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T-CELL SPECIFICITY AND REGULATION IN ATHEROSCLEROSIS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cardiovascular disease is the main cause of death in the world. The underlying cause in most cases is atherosclerosis, a chronic inflammatory disease. Subendothelial retention of lipoproteins triggers monocyte-derived macrophages and T-helper (Th) 1 cells to form lipid-laden atherosclerotic plaques in the artery wall. The Th1 cells react to autoantigens from the ApoB protein in low-density lipoprotein (LDL) perpetuating the inflammation initiated by the innate immune reactions to modified lipoproteins. Other T-helper cells are also active in the lesions with regulatory T cells (Treg) limiting the injurious inflammation, while the effects of Th17 cells are less clear.

The slow build-up of atherosclerotic plaques is asymptomatic, but eventually the plaque may cause symptoms. Plaque rupture or endothelial erosion induces thrombus formation that causes a heart attack or ischemic stroke. Advanced plaques usually contain large cholesterol-rich necrotic cores. This determines plaque stability along with a stable cap formation by smooth muscle cells and collagen. Prevention of risk factors has reduced mortality, but there is still a need for novel therapies to stabilize plaques and to treat arterial inflammation. The aim for this thesis is to investigate T-cell responses to LDL and regulation of Th cells during atherogenesis. Genetically modified mouse models were used to study LDL-reactive T cells, mechanisms involved in Th cell differentiation, and the subsequent influence on disease development.

Paper I shows how inflammatory signals from the atherosclerotic lesions contribute to Th17 cell differentiation by means of IL-6 and transforming growth factor β (TGF-β). Th17 cells produce IL-17A that promotes collagen synthesis by smooth muscle cells. This paper establishes a plaque-stabilizing role for Th17 cells and IL-17A, which is likely to operate in man and reduce incidence of myocardial infarctions.

Paper II establishes that Tregs have a protective role in atherosclerosis by modulating lipid metabolism. Depletion of Foxp3+ Tregs during atherogenesis impairs lipoprotein uptake by unleashing liver inflammation that downregulates the very low-density lipoprotein (VLDL)-regulating protein called sortilin. This leads to increased plasma cholesterol and development of large atherosclerotic plaques with lipid-filled necrotic cores.

Paper III shows how LDL-reactive T cells survive clonal selection in the thymus, differentiate into T follicular helper cells (Tfh), and promote a protective B-cell response with anti-LDL antibodies. These antibodies mediate lipoprotein clearance and lower plasma cholesterol, which protects against atherosclerosis.

All three papers presented in this thesis illustrate an intricate interplay between the immune system and lipoprotein metabolism, resulting in profound effects on atherosclerosis. These notions may lead to new therapies that stabilize atherosclerotic plaques through specific anti-inflammatory actions that are mirrored by lipid-lowering effects.

LIST OF SCIENTIFIC PAPERS

I. Gisterå A, Robertson AK, Andersson J, Ketelhuth DF, Ovchinnikova O, Nilsson SK, Lundberg AM, Li MO, Flavell RA, Hansson GK.
Transforming growth factor-beta signaling in T cells promotes stabilization of atherosclerotic plaques through an interleukin-17-dependent pathway.
Sci Transl Med. 2013; 5(196):196ra00.

II. Klingenberg R, Gerdes N, Badeau RM, Gisterå A, Strodthoff D, Ketelhuth DF, Lundberg AM, Rudling M, Nilsson SK, Olivecrona G, Zoller S, Lohmann C, Lüscher TF, Jauhiainen M, Sparwasser T, Hansson GK.
Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis.
J Clin Invest. 2013; 123(3):1323-34.

III. Gisterå A, Klement MR, Mailer RK, Polyzos KA, Duhlin A, Karlsson MC, Ketelhuth DF, Hansson GK.
LDL-reactive T cells protect against atherosclerosis by inducing lipid-lowering antibodies.
Manuscript.

Note: In Paper I and II the two first authors contributed equally. In Paper I, the two last authors contributed equally.

OTHER RELATED PUBLICATIONS

Polyzos KA, Ovchinnikova O, Berg M, Baumgartner R, Agardh H, Pirault J, Gisterå A, Assinger A, Laguna-Fernandez A, Bäck M, Hansson GK, Ketelhuth DF.
Inhibition of indoleamine 2,3-dioxygenase promotes vascular inflammation and increases atherosclerosis in Apoe-/- mice.
Cardiovasc Res. 2015; 106(2):295-302.

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Methods Mol Biol. 2015; 1339:149-59.

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
Apo	Apolipoprotein
BCR	B-cell receptor
BT	ApoB-reactive T cell
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CETP	Cholesteryl ester transfer protein
CRP	C-reactive protein
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXCR	C-X-C motif receptor
CYP7A1	Cholesterol 7 alpha-hydroxylase
Fc	Fragment, crystallizable
FH	Familial hypercholesterolemia
Foxp3	Forkhead box P3
FXR	Farnesoid X receptor
GFP	Green fluorescent protein
GPIHBP1	Glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1
HDL	High-density lipoprotein
HLA	Human leukocyte antigens
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HSP	Heat shock protein
HuBL	*Human APOB100-transgenic Ldlr$^{-/-}$*
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LDL	Low-density lipoprotein

Ldlr$^{-/-}$	Low-density lipoprotein receptor knockout
LPL	Lipoprotein lipase
LXR	Liver X receptor
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
oxLDL	Oxidized low-density lipoprotein
PCSK9	Proprotein convertase subtilisin/kexin type 9
PD-1	Programmed cell death protein 1
PLTP	Phospholipid transfer protein
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
Rag	Recombination-activating gene
RORγt	RAR-related orphan receptor gamma t
RXR	Retinoid X receptor
SMAD	Small mothers against decapentaplegic
SR	Scavenger receptor
SREBP	Sterol regulatory element-binding protein
T-bet	T-box expressed in T cells
TCR	T-cell receptor
Tfh	T follicular helper cell
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRA	Tissue-restricted antigen
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low-density lipoprotein

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system protects us against foreign pathogens such as bacteria, viruses, parasites, and fungi [1]. When an infection occurs, the immune system fights the pathogen and clears the infection. A hallmark of immunity is a specific memory that distinguishes self from non-self. The basis for this is antigen recognition. Physical and chemical barriers prevent potential pathogens from entering through the skin, lungs, and gut. Cells in these locations take an active part in the defense before the immune system is engaged and secrete antimicrobial peptides, produce mucus, and have cilia that impede infections. In addition, commensal bacteria exist in concordance with the host and prevent pathogens to take foothold [2].

The immune system is commonly divided into an innate and an adaptive part. The innate system is fast, but unspecific and calls for the attention of the slower adaptive system that is specific. The two systems communicate with cell-to-cell interactions and soluble molecules such as chemokines and cytokines. Activation of the immune system leads to inflammation. In the 1st century, Celsus described four signs of inflammation: calor, dolor, rubor, and tumor (heat, pain, redness, and swelling) [3]. Later, functio laesa, the disturbance of function, was added as a fifth cardinal sign of inflammation [4]. Apart from infections, the immune system has an important function to maintain host homeostasis through tissue repair and tumor surveillance. When the infection is cleared, inflammation resolves by regulatory mechanisms, one being wound-healing [5].

1.1.1 The innate immune system

The innate immune system recognizes common characteristics of pathogens [6]. This defense system is particularly present at sites where pathogens are expected, allowing a fast response to take place instantly when a pathogen eludes the first line of barrier defense. The innate immune system relies on pattern recognition receptors (PRR). These receptors are germ-line encoded and recognize pathogen-associated molecular patterns. Toll like receptors (TLR) on innate immune cells are PRRs that convey one of the first signals that start the immune response. These receptors belong to the most evolutionarily conserved and ancient part of the immune system [7]. Some TLRs recognize extracellular components of bacteria, such as lipopolysaccharides. Other TLRs are intracellular and control the endolysosomal compartment for invading viruses or intracellular bacteria [8]. Scavenger receptors are extracellular PRRs that recognize foreign material and initiate its internalization and destruction [9]. These receptors also take part in housekeeping duties in the body, such as clearing modified lipoproteins.

Soluble PRRs circulate in the blood as part of the complement system. These proteins are ready to directly lyse cells or activate cellular components of the innate system [10]. This network of proteins recognizes pathogen surfaces. A reaction cascade forms complexes that attack and puncture the membrane of pathogens. The complement system also facilitates

phagocytosis of bacteria by opsonization [11], activates inflammatory responses, and helps in clearing immune complexes that consist of antibodies bound to antigen.

Main cellular components of the innate immune system are monocytes, macrophages, granulocytes, mast cells, dendritic cells, and innate lymphoid cells. Monocytes and neutrophil granulocytes are phagocytic cells that are rapidly recruited to the site of infection. The initial inflammatory response consists of extravasation mediated by IL-1, tumor necrosis factor (TNF), and chemokines [12]. Endothelial cells upregulate adhesion molecules and circulating neutrophils and monocytes adhere to the endothelial surface and infiltrate the underlying tissue. Chemoattractants guide the infiltrating cells to the site of infection where the pathogen is attacked and ingested by phagocytosis [13]. The monocytes differentiate into macrophages after entering the tissue. Mast cells share common characteristics with basophil granulocytes, but reside locally in tissues close to potential infection sites. They express FcεRI receptors with high affinity for Immunoglobulin E (IgE) antibodies. IgE therefore coats mast cells. When antigen-specific IgE on the mast cell surface cross-links an antigen, a signal through FcεRI leads to degranulation and release of histamine as well as other pro-inflammatory mediators and enzymes. This occurs in a common allergic reaction when IgE antibodies are directed against an allergen [14].

