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# Development of a Passive Immunization Strategy Against Atherosclerosis

**Alexandru Şchiopu, M.D.**

AKADEMISK AVHANDLING

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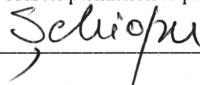
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Abstract  <p>Atherosclerosis is a chronic inflammatory disease, characterized by the accumulation of lipids and fibrous tissue in the wall of medium and large-sized arteries. The characteristic culprit of the disease is the atheroma, or atherosclerotic plaque, a patchy thickening of the arterial wall which affects the lumen, inducing various degrees of stenosis. The rupture of the atherosclerotic plaques, followed by local thrombosis, is the underlying cause of myocardial infarction and stroke, which claim millions of lives every year worldwide.</p> <p>Oxidized LDL and the immune system play very important roles in atherosclerosis. Several studies have demonstrated the existence of both atherogenic and atheroprotective immune responses against oxidized LDL. We have identified several of the epitopes in the oxidized LDL particle which trigger immune responses. These epitopes are aldehyde-modified peptide sequences of apoB-100, the main protein in LDL structure. Immunization of atherosclerosis-prone mice with some of the apoB-100 peptides reduced plaque area by up to 60% in the immunized mice compared to controls.</p> <p>We tested the effects of recombinant human IgG1 antibodies against two of these peptide sequences on the development of atherosclerosis in mice. Passive immunization with the IEI-E3 and 2D03 antibodies, specific for MDA-p45 (aa 661-680), significantly inhibited atherosclerosis progression and induced plaque regression in the descending aorta. 2D03 prevented constrictive remodeling after injury in the carotid arteries and potentially reduced lesion extent in the uninjured carotid arteries in mice. Additionally, antibody treatment decreased the local and systemic inflammatory responses.</p> <p>We have also found that plasma levels of human IgG1 autoantibodies which recognize the same aldehyde-modified apoB-100 peptide are inversely correlated with carotid stenosis in healthy individuals, which further supports the hypothesis of the potential atheroprotective role of these antibodies.</p> <p>The influence of the antibodies on human atherosclerosis and their potential side effects need to be carefully characterized. In the future, the oxidized LDL-specific recombinant human IgG1 antibodies could be developed into novel diagnostic and therapeutic tools for the management of atherosclerosis-related cardiovascular diseases.</p>		
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# **Development of a Passive Immunization Strategy Against Atherosclerosis**

**Alexandru Şchiopu, M.D.**



**LUND UNIVERSITY**  
Faculty of Medicine

**Cover figure:** Ribbon representation of the structure of a murine IgG.  
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# *To Anca*

*“Life is a journey, not a destination”*

*Steven Tyler*



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## LIST OF ORIGINAL ARTICLES

This thesis is based on the following articles, which will be referred to in the text by their roman numerals:

- I. Recombinant Human Antibodies Against Aldehyde-Modified Apolipoprotein B-100 Peptide Sequences Inhibit Atherosclerosis**  
Schiopu A., Bengtsson J., Söderberg I., Janciauskiene S., Lindgren S., Ares MPS., Shah PK., Carlsson R., Nilsson J., Fredrikson GN.  
*Circulation. 2004;110:2047-205*
- II. Human Recombinant Antibodies to an Oxidized LDL Epitope Induce Rapid Plaque Regression in LDL Receptor Apobec-1 Double Knockout Mice**  
Schiopu A., Jansson B., Söderberg I., Ljungcrantz I., Araya Z., Shah PK., Carlsson R., Nilsson J. & Fredrikson GN.  
*Submitted for publication*
- III. Inhibition of Injury-Induced Arterial Remodeling and Carotid Atherosclerosis by Recombinant Human Antibodies Against Aldehyde-Modified ApoB-100**  
Ström A., Fredrikson GN., Schiopu A., Ljungcrantz I., Söderberg I., Jansson B., Carlsson R., Hultgårdh Nilsson A. & Nilsson J.  
*Accepted for publication in Atherosclerosis*
- IV. Increased Levels of IgG1 Against an Aldehyde-Modified Peptide Sequence in ApoB-100 Are Associated With Decreased Severity of Carotid Stenosis**  
Fredrikson GN., Berglund G., Alm R., Nilsson J-Å., Schiopu A., Shah PK., Nilsson J.  
*Manuscript*

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## **ABBREVIATIONS**

<b>ABCA-1</b>	ATP-binding membrane cassette transporter
<b>ADCC</b>	Antibody-dependent cellular cytotoxicity
<b>AP-1</b>	Activator protein-1
<b>APC</b>	Antigen presenting cell
<b>apo</b>	Apolipoprotein
<b>apobec-1</b>	ApoB mRNA editing catalytic polypeptide-1 knockout
<b>BCR</b>	B cell receptor
<b>BSA</b>	Bovine serum albumine
<b>cAMP</b>	Cyclic AMP
<b>CDR</b>	Complementarity determining region
<b>CR</b>	Complement receptor
<b>CRP</b>	C-reactive protein
<b>CVD</b>	Cardiovascular disease
<b>DC</b>	Dendritic cell
<b>EC</b>	Endothelial cells
<b>Fab</b>	Fragment antigen binding
<b>Fc</b>	Fragment crystalline
<b>FcR</b>	Fragment crystalline receptor
<b>HDL</b>	High density lipoprotein
<b>Hsp</b>	Heat shock protein
<b>IC</b>	Immune complex
<b>ICAM-1</b>	Intercellular adhesion molecule-1
<b>IFN<math>\gamma</math></b>	Interferon $\gamma$
<b>IL</b>	Interleukin
<b>IMT</b>	Intima-media thickness
<b>IVIg</b>	Intravenous immunoglobulins

<b>LDL</b>	Low density lipoprotein
<b>LDLR</b>	LDL receptor
<b>MCP-1</b>	Monocyte chemoattractant protein-1
<b>M-CSF</b>	Macrophage-colony stimulating factor
<b>MDA</b>	Malondialdehyde
<b>MHC</b>	Major histocompatibility complex
<b>mmLDL</b>	Minimally modified LDL
<b>MMP</b>	Matrix metalloproteinase
<b>NF-κB</b>	Nuclear factor κB
<b>NK cells</b>	Natural killer cells
<b>oxLDL</b>	Oxidized LDL
<b>PAMPs</b>	Pathogen-associated molecular patterns
<b>PC</b>	Phosphorylcholine
<b>POVPC</b>	Palmitoyl oxoaleroyl glycerophosphorylcholine
<b>PPAR-γ</b>	Peroxisome proliferator activated receptor
<b>PRR</b>	Pathogen recognition receptor
<b>SAA</b>	Serum amyloid A
<b>ScR</b>	Scavenger receptors
<b>SMC</b>	Smooth muscle cells
<b>SR-B1</b>	Scavenger receptor B-1
<b>VCAM-1</b>	Vascular cell adhesion molecule-1
<b>TCR</b>	T cell receptor
<b>TF</b>	Tissue factor
<b>TG</b>	Triglycerides
<b>TGFβγ</b>	Transforming growth factor β
<b>Th</b>	T helper lymphocyte
<b>TLR</b>	Toll-like receptor
<b>TNFα</b>	Tumor necrosis factor α

## INTRODUCTION

### General considerations

Atherosclerosis is a chronic inflammatory disease, characterized by the accumulation of lipids and fibrous tissue in the tunica intima of medium and large-sized arteries. The characteristic culprit of the disease is the atheroma, or atherosclerotic plaque, a patchy thickening of the arterial wall which affects the lumen, inducing various degrees of stenosis. Atherosclerosis is the underlying cause of ischemic cardiovascular diseases (CVD), generated by reduction or disruption of blood flow in a certain vascular territory, causing cell death and loss of function. The clinical manifestations and outcome depend on the affected territory and the severity of the occlusion (Table 1).

**Table 1. Clinical manifestations of atherosclerosis**

Artery	Affected territory	Clinical manifestations
Coronary system	Heart	Angina (Chest pain) Arrhythmias Acute myocardial infarction Sudden death
Carotid artery	Brain	Transient ischemic attack Stroke
Peripheral arteries	Limbs (mostly inferior)	Loss of function Gangrene

The current statistics underline the importance of CVD and their impact on global health. In Europe almost half of all deaths are caused by CVD. Coronary heart disease and stroke are the two single most common death causes, claiming 1.95 and 1.28 million lives, respectively, in 2002 alone. In the USA, mortality rates from CVD equals the combined mortality rate from the next five causes of death (cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, influenza and pneumonia), accounting for 1 of every 2.6 deaths in 2002. Every 26 seconds a coronary event is registered and a stroke every 45 seconds. It was estimated that life expectancy in the USA would

rise by 7 years if every major form of CVD were eliminated. Due to the constant rising of mortality by CVD in the developing countries, coronary heart disease, stroke and the related diseases will constitute the leading cause of death worldwide by 2020, claiming over 20 million lives every year. In reality, the burden is much greater than shown by these figures. CVD are debilitating diseases, inducing dramatic decrease of life quality and work capability. For example, only one third of all patients who suffer a stroke recover completely, the other two thirds die or remain with lifetime disabilities. The total costs implied, including healthcare, loss of life years and work years, are enormous.<sup>1-3</sup>

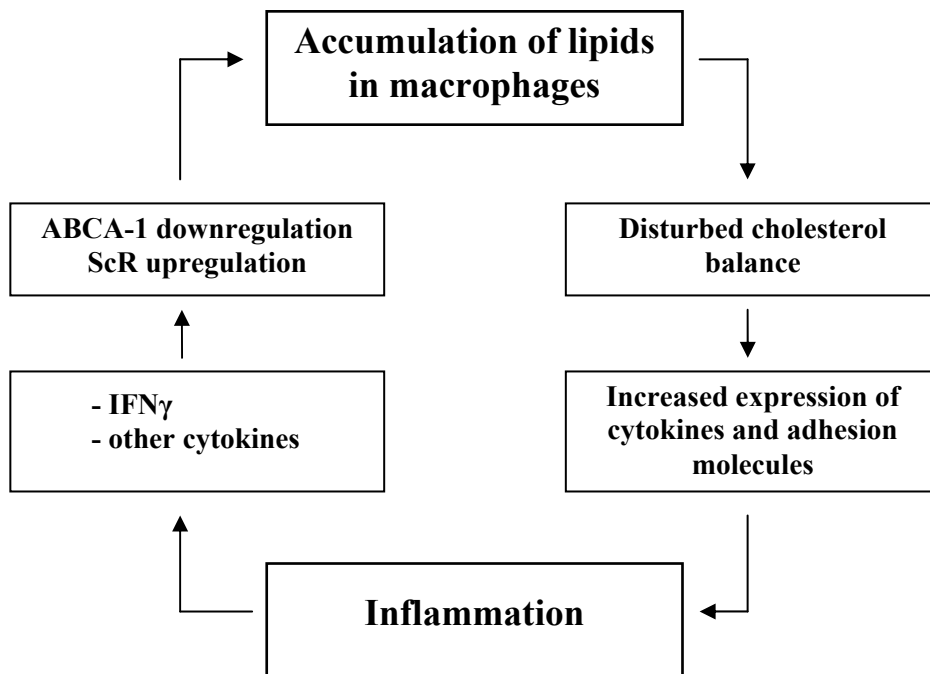
Atherosclerosis is often referred to as a “silent killer”. A recent study showed that 50% of all men and 64% of all women who died suddenly of coronary heart disease presented no previous symptoms.<sup>1</sup> The disease begins already in fetal life and slowly and silently progresses throughout the life at a pace dictated by the presence or absence of favoring conditions, called risk factors, such as dyslipidemia, hypertension, smoking or diabetes. Age, gender and genetic background also play an important role, as well as other determinants, such as physical inactivity, obesity, stress<sup>4</sup> and different infections (*Chlamydia Pneumoniae*, Cytomegalovirus, Herpes Virus).<sup>5</sup> The symptoms, if any, appear late during the development, at a 60-70% degree of lumen stenosis or when the plaques have already started to become eroded or ruptured. The disease is not detectable by routine investigations, and intracoronary ultrasound demonstrated a prevalence of atherosclerosis of 37% between 20-29 years of age, 60% between 30-39 years of age and 85% over 50 years of age in heart donors initially considered to be healthy.<sup>6</sup>

The high number of deaths by CVD throughout the world indicates that the current tools available for the management of atherosclerosis-related diseases are far from being sufficient. Most of the current diagnostic methods are invasive and expensive and therefore only used at advanced stages of the disease, when the symptoms are already present. So far, the largest breakthrough in the treatment of atherosclerosis has been the development of statins, cholesterol lowering drugs which also possess other anti-inflammatory properties, but which can only provide a 30-40% lowering of cardiovascular risk.<sup>7-10</sup>

There is an acute need for new and more effective non-invasive methods for diagnosis of atherosclerosis at early stages and for detecting patients at high risk to develop acute cardiovascular events, as well as new therapies for preventing atherosclerosis progression and plaque rupture. The ultimate goal is to reduce morbidity and mortality due to CVD worldwide, which would have a dramatic positive impact on global health.

## Pathogenesis of atherosclerosis

Atherosclerosis develops as a chronic inflammatory response to lipid retention in the arterial wall. This simple characterization reveals the two most important factors that drive the atherogenic process: lipid accumulation and inflammation (Figure 1).



**Figure 1. Interplay between lipid accumulation and inflammation in the intima**

The macrophage is the central player in atherosclerosis. Initially, the macrophages enter the subendothelial space in an attempt to remove oxidized LDL particles and apoptotic cells. However, under dysmetabolic conditions, lipid accumulation occurs as a result of a disturbed cholesterol metabolic balance in the cells. When cholesterol influx is greater than macrophage reverse cholesterol transport (the mechanism responsible for cholesterol transfer from the peripheral macrophage to the liver for excretion) cholesterol esters accumulate in the cytoplasm as lipid droplets, and the macrophage is transformed into a foam cell. Accumulation of foam cell clusters in the intima is the hallmark of the arterial lesion in atherosclerosis. Lipid retention



activates and sustains an inflammatory reaction which favors lipid influx and inhibits lipid efflux from the plaque. Thus, the two processes potentiate each other in a positive feed-back loop which gives the atherogenetic process its chronic character.<sup>11</sup>

## **LDL – structure and role in atherosclerosis**

During atherogenesis, under hyperlipidemic conditions, low density lipoproteins (LDL) enter the intima of the arterial wall at particular atherosclerosis-prone sites characterized by increased endothelial permeability, such as arterial branching and curvature.<sup>12</sup> These particles become trapped in the extracellular matrix by interactions with matrix proteins, particularly proteoglycans.<sup>13, 14</sup> The internal elastic lamina functions as a barrier between the intima and the media, limiting LDL penetration to the subendothelial intimal layer.<sup>15</sup> Studies in cholesterol-fed rabbits have shown that a 7.6 fold increase in plasma LDL concentrations led to a 22 fold focal increase in LDL concentration in lesion-susceptible areas, characterized by high LDL retention and diminished fractional rates of LDL degradation. LDL accumulation is a necessary first step towards generation of fatty streak lesions, which are characterized by foam cell clustering beneath the endothelium, and precede atheroma formation.<sup>16, 17</sup>

### **LDL structure**

LDL, the main carriers of cholesterol to peripheral tissues, are lipoprotein particles within the density limits of 1.019-1.063 g/mL. LDL particles have a spherical micellar structure<sup>18, 19</sup> with an average diameter of 22 nm, which allows them to enter the sub-endothelial space through intercellular clefts. Their complex structure includes a hydrophobic lipid core, consisting of approximately 170 molecules of triglycerides (TG) and 1600 molecules of cholesteryl ester (CE).<sup>20</sup> The core is surrounded by an amphipatic monolayer of about 700 phospholipid molecules, mainly phosphatidylcholine (PC) and sphingomyeline (SM).<sup>21</sup> LDL particles contain only one integrated protein molecule, the 4536 amino acids long apolipoprotein B-100 (ApoB-100)<sup>22</sup>, one of the largest proteins known, which is wrapped around the particles outer layer, and is responsible for the interactions between LDL and the extracellular matrix.<sup>23, 24</sup> Another important constituent of LDL is unesterified cholesterol (UC), about 600 molecules, a third of which lie in the core and the rest in the surface.<sup>25</sup> Traces of lipophilic antioxidants, such as  $\alpha$ -tocopherol,  $\gamma$ -tocopherol, carotenoids, oxycarotenoids and ubiquinol-10, are also included in the structure, preventing the particle from oxidation in the plasma.<sup>26</sup>

## **LDL oxidation**

Accumulation, aggregation and oxidation of LDL are key processes in disease development. LDL oxidation occurs gradually, and two biologically and morphologically distinct forms of oxidized LDL have been identified: minimally oxidized LDL (mmLDL) and highly oxidized LDL (oxLDL). They can be differentiated by their receptor binding ability: mmLDL binds to LDL receptors (LDLR), while oxLDL, due to extensive modifications of apoB-100, loses this ability and binds scavenger receptors (ScR).<sup>27</sup> The proatherosclerotic properties of oxLDL are summarized in table 2.

**Table 2. Roles of oxidized LDL in atherosclerosis**

<b>Activity</b>	<b>Effects</b>
VCAM-1, ICAM-1 upregulation on EC	Monocyte adhesion
Stimulation of MCP-1 production Direct chemotactic effects	Monocyte and lymphocyte chemotaxis
AP-1 activation	ScR-A expression by macrophages
PPAR $\gamma$ activation	CD36 expression by macrophages
Generation of ScR ligands upon oxidation	Increased oxLDL macrophage uptake, foam cell formation
AP-1, NF-kB activation Increased cAMP	Secretion of proinflammatory cytokines
Oxidation specific epitopes	Activation of innate and adaptive immunity
Activation of apoptosis	Enhanced apoptosis and necrosis
Induction of tissue factor	Increased procoagulant activity

### *Minimally modified LDL (mmLDL)*

In the very first stages of atherogenesis, the subendothelial space contains virtually no macrophages. LDL suffers a mild oxidation under the influence of reactive oxygen species (ROS), oxidative products of the vascular cells. It appears that 12/15 lipoxygenase (12/15-LO), and its oxidation products such as hydroperoxyeicosatetraenoic acid (HPETE), have a very important role in this process, as the lack of this enzyme decreases lipid peroxidation and atherogenesis in apoE<sup>-/-</sup> mice.<sup>28, 29</sup> Minimal oxidation of LDL induces oxidation of structural phospholipids (PL) and conformational rearrangement of the molecular structures on LDL surface, resulting in the presentation of phosphorylcholine (PC) towards the exterior.<sup>30</sup>

The oxidized phospholipids are responsible for the pro-inflammatory and pro-atherogenic properties of mmLDL.<sup>31, 32</sup> They have the ability to induce the second major event in disease progression, monocyte recruitment into the intima. mmLDL induces monocyte adhesion to the endothelium and endothelial production of monocyte chemoattractant protein-1 (MCP-1), macrophage colony stimulation factor (M-CSF) and tissue factor (TF), which results in stimulation of monocyte chemotaxis, transmigration through endothelial cells and maturation into macrophages.<sup>33-36</sup> A relatively recent report by Miller et al. indicated for the first time the ability of mmLDL to bind the lipopolysaccharide (LPS) receptor CD14 and toll-like receptor 4 (TLR-4) on mature macrophages, which induced macrophage spreading and inhibition of the phagocytosis of apoptotic cells.<sup>37</sup>

From an immunological point of view, the epitopes on mmLDL appear to function as pathogen associated molecular patterns (PAMPs), inducing an innate immune response including the production of T-cell independent natural antibodies.<sup>30</sup>

#### Extensively oxidized LDL (oxLDL) and the generation of oxidation specific epitopes

The second stage of LDL oxidation is marked by the presence of macrophages in the subendothelial space, and their huge oxidative capacity. LDL continues to undergo non-enzymatic modifications involving reactive oxygen species produced by macrophages and endothelial cells (EC), but also enzymatic oxidation, mainly under the influence of NO synthase (iNOS), secretory phospholipase A2 (sPLA2), myeloperoxidase (MPO) and sphingomyelinase (SMase).<sup>38-42</sup> Both the protein and the lipid moieties of LDL are degraded, with two major consequences: 1) oxLDL gains the ability to bind to ScR<sup>43</sup> and 2) oxLDL becomes immunogenic.<sup>30</sup>

Peroxidation of the polyunsaturated fatty acids (PUFA) present in phospholipids and cholesteryl esters occurs at the oxidation-prone *sn*-2 polyunsaturated fatty acid and generates highly reactive breakdown products, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE).<sup>26, 44</sup> In its turn, apoB-100 is degraded into a mixture of fragments of different sizes, ranging from 14 to 550 kDa.<sup>45</sup> The reactive aldehydes, free or still attached to the phospholipid backbone, have the ability to form Schiff base adducts with the approximately 360 lysine residues found on the apoB-100 molecule.<sup>46</sup> These processes generate hundreds of new structures, including aldehyde-modified apoB peptides, phospholipid-protein or phospholipid-lipid adducts, which are recognized as non-self by the immune system. Extensive work by Palinski et al.<sup>47-52</sup> and Fredrikson et al.<sup>53</sup> characterized the immune responses associated with these neo-epitopes, termed "oxidation-specific epitopes".

Other oxidation-specific products of LDL are oxidized phospholipids (oxPL) which contain phosphorylcholine (PC)-headgroups, such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine (POVPC). The same PC-containing epitopes can be found on the membrane of apoptotic cells and the bacterial cell wall. IgM antibodies specific for this epitope, initially called E0 antibodies, were shown to be produced by B cells isolated from the spleens of apoE<sup>-/-</sup> mice.<sup>52</sup> The antigen binding domains of one of these antibodies, E06, were found to be genetically, structurally and functionally identical to those of T15, a natural IgA antibody secreted by innate B1 cells in a T-cell independent manner.<sup>54</sup> It has been shown that E06/T15 binds to the PC epitopes on both oxLDL and apoptotic cells, but does not recognize native PC-containing unoxidized phospholipids on native LDL or viable cells<sup>54</sup>, suggesting that during oxidation and apoptosis, the PC moiety is exposed to scavenger receptor recognition, as a possible clearance mechanism. Indeed, E06 blocks oxLDL and apoptotic cell binding to macrophage scavenger receptors (SR) CD36 and SR-B1 and inhibits cellular uptake.<sup>55</sup> The same effects were obtained by using BSA-bound POVPC. In contrast, E0 antibodies selected for binding to MDA-LDL did not inhibit binding of oxLDL to macrophages. These results suggest that it is the PC-containing oxidized phospholipids that mediate binding and uptake of oxLDL and apoptotic cells in macrophages via the scavenger receptors<sup>56-59</sup> and that natural antibodies present in plasma can block this effect.<sup>60, 61</sup>

## **The development of fatty streaks**

The activated endothelial cells express adhesion molecules, such as E- and P-selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule (ICAM-1). Leukocyte "rolling" along the endothelium is mediated by the selectins, whereas binding of VCAM-1 to the VLA4 integrin on the surface of monocytes and T lymphocytes induces firm adhesion to the endothelial cells.<sup>62</sup> After they adhere, monocytes are attracted into the subendothelial space by the interaction between MCP-1 and its receptor, CCR2. Animal studies on atherosclerosis prone mice in which these molecules were deficient or blocked showed marked disease reduction, underlining the importance of this step for atherosclerosis development.<sup>62-65</sup> Additionally, oxLDL was shown to be able to directly attract monocytes<sup>66</sup>, and studies by Boisvert et al. indicated an additional role of interleukin-8 (IL-8) and its receptor CXCR2 in intimal monocyte recruitment.<sup>67</sup>

In the intima, under the influence of macrophage colony stimulating factor (M-CSF), monocytes proliferate and mature into scavenger receptor expressing-macrophages. apoE<sup>-/-</sup> mice deficient in M-CSF developed decreased atherosclerosis, which is suggestive for the importance of this process in atherogenesis.<sup>68</sup> The scavenger receptors (ScR) are a family of receptors which mediate macrophage binding of negatively charged macromolecules on oxLDL, apoptotic cells or microorganisms, as well as the

removal of these structures from the extracellular space by endocytosis or phagocytosis.<sup>69</sup> Macrophage scavenger receptors A (SR-A) and CD36, that recognize and bind oxidized phospholipids on oxLDL, have been shown to be of primary importance in atherosclerosis.<sup>70, 71</sup> Unlike LDL receptors<sup>72, 73</sup>, ScR are not regulated by the cholesterol content of the cell, thus cholesterol efflux appears to be the only mechanism regulating cholesterol homeostasis in the macrophages which express ScR.

In the macrophage, free cholesterol is esterified by acyl CoA:cholesterol acyltransferase (ACAT-1) into cholesterol esters (CE) and stored as lipid droplets, and can be remobilized by hormone-sensitive lipase for membrane synthesis and transport out of the cells. Cholesterol efflux from macrophages to the different acceptors (macrophage-reverse cholesterol transport), is mainly mediated by the ATP-binding membrane cassette transporter (ABCA-1)<sup>74, 75</sup> and by other mechanisms such as passive diffusion, the scavenger receptor B1 (SR-B1) or caveolins.<sup>76</sup> High density lipoprotein (HDL) is the main extracellular cholesterol acceptor and is responsible for its transport to the liver for excretion as biliary acids. Plasma concentration of HDL and ApoA-I, its major apolipoprotein, was inversely correlated with the risk of cardiovascular disease in population studies. This indicated the atheroprotective role of the reverse cholesterol transport and established low HDL as an independent risk factor for cardiovascular disease.<sup>77</sup> Moreover, hypercholesterolemic mice deficient in ApoA-I developed more severe atherosclerosis<sup>78</sup>, while adenovirus-mediated overexpression of ApoA-I<sub>Milano</sub> proved to be protective.<sup>79, 80</sup> Shah et al. have shown that treatment with recombinant ApoA-I<sub>Milano</sub>, a naturally occurring mutant of ApoA-I, inhibited progression of atherosclerosis in hypercholesterolemic rabbits and mice.<sup>81-83</sup> ApoA-I<sub>Milano</sub> treatment was also shown to induce coronary atheroma regression in patients with acute coronary syndrome.<sup>84</sup> These findings demonstrate the anti-atherosclerotic property of ApoA-I<sub>Milano</sub> and indicate its potential as a new form of therapy in cardiovascular diseases. Additionally, macrophage secreted apoE may also contribute to macrophage reverse cholesterol transport, as indicated by Fazio et al.<sup>85</sup>

Initially, cholesterol metabolism in the macrophage is balanced between influx, storage and efflux, but under sustained hyperlipidemic conditions the balance is disturbed and the cholesterol esters accumulate extensively as lipid droplets in the cytoplasm. The macrophages are transformed into foam cells, which represent the basic feature of the arterial lesions and the main form of lipid deposition in the plaques. Accumulation of foam cells in the intima at the atherosclerotic-prone sites constitutes the first recognizable form of atherosclerotic lesions, the fatty streaks.

## **Formation of advanced, fibrotic lesions**

The next step in disease development, fatty streak progression towards advanced fibromuscular atheromas, requires the migration of smooth muscle cells from the tunica media of the arterial wall, past the internal elastic lamina, into the intima. In the intima, the smooth muscle cells (SMC) proliferate and secrete matrix proteins, as a fibrous response to injury, in an attempt to stabilize the lesions. Migration and proliferation of SMC are stimulated by growth factors, such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) or insulin-like growth factor (IGF) secreted by activated macrophages, T lymphocytes, EC and SMC.<sup>86</sup> Production of PDGF and FGF by SMC is stimulated by interleukin-1 (IL-1), a cytokine secreted by activated T cells and macrophages.<sup>87, 88</sup> Interferon gamma (IFN- $\gamma$ ) and transforming growth factor beta (TGF- $\beta$ ) prevent excessive accumulation of SMC by inhibiting proliferation and migration.<sup>89-92</sup> TGF- $\beta$  is the most potent stimulator of collagen synthesis by SMC<sup>93, 94</sup>, whereas IFN- $\gamma$  inhibits matrix formation, bearing important consequences on plaque stability.<sup>95-97</sup> Other factors that stimulate SMC growth and matrix synthesis are homocysteine<sup>98</sup>, hypertension and angiotensin II.<sup>99</sup>

SMC and the secreted matrix proteins finally form a fibrous cap that separates the lesion from the lumen. The foam cells gradually become apoptotic and die, releasing the lipids into the extracellular space and forming the necrotic core, an accumulation of leukocytes, extracellular lipids and cellular debris situated in the center of the plaque. The necrotic core and the fibrous cap are characteristic for the advanced atherosclerotic plaque. The lesion continues to grow at the shoulder regions by continuous leukocyte adhesion and entry.<sup>100</sup> At this stage of the disease, the atheromas are large enough to protrude into the lumen and induce various degrees of stenosis. Stenosis of the coronary or peripheral arteries may induce symptoms, such as *angina pectoris* or *claudicatio intermitens*, generated by decreased blood flow in the irrigated territory, especially under conditions of increased demand.<sup>101</sup>

Two other processes characteristic for the advanced atherosclerotic lesions, influencing plaque stability, are calcification and neovascularization. Calcification is induced by pericyte-like cells and regulated by cytokines and oxysterols.<sup>102, 103</sup> Neovascularization represents capillary growth from the media into the lesion. Inflammatory and immune mediators can enter the plaque through the new formed vessels. These capillaries are rudimentary and very fragile, being the source of plaque intrahemorrhage, which is an additional stimulus for inflammation and subsequent plaque growth.<sup>104</sup>

The different participating cell types are gradually involved in the atherogenic process, and each of them is characteristic for a certain stage of the disease. Activation of EC

and expression of adhesion molecules are crucial in the initiation of atherogenesis, whereas the formation of fatty streaks requires recruitment and activation of macrophages. T lymphocytes and SMC are essential for the progression of fatty streaks to advanced plaques<sup>105</sup>. Together with the macrophages, the T lymphocytes modulate the development of atherosclerosis. Mast cells are also present in the plaques, secreting cytokines and proteases which contribute to plaque destabilization and rupture.<sup>106</sup>

## Plaque stability and disruption

Even though stenosis can induce cardiovascular symptoms, the incidence of life-threatening acute coronary or carotid events depends mainly on plaque morphology and composition, rather than on the severity of stenosis.<sup>107</sup> Stable or fibrous atheromas contain a small necrotic core covered by a thick fibrous cap. The unstable, active or vulnerable plaques are characterized by intense inflammatory activity, high cellularity, extensive necrosis and lipid deposits, and are covered by a thin fibrous cap.<sup>108</sup> The large lipid core often occupies more than 40% of the total volume of the plaque, and the fibrous cap is depleted of collagen, glucosaminoglycans and smooth muscle cells.<sup>109</sup> The rupture of the fibrous cap and the subsequent thrombosis and ischemia are the key events which link atherosclerotic lesions to their clinical manifestations, causing approximately three-quarters of all myocardial infarctions.<sup>101</sup>

The rupture-prone shoulder regions of the atheroma are characterized by accumulation of activated macrophages, mast cells and T cells.<sup>110-115</sup> Mast cells and macrophages secrete potent proteolytic enzymes, such as matrix metalloproteinases (MMPs) and cysteine proteases which degrade extracellular matrix proteins, weakening the fibrous cap and inducing plaque instability.<sup>116, 117</sup> Several collagenases (MMP-1, -8, -13) and gelatinases (MMP-2, -9) have been found to be overexpressed in the plaques and act synergistically to degrade collagen fibres.<sup>118, 119</sup> Excessive proteolytic activity in the plaque is suppressed by tissue inhibitors of metalloproteinases (TIMP)<sup>120</sup> and cystatins.<sup>121</sup> Inflammatory cytokines, produced by T cells and mast cells, potentiate the degradation process. IFN $\gamma$  is a potent inhibitor of matrix formation by SMC<sup>92</sup>, whereas TNF $\alpha$  and IL-1 $\beta$  augment MMP expression by macrophages and SMC.<sup>122</sup>

Under the hemodynamic stress forces induced by blood flow, the weakened fibrous cap can fracture, allowing contact between blood and highly thrombogenic plaque components, such as tissue factor (TF). TF, a major inducer of the coagulation pathway<sup>109</sup>, is secreted by EC and macrophages under the influence of oxLDL, infections or CD40/CD40L interactions between EC and inflammatory cells.<sup>123</sup> Exposure of blood components to TF, plaque lipids and pro-aggregant collagen fibers initiates coagulation. Activated platelets aggregate at the rupture site and are bound together by a fibrin cloth inducing the formation of a thrombus which can obstruct blood flow

through the respective arterial segment. Depending on the size of the thrombus, the affected territory, the collateral circulation and the speed of intervention, this event can remain silent (without symptoms) can produce temporary symptoms, or it can be fatal.

Thus, the lifespan of a plaque goes through several stages, induced by extremely complex interactions between lipids and inflammatory factors. A comprehensive review series published in 1994-95 and revised in 2000 introduced a detailed morphological classification of atherosclerotic lesions. According to this classification, there are 8 distinct types of plaques. Type I lesions are characterized by a focal increase in the number of macrophages in the intima and the appearance of the first foam cells. Type II include the fatty streaks and are the first macroscopic visible lesions. Extracellular lipid accumulation defines a type III lesion. Lesions I to III appear in children and young adults and at this stage regression and disappearance of the arterial modifications is still possible.<sup>124, 125</sup> The classic atheroma, containing a distinct lipid core and signs of neovascularization, is classified as type IV lesion and starts to form in adult life. Formation of fibrous tissue layers inside the plaque marks its transformation into a type Va lesion, the fibroatheroma. If the plaque includes additional calcified nodules, it is a calcified plaque and is classified as a Vb lesion, or VII according to the new classification. The fibrous plaques, type Vc or VIII lesions, are characterized by the predominance of fibrous tissue over a small lipid core. Finally, the unstable or vulnerable plaques, that trigger acute clinical events, present surface erosion, hematoma/hemorrhage or thrombosis, and represent type VI lesions. Lesions of types IV to VIII develop after the third and fourth decades of life, can remain silent or generate symptoms, and are irreversible, although some therapies have been reported to be able to induce reduction of plaque volume.<sup>84</sup>

## **The immune system: Innate and adaptive immune responses**

The physiological role of the immune system is to protect the organism against the invasion of pathogens and to prevent pathogen associated infectious diseases. The structures which are able to trigger an immune response are called antigens. A very important characteristic of the immune system is immune tolerance, the ability to distinguish between the individual's own (self) and non-self antigens, and only attack the foreign organisms carrying the non-self antigens on their surface. Conformational or compositional changes of self structures can break the immune tolerance, rendering these molecules immunogenic and triggering an autoimmune response. These newly formed structures are called endogenous antigens or neoantigens, and this mechanism



is responsible for generating organ specific (thyroiditis, diabetes) or systemic (lupus erythematosus) autoimmune diseases.<sup>126</sup>

The immune system has two subsystems that work synergistically in protecting the organism against foreign invasions: the innate (natural or native) and the adaptive (acquired) immune systems. Innate immunity is the first line of defense against pathogens, acting fast but with low specificity to a large spectrum of both exogenous and endogenous antigens. Adaptive immunity reacts specifically and much more effectively to each particular antigen, but it takes several days to develop a competent adaptive immune response.<sup>127</sup> The two systems collaborate and potentiate each other, sharing common cellular and humoral effectors for an optimal response to intrusion (Table 3).<sup>128</sup>

**Table 3. Innate and adaptive immunity - overview**

	<b>Innate immunity</b>	<b>Adaptive immunity</b>
Activation of response	Fast (hours)	Slow (several days)
Receptors	PRRs (Encoded in germline- unspecific)	BCRs, TCRs (Somatic rearrangement – epitope specific)
Pathogenic determinants	PAMPs	Unique antigenic epitopes
Cells	Macrophages, NK cells mast cells	T and B lymphocytes
Effector mechanisms	Alternative and lectin complement pathways; cytokines, chemokines, cell-mediated cytotoxicity	Antibodies, classical complement activation, cytokines, chemokines ADCC, cytotoxic T cells (CTL)

## Innate immunity

Innate immune recognition of antigens involves a limited set (several hundreds) of cellular and humoral germline encoded receptors, with genetically predetermined specificity, called pattern recognition receptors (PRRs). The existence of PRRs was first predicted by Janeway in 1989, revolutionizing our understanding of native immunity.<sup>129</sup> These receptors recognize PAMPs, highly conserved molecular motifs that are common for a large number of pathogens, but are not present on self structures.<sup>127, 130</sup> Thus, the innate immune system has the extremely important ability and role to distinguish between self and non self antigens, priming the adaptive immune system

to react only against foreign intruders.<sup>131</sup> The effectors of the innate immune system include endothelial barriers, phagocytic and antigen presenting cells (neutrophils, monocyte/macrophages, dendritic cells and B cells), natural killer (NK) cells, mast cells and plasma proteins (complement, cytokines, CRP).

The epithelium of the skin, gastrointestinal and respiratory tracts, the three main portals for the entry of microbes, constitute a physical barrier which protects against pathogen invasion. Additionally, the epithelium secretes antibacterial peptides that kill bacteria. The epithelium also includes a particular type of T cells expressing  $\gamma\delta$  receptors which, unlike  $\alpha\beta$  T cells, can directly recognize antigens without requiring major histocompatibility complex (MHC) antigen presentation.<sup>132-135</sup> The neutrophils are the most abundant leukocytes in the blood, 4 000 to 10 000 cells/mm<sup>3</sup>, and their number can rise rapidly to 20 000 cells/mm<sup>3</sup> as a response to an infection. They are the first cells to arrive at the site of infection and present a high phagocytic capacity both in the circulation and in the tissues.

Circulating monocytes enter the extravascular space and are transformed into tissue macrophages, the main effector cell of the innate immune response. Macrophages express two classes of PRRs on their surface: scavenger receptors (ScR) and toll-like receptors (TLR). Binding of pathogens to ScR such as CD36 and SR-A, leads to endocytosis and activation of the phagocyte to kill the ingested microbes.<sup>136</sup> Proteins derived from the microorganisms are processed and the resulting peptidic fragments form complexes with MHC class II molecules. The MHC-peptide complexes are recognized by receptors on T lymphocytes, which are activated and generate an adaptive immune response. Antigen presentation by the macrophages is one of the most important links between the innate and the adaptive immune responses.<sup>137, 138</sup>

TLRs are so called signaling PRRs, and are found not only on macrophages, but also on EC<sup>139</sup>, dendritic cells (DC) and possibly SMC. Linking of TLRs generates transmembrane signals which activate a common signal transduction mechanism for all TLRs, the NF-kB pathway<sup>140-142</sup>, which is also triggered by IL-1 ligation to its receptor.<sup>127, 143</sup> NF-kB is a transcription factor that induces the expression of several inflammatory mediators: cytokines (IL-1,-6,-12, TNF $\alpha$ ), adhesion molecules, chemokines (MCP-1), enzymes (phagocyte oxidase, iNOS), growth factors, angiogenic factors, MMPs, TF and costimulatory molecules required for T cell activation.<sup>144, 145</sup> These mediators are responsible for pathogen killing, leukocyte recruitment, tissue remodeling, apoptosis, thrombosis, enhanced antigen presentation and activation of adaptive immunity.<sup>128, 146-148</sup> Lipopolysaccharide (LPS), component of the cell wall of gram negative bacteria, is the most potent bacterial activator of macrophages, and its effects are mediated by ligation of TLR-4 and TLR-2.<sup>149-151</sup> Additionally, it has been suggested that, similar to ScRs, TLRs too can bind and internalize pathogens, leading to antigen presentation by macrophages and dendritic cells.<sup>152</sup>

Other macrophage receptors include cytokine receptors and receptors for complement components (CR) and antibodies (FcR). Among the cytokines, IFN $\gamma$ , produced by activated Th1 lymphocytes and NK cells, is the most powerful macrophage activator, inducing responses similar to TLR ligation and priming the macrophages for TLR-induced activation.<sup>128</sup> Circulating antibodies and complement factor C3b, a downstream product of complement activation, bind onto the surface of bacterial pathogens in a process called opsonization and mediate their uptake by the macrophages by binding to CR1 (CD35) and FcR.

The complement system is a complex of circulating and membrane associated proteins, mostly proteolytic enzymes, which sequentially activate each other, forming an enzymatic cascade. It has an important role in the elimination of pathogens, either directly or indirectly, by opsonization and phagocyte chemoattraction.<sup>153</sup> The direct effect of complement on pathogens is mediated through the membrane attack complex (MAC), which is inserted into their membrane, favoring water and ions influx and leading to the death of the microbe. MAC is formed by complement factors C5b-C9. There are three different pathways of complement activation, which differ in their early steps but share the same effectors, factors C3b and MAC. The classic pathway of complement activation is triggered by antibody binding to epitopes on the surface of the pathogens, and is considered to be a part of the humoral adaptive immune response. The alternative and lectin pathways, parts of the innate immunity are initiated by binding of C3b and mannose-binding lectin (MBL) to microbes, respectively. All three pathways lead to the activation of proteolytic enzymes that cleave complement factor C3, leading to the formation of C3b. The late steps of complement activation, from C3b to MAC, are identical for all three pathways.<sup>153</sup> Due to their ability to recognize PAMPs on pathogens, MBL and other complement factors (C1q, C3b) are considered to be secreted PRRs.

