PE (53-6.7), CD4-biotin (RM4-5) (BD-Pharmingen). The biotin-labelled antibodies were detected in a second step with Streptavidin-CyChrome (BD-Pharmingen). Data were collected of 1×10^4 CD3⁺ cells. Flowcytometry analysis was performed on a FACS Calibur and analysed with CellQuest software (BD-Bioscience, San Jose, California).

Lipid parameters

Plasma cholesterol and plasma total triglyceride levels were determined using standard colorimetric assays (Roche Diagnostics, Mannheim, Germany). Pooled plasma was fractionised over a Superose 6PC 3.2/30 column in an AKTABasic chromatography system (Amersham Biosciences, Roosendaal, the Netherlands) to separate the lipoproteins. The amount of cholesterol in the lipoprotein fractions was measured using standard colorimetric assays (Roche Diagnostics).

Tissue processing

The heart was removed from sacrificed mice by cutting the aorta just cranial from the atria. The heart was divided in two by cutting perpendicular to the heart axis just below the atrial tips. The upper part of the heart containing the aorta was embedded in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) with the base facing downward, frozen in isopentane that was cooled by dry ice and stored at -80°C . Serial sections of the aortic root area of the heart were cut at $7\ \mu\text{m}$. Sections showing the presence of the aortic valves were collected and used for analysis.

Histomorphometry and immunohistometry

Collected sections of the aortic root, $42\ \mu\text{m}$ apart, were stained with hematoxylin and eosin, Sirius red (collagen content) or CD68 (supernatant of clone FA-11, macrophage content). Atherosclerotic lesions of each mouse were classified for their severity. Based on the AHA classification,²²⁻²⁴ three categories were defined: 1) early lesions, which contained mainly

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foam cells, 2) intermediate lesions, which consisted of foam cells and a starting collagen cap, 3) advanced lesions, which were composed of foam cells, a necrotic core, a collagen cap and foam cells infiltrating the media with occasionally disruption of the elastin fibers. Morphometric analysis was performed using a microscope coupled to a computerised morphometry system (Quantimet Qwin version 3, Leica, Rijswijk, the Netherlands). Total plaque area was quantified as an average of at least 3 sections per mouse. Lipid core content, collagen content and macrophage content were determined in the central part of the aortic root. The content was expressed as positive area relative to the mean total plaque area.²¹ All analyses were performed without prior knowledge of the genotype.

MHCII deficiency was confirmed by staining aortic root sections with MHCII (supernatant of clone M5/114). To quantify the infiltrating T-lymphocytes in the aortic lesions, aortic root sections were stained for CD3 (supernatant of clone KT3, T-lymphocytes), CD4 (supernatant of GK1.5, T_H-lymphocytes) and CD8 (53-6.7 (BD Pharmingen), T_C-lymphocytes). The number of positive cells was counted in the same area of the aortic root, which was used for the total plaque area measurement and expressed as a percentage of the mean total plaque area. All analyses were performed blinded to the genotype of mice.

Anti-MDA-LDL antibody detection

Antibodies to MDA-LDL were measured by ELISA.²⁵ Briefly, LDL (d = 1.030-1.045 g/ml) was isolated from human plasma by density-gradient ultracentrifugation. Isolated LDL was modified by malondialdehyde (MDA, Merck, Hohenbrunn, Germany) in a final concentration of 169 mM. Native LDL and MDA-LDL were used freshly (*i.e.* within 14 days). MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 100 µg native LDL or MDA-LDL in 100 µl PBS and kept overnight at 4°C. Plates were washed 5 times with washing buffer. Mouse plasma was added in duplicate at a 1:50 dilution in incubation buffer and kept overnight at 4°C. After washing the plates 5 times with washing buffer, they were incubated with either alkaline phosphatase-labeled anti-mouse IgM or IgG (Jackson Immuno-Research Laboratory, Westgrove, Pennsylvania) both at a 1:4000 dilution in incubation buffer for 1 hour at 37°C. Subsequently, the plates were washed again 5 times with washing buffer and developed by adding freshly made substrate containing 1 mg/ml disodium p-nitrophenyl phosphate (Sigma, St. Louis, Missouri) in diethanolamine

buffer at pH 9.8. After 120 minutes on a shaking platform at room temperature, the absorbance of the wells was read at 405 nm in an ELISA reader. Anti-MDA-LDL antibody levels were expressed as net absorbance, which was calculated by subtracting the mean absorbance of wells coated with native LDL from that of wells coated with MDA-LDL.

Statistical analysis

Results are presented as mean $\pm$ SEM. The data were analysed with the Kolmogorov-Smirnov (KS) test to assess normality and the F-test to compare variances. When results passed both tests ($p < 0.05$) they were analysed with the unpaired T-test (two-sided). Distribution of lipoproteins and classification of the atherosclerotic lesions were tested by the Chi-squared test. Correlation of the cholesterol level with the total plaque area was tested by the Pearson test. A p-value of $p < 0.05$ was accepted as statistically significant.

6.4 Results

Characterisation of LDLR^{-/-}MHCII^{-/-} mice

LDLR^{-/-}MHCII^{-/-} mice were viable and healthy. Their bodyweight prior to the induction of atherosclerosis was equal to that of their LDLR^{-/-} littermates (17.0 ± 0.6 g, LDLR^{-/-}MHCII^{-/-} vs. 17.0 ± 0.4 g, LDLR^{-/-}) and remained the same during the high fat feeding. After 9 weeks of high fat feeding, the bodyweight had increased to 21.2 ± 0.4 g for the LDLR^{-/-}MHCII^{-/-} mice and 21.0 ± 0.7 g for the LDLR^{-/-} mice ($p > 0.05$).

Analysis of the MHCII expression on blood cells by flowcytometry revealed that leukocytes from LDLR^{-/-}MHCII^{-/-} mice were indeed deficient for the MHCII molecule (Figure 6.1). Similar results were obtained in spleen and lymph nodes (data not shown). Further analysis of the leukocyte subsets (granulocytes, monocytes and lymphocytes) in blood by flowcytometry revealed that the granulocyte population was significantly increased in the LDLR^{-/-}MHCII^{-/-} mice ($39.2 \pm 3.4\%$, LDLR^{-/-}MHCII^{-/-} vs. $25.3 \pm 2.1\%$, LDLR^{-/-}, $p < 0.005$) as shown in Table 6.1. Signs of granulocytosis were not found in sections of heart or lymphoid tissues (data not shown).

Figure 6.1 Characterisation of $LDLR^{-/-}MHCII^{-/-}$ and $LDLR^{-/-}$ mice by flowcytometry analysis. Blood cells were labelled with anti-MHCII mAb or with anti-CD4 mAbs (T_H -lymphocytes) and anti-CD8 mAbs (T_C -lymphocytes) and analysed by flowcytometry. FACS analysis of spleen and lymph node cells gave similar results. A, The histograms display the MHCII expression (solid line) on blood cells of a representative mouse. B, The dotplots represent the distribution of $CD4^{+}$ and $CD8^{+}$ T cells in blood of a representative mouse.

Table 6.1 Circulating leukocyte values of $LDLR^{-/-}MHCII^{-/-}$ and $LDLR^{-/-}$ mice

	$LDLR^{-/-}MHCII^{-/-}$ N = 6	$LDLR^{-/-}$ N = 7
Granulocytes, %	39.2 ± 3.4 *	25.3 ± 2.1
Monocytes, %	17.7 ± 1.5	18.9 ± 3.4
T cells, %	30.4 ± 1.9	29.4 ± 3.7
CD4:CD8	0.04 ± 0.01 *	1.51 ± 0.24

Values are mean $\pm$ SEM. * $p < 0.005$

The T-lymphocyte compartment was significantly decreased in spleens of LDLR^{-/-}MHCII^{-/-} mice (Table 6.2; $1.9 \times 10^6 \pm 0.1 \times 10^6$ cells, LDLR^{-/-}MHCII^{-/-} vs. $2.9 \times 10^6 \pm 0.2 \times 10^6$ cells, LDLR^{-/-}, $p < 0.005$). Additionally, the CD4:CD8 T cell ratio was significantly reduced in LDLR^{-/-}MHCII^{-/-} mice in comparison to their LDLR^{-/-} littermates in blood, spleen and lymph nodes (blood: 0.04 ± 0.01 , LDLR^{-/-}MHCII^{-/-} vs. 1.51 ± 0.24 , LDLR^{-/-}, spleen: 0.07 ± 0.01 , LDLR^{-/-}MHCII^{-/-} vs. 1.45 ± 0.13 , LDLR^{-/-}, lymph nodes: 0.03 ± 0.00 , LDLR^{-/-}MHCII^{-/-} vs. 1.11 ± 0.11 , LDLR^{-/-}, $p < 0.001$) confirming that MHCII deficiency results in the absence of the CD4⁺ T-lymphocyte population (Table 6.1, Table 6.2 and Figure 6.1).

