

Aus dem Institut für Biochemie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

**Evaluation of T cell response to native and oxidized
low-density lipoprotein (LDL) in atherosclerosis**

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Marion Irene Wurm

aus Stuttgart

Gutachter: 1. Prof. Dr.sc.H. Kühn

2. Prof. Dr. med. V. Stangl

3. Prof. Dr. med. U. Habenicht

Datum der Promotion: 08.04.2011

Table of contents

Abbreviations.....	4
1 Introduction.....	6
1.1 Clinical background on atherosclerosis.....	6
1.1.1 Epidemiology	6
1.1.2 Characteristics of arterial lesions	7
1.1.3 Diseases involved	8
1.2 The immune system.....	9
1.2.1 Innate immunity.....	9
1.2.2 Adaptive immunity.....	10
1.2.3 Antigen recognition by T cells.....	11
2.1 T cells as modulators in atherosclerosis.....	13
2.2 Candidate antigens in atherosclerosis.....	14
2.2.1 Low- density lipoprotein function.....	14
2.2.2 Low - density lipoprotein structure.....	15
3.1 The role of T cells, LDL and oxidized LDL in atherosclerosis.....	17
3.1.1 The role of CD4 + T cells.....	18
3.1.2 The "oxidation hypothesis".....	19
3.1.3 The controversial role of ox LDL.....	20
2 Aims.....	23
3 Methods.....	24
3.1 Animals and immunization	26
3.2 T cell hybridomas	26
3.2.1 Creation of the hybridoma	26
3.2.2 T- cell hybridoma culture	27
3.3 IL-2 assay.....	27
3.4 Enzyme- linked immunosorbent assay, ELISA.....	27
3.5 Preparation of the antigens	28
3.5.1 Preparation of LDL	28
3.5.2 Preparation of oxidized LDL	28
3.5.3 Preparation of soluble ApoB 100.....	29
3.6 mRNA and cDNA preparation	29
3.7 Polymerase chain reaction (PCR) amplification	30
3.8 Flow cytometer analysis	30
3.9 MHC restriction essay.....	31

3.10	Statistical analysis.....	31
4	Results.....	32
4.1	T cells recognize native LDL.....	32
4.1.1	The more oxidation, the less activation	33
4.2	MHC class II - restricted response	36
4.3	Decrease of activation with increase of oxidation.....	37
4.4	TRBV 31+ CD4+ T cells recognize native LDL.....	38
4.4.1	PCR results of the three positive clones.....	38
5	Discussion	43
5.1	Reaction of T cell hybridomas to different antigens	43
5.1.1	The more oxidation, the less activation	43
5.2	MHC class II- restricted manner.....	45
5.3	Decrease of activation with increase of oxidation.....	46
5.4	TRBV 31+ CD4+ T cells recognize native LDL	47
5.5	Atherosclerosis as an autoimmune disease	48
6	Summary and conclusion	53
7	Zusammenfassung	55
8	References.....	58
9	Curriculum vitae (CV).....	65

Abbreviations

Abbreviations

ACS	acute coronary syndrome
APC	antigen presenting cell
APC	Allophycocyanin (APC) (fluorochrome used for flow cytometer)
APS	antiphospholipidsyndrome
Apo B	apolipoprotein B
Apo E	apolipoprotein E
CAD	coronary artery disease
CE	cholesteryl ester
CHD	coronary heart disease
CD4	cluster of differentiation 4
Con A	concanavalin A
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate (fluorochrome used for flow cytometer)
HAT	Hypoxanthine-aminopterin- thymidine
huB 100 ^{tg}	human apolipoprotein B 100 transgenic mice
HDL	high density Lipoprotein
HLA	human leucocyte antigens
IL-2	interleukin-2
KO mice	knockout mice
LDL	low- density lipoprotein
LN	lymph node
MHC	major histocompatibility complex
MI	myocardial infarction
Ox LDL	oxidized lipoprotein
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
PC	phosphatidylcholine

Abbreviations

PE	R-Phycoerythrin (PE) (fluorochrome used for flow cytometer)
PCR	polymerase chain reaction
RA	rheumatoid arthritis
RT- PCR	reverse transcriptase polymerase chain reaction
SCID	severe combined immunodeficiency
SDS	sodium dodecylsulfate
SDS- PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLE	systemic lupus erythematosus
TBARS	thiobarbituric acid reactive substances
TCR	T cell receptor
TG	triglyceride
Th1	T helper cell type 1
Th2	T helper cell type 2
TIA	transient ischaemic attack
TNF	tumor necrosis factor
TRBV	T cell receptor beta variable
UC	unesterified cholesterol
VCAM	vascular cell adhesion molecule
VLDL	very low density lipids

1 Introduction

1.1 Clinical background on atherosclerosis

1.1.1 Epidemiology

Atherosclerosis, the cause of myocardial infarction, stroke and ischaemic gangrene, is an inflammatory disease predicted to become the major cause of death globally within the next 15 years [1].

Cardiovascular diseases are the most common cause of death in European men under 65 years of age and the second most common cause in women [2].

These facts highlight the importance of this disease worldwide.

The Framingham study and other epidemiologic studies have identified certain risk factors for atherosclerosis. These include age, gender, high levels of LDL, low levels of HDL, high triglycerides, cigarette smoking, hypertension and diabetes [3].

Hypercholesterolemia is a dominant risk factor for the development of atherosclerosis. New investigative tools, including genetically modified mouse models of disease, have resulted in a clearer understanding of the molecular mechanisms of plaque development. It is now clear that atherosclerosis is not simply an inevitable degenerative consequence of ageing, but rather a chronic inflammatory condition that can be converted into an acute clinical event by plaque rupture and thrombosis [1, 2]. The immune system is a key player in the progression and development of the disease. This thesis is focused on the role of the adaptive immune system in atherosclerosis, especially the role of T cells and their response to potential antigens causing the inflammatory response in atherosclerosis. I have chosen to first give an overview of the stages of atherosclerotic development and its clinical complications, and to then give a short overview of the immune system to provide some background information for better comprehension of the performed experiments. In the sections following the overview, I describe some studies on the immunomodulation in atherosclerosis and the role of LDL and oxidized LDL. I will then describe the experiments performed and discuss the results.

1.1.2 Characteristics of arterial lesions

I will first describe the structure of a normal large artery. A large artery consists of 3 morphologically distinct layers. The adventitia, the outer layer, consists of connective tissue with interspersed fibroblasts and smooth muscle cells. The media, the middle layer, consists of smooth muscle cells, vasa vasorum, which supply the vessel itself with oxygen, and immune cells. The normal intima, which is the innermost layer, is a very thin region, covered by a monolayer of endothelial cells on the luminal side [4].

This is where the formation of atherosclerotic lesions or plaques takes place. They are asymmetrical focal thickenings of the innermost layer of the artery, and contain inflammatory and immune cells, mainly macrophages and T-cells, endothelial cells, smooth muscle cells, extracellular matrix, lipids and debris [1].

Atherosclerosis proceeds slowly in different stages.

Inflammation plays a key role in the progression of the disease. Immune cells dominate early atherosclerotic lesions, their effector molecules accelerate progression of lesions, and activation of inflammation can lead to acute clinical symptoms [2].

Early lesions are known as fatty streaks. They are dominated by sub-endothelial accumulations of macrophages that have taken up modified cholesterol, so-called “foam cells”. Such fatty streak lesions in humans can usually be found in the aorta in the first decade of life, in the coronary arteries in the second decade, and in cerebral arteries in the third or fourth decade of life [4]. So, fatty streaks are prevalent in young people, never cause symptoms, and can either disappear or progress into the next stage of atherosclerosis, the formation of a mature plaque, which is known as atheroma [2]. In the center of an atheroma, foam cells and extracellular lipid droplets form a core region, which is surrounded by a cap of smooth muscle cells and collagen- rich matrix. T-cells, macrophages and mast cells infiltrate the lesion and are particularly abundant in the shoulder region of the plaque, which is where it grows [5].

Many of the immune cells show signs of activation and produce pro-inflammatory cytokines such as interferon- γ and tumor-necrosis factor (TNF) [6]. Cytokines are small soluble proteins secreted by one cell that can alter the behaviour or properties of the cell itself or another cell [7]. With time, the plaque can progress into an even more complex lesion, a lipid core with a pool of cholesterol deposits which is surrounded by

a fibrous cap of varying thickness. It is a structure composed of a dense collagen-rich extracellular matrix with some smooth muscle cells, macrophages and T-cells. This fibrous cap isolates the lesion from the blood flow, which is good, so that all the pro-thrombotic material in the lesion is securely separated from the blood flow [1]. See figure 1 below to picture the different stages of atherosclerosis.

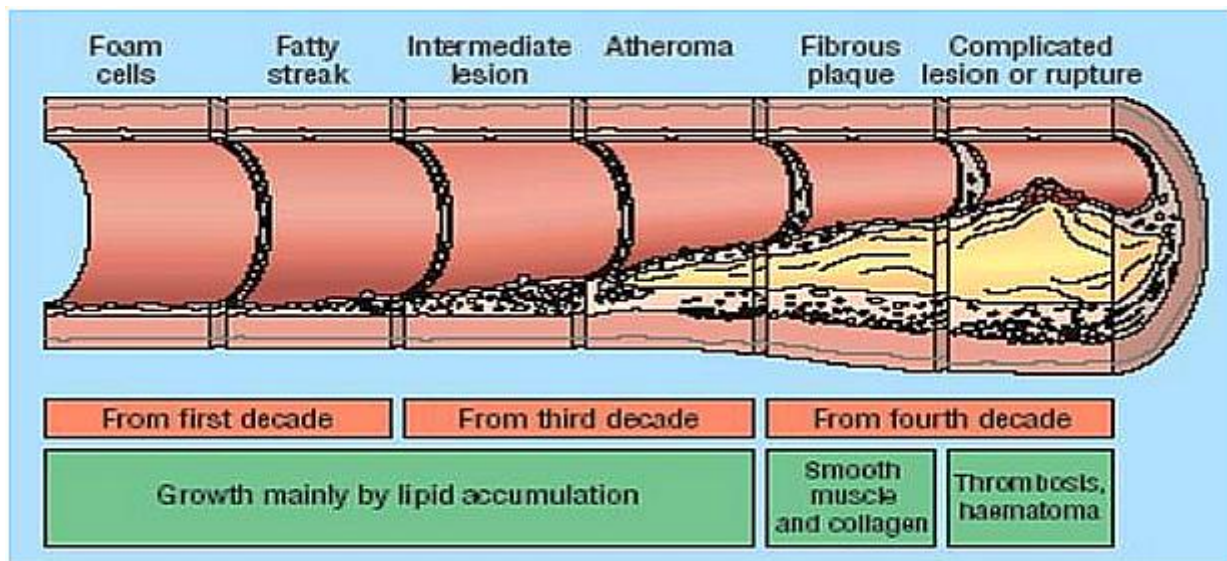


Figure 1. Stages in atherosclerosis. From [8]

Reprinted from: Stary, H.C., et al., A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*, 1995. 92(5): p. 1355-74.

1.1.3 Diseases involved

Clinical complications of atherosclerosis can appear as flow-limiting stenosis through the progressive luminal narrowing from growth of the plaque and cause coronary spasms, transient ischaemic attacks (TIAs) in cerebral arteries, or occlusion of peripheral arteries in the extremities, peripheral arterial occlusive disease, which appears clinically as pain. Yet the most severe clinical events follow the rupture of a plaque, so that the pro-thrombotic material causes sudden thrombotic occlusion of the artery. The major cause of coronary thrombosis is plaque rupture. Plaque rupture exposes the pro-thrombotic material from the core of the plaque to the blood. Activated immune cells within the plaque release certain molecules, such as proteases, which attack the fibrous cap, so that it becomes thinner, transforming the stable plaque into a vulnerable unstable structure.

The weakened plaque might fissure when subjected to the forces of arterial blood pressure. This can appear as myocardial infarction, stroke or ischaemic gangrene [1, 2]. The concept of the vulnerable plaque has generated much interest, and recent clinical data gave evidence that multiple vulnerable plaques coexist in symptomatic patients and that inflammation is a critical determinant of the stability of plaques. In patients with acute coronary syndromes (ACS), vulnerable coronary plaques are numerous, and active plaques extend beyond the culprit lesion in the coronary tree [9].

1.2 The immune system

The function of the immune system is to protect us from infection. It consists of cells and molecules with remarkable ability to distinguish between self and non-self. The immune system can be divided into two lines of defense, innate and adaptive immune response. Both systems communicate by means of cell-cell interactions through surface molecules and by soluble messenger molecules such as cytokines.

1.2.1 Innate immunity

The *innate response* is a rapid response to injury, initiated by molecular patterns that are commonly found on pathogens, so called pathogen associated molecular patterns (PAMPs) [10]. These are molecules like lipopolysaccharides, surface phosphatidyl-serine, protein, as well as modified LDL [11]. The innate immune response does not involve the recognition of specific structures or the use of memory cells [12, 13]. Macrophages express a set of pattern recognition receptors including various scavenger receptors and toll like receptors [14]. Ligands to these receptors include PAMPs [10]. Ligands bound to the scavenger receptors can be “eaten” by the macrophages through endocytosis and lysosomal degradation of the bound ligands [10]. Ligands that bind to the toll like receptors result in the activation of nuclear factor kappa b and mitogen activated protein kinase (MAPK) pathways [15, 16]. Ligation of toll like receptors can also heighten phagocytosis, production of reactive oxygen, release of cytokines and lipid mediators that coordinate and amplify the local inflammatory response [17-19]. The innate immune system is activated as an immediate immune response, and represents the acute immune phase.

1.2.2 Adaptive immunity

The *adaptive immune response* requires the recognition of specific molecular structures and depends on the generation of large numbers of antigen receptors (T cell receptors) and immunoglobulins by somatic rearrangement processes in blast cells [10,11]. When T cells recognize a foreign antigen presented to them, they target precisely that antigen, including a direct attack against the antigen-bearing cell by cytotoxic T cells, stimulation of antibody production by B cells, and induction of a local inflammatory response [10].

T cells can be differentiated into different subtypes of T helper cells, Th1 and Th2, Th 17 and regulatory T cells. Th1 cells elaborate a number of cytokines, for example INF- γ , which stimulate the macrophage. Th2 cells can stimulate humoral immunity by elaborating a number of cytokines that stimulate B cell maturation into antibody-producing plasma cells. Th2 cells can also aid recruitment and activation of mast cells. In addition to these specialized pro-inflammatory responses, Th 2 cells can dampen inflammatory response by elaborating cytokines with antiinflammatory properties such as interleukin 10 (IL-10) [10]. Th 17 cells are important for autoimmune disease and T regulatory cells control the effector population of T cells.

The adaptive immune system requires, in contrast to the immediate innate immune response, some time until it is activated, which is typical for chronic immune responses such as those in chronic inflammatory diseases or chronic infections.

Because the adaptive immune system requires the recognition of specific molecular structures, we wanted to have a closer look at the adaptive immune system, especially CD4⁺ T cells, and find out which molecular structures give rise to the adaptive immune response in atherosclerosis. Adaptive immune responses that involve clonally expanded T cell populations contribute to chronic inflammation in atherosclerosis, as do innate immune responses that are mounted by macrophages and other cell. Several studies have suggested that components of low-density lipoprotein (LDL) particles trigger vascular inflammation [82, 83]. In this research project, we wanted to investigate the role of oxidized LDL as an antigen for the specific T-cell dependent response.

1.2.3 Antigen recognition by T cells

In contrast to immunoglobulins, which interact with pathogens and their toxic products in extracellular spaces of the body, T cells only recognize foreign antigens that are displayed on the surface of the body's own cells. T cells can detect the presence of intracellular pathogens because infected cells display on their surface peptide fragments derived from the pathogens' proteins. These foreign peptides are delivered to the top of the cell surface by specialized glycoproteins, the MHC molecules. The MHC molecules are encoded in a large cluster of genes that were first identified by their potent effect on the immune response to transplanted tissues. For that reason, the gene complex was termed the major histocompatibility complex (MHC). Two different classes of MHC molecules deliver peptides from within the cell to the cell surface, MHC class I and MHC class II molecules [20].

Peptides that are bound to MHC class I molecules are typically derived from viruses and are recognized by CD 8+ T cells. Peptides bound to MHC class II molecules are derived from particles that replicate inside intracellular vesicles and are recognized by CD 4+ T cells. MHC class I and II genes have a great genetic variability, which extends the range of peptides that can be presented to T cells [20].

The T cells recognize these peptides that are presented by the MHC via the T cell receptor. The T cell receptor consists of two chains, named TCR α and TCR β chains, linked by a disulfide bond. These α : β heterodimers account for antigen recognition by T cells [21]. Both chains of the T cell receptor have an amino-terminal variable region (V region), a constant region (C region), and a short hinge region containing a cysteine residue that forms the interchain disulfide bond. The peptides that stimulate T cells are recognized only when bound to an MHC molecule. The ligand recognized by the T cell is thus a complex of peptide and MHC molecule. The T cell receptor interacts with this ligand by making contacts with both the MHC molecule and the antigen peptide [21]. The interactions are regulated by other molecules that either stimulate or inhibit the connection of the T cell to the antigen/ MHC complex.

