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Adaptive Immunity in Cardiovascular Disease

Engelbertsen, Daniel

2013

Link to publication

Citation for published version (APA):
Engelbertsen, D. (2013). *Adaptive Immunity in Cardiovascular Disease*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Malmö]. Department of Clinical Sciences, Lund University.

Total number of authors:
1

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Adaptive Immunity in Cardiovascular Disease

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DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended in the lecture hall at Kvinnokliniken, entrance 74, Skåne University Hospital, Malmö on May 17th 2013, at 9.00.

Faculty opponent
Professor Andrew Lichtman, Harvard Medical School

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
	Date of issue: May 17th, 2013
Author(s) Daniel Engelbertsen	Sponsoring organization

Title and subtitle:

Adaptive Immunity in Cardiovascular Disease

Abstract

Atherosclerosis, thickening of the major arteries, is a chronic disease leading to cardiovascular disease (CVD) such as myocardial infarction (MI) and stroke, both affecting millions worldwide. T-cells and antibodies have been implicated in atherosclerosis. This thesis contains studies on CD4+ helper subsets and studies on antibodies against methylglyoxal-modified apolipoprotein B100 (MGO-ApoB100).

In the prospective Malmö Diet and Cancer cohort we studied the relation of T helper 1 (Th1) and T helper 2 (Th2) cells to future cardiovacular events. We found that baseline-levels of Th2 cells, but not Th1 cells, were inversely associated with intima-media thickness in the cohort and future coronary events in women. In another cohort, the SUMMIT cohort, we found that type 2 diabetes patients with concomitant CVD were characterized by elevated levels of pro-inflammatory CD4+ T effector memory cells. Using a novel technique (the matrigel) for determining the immune responses *in vivo*, Th2 responses against human ApoB100 was characterized. In another study, genetic deletion of the signaling protein myeloid differentiation protein 88 (MyD88) in CD4+ T cells was found to reduce atherosclerosis. This reduction was associated with decreased production of the pro-inflammatory cytokine IL-17. In two separate studies, IgM antibodies against MGO-ApoB or MGO-ApoB-peptides were observed to be associated with reduced CVD. The mechanism mediating this protection against CVD remains to be clarified.

This thesis presents and discusses aspects of immunity, which are interesting targets for pharmaceutical intervention. Various components of adaptive immunity may be useful as biomarkers for determining patients with increased risk of developing CVD.

Key words Atherosclerosis, T cells, antibodies, MyD88, Th1, Th2, methylglyoxal, matrigel		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		Language: English
ISSN and key title 1652-8220		ISBN 978-91-87449-21-5
Recipient's notes	Number of pages 135	Price
	Security classification	

Signature _____ Date: 2013-04-11

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Cover from: Anitschkow, N. 1933. Experimental atherosclerosis in animals. In Arteriosclerosis: A Survey of the Problem. E. V. Cowdry, editor. Macmillan, New York. 271–322. Reprinted in Steinberg, D. Thematic review series: The Pathogenesis of Atherosclerosis. An interpretive history of the cholesterol controversy: part I. Journal of Lipid Research. 2004. Sep; 45(9):1583-93.

ISSN 1652-8220
ISBN 978-91-87449-21-5

Lund University, Faculty of Medicine Doctoral Dissertation Series 2013:51

Printed in Sweden by Media-Tryck, Lund University
Lund 2013

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Introduction/Prelude

The adaptive immune system, have been found to not only combat infectious agents, but also to affect the development of the cardiovascular disease. In this thesis I will present results derived from published articles and manuscripts concerning immunity and atherosclerosis.

In particular, I will present four articles that address the issue of $CD4^+$ T cells in atherosclerosis, both in mice and men. The two latter articles elucidate the role of antibodies against methylglyoxal-modified Apolipoprotein B100 and atherosclerosis in patients.

My ambition is that this thesis will be comprehensible to those who have a basic knowledge in cellular biology and biomedicine, not just specialists in cardiovascular medicine or immunology. However, for those of you who have found other pleasures in life than biomedicine, I encourage reading the "populärvetenskaplig sammanfattning".

If I had more time I would have written a shorter thesis

- Adapted from Blaise Pascal

Original papers

The thesis is based on the following papers (I-VI).

I. **Engelbertsen D**, Andersson L, Ljungcrantz I, Wigren M, Hedblad B, Nilsson J, Björkbacka H. T-helper 2 immunity is associated with reduced risk of myocardial infarction and stroke. *Arterioscler Thromb Vasc Biol*. 2013 Mar;33(3):637-44

II. **Engelbertsen D**, Ljungcrantz I, Östling G, Bengtsson E, Nilsson J, Björkbacka H. Evidence for a role of memory T cells in cardiovascular complications of diabetes. (manuscript)

III. **Engelbertsen D**, Rattik S, Knutsson A, Ketelhuth D, Björkbacka H, Bengtsson E, Nilsson J. Characterization of T-cell responses following immunization with human apolipoprotein B100 in mice. (manuscript)

IV. **Engelbertsen D**, Wigren M, To F, Björkbacka H, Nilsson J, Bengtsson E. Myd88 signaling in CD4+ T cells promotes atherosclerosis and Th17 responses. (manuscript)

V. **Engelbertsen D**, Anand DJ, Fredrikson GN, Hopkins D, Corder C, Shah PK, Lahiri A, Nilsson J, Bengtsson E. High levels of IgM against methylglyoxal-modified apolipoprotein B100 is associated with less coronary artery calcification in patients with type 2 diabetes. *Journal of Internal Medicine*, 2012, 271, 82-89

VI. **Engelbertsen D**, Nordin Fredrikson G, Alm R, Hedblad B, Björkbacka H, Nilsson J, Bengtsson E. Low levels of IgM auto-antibodies against a methylglyoxal-modified apolipoprotein B100 peptide predict cardiovascular events. (submitted)

List of published papers not included in the thesis

Engelbertsen D, To F, Dunér P, Kotova O, Söderberg I, Alm R, Gomez MF, Nilsson J, Bengtsson E. Increased inflammation in atherosclerotic lesions of diabetic Akita-LDLr-/- mice compared to nondiabetic LDLr-/- mice, *Exp Diabetes Res*. 2012;2012:176162

Abbreviations

ACS	Acute coronary syndrome
AGE	Advanced glycation end-product
AMI	Acute myocardial infarction
ApoB	Apolipoprotein B
APC	Antigen presenting cell
ApoE-/-	Apolipoprotein E deficient mouse
AMI	Acute myocardial infarction
BCR	B-cell receptor
BMT	Bone-marrow transfer
CAD	Coronary artery disease
CVD	Cardiovascular disease
CEL	Nε-(carboxyethyl) lysine
DC	Dendritic cells
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FoxP3	Forkhead box P3 (transcription factor)
HbA1c	Glycated haemoglobin A1c
HDL	High-density lipoprotein
HSP	Heat shock protein
IL	Interleukin
IMT	Intima-media thickness
IFN-γ	Interferon gamma
Ig	Immunoglobulin

LDL	Low-density lipoprotein
LDLr-/-	LDL receptor deficient mice
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MHC	Major Histocompatibility Complex
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation protein-88
MGO	Methylglyoxal
oxLDL	oxidized LDL
PAMP	Pathogen associated molecule pattern
PBML	Peripheral blood mononuclear leukocytes
PC	Phosphorylcholine
PRR	Pattern recognition receptor
PUFA	Polyunsaturated fatty acid
RAG	Recombination activation gene
SA	Stable angina
SMC	Smooth muscle cell
SLO	Secondary lymphoid organ
T2DM	Type 2 diabetes mellitus
TCR	T-cell receptor
Th	T helper cell
T_{EM}	Effector memory T cell
T_{CM}	Central memory T cell
T_N	Naïve T cell
TLR	Toll-like receptor
Treg	Regulatory T cell
UA	Unstable angina

Background

In this background I will (i) give an overview on the history, epidemiology and pathology of atherosclerosis and (ii) present aspects of immunology important for this thesis and finally (iii) review research on $CD4^+$ T cells and autoantibodies in cardiovascular disease.

Pathology of atherosclerosis

Epidemiology

Atherosclerosis, thickening of the major arteries, is a chronic disease leading to cardiovascular disease (CVD) such as myocardial infarction (MI) and stroke, both affecting millions worldwide[1].

There has been a great shift in the causes of mortality during the last 100 years. Previously, infections like tuberculosis and pneumonia were the most common causes of death, but the advent of antibiotics and vaccination greatly reduced such diseases[2]. Today, CVD is the most common cause of death worldwide[1] and the incidence of CVD is projected to increase in the future[3]. Also, due to changes in lifestyle and increased obesity during the last decades the incidence of diabetes mellitus has increased greatly, and is expected to continue to rise in the future[4]. Diabetes mellitus predisposes to CVD by mechanisms discussed in the coming chapters.

Although clinical manifestations of CVD are observed mainly in middle-aged and elderly individuals, early stages of atherosclerosis can be observed in young children[5]. Below, I will attempt to briefly describe the different stages of atherogenesis: the formation of the atheromatous plaque.

The healthy artery

The arterial wall consists of three layers: tunica intima, tunica media and tunica adventitia. The intima is defined as the endothelial cell layer, being layered on top of a basement membrane, and the subendothelial space which is rich in extracellular matrix (ECM) proteins. Human intima, as opposed to the mouse, contains smooth-muscle cells (SMCs). The internal elastic lamina constitutes the border between the intima and the media. The tunica media is situated between the internal and external elastic laminae and consists mainly of SMCs that regulate vessel contraction and vascular tone. The adventitia is the third and outer layer of the artery and consists of mesenchymal cells such as fibroblasts as well as mast cells[6].

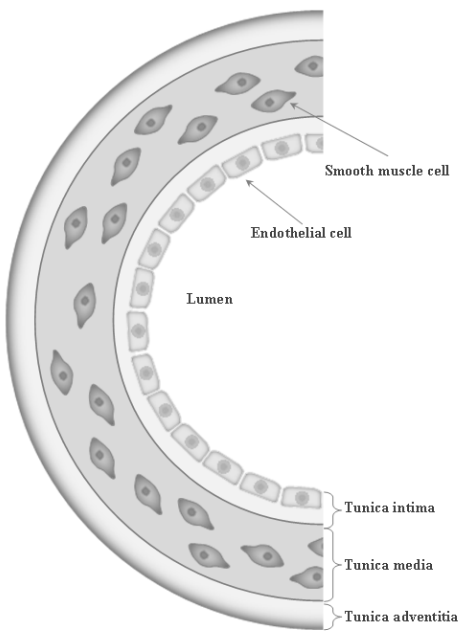

Figure 1. Schematic picture of the healthy arterial wall. *(Apapted from K.E Berg, Lyso-PC and macrophage interplay. ISBN 978-91-86671-98-3)*

Cholesterol

In 1910, Windaus discovered that atherosclerotic plaques were rich in cholesterol and calcified tissue[7]. The role of cholesterol in atherogenesis would become the topic for much research during the 20th century.

To understand the deposition of cholesterol in the plaque we must understand some basic facts about lipoprotein metabolism and the circulation of nutrients. In summary, triglycerides (TG) and cholesterol are taken up by the intestine and packed into chylomicron particles. The enzyme lipoprotein lipase (LPL) hydrolyzes triglycerides of chylomicrons turning the particles into chylomicron remnants, which are taken up by the liver. In the liver, cholesterol, TG and apolipoprotein B100 (apoB100) are combined to form very-low density lipoprotein (VLDL) particles that are released back into the circulation. VLDL particles like chylomicrons are subjected to LPL activity that strips the particle of TG content, eventually transforming VLDL to intermediate-density lipoprotein (IDL) particles. Hepatic lipase mediated TG hydrolysis of IDL particles generates the main antagonist of our story: low-density lipoprotein (LDL).

The LDL particle serves to supply peripheral tissues with cholesterol and cholesteryl esters needed for the synthesis of steroid hormones, cell membrane components and bile acids[8]. However, excess intake of saturated fat[9], lack of exercise[10] as well as genetic factors[11] increase levels of LDL above the level that is needed to supply tissues at homeostasis.

Anitschkow and cholesterol

During the 20th century several hypotheses was put forward trying to explain the initiating events causing atherosclerosis. In a groundbreaking study performed in 1913, Nikolaj Anitschkow fed rabbits purified cholesterol dissolved in sunflower oil and analyzed the development of atherosclerotic lesions. The rabbits fed cholesterol developed atherosclerotic lesions similar in morphology to those found in humans. Moreover, he observed that early lesions were characterized by lipid laden cells[7, 12].

However, the discoveries made by Anitschkow fell out of fashion for decades and the role of cholesterol in atherosclerosis was not pursued by the scientific community until the 1940s. Part of the reason for the skepticism was that the findings were not replicable in the animal models being used in the west: cats and dogs. In retrospect, the reason for this was that feeding of cholesterol to cats and dogs do not translate to elevated blood cholesterol since they efficiently convert excess cholesterol into bile acids[12].

Response-to-retention hypothesis of atherogenesis

There is still an ongoing debate regarding the sequence of events leading to formation of the atherosclerotic plaque. Several hypotheses have been put forward including Ross's "Response to injury hypothesis"[13], presented in 1977, in which the authors postulate that the event triggering atherogenesis is "some form of "injury" to arterial endothelium". In the 1990s, Tabas and Williams[14] proposed the response-to-retention hypothesis to explain the events of early atherogenesis. I will here describe the events of atherogenesis, as it is currently understood, based on the response-to-retention hypothesis. The influence of adaptive immunity will be left out of this discussion and discussed later on.

The first event in the atherogenic process is the formation of diffuse intimal thickening, possibly caused by shear stress, and production of ECM proteins like proteoglycans. Decorin and biglycan, two proteoglycans, contain negatively charged sulphate side chains that promote retention of LDL in the intima by interacting with electropositive residues on apoB100[15].

Trapped in the arterial intima, LDL gets oxidized by metal ions (Fe^{2+}, Cu^{2+}), lipoxygenases and myeloperoxidases[16]. Oxidation of LDL alters the structure of the particle and generates new epitopes such aldehyde-modified amino-acids and oxidized phospholipids. Such oxidation epitopes are recognized by scavenger receptors like SR-AI/II, CD36 and LOX-1 as well as on the toll-like receptor (TLR)-4/MD2-complex and promote production of pro-inflammatory cytokines and chemokines[17-19].

Pro-inflammatory cytokines act on endothelial cells to express vascular cell adhesion molecule-1 (VCAM-1)[20], P- and L-selectin[21] facilitating endothelial transmigration of monocytes and neutrophils from the circulation and into the growing plaque. The notion of lipid accumulation preceding macrophage infiltration is supported by studies on human plaques[22].

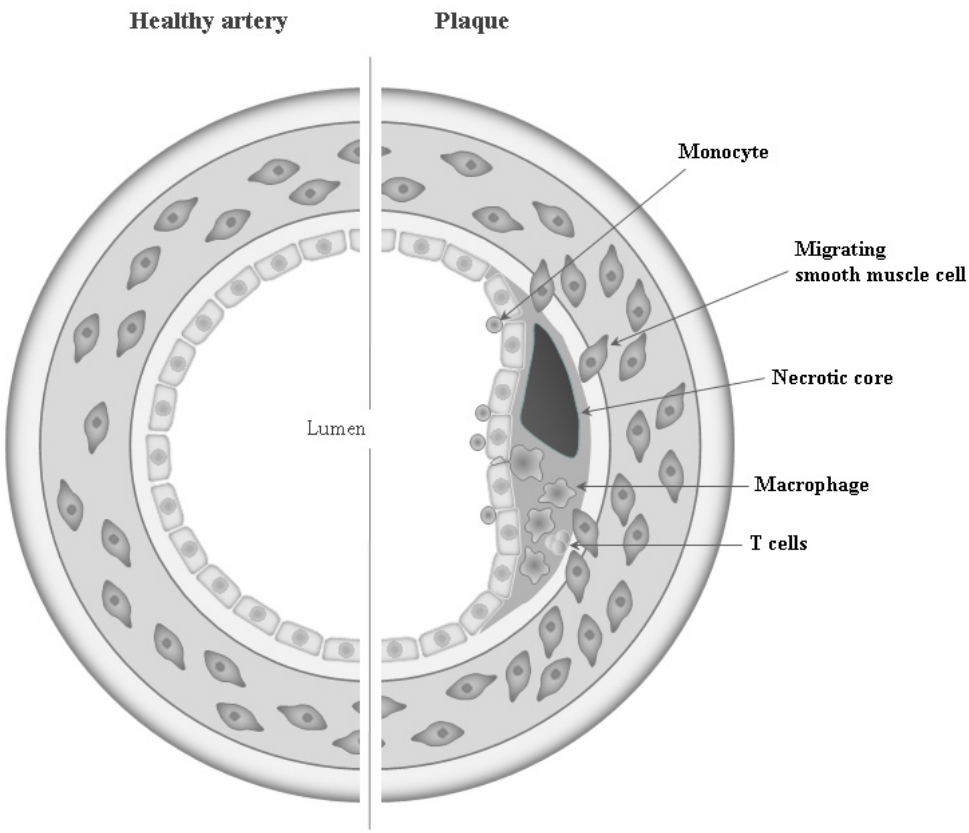

Figure 2. The atherosclerotic plaque (right) contrasted to the healthy artery(left). *(Apapted from K.E Berg, Lyso-PC and macrophage interplay. ISBN 978-91-86671-98-3)*

Plaque progression and rupture

Monocyte-derived macrophages encounter and engulf oxidized LDL attempting to 'clean up' this environmental insult. However, as the intimal LDL accumulation continues, the macrophages turn into lipid-laden foam cells (as previously described by Anitschkow). Necrosis of such foam cells generate additional inflammation and form the necrotic core of atherosclerotic plaques. Reduced ability of removing excess necrotic and apoptotic cells (efferocytosis) and thus resolving the ongoing inflammation has been postulated to be an important factor driving atherosclerotic disease[23].

Matrix-metalloproteinases (MMPs) released from activated macrophages degrade ECM and enable SMC migration from the media to the intima. In the intima,

SMCs produce ECM that forms a cap of collagen which is believed to protect the plaque from rupture. Recently, it has become evident that some plaques are more prone to rupture than others. These "vulnerable plaques" are characterized by thin fibrous caps and a large necrotic core[24].

When (if) the plaque ruptures, thrombogenic plaque material get exposed triggering the formation of a thrombus. Rupture of coronary plaques manifests as myocardial infarction (MI) or unstable angina (UA) while plaques located in the carotid artery are responsible for ischemic stroke. Non-ischemic stroke or hemorrhagic stroke is not caused by plaque rupture but is instead caused by arterial malformations or vessel damage. In our studies presented later in this thesis, we have excluded non-ischemic strokes from the analysis.

Another mechanism of thrombus formation is plaque erosion causing thrombus formation without plaque rupture. Such plaques are rich in proteoglycans and SMCs and do not display the typical superficial lipid pool often seen in ruptured plaques[25]. The mechanism triggering plaque erosions is still unknown, although endothelial dysfunction is believed to be of importance.

Macrovascular complications of diabetes

Diabetes mellitus (DM) can be separated into autoimmune type 1 diabetes mellitus (T1DM) and insulin resistant type 2 diabetes mellitus (T2DM). Both categories of diabetes have severely increased risk of developing cardiovascular disease[26]. Strikingly, patients with diabetes display a similar risk of developing a first cardiovascular event as non-diabetic patients who have had a previous cardiovascular event. As the incidence of T2DM is rising (and projected to increase in the future) this patient category is representing a growing part of all CVD mortality. The mechanism(s) responsible for the increased risk remains to be completely understood although several cardiovascular risk factors are affected in diabetic patients.

One hypothesis explaining the increased risk for CVD in patients with DM is diabetes-associated dyslipidemia[27]. In patients with diabetes (particularly T2DM), triglycerides, VLDL and LDL are elevated while HDL is decreased. Of note, diabetes is associated with increased levels of small-dense LDL which in turn is associated with elevated risk of CVD[28].

The role of hyperglycemia in CVD has been well studied and the subject of much debate. Intensive glucose control in diabetes patients has yielded inconsistent results, casting doubt on the direct role of glucose on diabetic atherosclerosis[29]. However, intensive glucose lowering treatment was associated with increased incidence of acute hypoglycemia. Follow-up studies on patients previously treated

with glucose lowering revealed long-term beneficial effects with regard to reduction of cardiovascular disease[30]. This "metabolic memory" has recently been attributed to hyperglycemic spikes affecting DNA methylation[31, 32].

Insulin is elevated in T2DM, as a compensatory mechanism reacting to persistent hyperglycemia, and insulin levels per se have been associated with CVD independently of hyperglycemia[33]. In mouse models, deletion of the insulin-receptor (IR) in endothelial cells reduces lesion size and endothelial VCAM-1 expression.

Reactive carbonyls – Lipoxidation and glucoxidation

The atherosclerotic plaque is characterized by inflammation, acidic pH[34] and impaired local antioxidant capacity[35]. All these factors contribute in forming an environment that facilitates the generation of reactive carbonyls, which non-enzymatically modify proteins. Peroxidation of polyunsaturated fatty acids (PUFA) generates reactive aldehydes like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These carbonyls react avidly with nucleophilic residues, e.g. primary amines, on ApoB100 and phospholipids and form various structures[36].

In a similar fashion, glucose may react directly with nucleophilic primary amines, through Amadori-rearrangement, to form advanced glycation end-products (AGEs). AGEs are a heterogeneous family of adducts formed mainly on nucleophilic side-chain residues on amino-acids. AGEs can be either smaller short-chain adducts (like Nε-(carboxyethyl) lysine; CEL) or larger more complex adducts (e.g. argpyrimidine). Glycated haemoglobin A1c (HbA1c) is an AGE that is commonly used as a marker of the glycemic load during the last 2-3 months. As the rate of a reaction is proportional to the concentrations of the reactants, patients with diabetes have increased AGE levels in tissues[37].

Methylglyoxal

A particular carbonyl, metylglyoxal (MGO) or pyruvaldehyde (figure 3), is derived from glucose (or glucose metabolites like triose phosphates) or from peroxidation of poly-unsaturated fatty acids (PUFA)[36]. MGO is highly reactive and generates several different adducts such as CEL, argpyrimidine and methylglyoxal-lysine-dimer (MOLD) when reacting with proteins. In healthy tissues, MGO is detoxified by aldol reductase to form acetol or by glyoxalase enzymes to form lactic acids. Detoxification is dependent on the presence of

glutathione (GSH), which is depleted by oxidative stress and is reduced in atherosclerotic plaques[35].

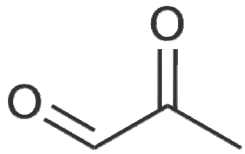

Figure 3. Chemical structure of methylglyoxal

Levels of methylglyoxal are elevated in diabetic patients compared to non-diabetic patients[38]. Patients with diabetes have increased levels of glycation and oxidative damage to ApoB100 compared to non-diabetics[39]. Further on, MGO-modification of LDL generates small-dense-like LDL that binds avidly to arterial proteglycans and accumulates in the intima[40]. Obese rats exhibit elevated levels of methylglyoxal in fat tissue and serum[41].

Immunity

Cells keep high levels of chemical energy, derived from metabolism of energy-rich compounds like sugars and fat, invested in energy-demanding complex biomolecules, like the protein translation machinery, and energy rich molecules like adenosine triphosphate. However, what if you could gain access to all this, without having to do any of the work? This is the aim of pathogens.

Parasites, fungi, viruses and bacteria are microorganisms that share the common feature of “stealing” chemical energy and biological infrastructure from multicellular organisms in order to replicate and spread. As is evident, this is rather problematic for the unwilling “host”.

Vertebrate animals have developed two main ways of dealing with pathogenic microorganisms: innate immunity and adaptive immunity. I will here, briefly, outline innate immunity and thereafter the concept of immunological memory and adaptive immunity relevant for this thesis.

Innate immunity

Innate immunity can be defined as germline encoded microbial defenses which are not dependent on previous exposure. As opposed to the more "modern" evolutionary invention of adaptive immunity, innate immunity is present in virtually all multicellular organisms. The cells of the innate immune system (e.g. monocytes, neutrophils, basophils, mast cells) are the first line of defense acting before the slower adaptive immunity.

Innate immunity can be summarized as a system for pathogen recognition and subsequent response to recognition. Pathogens evolve in order to escape from detection, but it appears as some structures are fundamental for function. Multicellular organisms have developed ways to distinguish these pathogen associated molecular patterns (PAMPs).

The existence of pattern recognition receptors (PRRs) was initially hypothesized by Charles Janeway in 1989[42], to account for the immunostimulatory effects of adjuvants. PRRs are either secreted, endocytic or signaling[43]. Mannan-binding lectin is a secreted PRR that recognizes carbohydrate structures present mainly on bacteria. Endocytic PRRs are transmembrane receptors that bind molecular structures on pathogens and facilitate phagocytosis without inciting a down-stream signaling cascade. The last class of PRRs is cellular receptors, which trigger intra-cellular signaling resulting in transcription of genes involved in anti-microbial activity. I will here focus on one such class of receptors; the Toll-like receptors (TLRs).

Toll-like receptors

Studies using *Drosophila melanogaster* as a model system to obtain genetic mutants with clear phenotypic traits have generated lots of information relevant to human biology. Of particular interest to us, mutation of the *toll* gene in *Drosophila melanogaster* was found to lead to severe fungal infections[44]. Human homologues of toll proteins, termed Toll-like receptors (TLRs), have been well studied and proved to be a key component of innate immunity

Transmembrane TLRs contain two distinct functional domains: extracellular leucine-rich repeats (LRR) recognizing PAMPs and an intracellular Toll/IL-1R (TIR) domain that signals downstream to notify the cell to respond to the threat. Different TLRs recognize different ligands. For example, TLR4 recognize lipopolysaccharide (LPS) and heat-shock proteins, TLR5 recognize flaggelin and TLR9 recognize CpG-containing DNA. After ligation and dimerization, TLRs

signal downstream via adaptor proteins. One such adaptor protein crucial for TLR function is myeloid differentiation protein-88 (MyD88)[45].

MyD88 signaling

MyD88 acts as an adaptor protein to propagate the signals initiated by activation of TLR2, TLR4, TLR5, TLR7, TLR9 and IL-1R. Ultimately, after several intermediary signaling molecules, MyD88 signaling promotes translocation of nuclear factor kappa-B (NF-κB) to the nucleus leading to transcription of pro-inflammatory mediators like TNF and IL-6[45]. Mice deficient in MyD88 do not respond to LPS and are resistant to LPS induced septic chock[46]. However, although MyD88 signaling is crucial for a rapid response against Toll agonists, recent studies of TLR4 signaling have demonstrated a parallel MyD88-independent pathway which is slower and results in transcription of interferon-inducible genes as well as NF-κB activation[47, 48]. Patients with a genetic MyD88-deficiency display recurrent, life-threatening, pyogenic bacterial infections[49]. Interestingly, these patients are otherwise healthy, indicating a redundancy.

Smallpox and the concept of "vaccination"

In the 18th century, small pox (smittkoppor in Swedish) caused by variola major or variola minor virus accounted for ~10% of all deaths[50]. At the time it was known that those who had survived small pox infection were immune to becoming infected again. The reason for this "immunity" towards infection was an object of much speculation and little science.

During the same century, physicians in Europe began inoculating small pox material (derived from a "donor" small pox macules) into the skin of uninfected subjects. This practise, called variolation, became widely used to prevent small pox infections. However, variolation was risky and often caused lethal small pox infections in the patients and made the disease endemic in Europe.

The physician Edward Jenner made a significant contribution to the emerging field of immunology by his studies on individuals infected with cow pox. Jenner observed that some patients were "resistant" to variolation (meaning not presenting with fever-like symptoms after variolation). He concluded that these patients had all previously been infected with the similar disease cow pox.

Jenner (1778) describes such a "resistant" patient:

> *" ..It is remarkable that variolous matter, when the system is disposed to reject it, should excite inflammation on the part to which it is applied more speedily than when it produces the Small Pox"*

Next, he tested whether he could variolate an unexposed patient using cow pox material instead of the usual small pox material otherwise used for variolation. He scratched the skin of a boy with a needle containing cow pox material. Six weeks later, the boy was given small pox by variolation, and like those previously infected with cow pox he did not present with symptoms and was thus immune to infection. Using "variolae vaccinae" cow pox material he could now effectively inhibit infection of this particular pathogen. The term "vaccination" (derived from the Latin word vacca meaning cow) was later coined by Louis Pasteur as an homage to the work of Jenner.

Not until the latter part of the 20th century, have we been given the tools necessary to study the mechanisms of how immunity is maintained and what cell populations are required. I will now discuss adaptive immunity with a focus on T lymphocytes expressing the CD4 antigen, termed T helper cells. In this section I will attempt to describe the events shaping the T-cell response against pathogens and self-proteins.

T cells – Birth and education

T cells are derived from bone-marrow resident hematopoietic stem cells (HSC), through intermediary stages of multipotent progenitor cells. Common lymphoid progenitor (CLP) cells become committed to the T-cell lineage after entry to the thymus[51]. In brief, CLP cells first become double-negative, i.e. not expressing CD4 or CD8. During thymocyte development, the cells first rearrange their T-cell receptor (TCR)-β-chain and then, if the rearrangement was successful, continue to rearrange the TCR-α-chain. This process of rearrangement requires the enzymes recombination-activation gene 1 and 2 (RAG1/2). Mice lacking RAG1 or RAG2 do not have any mature T cells or B cells, as B cells utilize RAG enzymes for B cell receptor (BCR) rearrangement needed for survival and generation of mature B cells[51, 52].

After successful rearrangement, thymocytes start co-expressing CD4 and CD8 that bind to MHC-II and MHC-I, respectively. At this point each thymocyte has its own unique TCR that may or may not recognize self-peptide presented on major histocompatibility complex (pMHC) on antigen presenting cells (APCs). Successful TCR-pMHC interaction promotes thymocyte survival (positive

selection), whereas thymocytes that interact insufficiently with pMHC undergo apoptosis[53]. At this stage, thymocytes commit to expressing either CD4 or CD8.

The last stage of thymocyte education is negative selection in the thymic medulla. Medullary thymic epithelial cells, expressing the gene autoimmune regulator (*aire*), which enables ectopic expression of tissue-restricted proteins, present self-peptides to thymocytes. Thymocytes expressing TCRs that bind self-pMHC with high affinity go into apoptosis. This process of negative selection eliminates thymocytes recognizing self-pMHC-I/II and is thus crucial for preventing autoimmunity[53].

T-cell homeostasis

As mentioned above, high affinity for self-pMHC leads to apoptosis of developing thymocytes. However, during positive selection T cells are selected to exhibit some reactivity against self-pMHC. For T-cell survival, such low-affinity self-pMHC interactions in addition with signals from IL-7 are required for survival[54]. Under lymphopenic conditions (e.g. the Rag1/2-/- or scid/scid-mouse), transferred T cells proliferate extensively and convert from a naïve to a memory phenotype. The exact mechanism for lymphopenia-induced proliferation is unclear, although reduced competition for self-pMHC-II and increased IL-7 is believed to be involved[54, 55].

What is the frequency of naïve T cells responding to a particular pMHC? Studies by Jenkins and coworkers[56] have estimated the precursor frequency for $CD4^+$ T cells to 1:200,000 – 1:2,000,000 and 1:20,000-1:1,300,000 for $CD8^+$ T cells. Calculations have estimated that the average T-cell clone responsive against a particular pMHC exists in 30 copies.

Recent advances have shown that while the naïve T-cell pool is sustained by thymic output in mice, maintenance of the naïve T-cell pool in humans mainly rests on homeostatic proliferation[57].

Initiation of an immune response – Antigen presenting cells

What is the chain of events leading to successful protective immunity? I will discuss four distinct phases: uptake and presentation of antigen by antigen presenting cells (APCs); activation of T cells; production of effector cells and the generation of long-lived memory type T cells. This discussion will be limited to $CD4^+$ T cells, well aware of the crucial role of $CD8^+$ cytotoxic T lymphocytes in immunity.

APCs are a diverse category of cells defined by their capability of priming T-cell responses. The main APC are dendritic cells (DCs). DCs can be categorized as conventional DCs, monocyte-derived DCs, Langerhans cells or plasmacytoid DCs[58]. DCs are located in various tissues where they continuously phagocytize antigens and present it on MHC-II (some DC subsets expressing $CD8\alpha^+$ are efficient in cross-presenting extracellular antigens on MHC-I).

Immature DCs normally express low levels of MHC and co-stimulatory molecules. Upon activation, by microbial or inflammatory stimuli, DCs upregulate MHC and co-stimulatory molecules (e.g. CD80 and CD86) as well as the chemokine receptor CCR7 which facilitates migration from peripheral tissues, via afferent lymphatic vessels, to T-cell zones of lymph nodes. Depending on the stimuli, DCs gain ability to secrete soluble factors affecting T cell priming and homing[59].

Activation of $CD4^+$ T

Naïve $CD4^+$ T cells express surface molecules like CD62L that enables them to circulate through secondary lymphoid organs where they sample pMHCs on APCs. Activated DCs localize to T cell zones of secondary lymphoid organs (SLOs) increasing the possibility of encountering T-cell clones recognizing one of the pMHCs expressed on the DC.

In brief, three signals are needed for T-cell activation[59]. The first signal is derived from stable interaction of the TCR with the pMHC complex. The second signal is the interaction of co-stimulatory molecules on DCs with their ligands on the T cell. Co-stimulatory molecules like CD80 and CD86 are up-regulated after DC activation and stimulate T-cell activation. The third signal is DC-derived cytokines and surface-bound receptors that skew T-cell differentiation.

Pathogens are sensed by DCs using PRRs, which induces production of certain cytokines and cell surface receptors depending on the pathogen. Simplified, intracellular pathogens induce IL-12 production whereas extracellular pathogens (like helminths) promote CCL2 production as well as OX40L-expression on DCs[59-61].

T helper subsets

$CD4^+$ T cells receiving these signals start producing the autocrine mitogen IL-2 as well as the alpha-chain of the IL-2 receptor (CD25). IL-2 signaling induces proliferation and expansion of the activated T-cell clone. Depending on the 'third signal', T cells decide between various lineages. The first two lineages discovered

was the T helper 1 (Th1) and T helper 2 (Th2) lineages. Th1 cells are characterized by IFN-γ-expression and are important for immunity against intracellular pathogens. Th2 cells produce IL-4 and IL-5, IL-10[62] and IL-13 and are crucial for responses against helminths and nematodes as well as for providing co-stimulatory signals for B cells. The transcription factor T-bet promotes Th1 lineage while GATA3 is the master-regulator for Th2 cells. T-bet and GATA3 are mutually exclusive and each inhibits initiation of the other, using DNA methylation and selective expression of cytokine receptors.

However, Th1 and Th2 are not the only lineage options available for $CD4^+$ T cells. Upon encountering IL-6, IL-1β and TGF-β $CD4^+$ T cells develop into T helper 17 cells that produce the signature cytokine IL-17, dependent on the transcription factor RORγt. Th17 cells have been shown to be important in combating Candida albicans[63]. Also, Th17 cells have been implicated in autoimmune diseases like psoriasis and rheumatoid arthritis[64]. This thesis will not discuss follicular helper, Th9 or Th22 cells.

Activated effector T cells express homing molecules that enables recruitment to inflamed tissues where they exert their function. Recently it has been appreciated that DCs may instruct T cells to home to certain tissues. For example, intestinal DCs produce retinoic acid that promotes expression of the integrin α4β7 that bind to MADCAM on intestinal endothelial cells[65]. T helper cell subsets differ in their ability to reach various tissues. For example, Th1 cells are recruited more efficiently than Th2 cells to sites of inflammation (exemplified by peritoneal inflammation) [66]. Th1 cells are characterized by expression of CXCR3 and CCR5 and Th2 cells by expression of CCR3 and CCR4[67, 68]. Chemokine receptors such as CXCR6[69] and CCR5[70] have been shown to increase recruitment of T cells to atherosclerotic plaques.