Table 6.2 T cell values of LDLR^{-/-}MHCII^{-/-} and LDLR^{-/-} mice

	LDLR ^{-/-} MHCII ^{-/-}	LDLR ^{-/-}
	N = 6	N = 6
# CD3 ⁺ cells ($\times 10^6$), spleen	1.9 ± 0.1 *	2.9 ± 0.2
CD4:CD8, spleen	0.07 ± 0.01 *	1.45 ± 0.13
# CD3 ⁺ cells ($\times 10^6$), lymph nodes	12.6 ± 1.9	7.6 ± 0.5
CD4:CD8, lymph nodes	0.03 ± 0.00 *	1.11 ± 0.11

Values are mean $\pm$ SEM. * $p < 0.005$

MHCII deficiency decreases plasma lipoprotein levels and reduces the severity of atherosclerosis

Analysis of plasma revealed that LDLR^{-/-}MHCII^{-/-} mice developed significantly lower cholesterol and triglyceride levels in comparison to their LDLR^{-/-} littermates after 9 weeks of high fat feeding (Cholesterol: 643 ± 85 mg/dl, LDLR^{-/-}MHCII^{-/-} vs. $1,214 \pm 217$ mg/dl, LDLR^{-/-}, $p < 0.05$ and triglycerides: 59 ± 10 mg/dl, LDLR^{-/-}MHCII^{-/-} vs. 283 ± 85 mg/dl, LDLR^{-/-}, $p < 0.05$). This difference was also reflected in a significantly altered distribution of lipoproteins of these mice: plasma of LDLR^{-/-}MHCII^{-/-} mice contained less VLDL and more HDL than their LDLR^{-/-} littermates (Figure 6.2; VLDL: 47.7%, LDLR^{-/-}MHCII^{-/-} vs. 68.0%, LDLR^{-/-} and HDL: 12.5%, LDLR^{-/-}MHCII^{-/-} vs. 1.3%, LDLR^{-/-}, $p < 0.0001$).

Figure 6.2 Plasma lipoprotein profiles of LDLR^{-/-}MHCII^{-/-} and LDLR^{-/-} mice after a HFD of 9 weeks. Plasma of individual mice was pooled and fractionised. A, Representation of the plasma cholesterol profile from LDLR^{-/-}MHCII^{-/-} (—●—) and LDLR^{-/-} (—■—) mice. B, Distribution of the individual plasma lipoprotein fractions from LDLR^{-/-}MHCII^{-/-} and LDLR^{-/-} mice.

Analysis of atherosclerotic lesion severity in the aortic root by classification of the lesions into early, intermediate or advanced showed that LDLR^{-/-}MHCII^{-/-} mice contained significantly more early lesions and less advanced lesions than their LDLR^{-/-} littermates (Figure 6.3A and 6.4A; early: 58%, LDLR^{-/-}MHCII^{-/-} vs. 11 %, LDLR^{-/-} and advanced: 17%, LDLR^{-/-}MHCII^{-/-} vs. 63%, LDLR^{-/-}, $p=0.0005$). This shows that MHCII deficiency decreases lesion progression from early to advanced lesions. Moreover, total plaque area in

the aortic root was reduced by 64% in LDLR^{-/-}MHCII^{-/-} mice when compared to their LDLR^{-/-} littermates (Figure 6.3B and 6.4A; 82,680±18,810 μm^2 , LDLR^{-/-}MHCII^{-/-} vs. 230,200±39,530 μm^2 , LDLR^{-/-}, $p=0.006$). Interestingly, cholesterol levels explained only 55% of the variation in total plaque area (Figure 6.3C; $R^2=0.547$, $p=0.0003$). This implies that the observed reduction in severity of atherosclerosis must have been for a great deal the result of another factor than cholesterol levels, probably MHCII deficiency.

Figure 6.3 Characterisation of atherosclerosis in LDLR^{-/-}MHCII^{-/-} and LDLR^{-/-} mice after a HFD of 9 weeks. Tissue sections of aortic roots were stained with hematoxylin and eosin. Atherosclerotic lesions were classified according to their severity (A). The total plaque area was measured by computer-assisted morphometric quantification (B). Data represent individual mice and the mean values (horizontal lines). C, Plasma cholesterol levels were correlated with the total plaque area ($R^2=0.547$). Data represents individual LDLR^{-/-}MHCII^{-/-} (▲) or LDLR^{-/-} (■) mice.

Figure 6.4 Atherosclerotic plaques of $LDLR^{-/-}MHCII^{-/-}$ and $LDLR^{-/-}$ mice after a HFD of 9 weeks. Tissue sections of aortic roots were stained with hematoxylin and eosin (A), anti-MHCII mAbs (B) or anti-CD4 mAbs (T_H -lymphocytes, C). See page 208 for full colour figures.

Quantitative analysis of the plaque phenotype revealed that lipid core content was significantly decreased, collagen content was comparable and macrophage content was significantly increased in $LDLR^{-/-}MHCII^{-/-}$ mice (Figure 6.5A-C; lipid core content: $2.5 \pm 1.5\%$, $LDLR^{-/-}MHCII^{-/-}$ vs. $17.6 \pm 4.1\%$, $LDLR^{-/-}$, $p=0.006$; collagen content: $17.8 \pm 2.7\%$, $LDLR^{-/-}MHCII^{-/-}$ vs. $24.5 \pm 2.8\%$, $LDLR^{-/-}$ and macrophage content: $71.8 \pm 4.6\%$,

LDLR^{-/-}MHCII^{-/-} vs. 49.2±2.8%, LDLR^{-/-}, p=0.002). Comparison of the plaque phenotype of early vs. early, intermediate vs. intermediate and advanced vs. advanced lesions of LDLR^{-/-} and LDLR^{-/-}MHCII^{-/-} mice showed no significant differences. This indicates that MHCII deficiency purely decreases plaque severity and does not result in the development of a different plaque phenotype.

Figure 6.5 Quantification of the atherosclerotic plaque characteristics in LDLR^{-/-}MHCII^{-/-} and LDLR^{-/-} mice after a HFD of 9 weeks. Tissue sections of aortic roots were analysed by computer-assisted morphometric quantification. The lipid core area (A) was determined and expressed relative to the mean total plaque area. The percent positive area in sections stained with Sirius red (collagen, B) or anti-CD68 mAbs (macrophages, C) was determined and expressed relative to the mean total plaque area. Data represent individual mice and the mean values (horizontal lines).

To further assess the effect of the MHCII-TCR interaction on plaque phenotype, immunoreactivity of MHCII was determined and the accumulation of T-lymphocytes was quantified. In LDLR^{-/-} mice, MHCII was abundantly expressed throughout the atherosclerotic lesions, but mostly in the macrophage-rich area. In contrast, the expression of MHCII was completely absent in atherosclerotic lesions of LDLR^{-/-}MHCII^{-/-} mice (Figure 6.4B). Quantitative analysis of the accumulation of T-lymphocytes demonstrated that the T-lymphocyte content was similar in LDLR^{-/-}MHCII^{-/-} mice (Figure 6.6A; $0.9 \pm 0.2 \times 10^{-4} \mu\text{m}^{-2}$, LDLR^{-/-}MHCII^{-/-} vs. $1.0 \pm 0.2 \times 10^{-4} \mu\text{m}^{-2}$, LDLR^{-/-}, p>0.05). However, the

accumulation of CD4⁺ T cells was reduced (Figure 6.4C and 6.6B; $0.3 \pm 0.1 \times 10^{-4} \mu\text{m}^{-2}$, LDLR^{-/-}MHCII^{-/-} vs. $0.7 \pm 0.1 \times 10^{-4} \mu\text{m}^{-2}$, LDLR^{-/-}, $p < 0.05$), while the CD8⁺ T cell content was enhanced (Figure 6.6C; $1.3 \pm 0.2 \times 10^{-4} \mu\text{m}^{-2}$, LDLR^{-/-}MHCII^{-/-} vs. $0.6 \pm 0.2 \times 10^{-4} \mu\text{m}^{-2}$, LDLR^{-/-}, $p < 0.05$).

Figure 6.6 Analysis of the atherosclerotic plaque phenotype in LDLR^{-/-}MHCII^{-/-} and LDLR^{-/-} mice after a HFD of 9 weeks. Tissue sections of the aortic roots were stained with anti-CD3 mAbs (T-lymphocytes, A), anti-CD4 mAbs (T_H-lymphocytes, B) or anti-CD8 mAbs (T_C-lymphocytes, C). Positive cells were counted and expressed relative to the mean total plaque area. Data represent individual mice and the mean values (horizontal lines).

T_H-lymphocytes are essential for isotype class switching of antibodies in B-lymphocytes. The reduced T_H-lymphocyte population in MHCII^{-/-} mice resulted in a hampered B-lymphocyte response,^{26,27} therefore we hypothesised that the anti-MDA-LDL antibody response would also be hindered in LDLR^{-/-}MHCII^{-/-} mice. In accordance with this hypothesis, anti-MDA-LDL IgG antibody levels were reduced in LDLR^{-/-}MHCII^{-/-} mice (Figure 6.7B; 0.10 ± 0.02 net absorbance at OD₄₀₅, LDLR^{-/-}MHCII^{-/-} vs. 0.19 ± 0.02 net absorbance at OD₄₀₅, LDLR^{-/-}, $p = 0.01$), while the anti-MDA-LDL IgM antibody levels were enhanced in LDLR^{-/-}MHCII^{-/-} mice (Figure 6.7A; 2.23 ± 0.10 net absorbance at OD₄₀₅, LDLR^{-/-}MHCII^{-/-} vs. 0.91 ± 0.07 net absorbance at OD₄₀₅, LDLR^{-/-}, $p < 0.0001$). These results

show that in the absence of MHCII-TCR interactions atherosclerosis is less severe, which may be the result of an attenuated T_H-lymphocyte response.