B1 cells are a particular type of long lived, self-replenishing B lymphocytes which reside in the peritoneal cavity.<sup>154-156</sup> As opposed to adaptive immune B2 cells, B1 cells secrete antibodies in complete absence of external antigenic stimulation, in a thymus-independent manner, without requiring T cell cognate help.<sup>157</sup> Because these antibodies are constitutively secreted and not specific for a particular antigen, they were termed natural antibodies, and are a link between the innate and the adaptive immune responses. Natural antibodies are mostly of IgM idiotype, and it has been shown that 80-95% of the IgM antibodies in uninfected mice are secreted by B1 cells.<sup>158</sup> These antibodies are polyreactive, binding to a broad range of epitopes, mostly on pathogens, but also on self structures.<sup>159</sup> They have an important role as a primitive layer of recognition and protection against pathogens.<sup>160</sup> The natural antibodies function as secreted PRRs, that recognize PAMPs on bacterial cell walls and opsonize them for recognition by the complement system and phagocytes.<sup>161-163</sup> They were also attributed an important “housekeeping” role, because of their crossreactivity with

modified self antigens found on senescent cells, cell debris and other oxidation generated epitopes on self structures, such as oxLDL. These oxidation specific epitopes are recognized as PAMPs, presenting molecular mimicry with those found on bacterial cell membranes, and they are removed in the same manner as the invading pathogens.<sup>54, 164</sup> For example, antigenic epitopes that carry the phosphorylcholine (PC) headgroup, common on the bacterial cell wall, apoptotic cells and modified LDL, can induce natural antibody production by B1 cells.<sup>47, 165-167</sup> The role of B1 cells in maintaining the cellular homeostasis of the organism is also suggested by studies indicating that, unlike the B2 cells which suffer negative selection, B1 lymphocytes are positively selected for their ability to bind to self-antigens.<sup>168, 169</sup>

Other cells involved in the innate immune response are dendritic cells (DC), natural killer (NK) cells and mast cells. DC are professional antigen presenting cells (APCs), expressing a large number of ScR and TLR on their surface. They present the antigen to lymphocytes in a MHC II dependent manner and are also capable of providing the co-stimulatory signals necessary for T cell activation, through B7-1 and B7-2 molecules (CD80, CD86). Activated mast cells release from their granules large amounts of histamine, proteases, cytokines, leukotrienes and platelet-activating factor, with an important role in immune and allergic reactions.<sup>137</sup> NK lymphocytes are responsible for killing tumor cells, virally infected cells and antibody-coated cells and they also produce IFN $\gamma$ , under the influence of IL-12 and IL-18, secreted by macrophages and DC. There is a cross-talk between NK cells and macrophages: macrophages secrete IL-12, stimulating NK cells to produce IFN $\gamma$ , which activates macrophages to phagocytose microbes and produce higher amounts of cytokines. A similar cross-talk mechanism between macrophages and T cells, mediated by the same cytokines, is central in the cell-mediated adaptive immune response. Because it is produced by both macrophages and T cells, IFN $\gamma$  is considered to be a cytokine of both innate and adaptive immunity. NK cells also express FcR, which mediates their binding to antibody coated cells. A particular type of NK cells express a limited diversity of  $\alpha\beta$ T cell receptors, and are able to recognize lipid antigens presented to them by a MHC-like compound, CD1 (CD1d-restricted NK 1.1+ cells).<sup>170</sup>

Under the influence of TNF $\alpha$ , IL-1 and IL-6 (cytokines released by macrophages and endothelial cells as a result of TLR ligation) the liver produces a group of proteins including C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen and ferritin. These proteins are called acute-phase plasma proteins and are systemic markers of inflammation. CRP is an important prognostic factor and an independent risk factor for cardiovascular disease.<sup>171</sup> It acts as a secreted PRR with a dual affinity, binding PC-containing epitopes on both *Streptococcus Pneumoniae* and on apoptotic cells and oxLDL<sup>172</sup>, coating them for phagocytosis by macrophages, which express receptors for CRP (Fc $\gamma$ RII).<sup>173</sup>

## Adaptive immunity

The adaptive immune system provides an immune response specifically tailored for the particular antigens that trigger its initiation. The antigens are pathogen related substances found on the surface of the microbes or secreted by pathogens, as well as noninfectious molecules. In order to maintain its specificity, the adaptive immune system needs to be able to generate receptors with the ability to respond to the challenge mounted by the enormous diversity of pathogens and their ability to mutate. Therefore, unlike the innate immune receptors, the receptors of the adaptive immune system cannot be encoded in the genome as such, because the human genome includes only a limited number of genes. Instead, they are generated through a process of random somatic rearrangements of the V, D and J-genes in the blastocytes, involving the recombination-activating genes RAG1 and 2, during the development of lymphocytes. Each lymphocyte bears a structurally unique receptor. The repertoire of antigen receptors in the entire lymphocyte population is very large and extremely diverse, including approximately  $10^{14}$  B cell receptors (BCR) and  $10^{18}$  T cell receptors (TCR), with the ability to recognize almost any antigenic structure. Because they are not part of the host genome as such, these receptors cannot be genetically transmitted and they have to be reinvented by each generation.<sup>174</sup> Lymphocytes that have not previously encountered antigen are called naïve lymphocytes.

T and B lymphocytes are the key mediators of adaptive immunity. Because of their almost unlimited diversity, TCRs and BCRs bind to antigens regardless of their origin, bacterial, environmental or self. Lymphocytes have to be activated first by components of the innate immune system, in order to be able to transform into effector cells. T lymphocytes only recognize antigens when they are presented to them by APCs, which also provide additional co-stimulatory or secondary signals necessary for lymphocyte activation. The innate immune system determines the origin of antigens and only provides the co-stimulatory signals if the antigens are pathogen related.<sup>175, 176</sup> Naïve lymphocytes recognizing antigens in the context of co-stimulatory signals are activated and suffer clonal expansion, transforming into effector cells.<sup>177, 178</sup> Clonal selection accounts for most of the basic properties of the adaptive immune system. T cells that do not receive co-stimulatory stimulation upon recognition of an MHC/peptide complex on APCs are permanently inactivated or suffer apoptosis.<sup>179</sup> The innate immune system also ensures the selection of the appropriate effector mechanisms, by controlling the differentiation of T cells into effector cells of a particular type.<sup>180-182</sup> IL-12 secretion by APCs, which determine differentiation of lymphocytes into Th1 subset, is such an example. B cells receive similar secondary signals from activated T cells by CD40L/CD40 interaction.

## **The cellular and humoral adaptive immune responses**

There are two types of adaptive immunity: cell-mediated immunity and humoral immunity. T lymphocytes are the effector cells of cell-mediated immunity, which is responsible for providing defense against intracellular pathogens, either by stimulating phagocytes to destroy the ingested microbes or by directly killing any other type of infected cells. B cells are the central cells of humoral immunity. They secrete antibodies, complex proteins which mediate the humoral immune response by neutralizing extra-cellular microbes and toxins and by flagging them for ingestion in the phagocytes.

All lymphocytes are produced in the bone marrow and they mature in the central (primary) lymphoid organs, B cells in the bone marrow and T cells in the thymus. They encounter the antigens mainly in the peripheral (secondary) lymphoid organs, spleen, lymph nodes, mucosal and cutaneous lymphoid tissues. The peripheral lymphatic organs are organized to concentrate antigens, APCs and lymphocytes, facilitating the interactions among the cells for optimal antigen presentation. T and B lymphocytes differ fundamentally by their antigen recognition mechanisms and by the types of antigens that they recognize. T cells recognize only peptide fragments of proteic antigens and only when they are presented by specific APCs together with MHC class II. B cells can recognize any antigenic structure, soluble or cell-associated macromolecules (proteins, polysaccharides, lipids and nucleic acids) or small chemicals.<sup>126</sup>

## **Antigen presentation to T cells**

The dendritic cells, professional APCs, capture, process and present peptides derived from microbial proteins to naïve T cells, in the context of MHC molecules.<sup>183</sup> Antigen presentation by DC takes place in the peripheral lymphoid organs. MHC molecules are specialized peptide display molecules which bind peptidic fragments inside the cells and are transported to the surface, where the MHC/peptide complex is recognized by TCRs.<sup>184</sup> The cells which are able to internalize extracellular antigens, mainly DC, macrophages and B cells, present antigen derived peptides on MHC class II molecules. All nucleated cells bearing an intracellular pathogen, such as a virus, are able to express peptide fragments of the microbial products on MHC class I molecules, signaling the presence of the intracellular infection to T cells.<sup>185, 186</sup> These two different signaling pathways involve different types of T cells, triggering the proper effector mechanisms for the elimination of pathogens.

Antigens captured in the tissues or those that enter the lymphatic vessels are transported to the lymphatic nodes, whereas blood-borne antigens are captured by APCs in the spleen. The macrophages are another example of efficient APCs. They do not have

the ability to transport antigens for presentation to the lymphatic organs, therefore antigen presentation by macrophages takes place in the tissues where they reside. A particular type of DC, the follicular dendritic cells (FDC), located in the B-cell rich lymphoid follicles of the lymph nodes and spleen, are the only specialized antigen presenters for B lymphocytes, which are generally able to recognize and bind antigens without the help of APCs.<sup>187</sup>

The population of T lymphocytes can be divided into two main subclasses, which differ by their surface markers and have distinct effector roles in the cellular immune response: CD4+ and CD8+ T lymphocytes.<sup>187</sup> CD4+ T cells are called T helper lymphocytes (Th), because they secrete cytokines which stimulate phagocytes to destroy ingested microbes and B cells to produce antibodies against proteic antigens. CD4+ T cells recognize peptide fragments presented on MHC class II molecules. CD8+ lymphocytes are cytotoxic T cells, having the ability to kill the cells which harbor intracellular microbes. They recognize the antigens loaded on MHC class I molecules. The CD4 and CD8 surface molecules function as co-receptors during the antigen recognition process, binding to invariant regions of the MHC molecules. T cell activation requires binding of both the TCR and the CD4 or CD8 to the MHC/peptide complex. CD4 can only bind MHC class II and CD8 only recognizes MHC class I, thus determining the specificity of Th and Tc lymphocyte receptors for a certain type of antigen presenting complex.<sup>187</sup>

### **Antigen recognition by T and B cells: BCRs and TCRs**

BCRs and TCRs are membrane complexes on the surface of lymphocytes, which recognize and bind specific antigens, triggering cell activation and transformation of naïve lymphocytes into effector cells. They consist of a proteic molecule specialized in antigen binding, covalently bound to other proteins which transmit the activation signals inside the cell. Each antigen receptor molecule consists of two regions: a constant region (C) and a variable region (V). The constant region of the receptors is responsible for the structural integrity and for the effector functions of the receptors, and is common for all clones of lymphocytes. The variable region, characterized by enormous diversity, is the one responsible for antigen binding and is specific for each particular clone. The variable regions of the different clones differ one from another only by the structure of the particular sites that bind antigens, called the complementarity determining regions (CDR). Cell activation requires the cross-linking of two or more antigen receptors that bind adjacent antigens, triggering signaling cascades induced by the enzymes attached to the cytoplasmic portions of their signaling proteins.

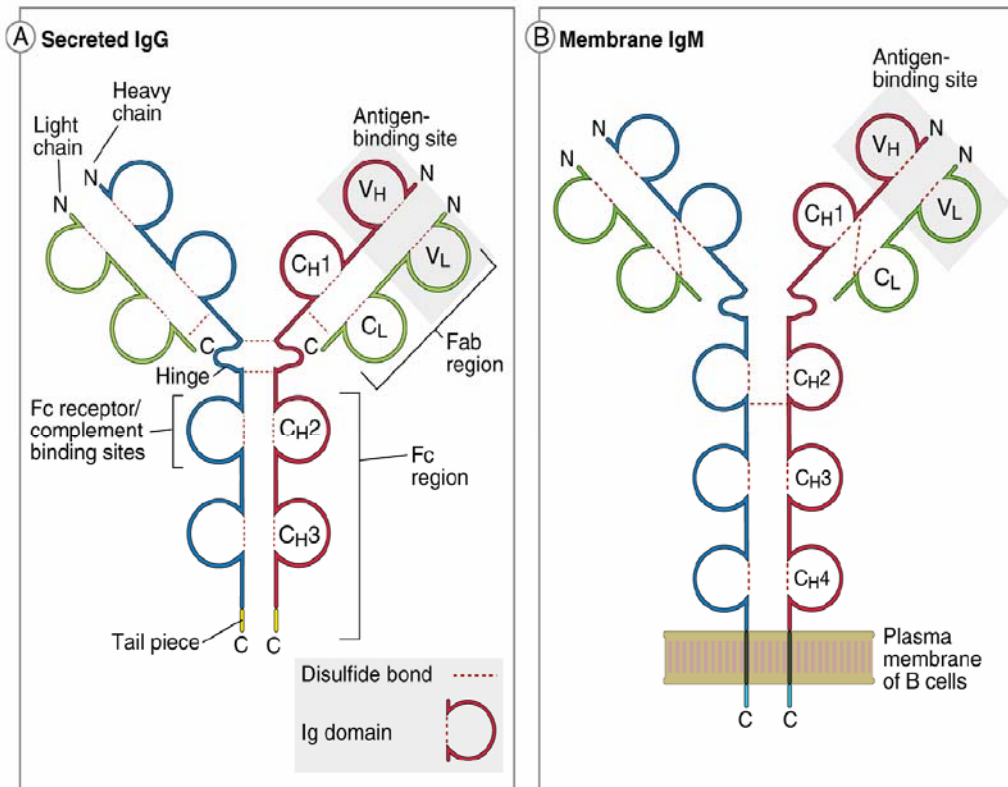
*BCRs and the structure of antibodies*

In humoral immunity, antibodies play important roles both in the antigen recognition step, as membrane-bound B cell receptors, and in the effector step, as secreted immunoglobulins. The structure of membrane bound and secreted antibodies of the same isotype is identical. The BCR complex contains one membrane-bound antibody molecule, for antigen recognition, covalently bound to two other proteins, Ig $\alpha$  and Ig $\beta$ , which transmit the activating signals inside the cell.<sup>188</sup>

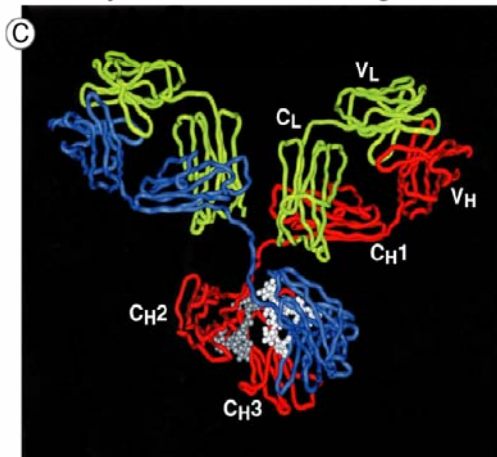
Antibodies are Y-shaped complex macromolecules, consisting of four polypeptide chains bound by disulfide bonds: two identical heavy (H) chains and two identical light (L) chains (Figure 2). Each light chain is attached to a heavy chain, and the two heavy chains are attached to each other.<sup>189</sup> The heavy chains are longer, and contain one V domain ( $V_H$ ) and three or four C domains ( $C_{H1,2,3,4}$ ), whereas the light chains contain only one V domain ( $V_L$ ) and one C domain ( $C_L$ ). Each domain has a characteristic three-dimensional structure, called the immunoglobulin (Ig) domain. Due to this structure, the antibodies are also called immunoglobulins. The antigen-binding site of the antibody is formed by one  $V_L$  and one  $V_H$  domain, which contain three hypervariable CDRs each. The H chain CDRs are located at the N-terminal end of the chain, and the L chain CDRs are evenly distributed along the chain. The CDR3 domain, located towards the junction with the C domains, is responsible for most of the variability of the antibody molecule and is the predominant antigen binding site.<sup>190, 191</sup> Each antibody molecule has three fragments: two identical Fab (fragment antigen binding) fragments, which contain the antigen-binding site, and one Fc (fragment crystalline) fragment. The  $V_L$  and  $C_L$  domains of the light chain face the corresponding  $V_H$  and  $C_{H1}$  domains of the heavy chain, forming the Fab fragment. The Fc fragment contains the remaining C domains of the heavy chains, being responsible for the effector functions of the antibodies, including signal transduction through the membrane and binding to complement and FcR. A flexible hinge region between the Fab and the Fc fragments allows the two Fab fragments to move and bind antigens situated at variable distances from one another. In the case of the BCRs, the Fc region is bound to the membrane by an anchor situated at the C-terminal end of the heavy chains.<sup>189</sup>

There are five types of heavy chains ( $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$  and  $\mu$ ) and two types of light chains ( $\kappa$  and  $\lambda$ ) which differ by the structure of their C domains. Depending on which type of heavy chains they contain, immunoglobulins are also classified in five classes, called isotypes: IgA, IgD, IgE, IgG and IgM. Each antibody isotype can contain any of the two light chain subtypes. The five immunoglobulin classes present different physical and biological properties and effector functions. BCR complexes on naïve B cells can only contain IgM or IgD immunoglobulins. The circulating antibodies are mainly IgA, IgE, IgG or IgM, with very low levels of IgD.<sup>192</sup> Initially, the naïve B cells only produce membrane bound IgD or IgM immunoglobulins, which function as BCRs. In order for an activated B cell to produce secreted IgA, IgE or IgG antibodies, it





Crystal structure of secreted IgG



**Figure 2. Antibody structure.** The diagrams show the structure of a secreted IgG (A) and a membrane-bound IgM (B). N and C refer to the amino-terminal and carboxy-terminal ends of the polypeptide chains, respectively. Panel C represents the crystal structure of a secreted IgG molecule. The heavy chains are colored blue and red, and the light chains are colored green; carbohydrates are represented in grey. (Reprinted from Basic Immunology 2<sup>nd</sup> ed., Abul K. Abbas and Andrew H. Lichtman, page 67, © 2003, with permission from Elsevier. Courtesy of Dr. Alex McPherson, University of California, Irvine.)

must first undergo a process called isotype (or heavy chain class) switching.<sup>193, 194</sup> The antibodies are secreted as monomers (IgA, IgE, IgG), dimers (IgA) or pentamers (IgM), containing one, two or five replicates of the above described immunoglobulin structures, bound together. IgG is the predominant antibody isotype in the peripheral blood.

The epitopes are specific regions of an antigen which are recognized by antibodies. They can be recognized by their sequence or spatial conformation. The term neoepitopes defines epitopes which are normally hidden inside the antigen molecules, and are only expressed as a result of changes in the antigenic structure, induced by different processes, such as oxidation. The interaction between the antibodies and their epitopes is characterized by affinity and avidity.<sup>190-192</sup> The affinity, or strength, of the interaction is quantitatively expressed by the dissociation constant ( $K_d$ ), which is defined as the molar concentration of an antigen which is needed to occupy half of the available antibody molecules in a solution. The lower the  $K_d$ , the higher the affinity of the antibody for the respective epitope. Successive encounters with the same antigen lead to production of antibodies with increased affinity for the respective antigen, lowering the  $K_d$  from  $10^{-6}$ - $10^{-9}$  to  $10^{-8}$ - $10^{-11}$  due to a process called affinity maturation.<sup>195, 196</sup> Depending on the number of immunoglobulin replicates in their structure, the antibodies contain from 2 to 10 antigen binding sites. The avidity of antibodies for a certain antigen characterizes the total strength of binding and is given by the affinity for the epitopes and the number of epitopes that one antibody molecule can bind at the same time.

### TCRs

T cell receptors for antigens are very similar to BCRs. They contain an antigen-binding molecule and a signaling part, formed by a three-protein complex, called CD3, and another proteic homodimer, the  $\zeta$  chain.<sup>197</sup>

The antigen binding part of the receptors found on the majority of T lymphocytes, called  $\alpha\beta$ T cells, is made up of two polypeptidic chains, the  $\alpha$  and the  $\beta$  chain, both anchored in the membrane by their C terminal end. Both chains contain one variable region ( $V\alpha$  and  $V\beta$ ) and one constant region ( $C\alpha$  and  $C\beta$ ), homologous to the V and C domains of the immunoglobulins. The greatest variability among TCRs is located in the CDR3 region of the two V domains. Despite their similar structure, there are some very important differences between TCRs and BCRs. The epitope affinity of TCRs is quite low ( $K_d 10^{-5}$ - $10^{-7}$ ) and they do not undergo affinity maturation. They can only bind a few amino acid residues on the MHC presented peptide, unlike antibodies, which offer a flat surface for antigen recognition, able to bind epitopes of different sizes and shapes. TCRs do not present isotype switching and are not secreted, thus do not participate in the effector function of T lymphocytes.<sup>197, 198</sup>

## T lymphocyte activation and effector functions

### *Activation of T lymphocytes*

T cells can only respond to antigens associated with other cells (macrophages, dendritic cells, B cells or infected host cells) which interact with the T lymphocytes, signaling the presence of infection. In order for a T cell to become activated, two or more TCRs need to be simultaneously bound for several minutes to the same antigen, or have multiple contacts with the respective antigen. Due to their low affinity for epitopes, TCRs need the additional binding of integrins on their surface, such as leukocyte function-associated antigen-1 (LFA-1) to ICAM-1 on the surface of the APCs, to strengthen the connection.<sup>199, 200</sup>

T cells need two signals for activation: signal 1 is provided by recognition of antigen/MHC complex and signal 2, also known as co-stimulatory signal, is induced by cell-cell communication between the antigen presenting cells and the T cells. The costimulatory signal is provided by ligation of B7-1 or B7-2 on the surface of the APCs to the CD28 receptor on the T lymphocyte.<sup>177</sup> As discussed previously, B7-1 or B7-2 are only expressed by APCs when these cell encounter and take up a pathogen. Since APCs continuously take up and present self antigens as well, both self and pathogenic antigens may be presented in connection with co-stimulatory molecules. In order to prevent them from reacting against self structures, T cells that recognize self antigens are deleted in the thymus.<sup>201</sup> At the same time, DCs that have taken up pathogens cease to take up new antigenic structures from the surroundings, in an attempt to reduce self-antigen presentation during the period when they express costimulatory molecules.<sup>202, 203</sup>

Thus, the complete set of interactions required for T cell activation includes binding of the MHC/peptide complex, ICAM-1 and B7-1/B7-2 on the surface of APCs to TCR, CD4/8, LFA-1 and CD28 on the T lymphocyte.<sup>204</sup> The cytoplasmic end of the CD3,  $\zeta$  and CD28 membrane proteins are linked to different proteins which initiate a signaling cascade, leading to the formation of several transcription factors, such as AP-1, NF- $\kappa$ B and NFAT. These factors stimulate the transcription of genes whose products mediate the responses of the activated T cell.<sup>205, 206</sup>

The activated T cells send a positive feed-back signal to APCs, by expressing a protein, called CD40 ligand (CD40L) on their surface, which binds to CD40 on APCs. The CD40/CD40L interaction stimulates APCs to produce more B7 molecules, and to secrete IL-12, a cytokine which enhances T cell differentiation. CD40 is also found on macrophages, B lymphocytes, EC and SMC, and intermediate cell-cell interactions and activation of these cells by T lymphocytes.<sup>207, 208</sup> The activated T cells stimulate

their own proliferation in an autocrine manner, by producing increasing amounts of IL-2, a cytokine which induces proliferation, and by expressing high affinity receptors for IL-2. Thus, within 1 to 2 days after activation, naïve T cells undergo clonal proliferation and differentiation, generating effector T cells, which eliminate the microbes, and memory T cells. The memory T cells are functionally inactive cells which circulate for months or years, ready to rapidly respond to a second challenge of the same pathogen.<sup>209</sup> The first effector T cells are generated 3 to 4 days after activation, and they either stay in the lymph nodes, killing pathogens and stimulating B cells to produce antibodies, or they migrate to the site of infection. The CD8+ cytotoxic T lymphocytes kill the infected cells, and the CD4+ helper T lymphocytes secrete cytokines, activating macrophages, eosinophils, mast cells and B cells to eradicate the infection.

#### *Different Th lymphocyte subsets and their effector functions*

The T helper lymphocyte population is not homogenous. There are different subsets of Th cells, which differ fundamentally by their secreted cytokines and by the effector mechanisms elicited by these cytokines. The predominant subsets of CD4+ T lymphocytes are the Th1 and Th2 cells.<sup>210</sup> Differentiation of a naïve T cell into either of these subclasses is dependent on the cytokine environment present upon its activation. There are several different subtypes of APCs, and they react differently depending on the nature of the ingested pathogens. If the APCs have ingested bacteria or viruses, they secrete IL-12 in association with antigen presentation. IL-12 stimulates differentiation of the naïve T cells into Th1 effector cells. In the case of helminths, for example, APCs cannot ingest the pathogen and do not secrete IL-12. In the absence of IL-12, the activated T cells themselves secrete IL-4, which induces their own differentiation into the Th2 subset of T lymphocytes.<sup>211-213</sup>

The Th1 and Th2 subsets of lymphocytes have different functions.<sup>210</sup> The main cytokine secreted by Th1 cells is IFN $\gamma$ , which has strong proinflammatory functions. IFN $\gamma$  promotes macrophage recruitment and activates them to kill the ingested pathogens and secrete additional inflammatory cytokines. It also stimulates B cells to produce opsonizing and complement-binding antibodies, IgG1 and IgG3, promoting the phagocytosis of pathogens. The Th2 subset secretes mainly IL-4, which stimulates production of IgE and IgG4 antibodies and IL-5, which activates eosinophils, triggering a mechanism suited to eliminate helminths. In mice, Th1 cells stimulate production of IgG2a antibodies and Th2 cells the production of IgG1. Other lymphocyte populations include the TGF $\beta$  secreting Th3 cells and the CD25 positive regulatory T cells (CD25+CD4+Treg), which also secrete TGF $\beta$ , in addition to IL-10.<sup>214, 215</sup> Both Th3 and Treg cytokines have anti-inflammatory effects.

Macrophages and Th1 lymphocytes activate and stimulate each other, propagating the inflammatory reaction. Th1 cells activate macrophages by CD40/CD40L inter-

actions and  $\text{IFN}\gamma$ , inducing IL-12 secretion and increasing the expression of MHC II and B7 surface molecules. This leads to activation of more T lymphocytes and their differentiation into  $\text{IFN}\gamma$  secreting Th1 cells. Macrophages and Th1 cells also secrete another potent proinflammatory cytokine,  $\text{TNF}\alpha$ , which induces additional leukocyte recruitment to the site of the inflammation.<sup>216</sup> The Th2 lymphocytes have an anti-inflammatory effect. Their cytokines, IL-4, IL-10 and IL-13 inhibit macrophage activation, Th1 cell proliferation and cytokine secretion. On the other hand,  $\text{IFN}\gamma$  is a potent inhibitor of Th2 activity. The Th1/Th2 balance regulates the local inflammatory reaction and determines the outcome of the cell-mediated response to a certain antigen. Macrophages use toxic substances, such as reactive oxygen intermediates, NO and proteolytic enzymes to kill the ingested microbes, upon activation. Chronic inflammatory cell-mediated immune reactions, such as atherosclerosis, may lead to the release of these compounds in the extracellular milieu, inducing tissue injury.

## **B lymphocyte activation and effector functions**

### *Activation of B lymphocytes*

The activation of B lymphocytes and their transformation into effector cells is similar to T cell activation. B cells are also activated in response to antigen recognition and second signals, and they also undergo clonal expansion and differentiation into antibody secreting cells, called plasma cells. Unlike T cells, B lymphocytes do not need antigen presentation by APCs, and therefore they can recognize a wide variety of antigenic structures, including proteins, polysaccharides, lipids and small chemicals.<sup>217</sup> In the case of proteic antigens, B cells need the help of Th lymphocytes that were activated by the same antigen, in order to produce antibodies and undergo isotype switching and affinity maturation.<sup>218</sup> Thus the B cell response to protein antigens is T cell-dependent. The B cell response to other antigenic structures is T cell-independent, and rarely characterized by isotype switching and affinity maturation.

Similar to T cells, activation of B cells requires binding of two or more BCRs to epitopes on the same antigen or on antigen aggregates. Binding of antigens to BCRs triggers activating signals which are transmitted inside the cell by the  $\text{Ig}\alpha$  and  $\text{Ig}\beta$  proteins attached to the immunoglobulin receptor. This activates signaling cascades, which lead to the expression of the same transcription factors as for T cells: NF- $\kappa$ B, AP-1 and NFAT.<sup>219</sup> Activation of the complement system leads to the production of the complement factor 3d (C3d), a breakdown product of C3 which binds to the surface of the microbes. C3d is recognized by CR2 (CD21), its receptor on the naïve B cells and provides the secondary signal which strongly enhances B cell activation by antigens.<sup>220</sup>

In T cell-independent humoral immune responses, activated B cells start secreting IgM directly.<sup>217</sup> The magnitude of the response depends on the number of epitopes cross-linked at the same time and on the strength of complement activation. Recognition of protein antigens prepares the B cell for a T cell-dependent immune response: the activated B cell expresses B7 co-stimulatory molecules, cytokine receptors, and starts migrating towards the periphery of the follicles, where the T cell layer is located. For a T cell-dependent humoral immune response to be initiated, the activated B cell has to encounter a T cell that was also activated by the same pathogen, and has TCRs specific for peptide fragments of its antigenic structure.<sup>218</sup> B cells internalize the proteic antigen and present it on MHC class II molecules to T cells<sup>221</sup>, together with co-stimulatory signals provided by the B7/CD28 interaction.<sup>222</sup> The CD4+ T lymphocyte reacts by expressing CD40L on its surface and secreting cytokines, which bind to their respective ligands on the B cell, initiating clonal expansion and antibody production.<sup>223</sup> CD40 ligation induces heavy chain class switching, and the T cell-secreted cytokines dictate which antibody isotype will be produced.<sup>224</sup> IgG1 and IgG3 antibody subclasses are produced under the influence of IFN $\gamma$ , whereas IL-4, associated with a Th2 response, stimulates IgE and IgG4 production.<sup>225, 226</sup> In the mucosal tissues, the predominant antibody isotype is IgA, secreted with the help of TGF $\beta$ , produced by Th3 cells.

#### *Effector functions of humoral immunity*

The effector functions of the antibodies are mediated by their Fab and Fc fragments. There are very important differences among the different isotypes with regard to their effector functions. IgG antibodies have the most widespread functions and are specialized in elimination of extracellular bacteria and viruses. They neutralize and opsonize microbes and their toxins with their Fab fragments and use the Fc fragments to initiate the classical way of complement activation.<sup>153</sup> The Fc fragments of human IgG1 and IgG3 promote pathogen phagocytosis by binding to Fc $\gamma$ RI on macrophages and neutrophils and Fc $\gamma$ RIII on NK cells. IgE mediates mast cell degranulation and killing of helminths by eosinophils, which express Fc $\epsilon$ Rs on their surface.<sup>227</sup> Activation of NK and eosinophils by IgG and IgE to kill infected cells is called antibody-dependent cellular cytotoxicity (ADCC).<sup>228</sup> IgM functions as complement activator and IgA is responsible for mucosal immunity, neutralizing microbes and toxins into the lumens of the gastrointestinal and respiratory tracts.

After activation, B cells evolve into antibody secreting plasma cells and memory B cells. Similar to memory T cells, memory B cells do not secrete antibodies and circulate in the blood and lymph for months or years, ready to rapidly respond to a second challenge from the same antigen. Circulating antibodies bind antigens in blood and tissues, forming immune complexes. When the BCR and the Fc $\gamma$ RII (CD32) of a B cell concomitantly bind the antigen part and the antibody part of an immune complex,

the FcR transmits a negative signal inside the cell, which blocks antigen-induced signals, inhibiting further B cell activation.<sup>229-231</sup> The effector B cells secrete antibodies as long as the antigen stimulation persists, and then suffer apoptosis, except for a few cells which migrate into the bone marrow and survive, continuing to secrete antibodies for long periods of time. These cells generate more than half of the total amount of circulating antibodies found at a certain time point in the blood of a normal healthy adult. When enough IgG antibodies have been produced, they shut down further antibody production in B cells, by a process called antibody feedback.<sup>232, 233</sup>

## **Immune mechanisms in atherosclerosis**

Atherosclerosis can be considered to be an autoimmune inflammatory disease, triggered by LDL oxidation and involving both the innate and the adaptive immune systems. The macrophage, effector cell of innate immunity, plays a central role in all phases of the atherosclerotic process: initiation, progression and complication (rupture and thrombosis). T lymphocytes, effectors of the adaptive immunity, are very important modulators of the disease. Thus, similar to the immune response to pathogens, the innate immune system is the first to react to the presence of the antigens, initiating plaque formation and activating the adaptive immune response, which modulates the later course the disease. The identification of the antigens which trigger immune reactions in atherosclerosis is of particular importance for understanding these mechanisms.

### **Antigenic determinants in atherosclerosis**

Three types of antigens were incriminated for their roles in atherogenesis: oxidation specific epitopes on oxLDL and apoptotic cells, heat shock proteins (Hsps) and infectious agents.

#### **Oxidized LDL**

LDL oxidation induces dramatic changes in its native structure, generating numerous oxidation specific epitopes, new compounds which are recognized as non-self by the immune system. These compounds are immunogenic and are responsible for most of the immune reactions in atherosclerosis. There are two types of oxLDL associated neo-epitopes which trigger immune reactions in atherosclerosis: proteic epitopes, mainly MDA-modified peptides of apoB-100, and lipidic epitopes, PC-containing

oxidized phospholipids, such as POVPC. The membrane of apoptotic cells and the bacterial cell wall also present PC-containing epitopes, this molecular mimicry being partly responsible for the initiation of natural immune reactions against oxLDL.

Substantial experimental evidence indicates LDL oxidation as being an obligatory step for atherosclerosis. Inhibition of oxidation by genetic and/or pharmacologic interventions, including antioxidant vitamins, potently inhibits atherogenesis, independent of lowering plasma cholesterol levels.<sup>234</sup> It is now well established that LDL oxidation occurs *in vivo* and that oxLDL is present in the atherosclerotic plaques. LDL extracted from atherosclerotic lesions of rabbits and humans proved to be physically, chemically and biologically identical with oxLDL prepared *in vitro* by copper oxidation or MDA modification.<sup>48, 235, 236</sup> The presence of oxLDL epitopes *in vivo* was also indirectly demonstrated by detecting the presence of IgM and IgG antibodies against MDA-lysine and the PC-containing oxidized phospholipids, both in the circulation<sup>31, 48, 51, 237</sup> and in the atherosclerotic plaques.<sup>51, 238</sup> These antibodies detected oxLDL epitopes in circulation and in aortic plaques of rabbits<sup>31, 48, 52, 239, 240</sup>, humans<sup>48, 52, 240</sup> and mice<sup>51</sup>, and the amount of antibodies was directly correlated with the atherosclerotic burden<sup>50, 241</sup> and the oxLDL content of the lesions<sup>241</sup>, indicating that LDL oxidation and the associated immune responses are key events in the development of the disease. The specificity of the stainings was accurate enough to determine that oxLDL is present in the macrophage rich areas in the early lesions, and as extracellular lipid bound to the matrix in the advanced plaques.<sup>239</sup> MDA2, a cloned monoclonal murine antibody specific for MDA-LDL epitopes<sup>47</sup>, was successfully used to detect the presence of atherosclerotic plaques in mice and rabbits.<sup>242</sup> Its uptake in the lesions was found to reflect the presence and changes in oxLDL content, the progressive or regressive character and the stability of the plaques.<sup>243, 244</sup> <sup>125</sup>I-MDA2 was also used to obtain *in vivo* gamma camera images of the location of atherosclerotic plaques in Watanabe heritable hyperlipidemic (WHHL) rabbits, indicating this method as a potential future tool for locating oxLDL-rich atherosclerotic lesions in humans.<sup>245, 246</sup>

Natural IgM murine antibodies against the PC-epitopes, termed E01-E17, were cloned from the spleens of hypercholesterolemic non-immunized mice<sup>52</sup>, and recognized the presence of these epitopes not only on oxLDL<sup>31, 166</sup>, but also on apoptotic cells<sup>61, 166</sup> and on the capsule of infectious pathogens, such as *Streptococcus Pneumoniae*.<sup>166</sup> Further studies showed that these antibodies inhibited macrophage uptake of oxLDL<sup>60</sup> and apoptotic cells<sup>61</sup>, indicating the functional role of PC-containing oxidized phospholipids as binders of macrophage ScRs.<sup>55, 247-249</sup> Similar antibodies, specific for MDA-LDL, failed to inhibit macrophage uptake of oxLDL<sup>60</sup>, but inhibited the uptake of apoptotic cells.<sup>61</sup> IK17, an MDA-LDL specific IgG Fab antibody cloned from a hypercholesterolemic patient, inhibited both the uptake of oxLDL and apoptotic cells.<sup>240</sup> A recent study by Chang et al. demonstrated the presence of oxidized phospholipid structures on apoptotic cells by mass spectrometry. Immunization of mice with apoptotic cells led



to the development of IgM and IgG antibodies specific for both PC and MDA-lysine epitopes in the plasma of these animals. In the same study, apoptotic cells were shown to activate endothelial cells and induce monocyte adhesion. This effect was inhibited by anti-PC antibodies.<sup>250</sup> These studies suggest the presence of complex antigenic structures on the surface of these cells and underline their atherosclerosis related immunogenic and proinflammatory properties.

The Fab fragment of E06, one of the PC-binding antibodies, was found to be identical to the Fab fragment of T15, a natural IgA antibody secreted by B1 cells, serving to protect mice from infections with encapsulated bacteria, such as *Streptococcus Pneumoniae*.<sup>54, 251</sup> Binding of these antibodies to oxLDL and apoptotic cells probably contributes to their “housekeeping” role, signaling the presence of oxidized structures which need to be removed. Hence, it is intriguing that they block instead of promote the binding of these structures to macrophages.

### Other antigens than oxLDL

Heat shock proteins (Hsp) are proteins normally involved in protein folding inside the cell.<sup>252</sup> They are believed to have cytoprotective effects and are produced and released in high amounts by stressed cells, such as injured EC or macrophages in response to oxLDL.<sup>253</sup> Hsps are immunogenic, and the immune responses against these proteins have been suggested to be involved in several inflammatory diseases with an autoimmune mechanism, such as Crohn’s disease and rheumatoid arthritis.<sup>254</sup> Human Hsp60 presents a remarkable structural and immunogenic mimicry with bacterial Hsps, such as mycobacterial Hsp65 and chlamydial Hsp60<sup>255, 256</sup> and the immune reactions to bacterial Hsps were identified as being able to induce endothelial injury and to represent links between infectious diseases and atherosclerosis.<sup>257, 258</sup> Further support for this theory are studies in which immunization of wild type<sup>259</sup> and LDLR<sup>-/-</sup> mice<sup>260</sup> and normocholesterolemic rabbits<sup>261</sup> with Hsp65 was found to induce early atherogenesis, with lesions rich in T cells specific for the respective protein. It has been demonstrated that Hsps are highly expressed in the atherosclerotic lesions of humans, rabbits, and apoE<sup>-/-</sup> mice.<sup>252</sup> Antibody levels against Hsp65 correlate with the progress of carotid disease in humans<sup>262, 263</sup> and are associated with the development of early atherosclerosis and with borderline hypertension.<sup>264</sup> Hsps 60 and 70 were also identified as PAMPs, elements of the innate immunity, showing LPS-like properties in binding and activating TLR4.<sup>258, 265-267</sup>

Infectious agents, such as *Chlamydia Pneumoniae*<sup>268</sup>, herpes simplex type I virus (HSV I)<sup>269</sup> and cytomegalovirus (CMV)<sup>270</sup> have been detected in human atherosclerotic lesions but their role is not yet fully understood.<sup>5, 271</sup> It has been suggested that the presence of infectious diseases is not necessary for murine atherosclerosis<sup>272</sup>, but they

may have an important role in disease modulation, directly or by the immune responses that they trigger. The studies trying to establish the importance and mechanisms of action of *Chlamydia pneumoniae* in atherosclerosis have been so far contradictory. While some investigators found a cholesterol-dependent atherogenic role for *Chlamydia pneumoniae* in mice, others failed to see such a connection.<sup>273-276</sup> In human studies, an increase in antibody titers to *Chlamydia pneumoniae* was found in patients suffering from chronic coronary heart disease and myocardial infarction.<sup>277</sup> Infection with CMV was associated with the development of coronary and carotid atherosclerosis and the risk of restenosis after coronary interventions.<sup>278-280</sup> A possible explanation for the implication of bacterial infections in atherosclerosis is offered by studies which demonstrate that *Streptococcus Pneumoniae*, oxLDL and apoptotic cells share the same PC-containing antigenic structures on their surface. Natural antibodies against these structures are present in mouse plasma.<sup>54, 251</sup> Immunization of mice with *Streptococcus Pneumoniae*<sup>251</sup> induced high titers of these antibodies and reduced atherosclerosis progression.<sup>167</sup> Immune crossreactivity between these epitopes could link bacterial pathogens to atherosclerosis. Nevertheless, these natural antibodies seem to have a protective rather than atherogenic influence.