Dendritic cells are professional antigen presenters and specialized in internalizing foreign antigens [15]. They patrol the tissue and take up potential pathogens that are then degraded in the phagolysosome and presented to T cells after migration to lymph nodes and spleen. Also macrophages act as antigen presenting cells (APC) and express MHC class II. Dendritic cells can be divided into three subgroups: classical, plasmacytoid, and monocyte-derived. Plasmacytoid dendritic cells are a rare subset that produce large amounts of type I interferons (IFN-α/β), which are important during virus infections [16].

Natural killer cells belong to the innate lymphoid cell family and are cytotoxic effector cells important for tumor surveillance and fighting viruses [17]. They identify and kill cells that have downregulated MHC class I to evade regular immune surveillance [18]. Other innate-like lymphocytes are B1 cells, marginal zone B cells, $\gamma\delta$ T cells, and natural killer T cells. These cells resemble adaptive immune cells, but usually reside in special compartments of the body ready to recognize and neutralize antigens or evoke immune reactions. The specificities of B1 cells and natural killer T cells are largely against evolutionary preserved antigens in pathogens, such as complex lipids [19].

1.1.2 The adaptive immune system

The adaptive immune system acquires immunity against pathogens and remembers this initial recognition for later encounters with the same pathogen. It is called upon when the innate system is incapable of handling the intruding pathogen alone. The adaptive response requires gene rearrangements that store the antigen specificity within the genetic code. This makes the system slow with a response peak usually one or two weeks after the first encounter with a foreign pathogen, but memory makes the time shorter the next time the host encounters the

same pathogen. The secondary immune response is much faster, and the pathogen is usually handled before general symptoms arise.

The adaptive immune system consists of lymphocytes developed from progenitor cells in the bone marrow. These cells carry either T-cell receptors (TCR) or B-cell receptors (BCR), which are epitope-specific antigen receptors. T cells with TCRs are the basis for the cellular immunity [20], and B cells with BCRs that can be secreted as antibodies, form the humoral immunity. T and B cells interact in an antigen-specific manner [21]. This potent system needs to be tightly controlled in order to avoid excessive tissue destruction when fighting an infection. The specificity of the lymphocytes ensures that the system only attacks infected tissues, and selection processes in the primary lymphoid organs eliminate self-reactive lymphocytes [22]. The neonatal period is especially important for these processes [23]. Selection might fail under certain conditions, which leads to uncontrolled immune responses against self-antigens. This occurs in autoimmune diseases, but there are a number of peripheral tolerance mechanisms that also limit the immune response to self-antigens.

1.1.2.1 Humoral immunity

The humoral immune response targets mainly extracellular microbes. B-lymphocytes release antibodies recognizing different protein, lipid, and carbohydrate antigens. B cells develop in the bone marrow, and their development in the bursa of Fabricius in birds has given them their name [24]. Conventional B cells, also called B2 cells, are derived from hematopoietic stem cells in bone marrow and fetal liver. A key regulator of B-cell development in the bone marrow is IL-7 [25]. This cytokine differentiates the hematopoietic stem cells into lymphoid progenitors, and it also has a key role later in the maturation process.

Antibodies contain two antigen-binding fragments, which are formed by an immunoglobulin heavy chain and an immunoglobulin light chain. The heavy chains also form a crystallizable fragment (Fc) that binds various cellular receptors or complement proteins, which ensure that the antibody elicits an appropriate immune response when bound to antigen. Somatic recombination of the antigen receptor genes first occurs in the immunoglobulin heavy chain locus [26]. Recombination by the RAG-1 and RAG-2 proteins induces a unique receptor through the assembly of a variable (V), diversity (D), and joining (J) gene segment, with addition of untemplated nucleotides in the junctions. After the V(D)J recombination, the immunoglobulin heavy chain is expressed to form a pre-BCR. Cells with a functional pre-BCR pass this positive selection process and move on to recombination of the immunoglobulin light chain locus.

Before the B cells exit the bone marrow, a negative selection process occurs to identify self-reactive cells [27]. Cells that pass the negative selection process can exit to the blood and circulate in secondary lymphoid organs as IgM+ immature B cells. Cells that fail the negative selection will undergo receptor editing of the immunoglobulin light chain. B cells presenting an altered BCR that remains autoreactive are eliminated.

Activation of B cells in secondary lymphoid organs depends on three signals: (i) recognition of the antigen by the BCR; (ii) interaction with T cells through MHC-TCR; and (iii) costimulatory receptors such as CD40L and cytokine stimulation such as IL-6, IL-21, B-cell activating factor, and a proliferation-inducing ligand. Activated B cells form germinal centers in order to develop high affinity BCRs through somatic hypermutation. This typically occurs in the white pulp in the spleen, where B cells interact with the antigen and Tfh cells. IL-4 and IL-21 from the Tfh cell provide survival signals to B cells presenting the highest affinity for the antigen. High-affinity B cells, with the best antigen-presenting capability, undergo clonal expansion, while lower-affinity B cells die. Germinal center B cells can be identified through the expression of CD95 and the GL7 ligand as well as by binding of peanut agglutinin. The antigen-binding fragment (Fab) of the BCR consists of three complementarity-determining regions (CDR). These regions have the highest frequency of mutations during somatic hypermutation. Activation-induced cytidine deaminase induces enzymatic mutations in these hypervariable regions, and an error-prone DNA polymerase repairs the segment causing modifications of the receptor [28].

The constant region of the immunoglobulin heavy chain defines the class of the antibody. Class switch recombination between different switch regions in the immunoglobulin heavy chain gene locus is determined by the cytokine milieu and changes the antibody isotype to IgA, IgE, or IgG. TGF-β induces a switch to IgA that is secreted at mucosal sites. IL-4 induces a switch to IgE that binds receptors on mast cells and is important in combating parasites. There are several IgG subtypes. IFN-γ gives an IgG3 switch in humans and an IgG2a switch in mice. IL-4 gives IgG1 and IgG4 in humans and IgG1 in mice. The isotype dictates the effector functions of the antibody [29]. The Fc-part of the IgG has different affinities for Fc-receptors and different abilities to activate the complement system.

Differentiation of B cells can finally lead to plasma cells that secrete large amounts of antibodies [30]. Plasma cells express CD138, and long-lived plasma cells reside in the bone marrow. A small percentage of activated B cells differentiate into memory B cells, which are ready to be activated upon a new infection by the previously encountered pathogen.

1.1.2.2 Cellular immunity

T cells develop in primary lymphoid organs. Common lymphocyte progenitors in the bone marrow mature and are recruited to the thymus where they develop into naïve T cells [31]. The TCR resembles immunoglobulin [32] and uses RAG-dependent recombination to induce diversification and specificity to the receptor. An α-chain and a β-chain form the αβ-TCR-heterodimer. The TCR on γδ T cells similarly consists of a γ and a δ-chain. Each α- and β-chain consists of a constant region and a variable region with three CDRs. CDR3 is the most important region for antigen recognition. The β-chain has an additional hypervariable area (HV4), but it does not take part in regular antigen recognition and is not considered a CDR. The TCR-β-chain is ontogenetically similar to the immunoglobulin heavy chain in the BCR, but lacks the Fc-part, and the TCR-α-chain is similar to the immunoglobulin light chain. The

generation of the TCR starts with V(D)J recombination of the β-chain locus. The generated β-chain is paired with a pre α-chain and if this pre-TCR is functional, the recombination continues with the α-chain locus. The successful formation of a β-chain also shuts down recombination of the second β-chain locus, a mechanism called allelic exclusion. When the αβ-TCR is successfully formed, the cell starts to express both CD4 and CD8 molecules, and enters the double positive phase of maturation in the thymus.

Cortical thymic epithelial cells (cTEC) express MHC class I and II. These molecules associate with self-peptides and interact with the T-cell precursors [33]. In a positive selection process, more than 90% of the immature thymocytes undergo apoptosis due to lack of affinity to MHC. The remaining cells with TCRs, with the ability to bind MHC, survive, and the continued expression of CD4 or CD8 is decided in this process. Depending on their TCR interaction with MHC class I or II, the cells develop into CD8-single positive or CD4-single positive T cells, respectively. These molecules are important co-receptors of the TCR-complex and take part in intracellular phosphorylation during T-cell activation [34].

Medullary thymic epithelial cells (mTEC) control the negative selection crucial for T-cell tolerance induction. These cells express the autoimmune regulator (AIRE) transcription factor, which enables expression of tissue-restricted antigens (TRA) [35]. Peptides from these TRAs are loaded on MHC, while exogenous loading pathways are hampered. This allows a screen and elimination of T cells that recognize self-peptides. Each TRA is only expressed by a minor subset of mTECs. The single positive thymocytes perform a thorough scan of several mTECs over a 5-day period in the medulla. Dendritic cells and cTECs also participate in the negative selection process. The TCR signaling strength decides the fate of the cells [36]. The majority of high affinity single positive cells undergo apoptosis. Natural Tregs develop from cells with intermediate TCR signaling strength that upregulates Foxp3. Tregs maintain homeostasis and prevent immune reactions to self-antigens. Autoimmunity may occur when this central tolerance mechanism fails. The cells with low TCR signaling do not recognize self-peptides and mature into conventional naïve T cells.