Th cells need to encounter cognate pMHC-II complexes on tissue macrophages or DCs in order to secrete cytokines[71]. An elegant study by Müller and co-workers demonstrated that IFN-γ may diffuse 80 μm away from the site of production and initiate inducible-nitric oxide synthase (iNOS) expression in bystander cells[72]. As a consequence, a low number of cells may control infection without individually instructing each infected cell.

Regulatory T cells – induced or natural

As mentioned earlier, self-reactive thymocytes are deleted by the process of negative selection. However, this process is not perfect and some autoreactive T-cell clones escape deletion in the thymus and are found in the periphery. In order to deal with auto-reactive T cells and to limit excessive tissue-damaging immune responses against pathogens, the immune system has evolved to include regulatory

T cells (Tregs). Tregs can be categorized as belonging to either of two classes: natural Tregs (nTregs) or induced Tregs (iTregs).

Natural Tregs develop in the thymus from thymocytes with high-affinity for self-antigens but not high enough affinity for them to receive death signals (as is usual for autoreactive T cells). Induced Tregs, conversely, are derived from naïve $CD4^+$ T cells that receive adequate signals (analogous to Th1/2/17). Signals that induce iTreg formation include TCR-stimulation, IL-2 and TGF-β[73]. Moreover, the vitamin A product retinoic acid further augments iTreg generation[74].

The mechanisms of Treg-mediated suppression is contended, but thought to include production of inhibitory cytokines (TGF-β, IL-10 and IL-35) and expression of inhibitory receptors (LAG-3 and CTLA-4)[75]. It is also unclear whether iTregs and nTregs utilize the same means of suppression.

Immunological memory

Levels of antigen-specific $CD4^+$ Th effectors increase after appearance of the pathogen/antigen. After antigen clearance, the 'contraction phase' begins and redundant Th effector cells perish. However, approximately 10% of the Th effector cells specific for the antigen survive as 'memory T cells'[76]. Memory T cells survive without cognate-pMHC signals, thus enabling immunity against antigens which are rarely encountered (like small pox).

$CD4^+$ Memory T cells can be divided into two broad categories: $CD62L^-CCR7^-CD45RO^+$ effector memory (T_{EM}) or $CD62L^+CCR7^+CD45RO^+$ central memory T cells (T_{CM}). T_{EM} express homing receptors that enable migration to sites of inflammation. Upon encountering its cognate pMHC, T_{EM} cells rapidly secrete large amounts of effector cytokines. T_{CM} cells are long-lived cells that express homing receptors that facilitate homing to lymphoid tissues (CD62L/CCR7). As opposed to T_{EM} cells, T_{CM} cells proliferate extensively upon pMHC stimulation to generate cells capable of cytokine production[77]. Thus, a common interpretation is that T_{CM} serve as long-lasting reservoirs of T_{EM} cells. It is still unclear to what extent memory T cells are derived from primary effector cells or from memory-committed cells generating at early stages of an immune response[76].

Recent studies have shown that unimmunized or pre-immune individuals may have T_{EM} cells responding against foreign antigens that has not been previously encountered[78]. The authors demonstrate that the same T-cell clone responding against a particular foreign antigen, a HIV-1 peptide, may also respond against another non-HIV bacterial antigen. Demonstrating the same principle, immunizing against influenza H1N1 resulted in proliferation of T-cell clones responsive for a commensal bacteria.

Humoral immunity – B cells and antibodies

Immunoglobulins or antibodies are crucial for protection against pathogens. Naïve B cells express unique B cell receptors (BCRs), as a consequence of V(D)J-recombination, generating a wide repertoire of potential antibodies. Activation of B cells requires (i) BCR-antigen-ligation, often facilitated by follicular dendritic cells capturing and presenting soluble antigen, and (ii) T-cell help (cytokines and co-stimulatory molecules). Activated B cells migrate to germinal centers and mature to become either memory B cells or antibody secreting plasma cells[79].

As mentioned, B cells require co-stimulatory signals from T helper cells (primarily Th2 and T follicular helper T cells) in order to mature. B cells take up antigen bound to its BCR and present the antigen on MHC-II to receive co-stimulation from T effector cells recognizing the pMHC-II on the B cell. Importantly, as antigens often are large and complex, antibodies against a particular epitope do not imply the necessity of a T-cell response against the same epitope. The odds of a small set of T-cell clones, responding to an epitope on the same antigen as the B cell, encountering the correct B cell would be abysmal unless for the architecture of SLOs with chemokine gradients guiding activated B cells to the boundary of T- and B-zones where activated T cells reside[80].

During maturation, B cells switch the Fc constant region of the immunoglobulin to produce IgA, IgG or IgE, each with particular functions. Moreover, germinal center B cells express the enzyme activation induced deaminase (AID) that introduce random mutations in the genes coding for the BCR. This process called affinity maturation ensures generation of B cells producing antibodies with enhanced affinity for the antigen.

Natural antibodies

Although most antibody responses against pathogens require T cell help, it is recognized that there are also *natural antibodies* that are found in the blood of unexposed individuals. These natural antibodies are mainly IgM and are produced by B-1 and, to a smaller extent, marginal zone B cells[81]. The relationship between B-1 cells and B-2 follicular B cells has been the focus of much debate[82]. The details of B-1 development are still under investigation, but it is known that B-1 cells express a more limited BCR repertoire as compared to B-2 cells[83]. Unlike B-2 cells, which undergo apoptosis when recognizing self, B-1 cells expand upon self-recognition[84].

Natural antibodies function as a first line of defense, before adaptive immunity kicks in, by neutralizing pathogens (e.g. *Streptococcus pneumoniae* and influenza A/B)[84, 85] but also by binding to oxidation specific epitopes (OSEs) present on

oxidized proteins, phosphorylcholine and apoptotic cells. Such antibodies are believed to be beneficial by facilitating removal of danger-associated molecular patterns (DAMPs) [86].

Autoimmunity and atherosclerosis

Autoimmunity refers to deleterious immune responses targeted against self-proteins resulting in tissue damage. Both $CD4^+$ T helper and $CD8^+$ cytotoxic T lymphocytes have been implicated to differentially affect different autoimmune diseases. While some autoimmune diseases are characterized by autoantibodies (e.g. systemic lupus erythematosus) other autoimmune diseases appear to be more dependent on cellular immunity (e.g. multiple sclerosis).

In my previous account of the pathogenesis of atherosclerosis, I did not mention any involvement of autoimmunity. In fact, it is possible to produce a coherent picture of atherosclerotic disease without involving any elements of autoimmunity. After all, it is conceivable that T cells present in atherosclerotic plaques are merely bystanders, recruited as a consequence of local inflammation. I will here present evidence for and against a role for T lymphocytes, in particular $CD4^+$ T lymphocytes, in atherosclerosis.

$CD4^+$ T lymphocytes in human plaques

The role of T cells in atherosclerosis began to be appreciated in the 1980s starting with a series of articles published by Hansson and coworkers. HLA-DR (a MHC-II molecule) was localized to atherosclerotic plaques[87]. Soon after, T cells were found in the atherosclerotic plaque, primarily localized to shoulder regions[88], and shown to exhibit mainly a memory phenotype (expressing $CD45RO^+$). According to recent estimates, $CD3^+$ T-cells represent ~10% of total plaque cells[89]. Spectratyping of coronary plaque material for TCR alpha- and beta-chains showed that patients with unstable plaques had enrichment of certain TCRs, indicating clonal expansion. This pattern was not seen in the control group with stable angina (SA) [90]. In line with these findings, the levels of CD69-expressing T cells (recently activated T cells) were increased in plaques from patients with acute myocardial infarction (AMI) compared to SA[91]. Indicating defective immune-regulation, Tregs are scarce in atherosclerotic plaques and represent only between 0.5- 5% of the T cells depending on the stage of the disease[92].

Anti(n) gen-eller?*

There is solid evidence for accumulation of T cells in mature plaques. However, a key question is whether these T cells recognize antigens presented in the plaque. Given that this statement is true, one asks: which antigen(s) are targeted?

Suggesting a role for modified LDL as an autoantigen, a fraction (15%) of T-cell clones isolated from atherosclerotic plaques proliferate in response to in vitro oxLDL stimulation[93]. Notably, all clones responding to oxLDL stimulation secreted IFN-γ, but only one of the 27 T-cell clones produced IL-4. Moreover, there have been reports of plaque T cells recognizing heat-shock proteins (HSP)[94, 95] or Chlamydia pneumonia[96].

Circulating $CD4^+$ T lymphocytes and CVD

Several studies have investigated whether immune activation of T cells in peripheral blood is associated with CVD. Percentages of IL-17^+ Th17 cells are increased and percentages of Tregs decreased in patients with AMI or unstable angina[97]. Th1 cells are increased in patients with acute coronary syndrome (ACS) [98]. Further on, Th1 and Th1/Th17 cells (defined as IFN-γ and IL-17 co-expression), but not IL-17 alone, were elevated in patients with ACS[99].

Also, in a study investigating the role of T memory subsets and CVD, Ammiratti and coworkers observed that T_{EM} (defined as $CD3^+CD4^+CD45RA^-CD45RO^+CCR7^-$) was increased in patients with SA or ACS compared to age- and sex-matched controls. In the same study, $CD4^+CD45RO^+CCR7^-$HLA-DR^+ activated memory T cells were associated with increased intima-media thickness.

It is important to keep in mind that changes in lymphocyte subsets may be a consequence of the acute event and not the other way around. The study presented later on in this thesis[100] is the first to investigate the role of Th1/Th2 subsets in a prospective cohort. The only other prospective study analyzed another peculiar $CD4^+$ T-cell subset that lacks CD28 ($CD4^+CD28^{null}$). This pro-inflammatory cell population, which produces IFN-γ and has cytolytic activity, was related to cardiovascular disease and event-free survival[101].

Translated as "Antigen-or" implying "is-there-an-antigen?" or "Either-or", referring to the novel written by 19th century Danish existentialist Sören Kierkegaard. The Swedish word "*antingen*" meaning "*either*" and the word *eller* meaning *or*. In his book, Kierkegaard explores themes of choice and responsibility; topics later picked up by french author Albert Camus in his novel "The plague", thus bringing the us back to our current theme of immunology.

Mouse models of atherosclerosis

Studies performed on mouse models of atherosclerosis have given us much insight on the mechanism of hyperlipidemia-induced atherosclerosis in mice. The two most common models for study of atherosclerosis are ApoE- and LDL-receptor (LDLr) deficient mice. Both display severe hyperlipidemia when fed a high-fat diet. In normal mice, ApoE is present in VLDL particles and is able to interact with LDLr on liver cells in order to clear VLDL from the circulation. Consequently, deletion of either LDLr or ApoE on a C57Bl/6 background generates severe hyperlipidemia with increased levels of ApoB-containing lipoprotein particles. Although differences in lipoprotein profiles between the two strains, LDLr-/- and ApoE-/- mice are interchangeably used as models of human atherosclerosis. Bone-marrow transfer (BMT) experiments requires the recipient to be LDLr-/-, since ApoE produced by bone-marrow cells may compensate for the inherent lack of ApoE in ApoE-/- and thus restore hyperlipidemia[102]. However, ApoE-/- mice may be bone-marrow recipients if the donor bone-marrow also is ApoE-/-.

Double-knockout mice have been widely used in order to evaluate the effect of deletion of specific genes on experimental atherosclerosis. Next, I will attempt to summarize important findings regarding the role of Th subsets in experimental atherosclerosis relevant for this thesis.

Th1 and Th2 immunity

Initial studies of the role of lymphocytes in atherosclerotic mice produced conflicting results[103-107], possibly due to the effect of lymphocyte deficiency being clouded by severe hyperlipidemia[108]. Transfer of $CD4^+$ T cells to scid/scid mice (lacking mature T- and B cells) greatly increased atherosclerosis[107] and CD4-depletion reduced plaque burden[103, 105].

Observations in human plaques have indicated a potential causal role of Th1 immunity in atherosclerosis[93]. To test this hypothesis several groups generated hyperlipidemic mice deficient in Th1 related functions. Deletion of IFN-γ[109], the prototypical Th1 cytokine, or the IFN-γR[110] resulted in decreased atherosclerosis. Moreover, injections of IFN-γ proved to be atherogenic. In line with these findings, deletion of the Th1 instructing transcription factor T-bet also reduced atherosclerotic burden[111].

The role of Th2 type immunity is not as clear-cut as for Th1. Studies of IL-4 function have yielded inconsistent results with regard to its role in

atherosclerosis[112, 113]. A study comparing IL-4 knock-outs on both ApoE-/- and LDLr-/- background found no effect of IL-4 deletion on atherosclerotic burden. However, BMT of IL-5-/- bone-marrow increased atherosclerosis compared with transfer of wild-type (WT) bone-marrow. The protective effect of IL-5 was linked to its ability to promote production of natural antibodies recognizing oxidized epitiopes[114].

It remains possible that different aspects of Th2 immunity produce divergent effects on atherosclerosis. For example, the role of Th2 cells in promoting antibody responses may be atheroprotective, while other effects of Th2 cells may be negligible. Deletion of T-bet abrogated Th1 responses but simultaneously increased Th2 cytokines and antibody production. One can conclude that the concomitant increase in Th2 cytokines caused by T-bet deletion was not detrimental regarding the effect on atherosclerosis.

Th17 and Tregs

Th17 cells and their signature cytokine IL-17 (IL-17A) have been studied using several different approaches. IL-17-/- ApoE-/- mice exhibited similar extent of atherosclerosis as IL-17+/+ controls but had less inflammation in the plaque, indicating a pro-inflammatory role of IL-17 in atherosclerosis[115]. In addition, reducing IL-17 levels by adenoviral production of soluble IL-17 receptor A (IL-17RA) reduces atherosclerosis and plaque inflammation[116]. In the same study, injections of recombinant IL-17A promoted adherence of monocytes to the aorta.

On the contrary, deletion of the transcription factor suppressor of cytokine signaling 3 (SOCS3) is related to increased levels of IL-17 and IL-10 resulting in decreased atherosclerosis. When giving anti-IL-17 blocking antibodies to these mice atherosclerosis was increased, suggesting a protective role for IL-17[117]. In the same study, injections of recombinant IL-17 reduced atherosclerosis and VCAM-1 expression.

While the proverbial jury is “still out” in the case of Th17 and IL-17, Tregs have repeatedly been shown to be athero-protective. Transfer of Tregs reduces atherosclerosis while deletion of Tregs (either by injection of anti-CD25 (PC-61) Treg-depleting antibodies[118] or by utilizing the DEREG mice[119], where Tregs express diphteria toxin receptor and are selectively depleted upon diphteria toxin injection) increase atherosclerosis.

Table 1. Mouse models investigating the role of adaptive immunity in atherosclerosis

	Model	Background	Effect on atherosclerosis	Reference
Lymphocyte	Rag2-/-	ApoE-/-	None	Daugherty et al[106]
	SCID	ApoE-/-	Decrease	Zhou et al[107]
	Rag1-/-	LDLr-/-	Decrease	Song et al[104]
CD4	CD4-/-	ApoE-/-	Increase/none	Elhage et al[120]
	CD4 depletion	C57BL/6	Decrease	Emeson et al[103]
	CD4 depletion	C57BL/6	Decrease	Huber et al[105]
	CD4 transfer	SCID-ApoE-/-	Increase	Zhou et al[107]
Th1	IFN-γ -/-	LDLr-/-	Decrease	Buono et al[109]
	IFN-γR-/-	ApoE-/-	Decrease	Gupta et al[110]
	T-bet-/-	LDLr-/-	Decrease	Buono et al[111]
	IFN-γ treatment	ApoE-/-	Increase	Whitman et al[121]
Th2	IL-4-/-	LDLr-/- (BMT)	Decrease	King et al[113]
	IL-4-/-	ApoE-/-	None	King et al[112]
	IL-4-/-	LDLr-/-	None	King et al[112]
	IL-5-/-	LDLr-/- (BMT)	Increase	Binder et al[114]
Th17	IL-17-/-	ApoE-/-	None	Madhur et al[115]
	IL-17 treatment	LDLr-/-	Increase	Taleb et al[117]
	IL-17 blockade	ApoE-/-	Decrease	Smith et al[116]
Treg	Treg transfer	ApoE-/-	Decreased	Ait-Oufella et al[118]
	Treg transfer	ApoE-/-	Decreased	Mor et al[122]
	Treg depletion (anti-CD25)	ApoE-/-	Increase	Ait-Oufella et al[118]
	Treg depletion (DEREG)	LDLr-/-	Increase	Klingenberg et al[119]

Vaccination against atherosclerosis

As outlined above, variolation of cow pox material (vaccination) generated protective adaptive immunity effectively curing small pox. In a similar manner, immunizing rabbits and mice with LDL reduced plaque size[123, 124]. Since then several studies have shown that LDL immunizations reduce atherosclerosis, although a study transferring $CD4^+$ T cells from MDA-LDL-immunized animals aggravated disease[107]. Immunizations against ApoB100-peptides have been shown to affect atherosclerosis in mice[125] and a peptide-based vaccine to treat

atherosclerosis in man is under development. It is apparent that both the type of antigen and adjuvant used influence the effect of immunization on atherosclerosis.

Antibodies against oxidized and native LDL

In autoimmune diseases like SLE and T1DM antibodies against certain antigens can be used to diagnose the patient. However, IgG and IgM antibodies against the putative atherosclerosis-antigens oxLDL and MDA-LDL are ubiquitously present in the population[126]. The levels of such antibodies have been shown to predict disease progression[127], although a recent large scale study[128] has cast doubt on the relationship between anti-ox/MDA-LDL and atherosclerosis.

While the role of anti-ox/MDA-LDL IgG antibodies remains uncertain, IgM antibodies appear to be more or less consistently related to reduced CVD[129-131]. Suggesting that IgM antibodies against oxLDL may be natural antibodies, the levels of anti-oxLDL IgM was associated to levels of plasma IL-5[132]. IL-5 has previously been shown to increase production of B-1 cell-derived natural antibodies in vitro[114].

Not only oxLDL but also native ApoB100 is targeted by antibodies in man. Screening a peptide library of the ApoB100 protein, consisting of 302 amino-acid peptides each 20 amino acids long (with 5 amino-acid overlap on both the amino- and carboxy-terminal end of the ApoB peptide), revealed several peptides targeted by high levels of antibodies (e.g p210, p45)[133]. The same study found that certain MDA-modified ApoB peptides are recognized by antibodies. Both native and MDA-modified ApoB100 peptides (p210 and p45) have been associated with CVD[134]. Injections of a recombinant antibody directed against an MDA-modified ApoB100 peptide were able to induce plaque regression in mice[135].

Antibodies against glycated LDL

Isolated human LDL contains both oxidation and glycation epitopes. As mentioned above, the content of certain AGEs (e.g. CML, CEL) are increased in diabetic patients. Antibodies against such adducts have been observed in both patients with diabetes as well as in non-diabetic CVD patients[136, 137].

The role of antibodies against glycated/AGE-LDL and atherosclerosis has not been studied as extensively as anti-oxLDL antibodies. In a study of T1DM patients, Lopes-Virella and colleagues[138] did not find any relationship between AGE-LDL antibodies and carotid IMT.

As is the case for ox/MDA-LDL, the glycated-LDL or AGE-LDL used in assays to measure antibodies contains a heterogeneous mix of various epitopes depending on the chemicals used (e.g. glucose, fructose-6-phosphate and methylglyoxal), degree of modification as well as the use of different LDL preparations that may be more or less oxidized. Such differences constitute a problem with regard to reproducibility and potential clinical utility of such assays.

Methods discussion

Population studies & Statistics

In this thesis I present three population studies of varying design. In general, one can classify population studies as either prospective or retrospective. Prospective studies can test whether baseline measurements predict incidence of future end-points (development of CVD) whereas retrospective studies test whether measurements differ between groups which are classified by events that have happened in the past (e.g. CVD vs. non-CVD). Case-control studies, which are generally retrospective, enroll patients based on a certain criteria, e.g. presence or absence of CVD. I will briefly describe the population studies analyzed in this thesis.

Malmö Diet and Cancer Study (MDCS)

The Malmö Diet and Cancer Study (MDSC) is a prospective cohort study consisting of 17000 women and 11000 men living in Malmö, Sweden. Within that larger study, 6000 individuals participated in a substudy to investigate the mechanisms of CVD (the cardiovascular arm of the MDSC). Baseline plasma and peripheral blood mononuclear leukocytes (PBMLs) were isolated and stored at -80˚C and -120˚C, respectively. In our studies (papers I and VI), we randomly selected 700 patients (mean age 65) from the cardiovascular arm of the MDCS and analyzed laboratory and immunological parameters. These individuals were followed for 15 years for assessment of CVD.

T2DM cohort (London)

Individuals living in London between 30 and 65 years of age with T2DM but without documented CAD, angina, peripheral artery disease or renal impairment were recruited from secondary care units to participate in this study. Coronary calcium was assessed by computed tomography (CT) at baseline and at follow-up (mean follow-up time 2.5 years) [139]. Plasma was stored at -80˚C before analysis of anti-MGO-ApoB100 autoantibody measurement.

SUMMIT study

The SUrrogate markers for Micro and Macrovascular hard endpoints for Innovative diabetes Tools (SUMMIT) study is a case-control study aimed at developing new markers for micro and macrovascular diseases. The study consisted of T2DM patients with and without CVD in addition to non-diabetics with and without CVD. In our substudy (paper II), we randomly selected ~50 patients from each group and measured T memory subsets in freshly drawn blood.

Statistics

All population studies share the problems of biased sampling and correlation versus causation. Biased sampling referred to the issue of the cohort being distorted and not being representative of the whole population. The problem of causation and correlation is illustrated by the comic by XKCD shown below[†].

A brief philosophical digression. This notion of causal sceptisism can be carried even further. The 18th century philosopher David Hume proved that the same could be said for all observations of causal relationships (A Treatise of Human Nature, Book I, part III, 1739):
"*We can never demonstrate the necessity of a cause to every new existence, or new modification of existence, without shewing (validifying) at the same time the impossibility there is, that any thing can ever begin to exist without some productive principle; and where the latter proposition cannot be prov'd, we must despair of ever being able to prove the former. Now that the latter proposition is utterly incapable of a demonstrative proof we may satisfy ourselves by considering, that as all distinct ideas are separable from each other, and as the ideas of cause and effect are evidently distinct, 'twill be easy for us to conceive any object to be non-existence this moment, and existent the next, without conjoining to it the distinct idea of a cause or productive principle*".
In essence, we cannot establish causal relationships between any phenomena, e.g. *fire-heat* or *beer-hangover*, since we are able to call them to mind separately. And if we can envision them separately, there is no *necessary* connection between the two. This argument can be used against those blindly opposed to all 'correlation studies', thus informing them of the shaky foundation of all inductive knowledge (including data gained from intervention studies). It may also be used as

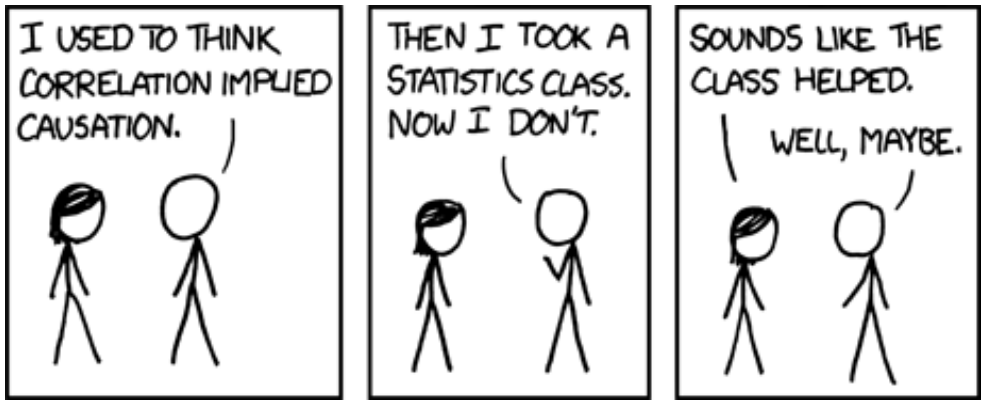

Figure 4. Illustration of the difficulties of correlation versus causation (© xkcd.com)

However, as is obvious to everyone, some correlations do in fact tell us important facts regarding causal relationships. The difficulty lies in figuring out which ones provide us information regarding the disease and which is merely correlation without any causation.

Statistical methods are employed in order to evaluate the statistical validity and magnitude of relations between measurements and end-points. In general, statistical tests generate p-values that answer the question: what is the likelihood of this outcome being a consequence of chance, even if the null hypothesis is true. The null hypothesis can be succinctly stated as: my treatment has no effect. If the p-value is low you may choose to 'reject the null hypothesis' and conclude that it is likely that there in fact is a difference between groups or that your treatment has had an effect. The p-value cut-off for such rejection depends on both scientific convention ($p<0.05$) and the numbers of tests performed. That is, statistically analyzing >20 measurements using a cut-off of $p<0.05$ will generate on average one statistically significant finding, even under circumstances where there is no true relationship. This would constitute a type 1 error or a false positive. On the other hand, due to variation or small sample size one may conclude that A and B are not related (based on the p-value) when in fact they are related. This is called a type 2 error and may be equally distorting as a type 1 error.

In all papers presented in this thesis various statistical methods are used in order to compare means between groups or to correlate variables to one another. Details regarding statistical methods will not be presented here but can be reviewed individually in the methods section of each paper.

Matrigel

The study of antigen specific responses in atherosclerosis has been the focus of much of my work during my PhD, although a small fraction of the efforts have made it into this thesis (paper III). I will here describe the matrigel method as used for the study of antigen-specific responses.

Several different methods have been employed to study antigen-specific responses *in vivo* including subcutaneous (s.c.), intradermal ear[71] or tail injections[140] and air pouches[141].Of particular interests, Corthay and co-workers published a method to monitor anti-tumor T-cell responses *in vivo* using a matrigel system[142].

The matrigel is a commercial product which is derived from Engelbreth-Holm-Swarm tumor cells. It is composed of ECM proteins such as collagen IV, laminin, entactin and perlecan[143]. The matrigel that we have utilized is 'growth factor reduced' and thus contains a reduced amount of TGF-β and other growth factors. At low temperatures (4 ˚C) there is not enough energy to form interactions between proteins and the matrigel appears as a viscous liquid, but at higher temperatures (room temperature/body temperature) protein-protein interactions between the ECM proteins form and the matrigel solution becomes a gel.

In brief, Corthay and co-workers mixed tumor cells with cold matrigel and injected the cell/matrigel mixture subcutaneously. At body temperature, the gel rapidly formed a plug containing tumor cells. Thereafter, they were able to monitor infiltrating T cells and macrophages, which had entered the matrigel plug. Infiltrating cells were isolated by enzymatic digestion of the matrigel followed by flow cytometric analysis of the released cells. Moreover, proteins released from matrigel-localized cells were measured in the supernatant after centrifugation of matrigel digests[144].

I adapted the method developed by Corthay and co-workers[144] in order to study immune responses against protein antigens. In paper III, human ApoB100 was mixed with matrigel and the mixture was injected under the skin of ApoE-/- mice. Matrigels containing either antigen or vehicle was injected to each flank of the mice generating an intrinsic control. After seven days we retrieved the matrigel, digested the matrigel and measured infiltration of cells and release of cytokines.

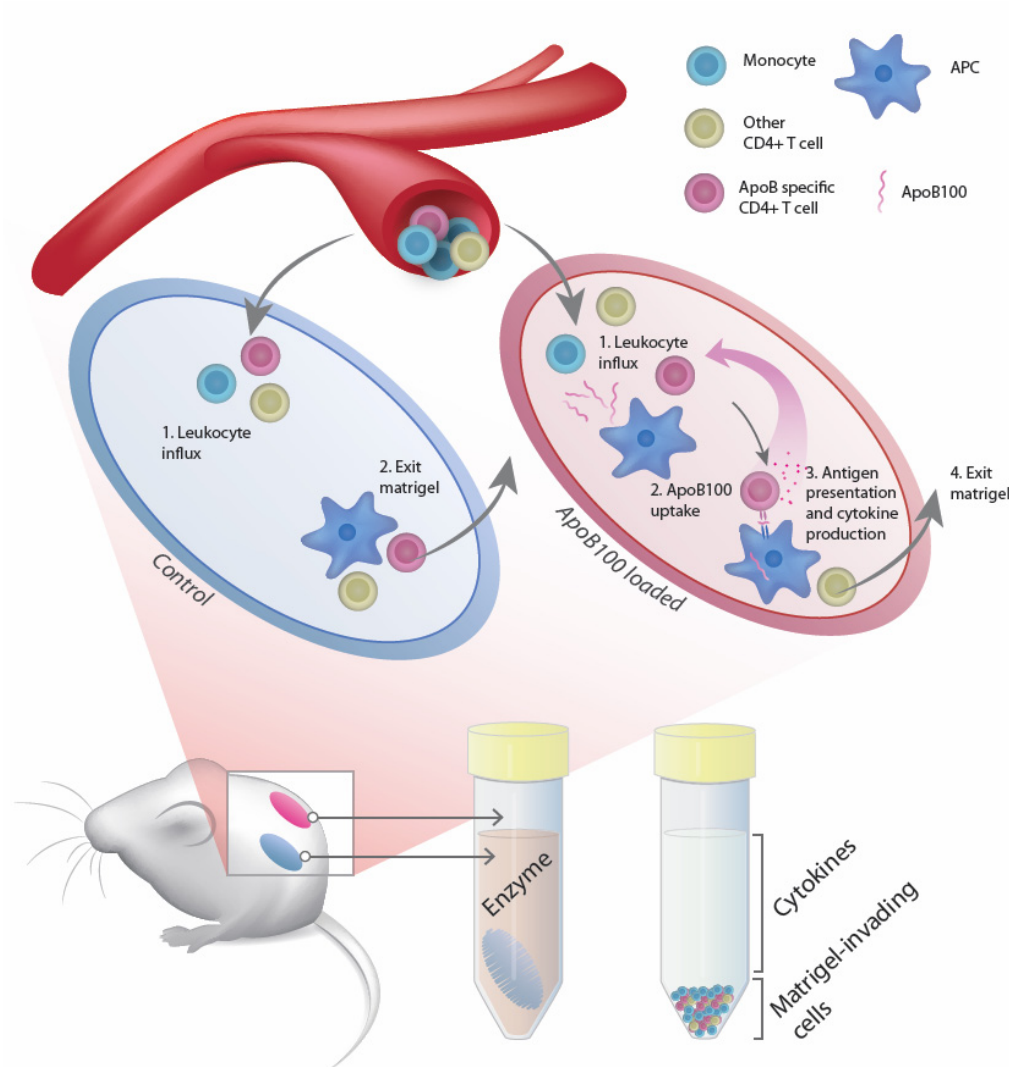

Figure 5. The matrigel model for the study of antigen-specific immune responses *in vivo*.

Here, I will describe my understanding of the cellular events leading to accumulation of cells and cytokines in the matrigel (Figure 5).

First, injection of the matrigel *per se* is an insult triggering influx of mononuclear leukocytes to the matrigel. Monocytes entering the matrigel plug encounter antigen, which they phagocytize and present on MHC-II. T lymphocytes traffic the

matrigel plug, continuously sampling pMHC-II on matrigel-resident APCs. If a T cell recognizes such a pMHC-II it will produce cytokines that will act on local monocytes/macrophages/APCs and endothelial cells to further recruit leukocytes. Moreover, as activated T cells upregulate the alpha-subunit of the IL-2 receptor upon activation, we expected activated effector T cells to be $CD25^+$; differing from the steady-state situation in which $CD25^+$ expression is almost exclusively expressed on Tregs along with FoxP3[145]. At the moment, we are not certain to what degree the accumulation of $CD25^+$ effector cells is influenced by local proliferation of activated T cells or increased retention of T cells.

ELISA – methylglyoxal modified epitopes

During the course of the last four years, we tested several strategies to generate AGE-modified epitopes on various proteins to measure autoantibodies‡ by ELISA. Most methods employed to measure antibodies against AGE-proteins modify the proteins prior to the ELISA procedure. In that case, glucose is incubated together with the protein to induce AGE formation. This approach generates a multitude of different AGE-epitopes on the protein (as described above) and also allows for protein fragmentation due to the lengthy incubation time at 37 ˚C.

We generated a set of AGE-epitopes by modification of the protein with MGO either of proteins in solution (paper V) or on platebound peptides (paper VI). Addition of the modifying agent directly to platebound peptides has previously been used for study autoantibodies against malondialdehyde (MDA)-modified ECM proteins[146].

Some AGE-adducts have fluorescent properties and emit light when excited at certain wavelengths. Argpyrimidine, generated by methyglyoxal-addition to arginine side-chains, is such a fluorescent AGE that emits light at 380 nm after excitement at 320 nm. We verified AGE-modification by means of both fluorescence and monoclonal antibodies against Nε-(carboxyethyl) lysine (CEL). However, although we detected CEL and argpyrimidine in our MGO-modified proteins/peptides, we cannot exclude that antibodies measured in patient plasma recognize other unknown adducts formed by MGO modification.

‡ Antibodies against AGE-proteins are usually termed autoantibodies, however it is perhaps more accurate to designate these antibodies as ‘antibodies against altered self’. However, for the sake of brevity (and consistency), I will continue the use of the term autoantibodies.

Results & Discussion

In this chapter I will briefly outline the results of each paper presented in this thesis. Also, I will attempt to discuss and clarify key findings from each paper. I will not address every finding, but will instead focus on what I believe to be the key aspects.

Antibodies against methylglyoxal-modified ApoB100

As detailed in the background section, IgM antibodies against oxLDL or MDA-LDL have been shown to be related with less CVD. Methylglyoxal (MGO) is a reactive carbonyl, similar to MDA, which is formed from glucoxidation and lipoxidation. Reactions of MGO with proteins generate various AGE-epitopes. In order to study antibody responses against AGE-modified ApoB100, we utilized MGO as the modifying agent. In two studies (paper V and VI) presented in this thesis, we observed that IgM antibodies against MGO-ApoB100 epitopes were associated with less CVD or markers thereof.

In paper V, high levels of MGO-ApoB100 IgM antibodies were associated with reduced coronary calcification score as well as reduced progression of calcification, in a cohort consisting exclusively of T2DM patients without previous CVD (see methods). Coronary calcification, as measured by computed tomography, identifies patients with risk of developing CVD[147]. It is likely that these antibodies are not connected to calcification *per se*, but rather reflect general atherosclerotic burden and cardiovascular risk. This study encouraged us to continue to investigate the role and specificity of such MGO-ApoB100 IgM antibodies.

In order to identify the immunodominant epitopes of MGO-ApoB100, we screened an ApoB100 peptide library for MGO-ApoB100 peptides targeted by antibodies present in plasma. The screening found several MGO-peptides targeted by relatively high levels of both IgM and IgG antibodies. We selected a candidate peptide based on two criteria: 1) at least a 2-fold increased absorbance value compared to a control peptide, and 2) an antibody response against modified/native peptide ratio above 2. Both peptide 211 (p211) and peptide 220

(p220) fulfilled the selection critera. Since p211 shares 5 amino acids with the well-characterized p210 we decided to continue with p220. In paper VI, IgM and IgG antibodies against MGO-p220 were measured in 700 individuals in order to investigate whether these antibodies were associated with incidence of CVD.