Finally,  $\beta_2$ -glycoprotein I ( $\beta_2$ GPI) and the advanced glycosylation end products (AGEs) are also targeted by immune responses involved in atherosclerosis and other auto-immune diseases.  $\beta_2$ GPI, a protein mainly present on platelets and ECs, colocalizes with oxLDL and activated T lymphocytes in atherosclerotic plaques.<sup>281</sup> It binds to phospholipids on oxLDL and forms complexes which are found in the blood of patients with chronic inflammatory immune diseases, such as systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), systemic sclerosis<sup>282</sup> or diabetes mellitus (DM).<sup>283</sup> These immune complexes and the antibodies formed against  $\beta_2$ GPI were demonstrated to have a proatherogenic role. Immunization with  $\beta_2$ GPI and transfer of lymphocytes reactive to  $\beta_2$ GPI induced early atherosclerosis in LDLR<sup>-/-</sup> mice.<sup>284, 285</sup> AGEs are formed in diabetic patients by constant protein exposure to high glucose concentrations<sup>286, 287</sup>, but also in normoglycemic rabbits in association with lipid oxidation.<sup>288</sup> They were found in atherosclerotic plaques of these rabbits, and are able to induce the formation of specific antibodies.<sup>288, 289</sup>

## The immunomodulatory role of macrophages and T cells in atherosclerosis

### Presence of activated immune cells in lesions

The presence of macrophages and T cells in atherosclerotic lesions has been well documented by immunohistochemical and morphological studies.<sup>290-294</sup> Both CD4+ and CD8+ T lymphocytes were detected in the lesions.<sup>295</sup> Two-thirds of all CD3+ cells in human plaques and 90% of T cells in apoE<sup>-/-</sup> mouse plaques are CD3+ CD4+ αβ T cells.<sup>296</sup> The population of T lymphocytes in the lesions is not uniform, indicated by the extremely diverse repertoire of TCRs, suggestive for a polyclonal expansion of T cells in response to multiple antigens present in the plaques.<sup>297-300</sup> Four out of twenty-seven T cell clones extracted from human atherosclerotic plaques were found to proliferate in response to oxLDL presented by autologous macrophages in a MHC class II dependent manner. Even if they were specific for the same antigen, these clones were not identical, three of them secreting IFNγ and one IL-4, cytokines specific for the Th1 and Th2 subtypes of T helper cells, respectively. These findings are suggestive for the presence of multiple T cell populations in the lesions, and for the predominance of proinflammatory Th1 cells.<sup>301</sup> B cells are rare in atherosclerotic lesions<sup>302, 303</sup>, but are present in the adventitia, in close vicinity to the plaques.<sup>304</sup>

The majority of the T lymphocytes present in human plaques are activated, expressing high levels of the HLA-DR (human leukocyte antigen-DR) and VLA-1 (very late activation antigen-1) cell surface proteins<sup>115, 305</sup>, as well as cytokines and cytokine receptors, such as IL-1, IL-6, TNF and IL-1R.<sup>306</sup> IFNγ, the main secreted cytokine of Th1 cells was also detected in the plaques<sup>115</sup>, as well as the presence of IFNγ-induced expression of HLA-DR on the surface of SMCs.<sup>307</sup> As previously presented, the interaction between IL-2 and its receptor IL-2R induces clonal proliferation of T lymphocytes. T cell populations tend to decrease in advanced atherosclerotic lesions<sup>308</sup>, associated with decreased expression of IL-2R.<sup>115, 306</sup> Thus, it appears that the T lymphocytes are mostly important for the progression of atherosclerosis from fatty streak to advanced lesions, since they are not required for disease initiation<sup>309, 310</sup>, and tend to decrease in the advanced stages.

Macrophages and T cells reciprocally activate each other by cell-cell interactions and paracrine mechanisms. Their secreted cytokines act on all the cells involved in the atherosclerotic process (ECs, SMCs, DCs, macrophages, lymphocytes, mast cells) leading to increased LDL oxidation and recruitment of more monocytes and naïve lymphocytes in the intima. They also stimulate SMC migration and secretion of matrix proteins, macrophage secretion of tissue modulatory MMPs and increased antigen

presentation by DCs and macrophages. These processes generate and maintain an inflammatory state in the lesions during all stages of the disease.<sup>128</sup>

## **Macrophages**

The initiation of atherosclerosis represents a response of the innate immune system to the accumulation and modification of lipoproteins in the arterial intima.<sup>128</sup> The macrophages, central cells of the innate immune responses in atherosclerosis, interact with the lipids that enter the artery wall. Considering their role in all the stages of the disease, as scavenger, secretory and antigen presenting cells, the macrophages are probably the most important cells in atherosclerosis. OxLDL is taken up by the macrophages, processed and presented in association with MHC molecules to T cells. Additionally, oxLDL activates macrophages<sup>311, 312</sup> by the activity of inflammatory PAF-like lipids contained in their composition<sup>313, 314</sup>, or by TLR ligation.<sup>37, 315, 316</sup> The proatherogenic role of TLRs in atherosclerosis has raised considerable interest during the past years, in studies showing that the lack of TLR4, or MyD88, a component of its signaling pathway, reduced atherosclerosis in apoE<sup>-/-</sup> mice.<sup>317, 318</sup> TLR2 was also proven to have proatherogenic effects.<sup>319</sup> TLRs are expressed on the surface of macrophages and endothelial cells present in the atherosclerotic lesions<sup>320, 321</sup> and TLR4 appears to be the most important TLR in atherosclerosis, its expression on the surface of macrophages being upregulated by oxLDL.<sup>320</sup> TLR4 is the classic receptor for lipopolysaccharide (LPS), the endotoxin of gram negative bacteria<sup>319</sup>, but also binds mmLDL<sup>37, 316</sup> and Hsp60<sup>322</sup>, thus having the ability to link all these PAMPs to macrophage activation and the development of atherosclerotic lesions.

Under the influence of these ligands or following oxLDL uptake, the activated macrophages produce inflammatory cytokines, such as IL-1<sup>323-325</sup>, TNF $\alpha$ <sup>326, 327</sup>, IL-6<sup>326</sup> and IL-12.<sup>328, 329</sup> Lack of IL-1 and inhibition of TNF $\alpha$  activity lead to decreased disease activity and progression<sup>330-332</sup>, suggesting the role of these cytokines as potent proatherogenic mediators. IL-1 and TNF $\alpha$  increase endothelial expression of adhesion molecules, MMP secretion in the macrophages and regulate SMC proliferation and IL-6 secretion.<sup>296, 333, 334</sup> Their pleiotropic inflammatory effects are not limited to the arterial wall environment, but also include systemic inflammatory responses and metabolic disturbances.<sup>335, 336</sup> IL-6 released in the circulation stimulates the liver to produce large amounts of acute-phase proteins, such as CRP, serum amyloid A (SAA) or fibrinogen.<sup>337</sup> These are used in the clinical practice as markers of systemic inflammatory activation. CRP has been shown to represent an independent marker for increased cardiovascular risk<sup>338</sup> and to have a direct proinflammatory effect on endothelial cells.<sup>339</sup>

## **Cross-talk between macrophages and T lymphocytes in atherosclerosis – the role of CD40/CD40L interaction**

The cellular immune responses in atherosclerosis are driven by a complex cross-talk between macrophages and T lymphocytes, mediated by paracrine mechanisms and cell-to-cell interactions. Electron-microscopic examination of atherosclerotic lesions demonstrated the direct apposition of lymphocytes to macrophages or macrophage foam cells.<sup>306</sup> The macrophage membrane molecules responsible for cell-to-cell communication between these two cell-types are B7-1/2 and CD40, which bind CD28 and CD40L on lymphocytes, respectively. Additionally, during antigen presentation, TCRs bind to the MHC-peptide complexes on the surface of macrophages.

The macrophages take up antigen and function as professional APCs in relation to T lymphocytes.<sup>138</sup> They are able to provide the second signal necessary for T cell activation, in addition to antigen presentation, by cytokine secretion and co-stimulatory molecules. IL-12 stimulates the differentiation of T lymphocytes into the Th1 subtype of T helper cells and was shown to be secreted by macrophages in the atherosclerotic plaque and to co-localize with the co-stimulatory molecules B7-1 and B7-2.<sup>328, 329</sup> Daily administration of IL-12 to ApoE<sup>-/-</sup> mice lead to increased titers of IgG2a (Th1 dependent antibodies) and accelerated atherosclerosis, indicating an atherogenic role of these mechanisms.<sup>328</sup> Macrophage ability to activate adaptive cellular immune responses functions as a link between innate and adaptive immunity in the pathogenesis of atherosclerosis.

In response to activation, T cells secrete IFN $\gamma$  and express CD40L on their surface. Binding of IFN $\gamma$  to its receptor and the CD40-CD40L interaction provides a double signal for further macrophage activation and increased expression of IL-12, MHC and B7 molecules. Thus, Th1 cells stimulate macrophages to become better APCs and to secrete more IL-12, leading to enhanced T cell differentiation and IFN $\gamma$  secretion.<sup>340-342</sup> CD40 and its ligand are present not only on macrophages and T cells, but also on ECs, SMCs and platelets, having a key role in the activation of inflammatory mechanisms implicated in atherosclerosis and its complications.<sup>207, 341, 343-346</sup> CD40-CD40L interaction has potent atherogenic effects, as demonstrated by studies in atherosclerosis prone mice. Blocking of CD40L (CD154) by antibody treatment or by genetic disruption decreased lesion formation by up to 60% and reduced the amount of lipids, macrophages and T lymphocytes in the plaques, leading to a more stable plaque phenotype.<sup>347-350</sup>

## **Different T lymphocyte subsets in atherosclerosis**

Several subtypes of T lymphocytes were demonstrated to be involved in atherosclerosis, including Th1, Th2, CD8+ T cells, NKT and Treg. The main T helper lymphocyte subsets, Th1 and Th2 have opposite effects and counteract each other's activity. IFN $\gamma$  and TNF $\alpha$ , cytokines secreted by Th1 cells, induce cellular immune responses, promoting lymphocyte recruitment, macrophage activation, inflammation and atherosclerosis. The Th2 cells secrete IL-5 and IL-10. IL-5 mediates antibody production and IL-10 is anti-inflammatory and anti-atherogenic by inhibiting Th1 activation.<sup>351</sup> The immune responses in the atherosclerotic plaque are modulated by the inhibitory cytokines IL-10 and TGF $\beta$ , secreted by regulatory T cells (Treg), which constitute 5-10% of peripheral CD4+ cells in mice and have also been found in the lesions.<sup>352</sup> Activation of CD8+ and NKT cells by antigen presentation accelerated atherosclerosis in mice<sup>353-355</sup>, indicating an atherogenic role of these cells, partly explained by additional *in vitro* studies showing that activated NKT cells are able to secrete IFN $\gamma$ .<sup>353</sup>

Studies on immunodeficient mice indicated a pronounced pro-atherogenic role of CD4+ cells. Crossing of atherosclerosis prone apoE<sup>-/-</sup> mice with *scid/scid* mice, lacking T and B cells<sup>356</sup>, or with CD4+ deficient mice (CD4/apoE dKO)<sup>357</sup> led to a 70% decrease in the extent of atherosclerosis. When the apoE<sup>-/-</sup> *scid/scid* mice received immunocompetent CD4+ cells from older apoE<sup>-/-</sup> mice, they developed lesions of the same size as the apoE<sup>-/-</sup> controls.<sup>356</sup> The circulating levels of IFN $\gamma$  were increased in these mice and the injected cells infiltrated the lesions. Thus, the net effect of the adaptive immune responses seems to be proatherogenic, due to the predominance of Th1 cells in the atherosclerotic intima over the other antiatherogenic lymphocyte subsets. Several studies demonstrated a predominance of IL-12 and IFN $\gamma$  over the Th2 cytokines IL-4 and IL-5, both in advanced human atherosclerotic lesions<sup>358, 359</sup> and in plaques collected from apoE<sup>-/-</sup> mice.<sup>328</sup> IL-1, IL-8 and M-CSF, which stimulate monocyte recruitment and activation, were also detected in the majority of the plaques, whereas IL-4 and IL-5 were rarely observed. Notably, significant amounts of TGF $\beta$  were found in almost all plaques, which is suggestive for a concomitant activation of immunosuppressive atheroprotective mechanisms.<sup>358</sup>

Further evidence supporting the major proatherogenic role of Th1 cells was provided by a recent study in LDLR<sup>-/-</sup> mice lacking T-bet, a transcription factor required for Th1 cell differentiation. Similar to the CD4/apoE dKO mice, the extent of atherosclerosis in these mice was reduced by almost 70% compared with the LDLR<sup>-/-</sup> control group.<sup>360</sup> IL-12 and IL-18 are cytokines required for Th1 cell differentiation and IFN $\gamma$  secretion. Generally, all studies in which Th1 differentiation was inhibited, either pharmacologically<sup>361</sup> or by knocking out the genes for IL-12 or IL-18<sup>362, 363</sup>, led to a decrease in plaque area, whereas treatments with either of these cytokines

enhanced atherosclerosis.<sup>328, 364</sup> These studies also show that both IL-12 and IL-18 are required for a complete Th1 response. IFN $\gamma$  and TNF $\alpha$  have pleiotropic proinflammatory and plaque destabilizing effects, by promoting leukocyte adhesion, macrophage activation, cytokine and MMP secretion and inhibiting collagen production and cholesterol efflux.<sup>296</sup> Expectedly, atherosclerosis is inhibited to a large extent in mice lacking these cytokines or their receptors.<sup>95, 96, 331, 365, 366</sup>

As previously discussed, the prevalence of Th2 cells and their cytokines in atherosclerotic plaques is reduced compared to Th1. It is possible that they are under constant suppression by the Th1 cytokines, mainly IFN $\gamma$ . Several studies in which Th1 differentiation and activity was inhibited detected a concomitant switch towards a Th2 mediated immune response, assessed by an increase in IL-10 secreting cells in the spleen<sup>361</sup> or increased titers of Th2 specific IgG1 antibodies.<sup>360</sup> Lee et al. demonstrated a relative decrease in IgG2a antibody titers in 6 month old mice compared with 3 month old mice, associated with the appearance of IL-10 mRNA in the lesions, which was absent after 3 months.<sup>328</sup> These studies suggest that a similar switch may be occurring naturally *in vivo* in atherosclerosis-prone mice, in the absence of any other intervention. The Th1/Th2 switch was also evident under severe hypercholesterolemic conditions, in apoE<sup>-/-</sup> mice fed a high cholesterol diet. These mice presented much higher IgG1 titers to MDA-LDL than IgG2a. IL-4 secreting T cells were also predominant in the spleen of these animals, and IL-4 mRNA could be detected in their lesions.<sup>367</sup>

The cytokines secreted by Th2 lymphocytes (IL-4, IL-5, IL-10, IL-13) are predominantly, but not exclusively, anti-atherogenic and are mostly associated with humoral immune responses.<sup>212</sup> IL-10 is a so called “cytokine secretion inhibiting cytokine”<sup>351</sup> with immunosuppressor activity, also secreted by Treg<sup>368</sup>, NK cells, macrophages and DCs. It is a potent inhibitor of IL-12<sup>359</sup> and IFN $\gamma$ <sup>369</sup> secretion from T cells and macrophages, and numerous studies have either directly or indirectly demonstrated its atheroprotective role.<sup>370-373</sup> IL-5 was shown to stimulate B1 cells to produce natural IgM antibodies to oxLDL epitopes, which have a protective role against atherosclerosis development. This effect was abrogated in IL-5 deficient mice and was associated with increased plaque area.<sup>374</sup> In contrast, IL-4 is considered to be atherogenic, as disease progression was reduced in both apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice lacking this cytokine.<sup>363, 375</sup> IL-4 up-regulates CD36<sup>376</sup>, induces mast cell degranulation and MMP secretion. The enzymes released by mast cells contribute to increased plaque instability, and the MMPs may be linked to the role of Th2 cells in aortic aneurisms.<sup>377</sup> IL-4 and IL-13 also stimulate antibody production in B cells.

Th1 and Th2 lymphocytes have crossregulatory roles. IFN $\gamma$  and IL-12 inhibit the Th2 pathway and the secretion of IL-4 and IL-10, whereas IL-10 inhibits antigen-dependent activation of Th1 cells and IFN $\gamma$  production.<sup>128, 378</sup> As previously discussed, the balance

between the proatherogenic Th1 and the atheroprotective Th2 lymphocyte subsets has a crucial influence on the development of atherosclerosis, and tilting the balance towards Th2 mediated atheroprotective humoral immune responses may represent a future therapeutic target for the treatment of CVD.

The regulatory T cells (Treg) are a particular CD4<sup>+</sup> T lymphocyte subpopulation with potent immunosuppressive effects, which is important in self-tolerance and the inhibition of autoimmunity.<sup>215</sup> The natural Treg cells, which are present in the blood at all times, characteristically express the IL-2 receptor  $\alpha$  chain (CD25) on their surface and the Foxp3 transcription factor, which is essential for their development.<sup>379</sup> These CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T lymphocytes are able to inhibit the activation of both Th1<sup>380</sup> and Th2 cells<sup>381</sup> through cell-cell contact<sup>382</sup> or TGF $\beta$  secretion.<sup>383</sup> Certain CD4<sup>+</sup>CD25<sup>-</sup>Treg subpopulations have also been shown to participate in adaptive antigen-specific immune responses.<sup>384</sup> Depending on their secreted cytokines, two of these subpopulations were differentiated: the type 1 Treg cells (Tr1) which produce high amounts of IL-10 and were shown to reduce atherosclerosis development<sup>385, 386</sup>, and the Th3 cells, which mainly secrete TGF $\beta$ .<sup>352</sup> TGF $\beta$  is a potent immunosuppressive cytokine secreted by Treg cells, as well as by macrophages, EC, SMC, DC and platelets. Its main role is to prevent excessive activation of the immune system. Mice lacking TGF $\beta$  presented massive inflammation and early death by 3-4 weeks of age.<sup>387</sup> Inhibition of the TGF $\beta$  signaling pathway, either by antibody blockade<sup>388</sup> or by specific genetic deletion of the TGF $\beta$  receptor type II in T cells of apoE<sup>-/-</sup> mice<sup>389, 390</sup>, led to a dramatic increase of up to 6 fold in plaque area and generation of an unstable plaque phenotype. These studies suggest that under normal conditions, TGF $\beta$  dampens the atherogenic pro-inflammatory T cell immune responses.

## **Atheroprotective immunity in atherosclerosis – the role of B cells.**

Despite the atheroprotective roles of Th2 and Treg cells, cellular immune responses are mostly pro-atherogenic. In contrast, several studies have demonstrated an atheroprotective role for B cells and antibodies, the effectors of humoral immunity. Atherosclerosis-prone apoE<sup>-/-</sup> or LDLR<sup>-/-</sup> mice lacking mature B cells, as a consequence of either splenectomy<sup>391</sup> or genetic manipulations<sup>392</sup>, present a dramatic increase in lesion size and a significant depletion of total serum antibodies. Transfer of mature B cells isolated from the spleens of fully immunocompetent apoE<sup>-/-</sup> control animals was shown to rescue this effect. These cells produced high amounts of oxLDL antibodies and also inhibited atherosclerosis progression in non-splenectomized apoE<sup>-/-</sup> mice.<sup>391</sup> B



cell transfer had similar effects on neointima formation following arterial injury in immunodeficient Rag-1 mice.<sup>393</sup>

The pro-atherogenic role of oxLDL and its involvement in all stages of the disease is well established and unanimously accepted. Considering this, it was hypothesized that injection of oxLDL in pro-atherogenic animals would lead to accelerated atherogenesis and increased plaque formation. Surprisingly, pioneering studies by Palinski et al.<sup>394</sup> and Ameli et al.<sup>395</sup> demonstrated the exact opposite effects, following immunization with homologous MDA-LDL in rabbits. Shortly thereafter, their results were confirmed by several other groups, which demonstrated an atheroprotective role of immunization with both oxLDL and native LDL on atherosclerosis development or neointima formation after balloon injury in hyperlipidemic rabbits<sup>396</sup> and mice.<sup>397-399</sup> In the majority of these studies the atheroprotective immunization was associated with the generation of high antibody titers against oxLDL epitopes. These antibodies were mostly IgM, IgG1 and IgG2a generated both in a T-dependent (TD) and in a T-independent manner (TI). Indeed, it was shown that MDA-LDL immunization can also elicit antibody responses and inhibit atherosclerosis in mice completely lacking CD4+ T lymphocytes.<sup>357</sup>

The existence of antibody-mediated atheroprotective immune responses was further confirmed by other studies in which the reduction of atherosclerosis was directly achieved by intravenous treatment with polyclonal immunoglobulins (IVIg).<sup>400-403</sup> These effects seemed to be mediated by the Fc fragment of the antibodies<sup>403</sup>, and required the presence of an intact complement system.<sup>400</sup>

Both the oxidized phospholipid and the proteic oxLDL epitopes were shown to trigger immune responses in hypercholesterolemic animals, with or without immunization. Immunization of LDLR<sup>-/-</sup> mice with *Streptococcus Pneumoniae*, which shares the same PC-containing epitopes on its surface as the oxidized phospholipids, induced high titers of natural T15 IgM antibodies secreted by B1 cells in the spleen, and reduced atherosclerosis.<sup>52, 251</sup> These antibodies, components of innate immunity, are naturally present at all times in plasma and are believed to have an atheroprotective role.<sup>52, 54, 251</sup> Interestingly, the T15 antibodies were also shown to be involved in atheroprotection following MDA-LDL immunization, through a mechanism involving IL-5 and the activation of B1 cells by Th2 lymphocytes.<sup>374</sup>

MDA-LDL immunization triggers both T cell-dependent and T cell-independent antibody production in relation to atheroprotection.<sup>357, 399</sup> Nevertheless, the apoB-100 molecule contains a large number of lysine residues able to bind MDA and can generate multiple fragments of different lengths and conformations upon oxidation. Therefore, in the perspective of using these atheroprotective immune responses against MDA-LDL as possible therapeutic strategies against atherosclerosis and atherosclerosis related diseases, it was necessary to define the exact immunogenic epitopes on

MDA-LDL. Using a peptide library covering the complete sequence of apoB-100, Fredrikson et al. found over 100 different epitopes recognized by IgM or IgG antibodies in human plasma.<sup>53</sup> Immunization of apoE<sup>-/-</sup> mice with some of these native or MDA-modified peptide sequences induced a 50-fold increase in Th2-specific IgG1 levels<sup>404</sup> and reduced atherosclerosis by up to 60%.<sup>404-406</sup> These effects were abolished in splenectomized mice and splenocyte transfer from immunized to non-immunized mice conveyed atheroprotection to these animals.<sup>406</sup> Thus, although the exact mechanisms involved are still unclear, they seem to be dependent on antibody production in the spleen.

In conclusion, atherosclerosis is a multifactorial chronic inflammatory disease initiated by the accumulation and oxidation of LDL in the intima. The innate and the adaptive immune systems have important roles in modulating disease activity and progression. Several lines of evidence demonstrated the existence of atheroprotective immune responses which appear to be antibody-mediated and selective activation of such immune mechanisms could represent a future therapeutic approach for prevention and treatment of atherosclerosis-related cardiovascular diseases. Nevertheless, the exact epitopes and effector mechanisms involved need to be thoroughly characterized before these methods can be used in clinical practice. The purpose of this thesis is to investigate the effects of a passive immunization strategy with antibodies directed against MDA-modified peptidic oxLDL epitopes on atherosclerosis development and plaque composition in atherosclerosis-prone mice and to determine the value of these antibodies as markers for disease progression in humans.



## **AIMS**

The aims of the present studies were:

- To assess the effects of passive immunization with recombinant human IgG1 antibodies specific for MDA-modified amino acid sequences in apoB-100 on the development of atherosclerosis in mice
- To explore the mechanisms involved in the putative protective effects of these antibodies against atherosclerosis
- To determine if recombinant human IgG1 antibodies specific for MDA-modified amino acid sequences in apoB-100 are able to induce regression of advanced atherosclerotic plaques
- To study the influence of these antibodies on arterial response to injury in mice
- To investigate if treatment with recombinant human IgG1 antibodies specific for MDA-modified peptide sequences of apoB-100 can induce a more stable plaque phenotype in mice
- To determine if plasma levels of the corresponding autoantibodies are associated with the severity of atherosclerosis and risk for development of acute coronary events in humans



## **METHODS**

We have previously detected over 100 different MDA-modified peptide epitopes recognized by antibodies in human plasma, by using a peptide library covering the complete amino acid sequence of human apoB-100.<sup>53</sup> The library contains 302 peptides which are 20 amino acids (aa) long and were synthesized with a 5 aa overlap. They are numbered 1-302 starting from the N-terminal end of the protein. The immune responses against 2 of these peptide sequences seem to have a particularly important role in atherosclerosis and cardiovascular disease. High levels of IgG against MDA-p45 (aa 661-680: IEIGL EGKGF EPTLE ALFGK) were detected in coronary heart disease patients and high IgM and IgG levels against MDA-p210 (aa 3136-3155: KTTKQ SFDLS VKAQY KKNKH) were present in healthy controls.<sup>53</sup> Additionally, active immunization with these peptide sequences, alone or in combination with other apoB-100 peptides, reduced atherosclerosis in apoE<sup>-/-</sup> mice.<sup>404, 405</sup> We therefore decided to synthesize recombinant human IgG1 antibodies against MDA-p45 and MDA-p210 and test their effects on atherosclerosis in different mouse models.

### **Generation and testing of antibodies**

Single-chain human antibody fragments specific for MDA-p45 and MDA-p210 were selected from the single-chain fragment-variable (scFv) n-CoDeR phage display library.<sup>407</sup> In brief, the complete phage library was selected on MDA-modified peptides presented in immunotubes. To minimize the number of unspecific binders, the phage pool was both preselected and exposed to a competitor antigen. An MDA-modified non-target peptide bound to immunotubes was used for preselection and the native (non-modified) target peptide or the MDA-modified non-target peptide were used in solution as competitors (Table 4). In this way a more narrow type of specific binders could be selected. The first round of selection did not include any preselection or competitor. Bound phages were eluted and amplified through infections of *E. Coli*, and the expanded phage pools were used for a new round of selection. We performed three rounds of selections. After the last round of selection DNA plasmids containing the sequence for the scFv antibodies, together with the c-myc and the 6xHis tags, were isolated from the phages and transformed into *E coli*. The scFv antibodies produced by the different *E coli* clones were used in the screening process.

**Table 4.** Phage selection

	Phage input	Pre-selection	Selection /Immunotube	Competition
<b>1st round of selection</b>				
1	n-CoDeR (complete library)	None	MDA-p45	None
2	n-CoDeR	None	MDA-p210	None
<b>2nd round of selection</b>				
1	Amplified phage from p45	MDA-p210	MDA-p45	p45
2	Amplified phage from p210	MDA-p45	MDA-p210	p210
<b>3rd round of selection</b>				
1	Eluate from p45	None	MDA-p45	p45 and MDA-p2
2	Eluate from p210	None	MDA-p210	p210 and MDA-p301

The primary screening was performed using an automated robotic system<sup>408</sup>, as a large number of clones were tested against target and non target peptides. The detection was performed by luminescence ELISA using an anti-c-myc antibody as primary antibody (Boehringer Mannheim, Germany) and alkaline phosphatase-conjugated anti-mouse secondary antibody (Applied Biosystems, USA). The scFv clones that scored positive in the primary screening (i.e. binding to MDA-modified peptide and no binding to native peptide) were further analyzed in the secondary screening, which was performed manually. Each clone was tested against four different reagents: MDA-modified target peptide, native peptide (non target), MDA-modified LDL (target) and native LDL (non target). The scFv were detected with a primary mouse anti-6xHis antibody (R&D systems, USA), and an alkaline phosphatase conjugated anti-mouse antibody secondary antibody (Applied Biosystems, USA) followed by chemiluminescence detection. In screening III, positive scFv clones from screen II were titrated against MDA modified LDL (target) and native LDL (non-target). Clones that did not show a dose response-like curve against target and low background signal were excluded.

Finally, the positive clones from screening III were further investigated by DNA sequencing and the identical clones were excluded. A total of 6 scFv clones, 4 specific for MDA-p45 and 2 for MDA-p210 were chosen to be converted to full-length IgG1 $\lambda$  format. Based on the first 3 amino acids of the peptide, the antibodies against

MDA-p45 and MDA-p210 were denominated IEI and KTT, respectively. Table 1 in paper I contains the different CDR sequences of the 6 antibodies. The scFv antibody fragments were transformed into IgG1 $\lambda$  format through cloning into a modified pcDNA3 vector.<sup>409</sup> The VH and VL fragments were digested and ligated into 2 different vectors, containing the  $\gamma$ 1 genomic constant region and the  $\lambda$  genomic constant region, respectively. The ligations were transformed into *E. coli* Top10 bacteria and the plasmids encoding for the heavy and light chain were prepared in small scale from one clone for each construct. These plasmids were sequenced in order to confirm the sequences of the converted clones. Heavy and light chain plasmids originating from the same scFv were digested, ligated and transformed into *E. coli* Top10 bacteria. Correctly ligated vectors were identified using colony PCR. Joined plasmids were prepared in large scale in order to obtain the amount of DNA required for stable transfection.

The day before transfection, 12-15 million NS0 cells were seeded into T-75 cell culture flasks, in DMEM (Dulbecco's Modified Eagle's Medium with Glutamax-I) supplemented with 10% Fetal Bovine Serum (FBS) and 1x non-essential amino acids. Linearized constructs were transfected into NS0 cells using Lipofectamine 2000 reagent (Invitrogen, Sweden). We used 40  $\mu$ g of linearized DNA for every T-75 flask with NS0 cells. The media was supplemented with 600 $\mu$ g/ml G418 sulfate (Invitrogen, Sweden) to select for stably transfected cells.<sup>409</sup> The cells producing high levels of antibodies were selected and grown in the same medium as above. Human IgG1 was purified from spent cultivation medium on a MabSelect Protein A column (Amersham Biosciences). The purity of the preparations exceeded 98% as determined from polyacrylamide gel electrophoresis analysis, and contained between 1 and 12 EU/mL endotoxin, as tested by a LAL-test (QCL-1000<sup>R</sup>, BioWhittaker).

The specificity of the purified ScFv and IgGs for MDA-LDL and MDA-peptides was demonstrated using a luminescence based ELISA. Antigens were coated to high binding luminescence 96 well test plates. Bound ScFv and IgG were detected using HRP-conjugated mouse anti-his (Man150, R & D) and rabbit anti-human IgG (gamma-chain) antibodies (P0214, DAKO, Denmark) respectively. The affinity of the IgG1 antibodies for human MDA-apoB-100 was measured in a Biacore system. Human MDA-modified apoB-100 (Academy Bio-Medical Co., USA) was immobilized to a total signal of 7000 RU using amino-coupling on a CM5 chip in a Biacore 3000 (Biacore, Sweden). Native human apoB-100 was used as a reference. Five different concentrations (100, 25, 6.25, 1.56, 0.39 nM) of each antibody were injected consecutively on the chip. The resulting binding curves were analyzed using the BiaEvaluation software (Biacore). Between each run the chip was regenerated with 10 mM NaOH.



## Mouse models of atherosclerosis

The effect of the IgG1 antibodies on atherosclerosis was studied in three different mouse models: apoE<sup>-/-</sup> mice, *apobec-1*<sup>-/-</sup>/LDLR<sup>-/-</sup> mice and LDLR<sup>-/-</sup>/human apoB-100<sup>+/-</sup> mice.

The apoE<sup>-/-</sup> mouse model was simultaneously created in 1992 in two different laboratories, by targeted gene inactivation.<sup>410,411</sup> Apolipoprotein E is a glycoprotein synthesized mainly in the liver and is a constituent of all lipoproteins, except LDL. ApoE has an important role as a ligand for the receptors which clear chylomicrons and VLDL remnants from the circulation. The impaired clearance of these lipid fractions in apoE<sup>-/-</sup> mice leads to a dramatic increase of plasma cholesterol levels up to 400-600 mg/dL even on a low-fat chow diet. Following these metabolic disturbances, the mice develop progressive atherosclerotic lesions ranging from fatty streaks to advanced fibrofatty plaques throughout their arterial tree. On a Western-type high fat diet (0.15% cholesterol and 21% fat), the cholesterol levels are 3-4 times higher and the progression of atherosclerosis is exacerbated.<sup>412,413</sup> The phenotype of these animals differs markedly from the wild type mice, which have a total serum cholesterol level of 85 mg/dL, mostly carried in the HDL particles, and do not develop atherosclerotic lesions. The morphology and the location of the plaques in apoE<sup>-/-</sup> mice mimic human atherosclerosis. The foam cell clusters characteristic for fatty streaks appear at 10 weeks of age, intermediate SMC containing lesions at 15 weeks, and the fibrofatty plaques are already present at 20 weeks of age. The plaques are mainly located in the aortic sinus or subvalvular area, the aortic arch, the branching points of the carotid, intercostal, renal, mesenteric and iliac arteries and the proximal segments of these branches.<sup>414</sup> The first lesions appear proximally and progress distally with age, so in general the subvalvular plaques are more advanced than the plaques found in the descending aorta.<sup>415</sup>

In study I we began the antibody treatment at 21 weeks of age, and assessed the extent of atherosclerosis in the descending aorta 4 weeks later. We used male apoE<sup>-/-</sup> mice on a C57BL/6 background purchased from B&M, Denmark. The mice were fed a Western diet (Lactamin AB, Sweden) provided *ad libitum* from 6 weeks of age and the average cholesterol levels at sacrifice in the different groups ranged between 770 and 1420 mg/dL (Paper I, table 3). We included 7 groups of 9 mice in the first experiment and 7 groups of 10 mice in the second experiment.

In the second study we used male *apobec-1*<sup>-/-</sup>/LDLR<sup>-/-</sup> mice on a C57BL/6 background produced by the Jackson Laboratories, USA. The binding of apoB in the LDL particles and of apoE in the IDL particles to the LDL receptor is the most important removal mechanism of these lipoproteins from the circulation. On a low-fat diet, the mice lacking LDLR present a 2-fold increase of total cholesterol in plasma, determined by substantially elevated LDL and IDL levels. These metabolic changes are not

sufficient to induce atherosclerosis in these animals. A high fat Western-type diet induces cholesterol levels of up to 1200 mg/dL in LDLR<sup>-/-</sup> mice already after one month and these values remain constant over time<sup>50</sup>, leading to the development of extensive atherosclerotic lesions. The lesions consist mainly of fatty streaks or advanced plaques characterized by a necrotic core capped with foam cells and are present at the same locations as the atherosclerotic plaques in apoE<sup>-/-</sup> mice.<sup>416</sup> Apobec-1 is a cytidine deaminase which binds apoB-100 mRNA and truncates the full-length apoB-100 to form apoB-48, by a site-specific cytidine-to-uridine editing reaction. *Apobec-1*<sup>-/-</sup> mice are viable and express elevated levels of apoB-100, while lacking apoB48, due to a mutation in the enzyme. There is no difference in total plasma cholesterol between these mice and wild-type mice, but the HDL lipoprotein fraction is significantly decreased in the *apobec-1*<sup>-/-</sup> strain.<sup>417</sup> The relative resistance of LDLR<sup>-/-</sup> mice to atherosclerosis was attributed to the presence of apoB-48 in these animals, which is able to mediate lipoprotein clearance from the plasma by mechanisms other than LDLR linkage.<sup>418</sup> These mechanisms are eliminated by cross-breeding the LDLR<sup>-/-</sup> mice with the *apobec-1*<sup>-/-</sup> strain. Plasma cholesterol levels are similar in the *apobec-1*<sup>-/-</sup>/LDLR<sup>-/-</sup> and the apoE<sup>-/-</sup> mice fed the high-fat Western diet. By changing the diet to a normal chow, the concentration of cholesterol in plasma dropped significantly from 967 mg/dL to 367 mg/dL during only one week (Paper II, table 2).

Considering that the IgG1 antibodies that we intended to test recognize oxLDL epitopes, we decided to use *apobec-1*<sup>-/-</sup>/LDLR<sup>-/-</sup> mice in the second study because the LDL lipoproteins are the atherogenic stimuli in these animals, compared to VLDL and chylomicrons in the apoE<sup>-/-</sup> strain. Additionally, the presence of apoB-100 in the LDL particles offer a wider array of potential ligands for the antibodies than apoB-48. These mice also have a functional apoE protein. ApoE has an important role in reverse cholesterol transport, one of the potential mechanisms of plaque regression. Study II included 6 groups of 9-12 male mice fed the high-fat Western diet from 4 weeks of age. One week before the immunizations the diet was changed to chow and the immunizations were started at 25 weeks of age, when the mice already presented advanced lesions, as demonstrated in the 25-weeks baseline group. Similar to the first study, the extent of atherosclerosis was measured in the descending aorta at 4 weeks after the beginning of the treatment.

LDLR<sup>-/-</sup> mice expressing human apoB-100 on a C57BL/6 background were kindly provided by professor Jan Borén from Gothenburg University, Sweden, and used in the third study. These mice have previously been described by Sanan et al.<sup>418</sup> Compared with the LDLR<sup>-/-</sup> mice, the LDLR<sup>-/-</sup>/human apoB-100<sup>+/+</sup> mice kept on a chow diet presented a more pro-atherogenic lipid profile, characterized by a 2.6 fold increase in plasma cholesterol, 5-fold higher triglyceride plasma levels, a dramatic increase in LDL-cholesterol and a concomitant drop in HDL-cholesterol. The athero-

sclerotic lesion area in these mice at 6 months of age was several fold higher than in the LDLR<sup>-/-</sup> mice fed a normal chow.

Besides the advantages of using an LDLR<sup>-/-</sup> mouse strain, the epitopes on the human apoB-100 expressed by the LDLR<sup>-/-</sup>/human apoB-100<sup>+/-</sup> mice present complete homology with the sequences recognized by our antibodies. Two groups of 9-11 female mice fed low-fat chow were used in study III to assess the effects of antibody treatment on neointima formation after carotid injury and on the extent of atherosclerosis in the uninjured contralateral carotid artery. The first round of injections and the carotid injury were performed when the mice were 21 weeks old and the animals were sacrificed at 24 weeks of age.

## Immunization strategies

Depending on the purpose of each study, the mice were immunized with different amounts of antibodies and at different ages. In all cases, the antibodies were diluted in 500 µL of sterile PBS and administered intraperitoneally (i.p.). In the first 2 studies we injected 3 antibody doses at one-week intervals and sacrificed the mice 2 weeks after the last injection. In study III, the mice received 4 immunizations and were sacrificed 8 days after the last immunization, 21 days after carotid injury. PBS and an IgG1 antibody specific for fluorescein isothiocyanate (FITC-8) were used as controls. FITC-8 did not present any binding activity to the native or MDA-modified forms of the peptides, apoB-100 or LDL. In study II, the Western diet was changed to chow at 24 weeks of age in all groups except for a 20-week control group fed high-fat diet until sacrificed. Two additional untreated control groups were added in this study: a 25 week old baseline control group, sacrificed at the beginning of the antibody treatment and a 29 week old control kept on normal chow from 24 weeks of age and sacrificed at the same time as the antibody treated groups. The experimental protocol used in all the studies was approved by the Animal Care and Use Committee of Lund University. The design of the different studies is outlined in table 5.

## Surgical interventions and tissue preparation

All mice were sacrificed by exsanguination through cardiac puncture under anesthesia with 300 µL of distilled water, fentanyl/fluanisone and midazolam (2:1:1, vol/vol/vol), administered intraperitoneally. The arterial tree was washed with PBS and the tissues were fixed by a 10-minute perfusion with Histochoice (Amresco, USA). The descending aorta was dissected free of external fat and connective tissue, cut longitudinally and mounted *en-face* lumen side-up on ovalbumin- (Sigma, USA) coated slides (termed

**Table 5.** Study design

Features	Study Ia	Study Ib	Study II	Study III
<b>Strain</b>	apoE <sup>-/-</sup>	apoE <sup>-/-</sup>	<i>Apobec-1</i> <sup>-/-</sup> /LDLR <sup>-/-</sup>	LDLR <sup>-/-</sup> /human apoB-100 <sup>+/-</sup>
<b>Sex</b>	Males	Males	Males	Females
<b>Number</b>	7 gr x 9 m	7 gr x 10 m	6 gr x 9-12 m	2 gr x 9-11 m
<b>Diet</b>	Western	Western	Western changed to chow at 24w	Low-fat chow
<b>Antibodies</b>	IEI-A8,D8,E3,G8 KTT-B8,D6	IEI-E3	IEI-E3 2D03	2D03
<b>Controls</b>	PBS	PBS, FITC-8	FITC-8	FITC-8
<b>Additional controls</b>			20 w, 25 w baseline, 29 w	
<b>Dose</b>	0.5 mg	0.25, 0.5, 2 mg	1mg	0.2 mg
<b>Antibody treatment</b>	21, 22, 23w	21, 22, 23w	25, 26, 27w	21w-1d before i 3, 6, 13d after i
<b>Sacrifice</b>	25w	25w	29w	24w, 21d after i

Abbreviations: gr = group; m = mice; w = week; d = day; i = injury

flat preparation).<sup>419</sup> The external fat has to be carefully and completely removed, because it stains with the same dye as the intra-plaque lipids and can interfere with plaque area measurements. The slides were stored in Histochoice at 4° until analysis. The heart and the aortic arch with the attached innominate artery were also removed and stored at 4°C in Histochoice. The innominate artery, or the brachiocephalic truncus, is the first artery which originates from the aortic arch and branches into the common carotid artery and the subclavian artery. It irrigates the brain, the right side of the neck and the right upper limb.