The TCR heterodimer has only a short intracellular part and needs to form a complex with CD3, which transmits the TCR activation signal intracellularly. The signal transduction starts a cascade of kinases and phosphatases leading to phosphorylation of Zap70, which attracts phospholipase C-γ. Next, transcription factors are activated. Nuclear factor of activated T-cells (NFAT) is released in the nucleus and starts transcription of a broad range of genes such as IL-2. IL-2 is needed for long-term T-cell activation and proliferation [37].

The priming of naïve T cells occurs in secondary lymphoid organs and is MHC-dependent [38]. Several TCR-MHC complexes need to signal simultaneously to activate the T cell, while adhesion molecules stabilize the interaction between the T cell and APC. The TCR-MHC interaction provides the first activation signal. The T cell also needs costimulation from ligands such as CD80 and CD86 expressed by the APC. These molecules ligate the costimulatory molecule CD28 on the T cell. This gives the second activation signal and

allows the T cell to initiate cell division and upregulate CD25, a high-affinity IL-2 receptor [39]. IL-2 is crucial for maintaining T-cell activation and drives clonal expansion. The T-cell production of IL-2 forms an autocrine loop [40]. This is the third signal that is needed for T-cell activation, along with cytokines provided by the APC, crucial for the activation and differentiation into effector T-cell subsets (Fig. 1).

CD8+ cytotoxic T cells screen nucleated cells for the presence of intracellular pathogens. Cytoplasmic proteins are digested in the proteasome and loaded on MHC class I, which is then transported to the cell surface. If the cell presents peptides on MHC class I that are identified as foreign, e.g., damaged self-peptides or derived from intracellular bacteria and viruses, the cell is killed. Damaged self-peptides are more common in tumor cells, and CD8+ T cells prevent tumor development. Normal self-peptides are tolerated through the thymic education process and are not recognized by the CD8+ T cells in the periphery. MHC molecules in man are called human leukocyte antigens (HLA). Three separate loci encode MHC class I: *HLA-A*, *HLA-B*, and *HLA-C*. The homologues in mice are *H2-D*, *H2-K*, and *H2-L*. MHC class I consists of an α-chain linked to the non-polymorphic protein, β_2-microglobulin. The α-chain contains a peptide-binding groove that fits an 8-11 amino acid long peptide. The groove needs to be occupied to stabilize the complex. Another domain of the α-chain recognizes the CD8 co-receptor of the TCR complex and restricts MHC class I activation to CD8+ T cells. CD1d is another antigen presenting molecule that resembles MHC class I, but presents glycolipids to natural killer T cells.

Dendritic cells, B cells, and macrophages are primarily responsible for presentation of extracellular antigens. These cells internalize microbes and foreign material by endocytosis. Internalized proteins are degraded in lysosomes and peptides generated in this process associate with MHC class II. The peptide-MHC complex is then transported to the cell surface where T cells can recognize the potential antigen. Humans have three MHC class II loci: *HLA-DP*, *HLA-DQ*, and *HLA-DR*. These loci contain polymorphic genes that ensure efficient antigen presentation of a broad range of peptides. The mouse homologues are *H2-M*, *H2-A*, and *H2-E* with the latter usually called *IA* and *IE* since they were initially named immune response genes. The MHC class II molecule consists of an α-chain and a β-chain, where a peptide groove is formed between the chains. The fitted peptides are usually 15 amino acids long, but there is no steric hindrance for longer peptides. Similar to MHC class I, only complexes with a bound peptide are stable on the cell surface. The β-chain contains a domain binding CD4, which restricts MHC class II presentation to CD4+ T-helper cells.

1.1.3 T-cell subsets

Naïve T cells express high levels of the surface molecule CD62L, a homing receptor for the T cells to enter secondary lymphoid tissues. CD62L binds glycosylation-dependent cell adhesion molecule-1 expressed in high endothelial venules. When a T cell finds its antigen, it differentiates into an effector T cell, proliferates, and secretes cytokines. CD4+ T cells differentiate into T-helper cells, and cytotoxic T cells are differentiated from CD8+ T cells.

The activated T cell downregulates CD62L and upregulates the cell adhesion molecule CD44, a marker for effector/memory T cells. Central memory T cells express both CD62L and CD44, together with CCR7, and are retained in the secondary lymphoid organs [41]. T effector/memory cells that lack CCR7 expression migrate to tissues. Integrins on their cell surface recruit them to inflammatory sites where the endothelium expresses adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1). The threshold for reactivation of effector T cells in the periphery is lower than for naïve T cells.

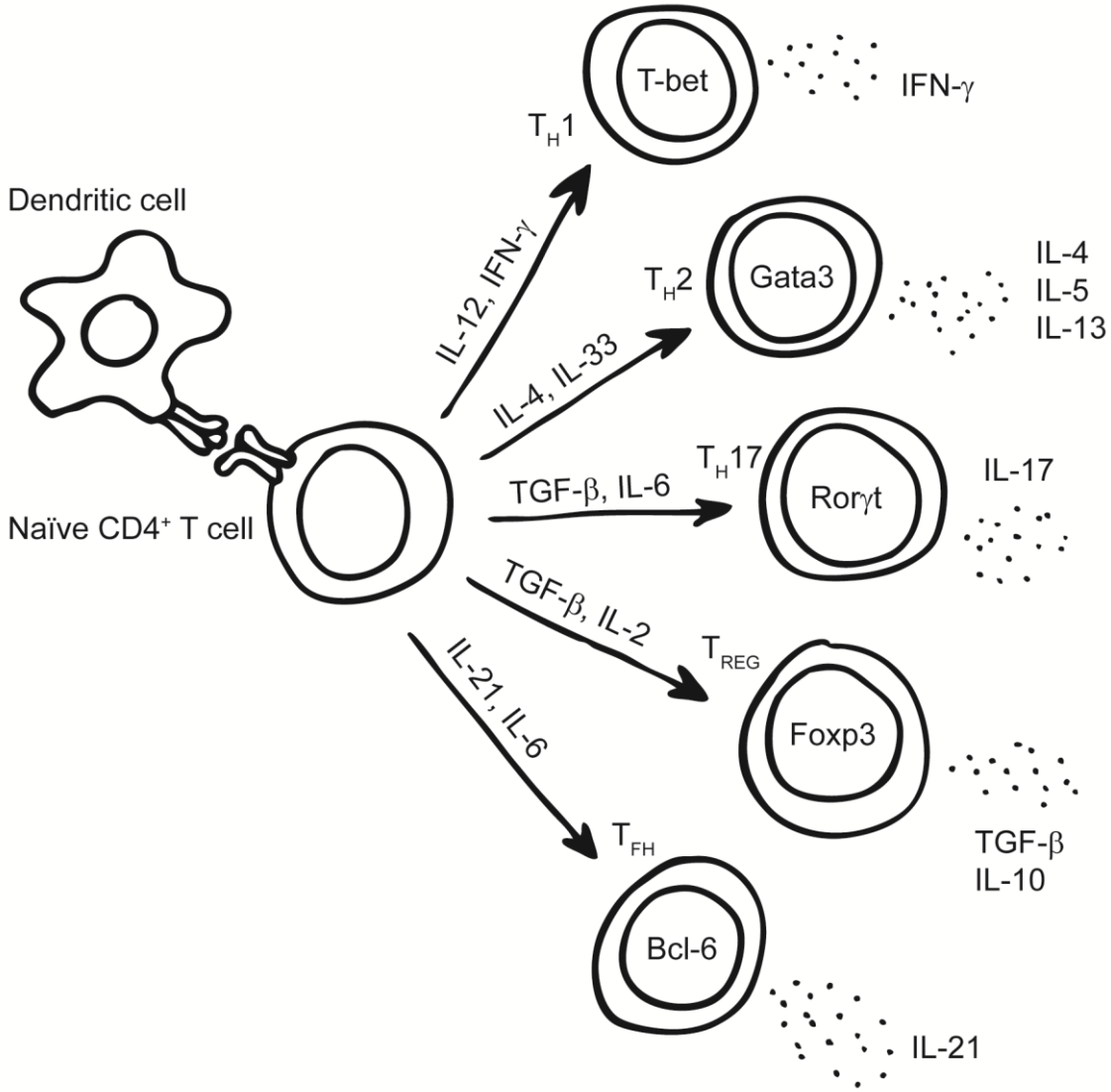

Figure 1. T-helper cell differentiation. Antigen is presented by a dendritic cell to a naïve CD4+ T-helper cell, which gets activated and differentiates into different subsets depending on the local cytokine milieu. Th1 cells are important for immunity to viruses, intracellular bacteria, and parasites. Th2 cells are important for immunity to extracellular parasites, including helminths. Th17 cells are important for immunity to extracellular bacterial and fungal infections. Tregs are involved in regulation of immunity and tolerance mechanisms. Tfh cells help B cells.

1.1.3.1 Cytotoxic T cells

CD8+ cytotoxic T cells are important in fighting viruses, intracellular bacteria, and tumor cells. They release perforin, granzymes, and granulysin when they identify an infected cell.