The experiments performed for this medical doctoral thesis aim at finding an antigen in the atherosclerotic process that initiates the T cell response via the T cell receptor/ MHC class II complex. In order to understand the experiments performed for this thesis,

Introduction

I would like to add that in mice, the homologous gene complex is called H-2 locus. MHC class II genes A and E in mouse are called I-A and I-E antigens, since the original name of MHC class II genes were immune response genes. These are encoded for in the I region of the mouse H-2 major histocompatibility gene complex, and play an essential role in antigen recognition by T lymphocytes involved in helper functions [22-24] and proliferation [25, 26]. The recognition by T cells of the I-A molecule is essential for T cell activation and results in H-2 restriction of responses [22]. See figure 2 below to picture interactions between T cells and antigen presenting cells.

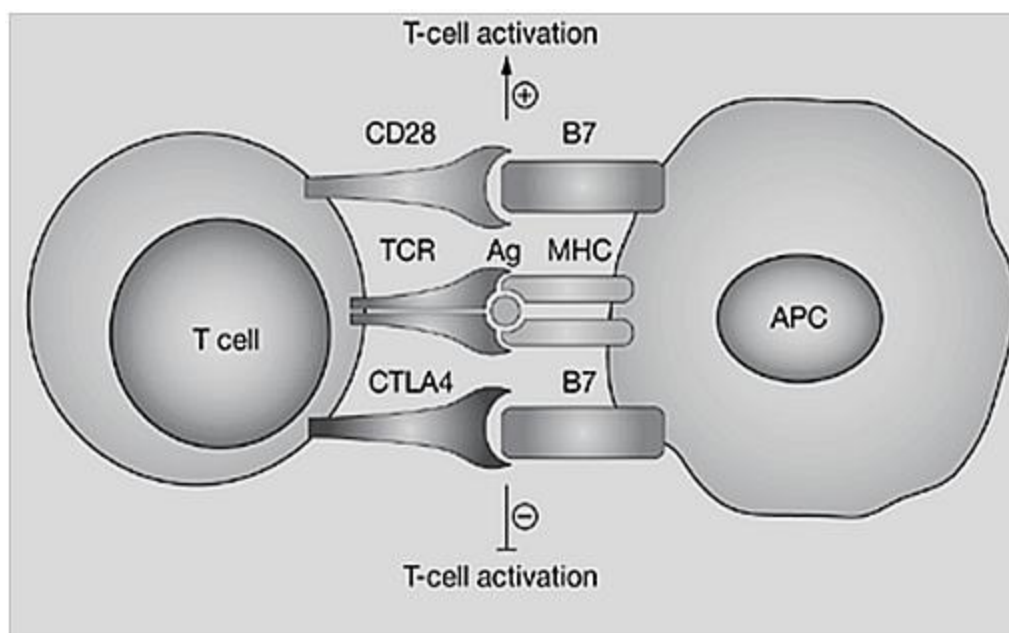


Figure 2. Interactions between T cells and antigen presenting cells.

The T cell receptor recognizes the antigen–major histocompatibility complex on the surface of antigen presenting cells. Interaction between CD28 on the T cell and B7 on the antigen presenting cell provides a positive costimulatory signal, whereas interaction between CTLA4 and B7 is inhibitory to T cell activation. If the positive signal through CD28–B7 interaction dominates, the T cell is activated resulting in cytokine production, provision of B cell help and inflammation. If the CTLA4–B7 interaction dominates, T cell activation is suppressed.

Figure modified from: Sakkas, L.I., I.C. Chikanza, and C.D. Platsoucas, Mechanisms of Disease: the role of immune cells in the pathogenesis of systemic sclerosis. Nat Clin Pract Rheumatol, 2006. 2(12): p. 679-85. [27]

2.1 T cells as modulators in atherosclerosis

The atherosclerotic process is initiated when cholesterol- containing lipoproteins accumulate in the intima and initiate a local inflammatory immune response involving different subsets of cells, endothelial cells, smooth muscle cells, macrophages and B and T cells [2]. It has been shown that T cells may be activated locally in atherosclerotic lesions presumably by antigens presented in the context of MHC class II expressing smooth muscle cells and/ or macrophages [28, 29]. We are particularly interested in the role of T cells in atherosclerosis, since they influence plaque development. See figure 3 below to picture the effects of T cell activation on plaque inflammation.

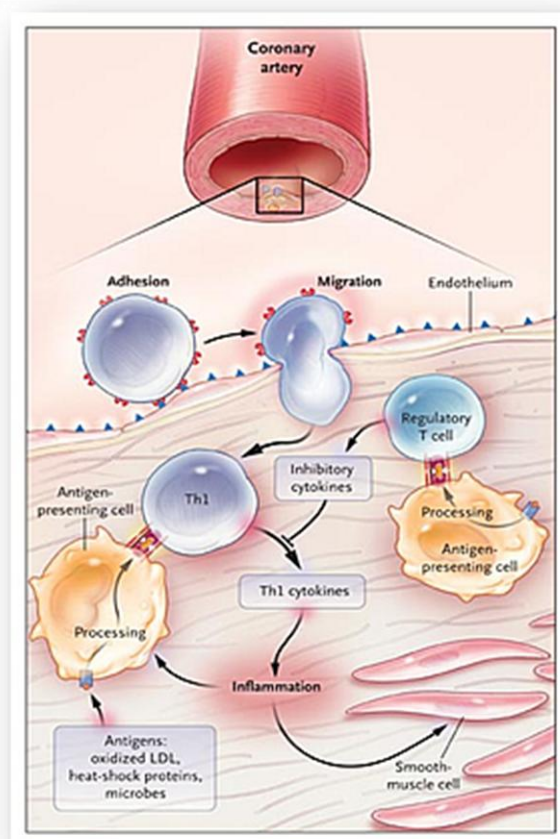


Figure 3. Effects of T cell activation on plaque inflammation

Antigens presented by macrophages and dendritic cells stimulate antigen specific T cells. Most of the activated T cells produce Th1 cytokines which in turn activate macrophages and vascular cells. This leads to inflammation [2].

Reprinted from: Hansson, G.K., Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med, 2005. 352(16): p. 1685-95.

2.2 Candidate antigens in atherosclerosis

There are several candidate antigens in atherosclerosis. They initiate disease through adaptive immunity. Immunodominant T cell epitopes are peptides of a given protein that best fit an MHC molecule. Even though the involvement of cellular immunity in atherosclerosis is well established, the specific T cell clones and antigens are still to a large extent unknown. Evidence of oligoclonal T cell expansion in lesions of atherosclerotic mice also points in the direction of a limited set of antigens giving rise to the immune response [30]. Such T cell epitopes have not yet been identified in atherosclerosis [31]. In this research project, we wanted to search for such an antigen. There are several approaches to gain insight into specific immune responses. One method is to measure circulating antibodies. Another approach is to expose T cells to potential antigens, and measure their reaction, which was our approach in the experiments. The antigen in atherosclerosis that has gained most attention is oxidized LDL. Let us therefore have a look at the molecular structure of LDL.

2.2 .1 Low- density lipoprotein function

LDL is one of the five major groups of lipoproteins; these groups include chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). LDL is a heterogenous particle that contains a protein part, apolipoprotein B -100, cholesteryl esters, cholesterol, triacylglycerol, and phospholipids. As the principal transporter for cholesterol in the blood, circulating LDL guarantees a constant supply of cholesterol for tissues and cells and is essential for human life. Cholesterol is an essential substance involved in many functions, which include maintaining cell membranes, manufacturing vitamin D and producing hormones. LDL delivers cholesterol to peripheral tissue. Homeostasis of cholesterol is centered on the metabolism of lipoproteins [32]. The cellular uptake of LDL is mediated by specific receptors (LDL- receptors) and is strongly dependent on the structure of LDL particles and on the apoB 100 located on the surface of LDL [33]. In case of lack of receptors for LDL uptake, as is the case in familial homozygote hypercholesterolemia, massive accumulation of LDL in the vascular system occurs, with an increased risk of cardiovascular disease [34].

2.2.2 LDL structure

Each native LDL particle contains a single protein part, apolipoprotein B 100 molecule (ApoB 100), that circulates the fatty acids. It has a highly hydrophobic core consisting of polyunsaturated fatty acid known as linoleate and about 1500 esterified cholesterol molecules. This core is surrounded by a shell of phospholipids and unesterified cholesterol as well as a single copy of B-100 large protein (514kD). In the process of atherosclerosis, the particles are modified and accumulate in the arterial wall. Although the composition and overall structure of LDL is well known, the fundamental molecular interactions and their impact on the structure of the LDL particle are not well understood [35]. Oxidative modification of the lipid moieties includes degradation of plasma unsaturated fatty acid residues and generation of highly reactive aldehydes and other products that can modify other lipids. The cholesterol moiety also undergoes oxidation, and apolipoprotein B undergoes nonenzymatic degradation, as well as derivatization of its lysine and other residues [36, 37]. See figures 4 and 5 below showing the approximate chemical structure of low density lipoprotein (LDL).

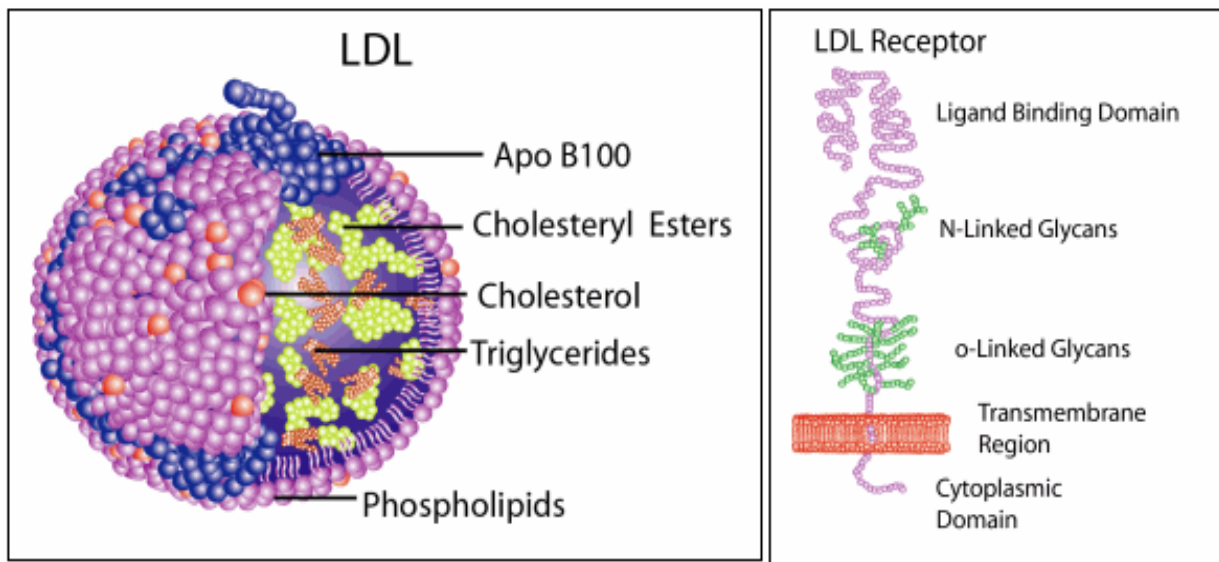


Figure 4. Structure of LDL

Each LDL particle consists of a protein part, ApoB 100, and cholesterol molecules. LDL is recognized via specific receptors, LDL receptors. The approximate structure is shown on the right side. From [38]

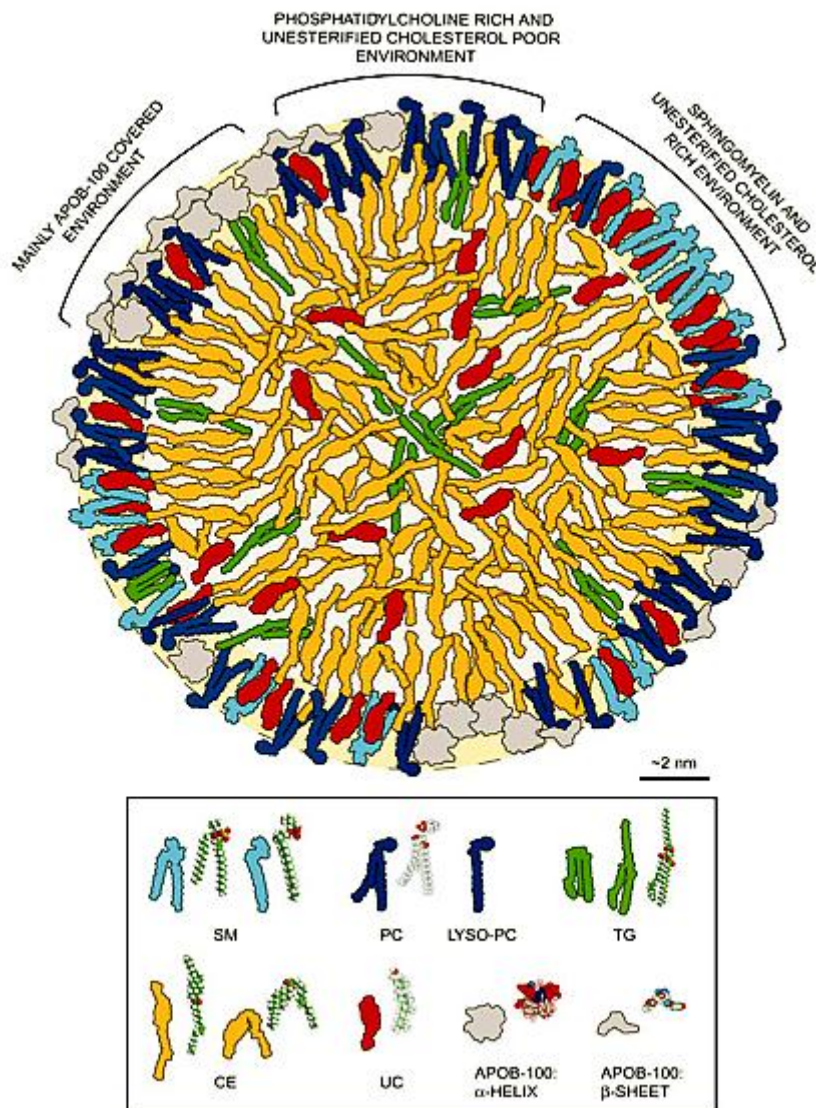


Figure 5. Structure of LDL

Schematic molecular model of an LDL particle. The average composition is 20% protein, 20% phospholipids, 40% cholesteryl esters, 10% unesterified cholesterol, and 5 % triglycerides.

Abbreviations: SM- sphingomyelinase, PC- phosphatidylcholine, lyso- PC- lyso- Phosphatidylcholine, TG- triglycerides, CE- cholesteryl esters, UC- unesterified cholesterol, Apo B 100- Apolipoprotein B 100.

Reprinted from: Hevonoja, T., et al., Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim Biophys Acta, 2000. 1488(3): p. 189-210.

[35]

3.1 The role of T cells, LDL and oxidized LDL in atherosclerosis

There is evidence that especially T cells play a crucial role in the development of atherosclerosis. T cells were isolated and cloned from atherosclerotic plaques obtained from patients undergoing carotid surgery. These T cell clones were then exposed to potential antigens, and a significant number of the clones responded to oxidized low density lipoprotein (ox LDL) by proliferation and cytokine secretion. This data showed that the inflammatory infiltrate in the atherosclerotic plaque is involved in a T cell dependent, autoimmune response to ox LDL [39]. It has been shown earlier that immune complexes consisting of ox LDL and antibodies to ox LDL can be found in atherosclerotic lesions of rabbits and humans [40].

The simplistic view of atherosclerosis as a disorder of a pathological lipid deposition has been redefined by the more complex concept of an ongoing inflammatory response. Apolipoprotein E and low density lipoprotein (LDL) - receptor-deficient mice develop accelerated atherosclerosis [41]. These basic experimental findings have partly been confirmed in studies of the human carotid artery system [41].

Inflammation is not only instrumental in the development of human atheromatous plaques, but, importantly, it plays a crucial role in the destabilization of internal carotid artery plaques, thus converting chronic atherosclerosis into an acute thromboembolic disorder. Humoral factors involved in internal carotid artery destabilization include cytokines, cyclooxygenase-2, matrix metalloproteinases (MMPs), and tissue factor. The impact of inflammation on the development of atherosclerotic plaques and their destabilization opens new avenues for treatment. The effects of statins, acetylsalicylic acid and angiotensin- converting enzyme inhibitors on stroke prevention may partly be attributable to their profound anti-inflammatory actions. [41]

The cause of the inflammatory adaptive immune response in atherosclerosis is very complex, which makes it so difficult to find a treatment. The identification of the specific antigen that initiates the inflammatory response could in the future lead to the development of a vaccine. The experiments performed for this thesis aimed at finding a possible antigen that causes the inflammatory adaptive immune response.