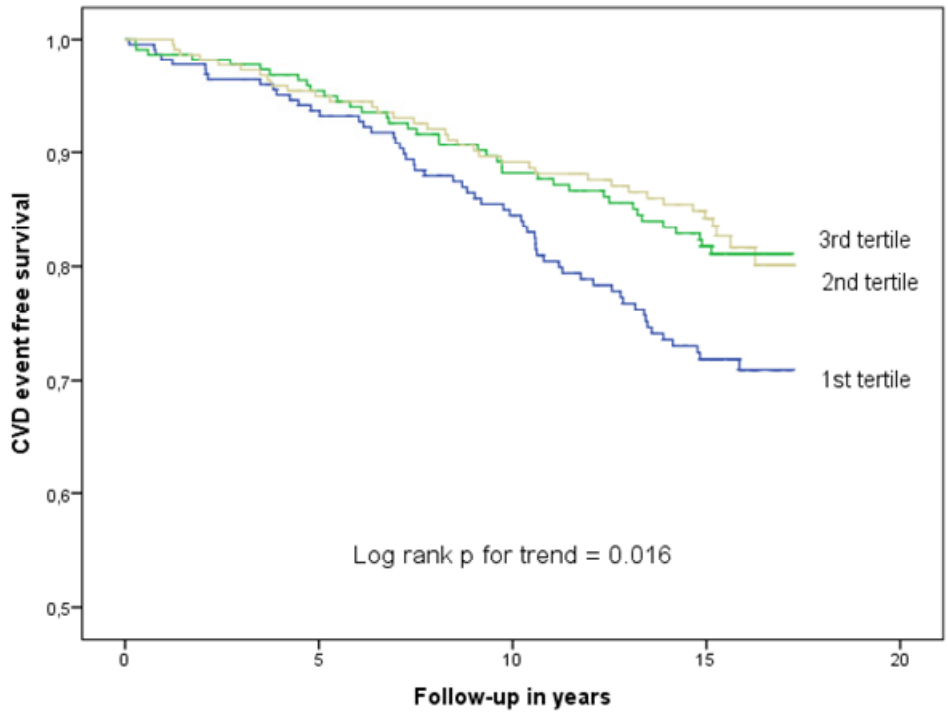

Figure 6. Tertiles of MGO-p220 IgM levels and cardiovascular (CVD) event free survival. Low levels (1st tertile) of MGO-p220 IgM antibodies is associated with increased risk of incident CVD events.

As was found in paper V (but for the entire ApoB100 protein), IgM against MGO-p220 was associated with reduced CVD. In brief, patients in the lowest tertile displayed an increased risk (Hazard ratio 1.8 compared to the top tertile, figure 6 above) of developing CVD during the follow-up. This relationship remained even after adjusting for risk factors such as age, gender, hyperlipidemia and diabetes.

Effect of diabetes on antibody levels

It can be argued that high levels of an antigen should elicit high levels of the corresponding antibody. However, in both cohorts we observed an inverse relationship between anti-MGO-ApoB100 IgM and glucose (paper VI) and

duration of diabetes (paper V). There are at least three possible, mutually non-exclusive, explanations for this inverse relationship.

First, the reduction may be due to increased levels of the antigen, MGO-ApoB100, leading to decreased levels of unbound circulating antibodies present for detection in our ELISA. The second explanation is that glucose exerts a direct inhibitory effect on antibody producing B cells. A third possibility is that the relationship is secondary and due to some common confounder, e.g. that glucose is highly related to another biological parameter (or drug treatment) that in turn is affecting or affected by circulating antibody levels. These studies are not able to settle this issue, but given that AGE-generation is increased in diabetic patients[37], it seems likely that formation of antigen-antibody immune complexes has some part in generating this inverse relationship between glucose and anti-MGO-ApoB IgM antibodies.

Natural antibodies?

As previously mentioned, mammals have naturally occurring IgM antibodies against viruses, bacteria and phosphorylcholine (PC), which arise without the need of T cell help. It has been shown that isolated human and murine B-1 cells produce IgM antibodies that bind to PC[148] and oxLDL[114]. In the study by Binder and coworkers, IL-5, but not IL-4 or IFN-γ promoted B-1 cells to produce such IgM antibodies against oxLDL and PC. Building on this finding, Sämpi and coworkers reported that IgM antibodies against oxLDL and MDA-LDL were related to plasma IL-5 levels.

In our study, we observed that IgM antibodies to MGO-p220 were correlated with several plasma cytokines (Paper VI, Table 4). In particular, anti-MGO-p220 appeared relatively strongly associated with Th2 related cytokines (IL-4 $r=0.174$, IL-5 $r=0.141$, IL-13 $r=0.228$; $p<0.005$ for all) compared to pro-inflammatory cytokines or IL-10. It is difficult to draw conclusions from association studies, but it is an interesting observation that IL-5 is less associated to IgM antibody levels compared to the two other Th2 cytokines measured (IL-4 and IL-13). Further studies are needed to elucidate the origin, natural or adaptive, of these anti-neo-self antibodies.

T-cell subsets and atherosclerosis in man

Th1 and Th2

During the last few decades, more than a few mice have been fed high-fat diet, killed and subsequently analyzed in order for us to gain knowledge about CVD. These studies have provided several hypotheses that ultimately have to be tested in the setting of human atherosclerosis and CVD. As is shown in the background section, there is evidence from mice that Th1 cells are atherogenic. In man, Th1 cells are present in the atherosclerotic plaque and are elevated in the blood in patients presenting with coronary events and unstable angina. On the contrary, there is less evidence pointing to a role of Th2 cells in human atherosclerosis and in animal models of atherosclerosis.

If T-cell subsets are indeed important orchestrators of the disease, then their overall levels may affect incidence of disease. In paper I we measured Th1 ($CD4^+$ IFN-γ^+), Th2 ($CD4^+$IL-4^+) and T-cell related cytokines at baseline in order to investigate whether these cell populations and cytokines could predict incident cardiovascular events during follow-up or correlate with baseline IMT.

Surprisingly, Th1 cells or IFN-γ secreted from stimulated peripheral blood mononuclear leukocytes (PBMLs) were not related to either IMT or incidence cardiovascular disease. On the other hand, Th2 immunity was associated with less CVD. High levels of Th2 cells were associated with reduced IMT in both men and women and reduced incidence of coronary events in women. IL-4 produced by PBMLs was inversely associated with incidence of cardiovascular events regardless of gender.

Mechanisms of Th2 mediated atheroprotection

Confronted with the data presented above, the astute reader will (probably) ask: by what mechanism do total Th2 cells affect CVD and how are Th2 levels regulated?

Th2 cells have in principle two ways of interacting with its environment: production of Th2 cytokines and by direct interaction with other cells via certain co-stimulatory molecules, like CD40L. Th2 cells may exert these effects either in lymphoid organs (e.g. mediating B cell help) or in peripheral tissues (producing Th2-cytokines upon pMHC encounter on tissue APCs). IL-4 has been shown to promote an alternative macrophage activation leading to an “M2” macrophage

phenotype[149]. However, studies of human plaques have found low levels of Th2 cells and Th2 related cytokines. If these studies are representative of human CVD, Th2 cells may instead affect atherosclerosis by promoting protective antibody responses. We observed that Th2 cells were directly related to levels of anti-MGO-p220 IgG (Paper VI, Table 4), but not with IgM. However, these IgG antibodies were not associated with reduced atherosclerosis.

It cannot be excluded that Th2 cells are merely guilty-by-association. Elevated levels of Th2 cells and enhanced IL-4 production in certain individuals may be due to genetic factors, gender and the environment (asthma, allergies, parasite infections etc). Also, increased propensity for Th2 may in turn reduce the formation of Th1 and Th17 cells and indirectly lead to less inflammation and atherosclerosis.

Th1

We can conclude from our study that in patients with already established atherosclerosis, the average participant being ~66 years of age at baseline, total Th1 cell levels are not associated with cardiovascular disease. However, our finding does not exclude that clones of Th1 cells specific for certain plaque-epitopes may affect CVD. Identification of such putative atherosclerosis-antigens in man is needed in order to test whether specific T-cell clones are relevant for the disease. Interestingly, the level of T-cell proliferation against HSP60, a proposed atherosclerosis-related antigen, was related to IMT in young but not in elderly[150]. It is possible that T-cell responses are mainly relevant in earlier stages of the disease. To test this, studies need to be designed to enroll younger individuals where the disease has not yet reached the tipping point where the inflammation becomes self-perpetuating.

Gender issues

Men and women differ with regard to incidence and severity of CVD. Women have a lower age-adjusted CVD death rate compared to men, but a similar rate of death due to stroke[151]. The cause for this difference is still unknown, although several risk factors for CVD are altered in women compared to men, e.g. smoking (reduced) and hypertension (increased).

It is noteworthy, that papers I, V and VI found distinct differences in anti-MGO-ApoB100 antibody levels and levels of Th1 and Th2 cells between men and women. Women had ~30% higher levels of both Th1 and Th2 cell numbers and ~20% higher levels of MGO-ApoB100 IgM. Studies have previously shown

differences in Th cell levels in men and women[152]. In addition, women have higher levels of anti-PC IgM compared to men[153]. It has been postulated that women are "Th2-biased" compared to men since women respond to infections and vaccination with increased antibody production and decreased inflammation compared to men[154]. Combined, it appears as if women in general display a beneficial "immunological profile" that may, at least in part, account for the observed reduced incidence of CVD.

T_{EM} / T_N imbalance in diabetes-associated atherosclerosis

T2DM is, as mentioned in previous chapters, a growing cause of CVD. In paper II we measured subsets of $CD4^+$ memory T cells in a total of 205 patients separated into four groups: T2DM with CVD, T2DM without CVD, non-diabetic with CVD and non-diabetic without CVD. Levels of memory T cell subsets were measured by flow cytometry. The purpose of the study was to test whether CVD in diabetic patients was in part caused by disturbed levels of certain types of memory T cells. We hypothesized that $T_{EM,}$ which secrete cytokines and express homing receptors that promote localization to inflamed tissues, would be associated with CVD.

Strikingly, individuals with both T2DM and CVD were characterized by elevated $CD4^+CD45RO^+CD62L^-$ T_{EM} (Figure 7) and reduced naïve $CD4^+CD45RA^+CD45RO^-CD62L^+$ T cells (T_N). There was no difference in T_{EM} or T_N levels comparing non-diabetics with and without CVD.

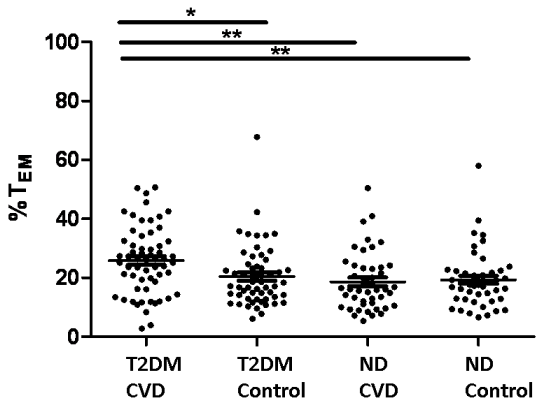

Figure 7. Kruskal-Wallis test with Dunn's multiple comparisons post-test comparing levels of T_{EM} between patient categories (T2DM, type 2 diabetes mellitus; ND, non-diabetic; CVD, cardiovascular disease)

$CD4^+CD45RO^+CD62L^+$ central memory T cells (T_{CM}) were elevated in diabetic compared to non-diabetic individuals, but did not differ between patients with and without CVD. It was recently shown that levels of T effector memory (T_{EM} cells) were associated with IMT and were elevated in patients with CAD and UA[155]. That study contained a small group of patients with T2DM (n=14, 17% of the total study population), and it was concluded that T2DM was associated with increased T_{EM}.

Interpretation

Similar to Paper I where we analyzed Th1/Th2, we compared total percentages and did not take antigen-specificity into account. Most T_{EM} are responsive to viral or bacterial antigens, and it is likely that only a very small percentage will recognize antigens presented in the atherosclerotic plaque. Thus, one can assume that the increase of T_{EM} seen in patients with both T2DM and CVD does not reflect activation of self-reactive clones. So, why do we see this striking difference between the groups? One possible explanation may be derived from the strong inverse association between HDL and T_{EM} in T2DM patients observed in our study ($r=0.323$, $p<0.0001$).

The basic metabolic function of HDL is to promote cholesterol efflux from peripheral tissues. However, HDL has also been found to have anti-inflammatory and anti-oxidative properties with beneficial effects on cardiovascular disease[156], although recent clinical trials of HDL-elevating drugs have shown poor results, potentially due to off-target effects[157].

Of particular interest to the findings in paper II, HDL has been proposed to alter adaptive immunity by affecting lipid raft structures on APCs or T cells[158]. Mice that lack ApoA-I, the main protein constituent of HDL, display lymphadenopathy with increased amounts of proliferating $CD4^{+}CD44^{hi}$ T_{EM}-like cells and concomitant increased atherosclerosis. These mice also display a SLE-like phenotype and produce antibodies against double-stranded DNA, suggesting a reduced threshold for autoimmune responses[159]. Increasing ApoA-I (thereby increasing HDL levels) reduces T_{EM} cells and boosts levels of Tregs[160]. Speculatively, low-HDL levels, as seen in some T2DM patients, may lead to cholesterol accumulation in lymphocytes and APCs. Alterations of lipid rafts, caused by cholesterol accumulation, may reduce the activation threshold and cause increased generation of T_{EM} cells.

Such "hypo-HDL-emia"-induced increases in T_{EM} cells might contribute to the increased risk of CVD seen in diabetic patients (figure 7, above). Future mechanistic studies are needed in order to clarify potential effects of low HDL and hyperactivation of T cells in T2DM patients. However, as the associations between T_{EM} and CVD in T2DM patients were independent of HDL, the sole reason for the interaction of T_{EM} is not linked to HDL-mediated effects.

Another possibility is that T_{EM} cells are elevated and T_N reduced in T2DM patients with CVD as a consequence of 'active' atherosclerosis triggering immune responses. However, as we did not observe any increase in T_{EM} cells comparing non-diabetic patients, this seems to be an insufficient explanation. Although, this study cannot exclude the possibility of T2DM and CVD acting in concert to increase atherosclerosis-specific T_{EM} clones, it seems unlikely that the increase (~5% of all T_{EM}) would reflect proliferation of plaque-antigen specific cells.

Th2 immunity against ApoB100

The previous studies presented in this thesis addressing the link between T cells and CVD did not measure antigen-specificity. This is mainly due to the difficulty of measuring such T-cell responses in man. Using mice it is possible to generate or augment immune responses by immunization. Immunizing against oxLDL and ApoB100 epitopes have yielded divergent results on atherosclerosis depending on

the means of immunization and the antigen being used[124]. However, the most common outcome of LDL immunization has been reduction in atherosclerosis.

Demonstrated in paper I, there is an association between the total levels of Th2 cells and reduced CVD. In paper III, we asked whether a Th2 response directed against human ApoB100 (humApoB) would affect atherosclerosis in mice.

To assess humApoB-specific immune responses *in vivo,* the matrigel model (described in methods) was utilized. Matrigels loaded with humApoB or vehicle were injected into human apoB immunized mice, adjuvant immunized mice or non-immunized mice. Matrigel-localized leukocytes, in particular $CD4^+$ T cell, were analyzed by flow cytometry and cytokines released inside the gel were measured by mesoscale multiplex technology. Subvalvular plaque size was measured to assess the effect of immunization on atherosclerotic disease. A Th2 response was generated by immunizing mice with humApoB mixed with the Th2-promoting adjuvant aluminum hydroxide (Alum). Also, the study compared T-cell responses against humApoB in young pre-atherosclerotic (8 weeks of age) and "old" atherosclerotic mice (18 weeks of age).

Our study demonstrated that (i) recruitment of T cells to humApoB is increased in "atherosclerotic" compared to pre-atherosclerotic mice, (ii) immunization with humApoB in Alum generated an antigen-specific Th2 response located to matrigel containing humApoB and (iii) Th2 immunity against humApoB does not reduce atherosclerosis compared to adjuvant alone.

Th2 cell accumulation in antigen-loaded matrigels

My understanding of how the matrigel system can be used to monitor antigen-specific immune responses has been outlined in methods (Figure 5). Briefly, matrigel-infiltrating monocytes will mature to macrophages/DCs and present antigen (e.g. humApoB) to tissue-circulating T cells. Upon cognate pMHC-TCR interaction, cytokines are released acting on local leukocytes and endothelial cells to promote further recruitment of leukocytes. T cell clones specific for antigen presented in the matrigel will be enriched due to both retention and local proliferation, although the relative contribution of these two mechanisms remains to be addressed. Mice having pre-existing immunity against the antigen that is localized inside the matrigel will thus exhibit increased levels of cells and cytokines in the matrigel compared to mice without pre-existing immunity.

By analyzing matrigels containing humApoB, retrieved after seven days, we were able to identify antigen-specific secretion of Th2 cytokines (IL-4, IL-5 and IL-10), but not the Th1 cytokine IFN-γ (Figure 8, below), as well as increased recruitment of $CD4^{+}CD25^{+}FoxP3$- T effector cells in mice immunized with humApoB in alum. Serum from the mice revealed high titers of antibodies against human LDL, and lower titers against mouse LDL in the humApoB immunized group. The antibody levels against both human and mouse LDL were significantly increased in ApoB-immunized mice compared to adjuvant only or non-immunized mice.

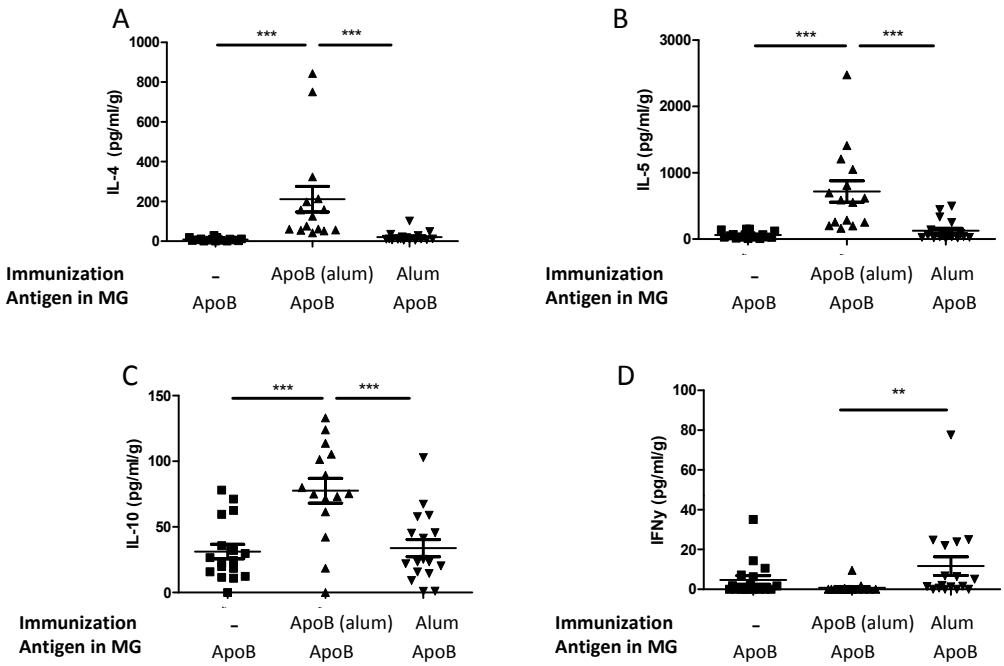

Figure 8. Cytokines measured in matrigel plugs. Comparison of mice immunized with ApoB in alum, alum only or un-immunized mice. Cytokine levels were normalized by matrigel weight.

Although we generated both cellular and humoral Th2 immune responses, we did not observe any effect on atherosclerosis compared to adjuvant control as both immunization with humApoB (in alum) and alum alone reduced plaque size to a similar extent compared to non-immunized mice.

Interpretation

A limitation of this study is that we used humApoB instead of mouse ApoB. The reason for this was that the amounts of ApoB needed for immunization and matrigel-loading was too great to be able to be isolated from mice.

Now, there are in principle two opposite interpretations of the results.

(a) Immunization with human ApoB generates sufficient T-cell cross-reactivity to mouse ApoB, but the Th2 cells reacting against ApoB do not affect atherosclerosis in mice.

(b) Immunization with human ApoB does not generate sufficient T-cell cross-reactivity to mouse ApoB. The role of Th2 cells reacting against ApoB cannot be addressed by this study.

Several lines of evidence support the statement made in (a). First, we observed increased recruitment of Tregs and T effector cells to matrigel-plugs containing humApoB in 18 week old atherosclerotic mice fed a high-fat diet compared to 8 week old chow-fed pre-atherosclerotic mice. These mice were not immunized, so the immunity against ApoB that occurs in these animals should be caused by atherosclerosis and hyperlipidemia. It could be argued that the ApoB-immunity (selective recruitment of T cells to ApoB-containing matrigel plugs) observed in these mice, may be due to leakage of ApoB from the matrigel causing a *de novo* immune response. This effect should then have been similar in young mice which did not exhibit the same increased infiltration of T cells.

On the other hand, arguing against a pre-existing, cross-reactive immune response against ApoB is the fact that hyperlipidemic mice contain more inflammatory $LyC6^{hi}$ monocytes than chow-fed normolipidemic mice[161]. High levels of inflammatory monocytes could augment immune reactions in the matrigel thus increasing influx of T cells.

The homology between mouse and human ApoB100 is ~85%[125]. Assuming, in a simplified manner, that the mutations are evenly distributed across the protein and that peptides loaded on MHC-II are on average 15 amino acids long, the likelihood of a mouse ApoB-derived peptide being identical to the human equivalent is 8.7 % (0.85^{15}). However, all amino-acid substitutions do not equally affect pMHC-TCR interaction. Substitution of a glutamine to glutamic acid, or vice versa, is likely to have a smaller effect compared to a tyrosine-to-glycine substitution. Differences in protein sequence together with the rather limited antibody response against mouse-LDL (paper III, figure 5) supports the opinion expressed in (b), i.e. that immunization against humApoB does not generate sufficient immunity against mouseApoB.

In order to solve this issue and elucidate the role of antigen-specific Th2 cells, we plan to perform these experiments in LDLr-/- mice expressing human ApoB100. Using this approach we circumvent the issues discussed above and determine the role of ApoB-specific Th2 responses in atherosclerosis.

MyD88 in T cells

An approach commonly employed to study the role of a particular entity is to remove the entity from the system and observe the effects of its removal. Knocking out a particular gene in a mouse and comparing the phenotype with a wild-type mouse is an example of this. The difficulty does not lie in assessing the phenotype of the knockout mouse, but rather in interpreting what mechanisms are responsible for the effects.

MyD88 is, as detailed in the background section, an adaptor protein that is required for TLR-signaling leading to activation of downstream transcription factors needed for responses against viruses and bacteria. Apart from TLR-ligands, MyD88 is also needed for IL-1β and IL-18 signaling. Studies of MyD88-/-ApoE-/- knockout mice revealed that MyD88 signaling promotes atherosclerosis[162, 163]. MyD88-/-ApoE-/- mice exhibited reduced plaque size, reduction of plaque macrophages and reduced levels of chemokines and cytokines. Given the dramatic effects on monocytes/macrophages the potential role of MyD88 on other cell populations was not further studied.

In paper IV, we asked whether MyD88 signaling in $CD4^+$ T cells would affect atherosclerosis. The rationale was that MyD88-signaling in T cells had recently been shown to promote autoimmunity in various experimental models[164, 165]. Abrogation of MyD88 signaling in $CD4^+$ T cells decreases the generation of Th17 cells by inhibiting IL-1β signals and inhibits Th17 proliferation by lowering mammalian target of rapamycin (mTOR)[165]. This reduction of Th17 cells and IL-17 has been proposed to account for the reduction in autoimmunity seen in animals given MyD88-deficient T cells, as compared to wild-type T cells.

We transferred isolated ApoE-/-MyD88-/- or ApoE-/-MyD88+/+ CD4+ T cells to lymphopenic ApoE-/-Rag1-/- recipient mice fed high-fat diet and assessed the effect on atherosclerosis. As has been shown before by others, transfer of WT MyD88+/+ CD4+ T cells resulted in increased plaque size. However, transfer of MyD88-deficient $CD4^+$ T cells did not increase atherosclerosis compared to control ApoE-/-Rag1-/- mice (Figure 9, below).

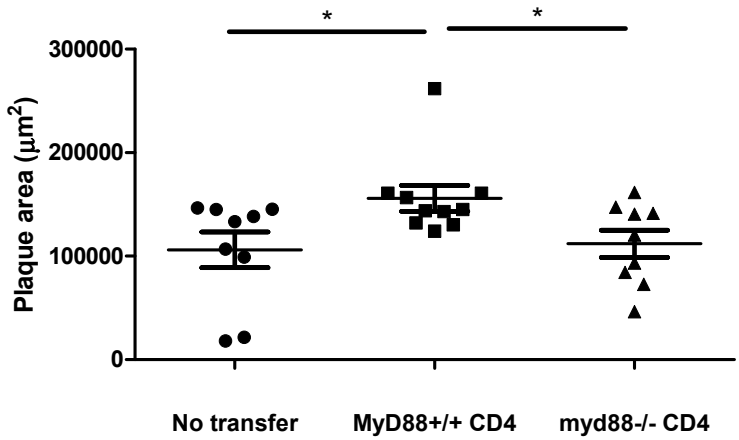

Figure 9. Plaque area comparing ApoE-/-Rag1-/- mice receiving MyD88+/+ or MyD88-/- CD4+ T cells and ApoE-/-Rag1-/- mice without transfer of cells. * = $p<0.05$

The reduction in plaque size was accompanied by reduced percentages of $CD4^+$IL-17^+ Th17 cells and reduced IL-17 production of PMA/ionomycin-stimulated splenocytes. There were no differences in released levels of IFN-γ or IL-10 from stimulated splenocytes.

IL-17 - An atherogenic cytokine?

As mentioned in the background section, the role of IL-17 and Th17 cells in atherosclerosis in debated. Although this study was not originally designed to demonstrate the role of IL-17 in atherosclerosis, it is striking that the decrease in IL-17 production is associated with an abrogation of $CD4^+$ T cell-transfer induced atherosclerosis. One could speculate that IL-17 production is needed for the pro-atherogenic effect $CD4^+$ T cells.

ApoE-/-Rag1-/- mice are lymphopenic and transferred $CD4^+$ T cells will receive strong signals to proliferate. As lymphopenia increases T-cell activation, it is likely that the effect of MyD88-deficiency, and the role of IL-17, is accentuated in this model system. Further studies, in mice and man, are needed to clarify the role of IL-17 and Th17 cells and atherosclerosis.

Upstream of MyD88

An intriguing question is what receptor-ligand interactions are disrupted in MyD88-/- T cells. The main two groups of ligands inciting MyD88 signaling are

TLR-ligands and cytokines. Since our mice are kept in a clean animal facility and did not show any signs of infection it seems unlikely that interactions of PAMPs with TLRs on $CD4^+$ T cells are of importance. Rather, it seems more likely that the lack of IL-1β signaling in MyD88-/- mice is responsible for the phenotype observed in these mice. It has been shown by others that IL-1β promotion of Th17 is dependent on MyD88[166]. Given the dramatic effect observed on IL-17 production and Th17 percentages, it is likely that IL-1β signaling is a key signal that is inactivated in MyD88-deficient T cells. However, we cannot exclude that TLR-ligands present in the atherosclerotic plaque influences T-cell activation that may promote atherosclerosis.

Ceteris Paribus?

In philosophy and law, the Latin term *ceteris paribus,* meaning *other things being equal,* is sometimes used to indicate that besides the indicated alteration, everything else is similar. For example: If I normally disapprove of cruelty to animals, then *ceteris paribus*, witnessing animal cruelty will elicit feelings of disapproval. The *ceteris paribus* condition, in the phrase above, is to eliminate those extraordinary instances where, animal cruelty is somehow justified or under those circumstances where my moral sentiment may have been altered.

All experimental science is inherently prone to errors of generalization. We can only account for the pathways and molecules we have knowledge of and our explanations will thus be limited to our current scientific vocabulary. In order to keep things simple, scientists (and readers of scientific papers) are prone to interpret results as if the *ceteris paribus* condition was fulfilled. Genetic knockout mice are a good example of this. However, there are several risks associated with this.

First, other cells than those we first expected may be affected by the genetic deletion. For example, the recently discovered group of innate lymphoid cells (ILCs) expresses many of the transcription factors previously believed to be, more or less, T-cell specific. Tbet-/- mice, which lack Th1 cells, also lacks certain ILC subpopulations[167]. Moreover, IL-17, IFN-γ, TNF-α and IL-5 may be produced by ILCs[168].

Second, compensatory or secondary effects of the treatment (e.g. knock-outs or vaccination) may cause the phenotype. Deletion of transcription factors controlling T-cell lineage commitment might not only cause deletion of a certain T-cell subset, but also cause increases in other subsets. In LDLr-/-Tbet-/- mice, which lacks Th1 cells, Th2 responses was noted to be increased[111].

In paper IV we demonstrate that MyD88-signaling in T-cells increases atherosclerosis, possibly acting through IL-17. Previous studies showing decreased atherosclerosis in systemically MyD88 deficient mice focused on myeloid cells, and did not address potential effects on adaptive immunity. Adding complexity, MyD88-signaling in DCs was shown to be atheroprotective, through activation of Tregs that reduces infiltration of myeloid-derived inflammatory cells to the plaque[169]. Altogether, these studies do not only highlight the importance of this signaling pathway, but also the utility of cell-specific deletions and the complexity of biological systems.

Conclusions and future perspective

Atherosclerosis is a chronic disease that accounts for most cases of MI and stroke. Pharmaceutical drugs used to treat CVD, like cholesterol-lowering statins, anti-coagulants and blood-pressure lowering medication, are efficient but do not come close to being a cure for the disease. The studies presented here, along with a multitude of other studies performed during the last three decades, point to the adaptive immune system as a target for the next generation of drugs treating CVD. I will here very briefly discuss potential implications of the findings presented in this thesis.

Enhancing the levels of IgM antibodies recognizing oxLDL and MGO-epitopes may be one possible approach for treating CVD. Of interest, a monoclonal anti-IL-5 antibody (mepolizumab) is being evaluated for treatment of patients with severe asthma[170]. Follow-up studies on patients treated with anti-IL-5 antibodies, to investigate effects on production of autoantibodies and incidence of CVD may provide mechanistic proof of IgM antibodies affecting CVD in man.

Canakinumab, a monoclonal antibody that neutralizes IL-β, is being tested in phase 3 trials for efficacy in treating CVD[171]. Our studies on MyD88-deficient T cells suggested a link between IL-1β signaling, Th17 generation and atherosclerosis.It will be interesting to see whether canakinumab treatment will affect CVD and whether this will be associated with decreased levels of Th17. Also, anti-IL-17 antibodies are being evaluated for treatment of psoriasis[172]. Quantification and phenotypical analysis of rare plaque-antigen specific T-cells in blood (and plaque) will answer important questions regarding their relation to CVD. As noted by a recent publication[72], T cells act by producing a cytokine gradient that may affect cells at a distance. It remains an open question how large quantity (or density) of T cells in the plaque is needed to produce “enough” cytokines that will cause deleterious effects on macrophages, SMCs and endothelial cells.

Apart from the fact that immune cells, and proteins released from immune cells, could be potential drug-targets, they may also be useful as biomarkers to predict disease or as surrogate markers of drug-efficacy. Paper I demonstrated that total Th1 and Th2 levels do not efficiently identify patients at risk, although increased levels of Th2 cells in women are related to a reduced incidence of MI. In T2DM patients, T_{EM} cells may prove an important biomarker of CVD. Prospective studies

are needed in order to evaluate the predictive value of T_{EM} cells. Further studies are needed to validate our findings and better characterize which subpopulations (if any) that may better identify high risk patients than the biomarkers currently being used (e.g. cholesterol, blood pressure). It is an interesting possibility that adaptive immune responses may be of importance in some, but not all, patients.

Using the matrigel, we were able to measure a spatially confined antigen-specific T-cell response in mice. We used the matrigel to study responses against the LDL-component ApoB100, but the matrigel may as well be used to study responses against any antigen in other disease models. Moreover, the matrigel-model for the study of antigen-specific T-cell responses might prove useful to determine the efficacy of adjuvants and vaccine formulations. Future work using the matrigel will determine the role of antigen-specific responses in animal models of atherosclerosis.

Populärvetenskaplig sammanfattning

Hjärtkärlsjukdomar är den vanligaste dödsorsaken i världen. Vi har idag en god överblick av vad som orsakar sjukdomens akuta symptom: en blodpropp bildas och täpper till blodflödet till hjärtats kranskärl eller de kärl som försörjer hjärnan med blod. Hjärtat eller hjärnan, beroende på var blodproppen befinner sig, drabbas därefter av akut syrebrist vilket leder till organsvikt och, i vissa fall, död om inte blodproppen avlägsnas. Högt LDL (”skadligt kolesterol”), högt blodtryck, rökning, övervikt och diabetes är alla riskfaktorer som leder till hjärtkärlsjukdom. Diabetes, både barndiabetes (typ 1 diabetes) och ”åldersdiabetes” (typ 2 diabetes) är särskilt starkt kopplat till hjärtkärlsjukdom. Även om dessa riskfaktorer kan förutsäga stor del av insjuknandet så behövs ytterligare förståelse om sjukdomens mekanismer.

Forskningen som presenteras i den här avhandlingen syftar till att förstå hur aterosklerotiska plack uppkommer. Ett aterosklerotiskt plack kan liknas vid en växande sårskorpa, fylld av celler och fetter, som sitter på kärlets insida (likt löv och skräp som till hösten fastnar på insidan av en stupränna). Placket kan till slut växa sig så stort att blodet har problem att ta sig förbi förträngningen. Då uppstår ”kärlkramp” som yttrar sig som bröstsmärtor vid ansträngning. Om plackets hölje (sårskorpa) brister så kommer blodet omedelbart att levra sig, vilket leder till en blodpropp.

Åderförkalkning, eller åderförfettning, kan sammanfattas i tre stadier. I det första stadiet så ansamlas fetter i kärlväggens innersta lager (närmast blodbanan). I det andra stadiet, anländer en klass av vita blodkroppar, makrofager, till placket i syfte att rensa upp de fetter som ansamlats. Tyvärr misslyckas makrofagerna med att avlägsna allt fett, och blir istället fettfyllda ”skumceller” som till slut dör. Fettet som fastnat i placket härsknar (oxiderar) och producerar ytterligare inflammation. Inflammationen leder till att vita blodkroppar från blodbanan samt muskelceller från muskellager som omger kärlet attraheras till placket som nu expanderar i riktning mot kärlets mitt. I det tredje steget så blir påfrestningarna för stora och placket spricker, vilket leder till att en blodpropp bildas.

I den här avhandlingen söker jag svaret på frågan: vilken roll har immunförsvaret i åderförkalkningsprocessen?

T-celler och B-celler är vita blodkroppar som skyddar oss mot angrepp från virus, bakterier och andra inkräktare. Varje T eller B-cell vi har i blodet är unik i sin förmåga att ”känna igen” olika delar av virus och bakterier. Då vi blir infekterade av exempelvis influensa-virus så kommer T och B-celler som ”känner igen” olika delar av influensa-viruset att bli aktiverade. Aktiverade T- och B-celler kommer sedan att hjälpa kroppen att bli av med inkräktaren. T-cellen hjälper till att döda infekterade celler (och därmed förhindra spridning) och får vävnadsceller att vara på sin vakt från nya virusangrepp medan B-celler producerar antikroppar som binder till ytan på inkräktaren och förhindrar därmed viruset spridning.

Förutom dessa välvilliga funktioner så kan vissa T-celler få för sig att kroppens egna vävnader och proteiner är något farligt som ska bekämpas. De kommer då att försöka få bort det som upplevs som hotfullt i en process som kallas för ”auto-immunitet” (immunitet riktad mot sig själv). Som exempel inträffar detta i barndiabetes (typ 1 diabetes) då insulin i bukspottskörteln angrips av T och B-celler. Detta leder till att de celler som producerar insulin dör och patienten får insulin-brist och därmed diabetes.