Figure 6.7 Analysis of the antibody response to MDA-LDL in $LDLR^{-/-}MHCII^{-/-}$ and $LDLR^{-/-}$ mice after a HFD of 9 weeks. In plasma of $LDLR^{-/-}MHCII^{-/-}$ and $LDLR^{-/-}$ mice, binding of IgM (A) and IgG (B) antibodies directed against native LDL or anti-MDA-LDL were determined by ELISA. The net absorbance was calculated by subtracting the mean absorbance of native LDL from that of MDA-LDL. Data represent individual mice and the mean values (horizontal lines).

6.5 Discussion

In the present study, we generated $LDLR^{-/-}MHCII^{-/-}$ mice to provide new evidence on the role of the MHCII-TCR interaction in the development of atherosclerosis. Characterisation of the $LDLR^{-/-}MHCII^{-/-}$ mice confirmed the MHCII deficiency on cells in blood, lymphoid organs and aortic roots and revealed the presence of a markedly small CD4 T-lymphocyte population. Analysis of the $LDLR^{-/-}MHCII^{-/-}$ mice after high fat feeding revealed three main findings. First, the extent of atherosclerosis was markedly reduced in the absence of MHCII. Moreover, atherosclerotic lesion severity, reflected by a decreased number of advanced lesions, was considerably diminished in $LDLR^{-/-}MHCII^{-/-}$ mice. Second, $LDLR^{-/-}MHCII^{-/-}$ mice had a decreased accumulation of CD4⁺ T cells in the atherosclerotic lesions and a hampered anti-MDA-LDL antibody response. Finally, it was shown that $LDLR^{-/-}$

MHCII^{-/-} mice developed lower plasma cholesterol and triglyceride levels after high fat feeding than wild-type LDLR^{-/-} mice.

The effect of MHCII on the initiation of atherosclerosis was previously analysed in MHCII^{-/-} mice on a C57Bl/6 background, but the results were inconclusive. One study found little difference in the extent of atherosclerosis,²⁸ while another study showed a dramatically reduced lesion size in the absence of MHCII.¹¹ C57Bl/6 mice develop only early lesions full of foam cells after high fat feeding²⁹ and consequently lack the advanced stages of atherosclerosis seen in humans and in other mouse models, like APOE^{-/-} and LDLR^{-/-} mice.³⁰ In this study, we therefore used LDLR^{-/-}MHCII^{-/-} mice to specifically examine the effect of MHCII on the development of both early and advanced lesions. Our LDLR^{-/-}MHCII^{-/-} mice clearly developed atherosclerosis, but to a lesser extent and severity than their LDLR^{-/-} littermates. This finding confirms that the adaptive immune response is not essential for the initiation of atherosclerosis, but plays an important role in the development of advanced lesions.

Earlier studies already reported that MHCII^{-/-} mice have a reduced T_H-lymphocyte population as found in the current analysis of the LDLR^{-/-}MHCII^{-/-} mice. Despite the absence of circulating CD4 T-lymphocytes, there were still some CD4 T-lymphocytes present in the atherosclerotic lesions of LDLR^{-/-}MHCII^{-/-} mice. The almost complete absence of the CD4 T-lymphocyte population in MHCII^{-/-} mice is the result of the fact that CD4 T-lymphocytes require positive selection mediated by MHCII to survive their maturation in the thymus.^{20,26,27} Still some CD4 T-lymphocytes escape this positive selection by yet unknown mechanisms. It has been suggested that the surviving CD4 T-lymphocytes are actually restricted to MHCI instead of MHCII²⁰ or that they are positively selected by interacting with a molecule that resembles MHCII (H-2O), which is expressed on thymic epithelial cells.³¹ A third option is that CD4 T-lymphocytes escape programmed cell death and therefore were not positively selected.²⁰ In any case, the remaining circulating CD4 T-lymphocyte population appears to be reduced but normal, since it contains a heterogeneous TCR V β repertoire,^{20,26,27} whereas the CD4 T-lymphocytes expressing a $\gamma\delta$ TCR are not affected by the MHCII deficiency.³²

The impaired CD4 T-lymphocyte population has likely contributed to the remarkable reduced severity of atherosclerosis in LDLR^{-/-}MHCII^{-/-} mice, since atherosclerotic lesions of LDLR^{-/-}MHCII^{-/-} mice contained less CD4 T-lymphocytes. The remaining CD4 T-lymphocytes in the LDLR^{-/-}MHCII^{-/-} mice are likely to produce less cytokines, like IFN γ , thereby possibly resulting in less activation of macrophages and decreased atherosclerosis severity. Indeed, attenuation of atherosclerosis development has been found in IFN γ -receptor-deficient mice.³³ Additionally, an increased level of anti-MDA-LDL IgM antibodies was observed in LDLR^{-/-}MHCII^{-/-} mice. The anti-MDA-LDL IgM antibodies can capture formed oxidized LDL³⁴ and block the uptake of oxidized LDL by macrophages,^{35,36} thereby preventing the accumulation of oxidized LDL and foam cell formation in LDLR^{-/-}MHCII^{-/-} mice. Moreover, the anti-MDA-LDL IgG antibody response was reduced showing that the anti-MDA-LDL response is T-lymphocyte dependent and that T-lymphocyte help for isotype class switching was largely absent in LDLR^{-/-}MHCII^{-/-} mice. This absence of isotype class switching was already reported for MHCII^{-/-} mice, which were challenged with T-lymphocyte dependent antigens.^{26,27} A reduction of the anti-MDA-LDL IgG antibodies can also contribute to diminished atherosclerosis, because the macrophages will be less activated by signals from the Fc receptor through binding of anti-MDA-LDL IgG antibody complexes^{37,38} eventually decreasing the inflammatory response.

Surprisingly, LDLR^{-/-}MHCII^{-/-} mice developed lower plasma cholesterol and triglyceride levels than wild-type LDLR^{-/-} mice after a HFD for 9 weeks. Interestingly, a similar reduction of cholesterol levels was also found in other immunodeficient atherosclerosis-prone mice, like APOE^{-/-}RAG^{-/-} or nude/nude mice.^{28,39,40} In the current study the reduction of cholesterol could be mainly attributed to a decline of the VLDL fraction in LDLR^{-/-}MHCII^{-/-} mice, while the HDL fraction had increased. Atherosclerosis is a chronic inflammatory process resulting in a prolonged acute phase response due to the release of cytokines. Specific changes in the distribution of lipoproteins are reported to be induced upon chronic inflammation leading to increased VLDL levels and triglyceride levels and decreased HDL levels, resulting in an overall increase of the total cholesterol level in mice.⁴¹ Therefore, it can be postulated that LDLR^{-/-}MHCII^{-/-} mice develop a less chronic inflammatory response, not only resulting in the observed decrease in atherosclerosis extent

and severity, but also resulting in reduced levels of triglycerides and VLDL and in an increased level of HDL in comparison to LDLR^{-/-} mice.

Interestingly, one of the most potent cholesterol lowering drugs in cardiovascular disease, the HMG-CoA reductase inhibitors, also have proven anti-inflammatory effects. This anti-inflammatory effect partly seems to result from their ability to dampen MHCII expression upon IFN γ stimulation.⁴² In this respect, it was recently demonstrated that statins reduced the IFN γ -induced expression of MHCII in human endothelial cells and monocytes/macrophages.⁴³ Considering the results in the current paper and the published beneficial effects of HMG-CoA reductase inhibitors in cardiovascular disease, which are partly due to a reduction of MHCII expression, the inhibition of MHCII expression may be a novel therapeutic strategy for the treatment of atherosclerosis.

Overall, the main finding of this study is that MHCII deficiency reduces cholesterol levels and the inflammatory response, thereby hampering the progression of early atherosclerotic lesions into advanced plaques. Therefore, reduction of MHCII expression might be a potential target in patients with atherosclerosis.

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Chapter 7

General discussion

7.1 Main findings described in this thesis

The chapters of this thesis describe how adaptive immunity contributes to advanced atherosclerosis and what the effects of interfering with adaptive immunity are on the plaque phenotype of advanced atherosclerotic lesions. In Figure 7.1, the main findings of this thesis are schematically presented according to the three major requirements of T-lymphocyte responses: antigen specificity, co-stimulation and regulation.