3.1.1 The role of CD4+ T cells

The importance of CD4 + T cells has been demonstrated in previous studies. CD 4 + T cells from Apo E $\text{--}/\text{--}$ mice were transformed into immunodeficient ApoE $\text{--}/\text{--}$ scid/scid mice. This increased the number of atherosclerotic lesions by as much as 164 % [75]. The scid mouse strain lacks T and B cells, so it is a good model to study the effects of the immune system on atherosclerosis. These data indicate that CD4+ T cells play an important role in atherosclerosis [75]. Fatty streak lesions were significantly reduced in the Apo E $\text{--}/\text{--}$ scid/scid animals. After transfer of CD4+ T cells from atherosclerotic donors into immunodeficient recipients, the lesions significantly aggravated. It was proven that injecting CD4+ T cells into immunodeficient mice accelerated atherosclerosis to a level almost as high as that in immunocompetent apoE $\text{--}/\text{--}$ mice [75]. This shows that mice lacking adaptive immunity exhibit reduced development of fatty streak lesions, and that CD4+ T cells play a crucial role in aggravation of atherosclerosis. These findings are compatible with a pro-atherogenic role for CD 4+ T cells [75]. To test whether this effect was dependent on specific disease-associated antigens, the same group immunized Apo E $\text{--}/\text{--}$ mice first with oxidized LDL, and then transferred CD4+ T cells from immunocompetent mice to immunodeficient Apo E $\text{--}/\text{--}$ scid/scid mice. It was shown for the first time that adoptive transfer of cell-mediated immunity to ox LDL significantly accelerates atherosclerosis [76]. A global deficiency of adaptive immunity leads to reduced atherosclerosis in such mice [75, 77]. This shows that particularly CD4+ T cells must play a crucial pathogenic role. On the other hand, several studies have demonstrated that immunization with ox LDL and modified ApoB 100 peptides reduces atherosclerosis in animal models [80]. In order to clarify these dichotomic results of immunization with ox LDL derived antigens, we wanted to evaluate the T cell response of CD4+ T cells to oxidized LDL. I would like to summarize some facts that show our current knowledge of the role of CD4 + T cells in atherosclerosis.

- T cells can be found in the atherosclerotic plaque and most of them are of the CD4⁺ T helper cell subtype [5, 78]
- T cells can be activated by oxidized LDL [39, 79]
- Transfer of CD4⁺ T cells from ox LDL-immunized mice to ApoE $\text{--}/\text{--}$ scid/scid mice aggravates atherosclerosis [76]

Based on these findings, we wanted to go one step further and investigate the reaction of CD4⁺ T cells to possible antigens. We wanted to investigate what the T cells that react to antigens have in common, that is to further characterize the T cells that recognize antigens. In a further step, we wanted to specify the antigen that causes proliferation in T cells in atherosclerosis. So our project aimed to identify the epitopes of oxidized LDL which the T cell receptor recognizes and which induces the proliferation of T cells in atherosclerosis. The question we addressed was: Which molecular structures of plasma lipoproteins serve as autoantigens in atherosclerosis?

The research project was aimed towards finding T cells against oxidized LDL.

In order to study T cell recognition of oxidized LDL, we created T cell hybridomas from human ApoB 100 transgenic (hu100^{tg}) mice that were immunized subcutaneously (s.c.) with oxidized LDL. These mice generate high levels of ApoB 100 that is packaged into humanlike LDL particles [81]. Like other mice that produce nonmurine proteins, huB100^{tg} mice tolerate the orthologous transgene, neither producing antibodies to it nor developing spontaneous autoimmune disease. This model makes it possible to study cellular immune responses to human LDL- derived epitopes in a controlled model system.

3.1.2 The “oxidation hypothesis”

LDL oxidation affects both the lipid and protein components of LDL [42]. The hypothesis that the sub-endothelial retention of atherogenic ApoB –containing lipoproteins is the initiating factor in atherosclerosis was tested in 2002 [43]. There was experimental evidence that the atherogenicity of Apo-B containing low-density lipoproteins (LDL) is linked to their affinity for artery wall proteoglycans. So this led to the conclusion that sub-endothelial retention of apoB100-containing lipoprotein is an early step in atherogenesis [43]. Macrophages are capable of accumulating large amounts of cholesterol derivatives in the cytosol in form of lipid droplets. [44] This gives the cells a foamy, vacuolated appearance [44], [45]. The reason for such foam cell formation is excessive uptake of LDL via the scavenger receptor mediated pathway. The oxidation hypothesis of foam cell formation suggests that modification of LDL in vivo by oxidation may be the major modifying process and the trigger of activation for uptake of modified LDL by macrophages [44], [46, 47].

This finding clearly supports the idea that a primary initiating event in atherosclerosis is the accumulation of LDL in the sub-endothelial matrix, as mentioned above [4].

3.1.3 The controversial role of ox LDL

Atherosclerotic lesions develop slowly and may pass through many stages during their progression to complex plaques. Each stage of lesion progression may be influenced by a variety of artery wall events [48]. It is not surprising, then, that our understanding of the atherosclerotic process is incomplete [48]. Atherosclerosis is a chronic inflammatory disease that involves a complex interplay of circulating cellular and blood elements with the cells of the artery wall [49]. While many factors are involved, the “oxidation hypothesis” has been a central focus of investigations into the pathogenesis of the atherosclerotic process for the past 20 years [50]. This hypothesis states that the oxidative modification of LDL, or other lipoproteins, is central, if not obligatory in the atherogenic process. The original interest in oxidized LDL (ox LDL) stemmed from two basic sets of observations. The first was that ox LDL was cytotoxic to endothelial and other cells and thus could directly cause damage to arterial cells [50-52]. The second observation was, that uptake of native LDL by macrophages occurred at a sufficiently low rate to prevent foam cell formation, but uptake of ox LDL was unregulated and led to foam cell formation [51, 52].

It is now clear that ox LDL contributes to both the initiation and progression of the atherosclerotic lesion by many mechanisms, including its pro-inflammatory, immunogenic and cytotoxic properties [48, 50, 53, 54].

Oxidation of LDL results in the generation of a variety of modifications to the lipid and protein parts, including the covalent modification of ApoB [55].

These and presumably many other such changes generate immunogenic neo-epitopes on the modified LDL [50]. Oxidized lipids were significantly elevated in unstable atherosclerotic plaques obtained from endarterectomy samples [54]. Ox LDL is immunogenic and leads to autoantibody formation. Autoantibodies to epitopes of ox LDL can be found in the plasma of animals and humans [56]. Circulating ox LDL is elevated in patients with advanced atherosclerosis. A Japanese group even suggests ox LDL as a biomarker of cardiovascular disease [57].

An increased titer of such antibodies was found in animals with atherosclerosis, and it was shown that the titer reflects the extent of lesion formation [58, 59]. Such antibodies are also found in plaques, in part in complex with ox LDL [40]. One of the reasons why there has been so much interest in ox LDL is that it is taken up unregulated by macrophages, leading to cholesteryl ester accumulation and foam cell formation [50]. Yet the role of ox LDL in humans is controversial, since clinical trials using antioxidants in humans have not proven to prevent cardiovascular events [50]. Peroxidation of unsaturated fatty acids in lipoproteins and cell membrane phospholipids occurs in many situations in the body, both under normal and pathological conditions [60]. As mentioned above, both antibody responses and T cell responses to oxidatively modified lipoproteins have been demonstrated in humans as well as in animal models. However, little is known about how these responses arise or how T cells recognize these antigens [60]. In experimental animal models, antioxidants have been proven to be beneficial [61]. In humans, there is up to date no clinical trial study that proves the beneficial effects of antioxidant therapy [61]. Until now, no pharmacological intervention study exists that proves a relevant benefit from the treatment with antioxidants [61]. So the oxidation hypothesis might be valid for the animal model, in humans it is questionable whether antioxidants have a beneficial effect on the development of the disease.

Several large-scale, double-blind, placebo-controlled trials have shown convincingly that neither β -carotene [62-64] nor vitamin E, alone [64-66] or in combination with other antioxidant vitamins [67], reduces the risk of fatal or nonfatal infarction (or other hard clinical end points) in an unselected population of people with established coronary heart disease (CHD) or at high risk of CHD. Oxidative stress may contribute to atherogenesis by mechanisms that are not necessarily linked to LDL oxidation [61]. For example, free radical oxygen species can rapidly react with nitric oxide (NO) and inactivate it. This can lead to pro-atherogenic effects by leukocyte adherence to endothelium, impaired vasorelaxation and platelet aggregation [68].

Vitamin E is the antioxidant used in most of the clinical trials to date. In mouse models of atherosclerosis it has been effective alone [69] or in combination with other antioxidants [70, 71], but most of the studies in rabbits have been negative.

Most of the animal model studies demonstrating antioxidant inhibition of atherosclerosis have been done in small animals—rabbits, hamsters, or mice. Only one systematic study has been done in nonhuman primates [61, 72]. A study in humans [73] could not prove a beneficial effect. The results suggest that in normally nourished humans, additional vitamin E will not confer any additional antioxidant protection [61]. It should be noted that in the absence of an appropriate co-antioxidant such as Vitamin C, Vitamin E can, paradoxically, act as a pro-oxidant [74]. The available data suggest that Vitamin E is not an appropriate antioxidant with which to test the oxidation hypothesis. β -carotene is not very effective as a chain-breaking antioxidant, compared with Vitamin E [61]. Until we know where and how LDL is modified in vivo, we have no way to predict which antioxidant would be most effective.

This medical doctoral thesis is an attempt to investigate the role of LDL and modified LDL in the inflammatory process, with specific focus on T cells. It is an attempt to search for the “antigen” in atherosclerosis. The clinical long-term aim with this subject is to develop a vaccine against atherosclerosis. In order to identify mechanisms of recognition that govern T cell responses to LDL particles, we generated T cell hybridomas from human ApoB 100 transgenic (huB100^{tg}) mice that were immunized subcutaneously with human oxidized LDL. Surprisingly, none of the hybridomas responded to oxidized LDL, only to native LDL and ApoB 100, the protein part of LDL. ApoB 100 responding CD4⁺ T cell hybridomas were MHC class II restricted and expressed a single T cell receptor variable chain TRBV 31, with different Va chains.

2 Aims

The aim for this study was to investigate the role of adaptive cellular immunity in atherosclerosis.

Oxidized low density lipoprotein (ox LDL) is the antigen that gained most attention for the development of atherosclerosis, as mentioned above. LDL modification occurs through oxidation in the intima and leads to release of bioactive molecules, including apolipoprotein B 100 (ApoB 100) fragments, which could be taken up and processed by antigen-presenting cells (APC) and presented to T cells, eliciting the cellular immune response. Zhou et al. (2006) [76] reported that transfer of purified CD4⁺ T cells from Apo E ^{-/-} mice immunized with ox LDL to immunodeficient mice accelerated atherosclerosis, and that circulating levels of IFN- γ were increased in proportion to this acceleration. Based on these findings, we wanted to further investigate the role of CD4⁺ T cells and their response to oxidized LDL.

We wanted to have a closer look at the response of T cells to oxidized LDL.

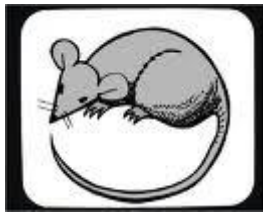
Aims in summary for my thesis

- 1 To characterize T cells reactive to oxidized LDL with regard to T cell receptors and effector responses.
- 2 To study T cell epitopes derived from human oxidized LDL.

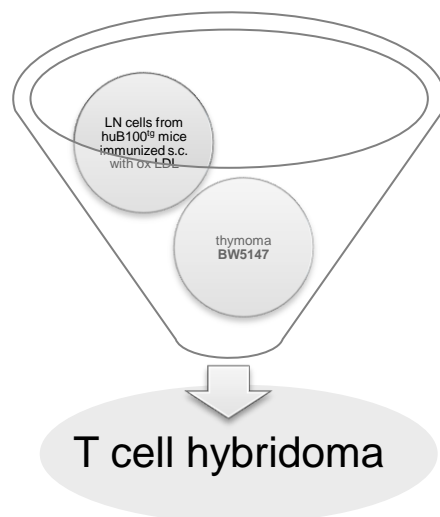
3 Methods

For better comprehension of the experimental approach used to address the above mentioned aims, I chose to give a short pictorial representation of the experimental setup. All experiments were performed on mice or on cells originating from mice.

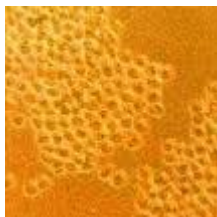
7 week old male human ApoB 100 transgenic mice (huB100^{tg})



- immunized subcutaneously (s.c.) with 50 ug copper- oxidized human LDL
- booster injection after 2 weeks with 50 ug copper- ox LDL
- † Sacrifice with CO₂ 10 weeks later
- LN cells of above mentioned sacrificed mice collected and fused with thymoma cells = hybridoma



- Selection of CD3, CD4 monoclonal T cell hybridomas, then held in cell culture



Methods

- Exposure of T cell hybridoma and antigen presenting cells (APC) to antigens:
a) human ox LDL, b) native LDL, c) purified unmodified ApoB 100 d) negative control

APC, T cell hybridoma cells +

- a)
- b)
- c)
- d)



- T cell reactivity measured as IL- 2 production by ELISA
- Hybridoma T cell clones that had strongest reaction on antigens, measured as strongest IL-2 response: mRNA -> cDNA by reverse transcriptase PCR to characterize the V α and V β chains of the T cell receptor
- Double- check of result with flow cytometer
- Check for MHC- class II restriction of response by blocking MHC class II with an antibody

Figure 6. Experimental setup

Pictures reprinted from:google.com

3.1 Animals and immunization

Seven week-old male human apoB 100 transgenic mice huB100^{tg} (C 57BL/6, 129-ApoB^{tm2Sgy}, DNX Transgenics, Princeton, USA) were used to generate T cell hybridomas. These mice carry the full- length human ApoB 100 gene, in which codon 2153 has been converted from leucine to glutamine to prevent formation of Apo B 48, generating only ApoB 100 [81, 84]. They were first immunized s.c. with 50 µg of copper-oxidized human LDL mixed with equal amounts of complete Freund's adjuvant (CFA). After 2 weeks the mice were boosted with 50 µg oxLDL mixed with incomplete Freund's adjuvant (IFA). The mice were fed a high- fat diet (maize starch, cocoa butter, casein, glucose, sucrose, cellulose flour, minerals, and vitamins; 17,2 % protein, 21% fat (62,9% saturated, 33,9% unsaturated, 3,4% polyunsaturated), 0,15 % cholesterol, 43% carbohydrates, 10% H₂O and 3,9% cellulose fibers) starting 5 days after the immunization until sacrifice 10 weeks later with CO₂. Irradiated splenocytes from C57/BL6 mice were used as antigen-presenting cells in the hybridoma experiments. All animal experiments were approved by the Stockholm North Committee for Experimental Animal Ethics.

3.2 T cell hybridomas

3.2.1 Creation of the hybridoma

T-cell hybridomas were generated by polyethylene glycol-induced fusion of LN cells (5×10^7 LN cells) with BW 5147 thymoma cells (3×10^7 cells), as previously described [85]. Lymph node cells from ox LDL-immunized apoB-transgenic mice were stimulated with 3 µg/ml ox LDL for 3 days before fusion. After fusion, 1×10^6 thymocytes/ ml were added as feeder cells and the cell suspensions were plated in 96-well plates and incubated at 37° C and 7.5 % CO₂. Hypoxanthine- aminopterin- thymidine (HAT) was added to the medium after 24 hours to select fused cells. This medium blocks the DNA synthesis in non- fused cells and so selects the fused cells. HAT-resistant hybridomas were then cloned by limiting dilution and screened for their reactivity against native LDL, copper oxidized LDL, and ApoB100, in the presence of irradiated spleen cells as antigen- presenting cells.

3.2.2 T- cell hybridoma culture

The T- cell hybridomas were held in cell culture in complete Dulbecco's minimal essential medium (DMEM), containing 5 % Fetal calf serum (FCS, Myoclone super plus) 10 mM sodium pyruvate, 100 U/ml Penicillin, 100 ug/ml streptomycin, 20 mM L- glutamine, all obtained from Gibco BRL (Life Technologies, Palo Alto, CA). The cell culture medium was changed to 10 % FCS before experiments were performed to increase the proliferation.

3.3 IL-2 assay

T-cells were harvested using an ordinary cell harvester and counted under the microscope in a chamber using Trypan blue stain to detect dead cells. Hybridoma cells were cultured together with irradiated mouse spleen cells as antigen-presenting cells in the presence of the antigen at indicated concentrations. Cells were prepared by meshing the tissues on nylon filters (100 μ m), followed by lysing of red blood cells and washing. T cell reactivity was measured in 96 well-plate assays with 10^5 T hybridoma cells, 4×10^5 irradiated (1,6 Gy) antigen- presenting cells and LDL preparations or pure Apo B 100. Concanavalin A, a polyclonal mitogen from Jackbean, was used as positive control. Cells were cultured for 24 hours at 37° C and 7.5% CO₂, in DMEM supplemented with 5 % FCS (Collaborative Biomedical, USA). Interleukin-2 (IL-2) was measured by a standard sandwich ELISA (R&D systems, USA) in the culture supernatants to determine T cell activation.