På liknande sätt tänker vi oss att T-celler ”feltolkar” proteiner som återfinns i placket som hotfulla och försöker rensa bort dem. Många forskare tror idag att denna ”auto-immunitet” förvärrar sjukdomen genom att öka inflammationen i placket.

Aterosklerotiska plack, utopererade från patienter, innehåller rikligt med T-celler. I möss som saknar vissa typer av särskilt inflammatoriska T-celler minskar åderförkalkningen avsevärt. Sådana fynd har lett till intensiv forskning kring olika typer av T-celler och dess inverkan på sjukdomen.

I papper I presenterar jag fynd som visar att höga nivåer i blodet av en viss typ av T-cell, kallad CD4-uttryckande ($CD4^+$) Th2 cell, är kopplat till mindre hjärtkärlsjukdom. Vidare visar vi i papper II att diabetiker med åderförkalkning skiljer sig från icke-diabetiska patienter med åderförkalkning genom förhöjda nivåer av en viss typ av aktiverad $CD4^+$ T-cell (minnes-effektor T-cell). Ytterligare studier behövs för att studera hur dessa T-celler påverkar åderförkalkning i patienter med diabetes och icke-diabetiker.

I två av de andra studierna i avhandlingen har jag använt musmodeller för att studera åderförkalkning. I den ena (papper III) utarbetar jag en modell för att studera T-cells reaktioner mot specifika proteiner. Den andra artikeln (papper IV) studerar inflytandet av ett visst signalerings-protein, MyD88, och dess betydelse för $CD4^+$ T-cellers förmåga att påverka åderförkalkning. Resultaten visar att MyD88-signalering får T-cellen att uttrycka större mängder av viktigt protein, cytokinen IL-17, och att åderförkalkningen minskar avsevärt om man tar bort denna MyD88-signalering i T-celler.

Härnäst mätte jag nivåerna av antikroppar mot en viss struktur som finns på LDL (det ”dåliga” kolesterolet). Strukturen bildas då en molekyl som kallas metylglyoxal (MGO), som bildas av nedbrytning av socker och vissa fetter, fäster sig till protein-delen av LDL-partikeln. Proteindelen heter Apolipoprotein B100 (ApoB100) och den nybildade sammansatta strukturen kallas således MGO-ApoB100. Genom att studera blodprover från sammanlagt ~1300 patienter, så fann jag att antikroppar mot MGO-ApoB100 är kopplade till minskad hjärtkärlskjukdom (papper V och VI). De patienter som hade höga nivåer av sådana antikroppar var avsevärt skyddade mot hjärtinfarkt och stroke. Vi vet idag inte varför antikropparna är skyddande, men en hypotes är att när antikropparna binder till MGO-LDL så främjas utrensning av sådana partiklar från placket.

Sammantaget visar avhandlingen att det adaptiva immunförsvaret, särskilt $CD4^+$ T-celler och antikroppar mot LDL-strukturer, påverkar åderförkalkningsprocessen. Dessa celler och antikroppar är förhoppningsvis möjliga mål för nästa generations läkemedel för att bekämpa hjärtkärlsjukdom.

Acknowledgements

Anyone familiar with experimental science is well aware of the fact that scientific knowledge is not produced by the singular scientist, but rather by groups of scientists. The results produced during the course of my PhD is a product of co-operation, including great many (great) people, some of which I will hereby acknowledge. My sincere apologies to those I will, probably, forget to mention.

First and foremost, I would like to acknowledge the infinite patience (with my many shortcomings and occacional "virrighet"), knowledge and helpfulness of my main supervisor **Eva Bengtsson**. I am especially grateful for Eva allowing and encouraging me to independently decide the topic of my research (trading MDA and ECM for MGO and T_{EM}).

My second supervisor, Professor **Jan Nilsson** has been crucial for this thesis. Jan has a certain skill in formulating the relevant questions and is highly skilled at designing experiments. Without his positive and encouraging attitude, many of the projects presented in this thesis would never have happened.

My third supervisor **Harry Björkbacka** has been involved in almost every aspect of this thesis. If someone would have gotten a cent for each time I asked Harry a question or presented him with an idea for a research project, that person would have earned… quite a lot of cents. Also, thanks to Professor **Gunilla Nordin-Fredrikson** for all help I've received over the years, especially regarding ELISA and the peptide library.

ELISA aficionado, music connoisseur and ornithologist extraordinaire **Ragnar Alm** has helped out a lot with the studies in this thesis (and introduced me to the poetry of John Prine and Townes van Zandt).

Fong To has helped out a great deal with almost every project I have worked since I started four years ago!

My fellow PhD-student lab-rats **Sara**, **Caitriona** and **Polyxeni,** who apart from being lovely people and brilliant scientists will excuse me for not writing anything more here (soon it will be your turn!). **Andreas**, you will write a great thesis this fall.

Thanks to my fellow roommate **Maria** and one-more-cup-of-coffee-**Pontus** for helping out with questions regarding the production of this thesis (not to mention

all the other questions I've asked during the years...). Shoutouts to former PhD students **Daniel** (for all the playlists) and **Katti** (for some of the figures in this thesis).

Also, I am fortunate to have worked with everyone else in the lab: **Isabel, Alex, Irena, Mihaela, Linda, Sinar, Ana, Marie, Helena**. A special thanks to **Ingrid** and **Lena** for isolating aortas from our brave mice. To **Gertud,** for countless lab-days and help with the arrangements surrounding this thesis. To **Anki, Filip** and **Parisa** for helping out with the matrigel project.

Maria Gomez and her research group (**Anna Z, Lisa, Olga, Fabiana, Eliana, Anna**) have been of great assistance, especially during the first two years of my PhD working to characterize the Akita mouse model. Thank you all!

My gratitude to all the mice as well as the the staff working in the animal facility.

I acknowledge the work of **Tommy "M.sc." Garting, Arvid "spelledaren" Wernersson, Filip "art director" Persson, Johan "GW" Lööf, Stefan "rymdsynth" Broselid** and **Anders "leg.läk."Ekström** in making sundays inferior to fridays and saturdays. Thanks to **Ahlmansgatan 17 (Patrik, Emilie, Mathias and Jönsson),** for helping me to find the way to Amarillo.

To my fellow musicians, friends at DMR, VSF and IFK Lund Bordtennis

Sist men inte minst, tack för allt stöd från min familj i Karlskrona och på andra platser! Vem kunde ana det här?

Till **Mikaela**

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Paper I

T-Helper 2 Immunity Is Associated With Reduced Risk of Myocardial Infarction and Stroke

Daniel Engelbertsen, Linda Andersson, Irena Ljungcrantz, Maria Wigren, Bo Hedblad, Jan Nilsson, Harry Björkbacka

Objective—Experimental studies in mice have attributed T-helper (Th) 1 and Th2 cells important roles in atherosclerosis, but the clinical importance of these cells in cardiovascular disease (CVD) remains to be clarified. Here, we investigated associations between Th1 and Th2 cells, carotid intima-media thickness, and cardiovascular risk.

Methods and Results—Blood drawn at baseline and incident cardiovascular events during 15-year follow-up were assessed in 700 participants. Baseline Th1 ($CD3^+CD4^+$interferon-γ^+) and Th2 ($CD3^+CD4^+$interleukin-4^+) cells were analyzed by flow cytometry, and cytokine-release from activated mononuclear leukocytes was measured by multiplex technology. High numbers of Th2 cells were independently associated with decreased mean common carotid intima-media thickness. High numbers of Th2 cells were also independently associated with a reduced risk of acute myocardial infarction in women (hazard ratio, 0.19; 95% confidence interval, 0.06–0.56; P=0.002 for the highest versus the lowest tertile of Th2 cells). Moreover, release of the Th2 cytokine interleukin-4 from activated mononuclear leukocytes was independently associated with a reduced risk of CVD. No independent associations between Th1 cells and carotid intima-media thickness or CVD risk were found.

Conclusion—Our observations provide the first clinical evidence for a protective role of Th2 immunity in CVD. They also suggest this protection is more prominent in women than in men. In spite of convincing evidence from experimental studies, we found no support for a role of Th1 immunity in CVD. **(*Arterioscler Thromb Vasc Biol*. 2013;33:637-644.)**

Key Words: cardiovascular diseases ■ atherosclerosis ■ cytokines ■ T helper cells

Atherosclerosis is a chronic inflammatory disease of large and midsized arteries incited by retention and subsequent oxidation of lipoproteins in the arterial wall and propagated by chronic inflammation.[1] Accumulating evidence indicates that this inflammation is controlled by both innate and adaptive immune responses against modified self-antigens in the vascular wall, such as oxidized low-density lipoprotein (LDL).[2–5] However, it is also possible that loss of tolerance against normal self-antigens, such as apolipoprotein B100, may be involved.[6] Extracellular antigens are presented to $CD4^+$ T-cells by dendritic cells and macrophages. $CD4^+$ T-cells that recognize their specific antigen will develop into T-helper (Th) 1, Th2, Th17, or regulatory T-cells (Tregs), depending on the costimulatory molecules and cytokines expressed by antigen-presenting cells. In general terms, Th1 and Th17 cells are proinflammatory, Th2 cells provide B-cell help and Tregs control and suppress the activity of autoreactive Th cells. In accordance, it is now considered that the mode of $CD4^+$ T-cell differentiation in response to plaque antigen-presentation plays a key role in determining plaque inflammation and disease progression.[3]

There is consistent evidence from animal models that Th1 cells are proatherogenic. Th1 cells are characterized by expression of the potent proinflammatory cytokine interferon-γ (IFN-γ). *Apoe*$^{-/-}$ mice lacking either IFN-γ or IFN-γ receptors develop less atherosclerosis,[7–9] whereas injection of recombinant IFN-γ aggravates the disease.[10] Reduced atherosclerosis is also observed in LDL receptor-deficient mice lacking T-bet, the transcription factor required for differentiation of naive T-cells into Th1 cells.[11] Analysis of cytokine expression in human atherosclerotic plaques suggests a predominance of Th1-promoting cytokines,[12] and T-cells isolated from plaques produce IFN-γ when challenged with oxidized LDL ex vivo.[13] The role of Th2 cells in atherosclerosis appears to be more multifaceted[14–16] than that of Th1 cells. Th2 cells are essential in inducing B-cell antibody isotype switching from IgM to IgG, and are typically characterized by expression of interleukin (IL)-4, IL-5, and IL-13. *Apoe*$^{-/-}$*Il4*$^{-/-}$ mice and LDL receptor–deficient mice transplanted with *Il4*$^{-/-}$ bone marrow develop less atherosclerosis indicating a proatherogenic role of IL-4.[17,18] IL-5, in contrast, has been attributed a protective role because of its ability to stimulate the release of oxidized phospholipid-specific natural antibodies from B1-cells. These antibodies are believed to protect against atherosclerosis by inhibiting the macrophage uptake of oxidized LDL and by facilitating the clearance of apoptotic cells.[19,20] Experimental studies have also attributed an atheroprotective role of certain oxidized LDL-specific IgG.[21,22] The role of Th17 cells in atherosclerosis remains to be fully understood,[23] whereas a body of experimental studies support a protective role of Tregs in atherosclerosis possibly

Received on: September 19, 2012; final version accepted on: December 13, 2012.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.112.300871**

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because of inhibition of autoreactive T-cells and release of antiinflammatory cytokines, such as IL-10 and transforming growth factor-β.[24,25] Although the role of Th1 and Th2 cells in atherosclerosis has been extensively studied in animal models of the disease, there are few studies addressing their potential clinical importance. It has been reported that Th1, but not Th2, cells are elevated in patients with acute coronary syndromes,[26,27] but it remains to be clarified whether this reflects an acute response to myocardial damage or the underlying coronary disease. In the present study, we have determined the levels of Th1 and Th2 cells, identified as IFN-γ– and IL-4–expressing $CD4^+$ cells, in mononuclear leukocytes from 700 subjects who participated in the Malmö Diet and Cancer Study,[28] and studied their association with baseline carotid intima-media thickness (IMT) and incidence of a first cardiovascular event during 15-years follow-up.

Materials and Methods

Study Population

The Malmö Diet and Cancer Study is a prospective cohort study examining the association between diet and cancer. Subjects born between 1926 and 1945, and living in Malmö were eligible for inclusion in the study. Between October 1991 and February 1994, every other participant was also invited to take part in a substudy focusing on cardiovascular risk (Malmö Diet and Cancer Study cardiovascular arm). In the present study, we randomly selected 700 participants, aged 63 to 68 years (mean age 65), from the cardiovascular arm of Malmö Diet and Cancer Study (n=6103). Participants underwent a medical history, physical examination, and laboratory assessment, as previously described in detail (Table 1).[29] The study was approved by the Ethics Committee of the University of Lund and was conducted in accordance with the Helsinki Declaration. All subjects gave written consent.

Isolation of Mononuclear Leukocytes

Blood (15 mL) was collected in heparin tubes and layered on top of 15 mL lymphoprep before centrifugation at 1350*g* for 12 minutes at room temperature. The cell interface layer was carefully harvested, and the cells were then washed twice with 0.9% NaCl. The cells were resuspended in 1.7 mL autologous serum and 1.6 mL cold Roswell Park Memorial Institute (RPMI) 1640 medium with 20% dimethyl sulfoxide added. The cells were frozen slowly by placing them in a Styrofoam box at –80°C overnight. Frozen mononuclear cells were stored at –140°C.

Staining of Mononuclear Leukocytes and Flow Cytometry

The antibodies used in this study were PE/Cy7-anti-CD3, biotin-PerCP-CD56, Pacific Blue-anti-CD4, PE-anti-IFN-γ, APC-anti-IL-4, biotinylated-anti-CD45/streptavidin coupled to CascadeYellow, and Alexa Fluor700-anti-CD8, all from Biolegend. Cell viability of $CD45^+$ leukocytes was assessed with the viability dye 7-aminoactinomycin D (BioLegend). To enable measurement of Th1 and Th2 cells, mononuclear cells (4×10^5 cells/cell culture well) were incubated with phorbol 12-myristate 13-acetate (10 ng), ionomycin (0.2 μg), and brefeldin A (1 μg, all from Sigma) for 4 hours at 37°C. Stained cells were fixed in 1% paraformadehyde and measured on a CyAn ADP flow cytometer (Beckman Coulter). The analysis was performed with FlowJo 7.6 software (Tree Star). Absolute cell numbers in blood were calculated by multiplying percentages of gated lymphocyte populations with counts obtained from a blood cell count analysis, using a Sysmex K-1000 with data unit DA 1000 (TOA Medical Electronics Co).

Table 1. Baseline Clinical Characteristics

	All, n=699	All Cases, n=150	All Noncases, n=549
Age at screening	65.6±1.2	65.6±1.2	65.6±1.1
Sex (% men)[a]	41.5%	53.3%***	38.3%
BMI	26.4	26.5±3.9	26.3±4.0
Current smoker[a]	22.1%	24.8%*	16.0%
Diabetes mellitus[a,b]	13.3%	21.3%***	11.1%
Hypertension[a,c]	81.1%	87.3%*	79.4%
Prevalent CVD events[a]	3.4%	6.7%*	2.6%
Medication[a]			
Antidiabetic	3.4%	7.3%***	2.3%
Lipid–lowering	3.5%	5.6%	2.9%
Blood pressure–lowering	24.9%	35.3%***	20.4%
Laboratory parameters			
Fasting venous blood glucose	5.4±1.5	5.6±1.9**	5.3±1.3
Triglycerides, mmol/L	1.5±0.8	1.5±0.8	1.5±0.8
HDL, mmol/L	1.4±0.4	1.3±0.04*	1.4±0.4
LDL, mmol/L	4.4±1.0	4.3±1.1	4.3±1.0
LDL/HDL ratio	3.5±1.2	3.7±1.4*	3.4±1.1
Systolic BP, mm Hg	151±20	154±19*	150±20
Diastolic BP, mm Hg	88±9,2	90±8.8*	88±9.2
hsCRP, mg/L	3.2±5.5	4.1±6.9	2.9±5.0
White blood cell count, $\times10^9$ cells/L	6.1±1.6	6.1±1.5	6.3±1.6
Lymphocyte count, $\times10^9$ cells/L	1.8±0.6	1.8±0.6	1.8±0.6
Granulocyte count, $\times10^9$ cells/L	3.8±1.3	4.0±1.3*	3.8±1.3
Mixed cell (monocyte rich) count, $\times10^9$ cells/L	0.47±0.23	0.51±0.23*	0.46±0.23

BMI indicates body mass index; BP, blood pressure; CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high-density lipoprotein; and LDL, low-density lipoprotein. *P<0.05, **P<0.01, and ***P<0.005 for case versus control or no event.

[a]Mann–Whitney test or χ^2 test for categorical data.

[b]History of diabetes mellitus, medication or fasting glucose ≥6.1 mmol/L

[c]Blood pressure ≥ 140/90 mm Hg or treatment.

Analysis of Cytokines in Cell Supernatants

Mononuclear cells (4×10^5 cells/cell culture well) were cultured in complete RPMI and stimulated with CD3/CD28 beads (1 bead per 2 cells; Miltenyi Biotec) for 72 hours at 37°C in a cell incubator (5% CO_2). Thereafter, the cell supernatants were stored at –80°C until analysis. The concentrations of released cytokine were determined with a multiplexed immunoassay (MesoScale Discovery).

B-Mode Ultrasound

Analysis of common and bulb carotid IMT was performed using an Acuson 128 CT system with a 7-MHz transducer, as described previously.[28,30] Common carotid artery IMT area was calculated, as described by Wendelhag et al,[29] as the difference between the total area inside the adventitia and the lumen area.

Clinical End Points

We examined the first outcome cardiovascular events. The procedure for ascertaining outcome events has been detailed previously.[31,32] Coronary events were defined as fatal or nonfatal myocardial infarction or death because of ischemic heart disease. Cardiovascular events were defined as coronary events or fatal or nonfatal stroke. Events were identified through linkage of the 10-digit personal identification number of each Swedish citizen with 3 registries: the

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Swedish Hospital Discharge Register, the Swedish Cause of Death Register, and the Stroke in Malmö Register. Ascertainment of cases and validity of the registries used (the Swedish Discharge Registry, the Stroke Register of Malmö, and the Cause of Death Registry of Sweden) have been proven to be high.[33] A cardiovascular disease (CVD) event was defined as a fatal or nonfatal myocardial infarction (ie, *International Classification of Diseases* 9th Revisions (410), fatal or nonfatal ischemic stroke (*International Classification of Diseases* 9th Revisions, 434), or death attributable to underlying CHD (*International Classification of Diseases* 9th Revisions, 412 or 414), whichever came first. Participants were followed from baseline examination until first event of CVD, emigration from Sweden, death, or until December 31, 2008.

Statistics

An independent sample *t* test was used to assess normally distributed continuous variables and a χ^2 test for proportions between cases and controls. Nonparametric test (Mann–Whitney) was used to assess nonnormally distributed continuous variables between cases and controls. Spearman correlation coefficients were used to examine the relationship among continuous variables as appropriate. Linear regression models were used to calculate independent associations. Kaplan–Meier curves were used to illustrate incidence of cardiovascular events in relation to T-cell tertiles, and differences was analyzed by log rank test. Cox proportional hazard regression was used to examine the association between T-cell tertiles and incident cardiovascular events. Plots of the hazard function in different groups over time did not indicate that the proportional-hazards assumption was violated.

Results

To determine viability, we analyzed the uptake of 7-aminoactinomycin D in thawed CD45$^+$ cells by flow cytometry. The results demonstrated that 95% of the cells remained viable. Comparing cell numbers registered at freezing and thawing confirmed that no loss of cells had occurred.[30]

Th1 cells were identified as CD3$^+$CD56$^-$CD4$^+$IFN-γ$^+$ cells and Th2 cells as CD3$^+$CD56$^-$CD4$^+$IL-4$^+$ cells (Figure 1), and presented as percentage of CD4$^+$ T-cells or as cell counts per μL blood. CD56 was used to exclude a subset of T-cells called natural killer T-cells that also can produce substantial amounts of IFN-γ and IL-4. To determine whether Th1 and Th2 cell levels were associated with the functional characteristics of the peripheral blood mononuclear leukocytes (PBML) population, we activated peripheral blood mononuclear leukocytes (PBML) from each subject with CD3/CD28 beads for 72 hours at 37°C and analyzed the release of cytokines into the medium. There were significant associations between both the percentage and absolute number of Th1 cells and the release of proinflammatory cytokines, such as IFN-γ, IL-1β, IL-6, and tumor necrosis factor-α, but also with the release of IL-5 and IL-10 (Table 2). Also, Th2 cells demonstrated associations with the release of both proinflammatory and antiinflammatory cytokines (Table 2), but these were generally weaker than those observed for Th1 cells. Notably, there were no significant associations between the percentage or absolute number of Th2 cells and bead-induced release of IL-4 and IL-5.

Th1 and Th2 cells were significantly higher in women compared with men (93±72 versus 71±64 Th1 cells/μL and 5.4±4.7 versus 4.1±3.8 Th2 cells/μL, respectively; Table 3). In contrast, Th1 and Th2 cells did not differ among individuals with and without diabetes mellitus or hypertension. Neither did smoking, antidiabetic medication, blood pressure (BP)-lowering medication, or lipid-lowering medication significantly alter Th1 and Th2 cell numbers. Bead-induced release of Th1 and Th2 cytokines did not differ among subjects with CVD risk factors,

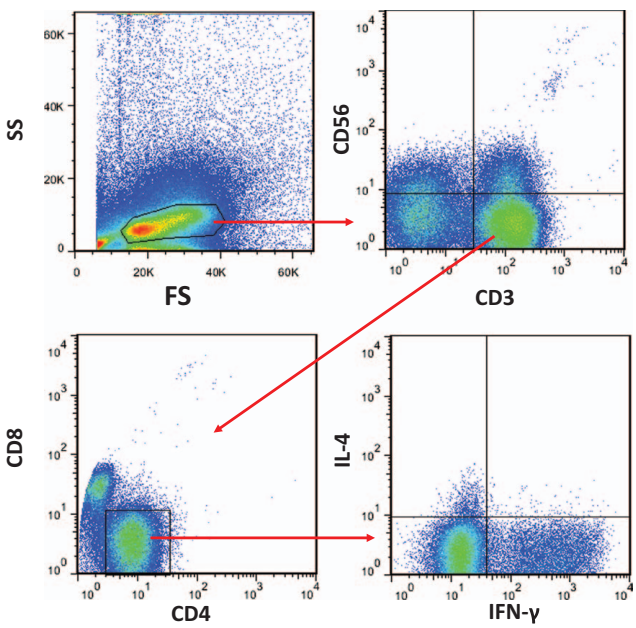

Figure 1. Gating strategy of mononuclear cells. Frozen peripheral blood mononuclear cells were thawed and subsequently stained with fluorescent antibodies. CD56$^-$ CD3$^+$CD4$^+$ T-cells were identified as shown and further divided into IFN-γ$^+$ T-helper (Th) 1 cells and IL-4$^+$ Th2 cells.

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Table 2. Associations Between Th1 Cells, Th2 Cells, and Release of Cytokines From Activated Mononuclear Leukocytes

	Spearman Correlation Coefficients			
	%Th1	Th1 Cell Numbers	%Th2	Th2 Cell Numbers
Th1-related cytokines				
IFN-γ	0.284***	0.338***	−0.015	0.087*
IL-12p70	0.115**	0.105**	−0.041	−0.014
Th2-related cytokines				
IL-4	0.023	0.066	−0.026	0.011
IL-5	0.077*	0.132***	−0.008	0.05
IL-10	0.237***	0.312***	0.087*	0.185***
Proinflammatory cytokines				
IL-6	0.354***	0.320***	0.223***	0.245***
IL-1β	0.366***	0.356***	0.053	0.115**
TNF-α	0.344***	0.363***	0.072	0.150***

TNF indicates tumor necrosis factor. Mononuclear leukocytes were cultured and stimulated with anti-CD3/CD28 beads for 72 hours in 37°C and the concentrations of released cytokines were determined with multiplex technology (see Materials and Methods). The Spearman test was used to calculate *r* values.
*$P<0.05$, **$P<0.01$, and ***$P<0.005$.

except for IL-5 which was significantly higher in smokers and lower in subjects with BP-lowering medication and IL-10 which was lower in subject on antidiabetic medication (Table 3). Th1 and Th2 cells did not appear to be affected by lipid profile, as there were no correlations between Th1 and Th2 cells (percentage and absolute numbers) and cholesterol, LDL, high-density lipoprotein (HDL), and triglycerides, except for a correlation between cholesterol and Th2 cell numbers (Spearman correlation coefficient=0.086; $P=0.03$), which was lost when adjusting for age and sex in a linear regression model.

We next determined the association between Th1 and Th2 cells, respectively, and baseline carotid artery IMT and area. The percentage of $CD4^+$ Th cells displayed significant inverse associations with common carotid mean IMT and area, but the associations were lost when taking age, sex, HDL, LDL, triglycerides, glucose, and systolic BP into account in a linear regression model (Table 4). Th2 cells (both percentage and absolute numbers) demonstrated significant inverse associations with the common carotid mean IMT and area, but not with the maximal IMT in the carotid bulb (Table 4). The inverse associations between both the percentage and total number of Th2 cells, respectively, and common carotid IMT area remained after taking age, sex, HDL, LDL, triglycerides, glucose, and systolic BP into account in a linear regression model ($P<0.05$ for both). Controlling for the same risk factors, only Th2 cell counts were independently and inversely associated with mean IMT in the common carotid artery ($P<0.05$). No association was observed between Th1 cells and mean IMT or area in the common carotid artery. There were no significant associations between bead-activated release of IL-4, IL-5, IL-6, IL-10, IL-12p70, or IFN-γ and carotid IMT or area (data not shown).

During follow-up, 150 subjects had an incident CVD event, of which 84 had a coronary event and 66 had strokes. Subjects who suffered a hemorrhagic stroke were excluded when evaluating risk of CVD events, as these were considered to have a

Table 3. Associations Between Cytokines and T-Helper Cell Subsets and CVD Risk Factors

	Sex		Diabetes Mellitus[a]		Antidiabetic Medication		Hypertension[b]		BP-Lowering Medication		Lipid-Lowering Medication		Smoking	
	Men	Women	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
IL-4, pg/mL	49±90	44±73	46±77	46±100	46±81	33±30	56±99	43±74	47±86	40±55	$1$3$5 78	65±116	42±70	47±73
IL-5, pg/mL	330±910	210±490	260±720	220±420	250±690	360±620	320±940	240±620	270±710	210±620**	250±640	620±1460	200±540	470±1000*
IL-10, pg/mL	620±610	550±530	610±580	500±440	610±570	340±270*	590±550	600±570	580±530	650±670	600±570	530±520	590±550	650±620
IFN-γ, pg/mL	35000±75000	34000±68000	35000±73000	33000±57000	34000±70000	55000±93000	38000±80000	34000±69000	36000±73000	31000±64000	34000±70000	44000±96000	36000±73000	30000±62000
Th1 cells, cells/μL	710±640	930±720***	820±710	910±590	820±700	1200±540	940±900	810±630	850±730	800±570	830±690	970±760	800±660	1000±780
Th2 cells, cells/μL	41±38	54±47 ***	48±43	54±49	48±44	61±53	51±45	48±44	49±44	49±43	49±44	45±32	50±45	48±41

CVD indicates cardiovascular disease; and Th, T-helper.
Values indicate pg/mL for cytokines and cells/μL for T-helper cell subsets (mean±SD). Statistical significance was calculated with a Student *t* test on natural logarithm transformed values.
*$P<0.05$, **$P<0.01$, and ***$P<0.005$.
[a]History of diabetes mellitus, medication or fasting glucose ≥6.1 mmol/L.
[b]Blood pressure ≥ 140/90 mm Hg or treatment.

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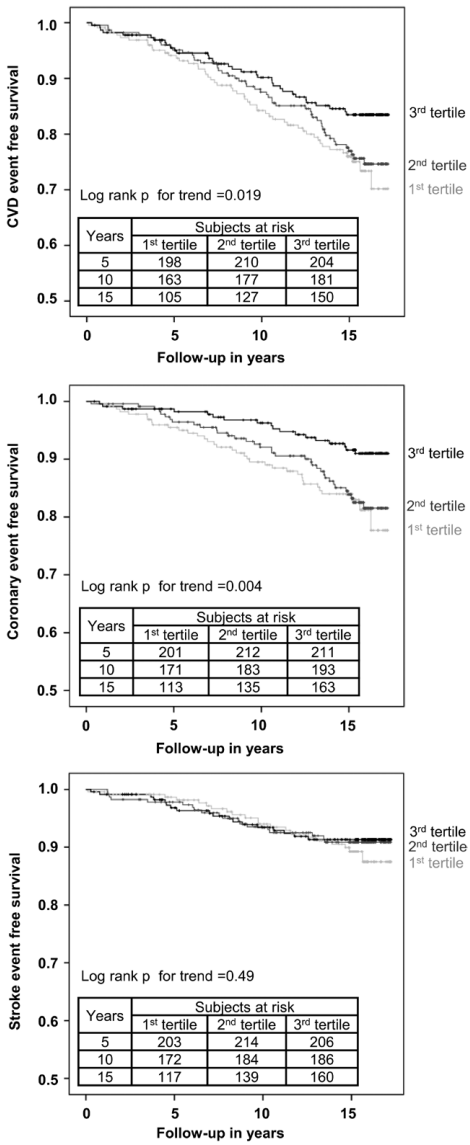

Figure 2. Event-free survival of the number of T-helper (Th) 2 cells in tertiles. Kaplan–Meier survival curves for tertiles of the number of Th2 cell vs event-free survival were analyzed by a log rank (Mantel–Cox) test for trend. Total ischemic cardiovascular events, coronary events, and stroke events are shown in separate graphs. The number of individuals still at risk after 5, 10, and 15 years are shown in each graph.

nonatherosclerotic pathogenesis (11 of 66 strokes were hemorrhagic). There were significant trends of reduced risk of CVD events with increased number of Th1 and Th2 cells (Th1 log rank test *P* for trend=0.025; Th2 log rank test *P* for trend=0.019). There were also significant trends of reduced risk of coronary events with increased number of Th1 and Th2 cells (Th1 log rank test *P* for trend=0.005; Th2 log rank test *P* for trend=0.004), whereas no associations were found with the risk for ischemic stroke. Kaplan–Meier survival curves for Th2 cell tertiles and total CVD, coronary, and stroke events are shown in Figure 2.

The numbers of Th1 and Th2 cells were significantly higher in women compared with men (Table 3). In a general linear model, including sex, incident CVD events, and the interaction between sex and incident CVD events, the interaction was significantly associated with the number of Th2 cells (*P*=0.023), but not with the number of Th1 cells, when adjusting for age, diabetes mellitus, hypertension, smoking, and LDL/HDL ratio. This indicates that the number of Th2 cells is associated with incident CVD events in a sex-specific way. Sex-stratified analyses of the association between the number of Th1 and Th2 cells, and incident coronary and stroke events revealed that women in the highest tertile of Th2 cells had a significantly reduced risk (5.3-fold) compared with women in the lowest tertile of Th2 cells (hazard ratio, 0.19; 95% confidence interval, 0.06–0.56; *P*=0.002), when adjusting for age, hypertension, diabetes mellitus, smoking, and LDL/HDL ratio (Table 5). No significant association was found in men, or between the number of Th1 cells and incident coronary or stroke events.

We next investigated the association between the release of Th1 and Th2 cytokines from CD3/CD28 bead-activated PBML and incident CVD. Only samples with detectable Th1- or Th2-related cytokine release were included in these analyses (n=440 for IL-4, n=373 for IL-5, n=612 for IL-10, n=307 for IL-12p70, and n=598 for IFN-γ). There were no significant differences in CVD events, sex, diabetes mellitus, antidiabetic medication, hypertension, BP-lowering medication, lipid-lowering medication, smoking between subjects with cytokine values below and above the detection limit, indicating that subjects with detectable cytokine levels are representative of the whole population with a similar risk profile.

Using a Cox regression model, adjusting for sex, hypertension, diabetes mellitus, smoking, and the HDL/LDL ratio, we tested whether release of the Th2 cytokine IL-4 was associated with a lower risk of developing

Table 4. Spearman Correlation Coefficients for Associations Between Th1 and Th2 Levels and Carotid IMT

	Spearman Correlation Coefficients		
	IMT (CCA)	IMT (Bulb)	IMT (CCA area)
%CD4 of CD3	−0.083*	−0.018	−0.084*
$CD3^+CD4^+$ cell numbers	−0.029	−0.011	−0.071
%Th2 of CD4	−0.080*	0.011	−0.085*
Th2 cell numbers	−0.090*	−0.014	−0.114**
%Th1 of CD4	−0.021	0.026	0.04
Th1 cell numbers	−0.040	0.055	−0.071

CCA indicates common carotid artery; IMT, intima-media thickness; and Th, T-helper.

IMT of the common carotid artery (CCA) and the carotid bulb, as well as the plaque area of the common carotid artery, was determined and correlated to Th1 and Th2 levels. The Spearman test was used to calculate *r* values. **P*<0.05; and ***P*<0.01.

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Table 5. Hazard Ratios and 95% Confidence Intervals for Incident Coronary Events or Stroke by Tertiles of Th1 and Th2 Cells

	1st Tertile	2nd Tertile	3rd Tertile	*P* for Trend
Th1 cell numbers				
Men	<397 cells/μL	397–785 cells/μL	>785 cells/μL	
Women	<581 cells/μL	581–1046 cells/μL	>1046 cells/μL	
Coronary events				
Men	17	16	15	n.s.
Women	18	11	11	n.s.
Coronary events HR (95% CI)				
Men	1.00	0.87 (0.42–1.79)	0.80 (0.38–1.65)	n.s.
Women	1.00	0.48 (0.20–1.20)	0.45 (0.18–1.10)	n.s.
Stroke events				
Men	13	11	9	n.s.
Women	6	6	13	n.s.
Stroke events HR (95% CI)				
Men	1.00	0.74 (0.30–1.84)	0.70 (0.28–1.76)	n.s.
Women	1.00	0.96 (0.27–3.40)	1.75 (0.59–5.20)	n.s.
Th2 cell numbers				
Men	<20 cells/μL	20–43 cells/μL	>43 cells/μL	
Women	<28 cells/μL	28–57 cells/μL	>57 cells/μL	
Coronary events				
Men	15	18	15	n.s.
Women	19	13	8	0.025
Coronary events HR (95% CI)				
Men	1.00	1.27 (0.60–2.71)	1.15 (0.52–2.54)	n.s.
Women	1.00	0.58 (0.26–1.30)	0.19 (0.06–0.56)*	0.002
Stroke events				
Men	9	13	11	n.s.
Women	10	6	9	n.s.
Stroke events HR (95% CI)				
Men	1.00	1.63 (0.61–4.33)	1.51 (0.54–4.18)	n.s.
Women	1.00	0.52 (0.17–1.62)	0.58 (0.26–2.14)	n.s.

CI indicates confidence interval; and Th, T-helper.

Associations between tertiles of T helper 1 (Th1) of T helper 2 (Th2) cells and incident cardiovascular disease was calculated using Cox proportional hazard regression and adjusting for age, hypertension, diabetes mellitus, smoking, and LDL/HDL. Significant associations between number of events and tertiles of cell numbers were determined with a χ^2 test for linear trend. *P<0.01; n.s., not significant.

cardiovascular events. Dividing into tertiles based on IL-4 release, the second tertile (hazard ratio, 0.456; 95% confidence interval, 0.257–0.809; P=0.007) and the third tertile (hazard ratio, 0.446; 95% confidence interval, 0.251–0.793; P=0.006) had significantly reduced incidence of CVD events compared with the lowest tertile. No associations were found for the release of IL-5, IL-10, IL-12p70, or IFN-γ and CVD events.