7.2 Adaptive immunity in atherosclerosis

It is now generally accepted that most cardiovascular events are caused by luminal thrombosis, which is the result of rupture or erosion of an advanced atherosclerotic lesion.¹ Histological characterisation of these lesions show that they are severely infiltrated with inflammatory cells, mainly macrophages and to a lesser extent T-lymphocytes, indicative for an aggravated immune response.¹ The participation of the immune system in the pathogenesis of atherosclerosis has been confirmed in several animal models as discussed in *Chapter 1*. Not only does innate immunity have a major role in the initiation and progression of atherosclerosis, it is now widely recognised that adaptive immunity largely determines the progression of atherosclerosis to the advanced stages mainly by acting as regulator of the activity of plaque macrophages.²⁻⁴ As depicted in *Chapter 1*, T-lymphocytes expressing activation markers are found in human and experimental atherosclerotic lesions. Additionally, studies in animal models reported that in the absence of T-lymphocytes the extent of advanced atherosclerosis is reduced,⁵⁻⁸ while transfer of T-lymphocytes aggravates atherosclerosis.^{9,10} These data clearly show that T-lymphocytes contribute to the progression of atherosclerosis. A remaining key question is how and to what extent T-lymphocytes participate in the development of advanced atherosclerotic plaques. For the activation of T-lymphocytes two signals are required: the first signal is recognition of antigen¹¹ and the second signal is co-stimulation.^{12,13} The importance of these two interactions in advanced atherosclerosis will be further discussed.

Figure 7.1 Schematic overview of the main results of this thesis.

7.3 Antigen recognition by T-lymphocytes in atherosclerosis

The first signal in the activation of T-lymphocytes is antigen recognition. Prerequisite for the recognition of antigen by T-lymphocytes is that antigen is presented in the context of an MHCII molecule, which is expressed by an APC. As such, the MHCII-antigen complex can bind to the TCR of CD4 T-lymphocytes. Strong evidence for the existence of antigen recognition in atherosclerosis is that activated T-lymphocytes are mainly found in the proximity of macrophages or smooth muscle cells that express MHCII molecules¹⁴⁻¹⁹ and thus can function as APCs.

In *Chapter 6*, we proved that antigen presentation is essential for the role of CD4 T-lymphocytes in the development of advanced atherosclerosis. We generated atherosclerosis-prone mice that were deficient for MHCII and found that advanced atherosclerosis was retarded in MHCII-deficient LDLR^{-/-} mice. To our surprise, we also found that the cholesterol levels were markedly lower in LDLR^{-/-}MHCII^{-/-} mice than in LDLR^{-/-} mice. This finding suggests that the cholesterol metabolism is somehow affected by the MHCII-deficiency. As discussed in *Chapter 6*, this could be due to a decreased chronic inflammation in LDLR^{-/-}MHCII^{-/-} mice when compared to LDLR^{-/-} mice. This might reduce the synthesis of hepatic cholesterol or adipose tissue lipolysis and thereby diminish the plasma cholesterol levels.²⁰ However, there may be another explanation for the lowered plasma cholesterol levels in LDLR^{-/-}MHCII^{-/-} mice. At the time that we backcrossed the MHCII^{-/-} mice into the LDLR^{-/-} mouse strain, we occasionally noticed that some mice of 3-4 months old spontaneously developed chronic diarrhea, resulting in a progressive wasting disease with an occasional anorectal prolapse. A publication by Mombaerts *et al.* reported similar observations and characterised this pathology as spontaneous development of inflammatory bowel disease.²¹ They postulated that this disease is probably caused by incorrect control of B-lymphocytes due to the lack of CD4 T-lymphocytes expressing a $\alpha\beta$ TCR.^{21,22} Subclinical presence of inflammatory bowel disease in the LDLR^{-/-}MHCII^{-/-} mice might have influenced the intestinal lipid uptake. Similar findings were reported in a recently published paper on the effects of chronic intestinal inflammation in APOE^{-/-} mice.²³ Chronic intestinal inflammation resulted in reduced plasma cholesterol levels and less atherosclerosis²³ like in our LDLR^{-/-}MHCII^{-/-} mice. Tous

et al. proposed that the observed lower plasma cholesterol levels were probably mediated by attenuated intestinal re-absorption of cholesterol.²³ Although the LDLR^{-/-}MHCII^{-/-} mice, which were included in the study described in *Chapter 6*, had similar weights as LDLR^{-/-} mice, macroscopic analysis of the intestines showed no signs of inflammatory bowel disease. However, it might be that the intestines of all LDLR^{-/-}MHCII^{-/-} mice were subclinically affected resulting in an impaired lipid uptake.

In *Chapter 6*, we demonstrated that despite the markedly dropped cholesterol levels the observed effect on atherosclerosis in LDLR^{-/-}MHCII^{-/-} mice must have been in part the result of a defective antigen-driven T-lymphocyte response. First of all, the cholesterol level could explain only 55% of the variation in total plaque area. Second, the accumulation of CD4⁺ T cells in the atherosclerotic lesions was reduced by 57% in LDLR^{-/-}MHCII^{-/-} mice when compared to LDLR^{-/-} mice. Finally, the anti-MDA-LDL IgG antibody response was significantly reduced, suggesting an impaired T_H-lymphocyte function. The importance of antigen-specific T-lymphocytes was recently established by a study of Zhou *et al.*²⁴ in which they showed that transfer of T-lymphocytes specific for oxLDL into APOE^{-/-} scid/scid mice aggravated atherosclerosis when compared to mice receiving T-lymphocytes specific for an irrelevant antigen.²⁴

Analysis of the LDLR^{-/-}MHCII^{-/-} mice gave a first proof that the T-lymphocyte response is antigen-driven in atherosclerosis. However, the T-lymphocyte deficiency in MHCII^{-/-} mice, which is possibly responsible for the lowered plasma cholesterol levels, seems to be a major drawback of this model. To overcome this unwanted deficiency in T-lymphocyte development, a possible strategy is to treat LDLR^{-/-}MHCII^{-/-} mice with anti-TCR antibodies. This treatment was reported to successfully restore the generation of CD4 T-lymphocytes in MHCII-deficient mice.²⁵ Since APCs originate from the BM, a different approach is to perform a BM transplantation with MHCII-deficient BM into LDLR^{-/-} mice. In our experience, this approach was not immediately successful, since in a pilot experiment LDLR^{-/-} mice receiving MHCII-deficient BM (n=5) died within 8 wk postengraftment of a progressive wasting disease. Similar observations were reported by others that found that mice receiving MHCII-deficient BM died from a systemic auto-immune disease.^{26,27} We hypothesised that this auto-immune disease is mediated by T-

lymphocytes and that the BM transplantation resulted in a deficient Treg population. If this is true an additional transfer of purified T-lymphocytes should rescue the mice from developing this auto-immune disease. In a pilot study, we tested this hypothesis and the mice receiving MHCII^{-/-} BM and 10⁷ T-lymphocytes (n=5) survived up to 12 wk postengraftment until they still had to be sacrificed because of developed illness. Our hypothesis was thereafter confirmed in a study by Teshima *et al.*, in which they transferred twice as many T-lymphocytes and could extend the life of the mice receiving MHCII^{-/-} BM up to 14 weeks without any signs of systemic auto-immune disease.²⁷ Examination of atherosclerotic lesion development in one of these two proposed mouse models will further establish the importance of antigen presentation for the T-lymphocyte response in atherosclerosis.

7.4 Co-stimulation by T-lymphocytes in atherosclerosis

The second signal required for the activation of T-lymphocytes is co-stimulation, which main effect is the amplification or counteraction of signals provided by the TCR. Without co-stimulation, antigen recognition by the TCR leads to death or anergy of the primed T-lymphocytes. The most important and best-characterised co-stimulatory interaction is CD28 on the surface of T-lymphocytes, which binds to CD80 (B7-1)/ CD86 (B7-2) expressed by APCs. CD28 and CD86 are constitutively expressed, whereas CD80 is inducible upon T-lymphocyte activation. Stimulation of CD28 on T-lymphocytes strongly upregulates CD40L on the T-lymphocyte surface, which can then bind to the constitutively expressed CD40 on APCs. Interaction between the CD40L and CD40 is a potent signal to further upregulate CD80 and CD86 on antigen presenting cells, thereby creating a positive feedback loop for the co-stimulatory action of CD28.²⁸⁻³⁰

At present, a number of reports describe the importance of the CD28-CD80/CD86 and CD40-CD40L interaction for the development of atherosclerosis. In CD80/CD86-deficient LDLR^{-/-} mice the extent of early atherosclerosis was markedly attenuated, whereas the development of advanced atherosclerosis was hardly affected.³¹ In contrast, transplantation of CD80/CD86^{-/-} BM into LDLR^{-/-} mice enhanced the lesion size of advanced atherosclerosis.³² This aggravating effect on advanced atherosclerosis was confirmed in LDLR^{-/-} mice receiving CD28^{-/-} BM.³² A disadvantage of the use of CD80/CD86^{-/-} or

CD28^{-/-} as a mouse model for co-stimulation is that the Treg population is largely absent in these mice. Still, these data do show that the CD28-CD80/CD86 interaction potentially participates in atherogenesis. However, the inconsistent data might suggest that the observed effects on atherosclerosis are rather the result of a deficient Treg population than a lack of co-stimulation. Therefore, the question remains whether interference with the co-stimulatory CD28-CD80/CD86 interaction in T-lymphocyte activation will be beneficial or detrimental for the development of atherosclerosis. Extensive investigations on the role of the CD40-CD40L interaction in experimental atherosclerosis have been more conclusive. In CD40L-deficient APOE^{-/-} or LDLR^{-/-} mice, advanced atherosclerotic lesions had a more stable plaque phenotype characterised by less inflammation and more fibrosis than APOE^{-/-} or LDLR^{-/-} mice.^{33,34} Moreover, LDLR^{-/-} or APOE^{-/-} mice treated with anti-CD40L antibodies developed advanced atherosclerotic lesions with a more stable plaque phenotype.^{35,36} These studies clearly showed beneficial effects of inhibition of CD40-CD40L signalling in atherosclerosis.