3.4 Enzyme- linked immunosorbent assay, ELISA

Briefly, 50 μ L of the different antigens (10 μ g/mL in PBS pH 7.4) were added to 96-well ELISA plates and incubated overnight at 37°C. Coated plates were washed with PBS and blocked with 1% gelatin (Gibco Invitrogen, Carlsbad, CA, USA) in PBS for 1 hr at room temperature. Next, plates were washed and incubated for 2 additional hours with mouse plasma, diluted in Tris-buffered saline (TBS)/gelatin 0.1%. For competition analysis, different concentrations of the competitors (1 to 100 μ g/mL) were added to the wells.

Methods

After washing, total IL-2 levels were measured using enzyme-conjugated anti-mouse antibodies (BD Biosciences, Franklin Lakes, NJ, USA). The plates were washed, and colorimetric reactions were developed using TMB (BD Biosciences, Franklin Lakes, NJ, USA). The absorbance was measured using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Plasma cholesterol and triglycerides were measured using enzymatic colorimetric kits (Randox Lab. Ltd. Crumlin, UK) according to the manufacturer's protocol.

3.5 Preparation of the antigens

3.5.1 Preparation of LDL

Human LDL ($d = 1.019\text{--}1.063\text{ g/mL}$) taken from blood samples from the blood bank at Karolinska Institute was isolated by ultracentrifugation from pooled plasma of healthy donors with 40 000 rpm for 24 hours, as previously described [86]. The VLDL was removed the following day. After isolation, LDL was dialyzed extensively against PBS to remove potassium bromide. The concentration was measured via the Bradford method. 1 mM EDTA was added to an aliquot of LDL to generate unmodified LDL.

3.5.2 Preparation of oxidized LDL

LDL was obtained using the method described above. Oxidized LDL was prepared by incubation of 1 mL of LDL (1 mg/mL protein content, determined by Bradford assay, Biorad, USA) in the presence of 20 μM CuSO_4 for 18 hours at 37 °C. Different degrees of oxidation were obtained by incubating LDL with 20 μM CuSO_4 for 1, 2, 4, 8, and 18 hours incubation time. The purpose of the oxidized LDL with different incubation times and therefore different degrees of oxidation was to determine whether there would be a stronger reaction of the hybridoma to stronger oxidized LDL, so we would have a quantification of the oxidation level and rule out cell toxicity at very strong levels of oxidation. CuSO_4 is typically used for experimental oxidation in vitro, because it is a very strong oxidant. The extent of oxidation was evaluated by TBARS, as previously described by Puhl et al. [87].

3.5.3 Preparation of soluble ApoB 100

To obtain soluble ultra- pure ApoB 100 to be used in the assay we first used a procedure for precipitating proteins from solution, as previously described by Wessel and Flügge (1984) [88, 89].

Briefly, 0.4 ml of methanol, 0.1 ml of chloroform and 0.3 ml of water were added to 0.1 ml of LDL (1 mg/ml). The suspension was vortexed and centrifuged at 9000 *g* for 1 min. The upper phase was removed and 0.3 ml of methanol added to the lower phase and interphase with precipitated protein, which was again vortexed and centrifuged at 9000 *g* for 2 minutes to pellet the protein. To obtain soluble and pure ApoB 100, the protein pellet was re-suspended in a minimum volume of 10 % SDS (Biorad Laboratories, USA) until it solubilized. These preparations first were filtered on a PD-10 column (Amersham, Sweden), to remove excess SDS. It was then subsequently purified using Superdex- 200 size- exclusion column (0.5 mL/ min, in TRIS-HCL, pH 7.4). The first peak containing mainly apoB 100 was collected. Extra peaks containing protein contaminants from LDL purification procedure were discarded. Apo B 100 preparations showed over 90% purity when evaluated in a second injection into a Superdex-200 column (GE Helathcare), and analyzed on SDS- PAGE. Finally, protein concentration was determined by Bradford assay (Biorad Laboratories, USA).

3.6 mRNA and cDNA preparation

The messenger RNA was isolated from each T- cell hybridoma using Rneasy Mini Kit (Qiagen, USA). The quality of the RNA was measured on a Bio Analyzer (Agilent Technologies). Concentration of mRNA was measured with Nanodrop. Single-stranded cDNA synthesis was performed by using Superscript II (invitrogen) and random hexanucleotide primers (pdN6, Pharmacia) in the presence of RNasin (Life technologies). The cDNA was amplified using appropriate V β family specific 5`primers with a constant region C β 3`primer, or relevant V α family specific 5`primers with a constant region C α 3`primer (Table III). The design of all primers was based on previously published sequences. (Table III) [90,91]. The cDNA was synthesized on the DNA thermal cycler using a standard protocol.

3.7 Polymerase chain reaction (PCR) amplification

The polymerase chain reaction (PCR) was performed in a master mix containing 10 mM Tris-HCL, 50 mM potassium chloride KCL, 1.5 mM magnesium chloride $MgCl_2$, 1 mM dNTP, 0.2 U/ml Taq polymerase (invitrogen). All primers were added to a final concentration of 0.2 μM . Each reaction was performed in a separate tube, so each PCR tube contained one of the alternative V primers together with the C primer. The reactions were carried out for 35 cycles using 94°C (40 seconds) for denaturation, 56°C (40 seconds) for annealing and 72°C (1 minute) for polymerization. PCR products were analysed on a 1.5% Agarose gel and visualized by ethidium bromide staining. Data were analysed with the Genotyper 2.0 software program.

See table III below in the last chapter for detailed information on primer sequences.

3.8 Flow cytometer analysis

In order to confirm the results from the PCR on protein level, positive clones were checked in the flow cytometer. Available antibodies for the specific receptor chains were used according to the supplier (BD PharMingen, San Diego, USA). The $V\alpha/V\beta$ anti-mouse monoclonal antibodies were conjugated to PE, FITC or Biotin/ Streptavidin Cy-5. Commercially available anti- mouse – TCR $V\alpha$ and TCR- $V\beta$ mouse antibodies (BD PharMingen, San Diego, USA) were used to detect TCR- $V\alpha$ and TCR- $V\beta$. They were combined with anti- CD3- Pacific blue and anti- CD4- APC to stain T cell hybridomas. Splenocytes from unimmunized mice were used as positive controls for all antibodies. The cells were analyzed on a CyAn ADP flow cytometer (Dako).

In combination with these antibodies we used anti-CD3- Pacific blue, anti- CD4- APC and anti- CD8- PE. All antibodies were verified to stain spleen cells from non-immunized mice. Briefly, the cells were stained for 15 minutes with Fc γ R III/II- blocking antibody followed by 30 minutes of staining with primary antibodies. For biotin- conjugated antibodies, an extra 30 minutes was included for the binding of streptavidin. Finally, cells were washed in PBS and analyzed on a CyAn ADP flow cytometry analyzer (Dako, Denmark).

3.9 MHC restriction essay

To evaluate MHC class II restriction, 10^5 hybridomal cells were incubated with different concentrations of ApoB 100 in the presence of 4×10^5 irradiated (1,6 Gy) antigen-presenting cells from syngeneic (C57BL/6; I-A^b) or allogeneic (BALB/c; I-A^d) donors. In a separate experiment, 10^5 hybridoma cells were incubated with ApoB 100 in the presence of 4×10^5 irradiated (1,6 Gy) APC from syngeneic donors in the presence or absence of blocking antibodies to MHC class II (BD). In both experiments, T cell activation was defined by increases in IL-2 concentration in the supernatant.

3.10 Statistical analysis.

Values are expressed as mean \pm SEM unless otherwise indicated.

4 Results

4.1 T cells recognize native LDL

After subcutaneous primary immunization with human oxidized LDL in complete Freund's adjuvant, followed by a booster injection in incomplete Freund's adjuvant, LN cells were collected and fused with thymoma cells to generate hybridomas. Among 268 hybridomas, 117 were found to express CD3 and CD4. Out of these, we chose randomly 23 for further characterization. We tested them against different antigens. Monoclonal hybridomas were screened for their response to human oxidized LDL, native LDL, and purified unmodified ApoB 100. T cell hybridoma activation was examined by measuring IL-2 production after exposure to the putative antigen in presence of irradiated antigen-presenting cells. IL-2 production was measured by an IL-2 ELISA, IL-2 being a marker for T cell activation. (See figure 7 A). T cells that are activated produce IL-2 in the presence of irradiated antigen-presenting cells (APCs) as a marker of activation and response to the antigen. Out of 23 tested hybridomas, seven reacted to native human LDL and pure ApoB100. Three subgroups were identified among clones responding to human LDL and pure ApoB 100. We chose representative clones from each subgroup, named 48-5, 45-1 and 15-2. We found a similar pattern of activation amongst the responding clones, where human LDL and pure ApoB 100 gave the strongest response. There was a clear dose-dependent response to unmodified ApoB 100 (See figure 7, B-D). To ensure that the response was not caused by a human – specific modification of ApoB 100, hybridomas were also exposed to ApoB 100 isolated from LDL of huB100^{tg} x Ldlr^{-/-} mice. This recombinant ApoB 100 also was recognized by the T cell hybridomas (Fig.7, B-D).

This is rather surprising, as it was believed that oxidized LDL was the culprit factor in the inflammatory process of atherosclerosis. As pointed out in the introduction, it was believed that inhibition of oxidation can inhibit atherosclerosis [50] and earlier data show that T lymphocytes from human atherosclerotic plaques recognize oxidized low density protein [39].

4.1.1 The more oxidation, the less activation

We then wanted to specify the reaction to native LDL and oxidized LDL. We exposed the hybridomas to native LDL or LDL oxidized to different extents. The surprising result was that the T cell hybridomas showed an inverse correlation between IL-2 levels and the degree of LDL oxidation. There was an inverse relationship between the degree of oxidation and the amplitude of activation (Fig. 7, E-G)

This dose dependent behaviour of the hybridoma leads to the hypothesis that the epitopes for recognition of the antigen are destroyed upon oxidation.

The hybridoma had the highest response when exposed to human ApoB 100, the protein part of LDL. The T-cell receptor recognizes only non- conformational epitopes that are exposed to them through the MHC class II complex on the antigen presenting cell surfaces. Native ApoB 100 elicited the highest proliferative T cell response, whereas highly oxidized LDL did not trigger activation.

This result was completely unexpected. The hybridomas were taken from a mouse strain which is transgenic for human ApoB 100. The mice should therefore be tolerant to LDL and ApoB 100. Yet, the T cells weren't, so this points in the direction of some kind of autoimmune reaction. The results show a cellular immune response to ApoB 100 protein of native LDL. This response seems to play an important role in atherosclerosis. The T cell reactivity is extinguished rather than induced by oxidative modification of LDL.

See figure 7 below showing the results.

Results

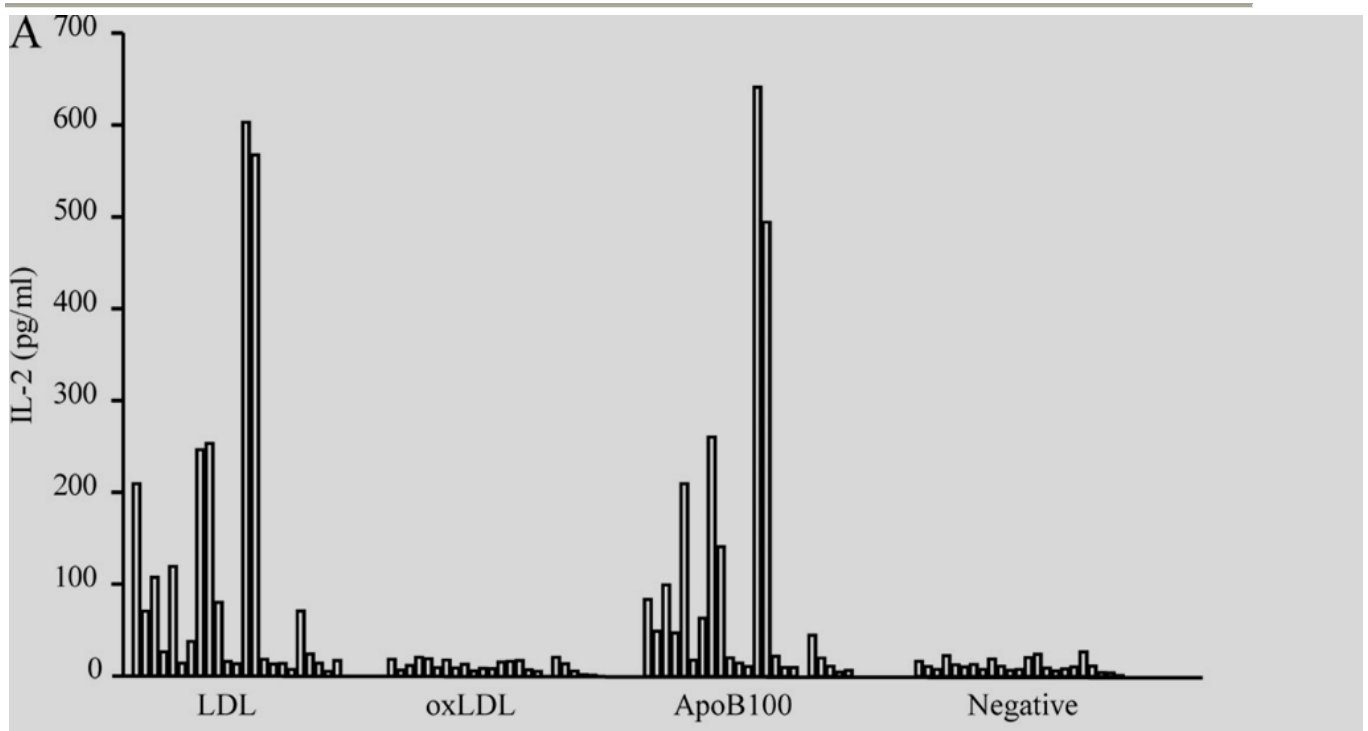


Figure 7 A. T cell hybridomas recognize native LDL and Apo B 100

(A) T cell hybridomas from human ApoB 1000 transgenic mice immunized s.c. with copper-oxidized LDL were exposed to different antigens in culture, and activation was measured in an IL-2 ELISA. 10^5 hybridoma T cells from each of 23 different clones were incubated with 4×10^5 irradiated spleen cells as antigen presenting cells (APCs) with 20 $\mu\text{g}/\text{mL}$ of LDL, ox LDL, and ApoB100. Each column represents 1 clone. Media without LDL were used as negative controls. The strongest response gave native LDL and ApoB 100. Results are from one experiment.

Results

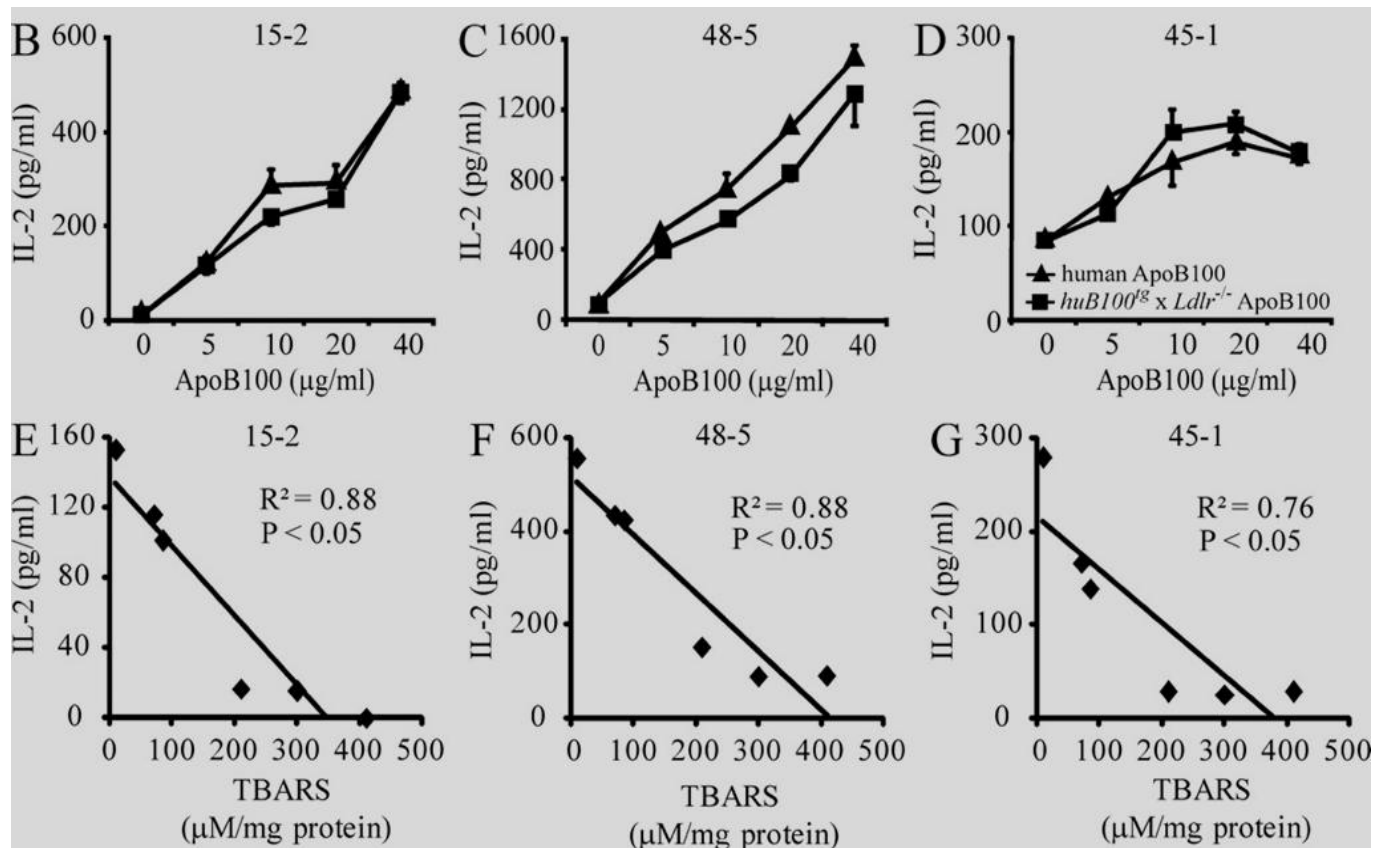


Figure 7 B-G. T cell hybridomas recognize native LDL and Apo B 100

(B-D) 10^5 hybridoma T cells of 3 different clones (B, hybridoma15-2, C, 48-5, D, 45-1) were incubated with 4×10^5 irradiated spleen cells as antigen presenting cells (APCs) together with different concentrations of human apolipoprotein B 100 from plasma LDL, or transgenic human ApoB100 from huB100^{tg} x Ldlr^{-/-} mice. Hybridomas 15-2, 48-5, and 45-1 (B-D) recognize purified as well as transgenic ApoB100 in a dose-dependent manner. In all experiments, IL-2 secretion was used as a measure of T cell activation. After 24 hours, IL-2 production from the supernatants was measured by ELISA. Data show means \pm SEM. Results are representative of three independent experiments.