These cytotoxins enter the targeted cell, which lead to apoptosis through serine protease activity. Cytotoxic T cells can also induce cell death through FAS ligand expression that binds CD95 (also known as FAS receptor) on the target cell.

1.1.3.2 T-helper 1 cells

CD4+ T-helper cells can differentiate into different subsets depending of factors in the local milieu. The properties of Th1 and Th2 cells were the first to be described [42]. Th1 cells fight intracellular bacteria, viruses, and protozoa. Differentiation is driven by IL-12 and IFN-γ and leads to expression of the transcription factor T-bet encoded by the *TBX21* gene. T-bet is a Th1 specific transcription factor that controls the hallmark cytokine of Th1 cells, IFN-γ [43]. IFN-γ can inhibit viral replication directly, but can also cause delayed type hypersensitivity and is associated with several autoimmune disorders [44, 45]. It upregulates MHC class II on APCs and activates pro-inflammatory responses of macrophages. Th1 cells can also provide help to B cells through CD40L expression and promote IgG isotypes efficient in opsonization.

1.1.3.3 T-helper 2 cells

Th2 cells are specialized in fighting extracellular parasites and cause eosinophilic inflammation. The influence of IL-4 on activated T cells leads to expression of the transcription factor GATA3, which induces differentiation to the Th2 lineage, while suppressing Th1 pathways [46]. This leads to production of IL-4, IL-5, and IL-13. IL-33 can also induce expression of these Th2-related cytokines [47]. Th2 cells are important for the humoral immune system and promote class-switching and production of neutralizing antibodies. Th2 cells are responsible for allergic inflammation, such as asthma, through the effects of IL-4 on B cells that promote IgE antibody production [48].

1.1.3.4 Th17 cells

A third important subset of T-helper cells was named Th17 cells for their ability to produce IL-17 isoforms [49, 50]. IL-6 together with TGF-β induces Th17 cell differentiation [51]. IL-23 has an important role in the expansion and survival of Th17 cells. IL-1β and TNF can further amplify Th17 differentiation. A specific isoform of retinoic acid-related orphan receptor gamma in T cells (RORγt) orchestrates Th17 cell differentiation [52]. Activated Th17 cells are specialized in fighting extracellular bacteria and parasites. Their signature cytokine is IL-17A, but also IL-17F, IL-21, and IL-22 are produced. IL-17A has pro-inflammatory actions. It induces local CCL20 expression, a chemoattractant that recruits more Th17 cells to the site due to their expression of the CCL20-chemokine receptor CCR6 [53]. Several studies have implicated involvement of Th17 cells in autoimmune diseases like multiple sclerosis, psoriasis, and rheumatoid arthritis [54-56]. Recently, IL-17A has been reported to have fibrogenic properties with an important role in wound-healing and liver fibrosis [57, 58].

1.1.3.5 Regulatory T cells and peripheral tolerance mechanisms

Tregs are important in immune homeostasis. Investigations initially described a suppressor activity that could restrain activation and proliferation of T cells [59]. The mechanism was difficult to pinpoint until CD25 was identified as a cell-surface marker of cells carrying this activity [60]. The cells could then be purified and studied in detail. Forkhead box P3 (Foxp3) is the key regulatory transcription factor of Tregs [61]. Mutations in the *FOXP3* gene leads to the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans [62] and mice with a mutation in the *Foxp3* gene also suffer from fatal autoimmunity [63]. Natural Tregs develop in the thymus and circulate in the body to prevent reactivity of the immune system to self-antigens. Tregs can also be induced in the periphery by activation in the presence of TGF-β and IL-2. Tregs secrete the anti-inflammatory cytokines: TGF-β and IL-10, with TGF-β influencing neighboring T cells to become induced Tregs [64]. Suppressive mechanisms include IL-2 deprivation that leads to apoptosis of the effector T cells [65]. Tregs also have various cell-mediated suppressive mechanisms and can decrease costimulation and antigen presentation by APCs [66]. Type 1 regulatory T (Tr1) cells are Treg-like cells, but lack Foxp3 expression. They mainly reside in the gut and have the ability to produce large amounts of IL-10, which makes them important in mucosal tolerance. IL-10 inhibits presentation of antigens, decreases expression of costimulatory molecules, and blocks cytokine and chemokine secretion [67].

T-cell activation in the absence of pro-inflammatory mediators may lead to induced tolerance in the periphery. APCs that are not activated by, e.g., TLR ligation, express low levels of costimulatory molecules. The recognition of its antigen without costimulation promotes T-cell anergy. Anergic T cells are unable to mount a normal immune response against their antigens even though presentation may occur with costimulation later. Tolerogenic dendritic cells are specialized in presenting self-peptides to induce antigen-specific Tregs. Their functionality is suggested to be mediated through cytokine secretion, such as IL-10 and TGF-β, and lack of costimulation [68]. In addition, indoleamine 2,3-dioxygenase (IDO) activity in tolerogenic dendritic cells leads to production of tryptophan metabolites that inhibit activated T cells.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is expressed on activated T cells to reduce the immune response and to restore homeostasis after clonal expansion. It binds to CD80 and CD86 on APCs and prevents their binding of the costimulatory molecule CD28 [69]. Tregs constitutively express CTLA-4, and it is critical for their ability to suppress immune responses [70]. Programmed cell death protein 1 (PD-1) is also an important inhibitory surface receptor on T cells. It binds PD-L1 and PD-L2 and prevents excessive T-cell activation and autoimmunity [71]. All these mechanisms act together to regulate the immune response, but immune tolerance is not indefectible. There are several examples of autoreactive T cells that escape control and cause autoimmune disorders.

1.1.3.6 T follicular helper cells

Tfh cells are a distinct subset of antigen-experienced T-helper cells that are specialized to assist follicular B cells in their formation of germinal centers [72]. Tfh cells depend on IL-6 and IL-21 to activate their lineage-specific transcriptional profile with Bcl-6 as the transcriptional master regulator [73]. Tfh cells secrete IL-21, which provides proliferative signals to activated B cells and also forms an autocrine loop during the Tfh cell differentiation. After antigen recognition, the Tfh cell upregulates CXCR5 to home to germinal centers. Through expression of CD40L, the cells trigger and maintain germinal center reactions. The ligation of CD40 on B cells by CD40L activates activation-induced cytidine deaminase that drives somatic hypermutation and causes antibody class-switching.

1.2 ATHEROSCLEROSIS

Cardiovascular disease is the main cause of death in the world, with ischemic heart disease and stroke accounting for one in every four deaths worldwide [74]. Atherosclerosis is the underlying cause in most cases. The disease progresses slowly with chronic inflammation and a build-up of lipid-laden plaques in large- and medium-sized arteries. Atherosclerosis typically remains unnoticed over several decades until a rupture of the plaque elicits thrombus formation that occludes the vessel and leads to ischemic tissue damage.

1.2.1 The pathogenesis of atherosclerosis

A normal artery consists of an intima layer with endothelial cells and sparsely distributed smooth muscle cells, a media layer with smooth muscle cells and elastic lamellae, and a surrounding adventitial layer with loose connective tissue [75]. An intimal accumulation of LDL offsets atherosclerosis development. Already in young adults, coronary atherosclerosis is evident as fatty streaks [76, 77]. The proteoglycan-binding and retention of LDL in the subendothelial space is the initiating event [78]. The trapped lipoproteins are biochemically modified by proteases and lipases, leading to aggregation and increased proteoglycan binding [79]. Oxidative modifications by myeloperoxidase, lipoxygenase, and reactive oxygen species lead to formation of oxidized LDL (oxLDL) that could elicit an innate inflammatory response [80].

In response to the trapped and modified lipoproteins and at sites in the arterial tree with turbulent blood flow, endothelial cells start to express adhesion molecules, such as VCAM-1 [81, 82]. Circulating monocytes and other leukocytes are recruited to these sites. The infiltrating monocytes differentiate into macrophages in response to M-CSF and GM-CSF produced by endothelial cells [83]. The monocyte-derived macrophages are a major cell population in atherosclerotic plaques and can proliferate locally [84, 85]. They express scavenger receptors, of which scavenger receptor class-A and CD36 have been identified to be the most important for uptake of modified LDL [86]. Scavenger receptors are not downregulated in response to intracellular cholesterol accumulation. The continued engulfment of lipids leads to macrophage foam cell formation [87]. The large and foamy cells are trapped within the arterial intima and have compromised migratory capacity [88].

1.2.1.1 Risk factors

The Framingham heart study started in 1948 with the aim to identify preventable risk factors for cardiovascular disease. After 6 years follow-up, elevated blood pressure, hypercholesterolemia and left ventricular hypertrophy were identified as independent predictors of risk for developing coronary heart disease [89]. Later, cigarette smoking, lack of exercise, obesity, diabetes, and low HDL levels were identified as risk factors.