Since both CD40 and CD40L are expressed on many cell types, long-term systemic interference with the CD40-CD40L interaction might give unwanted adverse effects, one of which is the occurrence of thrombotic events^{37,38} or infectious complications due to immuno-suppression. Therefore, a more specific therapy is needed. To design such a therapy, a better understanding of how anti-CD40L treatment acts on cells involved in the observed plaque stabilisation is required. In *Chapter 1*, we hypothesised that the CD40L expressing adaptive immune cells are essential for the stable plaque phenotype observed after anti-CD40L deficiency. To test this hypothesis, we analysed the severity of advanced atherosclerosis in LDLR^{-/-} mice transplanted with CD40L^{-/-} or wild-type BM in *Chapter 3*. Since the severity of advanced atherosclerosis in LDLR^{-/-} mice receiving CD40L^{-/-} or wild-type BM was comparable, we had to decline our hypothesis. These observations were confirmed by Bavendieck *et al.*³⁴ Since CD40L is not only expressed on haemopoietic cells, like T-lymphocytes, mast cells, NK cells, platelets, but also on non-haemopoietic cells,^{39,40} we postulated that CD40L expressing endothelial cells, fibroblasts or smooth muscle cells might be more important than leukocytes for the severity of advanced atherosclerosis. To test this hypothesis, we treated LDLR^{-/-} mice receiving CD40L^{-/-} BM with anti-CD40L or isotype antibodies in a pilot study described in *Chapter 3*. Again we found no difference in

severity of advanced atherosclerosis between the two mice groups. A possible explanation for these results might be the use of BM transplantation. Although we intended to only impair CD40L signalling in leukocytes, the CD40L^{-/-} BM transplantation also significantly reduced the natural Treg cell population, which in itself aggravates atherosclerosis as reported in *Chapter 5* and by the group of Mallat.³² Hence, we might have interfered in the delicate balance of stabilising and aggravating atherogenic effects in LDLR^{-/-} mice receiving CD40L^{-/-} BM. A balance that could not even be disturbed by the additional anti-CD40L antibody treatment. Altogether, the data presented in this thesis did not exclude or identify cells involved in the stable plaque phenotype observed after CD40L deficiency.

7.5 Diagnosis of atherosclerosis: the use of inflammatory markers

To efficiently prevent sudden cardiovascular events, high risk individuals harbouring unstable or rupture-prone atherosclerotic lesions need to be detected and treated as early as possible. At present, a lot of effort is undertaken to discover sensitive and specific markers that can discriminate individuals with stable, unstable or rupture-prone advanced atherosclerosis. Since rupture-prone atherosclerotic plaques are characterised by enhanced intraplaque inflammation,^{1,41} inflammatory markers may be sensitive and specific diagnostic targets. Currently, very promising inflammatory targets are being investigated. One inflammatory diagnostic target is CRP, an acute phase protein that is released primarily by the liver upon inflammatory stimuli. Large human screening studies have shown that elevated serum levels of CRP measured by a high sensitive assay (hsCRP) are predictive for future cardiovascular events in healthy individuals.⁴²⁻⁴⁴ In patients with stable or unstable angina, hsCRP levels were shown to discriminate between groups that are at higher and lower risk of sudden cardiovascular events.⁴²⁻⁴⁴ Although the clinical data support the general view that hsCRP should be added to the golden standard risk assessment, some precaution is required, since CRP is triggered by inflammation and therefore does not have to be related to cardiovascular events. Additionally, not all patients suffering from cardiovascular disease have elevated CRP levels. To overcome this disadvantage, measuring hsCRP at several serial time points is recommended. Another potential inflammatory marker for advanced atherosclerosis is the level of anti-oxLDL antibodies. Antibodies specific for oxLDL have been found in atherosclerotic lesions.⁴⁵

Over the past decade, a major effort has been undertaken to investigate whether these anti-oxLDL antibodies have a predictive value for the severity of human atherosclerosis.^{46,47}

In *Chapter 2*, we demonstrate that atherosclerosis-prone mice as well as wild-type mice harbour circulating IgM and IgG anti-oxLDL antibodies and that IgM anti-oxLDL antibody levels increase upon the progression of early to advanced atherosclerosis. However, anti-oxLDL antibody levels did not correlate with the severity of advanced atherosclerosis in mice. Also in healthy human subjects the presence of circulating IgG anti-oxLDL antibodies has been reported^{48,49} and a protective function of these antibodies in atherosclerosis has been postulated.⁴⁷ This would suggest that the measured level of anti-oxLDL antibodies represents a balance of both protective and pathogenic antibodies. Since the used ELISA measurement is unable to discriminate between the protective and pathogenic anti-oxLDL antibodies, the protective antibodies might distort the ELISA measurement. This could explain why the clinical data investigating the predictive value of anti-oxLDL antibodies in atherosclerosis are inconclusive (*Chapter 1*). Therefore, it might be useful to discriminate between the protective and pathogenic IgM and IgG anti-oxLDL antibodies. Recently, it was shown that the protective anti-oxLDL antibodies might be natural antibodies, which can be recognised by their T15 clonotype.⁴⁶ One possibility to estimate the pathogenic anti-oxLDL antibodies is to detect the anti-oxLDL antibodies in conjunction with a T15 clonotype analysis. Another possibility is to detect antibodies against a modification of LDL that is only induced by the inflammatory process in advanced atherosclerosis and therefore is more specific. At present, the majority of clinical studies used LDL that was oxidised with malondialdehyde or CuSO₄. However, possibly more inflammation-specific modifications are hypochlorite (HOCl) and 4-hydroxynol. Examination of one of these two proposed adjustments to measure anti-oxLDL antibodies might provide a useful marker for discriminating individuals with advanced atherosclerosis.

7.6 Treatment of atherosclerosis: new immunotherapeutic strategies

Myocardial infarction, peripheral arterial disease and stroke are the main complications of advanced atherosclerosis. Preventing progression of advanced atherosclerosis into rupture-prone lesions by effective treatment will tremendously reduce the number of patients suffering from cardiovascular disease or death. At present, very promising drugs and

therapeutic strategies are being investigated. One of these drugs is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-reductase inhibitors (*i.e.* statins). Since their introduction in the late 1980s, numerous clinical trials have shown that statins have been extremely successful in reducing coronary morbidity and mortality. This beneficial effect was first primarily attributed to lowering of serum cholesterol levels. Afterwards when clinical data also showed that patients with normal cholesterol levels benefited likewise from statin treatment, the clinical benefit of statins could not be entirely related to LDL reduction and it has been shown that statins also act through anti-inflammatory mechanisms. These anti-inflammatory effects include downregulation of CRP and serum amyloid A levels, a reduced expression of important immune interactive molecules present on antigen presenting cells, like MHCII, CD80, CD86 and CD40 and induction of anti-inflammatory cytokines.^{3,50,51} More recently, it was reported that another anti-immune effect of statin treatment is its interference with the T-lymphocyte response. It was shown that T-lymphocyte proliferation was attenuated⁵² and that the frequency of IFN γ -positive T-lymphocytes or CD28^{null} CD4 T-lymphocytes was reduced in patients suffering from coronary artery disease,^{53,54} although another study could not confirm these results.⁵⁵ The discovery of statins has revolutionised the treatment of atherosclerosis, both because of its tremendous lipid lowering effect and anti-inflammatory effect.