(E-G) 10^5 hybridoma T cells were incubated with 4×10^5 irradiated antigen presenting cells with 20 μ g/mL of LDL or LDL oxidized to different extents (copper oxidation (20 μ M CuSO₄) for varying lengths of time). After 24 h of incubation with the different preparations, IL-2 secretion was measured in the supernatant. X axis shows the mean of TBARS values and y axis shows the mean of IL-2 levels. All T cell hybridomas showed an inverse correlation between IL-2 levels and degree of LDL oxidation. Results are representative of three independent experiments.

Results

4.2 MHC class II - restricted response

Because purified ApoB 100 induced activation of the CD4⁺ T cell hybridomas, we hypothesized that the epitopes are peptides that are presented by MHC class II. In order to confirm that our CD4 positive T cell clones could recognize antigen in the context of the MHC class II complex, we tested all of the clones for MHC restriction. In mice, the MHC class II gene complex corresponds to the I- A^b haplotype. When we added an antibody to block I-A^b on antigen- presenting cells, T cell activation was suppressed in all clones. This shows clear evidence of an I-A- restricted response to the activating ApoB 100. I-A is a region in the H-2 major histocompatibility complex in mice and corresponds to the MHC class II gene complex in humans. These results show that ApoB 100 antigen is recognized by MHC class- II restricted CD4⁺ T cells. See figure 8 below to picture the result.

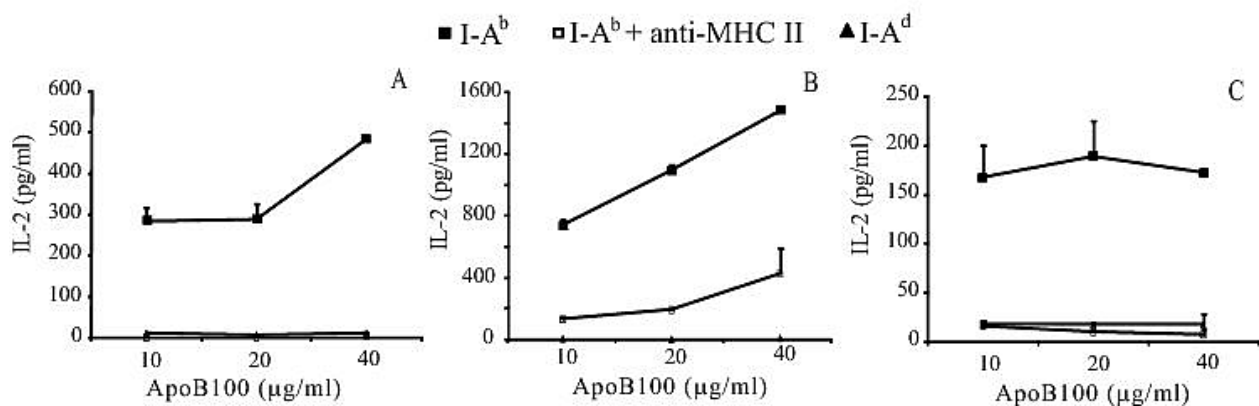


Figure 8. T cell responses to native LDL and ApoB100 are I-A restricted.

A.) clone 15-2 B.) clone 48-5 C.) clone 45-1

10⁵ hybridomal T cells were incubated with 4x 10⁵ irradiated spleen cells (APCs) taken from mice with either the I-A^b or I-A^d haplotype, along with 10μg/ml of human ApoB 100. One experimental group received excess of anti- MHC class II blocking antibody. After 24 hours, IL-2 production from the supernatants was measured by ELISA. There was no IL-2 production for I-Ab+anti MHC class II or I-Ad for clone 15.2, no IL-2 production for I –Ad in clone 48.5 and no IL-2 production for I-Ab + anti MHC class II for clone 45-1. Data show means +/- SEM. Results are from one experiment,

4.3 Decrease of activation with increase of oxidation

We then wanted to confirm the results that the hybridomas react stronger to native LDL, so we exposed the three clones that gave strongest reaction to LDL to native LDL and oxidized LDL in different degrees.

The T cell activation was inversely correlated with the degree of oxidation of LDL. The activation of the T cell hybridoma decreased already for 1h- oxidized LDL, and was weakest for 18h- oxidized LDL. The T cell clone response gradually disappeared with increasing degree of oxidation.

The clones that responded to LDL and ApoB 100 derived from 3 families, named 48, 45 and 15. (Fig.9). These data suggest that the T cell epitopes in LDL are destroyed upon oxidation. The stronger the oxidation, the weaker the reaction of the T cell hybridoma. This suggests that the “antigen” that T cells recognize is a non-modified form of LDL, and with increasing degree of oxidation, the T cells lose the ability to react to the antigen. Perhaps the T cell epitopes that connect with the T cell receptor are destroyed upon oxidation.

This also supports the theory of atherosclerosis being an autoimmune disease, as T cells react to the native form of LDL and to a protein (ApoB 100) that belongs to “self”. See figure 9 below representing the results.

Results

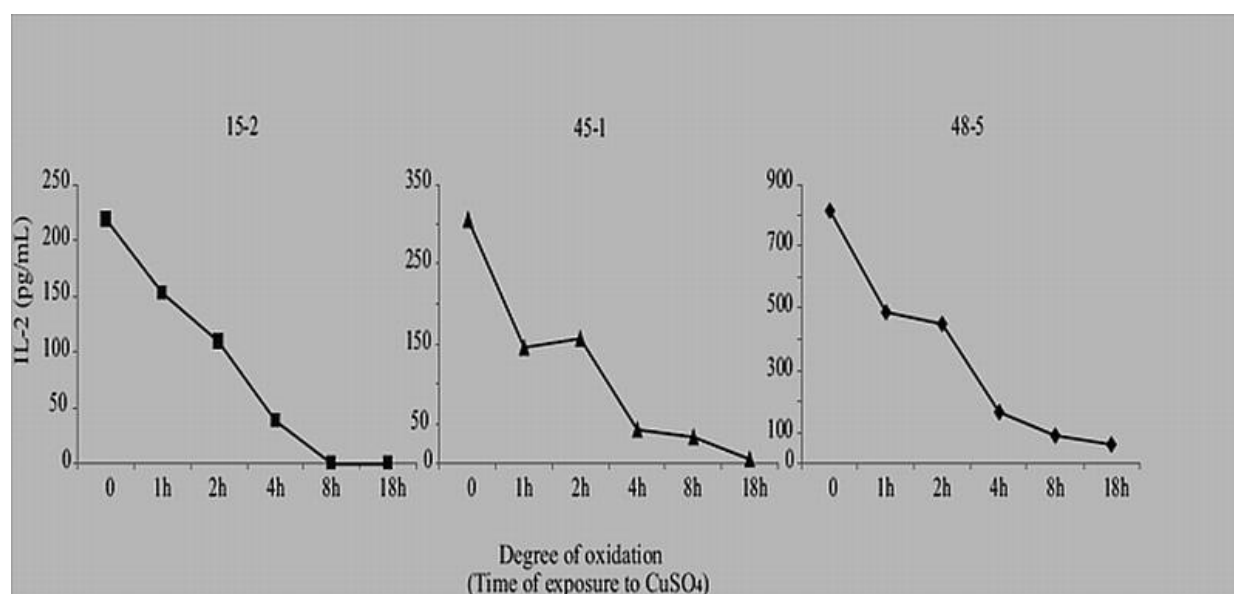


Figure 9. Decrease of activation in all clones with increasing oxidation.

Oxidation of LDL results in the destruction of the T cell epitopes. 1×10^5 hybridoma T cells were incubated with 4×10^5 irradiated spleen cells together with 40 $\mu\text{g}/\text{ml}$ of native human LDL and LDL that had been oxidized with 20 μM CuSO₄ for 1, 2, 4, 8 and 18 hours. After 24 hours, IL-2 production from the supernatants was measured by ELISA.

4.4 TRBV 31+ CD4+ T cells recognize native LDL

4.4.1 PCR results of the three positive clones

Reverse-transcript-PCR was performed for the 23 selected clones to characterize the variable regions. The fusion partner thymoma BW 5147 expressed the TRAV 20 and TRBV 12.1 variable chains. It was found that all clones positive for human LDL and ApoB 100 expressed a unique set of V β chains. The TCR V β chain among responding hybridoma clones were restricted to TRBV 31 alone. All clones negative for the antigens expressed other V β chains. The V α chains varied even among the positive clones, and could not be identified to a single common V α . The V alpha chains found in the PCR were V α 3,4,12,13,14, and 20 (See Table I below).

Results

Clone	PCR		Flow cytometry			Resulting phenotype
	V α gene	V β gene	Anti-TRAV14	Anti-TRAV12	Anti-TRBV31	
15-2	TRAV14, 3 and 20	TRBV31 and 12.1	negative	–	positive	TRAV3/TRBV31
45-1	TRAV4 and 20	TRBV31 and 12.1	–	–	positive	TRAV4/TRBV31
48-5	TRAV12, 13 and 20	TRBV31 and 12.1	–	negative	positive	TRAV13/TRBV31

Table I. The three positive clones and their V α / V β T cell receptor analyzed with PCR. All clones responding to native LDL and ApoB- 100 expressed one common V β T cell receptor, TRBV 31. The cDNA from reactive clones was amplified by PCR using appropriate V β - specific 5' primers with a constant region C β 3' primer, or relevant V α - specific 5' primers with a constant resion C α 3' primer. Further phenotype of V α and V β chain expression was analyzed by flow cytometry using antibodies to TRAV 14, TRAV12, and TRBV31.

All LDL responding T cell hybridomas expressed the common T cell receptor variable TRBV 31, formerly known as V β 14 in the old nomenclature. To confirm our finding on protein level, we stained selected clones with available TCR- mouse antibodies, and performed an experiment in the flow cytometer (FACS). All positive clones showed a strong expression of TRBV 31 also in the flow cytometer. (See fig. 10)

The V α could not be confirmed in the FACS, also due to the fact that not all antibodies are available.

To check if we would have the same results with the primers with new nomenclature, we performed PCR with the new primers. The previous results were confirmed also with the new nomenclature. See figure 10 A and B below to picture the results from the performed PCR and the flow cytometer, with new nomenclature.

Results

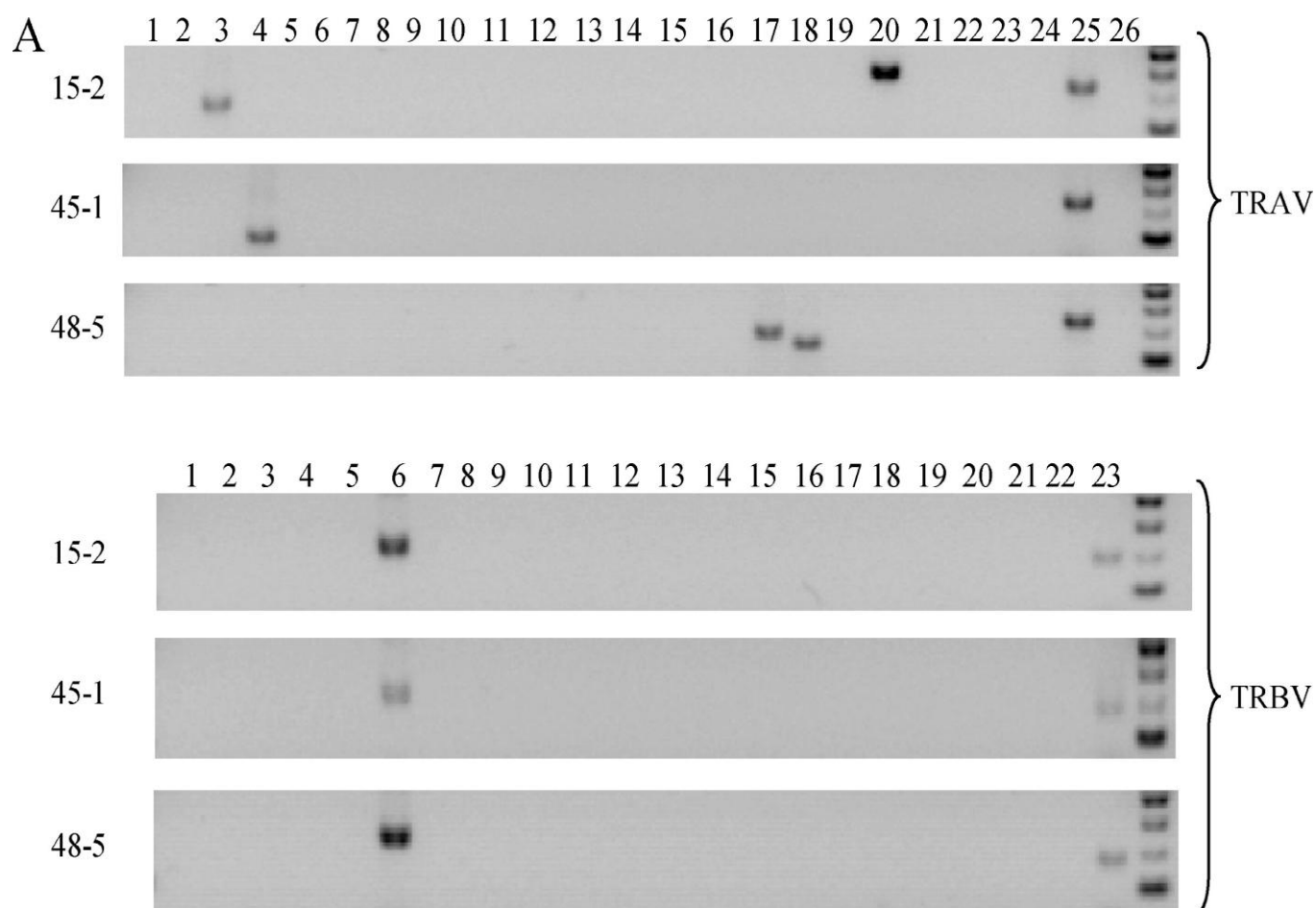


Figure 10 A. Characterization of ApoB100-responsive hybridomas, PCR

(A) Genotyping gel of LDL/ApoB 100 reactive clones. The cDNA from clones 15–2, 45–1, and 48–5 was amplified by PCR using either V α family-specific 5' primers together with a constant region C α 3' primer or V β family-specific 5' primers together with a constant-region C β 3' primer (primers are listed in Table III). The PCR products were analyzed on a 1.5% agarose gel and visualized by gel red staining. Results are representative of three independent experiments.