Inflammation was associated with myocardial ischemia through measurements of the acute phase reactant, C-reactive protein (CRP), in plasma [90]. Later it was recognized that elevated CRP levels, as an independent risk factor, could predict future cardiovascular events [91, 92]. Clinical symptoms of atherosclerosis typically do not arise until later in midlife illustrating how age is an important risk factor [93]. Lesion development is influenced by environmental factors, such as diet, but atherosclerosis is not a modern disease. It has existed in several ancient cultures with disparate eating habits [94]. Previously, males have been assumed to be at greater risk to develop cardiovascular disease. This is contradicted by current mortality statistics that show a small predominance of female deaths by cardiovascular disease [95]. Genetic factors are important for atherosclerosis and genome-wide association studies have identified several single nucleotide polymorphisms linked to increased risk for coronary artery disease [96]. The most robust genetic association is located in the chromosome 9p21 locus and has been implicated in trans-regulation of interferon signaling [97]. Some of the other identified loci are also linked to inflammation, but the associations are weak in comparison to other inflammatory diseases. This probably reflects a complex and multifactorial origin of the genetic contribution to atherosclerosis.

1.2.1.2 Symptoms of atherosclerosis

As the atherosclerotic plaque grows, it becomes more and more complex. Fatty streaks develop into fibro-fatty lesions that become advanced plaques. Plaques might even grow into a large stenosis that narrows the lumen. This could impair blood flow with symptoms such as angina pectoris or intermittent claudication. Plaque rupture or endothelial erosion may also occur. This leads to exposure of thrombogenic material, such as tissue factor, collagen, and phospholipids. Platelets rapidly aggregate, with ensuing coagulation, and thrombus formation. This blocks the blood flow and causes ischemia and tissue damage. Thrombus formation in a coronary artery may lead to myocardial infarction, and a peripheral thrombus may lead to gangrene. If a rupture occurs in a plaque located in a carotid artery, a stroke may be the consequence due to cerebral embolism.

1.2.1.3 Plaque stability

Plaque ruptures are estimated to account for around 70% of coronary thrombosis events [98]. The remaining 30% emphasize the importance of other mechanisms for clinical disease. Endothelial erosions are defined as the absence of endothelial lining leading to an acute thrombus formation without signs of cap rupture [99]. The underlying intima is usually rich in smooth muscle cells and proteoglycan matrix. Importantly, observations indicate that

endothelial erosions are becoming more frequent and modern pharmaceutical treatments with statins might drive this change [100, 101]. New successful therapies probably need to prevent both rupture and erosion or be given to substratified risk groups for either of these conditions.

Large and complex plaques contain a core with dying cells, both apoptotic and necrotic, cholesterol crystals, and other extracellular material. The necrotic core is covered by a fibrous cap, and at the shoulder regions of the plaque, accumulation of immune cells, such as T cells, are seen [102]. Symptoms of atherosclerosis typically arise when the cap fails to withstand the pulsatile force from the blood pressure and superficial fissures are formed, usually near the edges of the plaque [103]. Plaques vulnerable to rupture are characterized by a thin fibrous cap, a large lipid-filled necrotic core, and on-going inflammation [104, 105].

Smooth muscle cells and collagen have a central role in plaque stability. TGF-β stimulates extracellular collagen maturation and positively regulates collagen synthesis by smooth muscle cells [106, 107]. Mature collagen provides mechanical strength to the fibrous cap. IFN-γ is a powerful destabilizing agent and inhibits smooth muscle cell differentiation and proliferation as well as collagen production and maturation [106, 108, 109]. Matrix metalloproteinases degrade collagen fibers and promote plaque vulnerability [110]. As an illustration, mast cells that are commonly found at sites of plaque rupture, release proteases that degrade matrix and activate matrix metalloproteinases [111]. Atherosclerotic calcification usually provides stability to the plaques, but when the calcification occurs in small nodules, it adds instability. The latter is seen as spotty calcifications with ultrasonography and is associated with cardiovascular events [112]. Plaque stability can be estimated with imaging methods, such as ultrasonography, but there is a need for better techniques with higher resolution.

1.2.2 The immune response in atherosclerosis

Atherosclerosis is a chronic inflammatory disorder, although the contribution of cholesterol to the disease has been the main topic in the public debate [113]. The link between cholesterol and atherosclerosis became evident in 1913 when Nikolay Anichkov fed cholesterol to rabbits and investigated their aortas [114]. In 1856, Rudolf Virchow laid forth a hypothesis about how inflammation may initiate plaque formations in the arterial wall [115]. In support of this, recent knowledge has connected atherosclerotic cardiovascular disease with other inflammatory diseases. Patients with rheumatoid arthritis, psoriasis, or systemic lupus erythematosus all have increased risk for myocardial infarction [116, 117]. Low-grade inflammation, such as periodontitis, also gives an increased risk [118].

Associations between infections and atherosclerosis have led to a hypothesis about an infectious cause of atherosclerosis. *Chlamydia pneumoniae* could accelerate experimental atherosclerosis [119], but the effect depends on several factors [120] and antibiotics do not benefit coronary artery disease patients [121]. In addition, germ-free mice can develop atherosclerosis [122], but this study is far from conclusive. Taken together, there is no causal

link between an infectious agent and atherosclerosis. Instead, evidence points to atherosclerosis being an autoimmune disorder.

After the discovery of immune cells in atherosclerotic plaques using monoclonal antibodies [102], a great focus has been put on inflammation in cardiovascular research. This has led to major findings regarding the pathogenesis of atherosclerosis as well as new therapies such as drug-coated stents used for percutaneous coronary interventions. These metal stents are coated with an immuno-suppressive drug, such as rapamycin, which also inhibits proliferation of smooth muscle cells and decreases the restenosis frequency [123, 124]. Low dose aspirin is used clinically for platelet inhibition and not as an anti-inflammatory drug. For other anti-inflammatory drugs, e.g., cortisone and non-steroid anti-inflammatory drugs, adverse effects make them unsuitable for long-term treatment of cardiovascular inflammation. There is a need for more specific treatments. Several new anti-inflammatory treatments are under development [125], and hopefully a range of drugs will become available in the future.

1.2.2.1 Innate immune activation in atherosclerosis

In vitro studies have shown that oxLDL promotes innate immune activation in macrophages. In atherosclerotic lesions, macrophages have nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) translocated to their nuclei as a sign of innate immune activation [126]. Macrophages in the plaques express various TLRs. Modified LDL and products thereof might be endogenous ligands to TLR2 and TLR4 [127, 128]. Other ligands, both endogenous and exogenous for TLRs expressed by macrophages have been suggested as well [129]. The downstream signaling molecule of several TLRs, Myd88, confers an important pro-atherosclerotic signal, but also transmits IL1-β and IL-18 signals [130]. Lysophosphatidylcholine and oxidized non-esterified fatty acids generated during LDL oxidation by lipoprotein-associated phospholipase A2 could also activate the innate immune system [131]. All these events are likely to be important factors that initiate and contribute to the maintenance of the inflammation in the forming lesions. Nonetheless, preventive measurements against innate immune activation with anti-oxidants or selective lipoprotein-associated phospholipase A2 inhibition have failed to show benefit in patients [132-134].

Cholesterol crystals form in foam cells and can activate the NLRP3 inflammasome, leading to IL-1β release [135, 136]. The NLRP3 inflammasome contains leucine-rich repeats that sense intracellular danger signals. The activated inflammasome recruits caspase-1 that cleaves the pro-form of IL-1β to its functional and releasable form. This provides a clear link between cholesterol metabolism and innate immune activation. Inhibition of IL-1β, to prevent cardiovascular events, is currently under evaluation in clinical trials [137]. The released IL-1β acts on smooth muscle cells to produce IL-6 [138], which in turn signals to the liver to produce CRP [75].

Innate immune cells, such as neutrophils, mast cells, natural killer cells, and natural killer T cells have been suggested to play important roles during atherogenesis, but are minor

populations in the plaques in comparison to macrophages and CD4+ T cells. In hypercholesterolemic mice, neutrophils are recruited during the initiation of atherosclerosis, but they are not present at later stages [139]. Mast cells may play a role in plaque stability with their matrix degrading enzymes [111]. Natural killer cells and natural killer T cells aggravate atherosclerosis, possibly due to IFN-γ release [140, 141]. Natural killer T cells that produce IL-10, may on the other hand, limit disease development [142]. γδ T cells do not seem to impact atherosclerosis [143, 144]. Taken together, several small populations of innate immune cells play significant roles at different stages of disease development, but the main innate immune effector cell type in the plaques is macrophages.

1.2.2.2 Adaptive immune activation in atherosclerosis

HLA-DR is expressed by several cell types in the atherosclerotic plaque and presents antigen to CD4+ T cells [145]. Genome-wide association studies have implicated associations between MHC and coronary artery disease [146]. The regulation of MHC expression has also been associated with myocardial infarctions [147], and certain HLA haplotypes are associated with either increasing risk or conferring protection, although the associations are weak [148]. MHC class II expressing cells, together with the significant number of T cells present in the atherosclerotic plaques at all stages of the disease, form the basis of the adaptive immune response in atherosclerosis [80].

Dendritic cells are found in atherosclerotic plaques and in the adjacent adventitia [149]. They take up plaque-derived antigens and migrate to lymph nodes where they display these antigens to a large number of naïve T lymphocytes [150]. Autoantigens derived from the LDL particle have been shown to be important for atherosclerosis. The frequent presence of anti-oxLDL antibodies shows that B cells react to oxLDL [151]. In general, these antibodies are more prevalent in coronary artery disease patients than healthy controls [152]. As oxLDL is a complex particle with disparate properties and large heterogeneity, it harbors many potential epitopes of which lysophosphatidylcholine, phosphorylcholine, and different peptides from Apolipoprotein (Apo) B100 have been identified [153-155]. Anti-oxLDL antibodies can be either IgM or IgG, implicating that an isotype class-switch takes place, which, in turn, indicates T-cell help. Indeed, T cells from humans with atherosclerosis recognize LDL components presented by APCs [156].