More specific anti-inflammatory treatments aiming to prevent cardiovascular complications have been employed as well. In this strategy the adaptive immune response in advanced atherosclerosis is manipulated by preventive antigen-specific therapy. Since atherosclerosis affects the majority of the human population, a very delicate way to perform such a treatment will be to vaccinate all individuals against disease-specific antigens. The possibility of applying such a therapy in atherosclerosis has only been investigated in animal models so far. For atherosclerosis, a major antigen is oxLDL, which was explored for the use in vaccination. In *Chapter 1*, the results of immunising atherosclerosis-prone mice with oxLDL were reviewed. Vaccination with oxLDL had an inhibitory effect on the generation of advanced atherosclerotic lesions.⁵⁶⁻⁵⁸ However, this protective effect was not primarily related to the antigen, but rather to the adjuvant that was used.^{8,59,60} Although these results are very promising, there are some issues that have to be resolved before this strategy is ready for clinical trial. A major limitation of this strategy is that vaccination

requires immuno-dominant antigens that are disease-specific. Whether oxLDL answers to these requirements can be questioned, since the atheroprotective effect observed after oxLDL immunisation could not entirely be attributed to oxLDL. Because of the complexity of atherosclerosis it may be very difficult to find an immuno-dominant antigen. Another difficulty is the route of administration, since this was an important factor in determining whether the effect of immunisation on experimental atherosclerosis was beneficial or detrimental. Presently, an exciting new approach, which is already employed for cancer immunotherapy,^{61,62} may resolve most of the raised issues for vaccination based therapy in atherosclerosis. In this approach, DCs are used to skew the immune response to the presented antigen. In this way, DCs are used as adjuvant and because of the regulatory role of DCs in the adaptive immune response the aimed effect for cancer therapy would be an enhancing one,^{61,62} whereas for atherosclerosis this should be an immune regulatory effect.⁶³ Future research has to elucidate whether vaccination will be useful as preventive treatment for advanced atherosclerosis and its complications.

Another novel approach for the manipulation of the adaptive immune response in inflammatory diseases is to target T cell regulation. In this thesis, the effect of T cell regulation in advanced atherosclerosis was explored in *Chapter 4 and 5*. As described in *Chapter 1*, T cell regulation is maintained through the action of two major immunoregulatory cytokines: IL-10 and TGF- β and by direct communication with pathogenic T-lymphocytes. In this thesis, we investigated both ways of T cell regulation in advanced atherosclerosis. In *Chapter 4*, we showed that TGF- β signalling is essential for regulating the inflammatory response in advanced atherosclerosis. Analysis of advanced atherosclerotic lesions in APOE^{-/-} mice treated with TGF β RII:Fc, which abrogates TGF- β signalling, revealed that the therapy induced a rupture-prone plaque phenotype characterised by enhanced inflammation and diminished fibrosis. These results suggest that atherosclerotic treatment based on TGF- β should aim to enhance the cytokine, rather than inhibit it. One possible approach is to administer a TGF- β stimulating drug as was reported for TMX. In APOE^{-/-} mice, TMX treatment lowered plasma cholesterol levels in accordance with enhanced TGF- β levels, which eventually attenuated the extent of atherosclerosis.⁶⁴ Another approach may be to trigger or transfer Tregs thereby enhancing

the level of anti-inflammatory cytokines in atherosclerosis. This strategy has been applied in animal models of other auto-immune diseases with great success.⁶⁵ Also in APOE^{-/-} mice, transfer of IL-10 producing Tregs markedly reduced the severity of atherosclerosis.⁶⁶ In *Chapter 5*, we established a protective role for natural (CD4⁺CD25⁺) Tregs in advanced atherosclerosis. Treatment of LDLR^{-/-} mice with anti-CD25 antibodies resulted in a markedly declined CD4⁺CD25⁺ cell population and in atherosclerotic lesions with an inflammatory plaque phenotype. As discussed in *Chapter 5*, our therapy gave a less severe effect on advanced atherosclerosis than the study of Mallat³². If this difference is the result of using either LDLR^{-/-} or APOE^{-/-} mice as suggested in *Chapter 5*, this might have great consequences for future clinical application. That is, the two mouse strains may reflect the maximum variability in humans, which would suggest that the beneficial effect of a treatment aimed at the natural Tregs would not be the same for every individual. In March 2006, a phase I clinical trial showed that manipulation of regulatory T cells comes not without a risk. In the trial a regulatory T cell stimulating drug, CD28 (TGN1412), was tested and ended up in a disaster, since all of the participating healthy volunteers had to be hospitalised. The anti-CD28 drug had induced a cytokine storm with severe lymphopenia and monocytopenia, which resulted in multiorgan failure.⁶⁷ Therefore, the upcoming scientific challenge will be to implement this new basic knowledge into the clinic.

7.7 Concluding remarks

In the last decade strong evidence has been generated proving that atherosclerosis is an inflammatory disease of the vascular wall. More recently the implication of T-lymphocytes in advanced atherosclerosis is being recognised. This thesis has contributed to and expanded the knowledge of T-lymphocyte involvement in advanced atherosclerosis. The most important finding in this thesis is that manipulation of the T-lymphocyte response is not straightforward because of its strict regulatory mechanisms. It will require detailed characterisation of both disease-related and protective immune responses to be able to apply immuno-therapy in patients suffering from cardiovascular disease.

7.8 References

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Summary – Samenvatting

In developed countries cardiovascular events such as myocardial infarction, peripheral arterial disease and stroke are still the main cause of death. In most cases the cardiovascular event is caused by luminal thrombosis, which is the result of rupture or erosion of an advanced atherosclerotic lesion. Atherosclerotic lesions are formed in large and medium sized arteries and progress during a person's lifetime. The aetiology of atherosclerosis is clearly multifactorial and epidemiologic studies have identified many risk factors for atherosclerosis including smoking, lack of exercise, a high fat diet, hyperlipidemia and hypertension. Although reducing these risk factors will decrease cardiovascular events, it will not prevent atherosclerosis completely.

Over the past decades, many researchers have investigated the initiation and progression of atherosclerosis and the prerequisites needed for atherosclerosis to cause cardiovascular events. Our current knowledge on the pathogenesis of atherosclerosis is the result of combining the findings of clinical investigations, population studies, *in vitro* experiments and studies in animal models. At present, it is widely accepted that atherosclerosis is a chronic inflammatory disease and initiation of the disease is the result of an innate immune response. Innate immune cells, mostly macrophages, try to eradicate retained lipoproteins from the vascular wall, but fail and become trapped as foam cells. Further progression of atherosclerosis into the advanced stages is the result of a balance between inflammation and tissue healing. Both innate and adaptive immunity contribute to this inflammatory response. In this thesis the involvement of adaptive immunity in advanced atherosclerosis was investigated.

In *Chapter 1* of this thesis the current understanding on the pathogenesis of atherosclerosis and the role of innate and adaptive immunity in atherogenesis are described in detail. In the next experimental chapters (*Chapter 2-6*), the importance of three major requirements of T-lymphocyte responses: antigen specificity, co-stimulation and regulation in advanced atherosclerosis are reported.

Chapter 2 describes the anti-oxLDL antibody response in atherosclerosis-prone mice versus wild-type mice. This evaluation demonstrated that atherosclerosis-prone mice as well as wild-type mice harbour detectable levels of circulating IgM and IgG anti-oxLDL

antibodies. Additionally, it was shown that only the progression of atherosclerosis from the early phase to the advanced phase, but not the severity of advanced atherosclerosis, was reflected by an increase in IgM anti-oxLDL antibody levels.

In *Chapter 3*, the hypothesis was tested that adaptive immune cells are responsible for the observed stable plaque phenotype in APOE^{-/-}CD40L^{-/-} mice. Although analysis of LDLR^{-/-} mice receiving CD40L^{-/-} BM clearly showed a diminished natural Treg population, the severity of advanced atherosclerosis was similar in both mice groups. Therefore, we concluded that the stable plaque phenotype observed after CD40L inhibition is not dependent on CD40L expressing leukocytes. To confirm that indeed CD40L expressing non-haemopoietic cells are involved and not leukocytes, a pilot experiment was performed in which LDLR^{-/-} mice transplanted with CD40L^{-/-} BM were treated with either anti-CD40L antibodies or control IgG. Surprisingly, all mice developed comparable advanced atherosclerosis. In all, a role for CD40L expressing non-haemopoietic cells in plaque stability after CD40L deficiency could not be proven, but a role for leukocyte CD40L could not be excluded either.

In *Chapter 4 and 5* the role of natural Tregs in advanced atherosclerosis was studied. Effects of the immunoregulatory cytokine, TGF- β , on advanced atherosclerosis are described in *Chapter 4*. APOE^{-/-} mice were treated with TGF β RII:Fc to inhibit TGF- β signalling. This treatment resulted in a rupture-prone plaque phenotype characterised by increased plaque inflammation and decreased plaque fibrosis. In *Chapter 5* natural Tregs were depleted in LDLR^{-/-} mice by administering anti-CD25 antibodies. In the absence of natural Tregs, mice developed a more inflammatory plaque phenotype that was characterised by an enhanced T-lymphocyte response. These results show that natural Tregs have a protective role in the progression of atherosclerosis.

In the last experimental chapter, *Chapter 6*, MHCII-deficient LDLR^{-/-} mice were generated to analyse the involvement of antigen specificity in advanced atherosclerosis. LDLR^{-/-}MHCII^{-/-} mice had lower cholesterol levels after high fat feeding than their littermates, but when this was correlated to the reduced total plaque area, it was shown that another factor was contributing to the observed effect on plaque size. Further

characterisation revealed that the advanced atherosclerotic lesions were less severe and that the CD4 T-lymphocyte response was clearly hampered in LDLR^{-/-}MHCII^{-/-} mice. Hence, these results prove that antigen presentation is part of the processes contributing to the progression of atherosclerosis.