In study III, the carotid injury was performed on 21 week old mice under anesthesia with Avertin (0.016 mL/g of 2.5% solution i.p.). The right carotid artery was carefully isolated under a dissecting microscope and non-occlusive plastic collar (length, 3 mm; internal diameter, 0.51 mm; Cole-Parmer Instrument Co, USA) was placed around the vessel. The mice were sacrificed 21 days after the intervention and both carotid arteries were perfusion-fixed with Histochoice, dissected out and stored in Histochoice at 4° until analysis.

The heart and the innominate artery were frozen in OCT (Tissue-Tek, Japan) and sectioned into 10 µm thick sections. The right (injured) and left (uninjured) carotid arteries were embedded in parafin and sliced into 5µm thick sections. Depending on the study, we chose from each mouse 6 sections taken at 30 µm intervals of the aortic sinus and the innominate artery, 10 sections at 200 µm intervals of the injured carotid segment and 4 sections at 100 µm intervals of the uninjured carotid artery for further processing.

## Tissue staining

The *en face* preparations of the descending aorta were washed in distilled water, dipped in 78% methanol and stained for 40 minutes in a 0.16% Oil-Red-O (ICN Biochemicals, USA) solution in 78% methanol/0.2 mol/L NaOH, as described by Brånen et al.<sup>419</sup> Following this procedure, plaque lipids are stained in a dark wine-red color and the adventitial fat is colored in orange, allowing the observer to distinguish between the 2 components. Plaque area was quantified blindly by microscopy and computer aided morphometry using the Image Pro Plus software and the results were expressed as percentage of total area of the aorta.

The sections of the subvalvular plaques and innominate artery chosen for macrophage staining were fixed in 100% ice-cold acetone for 5 minutes. The cells were permeabilized with 0.5% Triton-X in PBS for 5 min, the endogenous peroxidase activity was neutralized with 3% H<sub>2</sub>O<sub>2</sub> in H<sub>2</sub>O for 5 minutes, and the sections were blocked with 10% mouse serum in PBS for 30 minutes. A Rat-anti-Mouse IgG2a MOMA-2 monoclonal antibody (BMA Biomedical, Switzerland) was used as primary antibody (1 µg/mL in 10% rabbit serum in PBS; overnight at 4°C), and a biotinylated Rabbit-anti-Rat IgG mouse (Vector Laboratories, USA) as secondary antibody (7 µg/mL in PBS; 50 min at room temperature). The sections were blocked with 10% rabbit serum in PBS for 30 min before the secondary antibody was added. ABC Elite peroxidase system (Vector Laboratories) and DAB peroxidase substrate (Vector Laboratories) were used for color development and the sections were counterstained with Harry's Hematoxylin (HistoLab AB, Sweden) for 20-30 sec. A similar protocol was used in study I to stain oxLDL epitopes in the subvalvular plaques. IEI-E3 (100 µg/mL) served as primary antibody, and a biotinylated mouse anti-human IgG1 antibody (25 µg/mL; ImmunKemi F&D AB, Sweden) diluted in PBS, as secondary antibody. The macrophage and oxLDL stained areas were quantified blindly by microscopy and computer aided morphometry using the Image Pro Plus software and the results were expressed as percentage of total plaque area.

The carotid artery sections were stained for elastin with accustain elastin stain (Sigma) to visualize the internal elastic lamina (IEL), external elastic lamina (EEL), the lumen and the atherosclerotic lesions. The different areas and circumferences were measured using the image software Zeiss Axiovision (Zeiss). The area of the lesions was calculated by

subtracting the lumen area from the area within the IEL, and the area of the media was calculated as the area between EEL and IEL. The perimeters of the lumen and EEL were measured for comparison as indicators of the size of the vessels. Smooth muscle cells were detected with a monoclonal anti-mouse alpha actin antibody (Sigma) and macrophages with rat anti-mouse Mac-2 (Cedarlanes Laboratories) in combination with appropriate secondary antibodies. The reaction products were visualized with Vectastain ABC elite kit (Vector Laboratories) using DAB as substrate (Vector Laboratories).

## **Analysis of lipids, SAA and antibodies in plasma**

The blood was collected by cardiac puncture in tubes containing 10 $\mu$ L 0.5M EDTA, centrifuged at 4°C and the plasma was frozen at -85 °C until processing. Commercially available kits were used to measure the concentration of total cholesterol (Thermo Electron, Australia), triglycerides (Thermo Electron), serum amyloid A (SAA; Bio-Source, USA) and oxLDL (Mercodia, Sweden). The plasma was diluted 1/8 for the analysis of cholesterol, 1/5 for triglycerides, 1/100 for SAA (1/300 for some samples) and 1/24000 for oxLDL. The concentration of circulating oxLDL was only measured in study III due to technical difficulties. The human apoB-100 transgenic LDLR<sup>-/-</sup> mice carry human apoB-100 in their LDL particles, and we were able to measure plasma oxLDL in these animals using a commercially available ELISA kit specific for human oxLDL (Mercodia, Uppsala, Sweden). There is currently no available kit which is able to detect mouse oxLDL particles in apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice. We are currently trying to develop an ELISA for measuring mouse oxLDL by using our antibodies. However, results will be unavailable before publication of the current work.

The amount of recombinant human IgG1 antibodies and mouse anti-human IgG1 antibodies in plasma at sacrifice were measured by luminescence ELISA. To detect the human antibodies in mouse plasma we used a rabbit anti-human lambda antibody (DAKO, Denmark) as catcher antibody, human IgG1 for standards and controls (G1 std and Human IgG, respectively, Sigma) and a HRP-conjugated rabbit anti-human IgG ( $\gamma$ -chain) as detection antibody. We coated ELISA plates with our recombinant IEL and KTT antibodies and used a HRP-conjugated rabbit anti-mouse Ig to measure mouse anti-human antibodies in the plasma of the immunized mice. Luminescence was developed by Super Signal<sup>®</sup>ELISA Femto Luminol/Enhancer and Super Signal<sup>®</sup>ELISA Femto Stable Peroxide (Pierce, USA) and read in a Victor2V, Perkin Elmer Wallac instrument. In order to determine if sera from atherosclerotic mice contain antibodies with the ability to bind oxidized apoB-100 and block binding of the 2D03 antibody to its target antigen, human MDA-apoB-100 (0.5  $\mu$ g/ml) was coated to test plates and then pre-incubated with increasing concentrations of mouse sera for 1 hour. After washing, the plates were incubated with 3 $\mu$ g/ml 2D03. Bound 2D03 antibody was detected with peroxidase-labeled anti-human IgG1, which does not cross-react with

murine IgG. The same procedure was used to detect the antibodies in human plasma that can compete with 2D03. We pre-incubated the plates with human plasma, followed by washing and incubation with 3 µg/ml of biotinylated 2D03, which was then detected by streptavidine-conjugated peroxidase. The controls were incubated with buffer alone.

## Binding and uptake assays

Human LDL was isolated from healthy donor plasma by sequential preparative ultracentrifugation in a narrow density range (1.034-1.054 kg/L) and incubated for 24 h at 37°C with a sterile solution of 10 µM CuCl<sub>2</sub> in PBS. The extent of LDL oxidation was assessed by comparing the electrophoretic mobility of Cu-oxLDL and native LDL. The electrophoresis was run in 1% agarose gels in barbital buffer (pH 8.6). Native LDL and oxLDL were labeled by the iodine monochloride method. Unbound <sup>125</sup>I was removed by chromatography on Sephadex G-25 columns PD-10 (Pharmacia, Sweden) followed by extensive dialysis against 0.15 mol/L NaCl, 1mmol/L EDTA and 0.03 mol/L KI, and further dialysis against 0.15 mol/L NaCl, 1mmol/L EDTA. The endotoxin levels in both preparations were below 0.015 EU/mL as determined by a limulus amoebocyte lysate test (Charles River Endosafe, USA).

Human monocytes were isolated from buffy coats from different donors using the Ficoll-Hypaque procedure, plated at a density of 4 x 10<sup>6</sup> cells/mL into 12-well plates (1 mL/well), and cultured at 37 °C in 5 % CO<sub>2</sub> in RPMI 1640 (Gibco, Life Technologies, UK). The medium was supplemented with 2 mmol/L N-acetyl-L-alanyl-L-glutamine, 100 U/mL penicillin, 100 µg/mL of streptomycin, 1% non-essential amino acids, 2% sodium pyruvate, and 20 mmol/L Hepes, without serum. The experiments were performed within 24 h after plating.

LDL binding to monocytes was assessed in experiments run at 4°C. At this temperature, the activity of the cells is strongly reduced and the LDL bound to surface receptors is not taken up inside the cell. The cells were washed 3 times with 1mL ice-cold PBS, followed by pre-cooling for 20 min at 4°C in 1mL of ice-cold RPMI medium containing 0.5% HSA. After addition of labeled nLDL (40 µg/mL) or oxLDL (50 µg/mL), alone or combined with the antibodies (100 µg/mL), the cells were incubated for 2 h at 4°C. After the incubation, the cells were washed 3 times with 1 mL ice-cold PBS and new medium containing 10 g/L dextran sulphate (Mw ~ 500 000, Pharmacia Biotech, Sweden) was added. Dextran sulphate is known to release receptor-bound LDL or oxLDL from the surface of the cell.<sup>420</sup> After 1 h incubation at 4°C on a rotatory shaker at 60 rpm, the radioactivity of released <sup>125</sup>I-nativeLDL or <sup>125</sup>I-oxLDL in the medium was measured in an LKB 1271 automatic gamma counter (Wallac, Finland). To determine the uptake of LDL into the cells, we incubated the monocytes for 24 h at 37 ° in 1mL RPMI medium without FCS, in the presence of the same <sup>125</sup>I-nLDL/oxLDL-antibody

combinations as for the binding assays. Following incubation, the cells were washed 3 times with PBS and scraped into 0.5 mol/L NaOH. The radioactivity of the cell lysate was measured as previously described.

## **Study IV – population and analysis**

The subjects included in study IV, born between 1926 and 1945, were recruited from the Malmö Diet and Cancer (MDC) study cohort. A random 50% of the individuals who entered the MDC study between November 1991 and February 1994 were invited to take part in a study on the epidemiology of carotid artery disease. Participants who had a history of myocardial infarction or stroke prior to enrolment were not eligible for our study. The 76 cases included in study IV were the first individuals who developed acute coronary heart events (acute myocardial infarction or sudden death due to CAD) during follow-up. Two controls matched for age, sex, smoking habits, presence of hypertension, month of participation in the screening examination and duration of follow-up were chosen for each case. Only one control was found for 4 cases, so the final study population consisted of 224 individuals, 76 cases and 148 controls, between 49 and 67 years of age. The ethical committee of Lund University, Sweden approved the study.

Blood samples were drawn after overnight fasting and plasma stored at -80°C. Total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol and whole blood glucose were measured using standard laboratory protocols. LDL cholesterol, expressed in mmol/L, was calculated according to the Friedewald formula. The amount of oxidized LDL in EDTA plasma supplemented with the antioxidants DTPA and BHT was measured using an ELISA kit (Mercodia, Sweden).

The plaques in the carotid artery were assessed by B-mode ultrasound vasculography using an Acuson 128 Computed Tomography System (Acuson, USA) with a 7 MHz transducer. The examination procedure and image analysis were performed by specially trained certified sonographers. Three centimeters of the distal common carotid artery, the bifurcation, and 1 centimeter of the internal and external carotid arteries were scanned for the presence of plaques. The plaques were defined as focal thickenings of the arterial wall, with an IMT >1.2 mm. The IMT was measured in the far wall according to the leading edge principle with a specially designed, computer-assisted image analyzing system based on automated detection of the echo structures but with the option for manual corrections by the operator.<sup>421</sup> The degree of stenosis was calculated based on blood flow velocity at the location of maximum lumen diameter reduction.



## Measurement of IgG subclasses in plasma

p45 was MDA-modified by treatment with a 0.5 M MDA solution (120 µL/mg peptide) for 3 hours at 37°C. The 0.5 M MDA solution was freshly prepared by incubation of 120 µL MDA (Sigma, USA) with 20 µL 4M HCl and 360 µL distilled H<sub>2</sub>O for 10 minutes at 37°C. The pH of the solution was increased to 7.4 by addition of 1 M NaOH to stop the reaction and the volume was brought to 1 mL with distilled H<sub>2</sub>O. The MDA-modified peptide was dialyzed against PBS containing 1 mM EDTA with several changes for 18 h at 4 °C to remove the unbound MDA, and the MDA content was measured using the thiobarbituric acid reactive substances (TBARS) assay. For the TBARS assay, 0.1 mL of MDA-p45 was incubated in a boiling water bath for 15 minutes with 0.1 mL of 1 mmol/L ferric chloride, 0.1 mL of 1 mmol/L butylated hydroxytoluene, 1.5 mL of 0.2 mol/L glycine buffer (pH 3.6) and 1.5 mL of 3.5 mmol/L thiobarbituric acid supplemented with 0.3% SDS. After allowing the solution to reach room temperature, the pink chromogen was extracted with acetic acid (1 mL) and chloroform (2 mL). The optical density of the upper layer was measured at 532 nm against water blank, using 1,1,3,3,-tetraethoxypropane as standard. The aldehyde content of the modified peptide was 0.022 nmol per µg peptide.

MaxiSorp microtiter plates (Nunc, Denmark) were coated with MDA-p45 (20 µg/mL in PBS), blocked with SuperBlock in TBS (Pierce, USA) and incubated with test plasma diluted 1/100 in TBS-0.1% Tween-20 (TBS-T) containing 10% Superblock. Mouse anti-human IgG1, IgG2, IgG3 and IgG4 antibodies (Sigma, St Louis, MO) diluted in TBS-T were used to detect the bound antibodies. Finally, the color reaction was developed by using an alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma) and a phosphatase substrate kit (Pierce).

## Statistical analysis

SPSS was used for the statistical analysis. In studies I-III, data are presented as mean ± standard deviation. Analysis of the data was performed using two-tailed Mann-Whitney test or the Students *t*-test when appropriate. Spearman's rho was used for correlation analysis. Statistical significance was considered at  $P \leq 0.05$ . In study IV, the results are presented as median and range and as proportions when appropriate. Pearson correlation with and without age adjustment was used to study association between risk factors and IgG subclasses. Differences between group means were tested by *t*-test. Chi-square test was used for comparing proportions. A general linear model was applied to examine the trend between carotid ultrasound measurements and quartiles of IgG.

## RESULTS

The aim of the studies included in the present thesis was to assess the associations between atherosclerosis and humoral immune responses against oxidized LDL, in humans and animal models. Previous results have indicated the presence of antibody-mediated immune responses which confer protection against the development of atherosclerosis. Several of the MDA-modified peptidic epitopes which trigger these immune responses were also characterized<sup>53</sup>. In **studies I-III** we tested the effects of recombinant human IgG1 antibodies against two of these epitopes on the extent and composition of atherosclerotic plaques in several mouse models of atherosclerosis. In **study IV** we used human material and data from the Malmö Diet and Cancer Study to assess the associations between carotid atherosclerosis, cardiovascular risk and the levels of different IgG isotypes against one of the MDA-modified apoB-100 peptide sequences.

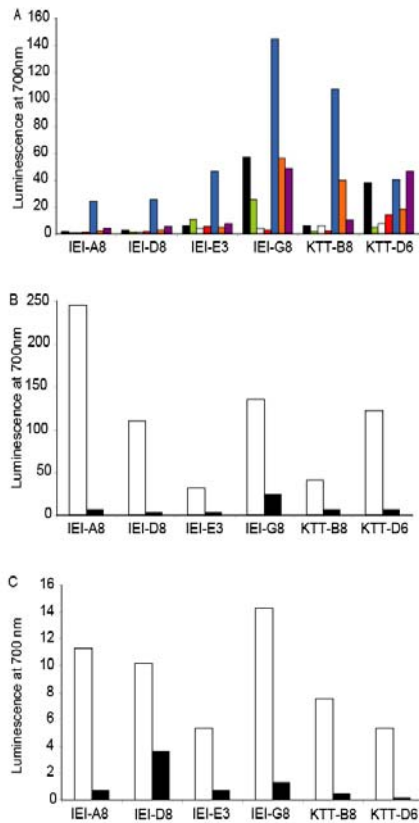
### Study I

Based on our previous results<sup>53, 405</sup>, we produced recombinant human IgG1 antibodies against 2 MDA-modified peptide sequences of apoB-100: p45 (aa 661 to 680) and p210 (aa 3136 to 3155). We selected human antibody fragments specific for the 2 peptides from the single-chain fragment variable (scFv) n-CoDeR library. These fragments were transferred into a full-length IgG1 $\lambda$  format by subsequent cloning into a pcDNA3 vector. Six antibodies were produced in this process, 4 against p45 (IEI-A8, IEI-D8, IEI-E3, IEI-G8) and 2 against p210 (KTT-B8, KTT-D6). The CDR sequences of these antibodies are presented in table 1 of paper I. The specificity and affinity of the antibodies for different MDA-apoB-100 epitopes were tested by luminescence-based ELISAs and the Biacore technique (Table 2 and Figure 1A, paper I). We also tested the binding of both the scFv and the IgG1 variants of the IEI and KTT antibodies to MDA-LDL compared with native LDL (Figure 1B and 1C, paper I). IEI-A8, IEI-D8 and IEI-E3 proved to be specific for p45. IEI-G8 and KTT-B8 bound stronger to p45, but they also cross-reacted with other MDA-modified peptides of apoB-100. IEI-G8 and KTT-D6 presented relatively high binding activities to a non-relevant MDA-modified control peptide. Both the scFv and the full-length IgG1 antibodies were specific for MDA-LDL compared with native LDL, demonstrating that the desired target specificity of the antibodies was achieved.

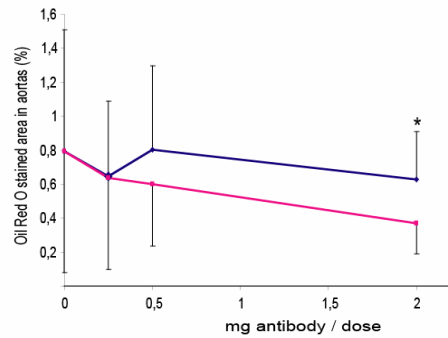
We tested the effects of the antibodies on atherosclerosis development in apoE<sup>-/-</sup> mice. The mice were kept on a high-fat diet starting from 6 weeks of age, in order to accelerate atherogenesis. Three 0.5mg IgG1 doses were injected intraperitoneally at 21, 22 and 23 weeks of age, and the mice were sacrificed at 25 weeks of age. Each mouse group was injected with a single antibody preparation. In a preliminary pilot study we demonstrated that the IgG1 antibodies administered via the peritoneal cavity penetrate into the blood stream and have a half-life of approximately 3 days. The extent of atherosclerosis was measured by ORO staining of *en face* preparations of the descending aorta and compared with plaque area in a control group injected with PBS alone. The treatment had no influence on the health status of the mice, which remained stable throughout the experiment. There were no differences in body weight and plasma levels of TG and HDL among the groups. The IEI-D8 and KTT-D6 groups had lower levels of total cholesterol compared with the PBS control, but this effect did not correlate with plaque area in these animals (Table 3, paper I). Average aortic plaque area in all antibody treated groups was lower than in the PBS group, but this effect was only significant for IEI-E3 (0.40±0.34 % compared to 0.86±0.58 % for PBS;  $P<0.05$ ; Table 3, paper I).

We then performed a dose-response study using IEI-E3 and a control IgG1 antibody specific for fluorescein isothiocyanate (FITC-8). PBS served as an additional control. Different mouse groups were treated with 0.25, 0.5 or 2 mg of antibody per dose of either IEI-E3 or FITC-8. The same layout was used as in the first experiment. FITC-8 had no influence on lesion area compared to PBS, whereas IEI-E3 reduced atherosclerosis by 2% in the 0.25-mg group, 25% in the 0.5-mg group and 41% in the 2-mg group, compared to the FITC-8 matching controls (Figure 2, paper I). The difference in plaque area between the groups treated with 2 mg of antibody per dose was significant ( $P<0.05$ ).

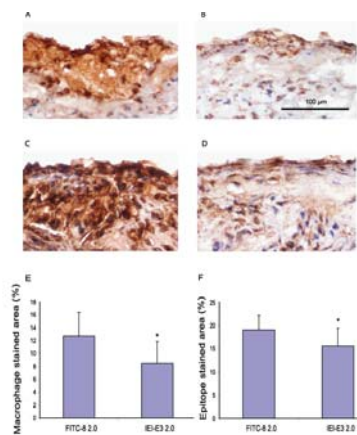
Immunohistochemical stainings of macrophages and oxLDL revealed significant differences in subvalvular plaque composition between the two groups. Using IEI-E3 as a detection antibody we were able to stain oxLDL epitopes in the atherosclerotic lesions. These epitopes were predominantly located close to the lumen. Preliminary inhibition studies with human oxLDL and native LDL demonstrated that the staining was specific for oxidation neopeptides on LDL. Macrophages were detected with a monocyte/macrophage (MOMA-2) antibody and were mostly shown to co-localize with oxLDL. There was a 33% reduction ( $P=0.02$ ) of macrophage stained area and a 20% reduction ( $P=0.04$ ) of oxLDL stained area in mice treated with 2 mg of IEI-E3 per dose, compared with their FITC-8 controls (Figure 3, paper I). These results suggest the ability of IEI-E3 to facilitate the removal of oxLDL particles bearing epitopes recognized by this antibody, followed by a reduction of the inflammatory activity inside the plaques.



**Figure 1, paper I.** Binding of selected scFv (A) to a number of different MDA modified antigens. P2 (aa no 16-31, red), P45 (aa no 661-680, blue), P143 (aa no 2131-2150, orange), P210 (aa no 3136-3155, purple) and P301 (aa no 4502-4521, white) are peptides corresponding to the human apoB-100 sequence. The control peptide is a non-relevant lysine containing peptide (MDA-modified, black; unmodified, green). (B) Illustrates the binding of scFv to native (black) and MDA modified human LDL (white), and (C) binding of cloned human IgG1 to native (black) and MDA-modified (white) human LDL. In figure A and B the luminescence ELISA data are presented as signal/buffer signal, while in figure C the data are plotted as signal/10<sup>5</sup>.



**Figure 2, paper I.** Dose-response curve showing increased reduction in plaque area in descending aortas of apoE<sup>-/-</sup> mice. Mice treated with different doses of IEI-E3 (red) or FITC-8 (blue) antibodies. Values on the Y-axis represent Oil Red O stained area in percent of total descending aorta area, values on the X-axis represent mg antibody per injected dose. \* P<0.05 versus FITC-8.



**Figure 3, paper I.** Staining of macrophages and oxLDL epitopes in subvalvular plaques of apoE<sup>-/-</sup> mice. Staining of macrophages in the groups injected with (A) FITC-8 and (B) IEI-E3 antibodies, respectively. IEI-E3 epitope staining in plaques from the same groups, (C) FITC-8 and (D) IEI-E3, respectively. The values represent percentage of stained area per total subvalvular plaque area, (E) macrophage staining and (F) epitope staining \* P<0.05 versus FITC-8.

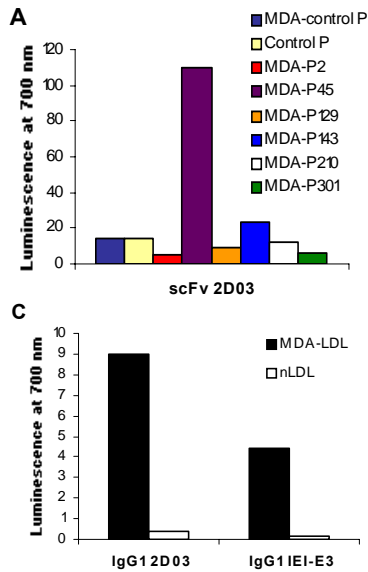
To support this hypothesis, we performed an *in vitro* experiment on human monocytes/macrophages cultured in the presence of different combinations between antibodies and <sup>125</sup>I-labeled native and oxidized LDL. Compared with FITC-8, IEI-E3 induced a 5-fold increase ( $P=0.001$ ) in binding and a 2-fold increase ( $P=0.006$ ) in the uptake of oxLDL in the cells. The IEI-D8 and the KTT-B8 antibodies had similar effects ( $P<0.01$ ), whereas there was no difference between IEI-A8, IEI-G8, KTT-D6 and FITC-8. None of the antibodies influenced binding and uptake of native LDL in macrophages (Figure 4, paper I).

## Study II

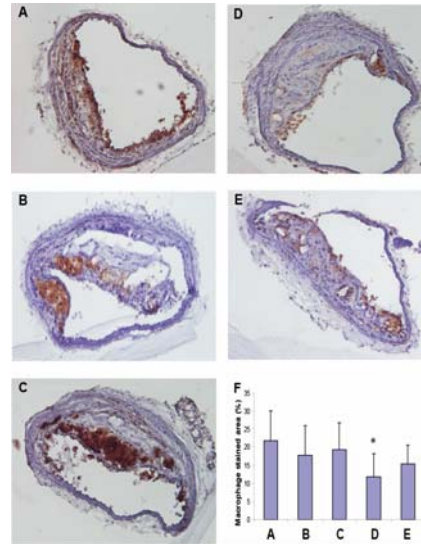
The major goal of the second study was to determine if the antibodies are able to induce regression of already present advanced atherosclerotic plaques. The transfer of hypercholesterolemic rabbits from high-fat diet to normal chow was previously reported to induce plaque regression.<sup>243</sup> We aimed to determine if the effects of the antibodies are additive to cholesterol-lowering dietary interventions. The association between the affinity of the antibodies for oxLDL epitopes and their effect on atherosclerosis was also assessed, by comparing IEI-E3 with 2D03, a similar IgG1 antibody characterized by a higher binding capacity to the same oxLDL epitope.

We chose to use *apobec-1*<sup>-/-</sup>LDLR<sup>-/-</sup> mice in this study for several reasons. These mice develop severe atherosclerosis when fed a high-cholesterol diet. Due to the lack of the apobec-1 enzyme, they carry mouse apoB-100 in their LDL particles, compared to wild type mice, which mainly express apoB-48. The presence of mouse apoB-100 provides the same array of epitopes as in human apoB-100, even if the homology is not perfect. Additionally, as reverse cholesterol transport is one of the potential mechanisms of plaque regression, it is important that these mice express functional apoE. The mice were 4 weeks older at the beginning of the treatment in the second study compared to study I (i.e. 25 weeks of age) in order to allow the development of advanced atherosclerotic plaques.

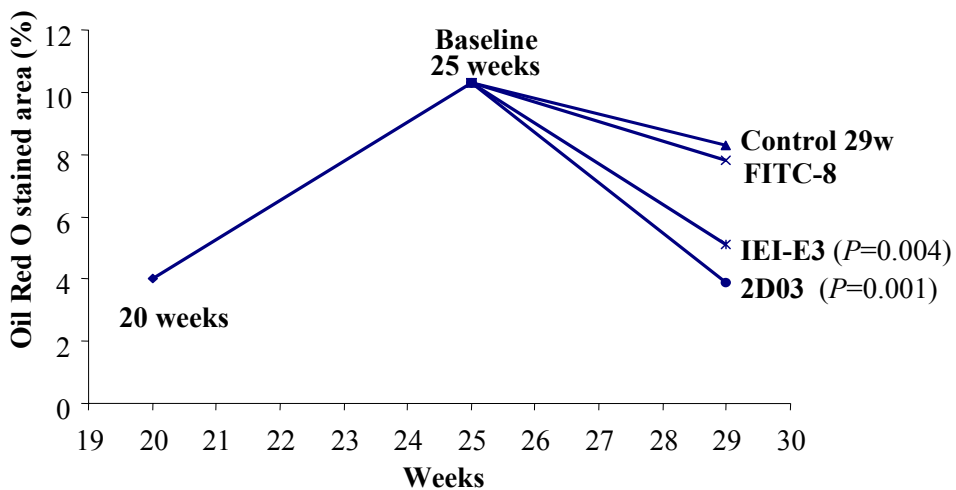
We tested the binding of the scFv 2D03 antibodies to several MDA-modified apoB-100 peptides. 2D03 bound to MDA-p45 and also demonstrated a weak binding to MDA-p129 (aa 2131 to 2150; Figure 1A, paper II). In contrast, as shown in the first study, IEI-E3 only binds to MDA-p45. The scFv 2D03 antibodies bound MDA-LDL more effectively than IEI-E3, and none of the antibodies recognized nLDL (Figure 1B, paper II). These specificities were preserved when the scFv were cloned into the full IgG1 format (Figure 1C, paper II). The affinity of the 2D03 antibodies for MDA-modified apoB-100, determined by the Biacore technique, was 10 times higher for 2D03 compared with IEI-E3 ( $3 \times 10^{-9}$ M versus  $3 \times 10^{-8}$ M, respectively). The binding of 2D03



**Figure 1, paper II.** Binding of single chain fragment (scFv) 2D03 to a number of different MDA modified antigens (A). P2 (amino acids [aa] 16-31), P45 (aa 661-680), P129 (aa 1921-1940), P143 (aa 2131-2150), P210 (aa 3136-3155) and P301 (aa 4502-4521) are peptides corresponding to human apoB-100 sequence. The control peptide was a non-relevant lysine-containing peptide. (C) binding of human IgG1 to native and MDA-modified human LDL. In (A) the luminescence ELISA data are presented as signal/buffer, whereas in (C) as signal/10<sup>5</sup>.



**Figure 4, paper II.** Macrophage content in plaques from the innominate artery in the 25 weeks old control (A), 29 weeks old control (B), FITC-8 (C), 2D03 (D) and IEI-E3 (E) groups. The values in (F) represent percentage of stained area per total plaque area. \*  $P < 0.05$  vs. FITC-8.



**Figure 3, paper II.** Plaque area in the descending aorta assessed by Oil Red O staining. The values are expressed as percentage of total plaque area per total area of the descending aorta. \*\*\*  $P < 0.001$ ,

to MDA-modified apoB-100 was not inhibited by mouse sera (from LDLR<sup>-/-</sup> or wild type mice) or human plasma (from healthy individuals or coronary heart disease patients) indicating lack of interference from potential corresponding autoantibodies with the binding of 2D03 to its target *in vivo* (Figure 2, paper II).

Lesion area in the descending aorta of the *apobec-1*<sup>-/-</sup>LDLR<sup>-/-</sup> mice kept on a high cholesterol diet increased from 3.98±1.46 % at 20 weeks of age to 10.31±3.73 % at 25 weeks (Figure 3, paper II). At 24 weeks of age the high-fat diet was changed to chow. We began the antibody treatment at 25 weeks and one group was sacrificed at this stage. The extent of atherosclerosis in these mice was used as baseline reference for the effects of the antibodies on already present atherosclerotic plaques. Three groups of mice received 1mg/dose of the IEI-E3, 2D03 or FITC-8 antibodies, respectively, injected intraperitoneally at 25, 26 and 27 weeks of age. The 29-week control group was also transferred to normal chow at 24 weeks of age, but did not suffer any additional intervention until sacrifice. Except for the 20-weeks and the 25-weeks baseline groups, all the mice were sacrificed at 29 weeks of age. Table 1 in paper II presents a detailed description of the experiment layout.

Plasma cholesterol levels dropped from 967±225 mg/dL in the 20-week group to 367±129 in the 25-week group, kept on chow diet for one week before sacrifice, and remained at approximately the same level in all the groups sacrificed at 29 weeks of age. A similar effect was observed for plasma TG. The antibody treatment did not induce any changes in weight, cholesterol and TG concentration compared with the baseline or the 29-week controls (Table 2, paper II).

The dietary intervention induced a modest non-significant plaque area decrease in the descending aorta, from the baseline value of 10.31±3.73 % at 25 weeks of age, to 8.28±4.36 % in the 29-week control group. Average lesion area was similar in the FITC-8 treated mice and in the 29-week control group, implying that the control unspecific antibody did not have any additional effect on atherosclerosis compared to the dietary intervention alone. The treatment with both IgG1 antibodies specific for oxLDL epitopes induced a strong regression of atherosclerotic lesions compared with the two control groups and with the baseline group (Figure 3, paper II). Plaque area regressed by more than 50% in the 2D03 group compared with the FITC-8 group (3.91±1.83 % vs. 8.01±2.52 %;  $P=0.001$ ). A similar but less pronounced effect was recorded in the IEI-E3 treated mice, which presented a 35% reduction in plaque area compared with the FITC-8 control (5.16±1.07 % vs. 8.01±2.52 %;  $P=0.004$ ).

The local and general inflammatory activity in mice was measured by immunostainings of macrophages in plaques of the innominate artery and of SAA levels in plasma, respectively. The innominate artery has previously been shown to present a highly consistent rate of lesion progression in apoE<sup>-/-</sup> mice between 24 and 60 weeks of age

and a high frequency of intraplaque hemorrhage in animals older than 42 weeks. Based on these results, the innominate artery was proposed as a suitable model for vulnerable plaques.<sup>422, 423</sup> The macrophage immunopositive area presented a slight decrease tendency as a result of the dietary intervention, alone or combined with the FITC-8 antibody, from 21.8±8.23 % at baseline to 17.8±8.16 % in the 29-week control group and to 19.3±7.43 % in the FITC-8 group (Figure 4, paper II). The differences between these groups were not significant. The 2D03 antibody induced a significant reduction by 38% of macrophage immunostained area (11.84±6.4 %;  $P=0.03$ ) compared with the FITC-8 control. IEI-E3 treated mice also had lower macrophage content in their innominate artery plaques compared with FITC-8 (15.38±5.1 %; n.s.). There were no significant differences in average innominate plaque area among the groups. Mouse SAA is considered to be an inflammatory marker equivalent to human CRP. SAA levels continuously increased in plasma from 20 to 29 weeks of age, regardless of the dietary replacement (Table 2, paper II). The treatment with IEI-E3 and 2D03 resulted in a dramatic decrease of SAA concentration in plasma compared to the isotype FITC-8 control. This difference was only significant for IEI-E3, which induced a more than 90% reduction in SAA levels ( $P=0.001$ ).

## Study III

In study III we assessed the effects of 2D03 on neointima formation and vessel remodeling following carotid injury in LDLR<sup>-/-</sup> mice over-expressing human apoB-100. In parallel, the effect of antibody treatment on plaque area in the uninjured contralateral carotid artery was also determined. FITC-8 served as isotype control antibody. The LDLR<sup>-/-</sup>/human apoB-100<sup>+/-</sup> were chosen because the sequence of the p45 peptide present in their LDL particles presents a complete homology to the p45 sequence that the 2D03 antibody was shown to recognize. The right common carotid artery was injured by placing a non-occlusive plastic collar around the vessel. This procedure was previously reported to induce neointima formation in the territory delimited by the collar.<sup>424</sup> A 200 µg dose of either 2D03 or FITC-8 were administered intraperitoneally to the mice one day before the intervention and at 3, 6 and 13 days following surgery. The mice were sacrificed at 21 days after surgery and both carotid arteries were collected for analysis.

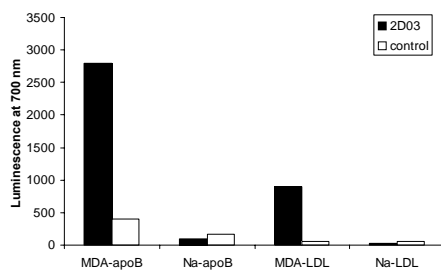
The ability of the 2D03 antibody to bind circulating oxLDL in plasma of the human apoB-100 transgenic mice was demonstrated by a sandwich ELISA in which 2D03 was used as catcher antibody and a rabbit anti-human apoB-100 as secondary antibody. The binding of 2D03 to oxLDL was inhibited by an excess of MDA-p45, demonstrating the specificity of the IgG1 antibody for this oxLDL epitope (data not shown). We have also shown the high affinity of 2D03 for MDA-apoB-100 and MDA-LDL compared to



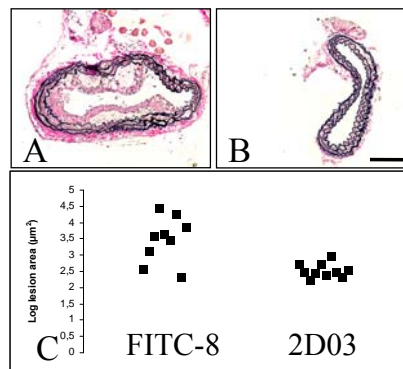
FITC-8 (Figures 1 and 2, paper III). Furthermore, the treatment with 2D03 significantly reduced plasma oxLDL content by 34% compared with FITC-8, while no differences were detected in plasma cholesterol levels between the groups. These results suggest the involvement of the 2D03 antibody in a more efficient clearance of oxLDL from the blood.

We measured plaque area, media area, lumen circumference and the length of the external elastic lamina (EEL) on serial elastin stained cross-sections of both the injured and the uninjured carotid arteries. Plaque area in the uninjured left carotid artery in 2D03 treated mice was minimal compared to plaque area in the FITC-8 group ( $397 \pm 235 \mu\text{m}^2$  versus  $7608 \pm 10304 \mu\text{m}^2$ ;  $P < 0.01$ ; Figure 3, paper III). In contrast, the uninjured vessels collected from mice injected with FITC-8 were larger, with an average EEL length of  $1293 \pm 274 \mu\text{m}$  compared to  $1041 \pm 88 \mu\text{m}$  in the 2D03 injected animals ( $P < 0.02$ ). This difference might be the result of a compensatory outward remodeling of the arteries in response to plaque formation. The area of the vessel media in the 2D03 group ( $32017 \pm 10304 \mu\text{m}^2$ ) did not differ from the FITC-8 group ( $26822 \pm 4501 \mu\text{m}^2$ ).

The circumference of the lumen, the medial area and the EEL were significantly larger in the injured segments of the mice treated with 2D03 compared to the control (Figure 4A and 4B, paper III). However, injury-induced neointima formation did not differ between the two groups (Figure 4A, paper III). Additionally, the length of the EEL in the injured segments of the 2D03 treated mice was inversely correlated with oxLDL plasma levels in these animals ( $r = -0.70$ ;  $P < 0.05$ ).



**Figure 2, paper III.** Luminescence ELISA illustrating the binding of 2D03 antibody and control (FITC-8) to MDA-modified human LDL (MDA-LDL), MDA modified human apoB-100 (MDA-ApoB), unmodified LDL (Na - LDL) and unmodified ApoB100 (Na-ApoB). Data are plotted as signal/10<sup>3</sup>.



**Figure 3, paper III.** Effect of antibody treatment on carotid atherosclerosis. Elastin stained sections of left uninjured carotid artery after injections with (A) anti-FITC-8 IgG1 and (B) 2D03 IgG1. Panel C demonstrates carotid artery lesion areas in individual mice. Scale bar = 100 µm.

We stained cross-sections from both the injured and the uninjured carotid arteries, for macrophages and SMC-specific alpha actin. In the uninjured carotid arteries of FITC-8 treated mice the plaques contained mostly macrophages, covered by a thin cap which stained positive for SMC. The media of these segments was rich in SMC (Figure 5A and 5B, paper III). The effects of 2D03 on plaque composition in the uninjured vessels could not be determined because of the lack of atherosclerotic lesions (Figures 5C and 5D, paper III). In the injured arteries, most of the macrophages were located in the outermost layers of the media and some macrophages could also be visualized in the neointima (Figures 5E and 5G, paper III). The neointima stained strongly for alpha-actin in both antibody treated groups (Figures 5F and 5H, paper III). Human IgG was not detectable in atherosclerotic plaques or neointima at the time of sacrifice.

## Study IV

In study IV we determined the associations between the levels of different IgG subtypes recognizing MDA-p45 and the extent of atherosclerosis in the carotid artery in human subjects. The value of these antibodies as predictors for acute coronary events was also assessed. We designed a nested case-control study, using baseline plasma samples and clinical data from 224 subjects included in the cardiovascular cohort of the Malmö Diet Cancer Study. All subjects were healthy at baseline. We chose the first 76 individuals who developed fatal or non-fatal acute coronary events during the follow up, referred to as cases, and 148 controls who remained healthy during this period. The cases and the controls were matched for age, sex, smoking, hypertension, month of inclusion in the study and the duration of follow-up. None of the subjects had a history of myocardial infarction or stroke previous to the enrolment in the study.