Heat shock protein (hsp) 60/65-reactive T cells have also been isolated from atherosclerotic plaques [157, 158]. Autoantibodies against hsp60/65 have been reported to be pathogenic, mediating cytotoxicity to endothelial cells, and evoking fatty streak formation [159, 160]. Heat-shock proteins are highly conserved, from bacteria to man, and produced in response to stressful conditions, such as hemodynamic strain and inflammation. Substantial antibody cross-reactivity exists between human hsp60 and the hsp60/65 counterparts in *Chlamydia* and *Mycobacteria*, which possibly explains these microbes' association with atherosclerosis. Induced mucosal tolerization to hsp65 protects against experimental atherosclerosis [161], but regular immunization against hsp65 could also induce such effect [162]. Further studies are needed to disentangle the role that heat shock proteins play as autoantigens. Other

immunogens that have been implicated in atherosclerosis are Apo-H (previously known as β_2-glycoprotein I) [163] and aldehyde-modified extracellular matrix proteins [164].

Vaccination against modified LDL confers a clear protective effect in experimental atherosclerosis models [165-168]. Proposed protective mechanisms include both cellular and humoral immunity as well as natural IgM antibodies [169]. Studies have focused on oxidation- or malondialdehyde-modified epitopes in LDL, but one of the initial findings was a superior protection by immunization with native LDL [166]. Protection from atherosclerosis can also be achieved through vaccination with native peptides from the ApoB-protein in LDL [170, 171]. Some protocols designed to induce mucosal tolerance implicate ApoB-specific Tregs with TGF-β and IL-10 secretion to confer the protection [172-174]. Dendritic cell vaccination that promotes development of ApoB-specific Tregs confirms this notion [175]. In humans, anti-oxLDL IgG levels in plasma are associated with coronary artery disease, while IgM levels show the inverse relationship [176].

The protective effect of antibodies could be: (i) neutralization of the pro-inflammatory properties of oxLDL, (ii) inhibited scavenger receptor uptake of oxLDL by foam cells, or (iii) Fc-mediated clearance of the particles [177]. Clearance of circulating particles would lower plasma cholesterol levels. An inverse association between serum cholesterol and oxLDL antibody titers in humans supports this notion [178]. This association has also been reported in LDL-vaccinated animals [168], and several other immunization studies show a decrease in total plasma cholesterol [179-181]. In addition, atheroprotection and LDL reduction are also seen in passive immunizations of hypercholesterolemic mice [182]. These studies have proposed that the protective mechanism to be neutralization of oxLDL [168, 182], inhibition of scavenger receptor uptake by foam cells [179, 181], or modulation of cellular immune responses [179, 180]. Reduction of LDL cholesterol mediated by anti-LDL antibodies seems to be a protective mechanism that has been overlooked or downplayed. Most studies have focused on modified LDL immunizations and disregard the clear protection from vaccination with native LDL [166, 180]. Together with the frequently seen autoantibodies against native ApoB peptides in humans [155], this suggests that adaptive immune responses to native LDL have a central role in atherosclerosis.

Interestingly, the atherosclerosis-associated immune response is not restricted to the intimal plaques and draining lymph nodes. Microvessels, lymphatics, and small conduits are formed that open a communication between the plaque and the adventitia [183, 184]. The media layer is normally immune-privileged, possibly through IDO expression [185], but tertiary lymphoid organs are formed in the adventitia located underneath the advanced plaques [183, 186, 187]. This is an interaction site for dendritic cells, B cells, and T cells. In these tertiary lymphoid structures, B cells are activated by antigens derived from atherosclerotic plaques and form germinal centers. More studies are needed to elucidate the specificity of the adaptive immune cells in these locations since aortic tertiary lymphoid organs seem to be of substantial importance for atherosclerosis development [188, 189].

1.2.2.3 B cells in atherosclerosis

B cells are only occasionally detected in the atherosclerotic plaques [102, 190]. A protective immunity mounted by B cells in spleen has been established through splenectomy and B cell transfer experiments in atherosclerotic mice [191], and this immunity is supported by effects seen in mice lacking B cells [192]. In addition, an increased risk to die from myocardial infarction is evident in splenectomized patients [193]. In contrast, B-cell depletion using anti-CD20 antibodies was reported to protect against experimental atherosclerosis [194, 195]. Indications of a beneficial effect on endothelial dysfunction exist in humans treated with anti-CD20 antibodies [196]. However, it should be considered that treatment with anti-CD20 only targets conventional B2 cells, and the mechanistic understanding of how these cells promote the disease remains largely elusive. B1 cells and antibody-producing plasma cells are unaffected by anti-CD20 treatment and could thus confer a protective effect. B1 cell transfer protects against atherosclerosis with production of germ-line encoded natural antibodies [197]. Especially the T15-idiotype, which is important to fight *Streptococcus pneumoniae*, has been suggested to have a beneficial effect during atherogenesis [154]. The proposed mechanism is molecular mimicry between the microbe and oxLDL. Indeed, pneumococcal vaccination protects against experimental atherosclerosis by blocking oxLDL uptake and lowering plasma cholesterol [181]. However, mice with a specific abrogation of T15-idiotype natural antibodies do not have increased atherogenesis [198]. Several different IgM antibodies with oxLDL reactivity probably have overlapping effects.

Similar to *S. pneumoniae*, apoptotic cells are immunogenic and share oxidation-specific epitopes with oxLDL particles [199]. Humoral immunity against these oxidation-specific epitopes occurs naturally and protects against atherosclerosis. The protective effect can be strengthened through apoptotic cell immunization, which lowers plasma cholesterol [200]. Possibly, IgM against (ox)LDL provides protection through neutralizing pro-inflammatory epitopes and by inhibiting scavenger receptor-mediated uptake, while anti-LDL IgG lowers cholesterol through immune complex formation.

1.2.3 T cells in atherosclerosis

Activated T cells are a significant cell population in atherosclerotic plaques [201, 202]. These T cells are antigen-experienced memory cells bearing evidence of an oligoclonal expansion [203, 204]. Mice with severe combined immunodeficiency, lacking both T and B cells, have a reduced development of atherosclerosis [205], and transfer of LDL-specific CD4+ T cells to these mice severely aggravates the disease [206]. A specific lack of CD4+ T cells inhibits atherosclerosis development [207, 208], although the *Cd4*$^{-/-}$ mouse model is far from optimal and still contain MHC class II restricted T cells [209]. CD8+ T cells do not have a major impact on experimental atherosclerosis in hyperlipidemic mice [144, 208], and are a minor population in comparison with CD4+ T cells, especially in mouse models of atherosclerosis. Treating *Apoe*$^{-/-}$ mice with a ligand for the costimulatory molecule CD137 increases CD8+ T-cell recruitment to the plaque and promotes atherosclerosis [210]. CD8+ Tregs may also influence atherosclerosis development [189]. Nonetheless, CD4+ T-helper cells are the main

adaptive effector cells in the atherosclerotic plaques. These T-helper cells react to peptide fragments from the ApoB100-protein in LDL when presented on MHC class II [156, 211].

Opposite to the innate response, oxidation of LDL blunts T-cell activation and possibly destroys the epitope. In immunized mice, several clones with an MHC class II restricted reaction to native LDL were shown to carry a TCR with the β-chain TRBV31 [211]. The mice were humanized in regard to their LDL particles, which made induction of an autoimmune response to human lipoproteins possible. Blocking the TRBV31+ T-helper cells by vaccinating against a TRBV31-derived peptide protects from atherosclerosis [211]. Clearly, LDL-reactive T cells exist both in humans [156] and mice [211], but are difficult to assess experimentally. Immunization protocols can expand these T cells, but their actions are then influenced by the adjuvant in the vaccine preparation. The different effects of CD4+ T-helper cell subsets on atherosclerosis are discussed in detail below.

1.2.3.1 Th1 cells in atherosclerosis

Th1 cells secrete IFN-γ, and this cytokine promotes monocyte infiltration, macrophage activation, and foam cell formation. IFN-γ also advances Th1-differentiation in synergy with IL-12. In human atherosclerotic plaques, a predominance of a Th1 immune response can be observed, and T cells isolated from human plaques respond in a Th1-fashion *ex vivo* [156, 212]. The pro-atherosclerotic effect of Th1 cells has been shown in several animal experiments. IFN-γ-deficient mice [213-215] and knockout mice of the Th1-trancription factor T-bet [216] have reduced atherosclerosis development. These studies also confirm the strong effect of IFN-γ on stimulating antigen-presentation with upregulation of MHC class II as well as the ability of IFN-γ to inhibit smooth muscle cell proliferation and expression of α-smooth muscle actin, effects that were initially described in [108, 145, 217, 218]. These effects make the plaque more vulnerable to rupture, the key event responsible for clinical manifestations of atherosclerosis, such as myocardial infarction and ischemic stroke. In conclusion, most evidence points to atherosclerosis being a Th1-driven disease, with a multitude of pro-atherosclerotic effects mediated by IFN-γ. However, several other cells in the plaque, such as macrophages and natural killer T cells, also have the ability to produce IFN-γ. The relative contribution of IFN-γ in the plaques from these cells compared to Th1 cells is not clarified [140, 219, 220].