Finally, in *Chapter 7* the presented results of this thesis are discussed and some future challenges are indicated.

In conclusion, this thesis has contributed to and expanded the knowledge of T-lymphocyte involvement in advanced atherosclerosis. Furthermore, the results described in this thesis show that manipulation of the T-lymphocyte response will not be straightforward because of its strict regulatory mechanisms. It will require detailed characterisation of both disease-related and protective immune responses to be able to apply immuno-therapy in patients suffering from cardiovascular disease.

Hart- en vaatziekten zijn nog steeds de meest voorkomende doodsoorzaak in Nederland en de meeste andere westerse landen. Cardiovasculaire complicaties (bijvoorbeeld een hartinfarct, perifere vaatlijden of een beroerte) zijn vaak het gevolg van verstopping van een bloedvat door een bloedstolsel. Dit bloedstolsel is meestal ontstaan als gevolg van aderverkalking (atherosclerose).

Elk mens ontwikkelt aderverkalking in meer of mindere mate. Dit hangt onder andere sterk af van de aanwezigheid van een of meerdere risicofactoren: roken, te weinig bewegen, een vetrijk dieet, een verhoogd cholesterolgehalte en een te hoge bloeddruk. Vroeger werd aderverkalking beschouwd als een opeenstapeling van cholesterol in de vaatwand die resulteerde in een verdikking (een lesie). Wanneer uiteindelijk de lesie zo groot werd dat deze een bloedvat afsloot, zou dit resulteren in een cardiovasculaire complicatie. Inmiddels heeft onderzoek aangetoond dat aderverkalking een chronische ontsteking van de vaatwand is. Het (geoxideerd) cholesterol (oxLDL) dat neerslaat in de vaatwand is een van de belangrijkste antigenen waartegen het afweersysteem reageert. In de beginfase van de ontstekingsreactie zijn met name cellen van het aangeboren afweermechanisme (in dit geval macrofagen) betrokken. Deze “stofzuigers” proberen het neergeslagen oxLDL op te ruimen, maar slagen hier niet volledig in en blijven gevangen in de vaatwand als schuimcellen. Door het aanhouden van de ontstekingsreactie raken ook de cellen van de specifieke immuniteit, met name T-lymfocyten, betrokken bij het ziekteproces. Deze chronische fase in het atherosclerotische proces is te classificeren in drie stadia: stabiel, instabiel of ruptuur-gevoelig. Vooral de instabiele en ruptuur-gevoelige lesies kunnen leiden tot het openscheuren van de vaatwand waardoor een bloedstolsel ontstaat en cardiovasculaire complicaties kunnen optreden. Opengescheurde lesies worden dan ook regelmatig gevonden bij patiënten met een acuut hart- of vaatprobleem.

Dit proefschrift beschrijft onderzoek naar de rol van specifieke immuniteit in de pathogenese van chronische aderverkalking. In *Hoofdstuk 1* wordt uiteengezet wat op dit moment bekend is over het ziekteverloop van aderverkalking en hoe aangeboren en specifieke afweermechanismen hieraan bijdragen. In de volgende experimentele hoofdstukken (*Hoofdstuk 2-6*), wordt gerapporteerd in hoeverre de drie belangrijkste vereisten van een T-lymphocyt reactie: antigeen specificiteit, co-stimulatie en regulatie,

bijdragen aan het ontstaan en de ernst van aderverkalking. Dit onderzoek is verricht in twee muismodellen (APOE^{-/-} of LDLR^{-/-} muizen), die zodanig gevoelig gemaakt zijn dat ze aderverkalking ontwikkelen.

De specifieke immuunreactie in aderverkalking bestaat uit twee componenten: T-lymfocyten en antilichamen, die geproduceerd worden door B-lymfocyten. In mensen is gevonden dat anti-oxLDL antilichamen correleren met de ernst van chronische aderverkalking. Dit kan mogelijk betekenen dat de hoeveelheid anti-oxLDL antilichamen in het bloed te gebruiken is als maat voor het verschil tussen stabiele en instabiele of kwetsbare lesies. Uit voorgaand onderzoek is gebleken dat atherosclerotische lesies in APOE^{-/-}CD40L^{-/-} muizen er stabiel uitzien dan die in controle (APOE^{-/-}) muizen. Daarnaast is de CD40-CD40L interactie van essentieel belang voor het verwisselen van het isotype (van IgM naar IgG) van een antilichaam in B-lymfocyten. Tevens is deze interactie belangrijk voor de inductie van T-lymfocyten met een helpende functie. *Hoofdstuk 2* beschrijft vervolgens hoe de anti-oxLDL antilichaam reactie zich ontwikkelt in muizen die aderverkalking hebben ten opzichte van controle muizen (zonder aderverkalking). Hieruit bleek dat de anti-oxLDL antilichaam reactie niet uniek was voor muizen met aderverkalking, omdat deze antilichamen ook aantoonbaar waren in controle muizen. Daarentegen hadden muizen met chronisch aderverkalking meer IgM anti-oxLDL antilichamen in hun bloed dan muizen met vroege aderverkalking.

In *Hoofdstuk 3* wordt de hypothese getest dat specifieke immuuncellen verantwoordelijk zijn voor het ontstaan van atherosclerotische lesies met een stabiel fenotype in APOE^{-/-}CD40L^{-/-} muizen. Hiervoor werden LDLR^{-/-} muizen getransplanteerd met beenmerg geïsoleerd uit CD40L-deficiente muizen, zodat alle immuuncellen geen CD40L meer tot expressie kunnen brengen. Bestudering van deze muizen toonde aan dat de ontstane chronische aderverkalking vergelijkbaar was met die in controle muizen. Op basis van deze resultaten werd geconcludeerd dat het stabiele lesie fenotype dat gevonden wordt na CD40L blokkering niet afhankelijk is van CD40L positieve immuuncellen. Om te bewijzen dat inderdaad niet-beenmerg afkomstige cellen (die CD40L tot expressie brengen) betrokken zijn bij het ontstaan van atherosclerotische lesies met een stabiel fenotype, werden LDLR^{-/-} muizen die getransplanteerd waren met CD40L^{-/-} beenmerg tevens

behandeld met anti-CD40L antilichamen. Uit dit experiment bleek echter dat de ontstane chronische aderverkalking vergelijkbaar was tussen de twee muizengroepen. Deze resultaten bevestigen daarom geen duidelijke rol voor niet-beenmerg afkomstige cellen in lesie stabiliteit. Tevens kan echter een rol voor immuuncellen niet worden uitgesloten.

Het tweede gedeelte van dit proefschrift gaat in op de T-lymfocyt reactie in aderverkalking. Aangetoond is dat T-lymfocyten bijdragen aan de pathogenese van chronische aderverkalking. Normaliter is een T-lymfocyt reactie specifiek voor de veroorzaker van een ontsteking, zodat deze zo efficiënt mogelijk kan worden uitgeschakeld. Nadat de veroorzaker van een ontsteking volledig is verwijderd, moet de T-lymfocyt reactie zo snel mogelijk worden beëindigd om onnodige schade aan het omliggende weefsel te voorkomen. Het beëindigen van de T-lymfocyt reactie vindt plaats doordat regulerende T cellen de T-lymfocyt reactie onderdrukken, waardoor er vervolgens herstel kan optreden. Aangezien er onvoldoende bekend is over de rol van regulerende T cellen in chronische aderverkalking, werd dit in *Hoofdstuk 4* en *5* nader onderzocht. Daarnaast is tot op heden nog niet onomstotelijk bewezen dat T-lymfocyten, die in de atherosclerotische lesies aanwezig zijn, antigeen gedreven cellen zijn. Dit werd verder bestudeerd in *Hoofdstuk 6*.

Hoofdstuk 4 beschrijft de effecten van het blokkeren van het immunoregulerende cytokine, TGF- β , in chronische aderverkalking. APOE^{-/-} muizen behandeld met TGF β RII:Fc kregen atherosclerotische lesies met een meer ruptuur-gevoelig fenotype, dat gekenmerkt wordt door meer ontsteking en minder bindweefsel. In *Hoofdstuk 5* werden de regulerende T cellen gedepleteerd in LDLR^{-/-} muizen door ze te behandelen met anti-CD25 antilichamen. In de afwezigheid van regulerende T cellen ontwikkelden de muizen chronische atherosclerose met een meer inflammatoir fenotype dat gepaard ging met een toegenomen T-lymfocyt reactie. Deze resultaten tonen aan dat regulerende T cellen een beschermende rol spelen in chronische aderverkalking.

In het laatste experimentele hoofdstuk, *Hoofdstuk 6*, werden MHCII-deficiënte LDLR^{-/-} muizen gemaakt om de invloed van antigeen specificiteit op chronische aderverkalking te bestuderen. LDLR^{-/-}MHCII^{-/-} muizen bleken een lager cholesterol gehalte te hebben na het eten van een vetrijk dieet dan controle LDLR^{-/-} muizen, maar correlatie van dit cholesterol

gehalte met het afgenomen lesie oppervlak toonde aan dat er nog een andere factor bijdroeg aan het waargenomen effect op lesie grootte. Verdere analyse van de atherosclerotische lesies in de LDLR^{-/-}MHCII^{-/-} muizen lieten zien dat de chronische aderverkalking minder ernstig was en dat de CD4 T-lymfocyt reactie belemmerd was. Op basis van deze resultaten werd geconcludeerd dat antigeen presentatie een onderdeel is van de processen die leiden tot chronische aderverkalking.