Discussion

There is convincing evidence from animal models of atherosclerosis that adaptive immune responses play a critical role in the disease process.[3,34] Mice lacking CD4+ T-cells develop less atherosclerosis,[15,35,36] whereas the disease is accelerated in severe combined immune-deficiency mice reconstituted with CD4+ T-cells.[37] Studies of the role of different CD4+ T-cell subsets have shown that Th1 cells are proatherogenic, Tregs atheroprotective, whereas the role of Th2 cells appears to be more complex with both proatherogenic and atheroprotective properties reported.[3] This has stimulated the development of novel therapies for prevention and treatment of CVD targeting the immune system.[38,39] However, an important limitation in this development has been the poor understanding of the role of the immune system in the development of atherosclerosis and acute cardiovascular events in humans. The present report is the first prospective study of associations between Th1 and Th2 cells with the risk for acute myocardial infarction and stroke. It is also the first large study of associations between Th1 and Th2 cells with carotid IMT in a non-HIV population. The aim of these studies was primarily not to investigate whether Th1 and Th2 cells are useful biomarkers for predicting cardiovascular risk, but to investigate whether immune responses shown to be involved in the development of atherosclerosis in experimental animals are of importance in humans also.

Our findings demonstrate that high levels of Th2 cells are associated with less carotid IMT. However, it should be noted that the relatively small r-values for the correlations between Th subpopulations and IMT explains <1% of IMT. Increased release of the Th2 cytokine IL-4 from activated PBMLs was associated with lower risk for incidence of acute cardiovascular events, and remained significant when controlling for sex, hypertension, diabetes mellitus, smoking, and HDL/LDL ratio. Additionally, there was a significant association between high levels of Th2 cells and a lower risk for incident coronary events in women. It is important to keep in mind that association studies like this one do not provide information about causal relationships. However, in line with experimental studies, our findings suggest that Th2 are involved in the development of CVD in humans also. Moreover, as high levels of Th2 cells were associated with less carotid IMT and a lower risk for cardiovascular events, it appears reasonable to assume that these cells have an atheroprotective role in humans. The protective effect of Th2 cells in experimental studies has been associated with IL-5.[19,20] However, we did not observe any association between the presence of Th2 cells in the PBML preparations and the release of IL-5 from these cell preparations, when stimulated with anti-CD3/CD28 beads. In line with our data, IL-5 deficiency in mice did not change production of IL-4, IL-10, and IFN-γ, indicating that IL-5 may not rely primarily on altered Th1/Th2 balance as a mechanism to facilitate protection in atherosclerosis.[19] It should also be noted that the correlation between Th2 cells and release of Th2 cytokines shown in Table 2 is poor at best, indicating that cytokine release may not be a surrogate measure of the number of Th2 cells.

Interestingly, we found increased numbers of Th1 and Th2 cells in women. It is known that Th cell numbers differ between healthy men and women.[40,41] It seems likely that the increased number of Th1 and Th2 cells found in our study

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reflects the generally increased numbers of Th cells in women. It is possible that the sex difference in CVD risk associated with Th2 cells found in our study can be explained by or is at least related to the fact that women generally have increased numbers of Th cells.

In apparent contrast with the experimental findings in mice, we were unable to demonstrate any associations between high levels of Th1 cells and carotid IMT or increased cardiovascular risk. This is clearly surprising in view of the marked association between the presence of Th1 cells and release of proinflammatory cytokines from activated PBML. However, high levels of Th1 cells were also associated with an increased release of the potentially atheroprotective cytokines IL-5 and IL-10, suggesting that other cells types may have counteracted any proatherogenic effect of Th1 cells. As discussed above, one should be careful to draw mechanistic conclusions based on clinical association studies, but the present study clearly does not provide support for a role of Th1 cells in CVD in humans. It should, however, be kept in mind that studying levels of Th1 cells in the circulation may not reflect their potential role inside the vascular wall and atherosclerotic lesions. Also, our data do not exclude the possibility that specific antigen-experienced effector memory Th1 cells, but not the entire Th1 population defined by their potential to release IFN-γ, are important for atherosclerosis development and clinical events.

It is also possible that to truly determine the role of Th1 cells in human atherosclerosis analysis of antigen-specificity need to be performed. For a $CD4^+$ Th cell to acquire its effector function and produce cytokines in nonlymphoid tissues, antigen recognition is required.[42] Accordingly, an antigen-specific T-cell reactive against any plaque-derived protein, for example, apolipoprotein B100,[6] microbial proteins, or heat-shock proteins,[43] is likely to promote atherosclerosis, if it releases proinflammatory cytokines on encounter with its cognate antigen being presented by dendritic cells and macrophages present in the plaque. Because our study addressed whether the entire population of Th1 or Th2 cells, regardless of antigen specificity, could predict atherosclerosis, it is possible that antigen-specific approaches may yield other results. Of note, this study does not exclude a role for plaque-antigen–reactive Th1 cells, although we could only conclude that the entire pool of Th1 cells does not appear to correlate with carotid IMT or predict cardiovascular risk. Future studies are needed to elucidate the potential role of plaque-antigen–specific T-cell responses and their relationship with CVD. Definite conclusions on the role of antigen-specific T-cells in CVD might only be possible when defined antigens and specific T-cell populations can be studied with tetramer technology. It can also not be excluded that other phenotypic definitions of Th1 cells may have provided a different outcome.

There are, to our best knowledge, no previous studies investigating whether levels of Th1 and Th2 cells can predict cardiovascular events. There are, however, several studies analyzing Th cell subsets in patients with various coronary syndromes. Methe et al observed elevations of Th1 levels in patients with acute coronary syndrome compared with patients with stable angina or unheralded myocardial infarction.[26] Similarly, others have reported that Th1, but not Th2, cells are increased in patients with acute myocardial infarction and unstable angina compared with patients with stable angina.[27,44] Cardiovascular complications are more common in HIV-infected individuals than in uninfected individuals and that antiretroviral therapy, which increases T-cell counts, reduces the cardiovascular risk.[45] We do, however, observe clear differences in risk associated with Th1 and Th2 cells comparing men and women, indicating that increased atherosclerosis cannot be explained by reduced levels of Th cells, independently of Th1 or Th2 subtype.

T-cell subsets have previously been shown to correlate with carotid IMT. Memory Th cells ($CD4^+CD45RO^+$) have been shown to positively correlate with carotid IMT, even after adjustment for traditional risk factors.[46] Particularly, the effector memory Th cell population ($CD4^+CD45RO^+CCR7^-$) correlates strongly to carotid IMT.[47] In contrast, Tregs ($CD4^+FoxP3^+$) do not correlate with carotid IMT.[30,48] Activated Th cells defined as $CD4^+CD69^+$ do not seem to correlate with carotid IMT.[48] However, activated Th cells defined as $CD4^+HLA^-DR^+$ did show a significant correlation to carotid IMT, but the significance was lost when adjusting for risk factors.[47] Our study is the first to show an inverse association between a T-cell subset (Th2 cells) and carotid IMT, even after adjustment for traditional risk factors.

There are some limitations of the present study that need to be considered. Most importantly, our analyses were performed on cells that had been stored at −140°C for several years. As compared with initiating new prospective studies, this has the obvious advantage of allowing studies to be completed within a relatively short period of time. However, it remains to be fully established how well-thawed cells are representative of the original cell population. Although we were unable to detect any loss of cells when comparing cell numbers at freezing and thawing, we cannot exclude the possibility of a selective loss of Th1 and Th2 cells, as these represent only a small fraction of all mononuclear cells.

In conclusion, our observations provide the first clinical evidence for a role of Th2 cells in CVD and are in line with experimental studies, suggesting that shifting adaptive immune responses toward Th2 is protective. However, in contrast with experimental animal data, we found no association between Th1 cells and CVD.

Sources of Funding

This work was supported by grants from the Swedish Research Council, the Swedish Heart–Lung foundation, the Knut and Alice Wallenberg foundation, VINNOVA, Swedish Foundation for Strategic Research, the Albert Påhlsson foundation, Skåne University Hospital foundation, and CardioVax and Söderberg foundation.

Disclosures

Jan Nilsson is signed as coinventor on patents describing the use of immune-modulatory therapy for atherosclerosis.

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Paper II

Evidence for a role of memory T cells in cardiovascular complications of diabetes

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Abstract

Introduction

Type 2 diabetes (T2DM) is associated with an elevated risk for cardiovascular disease (CVD). The mechanisms through which diabetes contributes to CVD development remains incompletely understood but have been proposed to involve increased inflammation. T-cell immunity has been shown to influence atherosclerosis and plaque inflammation in experimental models and has been linked with CVD in man. In this study we tested whether naïve and memory T cells are associated with CVD in diabetic patients enrolled in the SUMMIT study.

Methods & results

The SUMMIT study is a retrospective case-control study comparing 4 groups of patients: T2DM with CVD, T2DM without CVD, non-diabetics with CVD and non-diabetics without CVD. Blood samples were analyzed by flow cytometry and proportions of memory T cells determined. T effector memory cells (T_{EM}; $CD4^+CD45RO^+CD62L^-$, $p<0.001$) and central memory T cells (T_{CM}; $CD4^+CD45RO^+CD62L^+$, $p= 0.02$) were elevated in patients with diabetes compared to controls, whereas naïve T cells were reduced (T_N; $CD4^+CD45RO^-CD62L^+$, $p<0.001$). These differences between diabetics and non-diabetics were significant also when taking into account an interaction between diabetes and CVD in a 2-way ANOVA analysis. Notably, there was a significant interaction between diabetes and CVD for T_{EM} ($p^{interaction}=0.036$) and T_N ($p^{interaction}=0.029$) where the highest proportions of T_{EM} and the lowest proportions of T_N was observed in the group of T2DM patients with CVD.

Conclusions

Our studies identify T_{EM} as a novel cellular biomarker for CVD in diabetes. This suggests that a state of exacerbated immune activation with reduced naïve CD4+ T cells and elevated effector memory T cells may contribute to the increased risk of CVD in diabetes.

Introduction

Type 2 diabetes mellitus (T2DM) is associated with increased risk for cardiovascular disease (CVD) and cardiovascular mortality. The mechanisms responsible for this increased risk remains to be fully understood, although T2DM is associated with an atherogenic lipoprotein profile, platelet and endothelial dysfunction and increased low-grade inflammation[1]. Inflammation has been linked to CVD and atherosclerosis, and novel drugs targeting inflammation for the treatment of CVD are being evaluated in clinical trials[2].

The adaptive immune system has been shown to affect experimental atherosclerosis and it is hypothesized that autoimmune responses against plaque antigens (e.g. heat-shock proteins and apolipoprotein B100) are of importance[3, 4]. After activation, CD4 expressing T helper cells undergo differentiation into short-lived effector cells and memory cells.[5, 6] Several markers have been used to differentiate the different cellular components of T helper memory cells. $CD4^+CD45RO^+CD62L^+$ central memory T cells (T_{CM}) express markers which promote trafficking through lymphoid organs.[7] T_{CM} produce mainly IL-2 and proliferate extensively upon restimulation. $CD4^+CD45RO^+CD62L^-$ T effector cells (T_{EM}) do not express CD62L or CCR7 (CD197) and home to non-lymphoid tissue. T_{EM} are less long-lived, produce more cytokines and has less proliferative capacity as compared to T_{CM}. [5, 6] Recently, Ammirati et al reported that levels of T_{EM} were associated with carotid intima-media thickness as well as with myocardial infarction and chronic stable angina[8]. Naïve cells (T_N) that have not encountered their cognate antigen are characterized by surface expression of CD62L and absence of the memory associated CD45RO isoform. Instead naïve cells express the larger CD45RA isoform.

Here, we investigated the potential role of T memory cell fractions in diabetes-associated CVD in the SUMMIT cohort comparing patients with and without T2DM and CVD.

Methods

Study population

The study population consisted of individuals recruited in Malmö to be part of the SUMMIT (SUrrogate markers for vascular Micro- and Macrovascular hard endpoints for Innovative diabetes Tools) study cohort. The flow cytometry sub-study cohort included 62 diabetics without CVD, 60 diabetics with CVD, 47 non-diabetics with CVD and 46 non-diabetics without CVD (healthy) individuals. The participants had their anthropometric measures recorded and family medical history, drug usage, smoking and physical activity measures were obtained from questionnaires or registers. Blood samples were analyzed for laboratory parameters including HbA1c, serum creatinine, total cholesterol, LDL and HDL cholesterol, triglycerides and fasting glucose. The study was approved by the Ethics Committee of the University of Lund and was conducted in accordance with the Helsinki Declaration. All subjects gave written consent.

Disease definitions

Individuals in the diabetics with CVD group included those with type 2 diabetes or with non-diabetic hyperglycaemia who have had a major CVD event at any time from 5 years before the diagnosis of non-diabetic hyperglycaemia on wards or at any time from 10 years before the diagnosis of diabetes onwards. Non-diabetic hyperglycaemia included impaired glucose tolerance and impaired fasting glycaemia and was defined by any the following criteria: fasting glucose $\geq$ 6.1 mmol/L and < 7.0 mmol/L (impaired fasting glycaemia), 2 hour post load glucose $\geq$ 7.8 mmol/L and < 11.1 mmol/L (impaired glucose tolerance) or HbA1c $\geq$ 5.7% and no diagnosis of diabetes. Diabetics without CVD included individuals with non-diabetic hyperglycaemia or diabetes who have never had an event despite at least 5 years of either non-diabetic hyperglycaemia or diabetes.

Individuals with major CVD included individuals that had acute coronary heart disease (CHD) or ischaemic stroke. Acute coronary heart disease was defined by any of the following criteria: definite or possible fatal or non-fatal myocardial infarction (ICD9-code 410 ICD 10 code I21, I22), hospitalized unstable angina, resuscitated cardiac arrest not attributed to a non-CHD cause, acute CHD death, coronary artery bypass graft, or any other coronary re-vascularization procedure. Ischaemic stroke included fatal or non-fatal ischaemic stoke and was defined as rapidly developed clinical signs of focal or global disturbance of cerebral function lasting more than 24 hours (unless interrupted by surgery or death), with no apparent cause other than a vascular origin, including patients presenting clinical signs and symptoms suggestive of cerebral ischaemic necrosis. Imaging confirmed strokes without clinical signs were not included. Subarachnoid haemorrhage, stroke known to be due to intracerebral haemorrhage or transient cerebral ischaemia (TIA) i.e. focal deficits lasting < 24 hours without imaging confirmation of a stroke were not included. Stroke events in cases of blood disease (e.g.

leukemia, polycythaemia vera), brain tumour or brain metastases were not included. Secondary stroke caused by trauma or prior carotid artery surgery for atheromatous occlusion was not included.

Data sources of CVD phenotypic status included the Swedish Hospital Discharge Register, the Malmö Myocardial Register, the Stroke register of Malmö (STROMA) and the Cause of Death Registry of Sweden. International Statistical Classification of Diseases (ICD) codes used to define major CVD included: non-fatal CHD myocardial infarction (ICD-9: 410, ICD-10 I21, I22), non-fatal unstable angina (ICD-9: 411, ICD-10: I20), CHD deaths (ICD-9: 410-414, ICD-10: I20-I25), stroke cases (ICD-9: 433-434, ICD-10: I63-I64). Control individuals without CVD had the following codes excluded: acute CHD/ischaemic heart disease (ICD-9: 410-414, ICD-10: I20- I25, sudden death (ICD-9: 798, ICD-10: R96, R98). stroke/cerebrovascular disease (ICD-9: 430-438, ICD-10: I60-I69).

Blood collection and flow cytometry

Blood (1 mL) was collected in heparin tubes (BD) and T cells were enriched with RosetteSep Human T Cell Enrichment Cocktail (StemCell Technologies) following the manufacturer's protocol. Enriched T cells were incubated with anti-CD45RA (FITC), anti-CD45RO (PE), anti-CD44 (PerCP), anti-CD8 (PE-Cy7), anti-CD4 (PB), anti-CD197/CCR7 (APC) and anti-CD62L (APC-Cy7) for 30 minutes on ice (all antibodies were from BioLegend). Stained cells were washed twice with FACS buffer (0.5% w/v BSA, 2 mM EDTA in PBS pH 7.2) and 1% Fix/Lyse (BD) was added to preserve the cells until analysis on a CyAn ADP flow cytometer (Beckman Coulter). Data analysis was performed with FlowJo 7.6 software (Tree Star). CompBeads (BD) were used to correct for fluorescence spillover in multicolor analyses and gate boundaries were set by fluorescence-minus-one (FMO) controls.

Statistics

Differences between groups were tested using one-way ANOVA with Bonferroni post-tests, Mann-Whitney U or χ^2-tests, as indicated. Two-way ANOVA was used to test interactions between terms. Correlations between parameters were analyzed using Spearman's rho. $p<0.05$ was considered statistically significant.

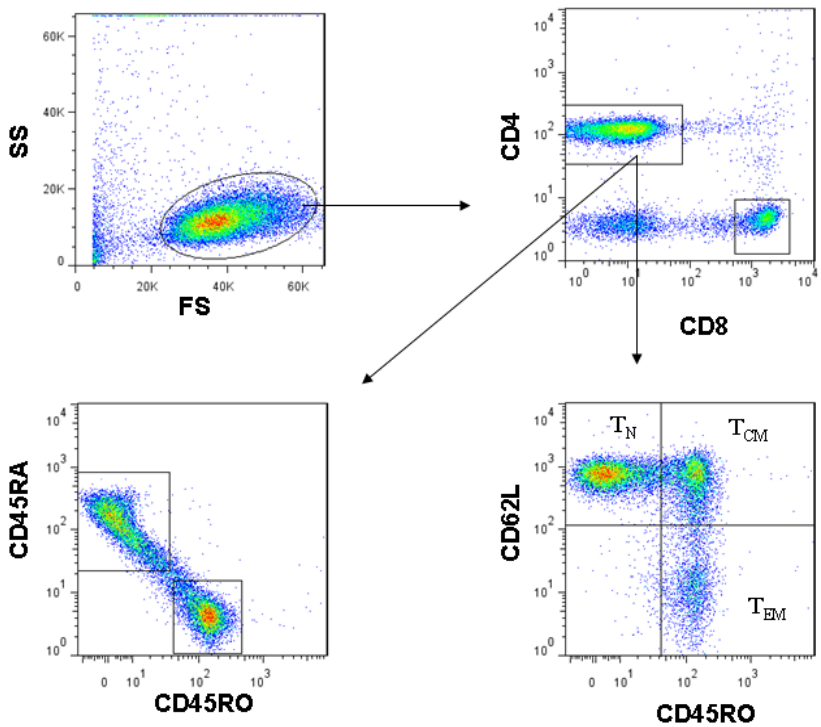

Figure 1. Gating of memory T cell populations. Naïve T cells (TN; $CD4^+CD45RO^-CD62L^+$), central memory T cells (TCM; $CD4^+CD45RO^+CD62L^-$) and T effector memory cells (TEM; $CD4^+CD45RO^+CD62L^-$) were gated from T cell enriched blood (see methods).

Results

Patient characteristics are shown in table 1. Blood was drawn from patients and enriched for T cells before $CD4^+$ T-cell subsets were analyzed by flow cytometry. The gating strategy used for the analysis is depicted in figure 1. Studies on $CD4^+$ memory T cells differ in their use of either CD62L or CD187/CCR7, both of which mark cells that home to lymphoid organs. In our study, the proportion of the T cell subsets gated on CD62L expression correlated strongly to the T cell subsets obtained by gating on CD197/CCR7 expression instead of CD62L (Spearman correlation coefficients: T_N r=0.965, p<0.0001; T_{CM} r=0.859 p<0.0001; T_{EM} r=0.918 p<0.0001).

First, we compared levels of antigen-experienced $CD4^+$ T cells ($CD45RA^-CD45RO^+$), total naïve ($CD45RA^+CD45RO^-$) T cells, T_N, T_{CM} and T_{Em} between patients with and without T2DM (Table 2). Strikingly, all cell populations analyzed differed between patients with diabetes and non-diabetic patients. T2DM was associated with elevated percentages of $CD45RO^+$, T_{EM} and T_{CM} and reduced percentages of $CD45RA^+$ and T_N.

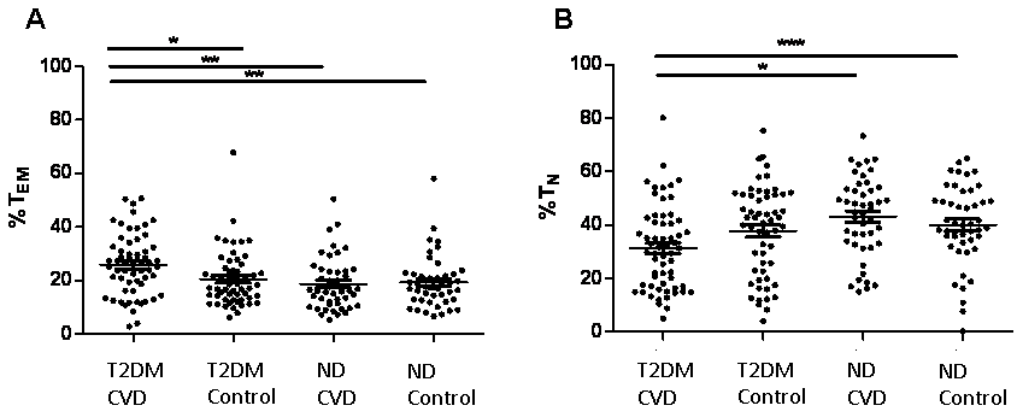

Figure 2. Kruskal-Wallis test with Dunn's multiple comparisons post-test comparing levels of T_{EM} (A) and T_N (B) between patient categories (T2DM, type 2 diabetes mellitus; ND, non-diabetic; CVD, cardiovascular disease). *p<0.05, ** p < 0.01 and *** p < 0.001.

Next we analyzed the proportions of T cell sub-populations between groups stratified on CVD and diabetes by 2-way ANOVA analysis. Notably, we observed an interaction between diabetes and CVD for T_{EM} ($p^{interaction}$=0.036) and T_N ($p^{interaction}$=0.029) (Table 3). The group exhibiting the highest levels of T_{EM} and the lowest levels of T_N was T2DM patients with CVD (Figure 2). All cell populations were significantly different between diabetics and non-diabetics also in the 2-way ANOVA analysis that considers the interaction with CVD. In contrast, we did not observe any relationship between memory T cell subsets and CVD in the 2-way ANOVA analysis. However, comparing all patients with and without manifest CVD (combining patients with diabetes with non-diabetics) we did observe a trend (p=0.057) towards T_{EM} being associated with CVD (Table 4). Adjusting for gender and age, T_{EM} was significantly associated with CVD in the total cohort (p=0.031, β=1.03) as well as in patients with diabetes (p=0.01, β=1.05) in binary logistic regression models. No association between T_{EM} and CVD was seen in non-diabetic patients (p=0.9, β=1.001).

Finally, we tested whether memory T cell subpopulations were associated with metabolic variables. Several T cell subsets were associated with diabetes related metabolic parameters (Table 5). T_{CM} and T_{EM} were inversely correlated with HDL whereas T_N was positively associated with HDL. T_{EM} (r=0.149) and T_N (r=-0.176) were associated with HbA1c. T_{EM} displayed a positive correlation with fasting glucose (r=0.157). Of note, comparing patients with and without diabetes separately, the relationship between HDL and T cell subsets was only observed in patients with diabetes (r=0.323, p<0.0001). Adjusting for HDL (including age and gender) in a binary logistic regression model did not remove the association between T_{EM} and CVD (p=0.035) seen in patients with diabetes.

Discussion

This is the first study, to our best knowledge, that has investigated CD4+ T memory cell subsets in T2DM patients, and the first to observe a relationship regarding memory T cells and diabetes-associated CVD. Demonstrated here, diabetes was associated with a state of exacerbated immune activation with a reduced percentage of naïve CD4+ T cells and increased levels of effector memory T cells. This finding is in agreement with diabetes being associated with increased inflammation[1] and raises the possibility that the increased inflammatory state in diabetes may be partly due to dysregulated activation of adaptive immunity. Remarkably, the exacerbated immune activation was most prominent in diabetics with CVD, whereas CVD patients without diabetes were indistinguishable from healthy individuals. The interaction between diabetes and CVD indicates that the effector memory / naïve T cell balance is of importance for the increased manifestation of CVD in T2DM patients. It also suggests that T cell mediated immune responses initiated during hyperglycemia might lead to CVD when the effector memory / naïve T cell balance is pushed too far and could at least in part explain the increased risk of CVD in diabetics. Further studies are needed to elucidate whether patients with diabetes have increased levels of T_{EM} recognizing plaque antigens such as Apolipoprotein B100.

Ammiratti and co-workers recently reported that T_{EM} (defined as $CD3^+CD4^+CD45RA^-CD45RO^+CCR7^-$) was associated with intima-media thickness as well as with chronic stable angina and acute myocardial infarction.[8] Interestingly, they reported that T_{EM} levels were increased in patients with diabetes (n=14, 17% of total patients) compared to controls. This is, although considering the small sample size, in line with our findings. However, our study differs in that we did not see any correlations with T_{EM} and LDL, triglycerides or systolic blood pressure. One possible explanation is the difference in age between the studies, since the patients included in our study was on average 20 years older than in the study by Ammirati and co-workers. Another difference is the composition of patients with regard to diabetes.

In our study, correlation analysis revealed a strong negative relationship between HDL and memory T cell populations in the entire cohort. Moreover, the relationship was significant also within patients with diabetes only, but not in the non-diabetic population. Recent studies have demonstrated that HDL might affect autoimmunity, possibly by influencing leukocyte cholesterol content[9]. Lipid rafts are important in organizing cellular components needed to come together upon T-cell receptor (TCR) interaction with MHC-II.[10] LDLr-/- mice also deficient in ApoA-I display an autoimmune phenotype and exacerbated atherosclerosis. [11] Administration of ApoA-I reduces the levels of T effector cells and increases levels of regulatory T cells in mice.[12] Further studies are needed to determine whether HDL influences lipid rafts and subsequent T-cell activation in man. Correcting for HDL in binary logistic

regression did not remove the relationship between CVD and T_{EM} comparing T2DM patients with and without CVD, showing that the atherogenic association observed for T_{EM} is not fully dependent on HDL.

In our study, the observed T_{EM}/T_N imbalance is most prominent in T2DM patients with diabetes (Figure 2). The statistically significant T_{EM}/T_N imbalance found when comparing all diabetics to all non-diabetics is likely driven by the prominent T_{EM}/T_N imbalance in T2DM patients with CVD. The 2-way ANOVA analysis does however indicate that diabetes alone as well as the interaction between diabetes and CVD is associated with a T_{EM}/T_N imbalance. It will be important in future studies to account for the interaction between diabetes and CVD, as comparing CVD versus non-CVD patients could might reveal false associations if the presence of diabetes is not accounted for. Similarly, comparing diabetics versus non-diabetics might reveal strong associations with diabetes that are in fact driven by the strong interaction between diabetes and CVD.

Little is known regarding the relationship between CD4+ T cells and atherosclerosis in T2DM. Giubilato and co-workers observed that $CD4^+CD28^{null}$ cells are increased in diabetes patients with acute coronary syndrome.[13] In the same study, this subpopulation of pro-atherogenic T cells also predicted event-free survival during a 3-year follow up. $CD4^+CD28^{null}$ T cells produce IFN-γ and have cytolytic properties, similar to cytotoxic $CD8^+$ T cells.[14]

A limitation of our study is that we do not have follow-up data on patients. It would be interesting to probe in prospective cohorts if an altered T_{EM}/T_N imbalance could predict CVD among diabetics. It would also be interesting to study the antigen specificity and the functional characteristics of the T_{EM} cells in T2DM patients with CVD. Definition of antigens associated with an altered T_{EM}/T_N imbalance could reveal important drug targets.

In summary, we demonstrate that T_{EM} are uniquely increased in patients with concomitant T2DM and CVD. Given the importance of T cell subsets in atherosclerosis, this finding warrants further research into potential mechanism by which immunity may affect diabetes-accelerated atherosclerosis.

Acknowledgements
We thank Lisette Hollender for her technical expertise in designing the flow cytometry measurements.

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Tables

Table 1. Laboratory and anthropometric parameters comparing patient categories

	T2DM		Non-diabetic (ND)		P-value
	CVD	Controls (C)	CVD	Controls (C)	
Number of patients	66	64	49	52	
Gender (M/F?)	39/25	45/19	29/19	34/14	p= 0.95
Age	74 ± 6	74 ± 6	75 ± 5	74 ± 5	P=0.76
LDL (mmol/l)	2.28 ± 0.77	2.32 ± 0.70	2.51 ± 0.77	3.34 ± 0.82	P<0.0001
HDL	1.14 ± 0.28	1.37 ± 0.41	1.52 ± 0.40	1.56 ± 0.45	P<0.0001
Triglycerides	1.52 ± 0.79	1.50 ±0.95	1.26 ± 0.63	1.27 ± 0.50	P=0.131
Glucose	8.75 ± 3.21	8.62 ± 2.61	5.56 ± 0.53	5.58 ± 0.77	P<0.0001
HbA1c (mmol/mol)	55.1 ± 11.7	54.1 ± 13.0	38.5 ± 3.80	38.1 ± 3.62	P<0.0001
Systolic BP	140 ± 21	140 ± 16	143 ± 16	139 ± 14	P=0.73
Diastolic BP	72 ± 10	76 ± 9	75 ± 8	78 ± 8	p= 0.006
Statin	53 (83%)	41 (64%)	12 (25%)	13 (27%)	p= 0.017
Metformin	44 (69%)	45 (70%)	0 (0%)	0 (0%)	p<0.0001
Duration of diabetes	14	12	-	-	P<0.45#

Differences between groups were tested using ANOVA with Bonferroni post-tests for continouus variables and χ^2 for catergorical variables.
LDL, low-density lipoprotein; HDL, high-density lipoprotein; BP, blood pressure; HbA1c, glycosylated hemoglobin A1c,
LDL: ND-C p<0.0001 vs all
HDL: T2DM-CVD <0.007 vs all.T2DM-C p=0.0503 vs ND-C
Glucose: T2DM vs. ND (p<0.00001)
HbA1c: T2DM vs. ND (p<0.00001)
Diastolic BP: T2DM-CVD vs. all (p<0.05)
= Mann-Whitney U comparing T2DM-CVD and T2DM-C

Table 2. T cell populations comparing T2DM patients with non-diabetics

	T2DM (n=116)	Non-Diabetic (n=93)
$CD45RA^+$	35 ± 15**	43 ± 15
$CD45RO^+$	57 ± 15***	48 ± 15
T_N	34 ± 17**	42 ±
T_{CM}	40 ± 10*	37 ±11
T_{EM}	23 ± 11**	19 ± 10

Mann-Whitney U test comparing T2DM and non-diabetics
* $p<0.05$
** $p < 0.01$
*** $p < 0.001$

Table 3 Two-way ANOVA of T cell subsets and diabetes and CVD

	T2DM		Non-diabetic				
	CVD	Controls	CVD	Controls	$p^{diabetes}$	p^{CVD}	$p^{interaction}$
$CD45RA^+$	32 (29-37)	37 (34-41)	43 (39-48)	42 (38-46)	0.001	n.s.	n.s.
$CD45RO^+$	58 (54-62)	55 (51-59)	47 (43-52)	47 (43-52)	<0.001	n.s.	n.s.
T_N	31 (37-35)	38 (34-42)	43 (39-48)	40 (36-45)	0.001	n.s.	0.029
T_{CM}	40 (37-42)	40 (37-43)	36 (23-39)	37 (34-40)	0.034	n.s.	n.s.
T_{EM}	26 (23-29)	20 (18-23)	18 (16-22)	19 (16-22)	0.004	n.s.	0.036

Mean (95% CI) of respective T cell population
n.s. non significant

Table 4. T cell populations comparing CVD with non-CVD controls

	CVD (n=106)	Non-CVD (n=103)
$CD45RA^+$	38 ± 16	40 ± 15
$CD45RO^+$	54 ±17	52 ± 15
T_N	36 ± 16	39 ± 16
T_{CM}	38 ± 10	39 ± 11
T_{EM}	23 ± 11#	20 ± 10

Mann-Whitney U test comparing CVD and non-CVD
p=0.057

Table 5. Associations between T cell subsets and metabolic parameters

	CD45RA+	CD45RO+	T_N	T_{CM}	T_{EM}
HbA1c	-0.179 *	0.203 **	-0.176 *	n.s.	0.149 *
Glucose	-0.147 *	0.164 *	n.s.	n.s.	0.157 *
LDL	n.s.	n.s.	n.s.	n.s.	n.s.
HDL	0.264 ***	-0.283 ***	0.266 ***	-0.164 *	-0.306 ***
Triglycerides	n.s.	n.s.	n.s.	n.s.	n.s.
Systolic BP	n.s.	n.s.	n.s.	n.s.	n.s.
Age	n.s.	n.s.	n.s.	n.s.	-0.140 *
Gender	n.s.	n.s.	n.s.	n.s.	n.s.

Spearman's rho correlations
* $p<0.05$
** $p < 0.01$
*** $p < 0.001$

Paper III

Characterization of T cell responses following immunization with human Apolipoprotein B100 in mice

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Introduction

Immune responses against LDL antigens have been found to play an important modulatory role in atherosclerosis. Immunization with homologous oxidized LDL, as well as human apolipoprotein B100 (ApoB)-derived peptides, has been demonstrated to inhibit atherosclerosis in hypercholesterolemic animal models of atherosclerosis. Several lines of evidence have implicated regulatory T cells as mediators of this protection, while the possible role of antigen-specific Th2 responses remains to be fully clarified.

Methods

Apo $E^{-/-}$ mice on high-fat diet were immunized with human ApoB using Alum as adjuvant at 12, 14 and 16 weeks of age. Alum and PBS alone were used as controls. A matrigel plug containing ApoB was placed subcutaneously one week after the last immunization and T cell infiltration into the plug as well as development of aortic root atherosclerotic lesions were analysed after an additional 7 days.

Results

Immunization with ApoB resulted in 4-fold increased accumulation of effector T cells in ApoB-containing matrigel plug as compared with the Alum and PBS controls. The levels of the Th2 cytokines IL-4, IL-5 and IL-10 were also increased in the matrigel plugs. Moreover, the levels of Th2-type IgG1 against human as well as mouse LDL were increased in plasma of ApoB immunized mice. As compared with the PBS treated group there was a significant reduction of atherosclerosis in both the ApoB immunized and Alum control groups. However, in spite of the induction of a Th2 response partially reacting also with the endogenous LDL in the ApoB immunized group there was no difference in atherosclerosis as compared with the Alum group.

Conclusion

This study describes a novel model to study antigen-specific T cell responses in mouse models of atherosclerosis. The results suggest that activation of Th2 immunity does not mediate the protective effect of immunization with LDL antigens described previously.