1.2.3.2 Th2 cells in atherosclerosis

From the classical dichotomy of dividing T-helper cell subsets in Th1 and Th2, it is clear that Th1 cells are more frequent in atherosclerotic plaques, with Th2 cells much less common [212]. Severe hyperlipidemia could switch the balance toward a Th2 phenotype in *Apoe*$^{-/-}$ mice, but the impact of this on atherosclerosis is uncertain [221]. Compound-knockout mice have been used to assess the separate effects of Th2-related cytokines. Most studies of IL-4 point toward a disease promoting effect [222, 223], while IL-5, IL-13, and IL-33 may limit the disease development [169, 224, 225]. The protective mechanism of IL-5 could be mediated through its stimulation of natural antibody production by B1 cells. A single

nucleotide polymorphism in the IL-5 locus is associated with human coronary heart disease, corroborating the experimental findings [226]. Nonetheless, Th2 cells are infrequent in human atherosclerotic plaques and have an undecided importance.

1.2.3.3 Th17 cells in atherosclerosis

The impact on atherosclerosis by Th17 cells has been difficult to assess clearly. Th17 cells are a minor population in the plaques [227], and IL-17A, the signature cytokine of Th17 cells, is also produced by other cells, e.g., mast cells and neutrophils [228]. Several experimental studies have described conflicting effects on atherosclerosis development. Publications investigating IL-17A deficient *Apoe*$^{-/-}$ mice have reported increased [229], decreased [230], or no effect at all on lesion size [231]. A proposed explanation was an upregulation of the family member gene IL-17F in the absence of IL-17A, but neutralization of IL-17F in the IL-17A deficient animals did not impact atherosclerosis [231]. The different effects might be attributed to different effects of IL-17A at different stages of the disease development and other parameters.

The use of anti-IL17A neutralizing antibodies and other methods of IL-17A blockade have shown more coherent results with a reduction in lesion size [232-234]. Conflicting results have been reported and are suggested to be due to different antibodies and treatment protocols [235, 236]. In accordance with the results of a proatherogenic role for IL-17A, knocking down the IL17-receptor leads to less atherosclerosis [237, 238], and administering recombinant IL-17A promotes the disease [232]. These various effects of IL-17A have been reported to be mediated through both pro- and anti-inflammatory cytokines, effects on chemokines, matrix metalloproteinases, and adhesion molecules. Interestingly, proatherogenic conditions and oxLDL might induce Th17 cell differentiation [239].

Apart from the various reports on lesion size, investigations of plaque composition and other experimental evidence point toward a plaque-stabilizing role for IL-17A [229, 231]. In response to acute coronary syndrome, Th17 cell percentage and IL-17A cytokine production are elevated in the blood [240]. Patients without this response due to low IL-17A levels in sera have a higher risk for recurrent cardiovascular events [241]. This supports the notion of IL-17A's involvement in plaque stability and prompts for a study that establishes this connection mechanistically. Such explorations were undertaken in **paper I** of this thesis. In summary, experimental evidence points toward a complex role of IL-17A in atherosclerosis with diverse effects on different cell types in a multifaceted interplay with other cytokines.

1.2.3.4 Tregs in atherosclerosis

Minor populations of Foxp3+ Tregs are found in atherosclerotic plaques at all stages of the disease [242]. In experimental atherosclerosis, Treg number in lesions may vary [243], but Tregs have an important role to decrease inflammation and disease progression. Depletion of Tregs with anti-CD25 antibodies increases atherosclerosis in *Apoe*$^{-/-}$ mice [244]. Dendritic cell vaccination that evokes a cytotoxic response specifically to Foxp3+ T cells repeats this finding [245]. Transfer of Tregs is protective [244, 246] and similarly, expanding the Treg

population by using experimental protocols reduces atherosclerosis [247-249]. Treg expansion has been shown to induce regression of atherosclerosis [250] or create a plaque stabilization effect [251]. Antigen-specific Tregs may have an important protective role during atherogenesis as indicated by experimental vaccination studies [173, 174, 252].

Several studies have pointed out an important role for the anti-inflammatory cytokine IL-10 during atherogenesis with its ability to prevent disease progression [253-255]. The other Treg-associated cytokine, TGF-β, has a more complex role being a potent regulatory cytokine with diverse effects. Posttranscriptional and posttranslational modifications regulate TGF-β activity [256]. For immune cells, TGF-β regulates differentiation, tolerance, and proliferation. Disrupting TGF-β signaling in T cells leads to large, inflamed lesions in *Apoe*$^{-/-}$ mice [257]. However, the role of TGF-β in atherosclerosis lesion development is not conclusive [258, 259]. Various outcomes regarding atherosclerotic burden are evident when studying the general effect of TGF-β without targeting a special cell type [260-264]. Nonetheless, all these studies agree on a plaque stabilizing effect of TGF-β with stimulation of collagen production.

Tregs and their effector molecules have been implicated in the regulation of cholesterol metabolism. Overexpression of IL-10 lowers VLDL and LDL [265] and *Il10*$^{-/-}$*xApoe*$^{-/-}$ mice have elevated LDL levels [255]. In addition, Treg expansion in a regression model associates with reduced serum cholesterol [251]. Altogether, experimental evidence indicates that Tregs protect from atherosclerosis by dampening inflammation, with a possible involvement in the stabilization of plaques and regulation of serum cholesterol.

1.2.3.5 Tfh cells in atherosclerosis

In atherosclerosis, Tfh cells are not well-characterized. Tfh cells provide help to B cells, and both pro- and anti-atherosclerotic effects of B cells have been reported. Tertiary lymph structures in the proximity of the aorta contain germinal center B cells and effector T cells likely to be Tfh cells [183]. These lymphoid structures have been suggested to protect against atherosclerosis development [188]. Conversely, pro-atherosclerotic effects of Tfh cells have been suggested [189]. Further studies are needed to elucidate the mechanisms of action and effect of Tfh cells on atherosclerosis.

No comprehensive report on the effects and role of IL-21 in atherosclerosis exist to this date. The exploration of Tfh-cell specificity, preferably isolated from tertiary lymphoid structures adjacent to the plaques, will be of great interest to understand Tfh function. Investigations into the specificity and effector functions of B cells are also crucial for the interpretation of the role that Tfh cells play in atherosclerosis.

1.3 LIPID METABOLISM

All living organisms continuously consume energy, mainly in the form of adenosine triphosphate (ATP), to maintain structure and run biological processes. ATP is synthesized during oxidation of carbohydrates, lipids, and proteins. Cholesterol is one of the most abundant lipids in cells of higher eukaryotes constituting of up to 25% of the cell membrane

[266]. Cholesterol has a rigid and hydrophobic structure contributing to membrane stiffness and cholesterol-rich microdomains within the cell membrane are important for transmembrane receptors and their signaling [267]. Metabolites of cholesterol, such as steroids and vitamin D, are important cell signaling molecules. Bile acids are also derived from cholesterol and important in forming micelles with dietary lipids to promote intestinal uptake. Of the cholesterol in the body, approximately 30% is derived from *de novo* synthesis and 70% is absorbed from the diet [268]. This relationship varies depending on genetic factors and diet.

All nucleated cells can synthesize cholesterol from Acetyl-CoA through the mevalonate pathway. The rate-limiting step is the conversion of hydroxymethylglutaryl CoA (HMG-CoA) to mevalonate by HMG-CoA reductase. The lipid-lowering statin drugs inhibit HMG-CoA reductase and reduces incidence of cardiovascular events in primary prevention to high-risk individuals [269] and in secondary prevention to coronary heart disease patients [270]. Statins typically decrease plasma cholesterol by around 30%. The lipid-lowering effect is not mainly caused by a reduction in biosynthesis of cholesterol, but a concomitant upregulation of LDL receptors in the liver [271, 272].

Cholesterol esterification with addition of a fatty acid residue makes the cholesterol molecule more hydrophobic. Cholesterol esters fit less well in the cell membrane and form intracellular lipid droplets. The cholesterol synthesis, uptake, and processing is controlled by sterol regulatory element binding proteins (SREBP) [273]. SREBPs are sterol-sensing transcription factors that are activated to increase cellular cholesterol levels through uptake and *de novo* synthesis of cholesterol.

The main lipid content in food is triglycerides that consist of a glycerol molecule and three fatty acid residues. Fatty acids exist in a saturated form with the maximal amount of hydrogen bound to the carbon backbone and in unsaturated forms with carbon double bounds in the tail structure. Triglycerides cannot pass through cell membranes, and they need to be broken down by lipases into free fatty acids and glycerol. In adipocytes, they are assembled into triglycerides again, and this is the main form of energy stored in the body.