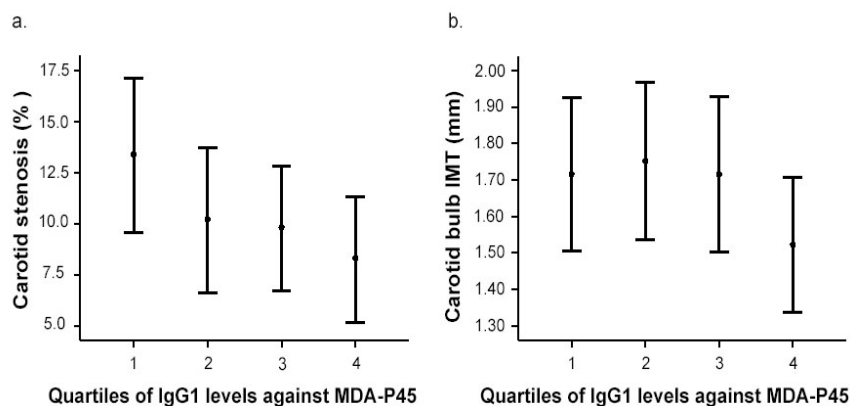


Figure 2, paper IV. Quartiles of IgG1 levels against MDA-p45 and severity of carotid disease. Inverse associations between MDA-p45 IgG1 levels and degree of carotid stenosis (a;  $P=0.006$  for trend) and IMT in the carotid bulb (b;  $P=0.064$  for trend).

Carotid atherosclerosis was assessed by B-mode ultrasound measurements of the IMT in the common carotid artery and in the bulb at baseline, as well as the degree of carotid stenosis. The cases were characterized by a higher IMT in the bulb and a higher degree of stenosis. There was also a slight increase in plasma TG concentration in the cases (Table, paper IV). No other differences were recorded between cases and controls regarding plasma levels of total cholesterol, LDL and HDL lipoproteins, oxLDL, glucose or body mass index (BMI).

None of the IgG1, IgG2, IgG3 and IgG4 subclasses was found to differ between cases and controls. IgG1 presented the highest concentration in both groups (Figure 1, paper IV). The 224 subjects were pooled together and divided into quartiles according to IgG levels. The different IgG subclasses were analyzed separately. We found a significant correlation between high levels of IgG1 and a lower degree of carotid stenosis (Figure 2a, paper IV). The association remained significant after adjusting for age and sex ( $P=0.008$  for the trend) and for the influence of systolic blood pressure, LDL and HDL cholesterol ( $P=0.006$  for the trend). There was also a weak negative correlation between IgG1 levels and carotid bulb IMT after adjusting for age and sex ( $P=0.064$  for the trend; Figure 2b, paper IV). No other associations were recorded between IgG1 and common carotid IMT (Figure 2c, paper IV) or between the other IgG subclasses and the measured parameters for carotid atherosclerosis (data not shown). The levels of the different MDA-p45 IgG subclasses did not correlate with age, sex, lipoproteins, plasma oxLDL, glucose, smoking or blood pressure.

## DISCUSSION

### Atheroprotective immune responses

The involvement of oxLDL in atherosclerosis and the existence of oxLDL-associated cellular and humoral immune responses have clearly been demonstrated and are now well accepted. Initially, these immune mechanisms were thought to be proatherogenic, stimulating inflammation and plaque progression. In the late '90s a series of consecutive studies by different groups demonstrated that immunization of hypercholesterolemic rabbits or mice with plaque homogenates or with native or oxidized LDL led to decreased disease progression and a more stable plaque phenotype.<sup>394-396, 398</sup> These findings revealed the existence of atheroprotective immune responses directed against oxLDL and suggested that immune modulation may constitute the basis for the development of new anti-atherogenic therapies for clinical practice.

A marked increase in antibody titers against oxLDL epitopes after immunization was detected in the majority of these studies, and some authors could even directly correlate these titers with the extent of plaque reduction.<sup>399</sup> These results were suggestive for an antibody-mediated atheroprotective mechanism underlying the observed effects on plaque development. However, elements of the cellular immunity are likely to participate in the protection against atherosclerosis as well. Freigang et al. found that immunization of LDLR<sup>-/-</sup> mice with native LDL reduced the extent of atherosclerosis without increasing the levels of IgG or IgM antibodies against nLDL or oxLDL.<sup>397</sup> It is also known that Freund's adjuvant alone has atheroprotective effects<sup>425</sup>, and it has been demonstrated that these effects are dependent on the presence of CD4<sup>+</sup> T cells in the immunized animals.<sup>357</sup>

As discussed by Zhou et al.<sup>399</sup>, the humoral atheroprotective hypothesis had to be demonstrated by direct B cell transfer as well as passive immunization with specific antibodies, and the exact epitopes triggering these immune mechanisms needed to be characterized. Three papers, all published in 2002 by different groups, pointed towards an atheroprotective role of B cells in atherosclerosis. The spleen is a very important reservoir of B cells. Splenectomized apoE<sup>-/-</sup> mice develop more aggressive atherosclerosis than their immunocompetent controls. The transfer of B cells increased antibody titers to oxLDL and reduced atherosclerosis both in the splenectomized mice and in the control group.<sup>391</sup> An increase in atherosclerosis was observed in mice trans-

planted with B cell-deficient bone marrow or lacking mature T and B cells due to RAG-1 deletion.<sup>392, 393</sup>

Additional evidence was provided by studies using passive immunization with intravenous immunoglobulins (IVIg) in mice. IVIg are concentrated preparations of normal polyspecific antibodies, purified from healthy blood donors. These preparations contain mainly IgG, which is the predominant circulatory antibody isotype. IVIg are widely used in current clinical practice for treatment of antibody deficiencies, autoimmune and systemic inflammatory diseases.<sup>426-428</sup> Several studies indicated that IVIg treatment in apoE<sup>-/-</sup> mice significantly reduced the extent of atherosclerosis at different stages of the disease.<sup>401-403</sup> The atheroprotective effect was associated with reduced T-cell activation in spleen<sup>403</sup> and lymph nodes<sup>402</sup>, reduced macrophage accumulation in the lesions<sup>402, 403</sup> and a decrease in anti-oxLDL IgM titers.<sup>402</sup> The authors suggested unspecific mechanisms for IVIg activity, supported by other studies, including FcR blockade, downregulation of T and B cell activation and regulation of antibody production<sup>429</sup>, direct influences on cytokine and chemokine levels<sup>430, 431</sup>, inhibition of cell adhesion<sup>432</sup>, interference with complement-induced tissue damage.<sup>402</sup> However, Wu et al. found specific anti-oxLDL antibodies and anti-anti-oxLDL antibodies in 6 different IVIg preparations<sup>433</sup>, indicating that there may also be specific mechanisms involved in the reduction of atherosclerosis by IVIg.

Thus, the oxLDL immunization, B cell substitution and passive IVIg immunization studies clearly demonstrated the existence of antibody mediated atheroprotection. Nevertheless, the LDL particle is very complex and the different antigenic structures generated during oxidation can be extremely heterogeneous. In order to fully understand and to be able to use specific immunomodulation for the prevention and treatment of atherosclerosis, the exact epitopes triggering these immune responses needed to be characterized. Using a polypeptide library covering the entire amino acid sequence of human apoB-100, we have identified over 100 different native or MDA-modified peptidic epitopes recognized by IgM and IgG antibodies in plasma of healthy individuals.<sup>53</sup> The 302 peptides of the library were 20 amino acids long and were produced with a 5 amino acids overlap. The levels of IgM antibodies against several of these MDA-peptides were correlated with the extent of atherosclerosis assessed by measurements of carotid intima-media thickness (IMT) and to the development of acute cardiovascular events in subjects under 60 years of age. The plasma concentration of these antibodies was shown to decrease with age, especially in individuals who developed acute coronary heart events during the follow-up, and to be inversely associated with the levels of oxLDL in plasma.<sup>53</sup> Similar but weaker associations could also be detected for IgG antibodies. These antibodies could be proatherogenic, or they may be produced as a response to increased LDL oxidation and have an atheroprotective role.

Subsequent studies evaluated the importance of the immune responses against some of these apoB-100-related oxLDL neoepitopes for the development of atherosclerosis. Different groups of hypercholesterolemic apoE<sup>-/-</sup> mice were immunized with native or MDA-modified peptides against which high levels of IgM or IgG antibodies were found either in the individuals which developed a coronary event or in their healthy controls. The immunizations inhibited atherosclerosis progression by up to 60%<sup>404-406</sup> and induced a more stable plaque phenotype, characterized by increased collagen<sup>405</sup> or reduced macrophage content.<sup>404, 406</sup> The inhibition of atherosclerosis was associated with a several fold increase in specific IgG antibodies in the plasma of the immunized animals. Thus, these apoB-100 epitopes seem to trigger atheroprotective antibody-mediated immune responses and may represent potential specific targets for prevention of atherosclerosis-related diseases by immunomodulation.

## **Anti-atherogenic effects of MDA-LDL IgG1 antibodies**

In order to study whether the IgG antibodies secreted in response to immunization served as atheroprotective mediators, we produced recombinant human IgG1 antibodies against 2 of the MDA-modified peptide sequences in apoB-100, in collaboration with BioInvent International AB, Lund, Sweden. We decided to produce antibodies against peptides 45 (aa 661-680) and 210 (aa 3136-3155), counted from the N-terminal end of the apoB-100 protein. p45 was chosen because we found marked increase in IgG antibody titers against this peptide in plasma from CHD patients compared to the healthy controls.<sup>53</sup> Additionally, immunization with p45 induced a 10 fold increase in specific IgG, reduced atherosclerotic plaque area by 48% and plaque macrophage content by 33% in apoE<sup>-/-</sup> mice.<sup>404</sup> We selected p210 as a counterpart to p45, because we found high levels of both IgG and IgM antibodies against this peptide in plasma from healthy individuals. P210 also reduced the extent of atherosclerosis in apoE<sup>-/-</sup> mice by 60% in combination with p143.<sup>405</sup>

The purpose of the studies described in paper I was to assess the influence of passive immunization with the IgG1 antibodies on atherosclerosis development in apoE<sup>-/-</sup> mice and to compare the effects of the different antibodies. Following 3 rounds of immunizations at 1 week intervals, all 6 antibodies used, 4 against p45 and 2 against p210, showed a tendency to inhibit atherosclerosis progression. IEI-E3, an antibody with high specificity for p45, had the most potent effect. IEI-E3 induced a significant and dose-dependent reduction of atherosclerosis by up to 41% compared with FITC-8, an isotype control IgG1 antibody with no binding activity to oxLDL. The content of macrophages and oxLDL epitopes in the plaques was also decreased. This study demonstrated the ability of passive immunization with IgG1 antibodies against MDA-apoB-100 epitopes to rapidly inhibit atherosclerosis and promote a less inflammatory plaque

phenotype. The findings support the hypothesis that specific antibodies are among the factors mediating the atheroprotective responses induced by different active immunization strategies. Theoretically, these antibodies may represent a future fast acting therapy for plaque stabilization and prevention of acute cardiovascular events in humans.

When considering the potential uses of the antibodies in clinical practice, it was of considerable interest to determine if the observed effects were due to inhibition of disease progression or to an additional regression of already established atherosclerotic plaques. Atherosclerosis regression was previously demonstrated both in animal and human studies, and was mainly achieved by lipid lowering therapies and stimulation of reverse cholesterol transport.<sup>434, 435</sup> In study II, the extent of atherosclerosis in the groups treated with IgG1 antibodies was compared both with an isotype control group and a baseline group sacrificed at the beginning of the treatment. We used *apobec-1<sup>-/-</sup>/LDLR<sup>-/-</sup>* mice on high fat diet and began the treatment at 25 weeks of age, when these animals already had advanced plaques throughout their arterial tree. One week before the treatment, the diet was changed to chow. A control group was also transferred to chow diet and sacrificed at the same age as the antibody treated groups. The IEI-E3 antibody was used in parallel with 2D03, another recombinant human IgG1 antibody with higher affinity for p45. Both antibodies induced a marked regression of atherosclerotic plaques over a 4 week period compared to the baseline, low-fat diet and FITC-8 control groups. The effect was more pronounced in the mice treated with the high affinity 2D03 antibody than in the IEI-E3 treated mice, suggesting that the specific binding of antibodies to oxLDL is important for atheroprotection. Transferring the mice from a high fat diet to normal chow did not induce significant plaque regression and there were no differences in plasma lipid levels between the antibody treated and the control groups. Thus, the mechanisms underlying antibody induced plaque regression do not seem to involve general changes in the lipid metabolism. These findings are important, as they suggest that immune and lipid lowering therapies could have additive effects on atherosclerosis.

The third study presented in this thesis was designed to test the influence of passive immunization with the 2D03 antibody on neointima formation and vascular remodeling following carotid injury. Previous studies have shown that neointima formation in response to vascular injury is accelerated in mice lacking functional T and B cells and that this effect can be rescued by B cell transfer.<sup>393</sup> Positive results were also obtained by antioxidant treatment in preventing neointima formation and restenosis after angioplasty in both animal and human studies.<sup>436, 437</sup> These results indicate that LDL oxidation may have an important role in vascular remodeling and restenosis following arterial injury, and suggest the presence of humoral immune responses which confer protection against these effects. We used transgenic *LDLR<sup>-/-</sup>* mice which over-express human apoB-100 in their LDL particles. There is a complete homology

between the apoB-100 related structures present *in vivo* in these mice and the epitopes recognized by the recombinant IgG1 antibodies. The 2D03 antibody did not inhibit neointima formation, but prevented the constrictive remodeling of the injured carotid segments. This effect, measured by the circumference of the external elastic lamina of the injured segments, was inversely correlated with the amount of oxLDL in plasma. Additionally, the antibodies had a marked effect on plaque formation in the uninjured contralateral carotid artery. These arteries were essentially free of plaques in the 2D03 treated mice compared with the isotype control group, in which 8 out of 10 mice developed substantial atherosclerotic lesions. However, a baseline control group was absent in this study, and therefore it was not possible to determine whether the antibodies inhibited atherosclerosis progression or induced plaque regression in the carotid artery. Reports focusing on different antiatherosclerotic interventions have proven these methods to be effective at different locations of the arterial tree. Thus, the development of atherosclerotic lesions and the effects of atheroprotective therapy may be site-specific. In studies I and II we demonstrated reduction of atherosclerosis in the thoracic and abdominal segments of the descending aorta. It was therefore important to prove the efficacy of the 2D03 antibody in another atherosclerosis-prone location of the arterial tree.

## **Potential antibody-mediated atheroprotective mechanisms**

### *The importance of Fc fragments*

These three studies, together with studies published by other groups, have provided a partial insight into the potential mechanisms involved in the observed atheroprotective effects. Focusing on the atheroprotection induced by passive IVIg immunization, several findings suggest that these effects are dependent on the Fc fragment of the antibodies, and that they require an intact complement system. Two recent studies confirmed the atheroprotective role of IVIg treatment in apoE<sup>-/-</sup> mice at different stages of disease progression. Immunizations using only the Fab part of the Ig had no effect on atherosclerosis, indicating that the Fc fragment of the antibodies mediates the atheroprotection.<sup>401, 403</sup> The Fc fragment of IgG antibodies has 2 important properties which could contribute to its role in atherosclerosis: binding to FcγR and activation of the complement system. The heterogeneous family of FcγR can be found mainly on T and B lymphocytes, macrophages and neutrophils. FcγRI (CD64) is a low-affinity activating receptor, FcγRIII (CD16) is a high-affinity activating receptor and FcγRII (CD32) is an inhibiting receptor. FcγRI and FcγRIII induce cell activation and cytokine secretion, while antibody binding to FcγRII inhibits cell activation and further antibody production in B cells. It is likely that the unspecific anti-inflammatory effects of the IgG antibodies in IVIg preparations partly contribute to their effects on atherosclerosis. For example, in a murine model of immune thrombocytopenia, the same protective



effect could be induced by immunizations with whole IVIg or by using only their Fc parts, and was also associated with Fc $\gamma$ RII upregulation on splenic macrophages.<sup>438</sup> Nevertheless, in our studies we have used an isotype control antibody, bearing a Fc fragment identical with the Fc fragment of the antibodies specific for the MDA-modified apoB-100 sequences, which presented no binding activity to oxLDL. This antibody had no influence on atherosclerosis, indicating that the mechanisms responsible for atheroprotection in our experiments were oxLDL specific. Further support for this hypothesis comes from comparisons between the doses used for treatment in our experiments and in the IVIg studies. While we achieved inhibition of atherosclerosis progression with a dose of 1.5 mg of antibodies and lesion regression with 3 mg of antibodies, both divided into three immunizations, the minimum IVIg dose that reduced atherosclerosis was more than 10 times higher (50 mg divided into 5 immunizations).

#### Clearance of oxidized LDL from plasma

One of the most important mechanisms possibly mediating the atheroprotective effects of the recombinant IgG1 antibodies used in our studies is the enhanced clearance of oxLDL from the circulation. In paper III we have shown that treatment with the 2D03 antibody reduced plasma levels of oxLDL by more than 30%. The proatherogenic role of oxLDL in itself has been clearly demonstrated in mouse models of atherosclerosis. Our group<sup>439</sup> and others<sup>440</sup> have shown a positive correlation between the amount of oxLDL in human plasma, total cholesterol and LDL-cholesterol levels. A high plasma oxLDL/total cholesterol ratio was correlated with an increased risk for acute myocardial infarction.<sup>439</sup> An inverse relationship was found between circulating oxLDL and the levels of IgG anti-oxLDL antibodies in healthy subjects.<sup>440</sup> In a prospective case-control study, we have demonstrated that the amount of IgM antibodies against some of the peptide sequences in apoB-100 (including p45) decrease with age, in parallel with an increase in oxLDL concentration. This effect was more pronounced in the subjects who suffered an acute coronary event during the follow-up than in the control group, who remained healthy throughout the study. Additionally, Fukumoto et al. demonstrated that carotid IMT in healthy individuals negatively correlates with HDL levels and the amount of anti-oxLDL IgG in plasma in a multiple regression analysis, independent of other risk factors.<sup>441</sup> Taken together, the findings presented above support the hypothesis that the humoral immune responses to oxLDL may have a protective role against atherosclerosis in humans, at least partly mediated by the removal of oxLDL from the circulation.

The complement system seems to be involved in the atheroprotective humoral immune responses as an additional mediator of antibody activity. LDLR<sup>-/-</sup> mice deficient in complement factor 3 (C3) had increased atherosclerosis in the aorta and a vulnerable plaque phenotype, characterized by large amounts of macrophages and few SMCs.<sup>442</sup> These results were later confirmed by a different group, by crossing LDLR<sup>-/-</sup>/apoE<sup>-/-</sup>

mice with C3<sup>-/-</sup> mice. In contrast, deficiency in factor B, which is required for the activation of the alternative complement pathway, did not influence the course of atherosclerosis.<sup>443</sup> These interesting findings suggest that the protective effects of complement in atherosclerosis occur via the lectin pathway or the antibody-mediated classical complement pathway. Further support in favor of the involvement of the classical complement pathway in atherosclerosis was provided by a consecutive study, showing that atheroprotection by passive IVIg immunization required the presence of an intact complement system.<sup>400</sup> The complement could have pleiotropic modulatory effects on atherosclerosis, both systemically and locally in the arterial wall, by binding to complement receptors (CR) and regulating phagocytosis, cytotoxicity, cell adhesion, activation and proliferation of B lymphocytes and antibody production. Different complement components, including C3 and its cleavage products, have been found in human atherosclerotic lesions and colocalize with Ig and modified LDL.<sup>444-447</sup> The complement system also has an important role as a modulator of the adaptive immune response. Antigens coated with antibodies and complement factors induce a 10 000 fold higher antibody production in B cells than the antigens alone. The BCRs bind the antigen in the immune complexes (IC) and C3d attached to the Fc fragment of the antibodies binds CR2 on the B cell, reducing the threshold for B cell activation.<sup>448, 449</sup> The complement also marks antigens for phagocytosis in the follicular DCs and further presentation to B cells in the follicular center of the lymph nodes and has an important role in the selection or maintenance of natural IgM producing B1 cells.<sup>450</sup>

The mechanism of antibody-mediated clearance of oxLDL from plasma involves the formation of circulating immune complexes (CIC) and complement activation. Complement factor C3b binds to the Fc fragment of the antibodies and links the CIC to human erythrocytes, which express complement receptor 1 (CR1) on their surface. CR1 is only present on red blood cells of primates and humans. In mice, a similar mechanism seems to be mediated by adherent platelets, which bind CIC through platelet-associated factor H<sup>451</sup>, but the IC have also been shown to be able to bind to sites on erythrocytes other than CR1.<sup>452</sup> The erythrocytes transport the IC to the liver, where it is taken up by liver macrophages, the Kupffer cells.<sup>453, 454</sup> IC binding to erythrocytes was termed "immune adherence"<sup>455</sup> and the phagocytosis of IC by macrophages "transfer reaction".<sup>456</sup> The transfer reaction is mediated by the Fc part of the antibodies and occurs with loss of CR1 from the surface of the erythrocyte, which is ingested by the macrophage together with the IC.<sup>456</sup> Loss of CR1 from the red blood cells could explain the impaired clearance of oxLDL from the blood of systemic lupus erythematosus (SLE) patients<sup>457, 458</sup>, which in turn could contribute to the increased levels of atherosclerosis found in these individuals.<sup>459, 460</sup>

Our findings are supported by previous work from another group which demonstrated that the clearance of glucosylated LDL from plasma is increased by up to 100 fold in rabbits previously immunized with the same LDL preparation. Extensive LDL

modification was demonstrated to inhibit oxLDL binding to LDLR. The antibodies appeared to redirect uptake and degradation of modified LDL to the Kupffer cells in the liver.<sup>461</sup> Nevertheless, in a recently published study, the same group failed to demonstrate differences in the clearance rates of injected human LDL between immunocompetent apoE<sup>-/-</sup> and immunodeficient apoE<sup>-/-</sup> RAG2<sup>-/-</sup> mice.<sup>462</sup> These apparent discrepancies may be explained by several differences in the experimental settings between the studies. In the above mentioned study by Reardon et al.<sup>462</sup>, acute clearance of human oxLDL from mouse plasma was measured at different time points of up to 6 hours after intravenous administration. In contrast, we have demonstrated a lasting reduction of the endogenous circulating oxLDL 2 weeks after the final antibody administration. The plasma cholesterol levels in the high-fat diet mice used in the above mentioned study were extremely high (1200-1500 mg/dL compared to 500 mg/dL in our mice). The high levels of circulating oxLDL particles which are likely to occur in these animals could inhibit the binding of antibodies to human oxLDL, since it is expected that mouse antibodies would have higher affinity for mouse oxLDL epitopes than for epitopes on human oxLDL. It is to be expected that both in our study and in the study performed on immunized rabbits<sup>461</sup>, the high amount of antibodies specific for modified LDL, injected or produced in response to immunization, was exceeding the binding capacity of circulating oxLDL in plasma. Finally, Reardon et al.<sup>462</sup> also recorded a faster oxLDL clearance immediately after administration in the immunocompetent mice and a more effective clearance of oxLDL particles bearing high amounts of neo-epitopes, suggesting that the antibodies may play a role in the removal of extensively modified oxLDL particles from the blood, even in their experiment.

#### Potential mechanisms of plaque regression

An enhanced clearance of oxLDL particles from plasma by the recombinant IgG1 antibodies may inhibit the progression of atherosclerotic lesions but is less likely to explain the regression of already present, advanced atherosclerotic plaques. Early fatty streaks in children and young adults are able to spontaneously disappear and plaque regression was previously induced either in humans or in animal models by treatment with apoA-I<sup>80</sup>, apoA-I<sub>Milano</sub><sup>82, 84, 463</sup>, apoE<sup>464-467</sup>, statins<sup>468-471</sup> or by HDL elevation.<sup>435</sup> Thus, plaque regression is possible and there is an “in and out” traffic of macrophages and lipids through the atherosclerotic plaque.<sup>472</sup> ApoA-I and apoE are recognized acceptors of free cholesterol, mediating the reverse cholesterol transport from the peripheral macrophages to the liver.<sup>473, 474</sup> In the experiments described in paper II, the regression of plaque area was assessed by Oil Red O staining of neutral lipids in the atherosclerotic lesions of the descending aorta. Therefore the assumption can be made that the observed effects are due to a decrease in the lipid content of the plaques. The IgG1 antibodies may induce atherosclerosis regression both by stimulation of reverse cholesterol transport and/or by promoting the migration of foam cells from the plaques to the liver. Our *in vitro* experiments, described in paper I, have demonstrated

a 5 fold increase in binding and uptake of oxLDL in cultured macrophages in the presence of antibodies. Additionally, less oxLDL epitopes were found in the remaining atherosclerotic plaques following antibody treatment in mice. It has been shown that antibodies mediate oxLDL uptake in the macrophages by binding to Fc $\gamma$ RI and internalization of the immune complex.<sup>475</sup> This mechanism may be in addition to oxLDL uptake via the ScR, or it could occur at a different stage of LDL oxidation, considering that the ScR and the recombinant IgG1 antibodies that we have used do not recognize the same epitopes on the oxLDL particles. Lipid uptake in the macrophages may stimulate free cholesterol transport to the surface of the cells by the ABCA-1 transporter and subsequent transfer to apoA-I or apoE. Thus, in order to be removed from the plaques, extracellular oxLDL has to be taken up by the macrophages. This mechanism is likely to be more efficient in the *apobec-1*<sup>-/-</sup>LDLR<sup>-/-</sup> and LDLR<sup>-/-</sup>/human apoB-100<sup>+/-</sup> mice used in studies II and III than in the mice used in the first study, which lack the cholesterol acceptor apoE.

In papers I and II we have shown a significant decrease in plaque macrophage content in the mice receiving passive immunization with the recombinant human antibodies. This effect may reflect a decrease of monocyte recruitment into the artery wall, but also increased foam cell efflux from the lesions. A significant proportion of the foam cells in the atherosclerotic plaques have a DC-like phenotype<sup>476</sup> and have been demonstrated to be able to migrate and present antigens to T cells in the peripheral lymph nodes.<sup>477, 478</sup> The migratory capacity of DC-like foam cells was shown to be impaired by proatherogenic lipid mediators such as platelet activating factor and lysophosphatidic acid.<sup>479</sup> The regression of atherosclerotic lesions in a diseased arterial segment transplanted from apoE<sup>-/-</sup> to wild type mice was associated with enhanced migration of foam cells bearing a DC-like phenotype to the draining lymph nodes. The migration of host monocytes into the lesions of the graft was not inhibited, suggesting that the regression is mediated by increased cellular efflux rather than by decreased entry of monocytes into the subendothelial space.<sup>479</sup> Uptake of antigen-antibody complexes by monocytes in the lesion could stimulate their differentiation into DC and induce cellular migration out of the plaque.

Further evidence for the potential involvement of antibodies in atherosclerosis was provided by our recent *in vitro* studies on human monocytes and macrophages cultured in the presence of oxLDL-containing human serum. Our data indicates that IgG1 antibodies against MDA-modified apoB-100 epitopes stimulate monocyte maturation and inhibit MCP-1 release from the cells (Freundus et al. – unpublished data). Binding of oxLDL-IgG immune complexes to Fc $\gamma$ RI was previously shown to be able to reduce apoptosis and promote survival of human monocytes and to stimulate the production of M-CSF.<sup>480</sup> The anti-inflammatory role of IgG antibodies in cell culture experiments is also supported by data showing that the activation of EC by oxLDL is inhibited by IVIg preparations.<sup>481</sup> Thus, the antibodies may stimulate macrophage

survival and oxLDL removal, inhibiting at the same time further macrophage recruitment to the intima. These observations are in apparent conflict with studies by Virella et al. who indicated that oxLDL-IgG immune complexes are pro-inflammatory, inducing macrophage activation<sup>482-484</sup>, upregulation of LDLR<sup>485, 486</sup>, foam cell formation<sup>483, 487</sup> and secretion of pro-inflammatory cytokines.<sup>483, 487, 488</sup> The same group found significant correlations between the circulating levels of oxLDL-IC and the development of coronary heart disease over a 8 year follow-up period, in a prospective study on diabetic subjects.<sup>489</sup> However, as discussed above, the increased uptake of cholesterol in macrophages does not necessarily have a negative influence on atherosclerosis development. It remains to be demonstrated whether the pro-inflammatory characteristics of the IC *in vitro* translate into proatherogenic properties *in vivo*, and it is not clear if the oxLDL-IgG IC are disease markers or active participants in the atherogenic processes. The role of immune complexes formed after passive immunization with IgG1 antibodies remains to be determined, but so far our studies have demonstrated that the net effect of IgG1 treatment seems to be atheroprotective.

#### Immuno-modulatory effects of the antibodies

Besides promoting the clearance of oxLDL from plasma and atherosclerotic plaques, the IgG1 antibodies may also have modulatory effects on both humoral and cellular immune responses in the immunized mice. The antibodies can either strongly enhance or inhibit further antibody response to the antigen that they are specific for, by a process called “antibody feedback regulation”.<sup>490</sup> IgG antibodies potentiate both primary IgM and IgG antibody responses to the soluble protein antigens that they are specific for<sup>491, 492</sup>, and are efficient inducers of immunological memory.<sup>493</sup> These effects are mainly mediated by Fc binding to the activating receptor FcγRI on APCs.<sup>494</sup> APCs take up IgG-immune complexes more efficiently than when they encounter the antigen alone and are able to present antigen found at concentrations over 100 fold lower.<sup>495, 496</sup> Additionally, FcγRs crosslinking by IgG/antigen complexes induce DC maturation, improving the ability of these cells to present antigen.<sup>497</sup> Thus, IgG-oxLDL immune complexes may increase antigen presentation, T cell activation<sup>498</sup> and subsequent antibody production to oxLDL epitopes, by cognate help provided to B lymphocytes.

IgG1 antibodies are also relatively potent complement activators. Animals lacking different complement factors<sup>499-501</sup> or complement receptors CR1/2<sup>502-504</sup> have impaired antibody responses. The immune responses were not affected in mice lacking complement factor B, indicating that complement enhancement of antibody production is likely to occur via the classical activation pathway.<sup>505</sup> Interestingly, mice deficient in complement factor 3, but not factor B, develop increased hyperlipidemia and atherosclerosis<sup>443</sup>, providing more support for the importance of antibody-mediated immune responses in atheroprotection. The complement acts directly on antibody-producing B cells, by CR2 linkage. IgG antibodies are not as potent complement activators as IgM,

and 2 or more antibody molecules have to bind to epitopes within close range to each other and aggregate in order to activate complement.<sup>506</sup> MDA-modifications of the lysine rich apoB-100 molecules in oxLDL particles, which are recognized by our IgG1 antibodies, are likely to provide the binding substrate for such a phenomena to occur.

Theoretically, the IgG-containing immune complexes may also reduce the humoral immune responses against oxLDL in the mice, by concomitant binding of antigens to the BCR and of the Fc part of the IgG to the inhibitory Fc $\gamma$ RIIB on the same B cell. Co-crosslinking of Fc $\gamma$ RIIB with the B cell receptor inhibits B cell activation<sup>507, 508</sup> and antigen presentation by the B lymphocyte.<sup>509, 510</sup> Antibody production in Fc $\gamma$ RIIB-deficient mice is up to 50 times higher in response to IgG/antigen immunizations, compared to the wild type mice. The T cell response in these animals was not affected, indicating that the Fc $\gamma$ RIIB is not important for the regulation of T cell activation.<sup>494, 511</sup> Nevertheless, the Fc $\gamma$ RIIB linkage only attenuates antibody responses, without exerting a complete inhibition, as the wild type mice which carry fully functional Fc $\gamma$ RIIB receptors are still able to develop strong humoral immune responses after immunization with IgG-immune complexes.

With regard to the potential immuno-modulatory effects of the recombinant human IgG1 antibodies against oxLDL, it is unclear which one of these mechanisms occurs in response to the passive immunization and what influence they have on atherosclerosis. One theoretical possibility is that the predominance of the stimulatory or the inhibitory effect is dictated by the ratio between the antibodies and the available antigen amounts. An antigen surplus would stimulate B cells to produce more antibodies, in an attempt to neutralize the antigens. On the other hand, an increased amount of unbound circulating antibodies may signal the B cell that there are enough antibodies to that particular antigen, inhibiting further antibody production and inducing B cell apoptosis. The latter mechanism is probably more likely to occur in our experiments, because we inject a relatively high amount of antibodies at the same time. Indeed, Nicoletti and others reported a drop in IgM antibody titers following IgG-containing IVIg treatment.<sup>402, 427</sup> However, we have not determined the outcome of our treatments on the level of mouse IgM and IgG anti-oxLDL antibodies in the immunized animals. Future studies will focus on a more extensive characterization of the immuno-modulatory effects of the IgG1 treatment in mice. Either way, the potentiation or suppression of humoral immune responses in mice and their influence on the development of atherosclerosis will have to be carefully interpreted in correlation with the complex local and systemic effects of recombinant human IgG1 antibody treatment in mice.

In recent years, the role of regulatory T cells in atherosclerosis has raised considerable interest.<sup>352</sup> As discussed in the introduction, the regulatory T cells secrete the anti-inflammatory cytokines IL-10 and TGF $\beta$ , which suppress the activity of the Th1 and Th2 subtypes of helper T cells. Both the natural regulatory CD4+CD25+Foxp3+T<sub>reg</sub>

cells<sup>512</sup> and the antigen-specific Tr1 regulatory cells<sup>386</sup> have been shown to reduce atherosclerosis in mice. The mechanisms of Treg activation and regulation have not yet been characterized. A very interesting study by Kemper et al.<sup>513</sup> suggests the involvement of complement and a transmembrane complement-regulatory glycoprotein, membrane cofactor protein (MCP; CD46) in the development of regulatory T cells. Concomitant binding of CD3 and CD46 on the surface of CD4+ T cells leads to differentiation into IL-10 secreting Tr1 cells *in vitro*. It has previously been shown that the measles virus ligates CD46 on human monocytes leading to an impairment in the expression of IL-12.<sup>514</sup> IL-12 is necessary for activation and differentiation of the pro-inflammatory and pro-atherogenic Th1 lymphocyte subtype. The role of antibodies and immune complexes in Treg differentiation remains to be investigated, but links between the humoral immune responses and this important T lymphocyte subtype are likely to be demonstrated in the future.

## Drawbacks to using human antibodies in a mouse model

To summarize, the recombinant human IgG1 antibodies against MDA-modified peptide sequences of apoB-100 inhibit atherosclerosis progression and induce plaque regression. Inhibition of disease progression in mice by passive immunization was also reported by 2 other groups, using IgG or IgM antibodies against oxidized phospholipids.<sup>515, 516</sup> The atheroprotective mechanisms triggered by the antibodies remain to be thoroughly characterized. The antibodies may enhance the clearance of oxLDL from plasma and lesions, stimulate foam cell migration, increase reverse cholesterol transport and modulate the cellular and humoral immune responses against oxLDL. However, the results obtained in animal studies cannot be directly extrapolated to humans. Under hypercholesterolemic conditions, apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice develop atherosclerosis extremely rapidly and advanced atherosclerotic lesions are present in their arterial tree already after a few months of diet. In contrast, disease progression in humans is very slow and it takes several years for advanced lesions to form. Therefore, it would be unreasonable to expect effects of the same magnitude following passive immunization in humans. Lipid metabolism and the genetic factors are very different in mice and humans, and it is unclear how switching the high fat diet to normal chow in mice would parallel diet or lipid-lowering therapies in humans. The effects of recombinant human antibodies in mouse models are impaired to a certain extent. There is only an 85% homology between the peptide sequence of human apoB-100 against which the antibodies were produced, and the correspondent sequence of mouse apoB-100. This could reduce the binding affinity of the antibodies to mouse oxLDL and impair to a certain extent their effects, since we have shown in paper II that the affinity for oxLDL influences the effects of the antibodies. Additionally, apoE<sup>-/-</sup> mice mostly express mouse apoB-48 in their LDL particles and lack apoE, which functions as cholesterol

acceptor in the reverse cholesterol transport mechanism. The mice also developed a strong immunological response to the foreign human antibodies. There was a significant negative correlation between the amount of human antibodies still present in plasma at the time of sacrifice and the levels of mouse anti-human IgG1. Mouse antibodies may form immune complexes with the foreign antibodies, removing them from the circulation or blocking their Fc or Fab parts. Nevertheless, there was no association between human or mouse anti-human antibodies in plasma and plaque area upon sacrifice. The effects and the mechanisms of antibody therapy in humans will have to be carefully characterized. If similar atheroprotective effects are recorded, passive immunization with recombinant IgG1 antibodies against MDA-apoB-100 would constitute a novel therapeutical approach for prevention and treatment of atherosclerosis-related cardiovascular diseases.

## **MDA-LDL autoantibodies and human atherosclerosis**

The purpose of the fourth study was to determine the importance of IgG antibodies against a specific oxLDL epitope as diagnostic markers for atherosclerosis. The immune responses against oxLDL are potent modulators of atherosclerosis and autoantibodies against different oxLDL epitopes are present in the plasma of healthy subjects and cardiovascular disease patients. Several studies have tried to assess the value of these antibodies as markers of disease extent and activity and predictors of acute cardiovascular events. Analyzed together, the results are so far inconclusive. Regarding the extent of atherosclerosis in the carotid artery, both positive<sup>517, 518</sup> and negative<sup>441, 519</sup> correlations have been reported between the amount of IgG antibodies and IMT of the common carotid artery or the carotid bulb. As reported by some studies, patients with angiographically demonstrated coronary artery disease had higher oxLDL-IgG levels compared with their healthy controls.<sup>520-522</sup> However, others could not demonstrate such a correlation.<sup>523-525</sup> The development of acute myocardial infarction appears to be predicted by high levels of circulating IgG antibodies<sup>526, 527</sup> or immune complexes<sup>489, 528</sup>, which were also shown to drop after the acute event.<sup>529, 530</sup> We have previously demonstrated that the titers of IgM antibodies against different MDA-modified peptide sequences of apoB-100 correlated with carotid IMT in healthy subjects under 62 years of age.<sup>53</sup> These results contradict the findings of other studies, which established inverse correlations between the two variables.<sup>531, 532</sup> These discrepancies could have resulted from a pronounced heterogeneity in the experimental settings of these studies. The extent of atherosclerosis was studied at different stages, either in healthy subjects or in patients who have suffered an acute event or have symptomatic carotid or coronary artery disease. Men and women were analyzed separately or pooled together and the size of the groups and the age of the populations differed significantly. There may also be a great variability between dietary habits and genetic background between the different countries



where these studies were performed. Additionally, there are important differences among the laboratories regarding the MDA-LDL and the copper oxidized LDL (Cu-oxLDL) preparations used to capture the antibodies present in the plasma. Last but not least, the statistical methods and the criteria used to divide the populations into different groups vary among the studies. Thus, as underlined by several reviews on the roles of oxLDL antibodies as disease markers<sup>533-535</sup>, the size of the populations has to be significantly increased, the methods have to be standardized and the different epitopes carefully characterized before clear conclusions can be drawn and the different findings associated.

In paper IV of the present thesis we propose a slightly different approach to assess the connection between oxLDL antibodies and cardiovascular disease. In this study we have determined the correlations between the different IgG subclasses against a specific oxLDL epitope, an MDA-modified peptide sequence of apoB-100 (MDA-p45), and the extent of carotid stenosis and IMT. The value of these antibodies as predictors of myocardial infarction was also assessed. There are 4 different IgG isotypes (IgG1, IgG2, IgG3 and IgG4), which differ markedly in their characteristics and functions. IgG1 are the most abundant IgG antibodies in plasma, and are thought to be produced under the influence of IFN $\gamma$ , secreted by Th1 lymphocytes. IgG4 require Th2-secreted IL-4 for their synthesis. IgG1 and IgG3 are potent complement activators (although not as effective as IgM), while IgG4 lacks this ability. Thus, considering the different roles of Th1 and Th2 cells in atherosclerosis and the differences among IgG antibody isotypes, we hypothesized that it may be more appropriate to measure these isotypes separately instead of considering the entire IgG altogether. The population selected for this study consisted of 76 subjects who have developed acute myocardial infarction or death due to coronary artery disease, and 148 healthy controls matched for age, sex, smoking and hypertension. These subjects were part of the large Malmö Diet and Cancer Study. MDA-p45 was chosen because we have previously shown that IgM against this epitope positively correlates with carotid IMT in the same population.<sup>53</sup> We found no difference in any of the IgG isotypes between the patients and the controls, but the patients were shown to have a higher degree of carotid artery disease. When the 2 groups were pooled together, only the levels of IgG1 significantly correlated with the extent of atherosclerosis in the carotid artery, before and after adjusting for age, sex, systolic blood pressure, LDL and HDL cholesterol. Subjects with high MDA-p45 IgG1 levels had a lower degree of carotid stenosis and lower IMT in the carotid bulb. We found no associations between any of the antibodies and other cardiovascular risk factors. This study supports previous results by other groups<sup>441, 531</sup> and conflicts with the findings of Hulthe et al.<sup>517</sup>, who found a positive correlation between oxLDL-IgG and carotid IMT in healthy 58-year old men. However, it has to be stressed that, according to our results, only IgG1 correlated with disease extent. Additionally, we measured IgG against a specific epitope instead of the multiple-epitope MDA-LDL and Cu-oxLDL preparations used by others. IgG1 and IgM presented opposite trends

of association with the same parameters in this population, implying that they may have different roles in atherosclerosis or reflect different aspects of the disease.