Introduction

A multitude of studies have demonstrated the involvement of both humoral and cellular immune responses in atherosclerotic disease.[1, 2] According to a current paradigm, autoimmune responses against self or modified-self proteins in the atherosclerotic plaque contribute to local inflammation, recruitment of macrophages and subsequent destabilization of the plaque.[3, 4] The clinical outcome is believed to depend, at least partly, on the balance between pro-inflammatory autoimmune responses and anti-inflammatory immune dampening counter-responses.[5]

CD4+ T helper (Th1) cells, a key orchestrator of adaptive immune responses, differentiate towards different lineages depending on cues from locally released cytokines and co-stimulatory molecules.[6, 7] Studies in mice suggest that IFNγ+ Th1 responses are pro-atherogenic[8] whereas immune-modulating regulatory T cells (Tregs) appear to mediate athero-protection.[9-11] The role of Th2 cells in atherosclerosis remains more controversial. Mice deficient in IL-4 have been shown to have reduced or unaltered atherosclerosis,[12, 13] while IL-5 have been suggested to protect against atherosclerosis by stimulating the release of so called natural antibodies from B-1 cells.[14, 15] Moreover, Th2 cells can produce the anti-inflammatory cytokine IL-10 which reduces atherosclerosis. [16] In a recent prospective clinical study we found an association between low levels of Th2 cells in the circulation and an increased risk for development of ischemic cardiovascular events.[17]

LDL has been identified as one of the most important autoantigens in atherosclerosis.[18] Oxidized LDL-specific antibodies and T cells have been isolated from atherosclerotic plaques and are also present in the circulation.[19-22] Immunization of hypercholesterolemic mice and rabbits with oxidized LDL inhibits the development of atherosclerosis.[23-28] These observations demonstrated that athero-protective immune responses against antigens in LDL exist and suggested the possibility of developing a vaccine-based treatment for atherosclerosis. Subsequent studies by Hermansson et al[29] identified apolipoprotein B100 (ApoB) as the most important LDL target for autoreactive T effector cells and showed that deletion of these cells resulted in reduced development of atherosclerosis. Moreover, they have also shown that treatment of human ApoB transgenic LDLr$^{-/-}$ mice with ApoB-pulsed tolerogenic DCs led to a significant reduction of atherosclerotic lesions.[30] We and others have demonstrated that immunization with peptides derived from human ApoB inhibits atherosclerosis in apoE$^{-/-}$ and LDL$^{-/-}$ mice.[31-34] Studies based on immunization with MDA-modified ApoB peptides and treatment with MDA-ApoB peptide IgG have provided indirect support for a protective role of antigen-specific Th2 responses.[35-37] However, other studies immunizing LDLr$^{-/-}$ mice transgenic for human ApoB with native ApoB peptides show that athero-protection can be achieved also in the absence of activation of an antibody response.[38]

The possibility to characterize the exact role of antigen-specific T cells subsets in the atherosclerotic disease process and the response to immunization therapy has been limited by the lack of in vivo models to study the activity of antigen-specific T cells. To address this problem we have developed a model based on subcutaneous implantation of an antigen-containing matrigel plug adapted from a previously described model by Corthay et al[39] to study tumor-specific CD8+ T cells. We then used this model to determine antigen-specific T cell responses to immunization with human ApoB.

Methods

Proteins

Human apolipoprotein B100 (Yoproteins, Stockholm, Sweden) was used for immunizations and loaded into matrigel solutions (BD Biosciences, Growth Factor Reduced). LDL was isolated from mouse plasma or from human plasma.

Mice and injections

Apoe-/- mice were given high-fat diet (HFD) containing 21% cocoa fat, 0.15 % cholesterol at 8 weeks of age. Mice were immunized subcutaneously in the neck with 100 µg ApoB in Alum (protein to adjuvant ratio 1:2; Pierce) or Alum alone at 12, 14 and 16 weeks of age. A control group was left un-immunized to account for the effect of adjuvant. Matrigel was injected subcutaneously in the back one week prior sacrifice of the mice. After 10 weeks on HFD mice were euthanized and organs were harvested.

Matrigel

The matrigel system for measuring T cell responses was adapted from Corthay et al[39]. One week before the end of the experiment, mice were injected with matrigel mixed with either ApoB (0.1 mg/ml) or PBS. Briefly, matrigel was thawed at 4˚C and mixed with ApoB/PBS before injection of matrigel-mixture to each flank of the mouse. The matrigel rapidly forms a gel at body temperature and is stable *in vivo* for >1 week. At sacrifice, matrigel plugs were dissected, and incubated with Cell Recovery Solution (BD Biosciences) at 4^{o}C for 2h. After digestion, cells were centrifuged and the supernatant was stored at -80˚C. The cell pellet was resuspended, washed and used for flow cytometry analysis.

Flow cytometry

Matrigel residing cells (isolated as above) were blocked with anti-CD16/32 before staining with anti-CD3, -CD4, -CD8, -CD25, -CD45 and -CD11b (Biolegend). Cells were fixed and permeabilized (Ebioscience), whereafter anti-FoxP3 antibody (Biolegend) was added. T cell populations were measured on a CyAN ADP flow cytometer (Beckman Coulter). Flow cytometry analysis was performed with FlowJo 7.6 software (Tree Star). PBS containing matrigels were used as controls.

ELISA

Antibodies against human or mouse LDL was measured using ELISA. Briefly, microtiter-plates (Nunc Maxisorp) were coated with 10 µg/ml mouse LDL, human LDL or buffer alone over night at 4ºC. Wells were blocked with 1% BSA in PBS and mouse plasma (diluted 1:100) in 0.1% BSA in PBS was added and incubated for 2 hours. Alkaline-phosphatase-conjugated anti-mouse IgG1 or anti-mouse IgG2A produced in rat (BD Biosciences) was added. Bound antibodies were detected by addition of substrate and absorbance was measured at 405 nm.

Immunohistochemistry

Subvalvular sections were stained with MOMA-2 antibody staining monocyte/macrophages. Sections were scanned, and positive areas as well as total lesion area was quantified using Biopix software (Biopix AB, Gothenburg, Sweden).

Statistics

Differences between groups were tested using unpaired t-tests or Mann-Whitney U, when applicable.

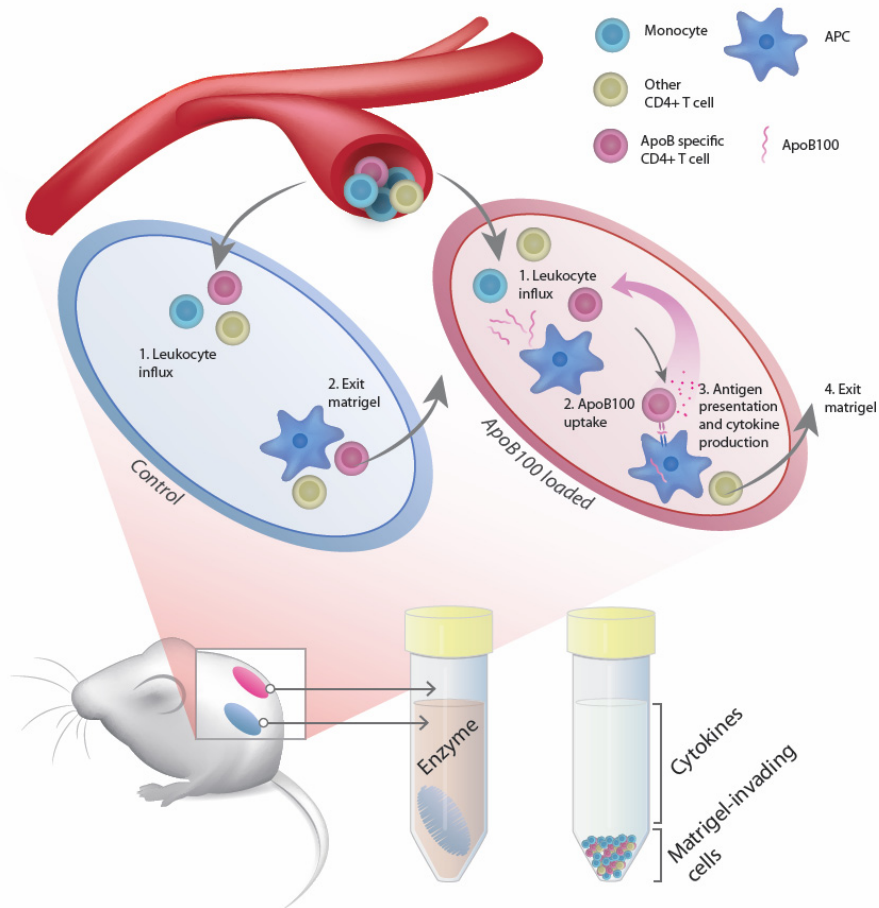

Figure 1. Summary of the matrigel model for the study of antigen-specific immune responses. Matrigel mixed with either ApoB or vehicle was injected s.c., whereafter leukocytes enter the matrigels. In ApoB-containing matrigels, monocytes take up ApoB and present it to T cells. ApoB-specific T cells produce cytokines upon antigen-recognition, further promoting leukocyte influx.

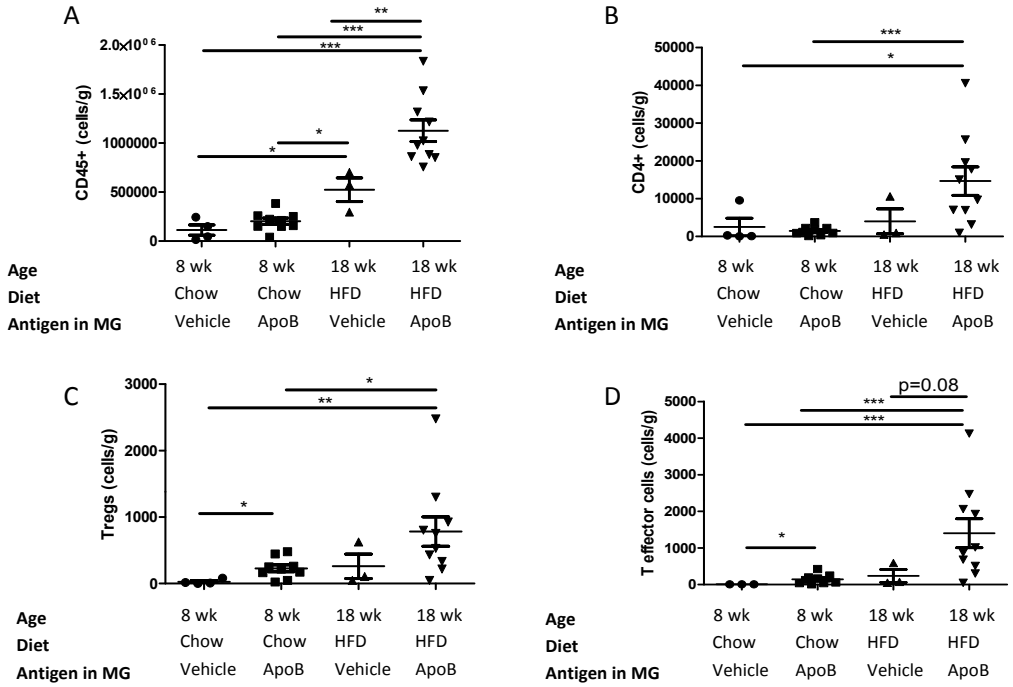

Figure 2. Accumulation of leukocytes to matrigel plugs containing ApoB or vehicle**.** 8 week (wk) old mice (chow diet) and 18 wk old mice (high-fat diet, HFD), both non-immunized, were injected with matrigel containing either apolipoprotein B100 (ApoB) or vehicle. Matrigel-invading cells were quantified by flow cytometry. Cell counts of CD45+ (A), CD4+ (B), $FoxP3^{+}CD25^{+}$ regulatory T cells (Tregs) (C) and $FoxP3^{-}CD25^{+}$ T effector cells (D) were normalized by matrigel weight and presented as cells/g.

Results

The general principles of the model to study antigen-specific T cells are outlined in figure 1. Briefly, $apoE^{-/-}$ mice on high-fat diet were first immunized with 100 µg ApoB in Alum followed by booster immunizations after 2 and 4 weeks. One week after the last booster immunization, matrigel containing ApoB or vehicle was injected subcutaneously in the back of all mice. The matrigel forms a plug that was recovered one week later. The cell content of the plug was then analysed by flow cytometry and the cytokine content by multiplex technology.

ApoE-/- mice have T cells that recognize human ApoB

First, we used the model to determine if non-immunized $apoE^{-/-}$ mice have T cells that recognize human ApoB. In 8 week old $apoE^{-/-}$ mice on chow diet, we observed a minor accumulation of both $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Tregs) and $CD4^{+}CD25^{+}FoxP3^{-}$ T cells in ApoB-containing

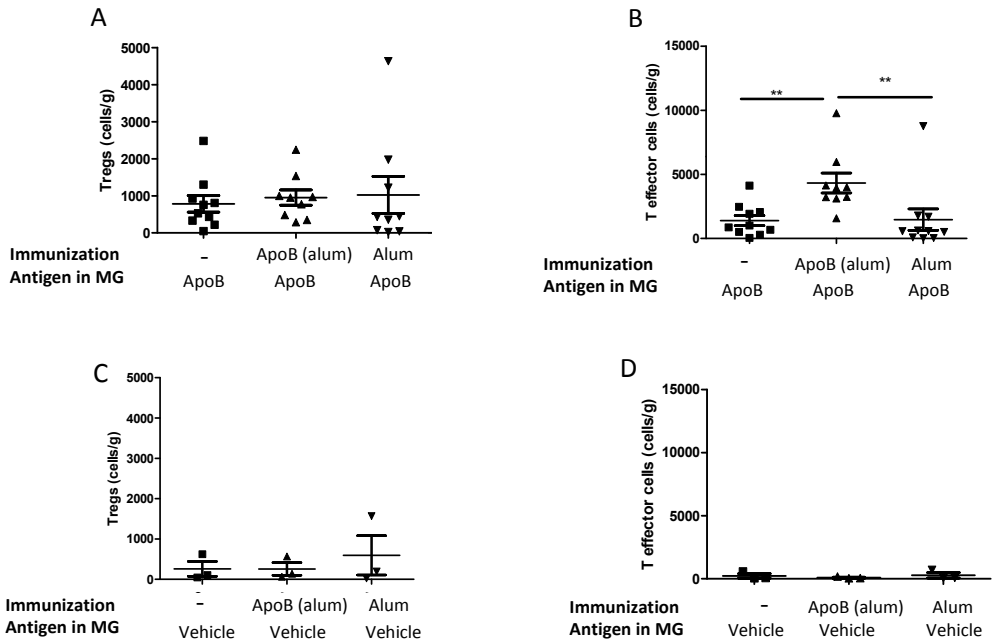

Figure 3. Immunization with human apoB results in increased non-regulatory Teffector cells in apoB containing matrigel. Apoe-/- mice were immunized with apoB in alum, alum alone or left un-immunized. Accumulation of Tregs (A, C) and T effector cells (B, D) in matrigel plugs containing either apolipoprotein B100 (ApoB) (A-B) or vehicle (C-D) were quantified by flow cytometry (values were normalized by matrigel weight).

matrigel plugs compared to vehicle-loaded matrigel plugs (figure 2C-D). As T cells up-regulate CD25 upon activation, this FoxP3$^-$ population was defined as non-regulatory CD4$^+$ T effector cells. In 18 week old apoE$^{-/-}$ mice that had been fed a high-fat diet for 10 weeks there was a larger accumulation of both total CD45$^+$ leukocytes, CD4$^+$ T helper cells, Tregs and T effector cells in the ApoB containing matrigel (figure 2A-D). Accordingly, the observations suggest that apoE$^{-/-}$ mice on high-fat diet have T cells that recognize epitopes in human ApoB.

Immunization with ApoB in Alum induces the generation of antigen-specific Th2 cells

We next determined the ability of the model to study generation of antigen-specific T cells in response to immunization. Immunization with ApoB in Alum dramatically increased the numbers of CD4$^+$CD25$^+$FoxP3$^-$ T cells recruited to the ApoB-containg matrigel plug (figure 3B) while no or only few CD4$^+$CD25$^+$FoxP3$^-$ T cells accumulated in the control plugs (figure 3D). Immunization with ApoB did not result in an increased recruitment of CD4+CD25+FoxP3+ Tregs to the ApoB-containing matrigel plug (figure 3A).

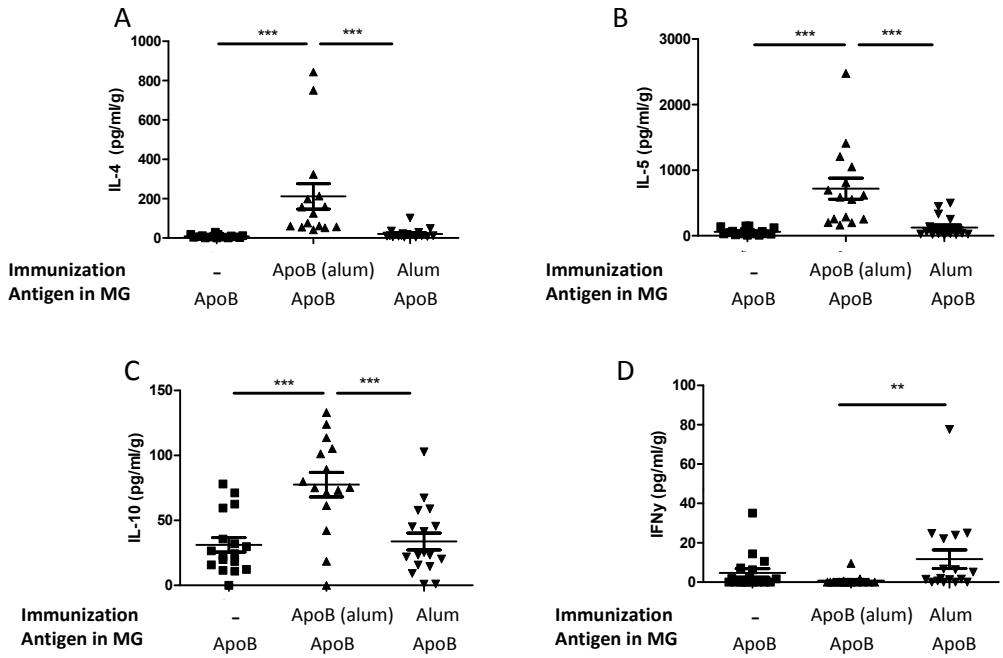

Figure 4. Immunization with human apoB results in increased Th2 cytokines in apoB containing matrigel. Apoe-/- mice were immunized with apoB in alum, alum alone or left un-immunized. One week prior euthanization, matrigel containing ApoB (A,B) or PBS (vehicle) were injected in the back, and retrieved one week later. IL-4 (A), IL-5 (B), IL-10 (C), and IFNγ (D) levels in the matrigel were analysed by multiplex technology.

We then measured cytokines isolated from the supernatants of enzymatically digested matrigels containing ApoB. We observed a typical Th2 cytokine profile in mice immunized with ApoB, with increased IL-4, IL-5 and IL-10 compared to alum alone or non-immunized mice (figure 4A-C). Notably, there was a reduction of IFN-γ in ApoB immunized mice compared to mice in the Alum control group further establishing that immunization with ApoB in alum generates an antigen-specific Th2-exclusive response (figure 4D).

High levels of class-switched anti-human-LDL IgG1 in ApoB immunized mice

One of the main functions of Th2 cells is to provide signals to B cells that promote class switching and antibody generation. To establish that the immunization had generated a functional Th2 response we measured IgG1 (related to Th2 responses) and IgG2a (related to Th1 responses) antibodies against both human and mouse-derived LDL. We observed high titres of anti-human-LDL IgG1 antibodies in mice immunized with ApoB (figure 5A, $p<0.001$), whereas no antibody response against human LDL

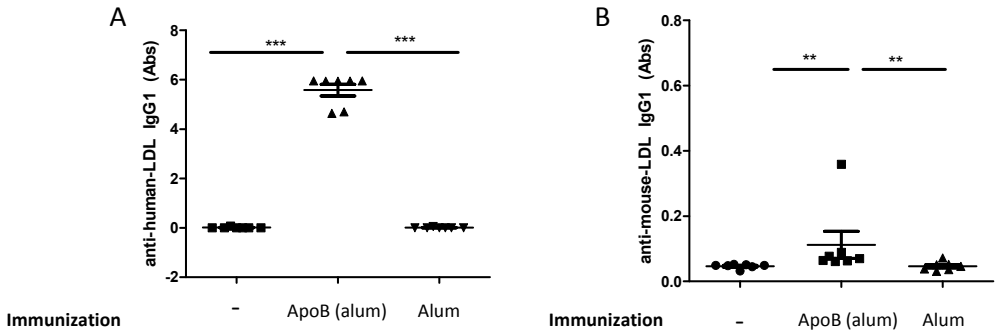

Figure 5. Immunization with human ApoB induces IgG1 antibodies. Apoe-/- mice were immunized with apoB in alum, alum alone or left un-immunized. IgG1 antibodies recognizing human LDL (A) or mouse LDL (B) were analysed by ELISA.

was present in alum or non-immunized mice. The homology of human ApoB compared to mouse ApoB is ~85%. To discern the amount of inter-species cross-reactivity we also measured antibodies against mouse-LDL. Mice immunized with ApoB were the only group with measureable anti-mouse-LDL IgG1 antibodies (figure 5B). However, the absorbance of antibodies recognizing mouse LDL was considerably lower than the absorbance of antibodies against human LDL. There was no difference in IgG2a antibodies against human or mouse LDL between groups (data not shown).

Immunization with human ApoB does not reduce atherosclerosis compared to Alum alone

Finally, we addressed whether immunization against ApoB affected plaque size or plaque inflammation (as estimated by MOMA macrophage/monocyte staining), compared to Alum alone or non-immunized mice. Immunization with ApoB resulted in a significant reduction in atherosclerotic plaque size as compared to non-immunized mice (figure 6A). However, as a similar reduction in plaque size was observed also in the Alum control group, protection achieved by ApoB immunization appeared to be unrelated to the activation of a Th2 response against ApoB. There was no difference in total macrophage-stained area between the groups (figure 6B), but the percent macrophage-stained area was higher in mice given Alum than in non-immunized mice (data not shown).

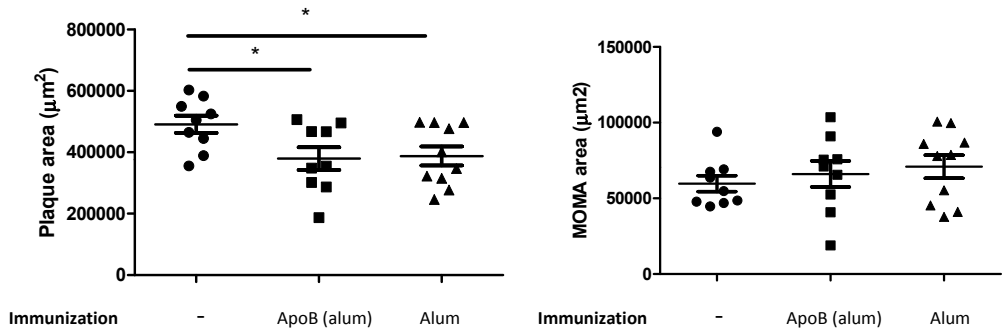

Figure 6. Immunization with human ApoB does not reduce atherosclerosis compared to alum. Apoe-/- mice were immunized with apoB in alum, alum alone or left un-immunized. Subvalvular lesions were stained with MOMA, recognizing monocytes and macrophages, and with haematoxylin. Subvalvular plaque area (A) and MOMA-positive area (B) were quantified.

Discussion

The present study describes an *in vivo* model to assess the generation and activation of antigen-specific T cells in atherosclerosis. The activation of T cells was determined by flow cytometry analysis of cells recovered from the matrigel as well as by analysing the cytokine content of the matrigel plug. Using this model we demonstrate accumulation of T cells to sites containing ApoB in ApoE-/- mice. Immunization with human ApoB in alum induces generation of antigen-specific Th2 cells without any detectable formation of antigen specific Th1 or Tregs. This was associated with a reduced expression of IFN-γ in the matrigel plug suggesting that the activated Th2 cells had an inhibitory effect on Th1 cells that concomitantly had been recruited to the plug. Since high-fat diet is associated with generation of T effector cells recognizing ApoB, these Th1 cells are most likely also ApoB specific. ApoB-specific T cells secreted IL-10 when accumulating in the matrigel plug. Since we could not detect any antigen-specific Tregs in the matrigel plug this IL-10 should come from activated Th2 cells. This notion was further supported by the increased levels of IL4 and IL-5 in ApoB-containing matrigel plugs from ApoB immunized mice. Taken together these observations demonstrate the ability of the model to assess antigen-specific T cells *in vivo.*

However, although a proportion of the T cells found in the plug are likely to be specific for ApoB, it is possible that some T cells found in the plug respond to other antigens. Moreover, as antigen in the matrigel may leak or be actively transported to lymph nodes by antigen-presenting cells, we cannot exclude that we are measuring both pre-existing immunity (and immunity induzed by immunization) as well as "matrigel-induced" immunity. However, the contribution of such *de novo* activation caused by antigen-leakage from matrigel can be assumed to be of minor importance, given the differences seen between young pre-atherosclerotic mice and atherosclerotic mice, both receiving matrigels loaded with ApoB (figure 2).

As discussed above, the present study shows that immunization with human ApoB results in generation of Th2 cells that secrete the anti-inflammatory cytokine IL-10 and suppress the release of IFN-γ from co-localized Th1 cells when encountering their cognate antigen. It has previously been shown that IL-10 inhibits the development of atherosclerosis[16] while IFN-γ promotes progression of the disease.[8] Accordingly, it can be assumed that activation of a corresponding Th2 response in atherosclerotic lesions should have an inhibitory effect on the disease process. However, in spite of this, immunization with ApoB did not result in decreased atherosclerosis as compared with Alum control group. It is possible that the study was too short to allow identification of any protective effects. Another explanation could be that the mouse apoB reactivity of the Th2 cells generated in response to immunization with human ApoB was insufficient to activate a protective response in the mice. Immunization with different human ApoB-derived peptides (primarily amino acids 3136-3155 also referred to as p210) has in previous studies been found to inhibit the development of atherosclerosis in apoE$^{-/-}$ and LDLr$^{-/-}$mice suggesting that at least some mouse and human apoB epitopes are sufficiently similar.[31, 34, 35]

Reduced atherosclerosis, compared with non-immunized mice, was observed in mice immunized with ApoB in alum as well as wuth alum alone. Inhibition of atherosclerosis in response to Alum has been reported in several previous studies.[40, 41] In a study on apoE$^{-/-}$ mice we found that the athero-protective effect of Alum was associated with an expansion of the Treg pool in the spleen and suggested that this was explained by a presentation of LDL antigens captured by Alum at the injection site. Interestingly, the present observation indicates that Alum is not associated with generation of ApoB specific Tregs. However, it cannot be excluded that Alum induced the formation of Tregs reacting with mouse but not with human apoB. Alternatively, Alum results in a polyclonal expansion of Tregs that is sufficient to inhibit atherosclerosis.

The present observations suggest that activation of a Th2 response against ApoB is associated with several potential athero-protective effects including increased release of IL-5 and IL-10 as well as an inhibition of IFN-γ expression. However, the possible role of Th2 immunity in atherosclerosis remains controversial. Th2 cell differentiation is dependent on IL-4 and hypercholesterolemic mice deficient in IL-4 have been reported to have reduced as well as unaltered atherosclerosis.[12, 13] IL-5 is another signature cytokine of activated Th2 cells. IL-5 has a protective effect on atherosclerosis in hypercholesterolemic mice, a phenomenon that has been attributed to the ability of IL-5 to stimulate the synthesis of so called natural antibodies from B cells of the B1 type [14, 15]. The clinical importance of Th2 cells in cardiovascular disease has not been studied in detail but in a recent study we could demonstrate subjects with high levels of circulating Th2 cells had less severe carotid disease and had a lower risk for development of acute cardiovascular events.[17]

In summary, the present study demonstrates that implantation of subcutaneous antigen-containing matrigel plugs can be used to study antigen-specific T cell responses in atherosclerosis *in vivo*. Using this model we demonstrate that immunization with human ApoB using Alum as adjuvant results in an antigen-specific Th2 cell response including increased release of IL-4, IL-5 and IL-10 as well as an inhibition of IFN-γ release when these cells encounter ApoB in the matrigel.

Acknowledgements

The authors thank Filip Persson for graphical design expertise.

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Paper IV

Myd88 signaling in CD4+ T cells promotes atherosclerosis and Th17 responses

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Abstract

Background

MyD88 is a transducer of TLRs and IL-1R signaling and crucial for responses against pathogen associated molecular patterns. MyD88 deficiency reduces atherosclerosis, an effect that primarily has been attributed to inhibition of macrophage activation. However, recent studies in autoimmune diseases have highlighted the importance of MyD88 signaling in T helper cells. Here, we tested whether MyD88-dependent signaling in CD4+ T cells affects atherosclerosis.

Methods

Apoe-/-Myd88-/- or *Apoe-/-Myd88+/+* $CD4^+$ T cells were transferred to lymphocyte deficient *Apoe-/-Rag1-/-* mice. The mice were fed high-fat diet for 7 weeks and atherosclerosis at the aortic root quantified by Oil Red-O staining and immunohistochemistry. Flow cytometry and multiplex analysis of cytokine release in isolated splenocytes was performed to assess T cell function.

Results

Apoe-/-Rag1-/- mice receiving MyD88-deficient $CD4^+$ T cells developed significantly smaller aortic root lesions compared to mice receiving MyD88-competent $CD4^+$ cells (108 000 ± 14 000 μm^2 vs 157 000 ± 17 000 μm^2, $p<0.05$). Total plaque area for lipids and collagen were lower in mice receiving MyD88-deficient $CD4^+$ T cells and there was also a trend towards less macrophage accumulation. However, the relative amounts of lipids, macrophages and collagen did not differ between the genotypes. Moreover, we observed lower levels of IL-17^+ Th17 cells and double-positive IFN-γ^+IL-17^+ cells in spleens from mice receiving $CD4^+$ T cells lacking MyD88. There were no differences in IL-10^+ or IFNγ^+ $CD4^+$ T cells comparing genotypes. In accordance, splenocytes from mice receiving MyD88-deficient $CD4^+$ T cells produced much less IL-17 but similar amounts of IFNγ and IL-10 compared to MyD88+/+ $CD4^+$ T cells.

Conclusion

This study demonstrates that MyD88 signaling in T cells contributes to development of atherosclerotic plaques and suggests that this involves activation of IL-17 expression.

Introduction

Pro-inflammatory cytokines and signaling pathways contribute to atherosclerotic disease and are associated with vulnerable plaques. Pathogen associated molecular patterns signal through a class of evolutionary conserved receptors called Toll-like receptors (TLRs) (1). Signaling through TLRs, and the IL-1R, is propagated by the adaptor protein MyD88, leading ultimately to nuclear translocation of NF-κB and transcription of pro-inflammatory genes. Aside from a severely reduced response to TLR-ligands, *Myd88*-knockout mice are unresponsive to IL-1β and IL-18. *Apoe-/-Myd88-/-* mice exhibited markedly reduced atherosclerosis demonstrating a role for MyD88 signaling in atherosclerosis (2,3). The reduced atherosclerosis was associated with lower levels of plaque macrophages and chemokines.

CD4+ T helper (Th) cells of the Th17 and Th1 subsets have been shown to promote experimental autoimmunity and are associated with autoimmunity in humans. In atherosclerosis, Th1 responses are considered atherogenic, whereas investigations of Th17 cells have yielded divergent results (4). Recently, several studies have suggested that MyD88 signaling in $CD4^+$ T cells is contributing to autoimmune and inflammatory diseases. In a model of T cell mediated colitis, *Myd88-/-* $CD4^+$ T cells fail to mediate disease (5). In another model of autoimmune inflammation, the lack of TLR4 or Myd88 in $CD4^+$ T cells significantly reduced experimental autoimmune encephalitis (6,7). In addition, MyD88 in T cells were shown to promote differentiation and proliferation of Th17 cells (6).

To investigate the influence of MyD88 signaling in $CD4^+$ T cells on atherosclerosis in a MyD88-competent host, we transferred *Apoe-/-Myd88-/-* or *Apoe-/-Myd88+/+* CD4+ T cells to B and T cell deficient hyperlipidemic *Apoe-/-Rag1-/-* mice.

Methods

Mice

*Apoe-/-Rag1-/-, Apoe-/-Myd88-/-*or littermate *Apoe-/-Myd88+/+* were obtained by breeding and genotypes were verified by PCR genotyping. To promote atherosclerosis, *Apoe-/-Rag1-/-* mice were given high fat diet containing 21% cocoa fat, 0.15% cholesterol, starting at 6 weeks of age. At 9 weeks of age, mice were injected into the tail vein with 1.5 million CD4+ T cells isolated from *Apoe-/-Myd88-/-* or *Apoe-/-Myd88+/+* mice. Four weeks after T cell transfer, mice were killed and organs harvested. All experiments were approved by the local ethics committee.

T cell isolation

To eliminate the possibility of anti-atherogenic ApoE-production from leukocytes, we utilized CD4+ T cells from either *Apoe-/-Myd88+/+* or *Apoe-/-Myd88-/-* mice. CD4+ T cells were purified from splenocytes by Easy Sep Mouse CD4+ T Cell Enrichment Kit (#19752). Cells were injected to recipient mice within four hours after isolation.

Flow Cytometry

Briefly, cells were blocked with anti-CD16/32 antibodies (FcR;CD16/32; clone 93, Biolegend) and stained with antibodies against the extracellular epitopes (anti-CD3, anti-CD4 and anti-CD25). Subsequently, cells were fixed, blocked as above and stained with intracellular antibodies against IFN-γ, IL-17, FoxP3 and IL-10. Cells were analyzed with CyAN ADP Flow cytometer (Beckman Coulter). For assessment of IFN-γ, IL-17 or IL-10 production, cells were stimulated with PMA and ionomycin supplemented with Brefeldin A and cultured for 24 hours prior to staining. Supernatants from splenocytes stimulated with PMA and ionomycin without Brefeldin A were used for multiplex cytokine analysis using Mesoscale technology.

Immunohistochemistry

Subvalvular sections were used for immunohistochemical analysis. Neutral lipids were quantified using Oil Red-O staining and MOMA-2 staining was used to evaluate monocyte/macrophage content. Collagen was stained using Van Gieson collagen staining. Sections were scanned and positive areas as well as total lesion area was quantified using Biopix software (Biopix AB, Gothenburg, Sweden).

Statistics

Differences between groups were tested using unpaired t-tests or Mann-Whitney U, when applicable. In some cases, values were log-transformed before statistical analysis. Values are presented as mean ± standard error of mean, unless otherwise stated.

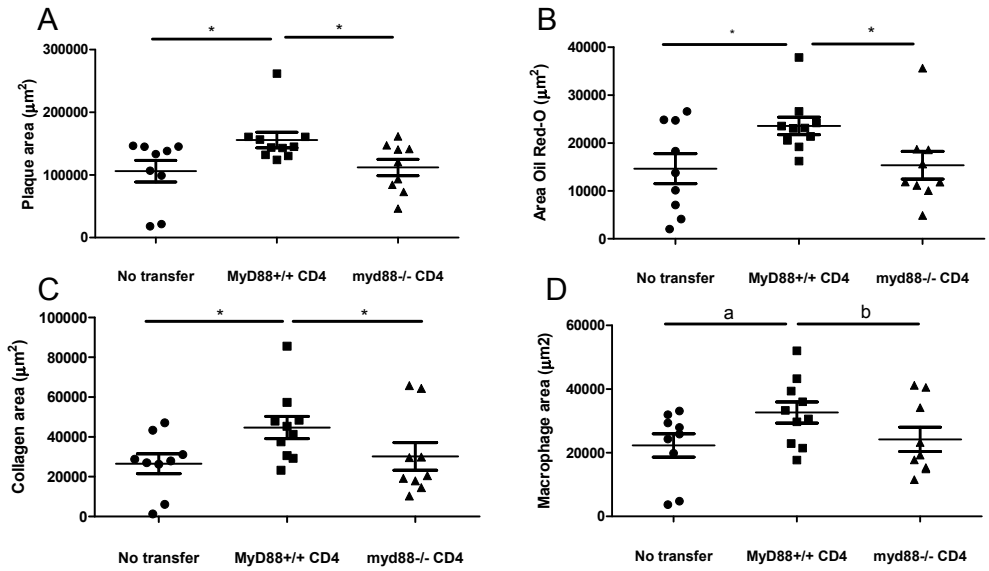

Figure 1. MyD88 signaling in $CD4^+$ T cells results in increased atherosclerosis. Transfer of *Myd88-/-* CD4+ T cells into *Apoe-/-Rag1-/-* mice resulted in decreased plaque area (A), lipid (B) and collagen content (C) as well as a strong trend to increased macrophage content (D) in lesions compared to transfer of *Myd88+/+* CD4+ T cells. *a*: $p<0.07$, *b*: $p<0.08$.