Dit proefschrift heeft bijgedragen aan de verbreding van de kennis ten aanzien van de rol van T-lymfocyten in aderverkalking. De belangrijkste uitkomst van dit proefschrift is dat manipulatie van de T-lymfocyt reactie in chronische aderverkalking niet eenvoudig te realiseren is vanwege sterk regulerende mechanismen in het afweersysteem. Om uiteindelijk in staat te zijn patiënten die lijden aan hart- en vaatziekten te genezen of preventief te behandelen met immuno-therapie zal daarom meer onderzoek nodig zijn naar zowel ziekte gerelateerde als beschermende afweerreacties.

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Marjan

Curriculum vitae

Marjan (Louise Fransesca) Smook was born in Feerwerd (the Netherlands) on January 22nd, 1977. Her family moved to Groningen in 1986. In 1995 she graduated from secondary school and continued her education studying Biology at the University of Groningen. In order to differentiate as a Medical Biologist and expand her knowledge on experimental techniques and immunology, she completed two scientific trainings of six months each: first under the supervision of Professor Dr. C. Kallenberg at the department of Clinical Immunology of the University Hospital Groningen and subsequently at the department of Immunology of the Dutch Cancerinstitute in Amsterdam under the guidance of Professor Dr. A. Kruijsbeek. The former research project involved the expression of recombinant proteinase 3 (the auto-antigen of Wegener's granulomatosis) in insect cells and its characterization. The latter training involved an investigation into the T-lymphocyte tolerance in mice against myelin basic protein (the auto-antigen of multiple sclerosis). In 2000, Marjan graduated as MSc in Medical Biology and moved to Geleen to start as a Ph.D. student at the department of Clinical and Experimental Immunology in cooperation with the department of Pathology at the University of Maastricht. The results of this research are described in this thesis. Currently, Marjan is employed as scientist at the department of Regulatory Affairs, NV Organon in Oss.

Marjan (Louise Fransesca) Smook werd geboren op 22 januari 1977 te Feerwerd. Samen met haar ouders en zusje verhuisde ze in 1986 naar Groningen. In 1995 behaalde ze haar VWO diploma en startte vervolgens met de studie Biologie aan de Rijksuniversiteit Groningen. In het kader van de afstudeerrichting Medische Biologie volgde zij twee wetenschappelijke stages van zes maanden elk om haar kennis op het vakgebied van de immunologie en de daarin gebruikte technieken te vergroten. De eerste stage werd verricht bij de afdeling Klinische Immunologie van het Academisch ziekenhuis Groningen onder begeleiding van prof. dr. C. Kallenberg. Hier bracht ze recombinant proteinase 3 (het auto-antigen in de ziekte van Wegener) tot expressie in inktencellen en karakteriseerde dit eiwit. Daaropvolgend verrichtte zij onderzoek naar T-lymfocyt tolerantie gericht tegen myelin basic protein (het auto-antigen bij multiple sclerose) in muizen op de afdeling Immunologie van het Nederlands Kanker Instituut te Amsterdam onder begeleiding van prof. Dr. A. Kruijsbeek. De studie Medische Biologie voltooide Marjan in 2000, waarna ze verhuisde naar Geleen om te beginnen aan haar promotieonderzoek bij de afdeling Klinische en Experimentele Immunologie in samenwerking met de afdeling Pathologie van de Universiteit van Maastricht. De resultaten van dit onderzoeksproject zijn beschreven in dit proefschrift. Momenteel is Marjan als scientist aangesteld bij de afdeling Regulatory Affairs van NV Organon te Oss.

Full colour figures

Figure 1.1 *Distribution of the high-susceptible sites (gray) of the aorta (right): 1, aortic root; 2, aortic arch; 3, carotid artery; 4, thoracic aorta; 5, abdominal aorta and 6, iliac artery. LV, left ventricle. Schematic representation of atherosclerotic lesions in the aortic root (lower middle) and hematoxylin and eosin-stained section of an aortic root containing atherosclerotic lesions (left). AS, aortic sinus.*

Figure 1.2 *Schematic representation of an early atherosclerotic lesion (upper panel) and an advanced atherosclerotic lesion (lower panel). NC, necrotic core. Hematoxylin and eosin-stained section of an aortic sinus containing an early atherosclerotic lesion (upper panel) and an advanced atherosclerotic lesion (lower panel).*

Figure 1.3 Schematic overview of effects of oxLDL on atherosclerosis: 1, secretion of chemokines to attract leukocytes; 2, foam cell formation. ScR, scavenger receptor.

Figure 1.4 *Schematic overview of the generation of activated macrophages and their effects on atherosclerosis: 1, secretion of inflammatory products; 2, antigen presentation.* Ab, antibody; TLR, toll-like receptor; FcR, Fc receptor.

Figure 1.5 Schematic representation of molecules involved in antigen recognition by T-lymphocytes: the MHC I-TCR interaction (left panel); the MHC II-TCR interaction (right panel).

Figure 1.6 Schematic overview of CD4 T-lymphocyte differentiation into effector T-lymphocyte subsets. Treg, regulatory T cell; APC, antigen-presenting cell.

Figure 3.3 *Atherosclerotic plaques of $LDLR^{-/-}$ receiving either wild-type BM or $CD40L^{-/-}$ BM after a HFD of 20 weeks. Tissue sections of paraffin embedded mouse aortic roots were stained with A, hematoxylin and eosin or B, Sirius red (collagen).*

Figure 3.6 Atherosclerotic plaques of LDLR^{-/-} receiving CD40L^{-/-} BM and treated with either isotype or anti-CD40L antibodies after a HFD of 14 weeks. Tissue sections of aortic roots were stained with hematoxylin and eosin.

Figure 4.1 *Histological characterisation of systemic effects of TGFβRII:Fc treatment.* Tissue sections of heart (A+B), spleen (C+D), liver (E+F) and kidney (G+H) were stained with hematoxylin and eosin. Treatment with TGFβRII:Fc or control IgG showed no systemic effects.

Figure 4.2 *Histological characterisation of effects of TGFβRII:Fc treatment on arteries.* Tissue sections of normal arteries (A and B) and atherosclerotic arteries (C and D) were stained with hematoxylin and eosin. Treatment with TGFβRII:Fc or control showed no adventitial inflammation. A = adventitia, L = lumen.

Figure 4.4 *Immunoreactivity of p-Smad 2/3.* Tissue sections of atherosclerotic plaques of the delayed TGF β RII:Fc treatment groups were stained with anti-p-Smad 2/3. TGF β RII:Fc treatment significantly decreased p-Smad 2/3 signalling.

Figure 4.7 Histological characteristics of effects on atherosclerotic lesions after delayed *TGFβRII:Fc* treatment. Hematoxylin and eosin (HE)-stained section of advanced atherosclerotic lesions in the aortic arch, revealing an enlarged lipid core (arrow) after *TGFβRII:Fc* treatment (A) compared with control IgG treatment (B). CD45 staining of the shoulder region of advanced atherosclerotic lesions, revealing an increased amount of CD45-positive cells (arrows) after *TGFβRII:Fc* treatment (C) compared with control IgG treatment (D). CD40L staining of advanced atherosclerotic lesions. CD40L is most abundantly present after *TGFβRII:Fc* treatment (E) compared with control IgG treatment (F). Sirius red staining of advanced atherosclerotic lesions. The amount of collagen significantly decreased after *TGFβRII:Fc* treatment (G) compared with control IgG treatment (H).

Figure 4.8 *Histological characteristics of intraplaque hemorrhage in advanced atherosclerotic lesions after delayed treatment with TGFβRII:Fc in APOE^{-/-} mice. A, Overview of an advanced atherosclerotic lesion showing erythrocytes, iron deposition, and fibrin deposition (arrows). (HE staining). B, Panel A in more detail. C, Perls' iron staining combined with factor VIII staining showing iron deposition (in blue) and endothelium (in red) in an advanced atherosclerotic lesion. D, Frequency of erythrocytes and fibrin and iron deposition.*

Figure 5.3 Atherosclerotic plaques of *LDLR*^{-/-} treated with anti-CD25 antibodies or control IgG after a HFD of 10 weeks. Tissue sections of aortic roots were stained with hematoxylin and eosin (A), anti-CD3 mAbs (T-lymphocytes, B) or anti-CD25 mAbs (CD25⁺ cells, C).

Figure 6.4 Atherosclerotic plaques of $LDLR^{-/-}MHCII^{-/-}$ and $LDLR^{-/-}$ mice after a HFD of 9 weeks. Tissue sections of aortic roots were stained with hematoxylin and eosin (A), anti-MHCII mAbs (B) or anti-CD4 mAbs (T_H-lymphocytes, C).