Study IV brings further support for the atheroprotective role of IgG antibodies against MDA-p45, which was suggested by our animal studies. As previously discussed, both active immunization with the MDA-p45 peptide and passive transfer of recombinant human IgG1 antibodies against MDA-p45 reduced atherosclerosis in hypercholesterolemic mice. This study was too small to establish IgG1 antibodies against MDA-p45 as a marker to be used in clinical practice, but revealed one of the epitopes which seems to be involved in human atherosclerosis. Our assay is specific and highly reproducible and can represent a way for standardization of the different methods used to assess the extent of humoral immune responses against oxLDL. We also suggest that the different IgG isotypes are not equally involved and do not equally reflect the extent and progression of the disease. This hypothesis would have to be taken into account in future studies. However, the oxLDL particles and the atherogenetic process are very complex, and measuring antibodies against only one oxLDL epitope is unlikely to provide enough information for complete disease assessment. Multivariate analysis of the immune responses triggered by several of these epitopes may be required to determine disease activity and the risk for development of cardiovascular events.

## **Potential uses of the antibodies in clinical practice**

If proven to be effective in humans, the antibodies characterized in the present thesis could represent potential valuable tools for cardiovascular disease prevention, treatment, diagnosis and imaging. The possibility of using immunomodulation of atherosclerosis as a preventive treatment for cardiovascular diseases has raised considerable interest in the past few years. Different active immunization strategies have been proven to confer atheroprotection in mice and rabbits, following intravenous or intraperitoneal administration of MDA-LDL, MDA-modified apoB-100 peptide sequences, *Streptococcus Pneumoniae* or peptides containing a region of CETP. Additionally, mice exposed to nasal or oral administration of Hsp 65 developed less atherosclerosis, and it has been reported that vaccination against influenza appears to reduce hospitalization and death rate due to cardiovascular diseases in a general population.<sup>536</sup> The mechanisms triggered by these immunizations include production of atheroprotective IgG and IgM antibodies against MDA- or PC-containing oxLDL epitopes, activation of Th2-dependent immunity, inhibition of CETP or induction of immune tolerance.<sup>351</sup> There are important differences between the characteristics of active and passive immunization. The active immunization confers a long-term protection due to the development of memory T and B cells and production of antibodies against the

injected antigen, but it takes several weeks to develop such an immune response. Passive immunization offers rapid but limited protection by transfer of ready-made antibodies against the respective antigens. If demonstrated to be effective in humans, our antibodies could serve as a future therapeutic approach in patients at high risk to develop acute myocardial infarction or stroke. They may have additive effects to aggressive lipid-lowering therapies, which have only been shown to prevent approximately 30% of the acute cardiovascular events, because the two therapies act through different mechanisms. Because of the high costs implied by an antibody treatment, the target patient groups will have to be chosen based on carefully defined diagnostic parameters. Additionally, since they are specific for epitopes predominantly found in atherosclerotic lesions, the antibodies could also serve as carriers of different active principles for targeted activity inside the plaques.

In a series of studies, Tsimikas et al. demonstrated that  $^{125}\text{I}$ -labeled MDA2, a murine antibody specific for MDA-lysine epitopes on oxLDL, can provide accurate autoradiographic images of atherosclerotic plaques in mice and rabbits.<sup>242</sup> The uptake of  $^{125}\text{I}$ -labeled MDA2 into the plaques closely reflected diet-induced progressive or regressive changes in plaque oxLDL content.<sup>243, 244</sup> The method was proven to be superior to standard lipid staining procedures for detecting compositional changes of the lesions. By using  $^{99}\text{Tc}$ -MDA2 and a gamma-camera, the same group generated non-invasive *in vivo* scintigraphic images of atherosclerotic plaques in WHHL rabbits.<sup>245, 246</sup> The recombinant human IgG1 antibodies that we have used in our studies were also shown to recognize oxLDL epitopes in the atherosclerotic lesions. In a preliminary pilot imaging study, we have shown that the uptake of  $^{125}\text{I}$ -2D03 correlated with ORO-stained plaque area in the descending aorta of *apobec-1*<sup>-/-</sup>/*LDLR*<sup>-/-</sup> mice. The nature of these antibodies makes them suitable for human use. These studies open exciting new perspectives for the use of labeled antibodies in nuclear magnetic resonance or ultrasound *in vivo* imaging of oxLDL-rich, vulnerable atherosclerotic plaques in humans.

## Conclusion

In conclusion, the studies included in the present thesis demonstrate the ability of passive immunization with recombinant human IgG1 antibodies against a defined oxLDL epitope to reduce the extent of atherosclerosis in several mouse models. The remaining plaques presented a less inflammatory phenotype, with lower amounts of oxLDL epitopes and macrophages. These findings were further supported by the results of our clinical studies, showing a negative correlation between IgG1 antibody levels against the same epitope and carotid stenosis. If the same positive results will be obtained in humans, these antibodies could be developed into novel diagnostic and therapeutic tools for the management of atherosclerosis-related cardiovascular diseases.

## POPULÄRVETENSKAPLIG SAMMANFATTNING

Åderförfattning (även kallat åderförkalkning eller ateroskleros) börjar genom att det ”onda kolesterolet” LDL fastnar i väggen på ett blodkärl. Där kan LDL börja modifieras och orsaka inflammation. Inflammationen påskyndas av att vita blodceller kommer in i artärväggen och försöker ta bort fett. Om en stor mängd fett lagras in, kan det inte avlägsnas. Tillsammans med de inflammatoriska cellerna bildas en förtjockning av kärlväggen, ett så kallat aterosklerotiskt plack. Plack uppkommer mestadels i de stora artärerna, såsom aortan, halskärlen (karotiskärlen) och i kärlen i benen. De utvecklas också i mindre artärer, så som kranskärlen (koronarkärlen). Om placken växer till och blir mycket stora kan de förminska blodflödet genom kärlet. Den största risken med aterosklerotiska plack är att de kan brista och ge upphov till en blodpropp som kan stoppa blodflödet totalt, med hjärtinfarkt och stroke som följd.

Vårt immunsystem finns inte bara till för att skydda oss mot infektioner, utan det har också en mycket viktig roll i andra processer och sjukdomar, såsom t ex i ateroskleros. Det har visats att olika delar av immunsystemet kan både bidra till och skydda mot plackutveckling. Vi har lyckats identifiera flera strukturer på LDL partikeln som känns igen och aktiverar vårt immunsystem. Dessa strukturer finns i den modifierade formen av apoB-100, som är ett stort protein som håller samman LDL partikeln. När vi injicerade dessa proteindelar (s.k. peptider) i aterosklerosbenägna möss (som åt en fettrik kost för att öka plackutvecklingen), aktiverades immunsystemet och en stor mängd antikroppar bildades mot dessa. De behandlade mössen utvecklade upp till 60% mindre plack än obehandlade möss av samma typ. För att studera om det var antikropparna som bidrog till att djuren fick mindre ateroskleros, producerade vi konstgjorda IgG1 antikroppar som känner igen två av de skyddande peptiderna (p45 och p210).

Målen med de första tre studierna som inkluderats i denna avhandling var att testa om antikropparna kunde förminska placken i olika aterosklerosbenägna musmodeller, samt att försöka hitta vilka mekanismer som gav upphov till antikropparnas skyddande effekter.

I den första studien testade vi 6 olika antikroppar och fann att en av dem minskade plackutvecklingen i mössen med upp till 50%. Denna antikropp, IEI-E3, kände igen p45. Effekten var dosberoende, d.v.s. ju mer antikropp mössen fick ju mindre ateroskleros utvecklade de. I en andra studie testade vi om IEI-E3 antikroppen och även en annan antikropp, 2D03, kunde förminska de redan befintliga placken i aortan eller om de bara hade förmågan att förhindra vidareutveckling av placken. 2D03 känner igen samma peptid som IEI-E3 men binder mycket bättre till modifierat LDL. En grupp kontrollmöss avlivades vid samma ålder som när vi startade att injicera antikroppar

i övriga grupperna av möss, för att kunna jämföra omfattningen av plackytan efter behandlingen med den befintliga plackytan före behandlingen. Genom att både byta ut den feta kosten mot en vanlig muskost och samtidigt behandla mössen med antikroppar visade resultaten på en kraftig regression av aterosklerosen, d.v.s. de befintliga placken minskade med upp till 50%. 2D03 antikroppen hade en starkare effekt än IEI-E3, och en kontrollantikropp, som inte binder till modifierat LDL, hade ingen effekt på plackutvecklingen. Dessa resultat tyder på att antikropparnas påverkan på ateroskleros är beroende på deras förmåga att binda modifierat LDL. Vi studerade även plackkompositionen i dessa möss och såg att efter antikropsbehandlingen så var det betydligt mindre modifierat LDL och vita blodceller i de resterande aterosklerotiska placken.

Efter kärlkirurgiska ingrepp som tar bort plack eller utvidgar ett mycket förminskat kärl på grund av ateroskleros, så finns det en stor risk att kärlet täpps igen. Detta beror på att det snabbt kan återbildas plackliknande strukturer och även på att artären kan dra ihop sig p.g.a. den mekaniska skadan. I den tredje studien testade vi hur 2D03 antikroppen motverkar dessa processer i halspulsådern hos möss. I varje mus som fick antikropsbehandling skadades den högra halsartären medan den andra lämnades oskadad. Vi fann att 2D03 inte kunde motverka den nya plackbildningen i det skadade kärlet, men däremot så förhindrade den att artären drog ihop sig. Dessutom så minskade 2D03 kraftigt plackytan i den oskadade halsartären jämfört med hos kontrollmössen.

I den fjärde studien tittade vi på sambandet mellan antikropps mängden i humant plasma, storleken på befintliga plack i halspulsådern hos friska individer och risken för att utveckla kranskärlssjukdom. Det finns flera olika typer av antikroppar i blodet, varav IgG finns i störst mängd och i fyra olika subtyper: IgG1, IgG2, IgG3 och IgG4. Blod samlades in från 224 friska individer, mellan 49 och 67 år gamla, och mängden av varje subtyp, specifika för peptiden p45 uppmättes. När vi jämförde dessa värden med värdena på placktjockleken i halspulsådern i samma individer såg vi att ju mer IgG1 antikroppar som fanns i blodet, desto mindre plack. Dessa resultat tyder ännu en gång på att IgG1 antikropparna mot p45 har en skyddande effekt mot åderförkalkning. Av de 224 individerna i vår studie, utvecklade 76 stycken vid senare tidpunkt kranskärlssjukdom medan de andra 148 förblev friska. Vi kunde dock inte finna någon skillnad mellan de två grupperna vad det gäller mängden av de olika IgG antikroppar i plasma.

Sammanfattningsvis, vi har hittat strukturer i den modifierade LDL-partikeln som ger upphov till ett immunsvaret som kan skydda mot ateroskleros. De konstgjorda antikropparna som vi producerade mot dessa strukturer minskade kraftigt aterosklerosen i möss. Vanligt förekommande antikroppar riktade mot samma strukturer finns i blodet hos människa och tycks ha en skyddande effekt mot ateroskleros. Våra antikroppar kan i framtiden komma att utgöra en terapi som kan förhindra hjärtinfarkt och stroke. Emellertid, är det nödvändigt med ytterligare tester innan detta kan bli verklighet.

## REZUMAT

Ateroscleroza este o boală determinată de depunerea de colesterol, în principal sub formă de LDL, în peretele arterelor. În spațiul subendotelial particulele de LDL sunt oxidate și transformate în oxLDL. Prezența oxLDL constituie un puternic stimul pro-inflamator care activează celulele endoteliale și determină pătrunderea de leucocite, inclusiv macrofage, în *tunica intima*. Dacă influxul de colesterol depășește capacitatea macrofagelor de a fagocita și îndepărta particulele de oxLDL, inflamația se cronicizează și favorizează formarea plăcilor de aterom. Acestea sunt îngroșări ale peretelui arterial care prezintă un centru necrotic alcătuit din lipide extracelulare și celule moarte, acoperit înspre lumen de un înveliș fibros care conține collagen, fibre elastice și celule musculare netede. Plăcile ateromatoase sunt localizate cu predilecție în arterele mari și mijlocii (aortă, coronare, carotide, femurale). Principala complicație a aterosclerozei este ruptura plăcilor, urmată de tromboză locală. Trombul format poate bloca circulația sanguină local sau la distanță, prin formare de tromboemboli. Acest mecanism stă la baza majorității afecțiunilor ischemice, inclusiv a infarctului miocardic și cerebral.

Sistemul imunitar joacă un rol important în ateroscleroză. A fost demonstrat că diferitele tipuri de răspuns imun pot avea roluri opuse în ateroscleroză. În timp ce unele mecanisme favorizează dezvoltarea plăcilor de aterom, altele au un efect protector. Particulele de oxLDL constituie un important stimul imunogen. Studiile efectuate în laboratorul nostru au evidențiat epitopi proteici asociați acestor particule care sunt capabili să genereze un răspuns imun. Acești epitopi rezultă din fragmentarea și modificarea moleculei de apoB-100, principala proteină din structura LDL. Imunizarea unei anumite specii de șoareci, predispuși la ateroscleroză, cu peptide modificate provenind din apoB-100 activează sistemul imun, determinând creșterea sintezei de anticorpi IgG care reacționează specific cu aceste structuri. Suprafața plăcilor de aterom din aorta șoarecilor imunizați cu aceste peptide a fost cu până la 60% mai redusă comparativ cu cea a șoarecilor neimunizați.

Pentru a studia dacă transferul pasiv de anticorpi specifici pentru oxLDL are efect protector antiaterogen, am produs prin recombinare genetică 6 anticorpi IgG1 cu specificitate pentru două dintre peptidele din structura apoB-100, denumite p45 și p210. Scopul primelor trei studii incluse în lucrarea de față a fost testarea efectului acestor anticorpi asupra evoluției aterosclerozei la șoareci și evidențierea mecanismelor prin care poate fi influențată evoluția plăcilor de aterom.

Dintre cei 6 anticorpi testați în primul studiu, anticorpul IEI-E3 s-a evidențiat prin efectele sale asupra aterosclerozei, reducând dezvoltarea plăcilor de aterom cu până la 50%, în funcție de doza administrată. Într-un al doilea studiu am testat efectul anti-

corpului IEI-E3 comparativ cu cel al anticorpului 2D03, un anticorp cu specificitate pentru acelaşi epitop dar cu o afinitate crescută pentru oxLDL. Pentru a accelera dezvoltarea aterosclerozei, şoarecilor incluşi în acest studiu li s-a administrat într-o primă fază o dietă bogată în lipide, schimbată ulterior cu o dietă normală cu o săptămână înainte de prima administrare de anticorpi. Unul dintre loturile studiate a fost sacrificat înainte de începerea tratamentului, pentru a stabili dacă anticorpii administraţi reduc plăcile de aterom dezvoltate anterior sau doar încetinesc dezvoltarea ulterioară a aterosclerozei. Schimbarea dietei urmată de tratamentul cu anticorpi a determinat o regresie a plăcilor ateromatoase cu până la 50% comparativ cu grupul sacrificat la începutul tratamentului. Anticorpul 2D03 a avut un efect mai puternic decât IEI-E3. Utilizarea unui anticorp de control, căruia i-a lipsit capacitatea de a recunoaşte oxLDL, nu a avut nici un efect asupra aterosclerozei. Aceste rezultate sugerează că influenţa anticorpilor asupra evoluţiei plăcii de aterom este dependentă de capacitatea lor de a recunoaşte şi lega oxLDL. În plus, am demonstrat că plăcile rămase în urma tratamentului conţineau mai puţin oxLDL şi mai puţine macrofage, ceea ce sugerează că anticorpii pot determina un eflux de lipide şi macrofage din placă.

Una dintre principalele complicaţii ale angioplastiei, endarterectomiei sau a implantării de stenturi este restenozarea segmentului arterial supus intervenţiei. Restenozarea se datorează unei reacţii inflamatorii care determină dezvoltarea rapidă a unor structuri asemănătoare plăcilor de aterom, la care se adaugă îngustarea vasului ca rezultat al unei reacţii fibrotice cauzate de agresiunea mecanică. În al treilea studiu am cercetat influenţa anticorpului 2D03 asupra acestor procese în arterele carotide la şoareci. Efectele angioplastiei au fost simulate prin plasarea unui inel de plastic în jurul arterei carotide drepte, în timp ce carotida stângă nu a suferit nici o intervenţie. Anticorpul 2D03 nu a avut efect asupra aterogenezei în carotida dreaptă la locul intervenţiei, în schimb a redus semnificativ dezvoltarea plăcilor de aterom la nivelul carotidei stângi, comparativ cu grupul de control.

Într-un alt studiu am cercetat corelaţiile dintre cantitatea de anticorpi din sânge, mărimea plăcilor de aterom din artera carotidă şi riscul de a dezvolta ischemie coronariană acută, infarct miocardic sau moarte subită coronariană. În acest studiu am recoltat sânge de la 224 de subiecţi sănătoşi, cu vârsta cuprinsă între 49 şi 67 de ani, şi am determinat nivelul anticorpilor specifici pentru p45 din fiecare subclasă de IgG (IgG1, IgG2, IgG3, şi IgG4). Un nivel ridicat de IgG1 anti p45 a fost corelat cu un grad redus de stenoză carotidiană la aceşti subiecţi. Rezultatul studiului este, la rândul lui, sugestiv pentru efectul protector al IgG1 în ateroscleroză. Din cei 224 de subiecţi incluşi în studiu, 76 au suferit ulterior un episod coronarian acut. Cu toate acestea, nu am constatat diferenţe cantitative privind concentraţia de anticorpi IgG specifici pentru p45 între subiecţii acestui lot şi lotul de control.

În concluzie, studiile noastre au pus în evidență structuri antigenice prezente în compoziția oxLDL capabile să determine un răspuns imun cu efect protector antiaterogen. Folosind tehnici de recombinare genetică am produs anticorpi IgG1 specifici pentru epitopii evidențiați și am demonstrat că tratamentul cu acești anticorpi reduce semnificativ ateroscleroza la șoareci. Este posibil ca anticorpii IgG1 specifici pentru același epitop aflați în sângele uman să aibă de asemenea un efect protector antiaterogen. Anticorpii testai în laboratorul nostru ar putea să constituie în viitor un tratament rapid și eficient pentru prevenția accidentelor vasculare ischemice datorate rupturii plăcilor de aterom, inclusiv a infarctului miocardic sau cerebral. Pentru a putea implementa acest tratament în practica medicală, vor trebui evaluate cu atenție efectele anticorpilor asupra dezvoltării aterosclerozei umane, precum și eventualele efecte secundare ale tratamentului.





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# I

*The most exciting phrase to hear in science,  
the one that heralds the most discoveries, is not  
'Eureka!' (I found it!) but 'That's funny'*

*Isaac Asimov*



# Recombinant Human Antibodies Against Aldehyde-Modified Apolipoprotein B-100 Peptide Sequences Inhibit Atherosclerosis

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**Background**—Accumulation and oxidation of LDL are believed to be important initiating factors in atherosclerosis. Oxidized LDL is recognized by the immune system, and animal studies have suggested that these immune responses have a protective effect against atherosclerosis. Aldehyde-modified peptide sequences in apolipoprotein B-100 (apoB-100) are major targets for these immune responses.

**Methods and Results**—Human IgG1 antibodies against 2 malondialdehyde (MDA)-modified apoB-100 peptide sequences were produced through screening of a single-chain antibody-fragment library and subsequent cloning into a pcDNA3 vector. Three weekly doses of these antibodies were injected into male apoE<sup>-/-</sup> mice. Phosphate-buffered saline and human IgG1 antibodies against fluorescein isothiocyanate were used as controls. One of the IgG1 antibodies significantly and dose-dependently reduced the extent of atherosclerosis as well as the plaque content of oxidized LDL epitopes and macrophages. In cell culture studies, human monocytes were incubated with native LDL or oxidized LDL, in the presence of antibodies. The same antibody induced an increase in monocyte binding and uptake of oxidized LDL.

**Conclusions**—These findings suggest that antibodies are important mediators of atheroprotective immune responses directed to oxidized LDL. Thus, passive immunization against MDA-modified apoB-100 peptide sequences may represent a novel therapeutic approach for prevention and treatment of cardiovascular disease. (*Circulation*. 2004;110:2047-2052.)

**Key Words:** atherosclerosis ■ antibodies ■ apolipoproteins ■ immune system ■ plaque

Atherosclerosis develops as a result of chronic arterial inflammation.<sup>1</sup> Innate and adaptive immune responses against oxidized LDL (oxLDL) are believed to play important roles in this inflammatory process. The oxidation of aggregating LDL in the extracellular matrix of the artery wall leads to the formation of highly reactive lipid peroxides and aldehydes.<sup>2,3</sup> The LDL protein apolipoprotein B-100 (apoB-100) is degraded, and aldehydes bind to free amino groups on the peptide fragments. This is associated with activation of an inflammatory response, including endothelial expression of adhesion molecules and infiltration of monocytes/macrophages and T cells.<sup>4</sup> Macrophages express a family of scavenger receptors, which bind and ingest oxLDL particles.<sup>5</sup> Continuous activation of such innate immune responses is believed to be a major cause of atherosclerotic plaque development.<sup>6</sup>

The presence of oxLDL also leads to the activation of more specific adaptive immune responses.<sup>7</sup> T cells in atherosclerotic lesions have been shown to recognize epitopes on oxLDL when presented by macrophages in combination with

major histocompatibility class II molecules.<sup>8</sup> In atherosclerosis, the adaptive immune response has been suggested to provide atheroprotective effects. A number of studies have shown that immunization of hypercholesterolemic animals with native or oxLDL leads to a significant reduction of atherosclerosis development.<sup>9,10</sup>

Using a library of malondialdehyde (MDA)-modified polypeptides covering the complete amino acid sequence of human apoB-100, we have recently identified a large number of epitopes recognized by antibodies present in human plasma.<sup>11</sup> The levels of several of these antibodies show an inverse association with plasma oxLDL, suggesting that antibodies are involved in the clearance of these particles. Immunization of apoE<sup>-/-</sup> mice with the corresponding human apoB peptides was found to result in reduced plaque formation and a stable plaque phenotype, as indicated by increased collagen content.<sup>12</sup> This effect was associated with increased formation of IgG against the respective apoB-100 peptides. To further study the role of these IgG antibodies in the atheroprotective response and to test whether specific MDA-

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TABLE 1. CDR Sequences of the 6 Antibodies (Ab) Directed to Different MDA-ApoB-100 Peptide Sequences

Ab	H1	H2	H3	L1	L2	L3
IEI-A8	FNNAWMSWRQAPG	SSISSSSSYYIYADSVKGR	ARVSRYYYGPFYFDS	CSGSRNIGNNYVS	GNNNRPS	CAAWDDSLNGHWV
IEI-E3	FSDYYMSWRQAPG	SGVSWNGSRTHYADSVKGR	ARAARYSYYYGMVDV	CSGSSNIGNNAVN	GNDRRPS	CQWTGTGRGV
IEI-D8	FSNAWMSWRQVPG	STLGGSGGGSTYYADSVKGR	AKLGRRSRYGRWRPQFDY	CSGSSNIGNNYVS	SNNQRPS	CAAWDDSLSHWL
IEI-G8	FSSYMSWRQAPG	SSISGSGRRYYADSVQGR	ARLVSYGSGSPGFDY	CSGSSNIGSNYVS	GNYNRPS	CAAWDDSLSGWV
KTT-B8	FSSYMSWRQAPG	SSISGSGRFYYADSMKGR	TRLRRGSYFWAFDI	CSGSSNIGGESVS	CSGSSNIGGESVS	SNNQRPS
KTT-D6	FSDYYMSWRQAPG	SSISGRGSSYYADSVRGR	ARLSYSYVYEGAYYFDY	CSGSSNIGNNYVS	CSGSSNIGNNYVS	RNNQRPS

CDR indicates complementarity-determining region; H1, H2, and H3, CDR1, 2, and 3 in the heavy chain, respectively; L1, L2, and L3, CDR1, 2, and 3 in the light chain, respectively.

apoB-100 antibodies could be used for direct inhibition of atherosclerosis in apoE<sup>-/-</sup> mice, we produced recombinant human IgG1 that specifically recognizes 2 MDA-modified sequences in human apoB-100. Active immunization with these peptides has previously been shown to reduce atherosclerosis by ≈50% in mice.<sup>12</sup>

### Methods

#### Generation of Human Recombinant Antibodies to Human MDA-Modified ApoB-100-Derived Peptides

Previous studies have shown high levels of IgG in coronary heart disease patients (P45) or high IgM and IgG levels in healthy controls (P210) against the MDA-modified peptides used.<sup>11</sup> Single-chain human antibody fragments with specificity for MDA-modified apoB-100-derived peptides P45 (IEIGLEGKGFEPTEALFGK, amino acids 661 to 680) or P210 (KTTKQSFDSLVSVAQYKKNKH, amino acids 3136 to 3155) were selected from the single-chain fragment-variable (scFv) n-CoDeR library, essentially as described earlier.<sup>13</sup> In brief, 3 consecutive rounds of selection were performed with 10 pmol of MDA-modified peptide bound to a solid phase. Competitors comprising unmodified peptide and an MDA-modified nonrelated peptide were included at a concentration of  $4 \times 10^{-7}$  mol/L in the last selection round to secure specificity against the MDA-modified peptides. Selected scFv were screened for specific binding to MDA-modified peptide in an automated system with an ELISA format with luminescence as the readout.<sup>14</sup>

The sc antibody fragments identified as being specific for the MDA-modified variants of the peptides were then transferred from the scFv format to a full-length human IgG1 $\lambda$  format through cloning into a modified pcDNA3 vector.<sup>15</sup> The different complementary determining region sequences of these are presented in Table 1. The cloned sequences were then transfected into NS0 cells with Lipofectamin 2000 reagent (Invitrogen), and transfectants were selected by using G418 sulfate (Invitrogen) as described.<sup>15</sup> Human IgG1 was purified from spent cultivation medium on a MabSelect protein A column (Amersham Biosciences). The purity of the preparations exceeded 98%, as determined from polyacrylamide gel electrophoresis analysis, and contained between 1 and 12 endotoxin units/mL, as tested by a limulus amoebocyte lysate test (QCL-1000, BioWhittaker). The specificity of the purified IgGs for MDA-modified LDL was demonstrated with a luminescence-based ELISA, in which the wells were coated with 0.5  $\mu$ g/well LDL or MDA-modified LDL, and bound IgG was detected with horseradish peroxidase-conjugated rabbit anti-human IgG ( $\gamma$ -chain) antibody (DAKO).

#### Analysis of Clones With Biacore

The antigens were immobilized on a CM5 chip in a Biacore 3000 (Biacore). Human MDA-modified apoB-100 (Academy Bio-Medical Co) was immobilized to a total signal of 7000 Biacore relative units by amino coupling. As a reference, human apoB-100 was used. Five different concentrations (100, 25, 6.25, 1.56, and 0.39 nmol/L) of each antibody were injected consecutively on the chip. The resulting binding curves were analyzed with BiaEvaluation software (Bia-

core). Between each run, the chip was regenerated with 10 mmol/L NaOH.

#### Mice, Immunization, and Tissue Preparation

Male apoE<sup>-/-</sup> mice on a C57BL/6 background from B&M (Ry, Denmark) were used in the present studies (n=72, 7 groups of 9 mice for the first study and n=90, 9 groups of 10 mice for the second). From 6 weeks of age they were fed a high-cholesterol diet (0.15% cholesterol, 21% fat; Lactamin AB) provided ad libitum. At 21 weeks of age the mice were injected intraperitoneally with 0.5 mL (0.5 mg/dose in the first study; 0.25, 0.5, and 2.0 in the second) of the human IgG1 antibodies directed to MDA-modified apoB-100 peptides (see earlier sections). As controls, phosphate-buffered saline (PBS) or nonspecific human IgG1 antibodies directed to fluorescein isothiocyanate (FITC) were used. The injections were repeated 2 times at 1-week intervals.

All mice were humanely killed at 25 weeks of age by exsanguination through cardiac puncture under anesthesia with 300  $\mu$ L distilled water, fentanyl/fluanisone, and midazolam (2:1:1, vol/vol/vol), administered intraperitoneally. After whole-body perfusion with PBS followed by Histochoice (Amresco), the heart and the aortic arch were dissected out and stored in Histochoice at 4°C until processing. The descending aorta was dissected free of external fat and connective tissue, cut longitudinally, and mounted on face, lumen side up, on ovalbumin- (Sigma) coated slides (termed flat preparation).<sup>16</sup> The Animal Care and Use Committee approved the experimental protocol used in this study.

#### Analysis of Lipid, Macrophage, and oxLDL Epitopes in Plaques

Staining and quantification of plaque area in flat preparations of descending aorta and subvalvular plaque macrophage content were done as previously described.<sup>12</sup> A protocol similar to that for macrophage staining was used for detection of oxLDL epitopes in the plaques with IEI-E3 (100  $\mu$ g/mL) as the primary antibody and a biotinylated mouse anti-human IgG1 antibody (25  $\mu$ g/mL; ImmunKemi F&D AB) diluted in PBS as the secondary antibody.

#### Serum Cholesterol and Triglyceride

Total plasma cholesterol and plasma triglycerides were quantified by colorimetric assays (Infinity cholesterol and triglyceride, respectively; Sigma). ApoB-containing lipoproteins were precipitated with MgCl<sub>2</sub> and dextran sulfate as previously described.<sup>12</sup>

#### Preparation of Unlabeled and <sup>125</sup>I-Native LDL or oxLDL

LDL was isolated from blood by sequential preparative ultracentrifugation in a narrow density range (1.034 to 1.054 kg/L). Copper-mediated oxidation was achieved by incubating freshly prepared LDL in PBS with a sterile solution of CuCl<sub>2</sub> at a final concentration of 10  $\mu$ mol/L. The extent of LDL modification was assessed electrophoretically. Native LDL and oxLDL were labeled by the iodine monochloride method. The endotoxin levels in both preparations were <0.015 endotoxin units/mL.

**TABLE 2. Analysis of Clones With Biacore**

Antibody	On Rate ( $k_{on}$ )	Off Rate ( $k_{off}$ )	Equilibrium ( $K_D$ )
IgG1 IEI-A8	$4 \times 10^5$	$1 \times 10^{-3}$	$3 \times 10^{-9}$
IgG1 IEI-G8	$5 \times 10^4$	$3 \times 10^{-4}$	$6 \times 10^{-9}$
IgG1 KTT-D6	$3 \times 10^4$	$2 \times 10^{-4}$	$7 \times 10^{-9}$
IgG1 KTT-B8	$8 \times 10^3$	$2 \times 10^{-5}$	$3 \times 10^{-9}$
IgG1 IEI-E3	$2 \times 10^4$	$3 \times 10^{-4}$	$1 \times 10^{-8}$
IgG1 IEI-D8	$3 \times 10^4$	$2 \times 10^{-4}$	$5 \times 10^{-9}$

**Isolation and Culture of Monocytes**

Human monocytes were isolated from buffy coats from different donors by the Ficoll-Hypaque procedure, plated at a density of  $4 \times 10^6$  cells/mL into 12-wells plate (1 mL/well), and cultured in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 2 mmol/L *N*-acetyl-L-alanyl-L-glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 1% nonessential amino acids, 2% sodium pyruvate, and 20 mmol/L HEPES without serum at 37°C in 5% CO<sub>2</sub>. The experiments were performed within 24 hours after plating of monocytes.

**<sup>125</sup>I-Native LDL and <sup>125</sup>I-oxLDL Uptake Assay**

Monocytes were incubated in the absence or presence of labeled native LDL (40  $\mu$ g/mL) or oxLDL (50  $\mu$ g/mL), alone or combined with the antibodies (100  $\mu$ g/mL). Thereafter, the cells were washed with PBS and scraped into 0.5 mol/L NaOH for uptake measurement. The radioactivity was determined in an LKB 1271 automatic gamma counter.

**Native LDL and oxLDL Binding Assay**

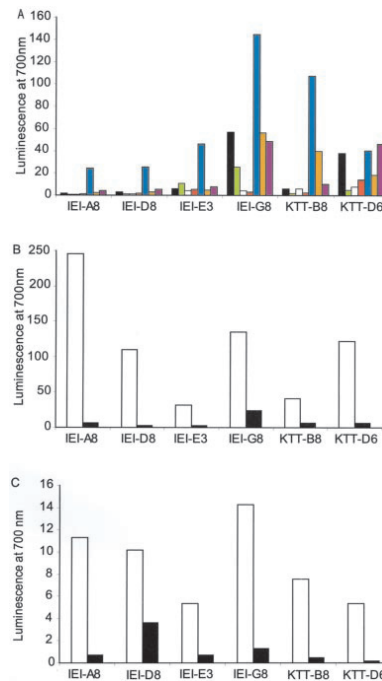
Monocytes were incubated in the absence or presence of antibodies and unlabeled native LDL or oxLDL, alone or in combination. LDL binding studies at 4°C were performed as previously described.<sup>17</sup> Radioactivity of released <sup>125</sup>I-native LDL or <sup>125</sup>I-oxLDL from monocytes was measured in a gamma counter.

**Statistical Analysis**

Data are presented as mean  $\pm$  SD. Analysis of the data was performed with the Mann-Whitney 2-tailed test. Statistical significance was considered at a level of  $P \leq 0.05$ .

**Results**

A total of 4 scFv with specificity for the MDA-modified apoB-100 peptide composed of amino acids 661 to 680 (IEI-A8, IEI-D8, IEI-E3, IEI-G8) and 2 for the peptide consisting of amino acids 3136 to 3155 (KTT-B8, KTT-D6) were identified and chosen to be transformed to the human IgG1 format after the selection and screening process. The affinity of the antibodies to human MDA-modified apoB-100 was compared with the Biacore technique (Table 2), and specificity was assessed by using a number of different MDA-modified antigens (Figure 1A). The IEI-E3 antibody had a lower affinity but was relatively more specific to the MDA-IEI peptide compared with a high-affinity binder such as IEI-G8 (Table 2 and Figure 1A). None of the scFv recognized the respective unmodified apoB-100 peptide (data not shown). Furthermore, the scFv bound to MDA-modified but not to native LDL (Figure 1B). Also, after the specificities had been transferred to the IgG1 format, this selectivity for MDA-modified LDL was evident (Figure 1C), demonstrating a desired target specificity of the antibodies.



**Figure 1.** Binding of selected scFv (A) to number of different MDA-modified antigens. P2 (amino acids [aa] 16 to 31, red), P45 (aa 661 to 680, blue), P143 (aa 2131 to 2150, orange), P210 (aa 3136 to 3155, purple), and P301 (aa 4502 to 4521, white) are peptides corresponding to human apoB-100 sequence. Control peptide is nonrelevant lysine-containing peptide (MDA-modified, black; unmodified, green). B illustrates binding of scFv to native (black) and MDA-modified (white) human LDL and (C) binding of cloned human IgG1 to native (black) and MDA-modified (white) human LDL. In A and B, luminescence ELISA data are presented as signal/buffer, whereas in C, data are plotted as signal/10<sup>5</sup>. Abbreviations are as defined in text.

The effect of the antibodies on the development of atherosclerosis was analyzed in apoE<sup>-/-</sup> mice fed a high-cholesterol diet. The mice were given 3 intraperitoneal injections of 0.5 mg antibody at 1-week intervals starting at 21 weeks of age, with PBS as a control. The mice were humanely killed 2 weeks after the last antibody injection. The characteristics of the different groups are presented in Table 3. The extent of atherosclerosis was assessed by oil red O staining of descending aorta flat preparations. The most pronounced effect was observed in mice treated with the IEI-E3 antibody, with a >50% reduction of atherosclerosis compared with the PBS group ( $P=0.02$ , Table 3). The mice tolerated the human antibodies well, and no effects on general health status of the mice were evident. The plasma levels of human IgG1 and murine anti-human IgG1 were measured at euthanization by ELISA (Table 3). There was no association between human IgG1 levels and total plaque area ( $r=0.08$ , NS) or between mouse anti-human IgG1 levels and total plaque area ( $r=0.04$ , NS). However, a strong inverse correlation between the levels



**TABLE 3. Overview of Results From the First Study**

Antibody	Plaque Area of Descending Aorta, % of Total Area	Macrophage-Stained Area, mm <sup>2</sup> /Section	Body Weight, g	Cholesterol, mg/mL	Triglycerides, mg/mL	HDL, mg/mL	Human IgG1, $\mu$ g/mL	Anti-Human IgG1, RLU
IEI-A8	0.60 $\pm$ 0.59	0.056 $\pm$ 0.023	31.25 $\pm$ 4.89	0.94 $\pm$ 0.30*	0.26 $\pm$ 0.08	0.35 $\pm$ 0.10	BDL	826.9 $\pm$ 194.5
IEI-G8	0.82 $\pm$ 0.85	0.054 $\pm$ 0.022	34.44 $\pm$ 4.44	1.09 $\pm$ 0.23	0.33 $\pm$ 0.11	0.33 $\pm$ 0.13	1.97 $\pm$ 2.96	140.7 $\pm$ 178.1
IEI-D8	0.56 $\pm$ 0.43	0.054 $\pm$ 0.015	32.44 $\pm$ 2.60	0.77 $\pm$ 0.31*	0.22 $\pm$ 0.05	0.31 $\pm$ 0.08	BDL	801.9 $\pm$ 224.6
IEI-E3	0.40 $\pm$ 0.34*	0.048 $\pm$ 0.017	38.00 $\pm$ 4.58	1.20 $\pm$ 0.32	0.36 $\pm$ 0.11	0.38 $\pm$ 0.10	8.35 $\pm$ 19.84	231.8 $\pm$ 223.9
KTT-D6	0.54 $\pm$ 0.41	0.054 $\pm$ 0.011	35.75 $\pm$ 6.62	0.97 $\pm$ 0.19*	0.24 $\pm$ 0.05	0.24 $\pm$ 0.09	47.88 $\pm$ 39.84	349.5 $\pm$ 394.1
KTT-B8	0.62 $\pm$ 0.50	0.043 $\pm$ 0.015	32.44 $\pm$ 3.57	1.19 $\pm$ 0.31	0.32 $\pm$ 0.12	0.26 $\pm$ 0.05	0.38 $\pm$ 0.70	284.4 $\pm$ 221.1
PBS	0.86 $\pm$ 0.58	0.052 $\pm$ 0.019	37.11 $\pm$ 7.81	1.42 $\pm$ 0.54	0.31 $\pm$ 0.01	0.33 $\pm$ 0.12	BDL	41.2 $\pm$ 13.8†

RLU indicates relative luminescence units; BDL, below detection level.

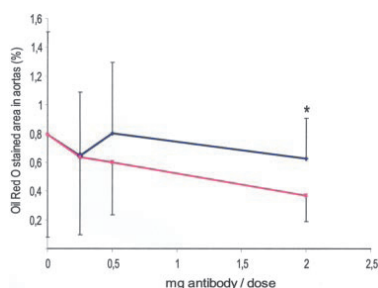
\* $P$ <0.05 vs PBS.

†Values represent background.

of mouse anti-human IgG1 and human IgG1 ( $r=-0.56$ ,  $P<0.001$ ) was observed.

To verify the inhibitory effect of the IEI-E3 antibody on the development of atherosclerosis, we then performed a dose-response study. The design was identical to that of the initial study with the exception that human IgG1 against FITC (FITC-8) was also used as a specificity control, in addition to PBS. In mice treated with IEI-E3 antibodies, atherosclerosis was reduced by 2% in the 0.25-mg group ( $P=NS$ ), by 25% in the 0.5-mg group ( $P=NS$ ), and by 41% in the 2.0-mg group ( $P=0.035$ ) compared with the corresponding FITC-8 antibody-treated groups (Figure 2). There was also a 33% reduction ( $P=0.02$ ) of macrophage immunostaining in atherosclerotic plaques in mice treated with 2 mg IEI-E3 antibody compared with the matching FITC-8 antibody group (Figure 3A, 3B, and 3E).