Results

MyD88 signaling in CD4+ T cells contribute to increased plaque size

To assess the role of MyD88 signaling in $CD4^+$ T cells in atherosclerosis, we transferred *Apoe-/-Myd88-/-* or *Apoe-/-Myd88+/+* CD4+ T cells to *Apoe-/-Rag1-/-* recipients being fed high-fat diet. Cells were transferred into 9-week-old mice, which were killed 4 weeks later. To be able to determine the effect of $CD4^+$ cell transfer into *Apoe-/-* mice, non-transferred *Apoe-/-Rag1-/-* were used as controls.

Transferred CD4+ T cells of both genotypes repopulated the spleen to a similar extent and the percent of $CD3^+CD4^+$ in the spleen did not differ (Data not shown). As expected, no B or T-cells were observed in untreated *Apoe-/-Rag1-/-* (data not shown). Next, we evaluated the effect of MyD88 signaling in $CD4^+$ T cells on subvalvular atherosclerosis. Mice receiving *Apoe-/-Myd88-/-* $CD4^+$ T cells exhibited significantly less atherosclerosis compared to *Apoe-/-Myd88+/+* recipient mice

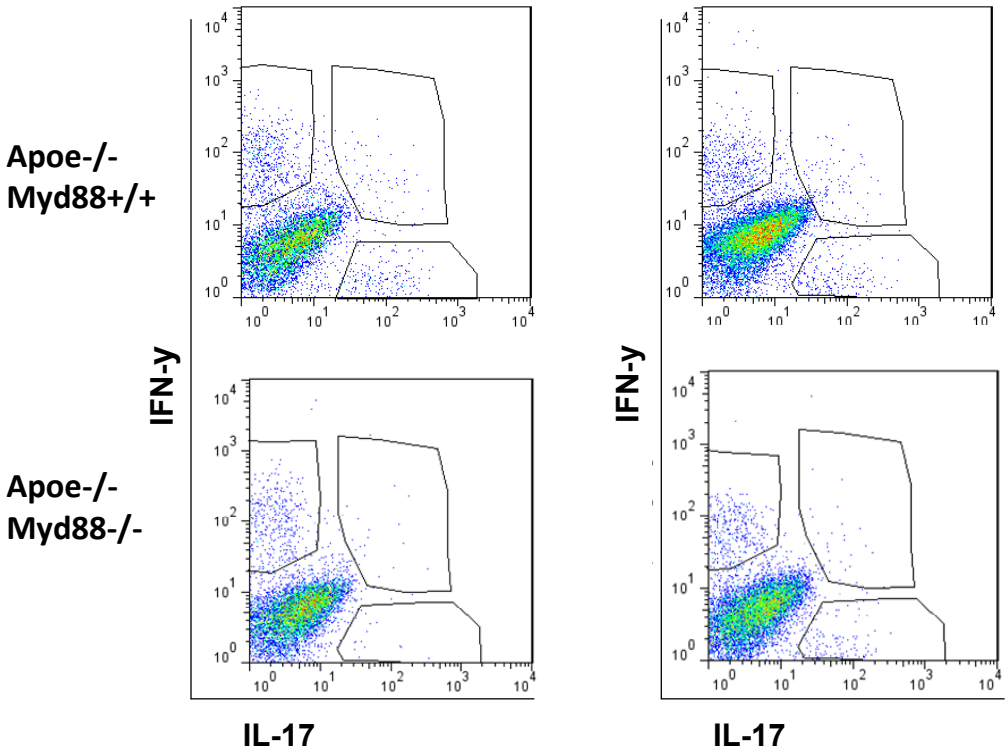

Figure 2. Transfer of MyD88-deficient T cells results in decreased $CD4^+IL\text{-}17^+$ cells in spleens.
Transfer of *Myd88-/-* $CD4^+$ T cells into *Apoe-/-Rag1-/-* mice resulted in decreased IL-17+ as well as $CD4^+IL\text{-}17^+IFN\gamma^+$ T cells compared to *Myd88+/+* $CD4^+$ T cells. Shown are two representative mice of each genotype.

(112 000 ± 13 000 μm^2 vs 156 000 ± 12 000 μm^2, $p<0.05$; Fig. 1A). We also observed increased atherosclerotic plaque size in *Apoe-/-Myd88+/+* recipients compared to untreated *Apoe-/-Rag1-/-* mice (156 000 ± 12 000 μm^2 vs 106 000 ± 17 000 μm^2, $p=0.09$; Fig 1A). These data confirm the previously described pathogenic role of $CD4^+$ T cells in atherosclerosis and indicates that this pathogenesis is partly mediated by MyD88 signaling.

Next, we tested whether the plaque composition differed between mice. Transfer of $CD4^+$ cells to *Apoe-/-Rag1-/-* mice increased lipid and collagen content in lesions (Fig. 1B, C). There was also a trend to increased macrophage content (Fig. 1D). Remarkably, these differences were absent in *Apoe-/-Rag1-/-* mice receiving MyD88-deficient T-cells. Thus, *Apoe-/-Myd88-/-* recipient mice displayed reduced lipid content, and a strong trend towards reduced macrophage content in lesions compared to

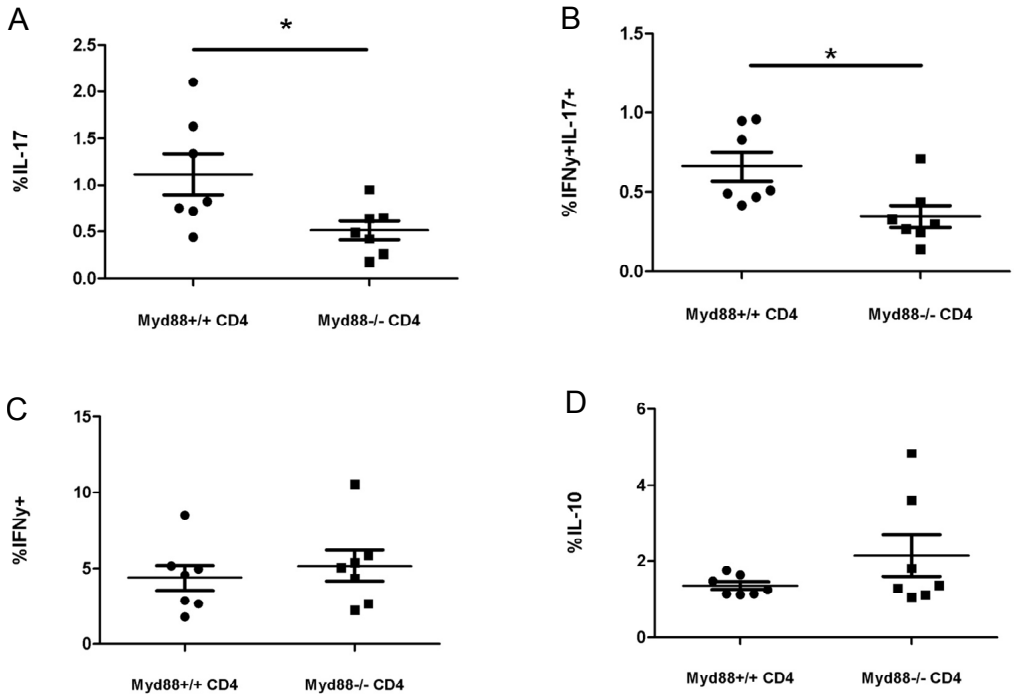

Figure 3. Transfer of MyD88-deficienct T cells results in decreased IL17+ CD4+ cells in spleens.
Transfer of *Myd88-/-* $CD4^+$ T cells into *Apoe-/-Rag1-/-* mice resulted in decreased IL-17^+ (A) as well as decreased double-positive IL-17^+IFNγ^+ CD4+ T cells (B), but no difference in IFNγ^+ (C) or IL-10^+ (D) T cells.

Apoe-/-Myd88+/+ recipient mice (Fig. 2B-D). No differences in plaque composition were observed between the groups, comparing relative contents of macrophages, lipids and collagen in the lesions (data not shown).

MyD88 signaling promotes Th17 generation

Various CD4 T helper cell subsets have been shown to differentially regulate atherosclerosis. To investigate potential mechanisms of the reduced atherosclerosis in mice receiving MyD88-deficient $CD4^+$ T cells, we analyzed T helper cell subsets in the spleen. There was a marked reduction of IL-17 producing Th17 in the spleen of *Myd88-/-* recipient mice compared to *Myd88+/+* recipients (Fig. 2, 3A). Also, double-positive IFNγ^+IL-17^+ cells were reduced in *Myd88-/-* recipient mice (Fig. 3B). IFNγ producing Th1 cells as well as IL-10 producing regulatory cells have been implicated in atherosclerotic disease. The lack of MyD88 in T cells did not influence either of these populations (Fig. 3C-D).

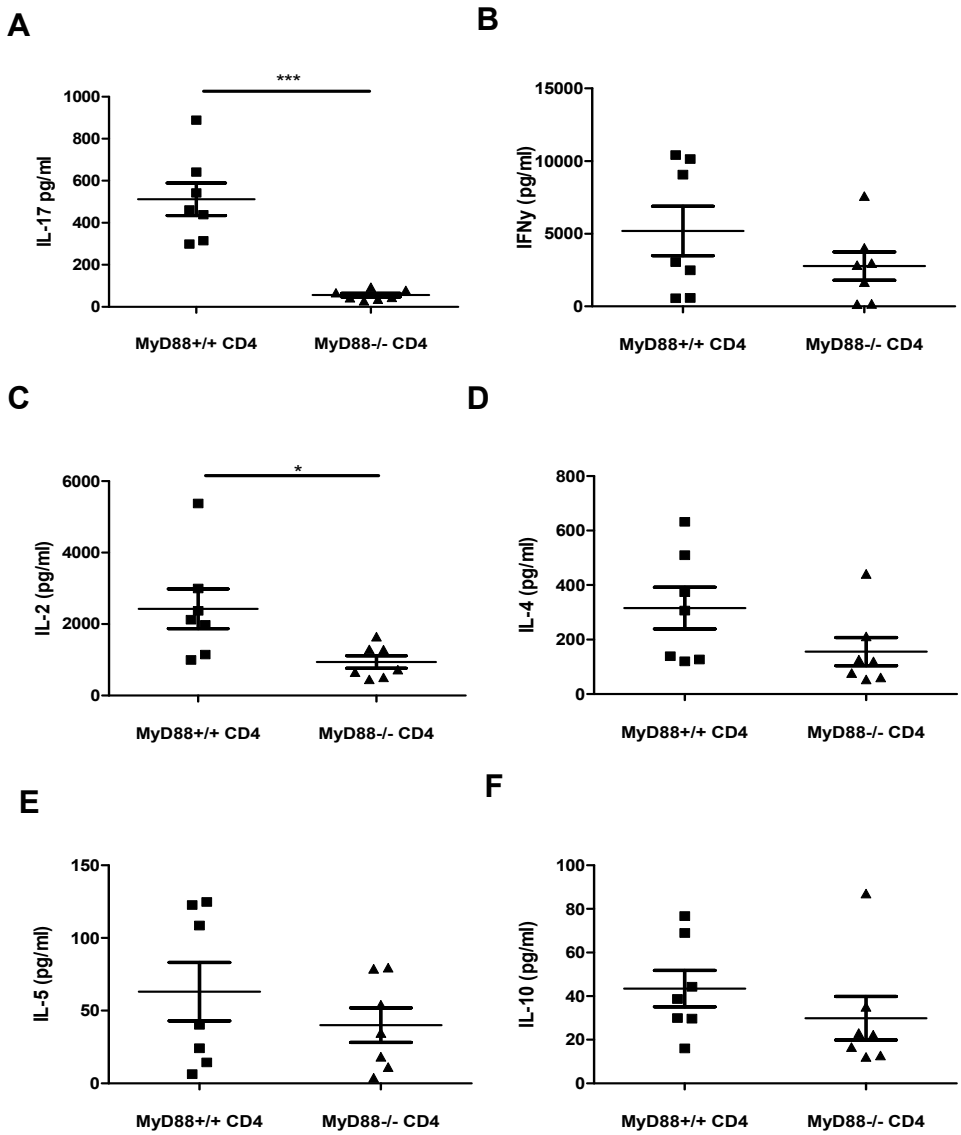

Figure 4. Transfer of MyD88-deficient CD4$^+$ T cells severely decreases IL-17 secretion of stimulated splenocytes. Transfer of *Myd88-/-* CD4+ T cells into *Apoe-/-Rag1-/-* mice resulted in decreased IL-17 secretion upon PMA/ionomycin stimulation, but did not affect Th1, Th2 or Treg cytokines.

In addition, the levels of Tregs in spleens were not influenced by MyD88 signaling (data not show). These findings were supported by an almost abolished IL-17 secretion of PMA and ionomycin stimulated splenocytes in mice receiving *Myd88-/-* T cells, whereas Th2 (IL-4, IL-5), Th1 (IFNγ) or Treg (IL-10) cytokines did not differ between *Myd88-/-* or *Myd88+/+* recipients. Moreover,

IL-2 levels were decreased in *Myd88-/-* recipient mice, indicating reduced T cell proliferation (Figure 4A-F).

MyD88 deficiency in T cells do not effect plasma lipid levels or mouse weight

Apoe-/-Rag1-/- mice receiving $CD4^+$ T cells of either phenotype exhibited reduced weight at sacrifice (20.67 ± 0.47 g for *Myd88+/+* recipient mice and 20.11 ± 0.45 g for *Myd88-/-* recipient mice) compared to untreated *Apoe-/-Rag1-/-* mice (23.15 ± 0.44 g). Similar weights have been reported in *Apoe-/-scid/scid* mice lacking B and T cells transferred with $CD4^+$ T cells (8). However, we did not observe any alterations in physical appearance or increased mortality in mice receiving $CD4^+$ T cells compared to untreated mice. Importantly, there was no difference in weight between *Myd88+/+* or *Myd88-/-* recipient mice.

There was no significant differences in plasma cholesterol or triglyceride levels in non-transferred *Apoe-/-Rag1-/-* mice, *Myd88-/-* or *Myd88+/+* recipient mice (data not shown).

Discussion

Mice lacking the TLR signaling protein MyD88 develop substantially reduced atherosclerosis (2,3). These studies emphasized an important role of MyD88 in macrophages and endothelial cells in atherosclerotic disease. Surprisingly, a recent publication by Subramanian et al reported that transplantation of MyD88-deficient $CD11c^+$ dendritic cells into irradiated *LDLr-/-* mice resulted in *increased* atherosclerotic lesions (9). This was explained by a loss of atheroprotective Tregs. Although MyD88 is a key signaling component of innate immune responses, it is also expressed in T cells. In this study we demonstrate that MyD88-deficiency in $CD4^+$ T cells significantly reduces atherosclerosis in a mouse model of T cell transfer into *Apoe-/-Rag1-/-* mice lacking mature T and B cells.

Previous studies have shown that transfer of $CD4^+$ T cells to *Apoe-/-scid/scid* mice lacking B and T cells results in an increase in early lesions (8,10). In agreement with those studies, we found that *Apoe-/-Rag1-/-* mice transferred with *Apoe-/-* $CD4^+$ T cells displayed increased atherosclerosis (Fig. 1A). Importantly, here we show that transfer of MyD88-deficient $CD4^+$ T cells abolishes the increase in atherosclerotic disease induced by $CD4^+$ T cells. Moreover, the decrease in plaque area induced in *Myd88-/-* recipients is accompanied by a significant reduction in splenic $CD4^+IL\text{-}17^+$ cells and blunted IL-17 secretion (Fig. 4). Thus, our data suggest that, at least in our model, the increased atherosclerosis of $CD4^+$ T cells in immune-deficient mice is dependent on MyD88 signaling in T cells associated with an increase in Th17 cells.

In the vascular wall endothelial cells, smooth muscle cells, macrophages and dendritic cells respond to IL-17 (11). IL-17 activates NF-κB resulting in expression of proinflammatory cytokines, chemokines and matrix metalloproteinases (MMPs). TGF-β, IL-6 and IL-23 are important for differentiation of Th17 cells (11). In the absence of TGF-β signaling, IL-1β in combination with IL-6 and IL-23 efficiently induce IL-17 production in naïve $CD4^+$ T cells (12). Recently, it was shown that MyD88 in T cells is necessary for Th17 cell differentiation and proliferation, whereas Th1 or Th2 cell differentiation was not affected by absence of MyD88 (6,13). In addition, MyD88-dependant IL-1R signaling in T cells was shown to be critical for the Th17 cell differentiation. In agreement, we found less IL-17 producing T cells in spleen and blunted IL-17 production of splenocytes from *Myd88-/-* CD4+ T cell recipient mice. Moreover, neither cell numbers nor cytokines of Th1, Th2 or Tregs differed between *Myd88-/-* or *Myd88+/+* T cell recipient mice. We also found that mice receiving *Myd88-/-* T cells exhibited less $IL\text{-}17^+IFN\gamma^+$ $CD4^+$ T cells. These double-positive cells have been previously described by Annunziato et al (14). In fact, Th17 cells have been shown to be a heterogeneous T cell population capable of producing IL-17 in combination with either IFNγ or IL-10

(11). It is possible that MyD88 signaling is important also for induction of IL17/IFNγ producing T cells.

Several studies have shown a pro-atherogenic effect of IL-17 (15-19), although anti-atherogenic effects of IL-17 also have been reported (20,21). Our data is in line with a pro-atherogenic effect of IL-17. This effect of IL-17 were in previous studies associated with increased inflammation including increased macrophage, T cell or neutrophil infiltration as well as increased levels of VCAM and IL-6. In our study lesions from *Myd88-/-* recipient mice contained less macrophages and lipids (Fig. 1C,D). On the other hand the same lesions also displayed decreased collagen content (Fig. 1B). The latter may be the result of decreased smooth muscle cell migration into the lesions, due to reduced MMP expression. IL-17 induces expression of several MMPs including MMP3 and MMP9 (11). Both MMP3- and MMP9- deficient mice on ApoE-deficient background develop larger lesions, but with reduced smooth muscle cell contents compared to their wildtype counterparts (22). Moreover, MMP3- or MMP9-deficiency reduced neointima formation in a carotid artery ligation model (23).

In conclusion, our data suggest that MyD88 signaling in $CD4^+$ T cells contributes to a proinflammatory Th17 cell differentiation, which increase atherosclerotic disease.

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Paper V

Original Article | Journal of INTERNAL MEDICINE

doi: 10.1111/j.1365-2796.2011.02411.x

High levels of IgM against methylglyoxal-modified apolipoprotein B100 are associated with less coronary artery calcification in patients with type 2 diabetes

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Abstract. Engelbertsen D, Anand DV, Fredrikson GN, Hopkins D, Corder R, Shah PK, Lahiri A, Nilsson J, Bengtsson E (Skåne University Hospital, Lund University, Malmö, Sweden; Cardiac Imaging and Research Centre, Wellington Hospital, London, UK; Malmö University, Malmö, Sweden; King's College Hospital, London, UK; William Harvey Research Institute, London, UK; Cedars-Sinai Medical Center, Los Angeles, CA, USA; David Geffen School of Medicine, UCLA School of Medicine, Los Angeles, CA, USA). High levels of IgM against methylglyoxal-modified apolipoprotein B100 is associated with less coronary artery calcification in patients with type 2 diabetes. *J Intern Med* 2012; **271**: 82–89.

Objective. Advanced glycation end products (AGE) have been implicated in diabetic vascular complications through activation of pro-inflammatory genes. AGE-modified proteins are also targeted by the immune system resulting in the generation of AGE-specific autoantibodies, but the association of these immune responses with diabetic vasculopathy remains to be fully elucidated. The aim of this study was to determine whether antibodies against apolipoprotein B100 modified by methylglyoxal (MGO-apoB100) are associated with coronary atherosclerosis in patients with type 2 diabetes.

Methods. We measured antibodies against MGO-apoB100 in plasma from 497 type 2 diabetic patients without clinical signs of cardiovascular disease. Severity of coronary disease was assessed as coronary artery calcium (CAC) imaging. Immunoglobulin (Ig)M and IgG levels recognizing MGO-apoB100 were determined by enzyme-linked immunosorbent assay.

Results. Anti-MGO-apoB100 IgM antibody levels were higher in subjects with a low to moderate CAC score (≤400 Agatston units) than in subjects with a high score (>400 Agatston units; 136.8 ± 4.4 vs. 101.6 ± 7.4 arbitrary units (AU), $P < 0.0001$) and in subjects demonstrating no progression of CAC during 30 months of follow-up (136.4 ± 5.7 vs. 113.9 ± 6.2 AU in subjects with progression, $P < 0.0001$). Subjects with a family history of premature myocardial infarction had lower levels of anti-MGO-apoB100 IgM. Female subjects had higher levels of anti-MGO-apoB100 antibodies and lower CAC than men. Accordingly, high levels of IgM against MGO-apoB100 are associated with less severe and a lower risk of progression of coronary disease in subjects with type 2 diabetes.

Conclusions. Although conclusions regarding causal relationships based on epidemiological observations need to be made with caution, our findings suggest the possibility that anti-MGO-apoB100 IgM may be protective in diabetic vasculopathy.

Keywords: atherosclerosis, autoantibodies, diabetes.

Abbreviations: AGE, advanced glycation end product; apoB100, apolipoprotein B100; CAC, coronary artery calcium; LDL, low-density lipoprotein; MGO, Methylglyoxal.

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Introduction

Coronary artery disease (CAD) is a major cause of mortality in patients with type 2 diabetes [1]. The mechanisms through which type 2 diabetes contributes to a more aggressive development of atherosclerosis remain to be fully understood, but considerable attention has focussed on the possible role of glucose-derived advanced glycation end products (AGE). The formation of AGE on nucleophilic side chain residues of amino acids (e.g. lysine and arginine side chains) is accelerated by hyperglycaemia [2], and animal studies have shown that the presence of AGE epitopes in atherosclerotic plaques is increased by diabetes [3]. AGE act as ligands for specific cell surface receptors (receptor for AGE; RAGE), and activation of RAGE is associated with enhanced expression of several NF-κB-regulated pro-inflammatory genes [4]. Inhibition of RAGE has been shown to suppress arterial inflammation and development of atherosclerosis in diabetic apolipoprotein E-null mice providing direct evidence for a role of AGE in diabetic vascular complications [5].

Advanced glycation end product–modified structures are also targeted by the immune system, and antibodies against various AGE-modified proteins have been observed in patients with diabetes [6, 7]. Whether immune responses against AGE epitopes in modified self-antigens are pathogenic and contribute to the development of vascular disease or have a protective role remains to be clarified. The role of immune responses against modified self-antigens, particularly oxidized low-density lipoprotein (LDL), in atherosclerosis has attracted increasing attention during recent years [8, 9]. Experimental studies have provided conclusive evidence that Th1-type immunity against self-antigens modified by hypercholesterolaemia is an important driving force in atherosclerosis [10]. However, these studies have also revealed the existence of a protective immunity largely mediated by antibodies and regulatory T cells [11]. Several lines of evidence have suggested that so-called natural phosphorylcholine-specific immunoglobulin (Ig)M antibodies have a protective role [12] and a similar function has been attributed to IgG against certain peptide sequences in apolipoprotein B100 (apoB100) [13]. We have recently shown that high IgG levels against two such sequences, apoB p45 and p210, are associated with less coronary disease in patients with type 2 diabetes [14].

Reactive α-dicarbonyls such as methylglyoxal (MGO) are, along with glucose, key producers of AGE. MGO, which is elevated in individuals with type 2 diabetes [15], is generated by autooxidation of glucose, fragmentation of triosephosphates and catabolism of ketone bodies [2, 16]. Accumulation of an AGE adduct generated by MGO modification of the lysine residue *N*-ε-carboxyethyl-lysine (CEL) has been shown to be increased in plaques of diabetic hyperlipidaemic apoE-null mice [17], and CEL-modified LDL has been detected in immune complexes isolated from patients with diabetes [7]. To study the possible role of immune responses against MGO-modified self-antigens in diabetic macrovascular complications, we determined the relation between IgG and IgM autoantibody levels against MGO-apoB100 and the severity of coronary atherosclerosis as assessed by the degree of coronary calcification in a cohort of 497 subjects with type 2 diabetes.

Materials and methods

MGO-apoB100 antibody enzyme-linked immunosorbent assay (ELISA)

Methylglyoxal-apoB100 was generated by co-incubation of apoB100 (Calbiochem, La Jolla, CA, USA) with 100 mmol L^{-1} MGO (Sigma, St. Louis, MO, USA) in 0.2 mol L^{-1} phosphate-buffered saline (PBS) at 37 °C for 24 h. The protein was subsequently dialysed against 0.15 mol L^{-1} PBS (pH 7.4) before storage at −20 °C. AGE modification was verified by demonstrating a 32-fold increase in AGE-specific fluorescence (excitation 370 nm, emission 440 nm) of MGO-apoB100 relative to native protein and by argpyrimidine fluorescence (excitation 320 nm, emission 380 nm) demonstrating an increase in MGO-apoB100 relative to native protein (106 vs. 0 fluorescence units). CEL epitopes on MGO-apoB100 were measured by ELISA using a monoclonal anti-CEL antibody (KNH-30; Cosmo Bio, Tokyo, Japan). Briefly, MGO-apoB100 was coated onto microtitre plates, and then wells were blocked with Superblock (ThermoScientific, Rockford, IL, USA) and washed, and anti-CEL followed by biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) and alkaline phosphatase-conjugated streptavidin was added. AGE modifications were also assayed using anti-MGO-AGE, primarily recognizing argpyrimidine (antibodies-online, clone 6B) in ELISA as described earlier.

An ELISA measuring IgM and IgG in plasma against MGO-apoB100 was performed essentially according to Fredrikson *et al.* [18], except for coating with

10 μg mL^{-1} protein and the use of streptavidin–alkaline phosphatase from Biolegend (San Diego, CA, USA). Absorbance values were normalized to a control plasma pool (pooled from 11 individuals without type 2 diabetes). The intra-assay coefficient of variation was 11.7% for IgG and 3.6% for IgM, and the inter-assay coefficient of variation was 14% and 16% for IgG and IgM, respectively. Antigen specificity was determined by a soluble-phase competitive ELISA. In brief, native and MGO-modified proteins (apoB100, bovine serum albumin (BSA), human serum albumin or human plasma fibronectin) were added to control plasma (diluted 1 : 5). After 2-h incubation at room temperature followed by overnight incubation at 4 °C, immune complexes were removed by centrifugation at 1000 ***g***. Subsequently, the plasma supernatant was collected, diluted to 1 : 100, added to wells coated with MGO-apoB100 and analysed as above. Antibodies recognizing glyoxal-modified apoB100 were measured in five patients, and the levels were similar to those of anti-MGO-apoB100.

Analysis of AGE proteins in plasma

An ELISA was developed to measure AGE-modified apoB100 in plasma. Wells were coated with polyclonal anti-apoB100 (Abcam, ab 20737-1) and blocked with Superblock. Wells were then incubated with test plasma overnight. Bound AGE-apoB100 was detected with mouse anti-CEL (KNH-30; Cosmo Bio), followed by biotinylated goat anti-mouse IgG (Vector Laboratories) and alkaline phosphatase-conjugated streptavidin. To measure total AGE-modified proteins in plasma, wells were coated with test plasma overnight and blocked with Superblock, and AGE proteins were detected with anti-CEL as described above. In both cases, the amount of AGE proteins was below the detection limit of our assays.

Study population

Plasma samples were derived from 510 type 2 diabetic individuals without pre-existing cardiovascular disease. Thirteen patients were excluded from the analysis because of sample unavailability. Inclusion and exclusion criteria have been described elsewhere [19]. The study was approved by the local research ethics committees of the participating institutions, and all subjects gave informed consent.

Coronary artery calcium imaging

Coronary artery calcium (CAC) imaging was performed using electron beam computed tomography, and coronary calcium levels are given in Agatston units. The CAC imaging protocol [19] and CAC stratification [14, 20] have been previously described. Determination of CAC progression has been previously defined [14].

Statistical analysis

Differences in baseline characteristics were tested using χ^2- or *t*-tests, as applicable. Skewed variables were log-transformed before statistical analysis. Spearman's and Pearson's correlation coefficients were used, as appropriate, to examine associations between continuous variables. Linear regression analysis was used to correct for interferences. $P \leq 0.05$ was considered significant.

Results

Methylglyoxal modification was verified by demonstrating relative increases in both AGE-dependent (370/440 nm) and argpyrimidine-dependent fluorescence (320/380 nm) and antibodies recognizing CEL and argpyrimidine epitopes (Fig. 1a, b) in MGO-apoB100 compared to native apoB100. Next, we determined the specificity of our anti-MGO-apoB100 ELISA. The binding of pooled control plasma to MGO-apoB100-coated ELISA plates was reduced by pre-incubation of the plasma with MGO-apoB100, but not by pre-incubation with native apoB100, MGO-modified BSA, native BSA (Fig. 1c,d) or native and MGO-modified human serum albumin and human plasma fibronectin (Fig. S1), demonstrating the specificity of the assay.

The subjects included in the present analysis were recruited from a cohort of patients with type 2 diabetes participating in a study of the ability of coronary calcium score to predict silent myocardial ischaemia and short-term cardiovascular events. The characteristics of the study group are described in Table 1. All subjects were free of clinical manifestations of CAD at the time of investigation.

Both IgG and IgM against MGO-apoB100 could be detected in almost all subjects (Fig. 2). Anti-MGO-apoB100 IgM antibody levels were higher in subjects with a low to moderate CAC score (≤400 Agatston units) than in subjects with a high CAC score (>400 Agatston units; 136.8 ± 4.4 vs. 101.6 ± 7.4 arbitrary units (AU), $P < 0.0001$, Fig. 3a). Accordingly, there was a significant inverse association between anti-MGO-apoB100 IgM levels and coronary calcification expressed as log CAC ($r = -0.164$, $P < 0.0001$)

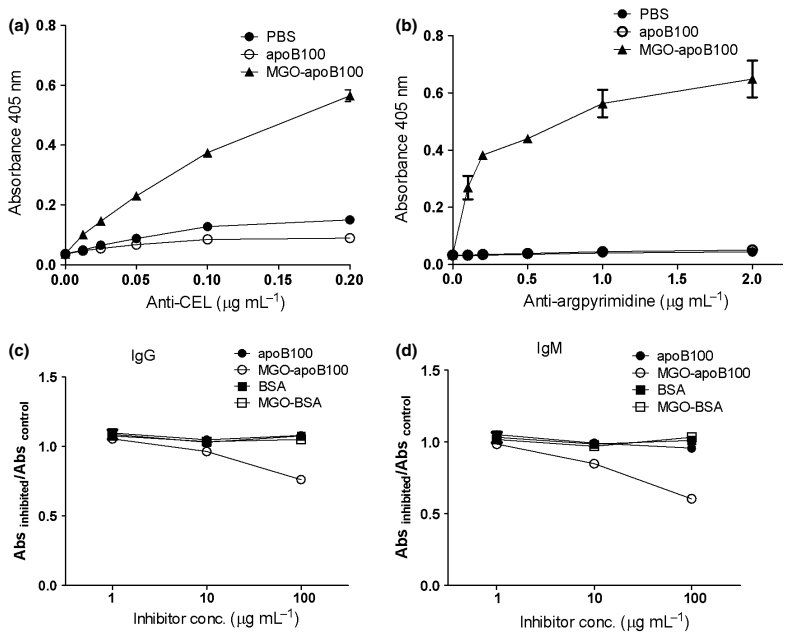

Fig. 1 *Anti-advanced glycation end products ELISA and specificity assessment of anti-methylglyoxal (MGO)-apolipoprotein B100 (apoB100) plasma antibodies. N-ε-carboxyethyl-lysine (CEL) epitopes (a) and argpyrimidine (b) on MGO-modified apolipoprotein B100 (MGO-apoB100) and apolipoprotein B 100 (apoB100) were measured by ELISA. (c, d) Comparison of anti-MGO-apoB100 signal inhibition by pre-incubation of pooled plasma from type 2 diabetic subjects (n = 11) with designated concentrations of apoB100, MGO-apoB100, BSA or MGO-modified BSA. Values are given as the fraction of the absorbance of plasma pre-incubated with protein (Abs inhibited) divided by the absorbance of control plasma (Abs control), for IgM (c) and IgG (d). Each data point (a–d) represents the mean of triplicate wells ± SD.*

(Fig. S2). This association remained significant after controlling for age, body mass index, hypertension, systolic blood pressure, duration of diabetes, HbA1c, LDL cholesterol, high-density lipoprotein (HDL) cholesterol and triacylglycerol using a linear regression model. Follow-up CAC imaging was performed in 398 patients after a mean follow-up time of 2.5 ± 0.4 years. Patients with no progression of coronary calcification had higher levels of anti-MGO-apoB100 IgM antibodies than patients demonstrating progression of coronary calcification (136.5 ± 5.8 vs. 113.9 ± 6.2 AU, $P = 0.008$; Fig. 3b). Patients reporting a family history of premature CAD had lower levels of IgM anti-MGO-apoB100 than subjects with no history of CAD (118.0 ± 7.0 vs. 139.3 ± 4.9 AU, $P = 0.014$). There was no association between the levels of anti-MGO-apoB100 IgG and either severity or progression of CAC (data not shown).

The level of anti-MGO-apoB100 IgM in plasma correlated with age ($r = -0.165$, $P < 0.0001$) and LDL ($r = -0.094$, $P < 0.05$), but neither IgM nor IgG correlated with systolic blood pressure, HDL, triglycerides or HbA1c. Both IgM and IgG anti-MGO-apoB100 antibody levels were associated with the duration of diabetes ($r = -0.109$, $P = 0.015$ and $r = -0.106$, $P = 0.018$, respectively). Female subjects had significantly higher levels of anti-MGO-apoB100 IgM than male subjects (147.5 ± 6.8 vs. 123.0 ± 4.9, $P = 0.003$). When gender was included as a variable in the linear regression model, the association between IgM anti-MGO-apoB100 antibodies and log CAC was no longer significant ($P = 0.079$). Analysing male and female subjects separately revealed that the correlation between IgM and log CAC was significant amongst men ($r = -0.173$, $P = 0.002$; $n = 302$), but not amongst women ($r = -0.071$, $P =$ ns; $n = 195$).

Table 1 *Characteristics of the study group*

Age	52.7 ± 8.4
Sex	195 (39%) women/302 (61%) men
Duration of diabetes (years)	8.1 ± 6.0
HbA1c (%)	8.2 ± 1.7
BMI (kg m^{-2})	28.5 ± 5.0
Systolic blood pressure (mmHg)	137 ± 16
Diastolic blood pressure (mmHg)	84 ± 12
Total cholesterol (mmol L^{-1})	4.8 ± 0.9
LDL cholesterol (mmol L^{-1})	2.7 ± 0.8
HDL cholesterol (mmol L^{-1})	1.3 ± 0.4
Triacylglycerol (mmol L^{-1})	1.9 ± 1.1
C-reactive protein (mg L^{-1})	8.1 ± 30
Current smokers	96 (19%)
Insulin therapy	105 (21%)
Statin therapy	195 (39%)

HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Values are expressed as mean ± standard deviation or number of individuals (percentage of the total group).

Women were also characterized by a lower CAC score than men (95.7 ± 423.6 vs. 278.0 ± 680.9 Agatston units, $P < 0.001$). There was no difference in IgM or IgG antibody levels between pre- and postmenopausal women. Postmenopausal women ($n = 104$) had higher CAC than premenopausal women ($n = 91$) (160 AU ± 57 vs. 21 AU ± 77, $P < 0.0001$), whereas there was no difference in progression of the disease.