Note: All clones contain the TRAV20 and TRBV12.1 mRNA from the hybridomal fusion partner BW5147

Results

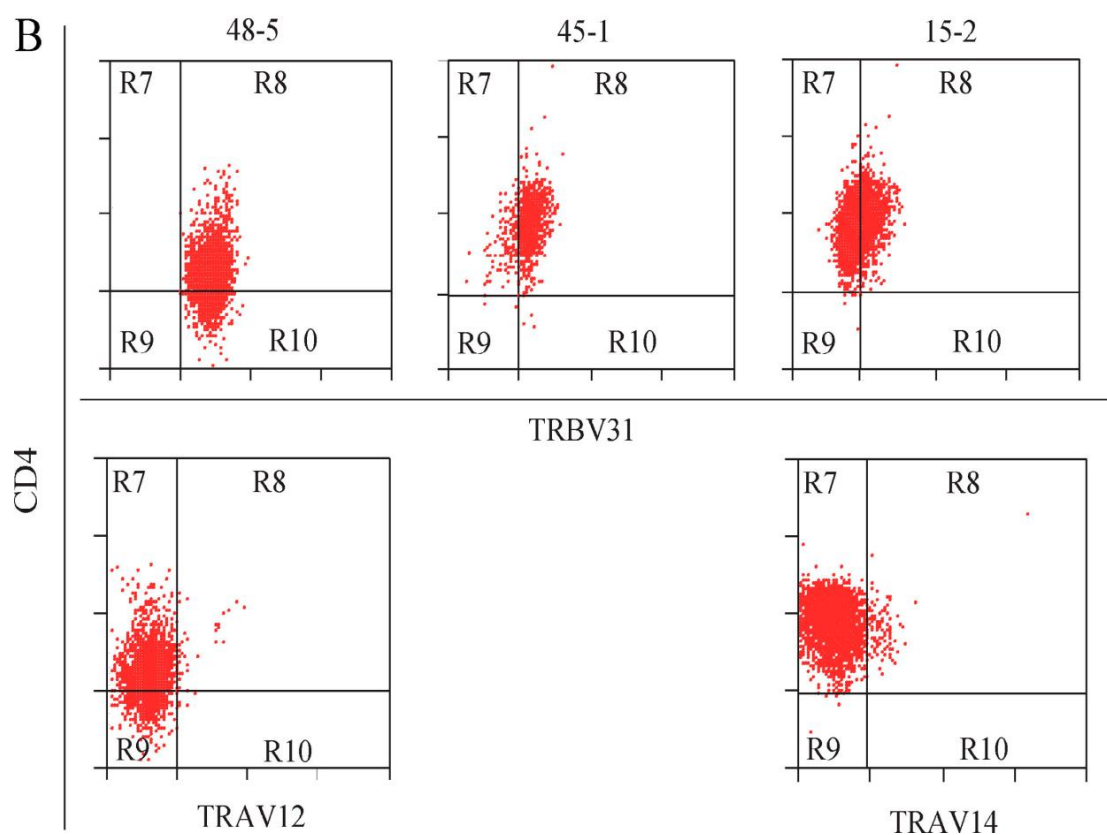


Figure 10 B. Characterization of ApoB 100-responsive hybridomas, flow-cytometer

(B) TCR expression evaluated by flow cytometry. Three representative T cell hybridoma clones were stained for CD3, CD4, and available commercial antibodies to TRBV and TRBV 31. Plots show CD4 and specific TRAV/TRBV staining on CD3 + -gated cells. Results are representative from two independent experiments.

Results

Table II. Genotyping of CD3 + CD4 + T cell hybridomas.

Clone	Clones recognizing LDL/ApoB100		Clone	Clones not recognizing LDL/ApoB100	
	<i>Vα gene</i>	<i>Vβ gene</i>		<i>Vα gene</i>	<i>Vβ gene</i>
15-2	TRAV14 and 3	TRBV31	96-7	TRAV13	TRBV19
15-3	TRAV14 and 3	TRBV31	97-3	TRAV13	TRBV19
15-4	TRAV14 and 3	TRBV31	32-1	TRAV20	TRBV19
15-5	TRAV14 and 3	TRBV31	32-4	TRAV20	TRBV19
15-6	TRAV14 and 3	TRBV31	117-1	TRAV5 and 3	TRBV3
45-1	TRAV4	TRBV31	117-3	TRAV5 and 3	TRBV3
45-2	TRAV4	TRBV31	117-5	TRAV5 and 3	TRBV3
45-4	TRAV4	TRBV31	117-7	TRAV5 and 3	TRBV3
48-5	TRAV12 and 13	TRBV31	50-0	TRAV20	TRBV29
48-8	TRAV12 and 13	TRBV31	50-2	TRAV20	TRBV29
			50-3	TRAV20	TRBV29
			50-4	TRAV20	TRBV29
			50-5	TRAV20	TRBV29

Note: All clones contain the TRAV20 and TRBV12.1 mRNA from the hybridomal fusion partner BW5147

Table II. The different Vα and Vβ receptors of T cell hybridoma clones recognizing LDL and ApoB 100 are shown versus the clones not recognizing LDL and native ApoB 100. All clones responding to native LDL and ApoB 100 express one single common Vβ T cell receptor, namely TRBV 31. The fusion partner thymoma BW 5147 expressed rearranged TRAV 20 and TRBV12.1 variable chains. All hybridomas expressed mRNA for these TCR chains.

Native human LDL- and ApoB 100 – specific T cell hybridomas uniformly expressed TCR TRBV31, previously called TCR- Vβ14 (Table and fig. 10). In contrast, the reactive hybridomas used several families of Vα chains, including TRAV3 (hybridoma 15-2), 4 (45-1), and 13 (48-5) (Table I). In unreactive hybridomas, Vα and Vβ TCR chains were expressed in a nonrestricted fashion, not including TRBV31 (table III). For each LDL- responsive hybridoma, the surface expression of TRBV31 was confirmed by flow cytometry (Table I and fig.10).

Our results lead to the hypothesis of a MHC class II restricted response of native ApoB 100, which is recognized by TCR TRBV31- expressing CD4+ T cells that recognize native ApoB 100 as proatherogenic. The ensuing T cell activation may lead to cytokine secretion that can promote macrophage activation and inflammation.

5 Discussion

5. 1 Reaction of T cell hybridomas to different antigens

5.1.1 The more oxidation, the less activation

We created hybridomas from T cells from human ApoB 100 transgenic mice. A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. Similar to other transgenic mice that produce nonmouse proteins, huApoB 100^{tg} mice tolerate the orthologous transgene, neither producing antibodies to it nor developing spontaneous autoimmune disease. We had therefore expected that the mice would respond to immunization by mounting cellular and humoral immune responses toward oxidation – induced epitopes on the LDL particles. Unexpectedly, oxidized- LDL immunized huApoB 100^{tg} mice developed T cell responses against native LDL and purified ApoB 100. The responding T cells were MHC class II restricted CD4+ cells and expressed T cell receptors that contained the single variable domain TRBV 31.

Several of the T cell hybridomas created from the human apoB 100 transgenic mouse strain were reactive to LDL and ApoB 100. Because this mouse strain is transgenic for human ApoB, the mice should be tolerant to LDL and ApoB. The T cell response to the antigens that the hybridomas were exposed to gives evidence of autoimmune reactions. Native epitopes of ApoB 100 seem to have an important role for T cell activation in atherosclerosis. Reactive T cell clones have passed through negative selection in the thymus during early T cell development. It was a great surprise when it was discovered that the hybridomas obtained from mice immunized s.c. with oxidized LDL reacted stronger to native LDL than to oxidized LDL (ox LDL), as this is against the general theories of atherosclerosis. Oxidized LDL is generally seen as one of the culprit factors in atherosclerosis. A number of studies suggest that the oxidized low density lipoprotein (ox LDL) is a more potent pro- atherosclerotic stimulus than the native unmodified LDL [3]. The results are contradictory to the “oxidation hypothesis”, where oxidized LDL is seen as crucial for initiation and progression of the atherosclerotic lesion, as explained in the introduction.

Low density lipoproteins (LDL) are the major carriers of cholesterol in plasma, and their increased concentration in circulation is correlated with the development of atherosclerosis [93]. As pointed out in the introduction, inflammation is crucial to cardiovascular disease. It often begins with inflammatory changes in the endothelium, which begins to express the adhesion molecule VCAM-1 (vascular cell adhesion molecule). This molecule attracts monocytes, which then migrate through the endothelial layer under the influence of various proinflammatory chemoattractants. Once within the arterial intima, the monocytes transform into macrophages, eat up lipids and become foam cells. T lymphocytes also migrate into the intima, where they release proinflammatory cytokines that amplify the inflammatory activity. Through these inflammatory processes, the initial lesion of atherosclerosis, the fatty streak, is formed [94]. To identify the particle that T cells recognize, the antigen, could lead to interruption of the inflammatory process at its crucial phase. In a review [95], regression and remodelling of atherosclerotic lesions is discussed. Factors promoting regression include decreased apolipoprotein B-lipoprotein retention within the arterial wall, efflux of cholesterol and other harmful lipids from plaques, and emigration of lesional foam cells followed by entry of healthy phagocytes that remove necrotic debris and other plaque components. The essential prerequisite for regression is robust improvement in plaque milieu, meaning large plasma reductions in atherogenic apolipoprotein B-lipoproteins. Our mice were transgenic to human ApoB 100. The human ApoB 100 transgenic mice should be tolerant to human ApoB 100. An increasing oxidative modification of the LDL particle diminished the response in the T cells, which could be explained by destruction of the T cell epitope when modified. This finding is quite surprising, as oxidized LDL was believed to be the culprit factor in atherosclerosis. The above mentioned results suggest that the T cells recognize the protein part of LDL, apolipoprotein B 100. Even clinically, the lowering of apolipoprotein B-lipoprotein levels is crucial for the regression of atherosclerotic plaques [95]. Taken together, the human studies parallel the animal work; intensive lowering of plasma apolipoprotein B- lipoprotein (apo B) concentrations simultaneously with elevation of functional HDL levels may achieve rapid plaque regression and stabilization [95]. The inflammatory process might much rather be initiated by the recognition of the protein part of low- density lipoprotein (LDL) than the oxidized parts of the low- density lipoprotein (ox LDL).

The results strongly suggest that autoimmune T cells that recognize protein epitopes from native ApoB 100 promote atherosclerosis.

We have identified a cellular immune response to ApoB100 protein of native LDL, and show that this response plays an important role in atherosclerosis. T cells from ox LDL immunized mice recognize unmodified ApoB 100 from native LDL rather than oxidized LDL. This response is clonotypic and involves TRBV31 TCR. Oxidized LDL triggers a strong humoral immune response that generates antibodies to oxidation- induced epitopes on LDL particles. [56, 96]

5.2. MHC class II- restricted manner

T cells recognize antigens via their receptor, the T cell receptor, which recognizes the antigen- major histocompatibility complex on the surface of antigen presenting cells. In order to prove that the T cell response in the used hybridomas is associated with MHC class II, the mouse MHC class II complex was blocked with an antibody; this led to suppression of the T cell response, thereby proving MHC class II restriction of response.

The cellular immune response to LDL that we observed was mounted by CD4+ T cells and showed MHC class II restriction. In conjunction with data that demonstrate that purified Apo B 100 protein elicits an identical response to that of intact LDL, the above mentioned results strongly suggest that intracellular processing of ApoB 100 in the antigen- presenting cell generates oligopeptide epitopes that are recognized by the T cells as peptide- MHC complexes. The finding that I A^b (corresponding to MHC class II in humans) is required for the T cell response and cannot be substituted by another MHC class II molecule further supports the model that specific oligopeptides that are bound to MHC class II constitute the ligand with which clonotypic T cell receptors interact. No studies have ever shown that oxidized peptides from LDL are presented via the MHC complex.

Even though the innate and humoral immune responses toward ox LDL are based on firm evidence, the notion that T cells can recognize and become activated by ox LDL needs to be changed.

5.3 Decrease of activation with increase of oxidation

The T cell recognition was restricted to the MHC class II antigen I -A. When challenging the reactive T cell clones with oxidized LDL, the response gradually decreased with increasing degree of oxidation.

With the use of hybridomas, we showed that native epitopes of Apo B 100 seem to have an important role for T cell activation in atherosclerosis.

The T cells reacted more strongly to native LDL than to oxidized LDL, and the T cell hybridomas could respond to pure ApoB 100 protein.

This indicates that the protein can give rise to autoimmune responses despite the fact that its reactive T cell clones have passed through negative selection in the thymus during early T cell development. It could be possible that the antigen is modified in such a way that it breaks tolerance. That means the protein is perceived as being non- self. Results obtained in an earlier study pointed in the same direction.

Wuttge et al. immunized mice with homologous albumin covalently modified with a series of defined aldehydes which are known to be generated during lipid peroxidation.

In addition to the response to modified epitopes, some aldehyde modifications resulted in strong antibody responses to the non-modified protein.

This T cell-dependent break of tolerance constitutes a novel pathway for induction of autoimmunity by lipid peroxidation [60].

In the current study we have clearly demonstrated that an increasing oxidative modification of the LDL particle leads to a lower degree of T cell recognition, which then speaks for a destruction of the T cell epitope when modified.

Recent identification of specific immunoreactive antigenic epitopes in apolipoprotein B-100 component of low-density-lipoprotein and early experimental observations have provided proof of the concept that active vaccination using specific apolipoprotein B-100- related antigens may emerge as a novel immunomodulating atheroprotective strategy [97].

Another study supports this theory, where the authors stated that autoantibodies recognizing peptide sequences in the LDL- receptor- binding region of ApoB 100 could potentially affect both cholesterol metabolism and atherosclerosis [98].

Another article supports the theory that it is not oxidized LDL, but apolipoprotein B 100, that is the crucial factor for the risk to develop atherosclerosis. The study was done by Wu et al. [99]. They identified 266 men and 235 women from the Health Professional Follow- up study and the Nurses Health study. These individuals had incident MI or fatal CHD, and each index patient was matched with two control subjects by age and smoking status. Plasma ox LDL levels in these individuals were significantly related to the risk of coronary artery disease in multivariate analysis. However, when ox LDL, LDL cholesterol, HDL cholesterol and triglyceride levels were mutually adjusted, ox LDL levels were no longer predictive.

Importantly, apolipoprotein B, total HDL cholesterol, and other conventional lipid markers, including LDL cholesterol, HDL cholesterol, and triglycerides, remained powerful differentiating factors [100].

Only elevated levels of lipoproteins containing apolipoprotein B (apo B) can drive the development of atherosclerosis of humans and experimental animals even in the absence of other risk factors [34]. In another review, the role of oxidized low-density-lipoprotein and its role in atherogenesis is discussed [101]. Ox LDL is not uniform, but rather contains complex structures, ranging from small conformational change in surface lipids to the breakdown of the peptide chain. Therefore, the immune responses to the variety of ox LDL and their association to atherosclerosis progression are very different [101].

5.4 TRBV 31+ CD4+ T cells recognize native LDL

The T cell receptor (TCR) expressed on most T cells is a protein complex consisting of TCR alpha/ beta heterodimers that bind antigen and cluster of differentiation (CD) that initiate signalling [102].

We characterized the T cell receptors of the ApoB 100 responsive clones. They all expressed one common variable chain, TRBV31, formerly known as V β 14, in combination with different V α chains.

The surface expression of the receptor chain TRBV 31 was also confirmed by flow cytometry. This suggests that TRBV 31 + CD4+ T cells participate in the atherogenic process. TRBV 31 plays an important role in other autoimmune diseases, for example in rheumatoid arthritis (RA), and in aplastic anaemia [103]. Immune reactions are highly relevant in atherosclerosis, and patients with other autoimmune diseases such as systemic lupus erythematosus (SLE) are at high risk for cardiovascular disease [104]. As patients with SLE have a higher life expectancy due to improved therapies and preventive measures, complications from cardiovascular events are increasing [105]. Patients with SLE have an increased risk of atherosclerosis that persists even after allowing for traditional cardiac risk factors. This is likely due to the complex interplay of many of the inflammatory and immune mediators, [105]. In several human T-cell mediated autoimmune diseases and animal models of such illnesses, T-cell receptors (TCR) specific for antigens that initiate or perpetuate the disease share a limited number of variable region determinants. Vaccinations with peptides derived from over-represented TCRs are effective treatment for some of these disorders. Rheumatoid arthritis (RA) is a chronic inflammatory disease in which there is prominent T cell infiltration in the synovial lining layer. TRBV 31 has been found to be overrepresented among IL-2 receptor-positive T cells from patients with RA [106]. A medical target for the treatment of atherosclerosis could be to have a vaccine with peptides derived from over-represented T-cell receptors.