Immunohistochemical staining with IEI-E3 as the primary antibody demonstrated the presence of the IEI-E3 epitope predominantly close to the lumen (Figure 3C and 3D). Blocking experiments by preincubation of the IEI-E3 antibody with human oxLDL and native LDL confirmed that the staining was specific for oxLDL (data not shown). There was



**Figure 2.** Dose-response curve showing increased reduction in plaque area in descending aortas of apoE<sup>-/-</sup> mice. Mice were treated with different doses of IEI-E3 (red) or FITC-8 (blue) antibodies. Values on y axis represent oil red O-stained areas as percentage of total descending aorta area; values on x axis represent milligrams of antibody per injected dose. \* $P$ <0.05 vs FITC-8. Abbreviations are as defined in text.

a 20% reduction ( $P=0.04$ ) in IEI-E3 immunostaining in plaques of mice treated with 2 mg IEI-E3 antibody compared with the FITC-8 controls (Figure 3F). However, no human IgG1 was detected in the atherosclerotic plaques at the time of euthanization (data not shown).

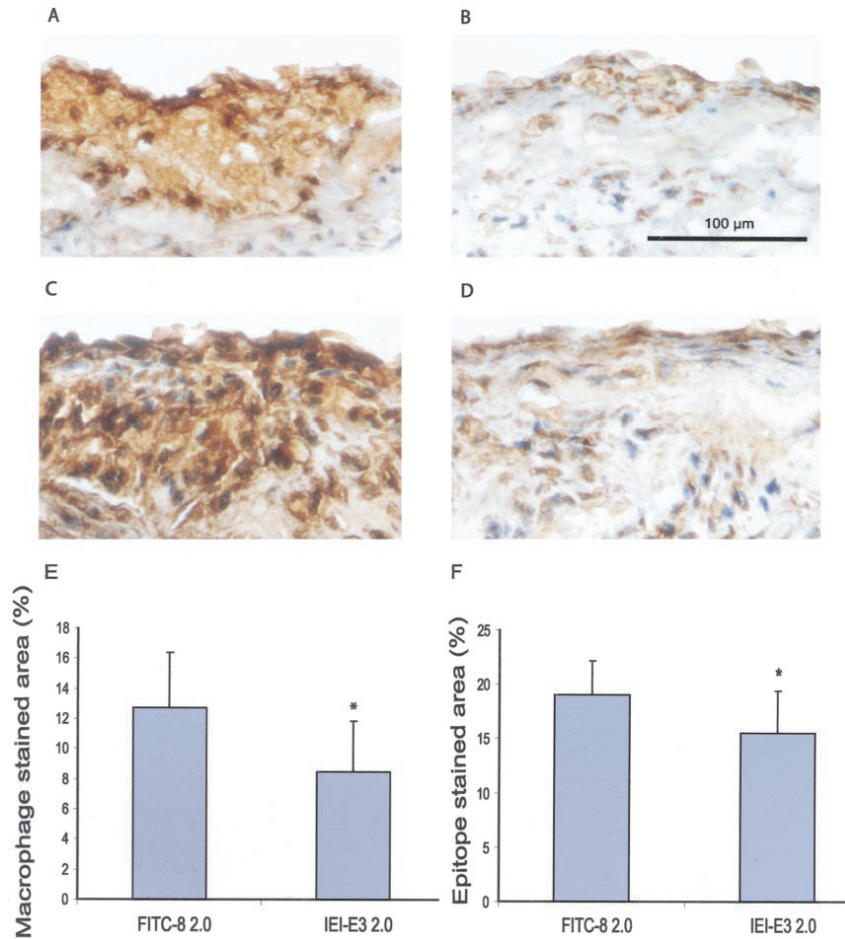
We also studied how the antibodies influenced the metabolism of oxLDL by analyzing the binding and uptake of oxLDL in cultured human monocytes/macrophages. Addition of IEI-E3 antibodies resulted in an increase in the binding ( $P=0.001$ ) and uptake ( $P=0.006$ ) of oxLDL compared with FITC-8. Similar observations were also made after incubation with IEI-D8 ( $P=0.004$  and  $P=0.001$ , respectively) and KTT-B8 ( $P=0.004$  and  $P=0.001$ , respectively) antibodies, whereas there was no effect of the antibodies on the binding and uptake of native LDL (Figure 4A and 4B).

## Discussion

oxLDL particles contain MDA-modified peptide fragments derived from degradation of apoB-100.<sup>2</sup> Autoantibodies against several such MDA-modified apoB-100 peptides have been found in humans.<sup>11</sup> The present studies show that human IgG1 generated against one of these MDA peptide sequences reduces atherosclerosis in apoE<sup>-/-</sup> mice and that this is associated with reduced accumulation of the corresponding oxLDL-associated epitope and of macrophages in atherosclerotic plaques.

These observations are consistent with earlier studies demonstrating that immunization with oxLDL inhibits the development of atherosclerosis in mice and rabbits.<sup>9,10</sup> Activation of this protective immunity is associated with a marked increase in oxLDL-specific IgG. We have recently identified a large number of MDA-modified sequences in apoB-100 that are recognized by antibodies present in human plasma.<sup>11</sup> Immunization of apoE<sup>-/-</sup> mice with some of these peptide sequences resulted in inhibition of atherosclerosis to a similar extent as that observed after immunization with oxLDL and was also associated with an increase in peptide-specific IgG.<sup>12</sup>

The present findings suggest that specific antibodies constitute an important component of atheroprotective immunity but do not exclude the involvement of cell-mediated immunity. Support for the existence of atheroprotective humoral immunity also comes from studies in apoE<sup>-/-</sup> mice demonstrating inhibi-



**Figure 3.** Staining of macrophages and oxLDL epitopes in subvalvular plaques of apoE<sup>-/-</sup> mice. Staining of macrophages in groups injected with (A) FITC-8 and (B) IEI-E3 antibodies, respectively. IEI-E3 epitope staining in plaques from same groups, (C) FITC-8 and (D) IEI-E3, respectively. Values represent percentage of stained area per total subvalvular plaque area, (E) macrophage staining, and (F) epitope staining \*P<0.05 vs FITC-8. Abbreviations are as defined in text.

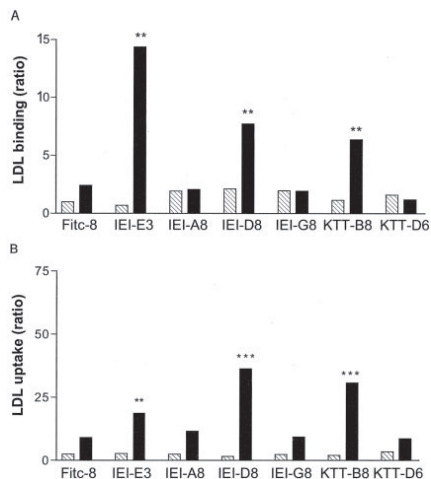
tion of atherosclerosis by repeated injections of polyclonal IgG and by B-cell rescue of splenectomized mice.<sup>18,19</sup>

The mouse model of atherosclerosis used in this study has some limitations when it comes to analyzing the effect of human antibodies against human oxLDL epitopes. Homology to the corresponding mouse apoB-100 sequences is not complete (95%), and the sequence recognized by the KTT antibodies is not expressed in the majority of mouse LDL particles in apoE<sup>-/-</sup> mice, because most of these are carrying apoB-48.<sup>20,21</sup> Moreover, the protective effect of human antibodies may be inhibited by expression of mouse antibodies against human IgG1, which were found to be present in all IgG-treated mice at the time of euthanization. These circum-

stances are likely to limit the effectiveness of the antibody treatments in mice by inducing clearance of the human IgG1.

Autoantibodies specific for the same epitopes as IEI and KTT antibodies are present in humans. IgM levels against these epitopes show significant correlations with plasma levels of oxLDL and carotid artery intima-media thickness, suggesting that they are involved in the disease process.<sup>11</sup> IgG against the same epitopes is present only at lower levels.

The mechanisms through which IgG1 directed to aldehyde-modified apoB-100 peptides sequences inhibits atherosclerosis in mice remains to be clarified. Low numbers of the MDA-apoB-100 epitope in plaques treated with the corresponding IgG1 suggest that these antibodies inhibit uptake of oxLDL in



**Figure 4.** Binding and uptake of  $^{125}$ I-labeled native (striped) or oxLDL (black) in monocytes. Binding (A) and uptake (B) properties are shown in presence of different human IgG1 antibodies directed to MDA-modified apoB-100 peptides and to FITC-8 as control antibodies. Values are given as ratio of counts per minute values of  $^{125}$ I-labeled native LDL or oxLDL and cpm values of control. \*\* $P < 0.01$  vs FITC-8; \*\*\* $P < 0.001$  vs FITC-8. Abbreviations are as defined in text.

plaques and/or facilitate the removal of oxLDL from the circulation or plaques. The decrease in plaque macrophage immunoreactivity observed with the highest IEI-E3 dose indicates reduced inflammation, which in turn could slow disease progression. There is also some support for a removal effect of oxLDL from clinical studies demonstrating an inverse relation between antibody levels and oxLDL in plasma.<sup>11</sup>

The IEI-E3 antibody effectively enhanced binding and uptake of oxLDL in cultured human monocytes/macrophages but did not affect the binding and uptake of native LDL. This mechanism represents a possible removal pathway of oxLDL, either by Kupffer cells in the liver or by macrophages in peripheral tissues. In contrast, Hörkkö et al<sup>22</sup> have shown that IgM directed to oxLDL phospholipids, but not IgM directed to MDA-LDL, inhibits oxLDL uptake by macrophages. Taken together, these observations suggest that IgG mediates uptake of oxLDL through binding to Fc receptors, whereas IgM may lack this effect.

The ability to induce an atheroprotective immunity by active or passive immunization against oxLDL epitopes has been clearly established in experimental animals. In this study, the antibodies found to inhibit atherosclerosis were human IgG1 specific for MDA-modified human apoB-100 sequences. However, it still remains to be determined whether a similar atheroprotective immunity can be induced in humans. If this is shown to be the case, it would represent a possible novel therapeutic approach for prevention and treatment of cardiovascular disease.

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# III

*If we knew what it was we were doing,  
it would not be called research, would it?  
Albert Einstein*



# Human Recombinant Antibodies to an Oxidized LDL Epitope Induce Rapid Plaque Regression in LDL Receptor *apobec-1* Double Knockout Mice

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**Objective:** A human recombinant antibody against a specific epitope in oxidized LDL was previously shown to reduce the development of early atherosclerosis in mice. This study tested the hypothesis that treatment with human recombinant antibodies against a specific epitope on oxidized LDL will induce regression of existing atherosclerotic lesions.

**Methods:** *Apobec-1*<sup>-/-</sup>/*LDLR*<sup>-/-</sup> mice were fed a high fat diet until 24 weeks of age and subsequently transferred to chow diet. Starting at 25 weeks mice were given three injections of two recombinant human IgG1 antibodies (IEI-E3 and 2D03) against a malondialdehyde-modified apoB-100 peptide sequence or control IgG1 (anti-FITC) with one week intervals and the effect on atherosclerosis in the aorta was assessed by *en face* Oil Red O staining at 29 weeks. Innominate artery plaque macrophage content was assessed by immunohistochemistry.

**Results:** At 25 weeks 10.3±3.7% of the aorta was covered by atherosclerotic lesions. Transfer to chow diet resulted in modest regression of atherosclerosis (8.28±4.36%, n.s.). Treatment with 2D03 and IEI-E3 IgG1 induced an additional regression in atherosclerosis by 50% (*P*=0.003) and 36% (*P*=0.003) respectively, whereas treatment with the control antibody had no effect. Administration of antibodies had no effect on body weight and circulating levels of cholesterol or triglyceride, but 2D03 significantly reduced the inflammatory phenotype of innominate artery plaques.

**Conclusions:** Our study demonstrates that human IgG1 against a specific oxidized LDL epitope reduces plaque inflammation and can induce rapid and substantial regression of atherosclerotic lesions over and above that induced by low fat diet.

**Key words:** atherosclerosis, antibodies, apolipoprotein B-100, oxidized LDL, regression

The increased understanding of the potential molecular mechanisms of atherosclerosis that has evolved over the last few years has focused attention of the role of the immune system in the disease process.<sup>1-3</sup> Oxidized LDL is one of the most important targets for the immune system in atherosclerosis.<sup>4</sup> Oxidation of LDL results in formation of neo-epitopes such as oxidized phospholipids and aldehyde-modified breakdown fragments of apoB-100, resulting in an escape from

self tolerance.<sup>5</sup> Autoantibodies against epitopes in oxidized LDL are common in man and have been associated with disease development.<sup>6,7</sup> Immunization of hypercholesterolemic rabbits and mice with oxidized LDL inhibits the development of atherosclerosis suggesting that immune responses against oxidized LDL may have an atheroprotective effect.<sup>8-14</sup> These observations point to the possibility of developing an immunomodulatory therapy for atherosclerosis.<sup>15,16</sup>

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We have recently characterized the apoB-100 peptide structures in oxidized LDL that give rise to autoantibody formation in humans<sup>17</sup> and demonstrated that immunization with some of these peptide structures significantly reduces the development of atherosclerosis in apoE<sup>-/-</sup> mice.<sup>18-20</sup> A peptide corresponding to the sequence between amino acids 661 and 680 of apoB-100 (peptide 45) was found to be one of the most effective in these immunization studies. Recently, we developed human recombinant IgG1 antibodies specific for this aldehyde-modified peptide.<sup>21</sup> These antibodies, including IEI-E3 used in this study, bind to oxidized but not to native LDL. Treatment with 3 injections of IEI-E3 IgG1, with one week intervals, was found to reduce early lesion development by up to 50% in apoE<sup>-/-</sup> mice in a dose dependent manner. The results pointed to the possibility of developing an antibody-based therapy for atherosclerosis. However, if such a therapy is to be of significant clinical relevance it would be desirable that the antibody treatment promotes stabilization or regression of pre-existing advanced atherosclerosis.

The aim of the present study was to test the hypothesis that a human recombinant IgG1 antibodies against the peptide 45 sequence in apoB-100 induces regression of existing atherosclerotic plaques in *apobec-1<sup>-/-</sup>/LDLR<sup>-/-</sup>* mice. Extensive atherosclerosis was allowed to develop by feeding *apobec-1<sup>-/-</sup>/LDLR<sup>-/-</sup>* mice a high fat diet until 24 weeks of age. The mice were subsequently transferred to chow diet in order to create conditions allowing regression to occur and received 3 injections with either of two different recombinant human IgG1 against peptide 45, with one week intervals.

## Methods

### *Human recombinant antibodies*

Antibodies were produced as previously described.<sup>21</sup> Briefly, single chain antibodies with specificity for MDA-modified apoB-100 derived peptides were selected from the n-CoDeR<sup>®</sup> library.<sup>22</sup> Full-length human IgG1 was produced through cloning into a modified pcDNA3 vector followed by subsequent transfection into NSO cells as previously described.<sup>23</sup> A human IgG1 antibody to fluorescein isothiocyanate, FITC-8, was used as an isotype control. The antibodies were purified from spent cultivation medium on a MabSelect protein A column (Amersham Bioscience). The purity of the preparations exceeded 98%, as determined from SDS-PAGE, and contained less than 2 endotoxin units/mL (limulus amoebocyte lysate test, QCL-1000, BioWhittaker). The binding specificity of the antibodies was tested using a luminescence-based ELISA where dilutions of the antibodies were incubated in test plate wells coated with MDA-modified apoB-100 peptides, MDA-modified LDL or unmodified LDL. Bound antibody was detected using horseradish peroxidase-conjugated rabbit anti-human IgG antibody (DAKO). Affinities of the antibodies for their target structures were determined using a Biacore 3000 instrument. Briefly, human MDA-modified apoB-100 (Academy Bio-Medical Co) was immobilized on a CM5 chip by amino coupling. Unmodified apoB-100 was used as a negative control and the resulting binding curves were analyzed with BioEvaluation software (Biacore). LDL was isolated from blood by sequential preparative ultracentrifugation and modified with MDA as previously described.<sup>24</sup>

## ***Plaque regression induced by IgG1 against MDA-apoB-100***

### *Mice, Antibody Treatment and Tissue Preparation*

Male *apobec-1<sup>-/-</sup>/LDLR<sup>-/-</sup>* mice on C57BL/6 background from Jackson Laboratories (Bar Harbor, ME, USA) were used in the present studies. From 4 weeks of age they were fed a high cholesterol diet (0.15% cholesterol, 21% fat, Lactamin AB, Kimstad, Sweden) provided ad libitum. One week before the first immunization the diet was changed to normal chow. At 25 weeks of age the mice were injected intraperitoneally with 1 mg/dose (0.5 mL) of the human IgG1 antibodies directed to MDA-modified apoB-100 peptides or the isotype control antibody. The injections were repeated twice at 1-week intervals.

All mice were sacrificed by exsanguination through cardiac puncture under anesthesia with 300  $\mu$ L of distilled water, fentanyl/fluanisone and midazolam (2:1:1, vol/vol/vol), administered intraperitoneally. After whole body perfusion with phosphate buffered saline (PBS) followed by Histochoice (Amresco, Solon, Ohio), the heart and innominate artery were dissected out and stored in Histochoice at 4 °C until processing. The descending aorta was dissected free of external fat and connective tissue, cut longitudinally and mounted *en-face* lumen side-up on ovalbumin- (Sigma, St. Louis, Missouri) coated slides. The Animal Care and Use Committee of Lund University approved the experimental protocol used in this study.

### *Analysis of Plaque Area and Macrophage Plaque Contents*

Staining and quantification of plaque area in flat preparations of descending aorta and of plaque macrophage content were done by Oil Red O staining or MOMA immunostaining, as previously described.<sup>21</sup>

### *Analysis of presence of competing antibodies in mouse and human plasma*

In order to determine if mouse and human plasma contain antibodies with the ability to bind oxidized apoB-100 and block binding of the 2D03 antibody to its target antigen, human MDA-apoB-100 (0.5  $\mu$ g/mL) was coated to test plates and then pre-incubated with increasing concentrations of mouse or human plasma for 1 hour. After washing, the plates were incubated with 3  $\mu$ g/mL 2D03 or 3  $\mu$ g/mL biotinylated 2D03, the latter when the plate had been pre-incubated with human plasma. Bound 2D03 antibody was detected with peroxidase-labeled anti-human IgG1, not cross-reacting with murine IgG, or peroxidase-streptavidine, respectively. Luminescence was developed by Super Signal<sup>®</sup>ELISA Femto Luminol/Enhancer and Super Signal<sup>®</sup>ELISA Femto Stable Peroxide (Pierce, USA) and read in a Victor2V, Perkin Elmer Wallac instrument.

### *Plasma Cholesterol, Triglyceride and Serum Amyloid A (SAA).*

Total plasma cholesterol and plasma triglycerides were quantified with colorimetric assays, using commercially available kits (Thermo Electron, Melbourne, Australia). SAA was determined by ELISA (BioSource, Camarillo, CA).

### *Statistical analysis*

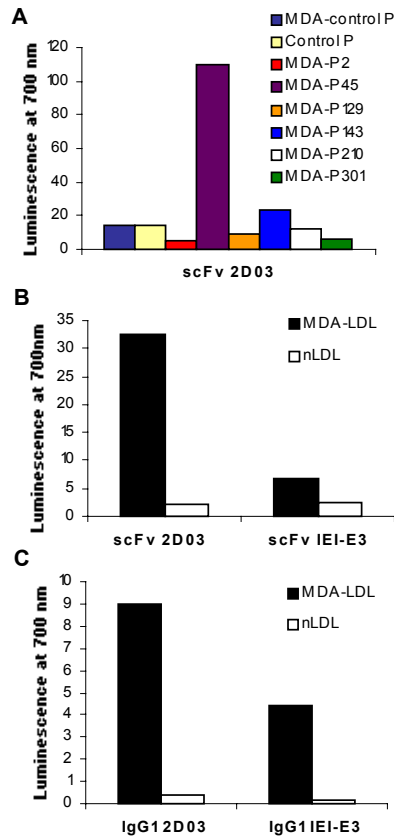
Data are presented as mean  $\pm$  standard deviation. Analysis of the data was performed using two-tailed Mann-Whitney test. Statistical significance was considered at  $P \leq 0.05$ .

## **Results**

### *Characterization of antibody binding*

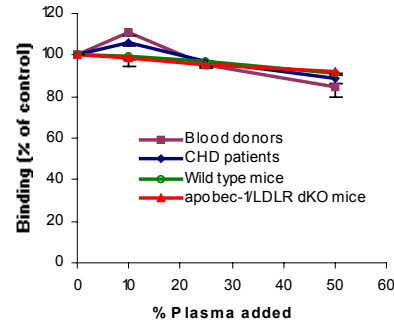
The single chain fragment (scFv) of 2D03 bound to MDA-modified apoB-100 peptide 45. 2D03 also demonstrated a weak binding to another MDA-modified peptide sequence in apoB-100 (amino





**Figure 1.** Binding of single chain fragment (scFv) 2D03 to a number of different MDA modified antigens (A). The P2 (amino acids [aa] 16-31), P45 (aa 661-680), P129 (aa 1921-1940), P143 (aa 2131-2150), P210 (aa 3136-3155) and P301 (aa 4502-4521) are peptides corresponding to human apo B-100 sequence. The control peptide was a non relevant lysine-containing peptide. (B) Illustrates the binding of 2D03 and IEI-E3 scFv to native (white) and MDA-modified (black) human LDL and (C) binding of human IgG1 to native (white) and MDA-modified (black) human LDL. In (A) and (B) the luminescence ELISA data are presented as signal/buffer, whereas in (C), data are plotted as signal/ $10^5$ . Abbreviations are as defined in text.

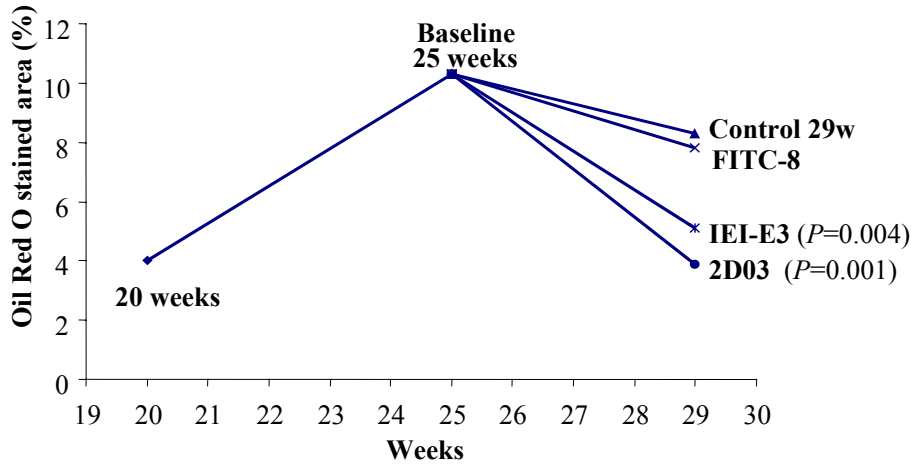
acids 2131 to 2150, Figure 1A) and differed in this respect from the IEI-E3 antibody which only recognizes the peptide 45 sequence of apoB-100.<sup>21</sup>



**Figure 2.** Analysis of presence of antibodies competing with 2D03 in mouse serum and human plasma. Human MDA-apo B-100 was coated on test plates and pre-incubated with increasing concentrations of mouse sera (*apobec-1*<sup>-/-</sup>/*LDLR*<sup>-/-</sup> or wild type C57BL/6) or human plasma (pooled from patients suffering from coronary heart disease (CHD) or healthy controls). The effect of this pre-incubation on binding of 2D03 to MDA-apo B-100 was determined as described in Methods. The results are expressed as percent of the values for the controls pre-incubated with buffer alone.

Moreover, the 2D03 scFv bound more effectively to MDA-modified LDL than IEI-E3, whereas both scFv showed only minimal binding to native LDL (Figure 1B). In accordance, analysis of the full length IgG1 showed a highly specific binding of both antibodies to MDA-LDL with little or no binding to native LDL. Again, the binding of 2D03 IgG1 to MDA-LDL was higher than that of IEI-E3 (Figure 1C). The control IgG1 against FITC did not bind to MDA-apoB-100, MDA-LDL or native LDL (data not shown). The affinity of 2D03 and IEI-E3 to MDA modified apoB-100 was determined to be  $3 \times 10^{-9}$  M and  $3 \times 10^{-8}$  M respectively using the Biacore technique. These observations demonstrate that both antibodies recognize the apoB-100 amino acid sequence between positions 661 and 680 as expressed in MDA-modified LDL and that the binding of the 2D03 antibody is ten times higher than that of IEI-E3. Binding of 2D03 to MDA-modified apoB-

*Plaque regression induced by IgG1 against MDA-apoB-100*



**Figure 3.** Plaque area in the descending aorta assessed by Oil Red O staining. The values are expressed as percentage of total plaque area per total area of the descending aorta. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  versus FITC-8, respectively.

**TABLE 1.** Experimental design and treatment groups

Group	Number of mice	Beginning of diet	End of diet	Antibody treatment	Sacrificed
20 weeks control	12	4w*		No	20w
25 weeks control (Baseline)	10	4w	24w	No	25w
29 weeks control	12	4w	24w	No	29w
FITC-8 (Isotype control)	12	4w	24w	25, 26, 27 w	29w
IEI-E3	9	4w	24w	25, 26, 27 w	29w
2D03	9	4w	24w	25, 26, 27 w	29w

Values are presented as mean±S.D., \* w indicates weeks of age

100 was not inhibited by sera from atherosclerotic mice or by plasma from normal human blood donors or patients diagnosed with coronary heart disease (Figure 2) indicating lack of interference from potential corresponding auto-antibodies with the binding of 2D03 to its target *in vivo*.

*Effect of antibody treatment on regression of atherosclerosis*

The design of the experiments and the different treatment groups are outlined in

table 1. The cholesterol level in 20 week old *apobec-1<sup>-/-</sup>/LDLR<sup>-/-</sup>* mice fed a high-fat diet was close to 1000 mg/dL (Table 2). At 24 weeks the mice were transferred to a low-fat chow diet resulting in a 60% reduction of plasma cholesterol one week later. Total plaque area in the descending aorta increased from 3.98±1.46% at 20 weeks of age to 10.31±3.73 % at 25 weeks (Figure 3). The latter group served as baseline control for studies of the effect of antibody treatment. As compared to the 25 week baseline control, a modest non-

TABLE 2. Weight, plasma lipids and SAA

Group	Weight (g)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	SAA ( $\mu$ g/mL)
20 weeks control	35.66 $\pm$ 3.6	967 $\pm$ 225	329 $\pm$ 105	225 $\pm$ 327
25 weeks control (Baseline)	31.6 $\pm$ 1.57	367 $\pm$ 129	105 $\pm$ 31	648 $\pm$ 1083
29 weeks control	32.83 $\pm$ 1.8	300 $\pm$ 34	115 $\pm$ 26	1235 $\pm$ 2126
FITC-8 (Isotype control)	34.16 $\pm$ 2.48	403 $\pm$ 59	145 $\pm$ 20	860 $\pm$ 1562
IEI-E3	36.44 $\pm$ 3.28	387 $\pm$ 65	163 $\pm$ 10	26 $\pm$ 20**
2D03	32.44 $\pm$ 5.45	353 $\pm$ 70	146 $\pm$ 25	194 $\pm$ 224

Values are presented as mean $\pm$ S.D., SAA; serum amyloid A. \*\* $P$ <0.001.

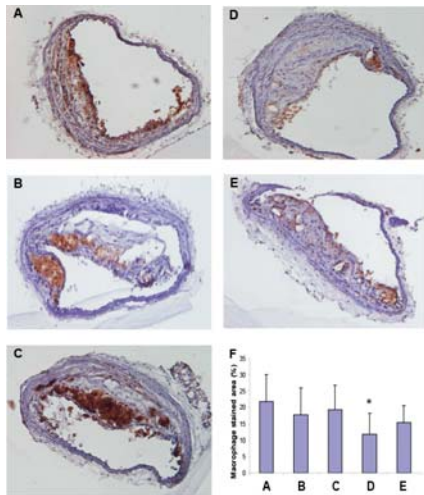
significant decrease was observed in animals transferred to chow diet alone and sacrificed at 29 weeks of age (8.28 $\pm$ 4.36%). The remaining 3 groups of mice received 3 intraperitoneal injections of 1 mg of 2D03, IEI-E3 or control FITC-8 antibodies at 25, 26 and 27 weeks. The general health status of the mice was not influenced by the antibody treatment. There were no significant differences among the treatment groups and the chow-fed control group with respect to weight and the plasma levels of cholesterol and triglycerides (Table 2). Treatment with the FITC-8 control antibody had no effect on atherosclerosis as compared to transfer to chow diet alone. In contrast, treatment with the 2D03 antibody resulted in a more than 50% regression of atherosclerosis (3.91 $\pm$ 1.83%) as compared with mice treated with control IgG1 ( $P$ =0.001, Figure 3). A less pronounced regression (36%) was observed in mice treated with the IEI-E3 antibody (5.16 $\pm$ 1.07%,  $P$ =0.004). (Figure 3).

#### *Effect of antibody treatment on plaque inflammatory activity and plasma SAA*

The effect of antibody treatment on plaque inflammatory activity was analyzed by macrophage immunostaining of cross-sections of the innominate artery. Transfer

of mice from high-fat to chow diet resulted in a non-significant reduction of macrophage immuno-reactive area from 21.8 $\pm$ 8.23% in the 25 weeks baseline group to 17.8 $\pm$ 8.16% at 29 weeks in mice given no antibody treatment and to 19.3 $\pm$ 7.43% in mice treated with the FITC-8 isotype antibody (Figure 4). Treatment with the 2D03 antibody resulted in a 38% reduction of macrophage immuno-reactivity (11.84 $\pm$ 6.4%,  $P$ =0.03) as compared with the FITC-8 isotype control group (Figure 4). A similar trend was also observed in mice treated with the IEI-E3 antibody (15.38 $\pm$ 5.1%, n.s.). To assess the effect of antibody treatment on the general inflammatory activity in mice, the plasma levels of serum amyloid A (SAA), the functional mouse equivalent to human CRP, were measured. Plasma SAA more than doubled between 20 and 25 weeks and continued to increase in mice given chow diet alone or treated with the FITC-8 control antibody (Table 2). Treatment with the IEI-E3 antibody resulted in a more than 90% decrease in SAA levels as compared with treatment with the FITC-8 control antibody ( $P$ =0.001), whereas a less pronounced reduction was observed in mice treated with the 2D03 antibody.

### Plaque regression induced by IgG1 against MDA-apoB-100



**Figure 4.** Macrophage content in plaques from the innominate artery in the 25 weeks old control (A), 29 weeks old control (B), FITC-8 (C), 2D03 (D) and IEI-E3 (E) groups. The values in (F) represent percentage of stained area per total plaque area. Abbreviations are as defined in text. \*  $P < 0.05$  vs. FITC-8.

#### Discussion

The present study demonstrates that treatment with recombinant human IgG1 antibodies against an aldehyde-modified peptide sequence in human apoB-100 induces regression of advanced atherosclerotic plaques in the aorta of *apobec-1*<sup>-/-</sup>/*LDLR*<sup>-/-</sup> mice by more than 50% over a 4 week period. This effect was paralleled by a decreased macrophage content of atherosclerotic plaques in the innominate artery, suggesting a less inflammatory phenotype of remaining plaques and occurred incrementally over and above the anti-atherogenic effect of transferring the mice to a low-fat diet. The LDO-D3 antibody bound more effectively to MDA-LDL than the IEI-E3 antibody and also tended to be more effective in inducing regression of atherosclerosis, whereas the FITC-8 control antibody did not bind to MDA-LDL or affected plaque regression. These observations suggest that the ability of the antibodies to bind to

modified LDL is of importance for the athero-protective effect.

The mechanisms through which IgG against epitopes in modified LDL induce regression of atherosclerosis remains to be fully clarified. The results of the present study demonstrates that transfer of cholesterol-fed *apobec-1*<sup>-/-</sup>/*LDLR*<sup>-/-</sup> mice to a low-fat diet at least temporarily halts further progression of atherosclerosis but is not in itself sufficient to induce a significant regression of aortic lesions. In contrast, a marked reduction in aortic lipid-rich plaques (as assessed by the *en face* Oil Red O staining) was found in 2D03 and IEI-E3 antibody-treated mice suggesting that the antibodies facilitated net removal of lipids from the aorta. Regression of lipid-rich atherosclerotic plaques has recently been shown to be potentially mediated by monocytes converting into a dendritic cell-like phenotype with increased migrating capacity.<sup>25</sup> Stimulation of this conversion represents one possible mechanism through which 2D03 and IEI-E3 antibodies could enhance arterial lipid efflux. Treatment with the IEI-E3 antibody resulted in a significant reduction of plasma SAA levels. This is of interest because the functional human equivalent of SAA, CRP, is an important marker for cardiovascular risk. However, since the 2D03 antibody reduced SAA levels less effectively in spite of a better effect on regression of atherosclerosis it remains to be elucidated how these processes are associated.

We have previously shown that IgG1 specific for MDA-modified apoB-100 peptides markedly increase the uptake of oxidized LDL in cultured human macrophages,<sup>21</sup> presumably by a Fc receptor-mediated process. Although this could potentially promote formation of macrophage foam cells, a recent study

demonstrating that mice deficient for the oxidized LDL scavenger receptor CD36 develop more atherosclerosis<sup>26</sup> suggests that macrophage uptake of oxidized LDL under certain conditions may be atheroprotective. Oxidized LDL taken up by macrophages is for example more likely to be removed through the ABCA1 – HDL reverse cholesterol transport pathway than oxidized LDL remaining in the vascular extracellular matrix. We have previously shown that treatment of apoE<sup>-/-</sup> mice with the IEI-E3 antibody reduces the amount of oxidized LDL epitopes in plaques.<sup>21</sup>

Autoantibodies specific for the same MDA-modified apoB-100 sequence as the 2D03 and IEI-E3 antibodies have been detected in humans.<sup>17,27</sup> These autoantibodies are primarily IgM and increase with the severity of atherosclerosis as assessed by carotid ultrasonography. High levels of these IgM are associated with low levels of oxidized LDL in plasma suggesting that the antibodies may help to clear modified LDL from the circulation. Low levels of IgG with the same specificity have also been identified in humans and in preliminary studies suggest an inverse association between these antibodies and the severity of carotid stenosis (Fredrikson GN et al, unpublished data). Interestingly, we found no evidence of interference between 2D03 and antibodies potentially present in mouse or human plasma, indicating that the recombinant antibody had either a much higher affinity and/or another epitope specificity than the naturally occurring antibodies.

There are some limitations to the present study that should be considered. The IEI-E3 and 2D03 antibodies are specific for the human apoB-100 sequence between amino acids 661 and 680. The homology to the corresponding mouse sequence is

only 85% suggesting that the affinity of the antibodies may be lower for mouse than for human oxidized LDL. Since the present results suggest that antibodies with higher binding affinity, such as 2D03 are more effective, it is most likely that this will limit the effectiveness of the antibodies when used in mice. Another limitation of the present study is that the immune system of the mice reacts to the human IgG1, generating mouse anti-human IgG1 antibodies.<sup>21</sup> These antibodies may reduce the effectiveness of the more prolonged human IgG1 treatment in mice by blocking their binding sites or by inducing their clearance from the circulation.

In conclusion, the present findings show that under conditions characterized by reduced plasma cholesterol levels, treatment with human IgG1 against a specific oxidized LDL antigenic epitope can induce a rapid and substantial regression of atherosclerotic lesions. Treatment with such recombinant human IgG1 represents a potentially novel approach for treatment of atherosclerosis in humans. However, in this respect the present study needs to be interpreted with due caution. The atherosclerotic disease process of *apobec-1<sup>-/-</sup>/LDLR<sup>-/-</sup>* mice is not likely to be identical to that of human atherosclerosis. It is also unclear how the conditions created in the present study by changing from a high-fat to a low-fat mouse diet relates to the changes that occur in humans in response to diet and/or lipid-lowering drugs.

#### **Acknowledgements**

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# III

*Somewhere, something incredible is waiting to be known.*  
*Carl Sagan*





# Inhibition of injury-induced arterial remodeling and carotid atherosclerosis by recombinant human antibodies against aldehyde-modified apoB-100

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**Objective:** The immune system plays an important regulatory role in the development of atherosclerotic plaques and neointima formation following various types of angioplasty. In the present study we investigated the effect of antibodies against aldehyde-modified apolipoprotein B-100 (apoB-100), a component of oxidized LDL, on atherosclerosis and response to arterial injury in mice.

**Methods:** The ability of a high affinity human recombinant antibody (2D03), specific for malondialdehyde-modified apoB-100, to influence formation of atherosclerosis as well as remodeling and neointima formation after a collar-induced injury of the carotid artery was studied in LDL receptor<sup>-/-</sup> mice over-expressing human apoB-100.

**Results:** The antibody recognized epitopes present in mouse plasma and reduced the plasma level of oxidized LDL by 34%. Antibody treatment inhibited injury-induced restrictive vascular remodeling but did not influence the size of the neointima. Atherosclerosis in the uninjured contra lateral carotid artery was determined by computerized image analysis and the mean plaque area in animals given control IgG1 was 7,608±10,336 μm<sup>2</sup>. In contrast, essentially no plaques were present in animals treated with the 2D03 antibody (397±235 μm<sup>2</sup>, *P*<0.01 versus control IgG1).

**Conclusions:** Treatment with antibodies against aldehyde-modified apoB-100 dramatically reduces atherosclerosis and inhibits restrictive vascular remodeling in mice expressing human apoB-100.

**Key words:** oxidized LDL, atherosclerosis, antibodies, vascular injury, apolipoprotein B-100

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## Introduction

Oxidized LDL has been attributed an important role in the development of atherosclerotic plaques.<sup>1</sup> Minor amounts of oxidized LDL are present in plasma but more extensive modifications are believed to occur as LDL becomes entrapped by extracellular matrix molecules in the vascular wall.<sup>2</sup> Oxidation of LDL results in the formation and release of oxidized phospholipids, lysophosphatidyl choline, aldehydes and oxysterols that injure and activate the expression of proinflammatory

genes in surrounding vascular cells. This inflammatory response stimulates the recruitment of leukocytes and contributes to plaque growth.<sup>3</sup> Oxidized LDL is removed by macrophage scavenger receptors, leading to formation of foam cells as well as to activation of adaptive immune responses against oxidized LDL. The latter includes stimulation of specific T cells and generation of oxidized LDL autoantibodies.<sup>4</sup> Immunization of hypercholesterolemic animals with oxidized LDL results in development of a partial

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protection against the progression of atherosclerosis<sup>5</sup>, demonstrating that some of these immune responses have an atheroprotective function. The epitopes in oxidized LDL that are responsible for activation of atheroprotective immunity have recently been characterized and found to include specific aldehyde-modified peptide sequences of apoB-100.<sup>6</sup> We have produced recombinant human IgG1 antibodies that specifically recognize these modified apoB-100 peptide sequences and shown that repeated injection of these IgG results in an inhibition of atherosclerosis development in apoE<sup>-/-</sup> mice.<sup>7</sup>

The role of oxidized LDL in neointima formation following mechanical injury is less clear. Low concentrations of oxidized LDL have been shown to stimulate the growth of cultured vascular smooth muscle cells, while high concentrations are cytotoxic.<sup>8</sup> There is also evidence from *in vitro* studies that induction of smooth muscle cell proliferation in response to mechanical injury is associated with generation of extracellular reactive oxygen species and LDL oxidation.<sup>9</sup> An increased oxidative stress has been demonstrated early after mechanical arterial injury and antioxidants inhibit injury-induced neointima formation in animal models.<sup>10</sup> The powerful antioxidant probucol has also been shown to prevent femoropopliteal restenosis after balloon angioplasty in clinical studies.<sup>11</sup> A marked increase in neointima formation has been observed in Rag-1 mice lacking functional T and B lymphocytes. This phenomenon that can be reversed by B cell transfer suggesting that humoral immunity is involved in regulating the injury response.<sup>12</sup>

The aim of the present study was to assess the role of antibodies against oxidized LDL in injury-induced neointima formation and atherosclerosis. LDL

receptor deficient mice expressing human apoB-100 were treated repeatedly with injections of recombinant human IgG specifically recognizing MDA-modified apoB-100 peptide sequences, and the effect on collar-induced neointima formation and vascular remodeling in the right carotid artery was determined. The effect on the development of atherosclerosis was analyzed in the uninjured left carotid artery.

## Materials and methods

### *Human recombinant antibodies*

The antibodies were produced as previously described.<sup>7</sup> Briefly, single chain antibodies with specificity for MDA-modified apoB-100 derived peptides<sup>6</sup> were selected from the n-CoDeR library.<sup>13</sup> High affinity specific single chain antibodies were transferred to a full-length human IgG1 lambda format through cloning into a modified pcDNA3 vector followed by subsequent transfection into NSO cells and cloning as previously described.<sup>14</sup> A human IgG1 antibody to fluorescein isothiocyanate, FITC-8, was used as an isotype control. The antibodies were purified from spent cultivation medium on a MabSelect protein A column (Amersham Bioscience). The purity of the preparations exceeded 98%, as determined from SDS-PAGE, and contained less than 2 endotoxin units/ml (limulus amoebocyte lysate test, QCL-1000, BioWhittaker).