We did not find any evidence for significant co-variations between IgM or IgG anti-MGO-apoB100 antibody levels and neuropathy, retinopathy or nephropathy. In addition, there were no significant correlations between microalbuminuria, levels of urea or creatinine and IgM or IgG anti-MGO-apoB100.

Discussion

The results of the present study suggest that patients with type 2 diabetes who have high plasma levels of IgM against the MGO-modified LDL-binding protein apoB100 are characterized by less-severe coronary disease and a lower risk of coronary disease progression. Although conclusions regarding causal relationships based on epidemiological associations need to be made with caution, our findings suggest a possible protective role of anti-MGO-apoB100 IgM in diabetic vasculopathy. This notion is in line with previous studies suggesting a protective role of IgM in atherosclerosis by recognizing modified phospholipids in oxidized LDL [21, 22] and by binding to oxidation-specific epitopes on apoptotic cells [12, 23]. The mechanisms through which these IgM antibodies exert their atheroprotective action are not fully understood but may include facilitation of the removal of damaged cells and lipoproteins. It is an interesting

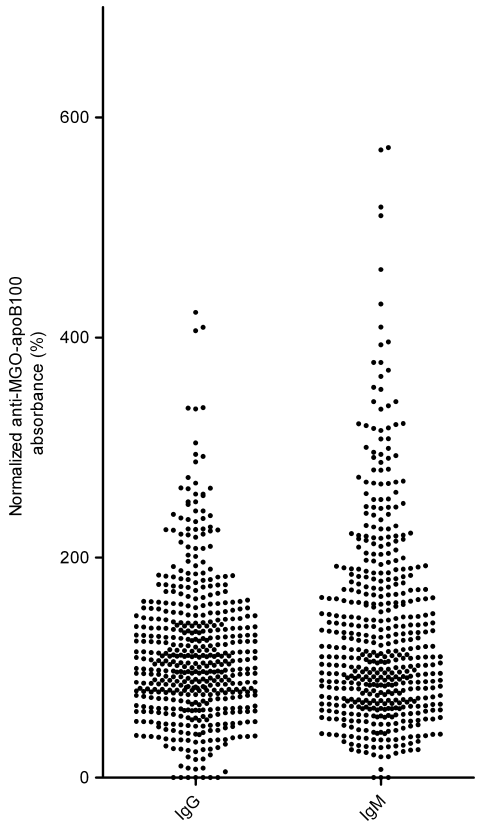

Fig. 2 *Levels of anti-methylglyoxal apolipoprotein B100 in patients with type 2 diabetes. Plasma IgG and IgM antibody levels were measured by ELISA. Absorbance level of each individual sample (n = 497) is expressed as percentage of nondiabetic control plasma (n = 11).*

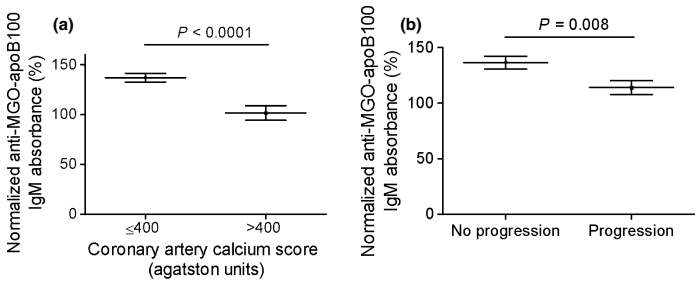

Fig. 3 *Association between anti-methylglyoxal (MGO)-apolipoprotein B100 (apoB100) IgM antibodies and coronary artery calcium score (CAC) in type 2 diabetic subjects. Levels of anti-MGO-apoB100 IgM antibodies in patients with low to moderate CAC (≤400 Agatston units) compared with patients with severe to extensive CAC (>400 Agatston units) (a). Anti-MGO-apoB100 levels in patients with progression of calcification compared to levels in nonprogressors (b). The values are mean ± SEM. Antibody levels are given as percentage of control.*

possibility that IgM recognizing AGE-modified self-antigens may have a similar function either by facilitating the removal of these antigens or by blocking the binding of AGE epitopes to RAGE.

The first evidence for the existence of autoantibodies against AGE-modified self-antigens came from Witztum *et al.* [24] who demonstrated that plasma from patients with diabetes could react with glycated LDL and albumin. Shibayama and colleagues [6] subsequently confirmed this finding and identified the *N*-ε-carboxymethyl-lysine (CML) epitope as a major target for these autoantibodies. They also reported increased levels of autoantibodies against CML-modified albumin in patients with diabetic nephropathy. In the only previous study addressing the association between AGE autoantibodies and cardiovascular disease in diabetes, Lopes-Virella *et al.* [25] found no relation between the level of anti-AGE-modified LDL antibodies and progression of carotid intima-media thickness amongst 1229 patients with type 1 diabetes participating in a follow-up study of the Diabetes Control and Complications Trial. Several factors may have contributed to the different outcomes of the present study and that of Lopes-Virella and colleagues. Whereas the latter study included only patients with type 1 diabetes, the present study was restricted to patients with type 2 diabetes. Accordingly, insulin sensitivity and other metabolic risk factors differ markedly. The epitope specificity of the ELISAs used to determine AGE antibodies was also not the same. Even though the spectra of AGE epitopes formed by glucose and MGO overlap, as MGO is generated during several steps of classical AGE formation by glucose [16], the frequency of MGO-generated epitopes would be greatly enhanced on proteins modified by MGO compared to those modified by glucose. Although LDL and apoB100 are comparable antigens, modification of LDL may generate additional epitopes compared to those formed on apoB100 alone. Finally, the severity of atherosclerosis was assessed by two different techniques at two different locations (i.e. the coronary vs. the carotid arteries) in the two studies.

The level of anti-MGO-apoB100 IgM was found to be significantly higher in female than in male subjects. Women were also characterized by less-severe coronary disease as assessed by the CAC score. Although the association between anti-MGO-apoB100 IgM and CAC was significant only amongst men, the general trend was the same in both groups. It may be that the lower and more narrowly distributed CAC values in the female group make it difficult to detect an association between anti-MGO-apoB100 IgM and CAC in women. In another study, Su *et al.* [22] analysed antibody levels against oxidized LDL and their correlation with atherosclerosis in patients with hypertension. It is interesting that they found higher levels of IgM against oxidized LDL in women than in men, and women also had less plaque.

Of note, we observed an association between family history of premature CAD and low anti-MGO-apoB100 IgM antibody levels, suggesting the possibility of genetic factors in the regulation of these antibodies.

In this study, we found no correlation between IgG antibodies against MGO-apoB100 and CAC. It is interesting to note that several studies have found discrepancies between IgM and IgG antibodies against oxidized LDL and an association with cardiovascular disease [21, 22, 26]. This isotype difference may have several explanations. One possibility could be that IgM antibodies are T-cell independent, whereas most IgG subtypes are T-cell dependent. T cells produce cytokines, which could have effects on plaque formation. In addition, studies in mice support a protective role of IgM in cardiovascular disease; *Ldlr–/–* mice deficient in serum IgM showed a 7-fold increase in plaque area compared with *Ldlr–/–*control mice [27].

Conclusions

In summary, we have demonstrated that high levels of IgM antibodies against MGO-apoB100 are associated with a less-severe coronary disease in patients with type 2 diabetes. These observations provide evidence for a role of immune response against AGE-modified self-antigens in diabetic vascular complications and suggest that IgM recognizing such structures may have a protective function.

Conflict of interest

The authors declare that there is no conflict of interest associated with this study.

Acknowledgements

We are grateful to Margaretha Persson Ph.D. for providing a control plasma pool from diabetic patients. This study was supported by grants from the Swedish Medical Research Council, the Swedish Heart-Lung Foundation, the Söderberg Foundation, the Albert Påhlsson Foundation, the Knut and Alice Wallenberg Foundation, the Malmö University Hospital Foundation, the Åke Wiberg Foundation, the Royal Physiographic Society Lund, the Magnus Bergvall Foundation, the Lundström Foundation, the Crafoord Foundation, VINNOVA, the Foundation for Strategic Research (SSF), the Harrow Cardiovascular Research Trust, the Michael Tabor Foundation, GE Healthcare Ltd, Bristol Myers Squibb Medical Imaging Inc, the Derrick Smith Research Grant and the European Community's Sixth Framework Programme contract ('IMMUNATH') LSHM-CT-2006-037400.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of anti-MGO-apoB100 inhibition by pre-incubation of pooled plasma from type 2 diabetic patients with 100 μg mL^{-1} native or MGO-modified apoB100, human serum albumin (HSA), or human plasma fibronectin. Values are the mean of triplicate wells ± SD.

Figure S2. Scatter plot showing log CAC and normalized levels of IgM antibody against MGO-apoB100.

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Paper VI

Low levels of IgM antibodies against a methylglyoxal-modified apolipoprotein B100 peptide predict cardiovascular events

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Abstract

Aims

Innate and adaptive immune responses play important roles in atherosclerosis and oxidized LDL is one of the major targets of these immune responses. Autoantibodies recognizing oxidized LDL particles have been found both in healthy subjects and in patients with cardiovascular disease. Methylglyoxal (MGO) is a reactive aldehyde formed from oxidation of glucose and polyunsaturated fatty acids. Recently, we demonstrated that IgM against MGO-modified apolipoprotein B100 (apoB100) was associated with less coronary calcium in type 2 diabetic patients. Here, we screened an apoB100 peptide library for peptides targeted by MGO-specific antibodies and determined if autoantibodies against these peptides were associated with the risk for development of cardiovascular events.

Methods and Results

The peptide p220 (amino acids 3286-3305) was identified as one of the major targets in apoB100 for MGO-autoantibodies. Baseline IgM and IgG against MGO-p220 were measured in 700 individuals from the Malmö Diet and Cancer Cohort. A total of 150 cardiovascular events were registered during the 15-year follow-up period. Subjects that developed a cardiovascular event had lower levels of IgM against MGO-p220 (107.7 ± 62.3 versus 121.0 ± 65.4 RU, p=0.014). Controlling for age, sex and major cardiovascular risk factors demonstrated that subjects in the lowest tertile of MGO-p220 IgM had an increased risk for cardiovascular events (hazard ratio 1.81, 95% confidence interval 1.12-2.94, p=0.027 for trend). IgM against MGO-p220 was associated with plasma levels of IL-2, IL-4, IL-5 and IL-13. In addition, IgM against MGO-p220 was inversely associated with plasma glucose.

Conclusion

Low levels of IgM against a MGO-modified apoB100 epitope are associated with a considerably increased risk of cardiovascular events.

Introduction

Atherosclerosis is characterized by retention, aggregation and oxidation of LDL in the arterial wall (1). This activates an inflammatory response and development of atherosclerotic lesions. It has been shown that both innate and adaptive immune responses play important roles in the atherosclerotic disease process (2). Oxidized LDL is one of the major targets of these immune responses. Oxidation of LDL includes oxidation of cholesterol and phospholipids as well as aldehyde-, (malondialdehyde (MDA) and 4-hydroxynonenal) modifications and fragmentation of apolipoprotein B100 (apoB100) (3). Autoantibodies recognizing different parts of the oxidized LDL particle have been identified both in healthy subjects and in patients with cardiovascular disease. Although the exact role of these autoantibodies remains to be fully understood, several studies have shown associations between antibodies against oxidized LDL and cardiovascular disease (3).

In particular, IgM antibodies against MDA-LDL and oxidized LDL have been associated with less severe atherosclerosis in several studies (4-7). However, a recent study by Ravandi et al failed to detect any relationship between anti-MDA-LDL antibodies and cardiovascular events (8). There is evidence from experimental studies that IgM against oxidized LDL has a protective role in atherosclerosis and that this is due to the ability of these antibodies to facilitate uptake and removal of apoptotic cells and modified LDL particles (9).

Diabetic patients have increased risk for cardiovascular disease. Diabetes is associated with increased glycation of many proteins (10). Accordingly glycated apoB100 was shown to be increased in serum of diabetic patients and positively associated both to glycated haemoglobin and fasting glucose concentration (11). Similar to oxidized LDL, autoantibodies against glycated LDL have been identified in immune complexes from diabetic patients (12, 13). In addition, IgG immune complexes containing advanced glycation end product (AGE)-LDL were associated with increased carotid intima-media thickness (IMT) in type 1 diabetic patients (14, 15).

LDL oxidation and LDL glycation are, however, strongly connected. Glycated proteins promote lipid peroxidation reactions. Oxidation of polyunsaturated fatty acids may result in reactive aldehydes inducing AGE-modifications (16). Moreover, small dense LDL, which is considered to be particularly atherogenic, contains an increased amount of glycated apoB100 in both non-diabetic and diabetic patients (17). Interestingly, glycation of LDL results in formation of small dense LDL-like particles (18). Thus, glycated LDL could be an important atherogenic factor also in non-diabetic patients, and autoantibodies recognizing glycated LDL could be important markers of cardiovascular disease also in non-diabetic individuals.

Methylglyoxal (MGO) is a highly reactive aldehyde metabolite arising from glucose, but may also be generated during oxidation of fatty acids (16). We have previously shown that IgM antibodies against MGO-modified apoB100 were associated with decreased coronary artery calcium score in type 2 diabetic patients (19). In this study we screened a library consisting of 302 peptides comprising the complete apoB100 molecule to identify the immunodominant epitopes of MGO-modified apoB100. Then we determined whether autoantibodies against these epitopes could predict cardiovascular events in a clinical cohort including mainly non-diabetic patients.

Methods

Peptide library

Antibodies against modified ApoB100 peptides were screened using a previously described peptide library (20). The library consisted of 302 peptides, each 20 amino acids long with 5 amino acid overlap.

ELISA

Antibodies against modified or native peptides were measured using ELISA as previously described (19). Briefly, ApoB100 peptides were coated to microtiter plates (Nunc MaxiSorp), washed, and subsequently MGO-modified by addition of 100 mM MGO in 0.2 M phosphate buffer (pH 7.4). After blocking with Superblock (Pierce), plasma (dilution 1:100) was added and incubated overnight. Bound antibodies were detected by biotinylated anti-IgM (ICN) and anti-human IgG (Abcam), followed by alkaline phosphatase conjugated streptavidin and absorbance at 405 nm was measured. Absorbance values were normalized against a plasma pool present on each plate and are presented as relative units (RU). Carboxyethyl-lysine (CEL) epitopes on MGO-modified peptides were measured in ELISA using anti-CEL (KNH-30; Cosmo Bio, Tokyo, Japan) as previously described (19).

Peptide selection critera

Native and MGO-modified peptides were screened for IgM and IgG antibody responses. Two selection critera were used for further evaluation of peptides 1) at least a 2-fold increased absorbance value compared to a control peptide (p8), and 2) an antibody response against modified/native peptide ratio above 2.

Study population

The Malmö Diet and Cancer Study (MDCS) is a prospective cohort study examining the relationship between diet and cancer. Subjects born between 1926 and 1945 and living in Malmö were eligible for inclusion. Between October 1991 and February 1994, every other participant was invited to take part in a sub-study focusing on cardiovascular risk (MDCS cardiovascular arm). In the present study, we randomly selected 700 participants, aged 63-68 years (mean age 65), from the cardiovascular arm of MDCS (n=6103). Participants underwent a medical history, physical examination and laboratory assessment as previously described in detail (21). The study was approved by the Regional Ethics Committee in Lund and was conducted in accordance with the Helsinki Declaration. All subjects gave written consent. Baseline characteristics of the study population are presented in Table 1.

B-mode ultrasound

Analysis of common and bulb carotid IMT was performed using an Acuson 128 CT system with a 7-MHz transducer as previously described (22). Common carotid artery IMT area was calculated as described by Wendelhag et al (23) as the difference between the total area inside the adventitia and the lumen area.

Clinical endpoints

We examined the outcome for first cardiovascular events. The procedure for ascertaining outcome events has been described previously (24). Events were identified through linkage of the 10-digit personal identification number of each Swedish citizen with three registries: the Swedish Hospital Discharge Register, the Swedish Cause of Death Register, and the Stroke in Malmö register. Ascertainment of cases and validity of the registries used have been shown to be high (25). A cardiovascular disease (CVD) event was defined as a fatal or nonfatal myocardial infarction (i.e. *International Classification of Diseases* 9th Revisions (ICD-9): 410), fatal or non-fatal ischemic stroke (ICD-9: 434), or death attributable to underlying CHD (ICD-9: 412 or 414), whichever came first. Participants were followed from baseline examination until first event of CVD, emigration from Sweden, death or until December 31st 2008.

Plasma cytokines and Th1/Th2 cells

Plasma cytokines were measured by Meso Scale Discovery multiplex technology. Th1/Th2 cells were determined as previously described (21).

Statistics

An independent sample *t* test was used to assess differences in means of normally distributed continuous variables and a Chi-square test for proportions between cases and controls. Spearman correlation coefficients were used to examine the relationship among continuous variables as appropriate. Linear regression models were used to calculate independent associations. Kaplan-Meier curves were used to illustrate incidence of cardiovascular events in relation to antibody tertiles and differences were analyzed by log rank test. Cox proportional hazard regression was used to examine the association between antibody tertiles and incident cardiovascular events. Plots of the hazard function in different groups over time did not indicate that the proportional-hazards assumption was violated.

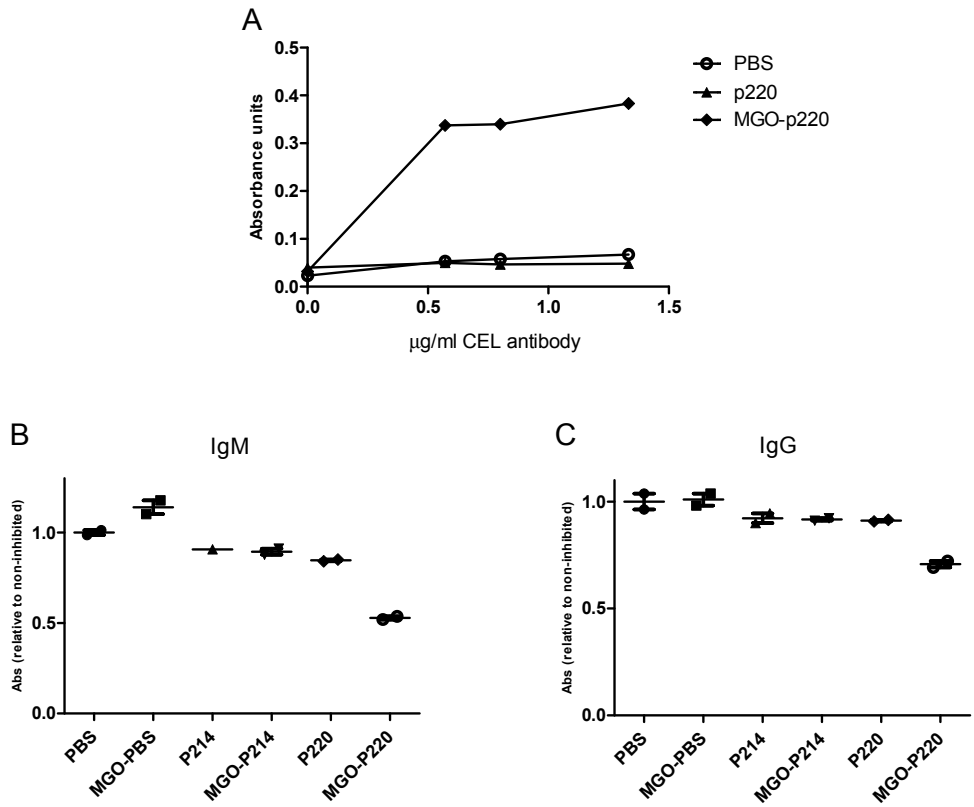

Figure 1. (A) MGO-modification of apoB100 peptide p220 generates carboxyethyl-lysine (CEL)-epitopes detectable by ELISA using anti-CEL antibody. (B-C) Binding of plasma autoantibodies to MGO-p220 could be inhibited using MGO-modified p220, but not native p220 or MGO-modified p214 of apoB100.

Results

MGO-modified apoB100 p220 is recognized by both IgM and IgG antibodies

To find key epitopes recognized by MGO autoantibodies, we screened an apoB100 peptide library, modified with MGO, for MGO-peptides recognized by IgM and IgG antibodies. We searched for MGO-apoB100-peptides targeted by relatively high levels of antibodies that also exhibited at least twice the absorbance compared to antibody levels against the corresponding native peptide. Five peptides (p211, p214, p220, p272 and p279) fulfilled both criteria for IgM binding, whereas four peptides (p14, p210, p211 and p220) fulfilled the same criteria for IgG (data not shown). Thus, two peptides fulfilled the criteria for both IgM and IgG: p211 and p220. Peptide p211 shares 5 amino acids with p210, which has been extensively studied in immune responses against both the native and MDA-modified form (26, 27), prompting us to continue with p220.

Peptide p220 (LKLSL PHFKE LCTIS HIFIP) contains two lysine residues (position 2 and 9), which may be modified by MGO generating CEL epitopes (28). Accordingly, CEL epitopes were detected on MGO-p220 by ELISA (Figure 1A). Anti-MGO-p220 antibodies were found to be specific and the binding could not be competed with another MGO-modified apoB100 peptide (Figure 1B-C).

Lower levels of IgM against MGO-modified apoB100 p220 predicts development of cardiovascular events

Next, we measured IgG and IgM recognizing MGO-p220 in 700 patients enrolled in the Malmö Diet and Cancer Cohort. The cohort has been previously described (see methods). During follow-up, 150 subjects had an incident cardiovascular disease event of which 84 had a coronary event and 66 were strokes. Subjects who suffered a haemorrhagic stroke were excluded when evaluating risk of CVD events because they were considered to have a non-atherosclerotic aetiology (11 out of 66 strokes were haemorrhagic).

Subjects that developed a cardiovascular event during follow-up had lower levels of anti-MGO-p220 IgM antibodies (108 ± 62 RU versus 121 ± 65 RU, p=0.014, Table 1). No association with cardiovascular events was observed for IgG against MGO-p220. Furthermore, incidence of cardiovascular events was associated with anti-MGO-p220 IgM tertiles (log rank test, p for trend=0.016, Figure 2). Subjects in the lowest tertile had an increased risk of developing cardiovascular events compared to the highest tertile, after adjustment for sex, hypertension, diabetes, smoking, prevalent CVD events, anti-diabetic medication, blood pressure lowering medication, fasting venous blood glucose, LDL/HDL, systolic and diastolic blood pressure (Hazard ratio 1.81, 95% CI 1.12-2.94, p=0.027 for trend, Table 2) in Cox regression analysis. This association remained significant also after excluding diabetic individuals (Hazard ratio 2.08, p=0.025) with adjustment for the same risk factors. There was no difference in levels of IgM MGO-p220 between diabetic and non-diabetic individuals (113.0 ± 68.2 RU vs 119.2 ± 26.8 RU, p=n.s.). No association was observed between anti-MGO-p220 IgG and cardiovascular events. Notably, neither anti-MGO-p220 IgM nor IgG were associated with baseline IMT (data not shown).

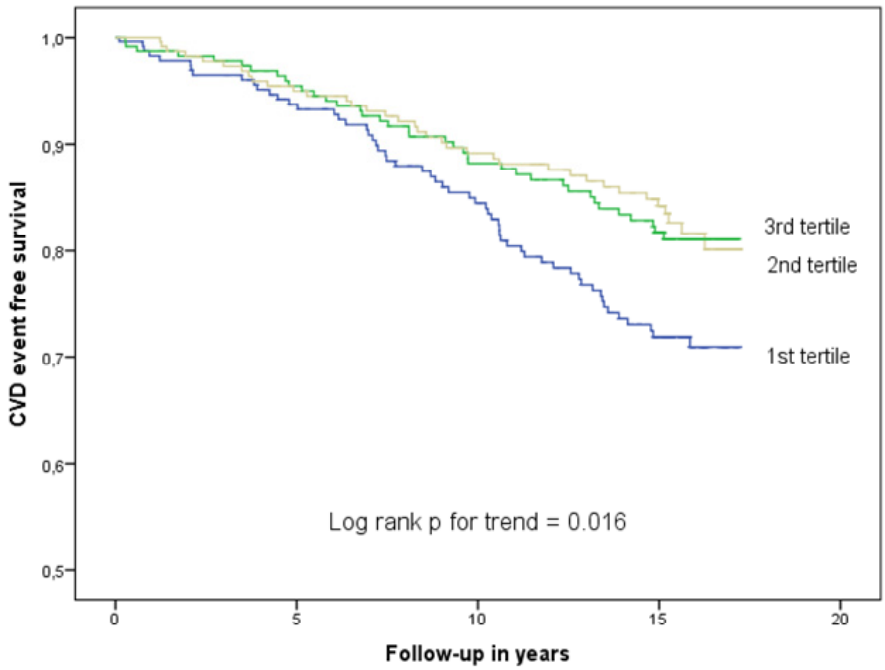

Figure 2. Kaplan-Meier survival curves for tertiles (1st tertile: blue, 2nd tertile: green, 3rd tertile: grey) of anti-MGO-p220 IgM and cardiovascular event-free survival were analysed by a log rank test for trend.

IgM against MGO-modified apoB100 p220 is associated with glucose and plasma cytokines

Methylglyoxal, and downstream adducts like CEL, is believed to be formed *in vivo* by both glycoxidation and lipoxidation (16). To investigate relationships between metabolic markers and antibody levels we performed correlation analysis. Strikingly, IgM antibodies against MGO-p220 were inversely related to glucose but not to LDL, cholesterol, or oxidized LDL (Table 3). In accordance with our previous study measuring IgM against the MGO-modified apoB100, IgM against MGO-p220 were higher in women than in men (data not shown). No gender effect was seen for anti-MGO-p220 IgG.

Previously, Sämpi et al demonstrated that IgM antibodies against MDA-LDL are correlated to IL-5 (29). These antibodies were hypothesized to be natural antibodies since IL-5 administration in mice increases IgM against MDA-LDL and phosphorylcholine (PC) (30). In the present study, IgM antibodies against MGO-p220 were strongly associated to the Th2 cytokines in plasma (IL-4, IL-5, IL-10 and IL-13) as well as IL-2 and IL-12p70, while weaker correlations with markers of classical

inflammation (IFN-γ, IL-1β and TNF-α) were observed. No associations between IgM against MGO-p220 and Th1 or Th2 cells were observed (Table 4). In contrast to IgM, anti-MGO-p220 IgG correlated with inflammatory cytokines TNF- α, IL-1β and IL-8 as well as IL-10 indicating differences in etiology. In addition, IgG against MGO-p220 correlated with number of Th2 cells.

Discussion

Accumulation and oxidation of LDL in the arterial intima is considered to play an important role in the development of atherosclerosis. Short-chain aldehydes like MDA and MGO, formed by lipoxidation and glucoxidation, respectively, modify the apoB100 protein of the LDL particle and generate neoepitopes recognized by the immune system. In this paper we investigated the relationship between autoantibodies recognizing an MGO-modified apoB100 peptide and cardiovascular events in 700 individuals followed for a mean of 15 years. We show that low levels of IgM, but not IgG, recognizing an epitope of MGO-modified apoB100 are associated with increased risk for developing acute cardiovascular events. This association remained significant after adjusting for risk factors (including sex, hypertension, diabetes, smoking, prevalent cardiovascular events, anti-diabetic medication, blood pressure lowering medication, fasting venous blood glucose, LDL/HDL, systolic and diastolic blood pressure), which differed between cases and non-cases in the cohort.

Whether IgM against MGO-p220 play a protective role in cardiovascular disease or merely are markers for the disease remains to be studied. However, there are several possibilities of how these autoantibodies could influence the risk for developing cardiovascular events. Vulnerable lesions are characterized by large lipid cores with foam cells in combination with thin fibrous caps. MGO-modified LDL has been shown to induce foam cell formation *in vitro* (31, 32). It has also been shown that antibodies recognizing oxidized LDL inhibit uptake of oxidized LDL by macrophage scavenger receptor *in vitro* and reduces atherosclerosis in mice (33, 34). Thus autoantibodies against MGO-apoB100 could be protective by blocking the uptake of MGO-LDL in macrophages, and thereby reducing plaque inflammation. About 20% of cardiovascular events are associated with plaque erosion instead of plaque rupture. Because glycated LDL has been shown to both induce apoptosis in endothelial cells (35) as well as to increase platelet aggregation (36) and plasminogen activator inhibitor (PAI-1) levels (37), antibodies against MGO-apoB100 may also protect against plaque erosions and thrombus formation. IgM recognizing MGO-p220 could possibly also mediate atheroprotection via inhibiting binding of MGO-modified LDL to the receptor for AGE (RAGE) present on vascular endothelial cells, smooth muscle cells and macrophages. Interaction of AGE-modified proteins to RAGE results in activation of the proinflammatory transcription factor NF-kβ and increased atherosclerosis. Interestingly, RAGE is proatherogenic also in non-diabetic *LDLr-/-* mice, and oxidized LDL was proposed as ligand for RAGE (38).

The levels of anti-MGO-p220 IgM antibodies were inversely associated with glucose. A possible explanation for this finding is that glucose levels are related to the amount of glycaemic stress and generation of MGO. Excess MGO in lesions reacting with LDL would generate MGO-p220 epitopes that bind antibodies, thus reducing anti-MGO-p220 levels in plasma. The lack of relationship between

anti-MGO-p220 IgM antibodies and LDL/oxLDL suggests that glycoxidation, but not lipoxidation, is primarily associated with the formation of MGO-p220 epitopes.

Although several studies have shown that IgM antibodies against oxLDL are associated with atheroprotection, studies involving IgM against peptides of apoB100 are instead correlated to increased cardiovascular disease. Thus, IgM against MDA-apoB100-peptide 210 was associated with a more rapid progression of atherosclerotic disease (26) and myocardial infarction patients had increased levels of IgM against MDA-apoB100-peptide 210 compared to controls (27). Moreover, IgM against several MDA-modified apoB100 peptides (P45, P102, P129, P210 and P240) were positively associated with intima media thickness of the carotid artery, and negatively associated with plasma oxidized LDL (20). Since the IgM against MGO-P220 in our study was negatively associated with glucose, but did not show any association with LDL or oxidized LDL, it is likely that the discrepancy regarding atherosclerotic disease in our study compared to previous studies of apoB100 peptides is due to differences both in type of modification and in apoB100 peptide sequence.

Several studies have inferred a relationship between IL-5 and natural IgM antibodies. IL-5 has been shown to promote release of natural antibodies in mice (30) and has since been viewed as a cytokine capable of promoting release of natural antibodies. A recent study demonstrated a relationship between IL-5 and anti-MDA-LDL IgM antibodies, strengthening this claim. In addition, IL-5 levels were associated with less atherosclerotic disease (29). In our study IgM recognizing MGO-p220 were strongly associated with plasma IL-5 levels, indicating that these antibodies could be natural antibodies recognizing modified LDL. However, IgM against MGO-p220 were also strongly associated with other Th2 cytokines: IL-4, IL-10 and IL-13. In agreement, both IL-10 and IL-13 have been shown to protect against atherosclerotic disease in mice (39, 40).

In conclusion, we show that low levels of IgM antibodies against an epitope present on MGO-modified ApoB100 is associated with a significant increased risk of cardiovascular disease in a prospective cohort after adjusting for risk factors.

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Tables

Table 1. Baseline clinical characteristics and IgM and IgG levels against MGO-p220

	All n=699	All cases n=150[a]	All non-cases n=549 [a]
Age at screening	65.6±1.2	65.6±1.2	65.6±1.1
Gender (% Male)	41.5%	53.3%***	38.3%
BMI	26.4	26.5±3.9	26.3±4.0
Current smoker	22.1%	24.8%*	16.0%
Diabetes[b]	13.3%	21.3%***	11.1%
Hypertension[c]	81.1%	87.3%*	79.4%
Prevalent CVD events	3.4%	6.7%*	2.6%
Medication			
Anti-diabetic	3.4%	7.3%***	2.3%
Lipid lowering	3.5%	5.6%	2.9%
Blood pressure lowering	24.9%	35.3%***	20.4%
Laboratory parameters			
Fasting venous blood glucose (mmol/L)	5.4±1.5	5.6±1.9**	5.3±1.3
Triglycerides (mmol/L)	1.5±0.8	1.5±0.8	1.5±0.8
HDL (mmol/L)	1.4±0.4	1.3±.04*	1.4±0.4
LDL (mmol/L)	4.4±1.0	4.3±1.1	4.3±1.0
LDL/HDL ratio	3.5±1.2	3.7±1.4*	3.4±1.1
Systolic BP (mmHg)	151±20	154±19*	150±20
Diastolic BP (mmHg)	88±9.2	90±8.8*	88±9.2
hsCRP (mg/L)	3.2±5.5	4.1±6.9	2.9±5.0
Antibodies			
Anti-MGO-p220 IgM (RU)	118±25	108±62*	121±65
Anti-MGO-p220 IgG (RU)	37±30	37±32	38±30

*p<0.05, **p<0.01 and ***p<0.005 for cases versus non-cases.

[a] Mann-Whitney test or χ^2 test for categorical data.

[b] History of diabetes, medication or fasting glucose ≥6.1 mmol/L

[c] Blood pressure ≥ 140/90 mmHg or treatment

Table 2. Hazard ratios and 95% confidence intervals for incident coronary events or ischemic stroke by tertiles of IgM against MGO-p220

Cardiovascular events Odds ratio (95%CI)	1st tertile	2nd tertile	3rd tertile	p for trend
Anti-MGO-p220 IgM				
Unadjusted	1.69 (1.17-2.57)	1.06 (0.67-1.67)	1.00	0.018
Risk factor adjusted	1.81 (1.12-2.94)	1.11 (0.66-1.87)	1.00	0.027
Anti-MGO-p220 IgG				
Unadjusted	1.04 (0.68-1.59)	1.11 (0.73-1.69)	1.00	n.s.
Risk factor adjusted	0.89 (0.56-1.42)	0.96 (0.60-1.53)	1.00	n.s

Associations between tertiles of anti-MGO-p220 IgM antibodies and incident cardiovascular disease was calculated using Cox proportional hazard regression and adjusting for sex, hypertension, diabetes, smoking, prevalent CVD events, anti-diabetic medication, blood pressure lowering medication, fasting venous blood glucose, LDL/HDL, systolic and diastolic blood pressure.

Table 3. Spearman correlation coefficients for laboratory parameters and MGO-p220 antibodies

	MGO-p220 IgM	MGO-p220 IgG
Glucose	-0.121 **	n.s.
Hb1Ac	n.s.	n.s.
Triglycerides	-0.086 *	n.s.
Cholesterol	n.s.	n.s.
LDL	n.s.	-0.087 *
HDL	n.s.	n.s.
oxLDL	n.s.	-0.085 *

The Spearman test was used to calculate r values.

*p<0.05, **p<0.01

n.s.= not significant

Table 4. Spearman correlation coefficients for plasma cytokines and MGO-p220 antibodies

	MGO-p220 IgM	MGO-p220 IgG
IL-1β	0.113 **	0.099 *
TNF-α	0.090 *	0.113 **
IFN-γ	0.085 *	n.s.
IL-2	0.186 ***	n.s.
IL-4	0.174 ***	n.s.
IL-5	0.141 ***	n.s.
IL-8	n.s.	0.100 **
IL-10	0.143 ***	0.098 *
IL-12p70	0.153 ***	n.s.
IL-13	0.228 ***	n.s.
Th1 numbers	n.s.	n.s.
Th2 numbers	n.s.	0.111**

Plasma cytokines were measured by multiplex analysis. The Spearman test was used to calculate r values. *p<0.05, **p<0.01, ***p<0.005. n.s.= not significant