5.5 Atherosclerosis as an autoimmune disease

As explained above, is adaptive immune response a critical component for defence against infection and essential for normal health. Unfortunately, it is also possible that adaptive immune responses to self tissue antigens occur, called autoimmunity. This can lead to autoimmune diseases characterized by tissue damage. Autoimmune disease occurs when a specific adaptive immune response is mounted against self antigens. Normally, the T cells of each individual are tolerant to self antigens. This tolerance to self is acquired by clonal deletion or inactivation of developing lymphocytes. This occurs during the fetal life in humans and around the time of birth in mice. There are two important mechanisms of self- tolerance; clonal deletion

by ubiquitous self antigens and clonal inactivation by tissue- specific antigens presented in the absence of co-stimulatory signals [107]. Normal consequence of an adaptive immune response against a foreign antigen is the clearance of the antigen from the body. However, when an adaptive immune response develops against self antigens, it is impossible for human effector mechanisms to eliminate the antigen completely, and so a sustained response occurs. The consequence is a chronic activity of the specific immune system, leading to chronic inflammation. Some common autoimmune diseases are for example rheumatoid arthritis, systemic lupus erythematosus, insulin-dependent diabetes mellitus, thyroid diseases such as M. Basedow or Hashimoto- thyroiditis, Pemphigus vulgaris, Goodpasture's syndrome and many more. It is not known what triggers autoimmunity, but both environmental and genetic factors, especially MHC genotype, are clearly important. Autoimmunity usually arises spontaneously. Some autoimmune disorders may be triggered by infectious agents, as is the case in rheumatic fever. Many autoimmune disorders occur through internal dysregulation of the immune system without the participation of infectious agents [107]. We can distinguish between "organ- specific" autoimmune diseases, such as thyroid diseases, and systemic autoimmune diseases, such as systemic lupus erythematosus, or, atherosclerosis. Organ specific autoimmune diseases frequently occur together in many combinations. Although anyone can develop an autoimmune disease, it seems that some individuals are more at risk than others of developing particular diseases. Results from both twin and family studies show an important role for both inherited and environmental factors [107]. To classify a disease as autoimmune, one must show that an adaptive immune response to a self antigen causes the observed pathology. Initially, the evidence of autoantibodies in serum was taken as evidence for autoimmune disease [107]. But autoantibodies can also result from tissue damage. One must show that the observed autoantibodies are pathogenic before classifying a disease as autoimmune [107]. The evolution of autoimmunity follows that of normal immune response. A normal immune response begins in a peripheral tissue wherein an antigen is endocytosed by a dendritic cell and then transferred to the regional lymph node. Here an immune response proceeds via the germinal centre reaction in lymphoid follicles wherein there occurs activation of T and B lymphocytes with return to the affected tissue of activated T cells and specific antibody to dispose of

the inciting antigen, so terminating the response [108]. Very likely, autoimmune responses proceed in the same way, given certain conditions [109]. In order for an autoimmune response to proceed, there needs to be coexisting inflammation such that antigen presenting cells (APCs) are appropriately activated, natural immune tolerance must be in some way compromised, and, finally, the response does not terminate because the auto-antigen cannot be eliminated [108].

With the results mentioned above, it was shown that the exposure of T cells to native LDL or ApoB 100 caused T cell activation and thus LDL and ApoB 100 could be seen as pathogenic for the development of the autoimmune response in atherosclerosis. The T cells were self- reactive upon exposure to LDL and ApoB 100. The exposure to non- modified LDL and ApoB 100 gave an adaptive immune response, leading to chronic inflammation.

Atherosclerosis can therefore be regarded as an autoimmune disease, Apo B 100 being a molecule of “self”. The obtained data demonstrate that clonotypic T cells that recognize ApoB 100 are needed for the development of advanced atherosclerosis. The results support the concept of atherosclerosis being an autoimmune disease, whereby ApoB 100 gives rise to the adaptive immune response.

Table III a). Primers for polymerase chain reaction and sequencing of the different T cell receptor (TCR) α - families

No.	TCR α chain family	Primer sequence
1	TRAV01	5`-TGGATGGTTTGAAGGACAGTG-3`
2	TRAV02	5`-CTGTTTATCTCTGCTGACCGG-3`
3	TRAV03-3	5`-ACGAAGGACAAGGATTCACTGT-3`
4	TRAV04	5`-CTGGAGGACTCAGGCACTTACT-3`
5	TRAV06	5`-GGTACCCGACTCTTTTCTGGT-3`
6	TRAV06D-4	5`-ACCCTTTCAGAAGATGACTTCC-3`
7	TRAV06D-5	5`-TTTAAAGTCCCAAAGGCCAA-3`
8	TRAV06-6	5`-TCCTGAAAGTCATTACGGCTG-3`
9	TRAV06-7	5`-AGAGCCTCAAGGGACAAAGAG-3`
10	TRAV07-3	5`-AGACTCCCAGCCCAGTGACT-3`
11	TRAV07-5	5`-ACATCAGAGAGCCGCAACC-3`
12	TRAV08D-1	5`-CCCTGCCCAGCTAATCTTAAT-3`
13	TRAV09-3	5`-CTGCAGCTGAGATGCAAGTATT-3`
14	TRAV09D-1	5`-TCCTATGGTGGATCCATTTACC-3`
15	TRAV10	5`-TGGACAGAAAACAGAGCCAA-3`
16	TRAV11	5`-CAGGCAAAGGTCTTGTGTCC-3`
17	TRAV12-1	5`-ACGCCACTCTCCATAAGAGCA-3`
18	TRAV13-1	5`-GCTCTTTGCACATTTCTCC-3`
19	TRAV14-1	5`-TGCAGTTATGAGGACAGCACTT-3`
20	TRAV14-3	5`-CTGCAGTTATGAGAACAGTGCTT-3`
21	TRAV15-1/DV6-1	5`-CCAGACGATTCGGGAAAGTA-3`
22	TRAV16	5`-TTCCATCGGACTCATCATCAC-3`
23	TRAV17	5`-AACCTGAAGAAATCCCCAGC-3`
24	TRAV19	5`-GGAAGACGGAAGATTCACAGTT-3`
25	TRAV20	5`-ACGCTCCTAATAGACATTGCT-3`
26	TRAV21	5`-GTTCTCTTCAGGGTCCAGA-3`
27	TRAC	5`-CACCAGCAGGTTCTGGGTTC-3`

Table III a). Primers for TCR genotyping, TCR α primer sequences

From: [92]

Table III b). Primers for polymerase chain reaction and sequencing of the different T cell receptor (TCR) β -families

No.	TCR β -chain family	Primer sequence
1	TRBV01	5` -ACACGGGTCACTGATACGGA-3`
2	TRBV02	5` -ATGGACAATCAGACTGCCTCA-3`
3	TRBV03	5` -TCACTCTGAAAATCCAACCCA-3`
4	TRBV04	5` -TAAACGAAACAGTTCCAAGGC-3`
5	TRBV05	5` -ACGGTGCCCAGTCGTTTTAT-3`
6	TRBV12-1	5` -GGATTCCTACCCAGCAGATTC-3`
7	TRBV12-2	5` -AGATAAAGGAAACCTGCCCAG-3`
8	TRBV13-1	5` -CCAGAACAACGCAAGAAGACT-3`
9	TRBV13-2	5` -GGCTACCCCCTCTCAGACAT-3`
10	TRBV13-3	5` -TGGCTTCCCTTTCTCAGACA-3`
11	TRBV14	5` -GCGACACAGCCACCTATCTC-3`
12	TRBV15	5` -CGCAGCAAGTCTCTTATGGAA-3`
13	TRBV16	5` -ATAGATGATTCAGGGATGCCC-3`
14	TRBV17	5` -TGAGAAGTTCCAATCCAGTCG-3`
15	TRBV19	5` -GAAGGCTATGATGCGTCTCG-3`
16	TRBV20	5` -TTCCCATCAGTCATCCCAAC-3`
17	TRBV21	5` -AAAATGCCCTGCTAAGAAACC-3`
18	TRBV23	5` -CAGCCTGGGAATCAGAACG-3`
19	TRBV24	5` -GCATCCTGGAAATCCTATCCT-3`
20	TRBV26	5` -AGTGTCTTTCAAACCTCACCTT-3`
21	TRBV29	5` -AAAGGATACAGGGTCTCACGG-3`
22	TRBV30	5` -GGACAAGTTTCCAATCAGCCG-3`
23	TRBV31	5` -TTCATCCTAAGCACGGAGAAG-3`
24	TRBC1	5` -TGCAATCTCTGCTTTTGATGGCTC-3`

Table III b). Primers for TCR genotyping, TCR β primer sequences

From: [92]

6 Summary and conclusion

Atherosclerosis is a chronic inflammatory disease in which lipoproteins accumulate, and cause an inflammatory response in the arterial wall [2]. When LDL particles infiltrate the innermost layer of the arterial wall, the intima, they are prone to oxidative modification, such as enzymatic attacks by myeloperoxidase and lipoxygenases, as well as nonenzymatic oxidative reactions [35]. Immune responses to oxidized low-density lipoprotein (ox LDL) are proposed to be important in atherosclerosis.

To study T cell recognition of oxidized LDL (ox LDL), we created T cell hybridomas from human ApoB 100 (huB100^{tg}) transgenic mice that were immunized subcutaneously with ox LDL. These mice generate high levels of ApoB 100 that are packaged into human-like LDL particles [81]. Similar to other transgenic mice that produce nonmurine proteins, huB100^{tg} mice tolerate the orthologous transgene, neither producing antibodies to it nor developing spontaneous autoimmune disease.

Surprisingly, none of the hybridomas responded to oxidized LDL, only to native LDL and the purified LDL apolipoprotein ApoB 100. Unexpectedly, ox LDL immunized huB100^{tg} mice developed T cell responses against native LDL and purified ApoB 100. The responding T cells were MHC class II restricted CD4⁺ cells and expressed T cell receptors (TCRs) that contained the variable β domain TRBV 31, formerly known as V β 14. These results strongly suggest that autoimmune T cells that recognize protein epitopes from native LDL promote atherosclerosis.

The results suggest that the inflammatory response in atherosclerosis is driven by a peptide sequence of the native Apo B 100 particle. In this study, we have identified a cellular immune response to Apo B 100 protein of native LDL, and show that this response plays an important role in atherosclerosis.

The cellular immune response to LDL that we observed was mounted by CD4⁺ T cells showing MHC class II restriction and expressing T cell receptors that contained the variable domain TRBV 31.

Summary and conclusion

Nowadays, diet and lifestyle can influence the process of atherosclerosis, and together with control of cardiovascular risk factors, can promote healthy ageing. To go one step further, to prevent atherosclerosis and develop a vaccine for atherosclerosis would make it possible to have extended life expectancy and rule out the most common cause of death in the western world.

In conclusion, the results show that ApoB 100 responding CD4+ T cell hybridomas were MHC class II–restricted and expressed a single T cell receptor (TCR) variable (V) chain, TRBV31, with different V α chains.

This could in the future lead to medical targets against atherosclerosis. The goal would be to develop a vaccine against atherosclerosis, preventing this progressive disease. Follow-up studies include an animal immunization study, where mice are immunized with different peptides. Then the lipoprotein profile, spleen cell proliferation, cytokine expression, immunoglobulin profiles and degree of atherosclerotic lesions are checked for the different groups.

Clearly, additional research is needed to contribute to our understanding of the complex process of autoimmunity in atherosclerosis.

7 Zusammenfassung

Atherosklerose ist eine der Haupttodesursachen weltweit. Klinische Manifestationen sind unter anderem periphere arterielle Verschlusskrankheit, akutes Koronarsyndrom, Herzinfarkt, Schlaganfall, Mesenterialinfarkt, und akute arterielle Verschlüsse der Extremitäten. Atherosklerose ist eine in Stadien verlaufende Erkrankung, der die Bildung von Plaques zugrunde liegt. Plaques sind Ansammlungen von Lipiden, Entzündungszellen, vaskulären Endothelzellen, glatten Muskelzellen, Bindegewebszellen und lipidreicher Debris in den Gefäßen. Plaques sind typischerweise asymmetrische fokale Verdickungen subendothelial unter der innersten Schicht in den Gefäßen, der Intima. Das Immunsystem spielt eine entscheidende Rolle an der Modulation der Erkrankung.

Im ersten Stadium der Atherosklerose sammeln sich immunkompetente Zellen und Fettzellen in der Intima an und bilden sogenannte „fatty streaks“, streifige Verfettungen der Arterienwand. Makrophagen nehmen die Lipide auf und bilden sich zu Schaumzellen um. Diese Schaumzellen sind besonders zahlreich im ersten Stadium der Atherosklerose. Dieses ist klinisch stumm. Die „fatty streaks“ können sich entweder zurückbilden oder ins nächste Stadium übergehen.

Reife Plaques haben einen komplexeren Aufbau. Im Zentrum befinden sich Schaumzellen und Lipidtropfen. Um dieses Zentrum befindet sich eine fibröse Kappe aus glatten Muskelzellen und Kollagenfasern, die den Plaque stabilisiert. In den meisten Fällen führt die Ruptur dieser Kappe zu den akuten klinischen Komplikationen.

Im Immunsystem werden zwei Komponenten unterschieden, das angeborene unspezifische und das erworbene spezifische Immunsystem. T Zellen gehören zum spezifischen Immunsystem, können Antigene erkennen und antigenabhängig stimuliert werden. Th1 Zellen haben einen entscheidenden Einfluss auf das Plaque-Wachstum, da sie inflammatorische Zytokine produzieren, die wiederum andere Zellen beeinflussen. So werden Makrophagen stimuliert, Endothelzellen aktiviert, glatte Muskelzellen inhibiert und die Thrombusbildung gefördert. T Zellen spielen also eine entscheidende Rolle an den Umbauvorgängen und damit der Stadienentwicklung in den Plaques.

Diese Doktorarbeit befasst sich mit der Reaktion der T Zellen auf oxidiertes LDL. Auf der Suche nach dem Antigen, das die T Zellen im atherosklerotischen Prozess aktiviert, wurden T Zellen von Hybridomen von für humanes ApoB 100 transgenen Mäusen, die mit oxidiertem LDL subkutan immunisiert wurden, verschiedenen potentiellen Antigenen exponiert und die T Zell Aktivierung durch Interleukin-2- Freisetzung gemessen. Die Ergebnisse waren überraschend, da die T Zellen stärker auf natives LDL als auf das oxidierte LDL reagierten. Es fand sich insbesondere eine starke Reaktion auf Apolipoprotein B 100, dem Proteinanteil von LDL.

Anhand dieser Ergebnisse wurde gezeigt, dass der Proteinanteil von LDL, Apo B 100, für die immunologische Reaktion der T Zellen im atherosklerotischen Prozess verantwortlich sein könnte.

Kurz zusammengefasst zeigen die Ergebnisse dieser Studie, dass CD4⁺ T Zellen Epitope des nativen LDL Protein erkennen, und diese Immunantwort mit einem bestimmten T Zell Rezeptor assoziiert ist, TRBV 31. Da ApoB 100 die Aktivierung von CD4 + T Zell Hybridomen induzierte, gingen wir davon aus, dass die Peptide über den MHC Klasse II Komplex präsentiert werden, was bei Mäusen dem I- A^b Haplotyp entspricht. Nach Blockierung des I- A^b mittels eines Antikörper zeigte sich keine T Zell Reaktion auf das Antigen, die immunogene Antwort war somit MHC Klasse II restriktiv. Aus den gewonnenen Resultaten lässt sich folgende Hypothese herleiten: natives LDL wird über den Scavenger- Rezeptor von der antigen- präsentierenden Zelle aufgenommen, Peptide des Proteinanteil von LDL, ApoB 100, werden an den MHC Klasse II- Komplex gebunden und den T- Zellen an der Zelloberfläche präsentiert. Diese erkennen den ApoB 100/ MHC Klasse II Komplex über einen bestimmten T- Zell- Rezeptor, TRBV 31, was die spezifische zelluläre Immunantwort auslöst. Atherosklerose kann somit als Autoimmunerkrankung betrachtet werden. Aus den gewonnen Resultaten schliessen wir, dass autoimmune T- Zellen, die den Proteinanteil von LDL erkennen, die Entwicklung von Atherosklerose fördern. Alle T Zell Klone, die auf das ApoB 100 und native LDL reagierten, exprimierten den gleichen β -T- Zell- Rezeptor, TRBV 31.

Dies könnte Angriffspunkt für zukünftige Therapien der Atherosklerose sein.

Erklärung

Erklärung

Ich, Marion Irene Wurm, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema „Evaluation of T cell response to native and oxidized low- density lipoprotein (LDL)“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Datum

Unterschrift

8 References

1. Hansson, G.K. and P. Libby, *The immune response in atherosclerosis: a double-edged sword*. Nat Rev Immunol, 2006. 6(7): p. 508-19.
2. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease*. N Engl J Med, 2005. 352(16): p. 1685-95.
3. Mehta, J.L., *Oxidized or native low-density lipoprotein cholesterol: which is more important in atherogenesis?* J Am Coll Cardiol, 2006. 48(5): p. 980-2.
4. Lusis, A.J., *Atherosclerosis*. Nature, 2000. 407(6801): p. 233-41.
5. Jonasson, L., et al., *Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque*. Arteriosclerosis, 1986. 6(2): p. 131-8.
6. Jonasson, L., et al., *Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis*. J Clin Invest, 1985. 76(1): p. 125-31.
7. Janeway, C.A., Jr., *Immunobiology. The immune system in health and disease*. 5th ed, ed. E.L. Penelope Austin. 2001, New York, NY 1001- 2299: Garland Publishing. 732. p.323.
8. Stary, H.C., et al., *A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association*. Circulation, 1995. 92(5): p. 1355-74.
9. Libby, P., *Atherosclerosis: disease biology affecting the coronary vasculature*. Am J Cardiol, 2006. 98(12A): p. 3Q-9Q.
10. Libby, P., *Inflammatory mechanisms: the molecular basis of inflammation and disease*. Nutr Rev, 2007. 65(12 Pt 2): p. S140-6.
11. Hansson, G.K., et al., *Innate and adaptive immunity in the pathogenesis of atherosclerosis*. Circ Res, 2002. 91(4): p. 281-91.
12. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition*. Annu Rev Immunol, 2002. 20: p. 197-216.
13. Medzhitov, R. and C. Janeway, Jr., *Innate immunity*. N Engl J Med, 2000. 343(5): p. 338-44.
14. Krieger, M., *The other side of scavenger receptors: pattern recognition for host defense*. Curr Opin Lipidol, 1997. 8(5): p. 275-80.
15. Faure, E., et al., *Bacterial lipopolysaccharide activates NF-kappaB through toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells*. J Biol Chem, 2000. 275(15): p. 11058-63.
16. Muzio, M., et al., *The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6)*. J Exp Med, 1998. 187(12): p. 2097-101.
17. Guha, M. and N. Mackman, *LPS induction of gene expression in human monocytes*. Cell Signal, 2001. 13(2): p. 85-94.
18. Libby, P., *Inflammation in atherosclerosis*. Nature, 2002. 420(6917): p. 868-74.