### *Biacore and recombinant antibody ELISA analysis*

The MDA-modified apoB-100 antigen (Academy Bio-medical Co) was immobilized by amino coupling on a CM5 chip in a Biacore 3000 (Biacore AB) to give a total signal of 2880 RU and the binding of the antibodies was analyzed using the BioEvaluation software as previously described.<sup>7</sup> The binding specificity of the 2D03 and FITC-8 antibodies was tested using a luminescence-based ELISA where

### *IgG1 against MDA-apoB-100 inhibits carotid atherosclerosis*

several dilutions of the antibodies were incubated in test plate wells coated with 0.5 µg/ml of MDA-modified apoB-100, MDA-modified LDL, unmodified LDL and unmodified apoB-100. Bound antibody was detected using horseradish peroxidase-conjugated rabbit anti-human IgG antibody (DAKO). LDL was isolated from blood by sequential preparative ultracentrifugation and modified with MDA as previously described.<sup>15</sup>

*Mice, immunization and tissue preparation*  
LDL receptor<sup>-/-</sup> mice that express human apoB-100 on a C57Bl/6 background were kindly provided by Jan Borén, Gothenburg University. At 21 weeks of age, female mice (n = 9-11) were given a first intraperitoneal injection with 0.5 ml (0.2 mg/dose) of the human IgG1 antibodies 1 day before the periadventitial collar injury. The injections were then repeated three times at 3, 6 and 13 days after the injury. FITC-8 was used as an isotype control antibody. The Animal Care and Use Committee approved the experimental protocol used in this study.

#### *Periadventitial collar injury*

At the age of 21 weeks, the mice were anaesthetised with Avertin (0.016 ml/g of 2.5% solution IP), and the right carotid artery was carefully isolated under a dissecting microscope. A nonocclusive plastic collar was placed around the right carotid artery, and the skin incision was closed, as described previously.<sup>16</sup> The mice were killed 21 days after collar placement and the carotid arteries were perfusion-fixed with Histochoice (Amresco), dissected out and stored in Histochoice at 4° until analysis.

#### *Carotid artery morphometric measurements*

The right (injured) and left (uninjured) carotid arteries were sectioned in 5 µm thick sections. From the injured artery,

1 section every 200 µm was used for measurements (approximately 10 sections per animal). From the uninjured artery, sections were collected every 100 µm from the distal end of the segment (4 sections per mouse). The slides were stained for elastin with Accustain elastic stain (Sigma). The areas of the different regions and the circumferences were calculated using the imaging software Zeiss Axiovision (Zeiss). Medial area represents the area between the external elastic lamina (EEL) and the internal elastic lamina (IEL). The lesion area was calculated by subtracting lumen area from IEL area. Lumen and total vessel dimensions were determined by measuring lumen and EEL perimeter respectively.

#### *Immunohistochemistry*

Tissue sections from paraffin embedded right (injured) and left (uninjured) carotid arteries from 2D03 and FITC-8 treated mice were rehydrated and used for immunohistochemistry. Smooth muscle cells were detected with a monoclonal anti-mouse alpha actin antibody (Sigma) and macrophages with rat anti-mouse Mac-2 (Cedarlanes Laboratories) with appropriate secondary antibodies. The reaction products were visualised with Vectastain ABC elite kit (Vector Laboratories) using DAB as the substrate (Vector Laboratories).

#### *Plasma cholesterol and oxidized LDL analysis*

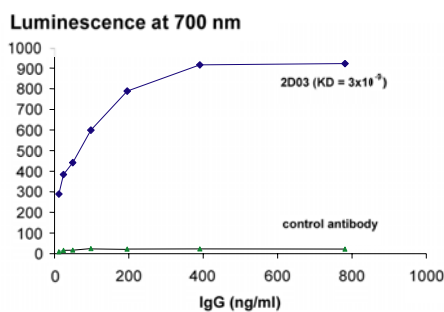
Total plasma cholesterol was quantified using a colorimetric assay (Thermo Electron, Melbourne, Australia). Oxidized LDL in EDTA plasma was measured using a commercially available ELISA kit (Mercodia, Uppsala, Sweden). The assay was not influenced by the human anti-oxApoB-100 antibody (data not shown).

### Statistics

Results are expressed as mean  $\pm$  standard deviation. The statistical difference between groups was determined using Student's *t*-test or the Mann-Whitney two-tailed test when appropriate. Spearman's rho was used for correlation analysis.  $P < 0.05$  was considered significant.

### Results

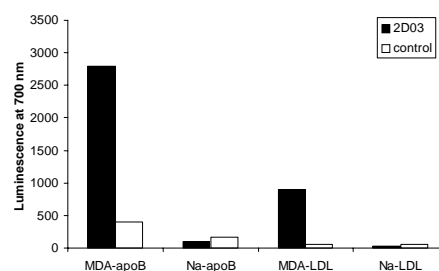
Several scFv (single chain fragment variable) with specificity for the MDA-modified apoB-100 peptide composed of amino acids 661-680 were identified and transformed into the human IgG1 format. After screening, an antibody (2D03) demonstrating high specificity for MDA-modified forms of apoB-100 and LDL was selected (figs. 1 and 2). The 2D03 antibody bound circulating oxidized LDL in plasma of LDL receptor<sup>-/-</sup>/human apoB-100<sup>+/-</sup> mice. A 96 well test plate was coated with 2D03 antibody and bound oxidized LDL could be detected via a rabbit anti human apoB-100 antibody. An excess of MDA labeled p45 peptide inhibited the binding (data not shown). At the age of 21 weeks LDL receptor<sup>-/-</sup>/human apoB-100<sup>+/-</sup> mice were given an intraperitoneal injection of 200  $\mu$ g of the



**Figure 1.** Binding of 2D03 to MDA-modified LDL. The MDA-LDL antigen was coated on test plates and bound antibody was detected with peroxidase-labeled anti-human IgG1.  $K_D$  values were estimated after Biacore measurements against MDA-modified apoB-100 as described in Material and methods. The IgG1 FITC-8 antibody was used as negative control. Data are plotted as signal/10<sup>3</sup>.

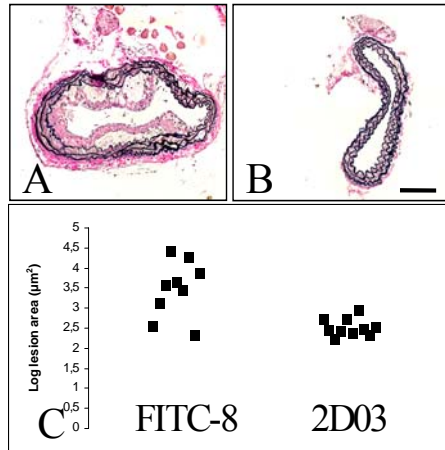
human recombinant 2D03 IgG1 antibody or recombinant control human IgG1 (anti-FITC-8). On the following day a non-occlusive plastic collar was placed around the right carotid artery. This procedure has previously been shown to result in neointima formation.<sup>16</sup> Repeated antibody injections were given 3, 6 and 13 days after the implantation of the collar and the mice were then sacrificed 21 days after the surgical procedure.

The extent of atherosclerosis in the uninjured carotid artery was determined by computerized image analysis of mean cross-sectional plaque area of 4 sections taken with 100  $\mu$ m intervals. The mean plaque area in animals given control IgG1 was  $7,608 \pm 10,304 \mu\text{m}^2$ . In contrast, essentially no plaques were present in animals treated with the 2D03 antibody (mean cross-sectional plaque area  $397 \pm 235 \mu\text{m}^2$ ,  $P < 0.01$  versus control IgG1, fig. 3). The uninjured carotid arteries of mice treated with control IgG1 were characterized by a larger circumference of the external elastic lamina ( $1293 \pm 274$  versus  $1041 \pm 88 \mu\text{m}$ ,  $P < 0.02$ ), whereas there was no significant difference in the medial area between mice treated with control and 2D03 IgG1



**Figure 2.** Luminescence ELISA illustrating the binding of 2D03 antibody and control (FITC-8) to MDA-modified human LDL (MDA-LDL), MDA-modified human apoB-100 (MDA-ApoB), unmodified LDL (Na-LDL) and unmodified ApoB100 (Na-ApoB). Data are plotted as signal/10<sup>3</sup>.

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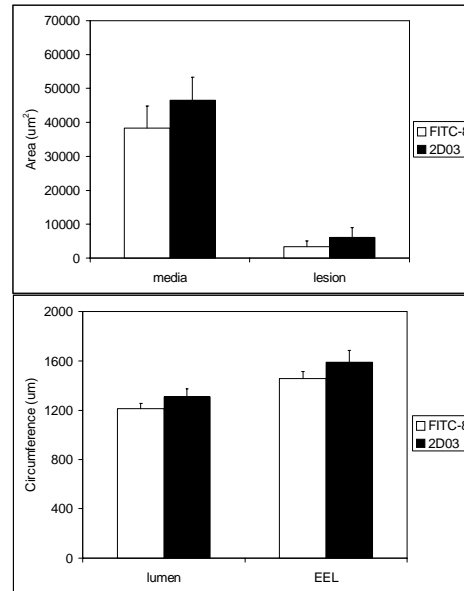


**Figure 3.** Effect of antibody treatment on carotid atherosclerosis. Elastin stained sections of left uninjured carotid artery after injections with (A) control (anti-FITC-8) IgG1 and (B) 2D03 IgG1 against MDA-modified apoB-100. Panel C demonstrates carotid artery lesion areas in individual mice after treatment with the FITC-8 and 2D03 antibodies respectively. Scale bar = 100 µm. Statistical difference between groups was determined by Mann-Whitney test. \*\*  $P < 0.01$ .

(32,017±10,304 µm<sup>2</sup> vs 26,822±4,501 µm<sup>2</sup>).

There was no significant difference in the formation of neointimal lesions in collar-injured carotid arteries between mice treated with control and 2D03 IgG1 (fig. 4A). However, mice treated with 2D03 IgG1 had a significantly larger inner lumen circumference, larger medial area and larger circumference of the external elastic lamina (fig. 4A and B).

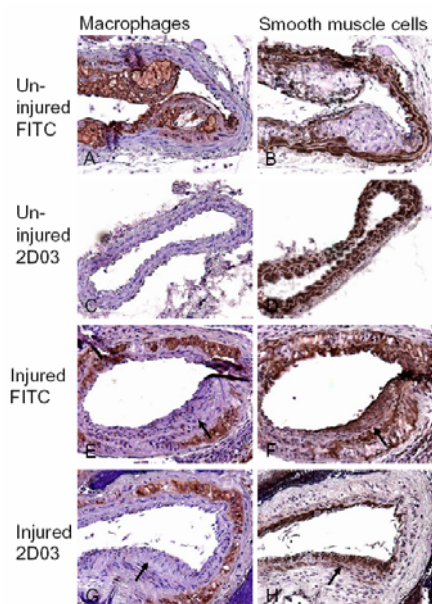
The cellular composition of the lesions was analyzed by immunohistochemical detection of smooth muscle cells and macrophages. In uninjured carotid arteries macrophages were localised to plaques and absent in the medial layer (fig 5A). Smooth muscle cell-specific alpha actin positive staining was present in the media and in the fibrous cap region of the lesions (fig 5B). In the injured arteries macrophages were detected in the



**Figure 4.** Effect of antibody treatment on vascular remodeling after collar injury. Morphometric measurements of the right injured carotid artery demonstrating (A) media and neointima area as well as (B) lumen and EEL circumference after treatment with control antibody (FITC-8, n = 9) and 2D03 (n = 11). EEL = external elastic lamina. Statistical difference between groups was determined by Mann-Whitney test. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ .

outermost layers of the media with a sparse distribution in the intimal area (fig 5E and G). The expression of alpha actin was reduced in the media in response to injury, whereas a strong staining was found in the neointima (fig 5F and H). There was no detectable immunoreactivity for human IgG in atherosclerotic plaques or neointimas at the time of sacrifice.

To determine if treatment with 2D03 antibodies affects the clearance of oxidized LDL we analyzed plasma levels of oxidized LDL by ELISA. The plasma level of oxidized LDL was found to be 34% lower in mice treated with 2D03 IgG1 as compared to mice receiving control IgG1. The total cholesterol levels did not differ between the groups (table).



**Figure 5.** Cellular composition of lesions. Immunohistochemical detection of macrophages and smooth muscle cells in uninjured carotid artery in mice treated with control (FITC-8) IgG (A and B) and 2D03 IgG (C and D) as well as in injured carotid artery treated with control IgG (E and F) and 2D03 IgG (G and H). Arrows indicate the position of internal elastic lamina in injured arteries.

There were no significant correlations between oxidized LDL or total cholesterol levels and cross-sectional plaque size in uninjured arteries. However, a significant inverse association was found between plasma levels of oxidized LDL and the external elastic lamina circumference of the collar-injured artery of 2D03 treated mice ( $r=-0.70$ ,  $P<0.05$ ).

### Discussion

The present study shows that treatment of LDL receptor<sup>-/-</sup> mice over-expressing human apoB-100 with human recombinant IgG1 recognizing an MDA-modified peptide sequence in apoB-100 inhibits the development of carotid atherosclerosis and restrictive vascular remodeling after mechanical injury, without affecting neointima formation. It also demonstrates

that treatment with anti-MDA apoB-100 IgG1 reduces the level of oxidized LDL in plasma, suggesting that the antibody may function by mediating the removal of oxidized LDL.

**TABLE.** Effect of antibody treatment on plasma levels of total cholesterol and oxidized LDL.

	FITC-8 n=9	2D03 n=10
Plasma cholesterol (mg/dL)	534±123	516±133
Oxidized LDL (mU/mL)	311±107	206±69

An atheroprotective effect of treatment with human recombinant anti-MDA apoB-100 IgG1 has previously been reported in apoE<sup>-/-</sup> mice.<sup>7</sup> There are, however, several disadvantages in using apoE<sup>-/-</sup> mice when studying the effect of antibodies against human MDA-modified apoB-100 peptide sequences. Human and mouse apoB-100 present only 85% sequence homology for the amino acids 661-680 against which the antibody was developed. Moreover, only about 30% of apoB-containing lipoproteins in mice carry apoB-100 and the remaining carry apoB-48.<sup>17</sup> This is in contrast to the human situation where all LDL particles contain apoB-100.<sup>18</sup> Accordingly, the use of LDL receptor<sup>-/-</sup>/human apoB-100<sup>+/-</sup> mice has several advantages as a model when using antibodies directed to human apoB-100 sequences. In comparison to the previously reported antibody (IEI-E3)<sup>7</sup>, the present antibody (2D03) has a higher affinity and binding capacity for human MDA-modified apoB-100 as well as for the equivalent epitopes in mouse plasma. This antibody was found to have a very pronounced effect on plaque formation in the uninjured carotid artery causing an almost complete inhibition (> 90% reduction) of plaque formation. Presently

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it is unknown if the effect on plaque size is a result of inhibition of plaque growth or if the antibodies can also reduce the size of already existing plaques.

The notion that antibodies against the MDA-modified 661-680 amino acids sequence of apoB-100 have an athero-protective effect is also supported by studies demonstrating that immunization of apoE<sup>-/-</sup> mice with this peptide sequence results in an inhibition of atherosclerosis associated with an increased expression of peptide-specific IgG.<sup>19</sup> Moreover, we have recently found that high plasma levels of IgG1 against this peptide sequence are associated with a decreased severity of carotid disease in humans (Fredrikson GN *et al.*, unpublished data).

The cellular composition of the atherosclerotic lesions in this particular model resembled lesions in other mouse models of atherosclerosis such as apoE<sup>-/-</sup> mice and with human atherosclerotic plaques. The staining pattern suggested a lesion core of macrophages, foam cells and debris covered by a smooth muscle cell fibrous cap layer. The neointimal lesions of injured arteries were mostly composed of smooth muscle cells and showed reduced levels of alpha actin in the media, which is consistent with previous studies showing decreased amounts of proteins associated with a de-differentiated SMC phenotype in the media after balloon injury in rats.<sup>20</sup> Injured arteries also showed an intense macrophage staining in the media, a finding that has also been observed after carotid artery wire injury in ApoE<sup>-/-</sup> mice and was suggested to influence the process of medial thickening.<sup>21</sup>

Treatment with 2D03 IgG1 significantly reduced plasma oxLDL levels, indicating that the antibodies mediate the clearance of oxidized LDL. These results are in line with previous clinical studies showing an

inverse relation between antibody levels and oxidized LDL in plasma.<sup>6</sup> The mechanism by which apoB-100 antibodies remove oxidized LDL from the circulation remains to be fully understood, but may involve the removal of the antibody/oxidized LDL complexes by Fc receptors. The reduced plasma oxLDL levels may also be a consequence of a direct effect of the antibodies on the plaques leading to a reduced net release of oxidized LDL from the remaining atherosclerotic lesions.

Treatment with 2D03 antibodies also resulted in an increased vessel size after mechanical vascular injury as compared to mice given control IgG1, suggesting an effect on vascular remodeling. Previous studies suggest that neointimal hyperplasia is not the major determinant of final lumen size after injury, but that all layers of the vessel wall contribute to this process.<sup>22</sup> Restenosis after angioplasty in humans and in experimental animal models is often associated with constrictive remodeling which is an important determinant of lumen narrowing.<sup>23</sup> Antibody treatment may have either prevented constrictive remodeling or promoted the positive outward remodeling.

The cuff injury model is an established method for studying neointima formation. Previous studies of cuff placement around rabbit carotid arteries have shown phases of inflammatory cells recruitment to the vessel wall, medial smooth muscle cell replication and subsequent migration into the neointima within 14 days.<sup>24</sup> However, the cuff injury model has also been used for studies of vascular remodeling. Drew *et al.* have identified significant compensatory medial remodeling after cuff injuries of mice femoral arteries.<sup>25</sup> Nevertheless, as is the case with all animal models, comparisons to the situation in human restenosis must be made with great caution.



The mechanisms involved in the process of vascular remodeling remain to be fully elucidated. Lafont *et al.*<sup>26</sup> have shown that constrictive remodeling is associated with endothelial dysfunction and collagen accumulation. Oxidized LDL has been suggested to impair endothelium dependent relaxation.<sup>27</sup> Recent studies have also demonstrated a significant association between the level of oxidized LDL in plasma and the severity of constrictive remodeling.<sup>28</sup> In addition it has been shown that hypercholesterolemia impairs compensatory enlargement after porcine angioplasty and that oxidized LDL could mimic this effect. These findings were suggested to be due to an inhibitory effect of cholesterol/oxLDL on SMC migration and collagen accumulation.<sup>29</sup> Hence it is possible that the observed differences in remodeling between 2D03 and FITC treated mice are due to the effects of oxLDLs on SMC and collagen.

Other studies suggest that constrictive remodeling is mediated by turnover of the extracellular matrix and several reports demonstrate the involvement of matrix metalloproteases (MMPs) in this process.<sup>30</sup> Oxidized LDL has been shown to induce the activation of MMP-1 and MMP-9, hence antibodies against oxidized LDL may limit constrictive remodeling via its effect on MMPs. Treatment with antioxidants (vitamin C and E) results in increased vessel and lumen area after balloon injury in pigs.<sup>31</sup> As treatment with apoB-100 antibodies gives similar effect it is possible that antibodies to oxidized LDL regulate the level of oxidative stress and/or its consequences. Accumulating evidence suggests that vascular injury is accompanied by oxidative stress, and redox processes have been found to contribute to post-angioplasty restenosis.<sup>32</sup> Oxidized LDL has been shown to limit the bioavailability of NO, which act as important scavengers for superoxide

radicals<sup>33</sup>, thus a reduction of the oxidized LDL levels may be beneficial for the degree of oxidant stress. Accordingly, it is possible that the effect of 2D03 treatment on vascular remodeling after injury in the present study is due to removal or blocking of oxidized LDL.

The observed effects on remodeling may be coupled to an altered haemodynamic status in the arterial wall between 2D03 and control treated mice.<sup>34</sup> It is possible that the artery proximal to the cuff site of FITC-8 treated mice contains more atherosclerotic lesions than in 2D03 treated mice, which in turn may affect shear stress and the remodeling process in response to the cuff injury.

The circumference of the external elastic lamina in the uninjured carotid artery was significantly larger in mice receiving control IgG1 than in mice treated with 2D03 IgG1. Although it cannot be excluded that 2D03 IgG1 has different effects on remodeling in injured and uninjured arteries this effect may be due to compensatory remodeling in response to plaque development in the carotid artery of the control group rather than a direct effect of the 2D03 antibody.

In conclusion, the present studies show that treatment with human recombinant IgG1 specifically recognizing the MDA-modified peptide sequence between amino acids 661 and 680 of human apoB-100 reduces atherosclerosis and constrictive injury-induced remodeling in the carotid artery of LDL receptor<sup>-/-</sup> mice over-expressing human apoB-100. The observation that antibody-treated mice have lower plasma levels of oxidized LDL suggests that the antibody may function by facilitating the removal of oxidized LDL. Passive immunization using human recombinant IgG1 recognizing MDA-modified peptide sequences in human

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apoB-100, such as the 2D03 IgG1, represents a possible novel approach for treatment of atherosclerosis and restenosis in humans.

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# IV

*Once we accept our limits, we go beyond them.*

*Albert Einstein*



# Increased levels of IgG1 against an aldehyde-modified peptide sequence in apoB-100 are associated with decreased severity of carotid stenosis

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**Background and Purpose:** Immunization with a malondialdehyde (MDA)-modified peptide corresponding to the amino acid sequence between 661 and 680 in apoB-100 (p45) has been shown to inhibit atherosclerosis in apoE knockout mice. Similar results were seen following treatment of mice with human recombinant anti-MDA-p45 IgG1. In the present study we tested the hypothesis that endogenous levels of IgG to MDA-p45 would be associated with reduced carotid atherosclerosis and cardiovascular events in humans.

**Methods:** Using a nested case control design we analyzed plasma MDA-p45 IgG1, IgG2, IgG3 and IgG4 levels in baseline samples from 76 subjects with coronary events and 148 matched controls recruited from the prospective Malmö Diet and Cancer study. Baseline percent carotid stenosis, common carotid artery and bulb intima-media thickness were determined by B-mode ultrasound.

**Results:** There were no differences in antibody levels between cases and controls. However, a significant association was found between high levels of MDA-p45 IgG1 and a low degree of carotid stenosis ( $p=0.006$  following adjustment for age, sex, blood pressure, low and high density lipoprotein cholesterol).

**Conclusions:** The finding of an inverse association between MDA-p45 IgG1 and severity of carotid stenosis in humans supports previous experimental studies suggesting that these antibodies have an atheroprotective effect.

**Key words:** apolipoproteins, antibodies, carotid stenosis, peptide, ultrasound

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Activation of adaptive immunity plays an important role in the development of atherosclerosis.<sup>1,2</sup> T cells in human atherosclerotic plaques recognize epitopes in oxidized LDL when presented by macrophage MHC class II molecules<sup>3</sup> and autoantibodies against oxidized LDL are commonly expressed in humans, suggesting that oxidized LDL is an important antigen in atherosclerosis.<sup>4,5</sup> Several lines of evidence favor the concept that adaptive immune responses are activated as part of the disease process and promote inflammation and plaque growth<sup>1,2</sup>. However, immunization with

oxidized LDL has been shown to reduce atherosclerosis, demonstrating that deviation towards protective immunity is possible.<sup>5-11</sup> Oxidized phospholipids<sup>12</sup> and aldehyde-modified peptide sequences in apoB-100<sup>13</sup> are the major targets in oxidized LDL for the immune system. We have previously demonstrated that high IgM levels against a number of different aldehyde-modified peptide sequences in apoB-100 are associated with increased carotid intima-media thickness (IMT) and risk for development of acute myocardial infarction.<sup>13</sup> Immunization of apoE knockout (KO) mice with some of these

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native and aldehyde-modified apoB-100 peptide sequences induces an immunoglobulin switch from IgM to IgG that is accompanied by an inhibition of atherosclerosis.<sup>14,15</sup> To study the possible atheroprotective effects of this IgG we produced human IgG1 specific for a malondialdehyde (MDA)-modified peptide corresponding to the sequence between amino acids 661 and 680 in apoB-100 (p45) by recombinant technique.<sup>16</sup> Subcutaneous immunization with MDA-p45 peptide has previously been shown to inhibit atherosclerosis by about 50% in apoE KO mice.<sup>15</sup> A similar inhibition of atherosclerosis was observed in apoE KO mice following three injections of recombinant anti-MDA-p45 IgG1, at 1 week intervals. Taken together these results suggest that IgG recognizing the MDA-modified peptide sequence between amino acids 661 and 680 in apoB-100 may protect against atherosclerosis.

The aim of the present study was to investigate the relationship between the levels of the different anti-MDA-p45 IgG subclasses and the risk for development of acute coronary events in humans, as well as the association between these antibodies and the severity of carotid disease determined by B-mode ultrasound. Since atheroprotective immunization with the MDA-p45 peptide in mice is associated with an IgG subclass switch from Th1 specific IgG2a to Th2 specific IgG1 we determined the expression of the IgG subclasses IgG1, IgG2, IgG3 and IgG4. An increased expression of IgG4 is characteristic for activation of Th2 immune responses in humans.

## **Material and Methods**

### *Study population*

The study subjects, born between 1926 and 1945, were recruited from the "Malmö Diet and Cancer" study cohort as previously described.<sup>13</sup> Participants who

had a history of myocardial infarction or stroke prior to enrolment were not eligible for the present study. The study population consisted of 224 subjects, 76 cases that developed acute coronary heart events, i.e. fatal or non-fatal myocardial infarction or deaths due to coronary heart disease during follow-up and 148 controls matched for age, sex, smoking habits, presence of hypertension, month of participation in the screening examination and duration of follow-up (Table). Only one control was available for four cases. The ethical committee of Lund University, Sweden approved the study.

### *Laboratory analyses*

After overnight fasting blood samples were drawn for the determination of serum values of total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol and whole blood glucose. LDL cholesterol, expressed in mM, was calculated according to the Friedewald formula. Oxidized LDL was measured using ELISA (Mercodia, Uppsala, Sweden) in EDTA plasma supplemented with the antioxidants DTPA and BHT.

### *B-mode ultrasound vasculography*

An Acuson 128 Computed Tomography System (Acuson, Mountain View, California) with a 7 MHz transducer was used for the assessment of plaques in the carotid artery as described previously.<sup>17</sup>

### *Determination of MDA-p45 IgG subclasses*

A 20 amino acid long peptide corresponding to the sequence between amino acids 661 and 680 in human apoB-100 (p45; IEIGLEGKGFPTLEALFGK) was produced (KJ Ross Petersen AS, Horsholm, Denmark) and used in an ELISA. The peptide was modified by treatment with 0.5 M MDA<sup>18</sup> for 3 h at 37 °C. The MDA-modified peptide was dialyzed against PBS containing 1 mM

*IgG1 against oxLDL epitopes is associated with decreased carotid stenosis*

EDTA with several changes for 18 h at 4°C. The MDA modification of peptides was assessed using the thiobarbituric acid reactive substances (TBARS) assay as described.<sup>13</sup> The aldehyde content of the modified peptide was 0.022 nmol per µg peptide. The MDA-modified peptides were diluted in PBS pH 7.4 (20 µg/ml) and absorbed to microtiter plate wells (Nunc

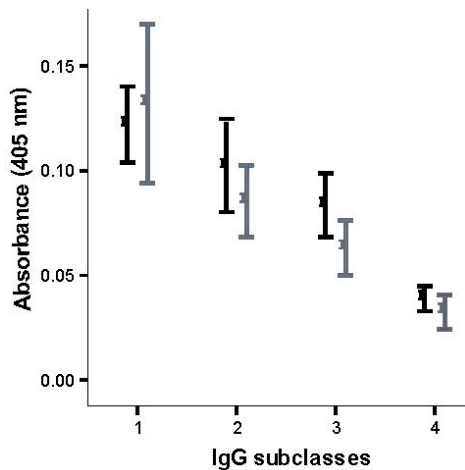
MaxiSorp, Nunc, Roskilde, Denmark) in an overnight incubation at 4 °C. After washing with PBS containing 0.1% Tween-20 (PBS-T), the coated plates were blocked with SuperBlock in TBS (Pierce, Rockford, Illinois) for 5 min at room temperature (RT) followed by incubation with test plasma, diluted 1/100 in TBS-0.1% Tween-20 (TBS-T) containing 10%

**TABLE.** Baseline characteristics of subjects with coronary events (myocardial infarction or deaths due to CHD), and controls matched for age, sex, smoking, hypertension and examination period. Values are expressed as median (range) or as proportions.

	Subjects	
	Cases	Controls
Number	76	148
Age (years)	61 (49 – 67)	61 (49 – 67)
Male sex (%)	70	69
<i>Life style factors</i>		
Never smoked (%)	29	26
Former smokers (%)	44	48
Current smokers (%)	27	26
<i>Anthropomorphic and blood glucose status</i>		
BMI (kg/m <sup>2</sup> )	26.4 (18.6 – 36.5)	26.3 (16.0 – 40.6)
Blood glucose (mM)	5.0 (3.6 – 21.4)	4.9 (3.8 – 12.0)
Diabetes mellitus (%)	18	11
Anti-diabetic medication (%)	13	3
<i>Blood pressure status</i>		
Diastolic blood pressure (mm Hg)	90 (74 – 126)	90 (70 – 130)
Systolic blood pressure (mm Hg)	150 (108 – 200)	154 (112 – 210)
Hypertension (%)	64	64
BP lowering medication (%)	30	23
<i>Blood lipid status</i>		
Total cholesterol (mM)	6.28 (3.47 – 8.24)	6.00 (4.08 – 9.90)
LDL-cholesterol (mM)	4.4 (1.7 – 6.2)	4.0 (1.6 – 7.6)
HDL-cholesterol (mM)	1.1 (0.6 – 2.5)	1.2 (0.6 – 2.9)
Oxidized LDL (U/L)	86.6 (34.9 - 184.0)	85.9 (32.7 – 163.4)
Triglycerides (mM)	1.5 (0.5 – 10.0)	1.2 (0.4 – 7.3)
Lipid-lowering medication (%)	12	6
<i>Carotid ultrasonography</i>		
Common carotid intima-media thickness (mm)	0.81 (0.36 – 1.67)	0.82 (0.47 – 1.58)
Carotid bulb intima-media thickness (mm)	1.79 (0.80 - 3.42)	1.45 (0.71 - 4.07)
Carotid stenosis, (%)	12.5 (0 - 60)	5.0 (0 - 60)

BMI, body mass index; LDL; low-density lipoprotein; HDL, high-density lipoprotein.





**Figure 1.** Subclass IgG levels against MDA-p45. No significant differences were detected between cases (grey) and controls (black) for any of the subclasses.

SuperBlock for 2 h at RT and overnight at 4°C. After rinsing, binding of autoantibodies directed to the peptides was detected using specific mouse anti-human IgG1, IgG2, IgG3 and IgG4 antibodies (Sigma, St Louis, MO) appropriately diluted in TBS-T. After another incubation for 3 h at RT the plates were washed and the bound antibodies were detected by alkaline phosphatase conjugated goat anti-mouse IgG (Sigma), by incubating for 2 h at RT. The color reaction was developed by using a phosphatase substrate kit (Pierce) and the absorbance at 405 nm was measured after 2 h of incubation at RT.

#### Statistics

SPSS was used for the statistical analyses. The results are presented as median and range and as proportions when appropriate. Pearson correlation with and without age adjustment was used to study association between risk factors and IgG subclasses. Differences between group means were tested by *t*-test. Chi-square test was used for comparing proportions. A general linear model was applied to

examine the trend between carotid ultrasound measurements and quartiles of IgG.

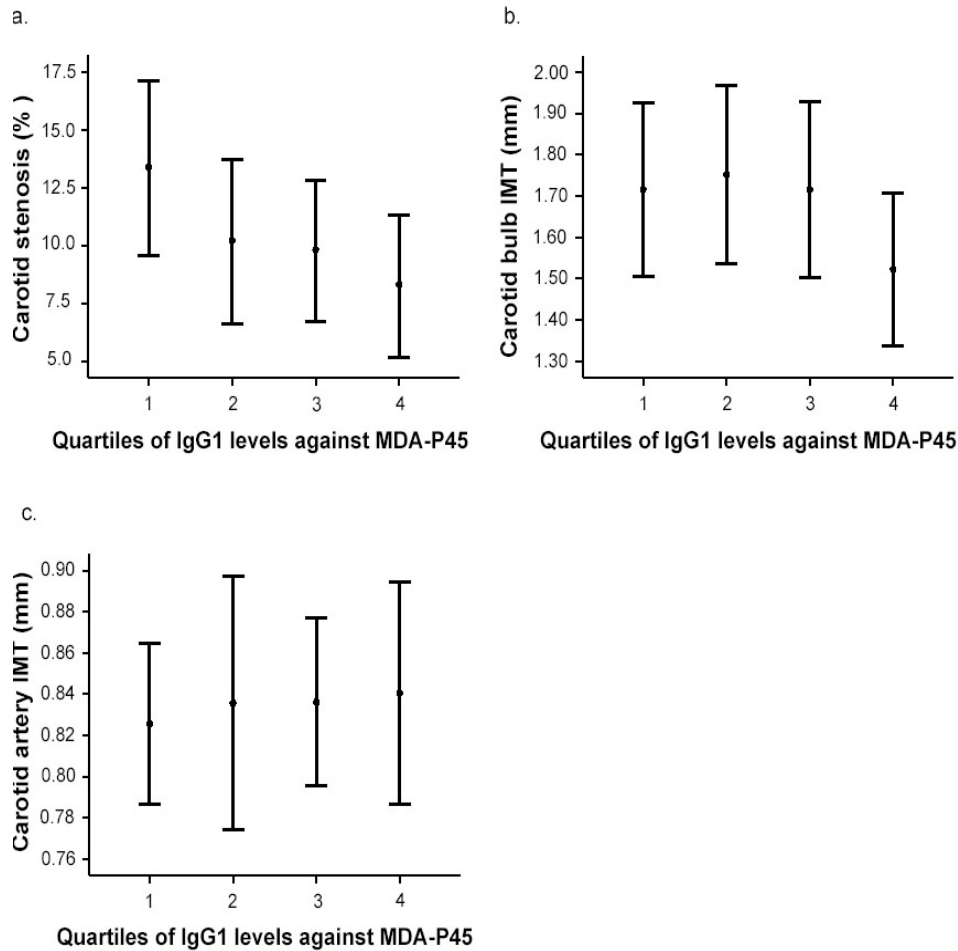
#### Results

Using a nested case control design we selected 76 subjects with coronary events (acute myocardial infarction or death due to coronary heart disease) and 148 controls matched for age, sex, smoking and hypertension from the Malmö Diet and Cancer Study. Neither cases nor control individuals had a history of previous myocardial infarction or stroke prior to enrolment in the study. The median time from inclusion to the acute coronary event was 2.8 years (range 0.1-5.9 years) among cases. The baseline characteristics of the study groups are shown in the table. Except for an increase in triglycerides among cases, there were no differences in lipoprotein lipids, plasma oxidized LDL or glucose between cases and controls. The severity of carotid disease was assessed by B-mode ultrasound by determining IMT in the common carotid artery and in the bulb, as well as the percent carotid stenosis at baseline. Cases were characterized by an increased IMT in the bulb and a trend towards more severe carotid stenosis (Table).

Baseline plasma levels of IgG1, IgG2, IgG3 and IgG4 against MDA-p45 were determined by ELISA. Antibodies recognizing this peptide sequence were detected in all individuals. IgG1 was the most common subclass while the lowest levels were found for IgG4 (fig.1). There was no significant difference between cases and controls for any of the subclasses.

For analysis of the relationship between MDA-p45 IgG and carotid disease the entire study cohort was divided into quartiles according to plasma levels of each IgG subclass. After adjusting for age

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**Figure 2.** Quartiles of IgG1 levels against MDA-p45 and severity of carotid disease. Inverse associations between MDA-p45 IgG1 levels and degree of carotid stenosis (a;  $P=0.006$  for trend, after adjusting for age, sex, systolic blood pressure, LDL and HDL cholesterol) and IMT in the carotid bulb (b;  $P=0.064$  for trend, after adjusting for age and sex). No association was found with IMT in the common carotid artery (c).

and sex, a significant association was found between high levels of MDA-p45 IgG1 and a low degree of carotid stenosis (fig. 2a;  $p=0.008$  for trend). This association remained significant also after adjusting for the influence of systolic blood pressure, LDL and HDL cholesterol ( $p=0.006$  for trend). A weaker inverse association was observed between MDA-p45 IgG1 and IMT in the carotid bulb (fig.

2b;  $p=0.064$  for trend after adjusting for age and sex), whereas no association was found between MDA-p45 IgG1 and IMT in the common carotid artery (fig. 2c). There were no associations between any of the other IgG subclasses and carotid disease. There were also no significant associations between MDA-p45 IgG subclass levels and age, sex, lipoprotein lipids, plasma oxidized LDL, glucose,

smoking or blood pressure (data not shown).

### **Discussion**

The present study demonstrates an association between high levels of IgG1 against a defined aldehyde-modified peptide sequence in apoB-100 (p45) and a lower degree of carotid stenosis. This association is independent of other major cardiovascular risk factors such as blood pressure, LDL and HDL cholesterol. Although this association in itself does not provide evidence for a protective role of MDA-p45 IgG1 in carotid plaque development, it does support previous experimental studies<sup>14,16</sup> suggesting the existence of an anti-atherosclerotic effect. Immunization of apoE KO mice with MDA-p45 peptide results in an increase in specific IgG associated with an almost 50% decrease in aortic plaque area and a 30% decrease in the plaque content of inflammatory cells.<sup>15</sup> Moreover, treatment of apoE KO mice with human recombinant IgG1 specific for the MDA-p45 sequences also reduced aortic plaque area and decreased plaque inflammation.<sup>16</sup>

We have previously shown that in humans most antibodies against MDA-p45 are of the IgM type and that high levels of these IgM are associated with increased carotid IMT.<sup>13</sup> The finding that IgG and IgM against MDA-p45 have opposite associations with the severity of carotid disease suggests the interesting possibility that switching antibody expression from IgM to IgG may be part of an endogenous defense mechanism against atherosclerosis.

Several other studies have provided support for the existence of an antibody-mediated protection against atherosclerosis. A much more aggressive development of atherosclerosis has been observed in apoE KO mice following

removal of the spleen and this effect was completely inhibited by transfer of isolated spleen B cells.<sup>19</sup> The increased intimal plaque development observed in Rag-1 mice in response to carotid cuff-injury is also diminished by transfer of spleen B cells from wild type mice.<sup>20</sup> In apoE KO mice immunized with MDA-LDL there is a significant association between the increase in specific IgG and inhibition of atherosclerosis.<sup>11</sup> Moreover, the development of atherosclerosis in these mice has been found to be reduced by repeated injections of polyclonal human IgG.<sup>21</sup>

The results of human studies regarding the association between IgG against epitopes in oxidized LDL and carotid disease are less clear. Karvonen et al<sup>22</sup> found inverse associations between IgG autoantibody titers to copper-oxidized LDL and carotid IMT, but the association was not statistically significant after adjustment for other major risk factors of atherosclerosis. Associations between low levels of IgG against oxidized LDL and increased carotid IMT have also been reported by Fukumoto et al<sup>23</sup> in a study on healthy Japanese subjects. In contrast, Hulthe et al<sup>24</sup> found a positive association between oxidized LDL IgG and carotid IMT in a study on healthy 58-year old Swedish men. The reason responsible for these discrepancies remains to be clarified. One possibility is that different antigenic structures present in oxidized LDL do not show the same association with disease severity and that the oxidized LDL preparations used in the analytical procedures differed in this respect.

The atheroprotective effect of immunization with MDA-p45 and MDA-LDL in mice is associated with a switch in IgG subclass from Th1 specific IgG2a to Th2 specific IgG1.<sup>15</sup> Activation of Th2 responses in humans results in an increased expression of IgG4. However,

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we could not identify any association between MDA-p45 IgG4 levels and carotid disease in the present study. It remains to be determined whether activation of Th2 responses to oxidized LDL antigens occurs exclusively when used in active immunization together with an adjuvant. Wu and Lefvert<sup>25</sup> have previously reported that oxidized LDL IgG antibodies are primarily of the IgG2 and IgG3 subclass. In contrast, the present study shows that the subclass distribution of IgG against MDA-p45 is the same as for that of total IgG in plasma, i.e. IgG1 is the most common and IgG4 is the least common.

In accordance with a previous study,<sup>26</sup> subjects that subsequently suffered an acute coronary event were characterized by an increased severity of carotid disease. Although low levels of MDA-p45 IgG1 were associated with an increased severity of carotid disease it did not predict risk for coronary events in the present study. However, it is possible that this study was too small to identify such an association.

### **Summary**

The present clinical findings support previous studies in experimental animals suggesting that IgG1 recognizing the aldehyde-modified peptide sequence between amino acids 661 and 680 in apoB-100 has a protective effect against atherosclerosis. Since such antibodies have been produced by recombinant technique it is an interesting possibility that they could be used for treatment of atherosclerosis.

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