References

19. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity*. Int Immunol, 2005. 17(1): p. 1-14.
20. Janeway, C.A., Jr., *Immunobiology. The immune system in health and disease*. 5th ed, ed. E.L. Penelope Austin. 2001, New York, NY: Garland publishing. 732. p 155-180.
21. Janeway, C.A., Jr., *Immunobiology. The immune system in health and disease*. 5th ed, ed. E.L. Penelope Austin. 2001, New York: Garland Publishing. 732. p 105-139.
22. Rock, K.L., *The role of Ia molecules in the activation of T lymphocytes. I. The activation of an IL 1-dependent IL 2-producing T cell hybridoma by Con A requires an interaction, which is not H-2-restricted, with an Ia-bearing accessory cell*. J Immunol, 1982. 129(4): p. 1360-6.
23. Swierkosz, J.E., et al., *The role of H-2 linked genes in helper T-cell function. II. Isolation on antigen-pulsed macrophages of two separate populations of F1 helper T cells each specific for antigen and one set of parental H-2 products*. J Exp Med, 1978. 147(2): p. 554-70.
24. Hodes, R.J., K.S. Hathcock, and A. Singer, *Major histocompatibility complex-restricted self recognition. A monoclonal anti-I-Ak reagent blocks helper T cell recognition of self major histocompatibility complex determinants*. J Exp Med, 1980. 152(6): p. 1779-94.
25. Schwartz, R.H., A. Yano, and W.E. Paul, *Interaction between antigen-presenting cells and primed T lymphocytes: an assessment of Ir gene expression in the antigen-presenting cell*. Immunol Rev, 1978. 40: p. 153-80.
26. Thomas, D.W., U. Yamashita, and E.M. Shevach, *The role of Ia antigens in T cell activation*. Immunol Rev, 1977. 35: p. 95-120.
27. Sakkas, L.I., I.C. Chikanza, and C.D. Platsoucas, *Mechanisms of Disease: the role of immune cells in the pathogenesis of systemic sclerosis*. Nat Clin Pract Rheumatol, 2006. 2(12): p. 679-85.
28. Hansson, G.K., J. Holm, and L. Jonasson, *Detection of activated T lymphocytes in the human atherosclerotic plaque*. Am J Pathol, 1989. 135(1): p. 169-75.
29. Hansson, G.K., et al., *Immunohistochemical detection of macrophages and T lymphocytes in atherosclerotic lesions of cholesterol-fed rabbits*. Arterioscler Thromb, 1991. 11(3): p. 745-50.
30. Paulsson, G., et al., *Oligoclonal T cell expansions in atherosclerotic lesions of apolipoprotein E-deficient mice*. Arterioscler Thromb Vasc Biol, 2000. 20(1): p. 10-7.
31. Robertson, A.K. and G.K. Hansson, *T cells in atherogenesis: for better or for worse?* Arterioscler Thromb Vasc Biol, 2006. 26(11): p. 2421-32.
32. Daniels, T.F., et al., *Lipoproteins, cholesterol homeostasis and cardiac health*. Int J Biol Sci, 2009. 5(5): p. 474-88.
33. Prassl, R. and P. Laggner, *Molecular structure of low density lipoprotein: current status and future challenges*. Eur Biophys J, 2008.
34. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead*. Cell, 2001. 104(4): p. 503-16.

References

35. Hevonoja, T., et al., *Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL*. Biochim Biophys Acta, 2000. 1488(3): p. 189-210.
36. Witztum, J.L., *Role of oxidised low density lipoprotein in atherogenesis*. Br Heart J, 1993. 69(1 Suppl): p. S12-8.
37. Steinbrecher, U.P., et al., *Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism*. Arteriosclerosis, 1987. 7(2): p. 135-43.
38. www.sigmaaldrich.com
39. Stemme, S., et al., *T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein*. Proc Natl Acad Sci U S A, 1995. 92(9): p. 3893-7.
40. Yla-Herttuala, S., et al., *Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL*. Arterioscler Thromb, 1994. 14(1): p. 32-40.
41. Stoll, G. and M. Bendszus, *Inflammation and atherosclerosis: novel insights into plaque formation and destabilization*. Stroke, 2006. 37(7): p. 1923-32.
42. Virella, G. and M.F. Lopes-Virella, *Lipoprotein autoantibodies: measurement and significance*. Clin Diagn Lab Immunol, 2003. 10(4): p. 499-505.
43. Skalen, K., et al., *Subendothelial retention of atherogenic lipoproteins in early atherosclerosis*. Nature, 2002. 417(6890): p. 750-4.
44. Kuhn, H., I. Romisch, and J. Belkner, *The role of lipoxxygenase-isoforms in atherogenesis*. Mol Nutr Food Res, 2005. 49(11): p. 1014-29.
45. Brown, M.S., et al., *Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins*. J Cell Biol, 1979. 82(3): p. 597-613.
46. Steinberg, D., et al., *Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity*. N Engl J Med, 1989. 320(14): p. 915-24.
47. Chisolm, G.M. and D. Steinberg, *The oxidative modification hypothesis of atherogenesis: an overview*. Free Radic Biol Med, 2000. 28(12): p. 1815-26.
48. Reaven, P.D. and J.L. Witztum, *Oxidized low density lipoproteins in atherogenesis: role of dietary modification*. Annu Rev Nutr, 1996. 16: p. 51-71.
49. Steinberg, D. and J.L. Witztum, *Lipoproteins and atherogenesis. Current concepts*. Jama, 1990. 264(23): p. 3047-52.
50. Witztum, J.L. and D. Steinberg, *The oxidative modification hypothesis of atherosclerosis: does it hold for humans?* Trends Cardiovasc Med, 2001. 11(3-4): p. 93-102.
51. Steinbrecher, U.P., et al., *Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids*. Proc Natl Acad Sci U S A, 1984. 81(12): p. 3883-7.

References

52. Heinecke, J.W., H. Rosen, and A. Chait, *Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture*. J Clin Invest, 1984. 74(5): p. 1890-4.
53. Steinberg, D., Witztum, J.L., *Lipoproteins, lipoprotein oxidation, and atherogenesis*. Chien KR ed. Molecular Basis of Cardiovascular Disease. Vol. Philadelphia, W.B. Saunders, Co. 1999. pp.458-475.
54. Mallat, Z., et al., *Protective role of interleukin-10 in atherosclerosis*. Circ Res, 1999. 85(8): p. e17-24.
55. Esterbauer, H., et al., *Biochemical, structural, and functional properties of oxidized low-density lipoprotein*. Chem Res Toxicol, 1990. 3(2): p. 77-92.
56. Palinski, W., et al., *Low density lipoprotein undergoes oxidative modification in vivo*. Proc Natl Acad Sci U S A, 1989. 86(4): p. 1372-6.
57. Ishigaki, Y., Y. Oka, and H. Katagiri, *Circulating oxidized LDL: a biomarker and a pathogenic factor*. Curr Opin Lipidol, 2009. 20(5): p. 363-9.
58. Tsimikas, S., W. Palinski, and J.L. Witztum, *Circulating autoantibodies to oxidized LDL correlate with arterial accumulation and depletion of oxidized LDL in LDL receptor-deficient mice*. Arterioscler Thromb Vasc Biol, 2001. 21(1): p. 95-100.
59. Palinski, W., et al., *Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor-deficient mice with increased atherosclerosis*. Arterioscler Thromb Vasc Biol, 1995. 15(10): p. 1569-76.
60. Wuttge, D.M., M. Bruzelius, and S. Stemme, *T-cell recognition of lipid peroxidation products breaks tolerance to self proteins*. Immunology, 1999. 98(2): p. 273-9.
61. Steinberg, D. and J.L. Witztum, *Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis?* Circulation, 2002. 105(17): p. 2107-11.
62. Omenn, G.S., et al., *Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease*. N Engl J Med, 1996. 334(18): p. 1150-5.
63. Hennekens, C.H., et al., *Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease*. N Engl J Med, 1996. 334(18): p. 1145-9.
64. *The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group*. N Engl J Med, 1994. 330(15): p. 1029-35.
65. *Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico*. Lancet, 1999. 354(9177): p. 447-55.
66. Yusuf, S., et al., *Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators*. N Engl J Med, 2000. 342(3): p. 154-60.
67. Brown, B.G., et al., *Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease*. N Engl J Med, 2001. 345(22): p. 1583-92.

References

68. Landmesser, U. and D.G. Harrison, *Oxidant stress as a marker for cardiovascular events: Ox marks the spot*. *Circulation*, 2001. 104(22): p. 2638-40.
69. Pratico, D., Tangirala R, Rader D et al., *Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in Apo-E deficient mice*. *Nat Med*, 1990. 10: p. 10:1189-1192.
70. Thomas, S.R., et al., *Dietary cosupplementation with vitamin E and coenzyme Q(10) inhibits atherosclerosis in apolipoprotein E gene knockout mice*. *Arterioscler Thromb Vasc Biol*, 2001. 21(4): p. 585-93.
71. Crawford, R.S., et al., *Dietary antioxidants inhibit development of fatty streak lesions in the LDL receptor-deficient mouse*. *Arterioscler Thromb Vasc Biol*, 1998. 18(9): p. 1506-13.
72. Sasahara, M., et al., *Inhibition of hypercholesterolemia-induced atherosclerosis in the nonhuman primate by probucol. I. Is the extent of atherosclerosis related to resistance of LDL to oxidation?* *J Clin Invest*, 1994. 94(1): p. 155-64.
73. Meagher, E.A., et al., *Effects of vitamin E on lipid peroxidation in healthy persons*. *JAMA*, 2001. 285(9): p. 1178-82.
74. Stocker, R., *Dietary and pharmacological antioxidants in atherosclerosis*. *Curr Opin Lipidol*, 1999. 10(6): p. 589-97.
75. Zhou, X., et al., *Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice*. *Circulation*, 2000. 102(24): p. 2919-22.
76. Zhou, X., et al., *Adoptive transfer of CD4+ T cells reactive to modified low-density lipoprotein aggravates atherosclerosis*. *Arterioscler Thromb Vasc Biol*, 2006. 26(4): p. 864-70.
77. Song, L., C. Leung, and C. Schindler, *Lymphocytes are important in early atherosclerosis*. *J Clin Invest*, 2001. 108(2): p. 251-9.
78. Zhou, X., S. Stemme, and G.K. Hansson, *Evidence for a local immune response in atherosclerosis. CD4+ T cells infiltrate lesions of apolipoprotein-E-deficient mice*. *Am J Pathol*, 1996. 149(2): p. 359-66.
79. Frostegard, J., et al., *Induction of T-cell activation by oxidized low density lipoprotein*. *Arterioscler Thromb*, 1992. 12(4): p. 461-7.
80. Zhou, X., et al., *LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis*. *Arterioscler Thromb Vasc Biol*, 2001. 21(1): p. 108-14.
81. Linton, M.F., et al., *Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein(a)*. *J Clin Invest*, 1993. 92(6): p. 3029-37.
82. Tabas, I., K.J. Williams, and J. Boren, *Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications*. *Circulation*, 2007. 116(16): p. 1832-44.
83. Hartvigsen, K., et al., *The role of innate immunity in atherogenesis*. *J Lipid Res*, 2009. 50 Suppl: p. S388-93.

References

84. Boren, J., et al., *Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100*. J Clin Invest, 1998. 101(5): p. 1084-93.
85. Kappler, J.W., et al., *Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition*. J Exp Med, 1981. 153(5): p. 1198-214.
86. Havel, R.J., H.A. Eder, and J.H. Bragdon, *The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum*. J Clin Invest, 1955. 34(9): p. 1345-53.
87. Puhl, H., G. Waeg, and H. Esterbauer, *Methods to determine oxidation of low-density lipoproteins*. Methods Enzymol, 1994. 233: p. 425-41.
88. Wessel, D. and U.I. Flugge, *A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids*. Anal Biochem, 1984. 138(1): p. 141-3.
89. Steele, J.C., Jr. and J.A. Reynolds, *Characterization of the apolipoprotein B polypeptide of human plasma low density lipoprotein in detergent and denaturation solutions*. J Biol Chem, 1979. 254(5): p. 1633-8.
90. Pannetier, C., et al., *The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments*. Proc Natl Acad Sci U S A, 1993. 90(9): p. 4319-23.
91. Yoshida, R., et al., *A new method for quantitative analysis of the mouse T-cell receptor V region repertoires: comparison of repertoires among strains*. Immunogenetics, 2000. 52(1-2): p. 35-45.
92. <http://imqt.cines.fr/>
93. Yang, C.Y., et al., *Structure and conformational analysis of lipid-associating peptides of apolipoprotein B-100 produced by trypsinolysis*. J Protein Chem, 1989. 8(6): p. 689-99.
94. Libby, P., *Inflammation and cardiovascular disease mechanisms*. Am J Clin Nutr, 2006. 83(2): p. 456S-460S.
95. Williams, K.J., J.E. Feig, and E.A. Fisher, *Cellular and molecular mechanisms for rapid regression of atherosclerosis: from bench top to potentially achievable clinical goal*. Curr Opin Lipidol, 2007. 18(4): p. 443-50.
96. Palinski, W., et al., *Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma*. J Clin Invest, 1996. 98(3): p. 800-14.
97. Shah, P.K., et al., *Vaccination for atherosclerosis: a novel therapeutic paradigm*. Expert Rev Vaccines, 2004. 3(6): p. 711-6.
98. Fredrikson, G.N., et al., *Autoimmune responses against the apo B-100 LDL receptor-binding site protect against arterial accumulation of lipids in LDL receptor deficient mice*. Autoimmunity, 2007. 40(2): p. 122-30.

References

99. Wu, T., et al., *Is plasma oxidized low-density lipoprotein, measured with the widely used antibody 4E6, an independent predictor of coronary heart disease among U.S. men and women?* *J Am Coll Cardiol*, 2006. 48(5): p. 973-9.
100. Mehta, J.L., *Oxidized or Native Low-Density Lipoprotein Cholesterol. Which Is More Important in Atherogenesis?* *Journal of the American College of Cardiology*, 2006. 48(No.5).
101. Shaw, P.X., *Rethinking oxidized low-density lipoprotein, its role in atherogenesis and the immune responses associated with it.* *Arch Immunol Ther Exp (Warsz)*, 2004. 52(4): p. 225-39.
102. James, J.R., et al., *Single-molecule level analysis of the subunit composition of the T cell receptor on live T cells.* *Proc Natl Acad Sci U S A*, 2007. 104(45): p. 17662-7.
103. Kook, H., et al., *Changes in T-cell receptor VB repertoire in aplastic anemia: effects of different immunosuppressive regimens.* *Blood*, 2002. 99(10): p. 3668-75.
104. Frostegard, J., *Autoimmunity, oxidized LDL and cardiovascular disease.* *Autoimmun Rev*, 2002. 1(4): p. 233-7.
105. McMahon, M. and B.H. Hahn, *Atherosclerosis and systemic lupus erythematosus: mechanistic basis of the association.* *Curr Opin Immunol*, 2007. 19(6): p. 633-9.
106. Bridges, S.L., Jr. and L.W. Moreland, *T-cell receptor peptide vaccination in the treatment of rheumatoid arthritis.* *Rheum Dis Clin North Am*, 1998. 24(3): p. 641-50.
107. Janeway, C.A., Jr., *Immunobiology. The immune system in health and disease.* 5th ed, ed. E.L. Penelope Austin. 2001, New York: Garland Publishing. 732. p 501-551.
108. Mackay, I.R., *Autoimmunity since the 1957 clonal selection theory: a little acorn to a large oak.* *Immunology and cell biology*, 2007. 86: p. 67-71.
109. Mackay, I.R., *The etiopathogenesis of autoimmunity.* *Semin Liver Dis*, 2005. 25(3): p. 239-50.

Aus datenschutzrechtlichen Gründen ist in der elektronischen Version der Doktorarbeit kein Lebenslauf enthalten.