1.3.1 Lipoproteins

The lipid transfer system that handles and distributes cholesterol in the body is tightly regulated. Hepatocytes in the liver orchestrate lipid metabolism and distribute cholesterol-rich lipoprotein particles around the body. Lipoproteins consist of a shell with a structural protein non-covalently linked to phospholipids and unesterified cholesterol and a core with cholesteryl esters and triglycerides. The various lipoproteins are classified according to their density (Table 1). Normal plasma cholesterol levels in humans are <5.0 mmol/l, and usually levels >6.0 mmol/l are classified as hypercholesterolemia. Total triglyceride levels are recommended to be below 1.7 mmol/l. Guidelines for risk assessments take several risk factors in consideration to determine suitable intervention [274]. Dyslipidemia refers to the presence of elevated levels of one or several fractions of plasma lipids.

Table 1. The characteristics of the main lipoprotein classes [275].

Particle	**Main apolipoprotein**	**Main component**	**Size** (nm)	**Density** (g/ml)
Chylomicron	B48, C, E	Triglycerides	100-1000	<0.95
VLDL	B100, C, E	Triglycerides	30-80	0.95-1.006
IDL	B100, E	Triglycerides and cholesterol	25-35	1.006-1.019
LDL	B100	Cholesterol	18-25	1.019-1.063
HDL	A1, A2	Protein	5-12	1.063-1.210

1.3.1.1 Chylomicrons

Dietary cholesterol and triglycerides are taken up in the gut and packaged by enterocytes into chylomicron particles. The structural protein of chylomicrons is ApoB48. Through RNA editing, the full-length ApoB100 protein is not expressed in the gut. Forty-eight percent of the sequence is translated due to a stop codon. The shortened ApoB48 lacks the C-terminal LDL-receptor binding domain of ApoB100. Nascent chylomicrons, up to 1000 nm in diameter, are released in the lymphatic system and eventually enter the blood via the left thoracic duct. ApoCII in the chylomicrons activates lipoprotein lipase (LPL) when the particles arrive to capillaries [276]. LPL is synthesized in peripheral tissue such as skeletal muscles and adipose tissue. It hydrolyzes triglyceride-rich lipoproteins in the lumen of capillaries bound to glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) on the endothelial surface. This releases free fatty acids that are taken up by the adjacent tissue. The chylomicron particle continuously shrinks in size to become a remnant particle with a size of 30-50 nm. ApoCII dissociates from the particle, while Apolipoprotein E (ApoE) instead is acquired from plasma. ApoE mediates clearance of the chylomicron remnants by hepatocytes.

During the fed state, chylomicron synthesis and LPL activity are high, as they are stimulated by insulin. This leads to storage of free fatty acids in the adipose tissue. In the fasted state, both chylomicron synthesis and LPL activity are low. The mobilization of stored triglycerides in the adipose tissue is mediated by adipose triglyceride lipase and hormone-sensitive lipase [277]. The liver has its specific hepatic lipase, which also hydrolyzes triglycerides and makes lipoprotein particles denser.

1.3.1.2 Very low-density lipoproteins and low-density lipoproteins

Hepatocytes repack cholesterol in VLDL particles that are secreted in circulation and distributed to muscles and adipose tissue. ApoB100 is the structural protein of these particles. Being 4563 amino acids long, it has an mRNA transcript larger than 14 kilobases [278].

Human livers express the full-length ApoB100 solely, while mice could have up to 65% edited mRNAs [279]. Microsomal triglyceride transfer protein allocates lipids to synthesized ApoB100 protein. The formed VLDL particles are 30-80 nm in diameter and rich in triglycerides. After secretion in the circulation, lipolysis shrinks the particle-size to form intermediate-density lipoprotein (IDL) particles. LDL particles are formed through the continued lipolysis in circulation. Other apolipoproteins, present in VLDL and IDL, dissociates from the particle, and makes ApoB100 the sole apolipoprotein of LDL. LDL is the main lipoprotein that delivers cholesterol to peripheral cells.

The ApoB protein contributes to innate immunity through protecting against invasive *Staphylococcus* infections [280]. A peptide fragment derived from ApoB has also been identified to convey pro-inflammatory signals and trigger platelet activation [281, 282].

1.3.1.3 High-density lipoprotein (HDL)

Cholesterol is transported back to the liver through reverse cholesterol transport. ApoA1 is formed in the liver and secreted in the blood. In the periphery, ApoA1 docks to cholesterol exporters on cells, loads up with cholesterol, and transports it back to the liver. ABC transporter A1 (ABCA1) controls the rate-limiting step in HDL formation. It forms discoidal HDL, which is further loaded with intracellular lipids by ABCG1, and the particle reaches a spherical diameter size of 5-12 nm. The spherical change is mediated by lecithin-cholesterol acyl transferase that esterifies free cholesterol. Foam cells have their excessive cholesterol stored as esters in lipid droplets. The impaired cholesterol efflux of these cells can partly be due to inflammation [283].

The cholesterol that reaches the liver is directly secreted to the bile or metabolized to bile acids, and then secreted. This secretion is ABCG5/8-dependent. The bile is excreted in the small intestine. From there, cholesterol can either be reabsorbed or further excreted in the feces. The reabsorption pathway forms an enterohepatic cycle. The absorption of cholesterol from the gut is incomplete, and usually around 50% of the dietary cholesterol is excreted.

Cholesterol-ester transfer protein (CETP) in the blood can shuffle cholesterol from HDL to other lipoproteins. This counteracts reverse cholesterol transport. Drugs that specifically inhibit CETP increase HDL-cholesterol, but do not reduce risk for cardiovascular events [284]. This has led to doubts whether raising HDL will ever translate into a therapy against cardiovascular disease in humans [285]. Another lipid transfer protein found in human plasma is phospholipid transfer protein (PLTP). It transfers phospholipids from triglyceride-rich lipoproteins to HDL. By this, it regulates the HDL particle size.

1.3.2 Regulation of lipid metabolism

The liver is the main organ regulating lipid metabolism, accounting for around 70% of the LDL uptake from plasma. LDL receptors are, however, present on most cells, and mediate uptake through endocytosis of lipoproteins carrying ApoB100 or ApoE proteins [286]. LDL receptors are regulated by SREBPs. When SREBPs sense a lack of cholesterol in the cell

membrane, they translocate to the nucleus and activate gene transcription of LDL receptors and cholesterol synthesis-related genes [273]. The *APOE* gene is polymorphic and exists in three major alleles in humans, *APOE2*, *APOE3*, and *APOE4*. ApoE2 and ApoE4 proteins have lower affinity to the LDL receptor than ApoE3, leading to slower clearance of chylomicron remnants. Especially *APOE4* is associated with hyperlipidemia and atherosclerosis development [287].

Other receptors important for hepatic uptake of LDL are low-density lipoprotein receptor-related protein 1 (LRP1), VLDL-receptor, sortilin, and scavenger receptor B type I (SR-BI). LRP1 is ubiquitously expressed, although most abundant in vascular smooth muscle cells, hepatocytes, and neurons. It mediates endocytosis of ApoE-containing lipoproteins. The interaction also leads to cell signaling, mediating an anti-atherosclerotic effect in vascular smooth muscle cells by maintaining the vascular wall organization [288]. The VLDL receptor is another LDL receptor family member, but it is usually not present in the liver. In **Paper II**, we report an upregulation of the mRNA level in the liver of *Ldlr*$^{-/-}$ mice after depletion of Tregs. If this results in increased protein expression remains unknown. The main function of the VLDL receptor is to mediate uptake of ApoE-containing lipoproteins in the periphery.

Sortilin is a multi-ligand receptor encoded by the *SORT1* gene. In the liver, it targets pre-secretory degradation of nascent VLDL particles. Reports have also indicated sortilin to function as a cell surface receptor that binds ApoB-containing lipoproteins [289]. The *SORT1* locus is strongly associated with serum lipoprotein levels as well as myocardial infarction in genome-wide association studies [290]. Heparan sulfate proteoglycans are present in the extracellular matrix and on the cell surface where they act as endocytosis receptors mediating uptake of various macromolecules, including lipoproteins. Syndecan-1 belongs to this family and clears ApoE-VLDL particles in the liver [291]. Yet another receptor that can mediate hepatic uptake of lipoproteins is SR-BI, mainly targeting HDL particles [292].

Proprotein convertase subtilisin/kexin 9 (PCSK9) is a serine protease that regulates recycling of LDL receptors. This is an interesting target for a new class of lipid-lowering drugs that increases the LDL receptors in the liver by inhibiting PCSK9. Gain-of-function mutations in the *PCSK9* gene are described to cause autosomal dominant hypercholesterolemia [293]. PCSK9 is produced in the liver and secreted in the circulation. It binds to the LDL receptor and targets it for degradation, preventing its re-use and therefore lowers the total amount of receptors present on the cell surface. This reduces cholesterol uptake by the liver and thus increases plasma cholesterol. Monoclonal PCSK9 antibodies have shown notable lipid-lowering effects with administration once per month [294].

As discussed in the lipoprotein section, apolipoproteins are fundamental regulators of lipid metabolism. ApoCIII is an important component of ApoB-containing lipoproteins [295]. It blocks the clearance of triglyceride-rich lipoproteins, opposing the effects of ApoE.

1.3.2.1 Nuclear hormone receptors sensing lipids

Liver X receptors (LXR) are important intracellular cholesterol sensors that regulate the cholesterol efflux pathway transcriptionally [296]. LXRs are activated by oxysterols. In macrophages, LXRs control reverse cholesterol transport and protect against foam cell formation. Activating these transcription factors inhibits inflammatory gene expression, mainly by negative regulation of NF-κB. Synthetic LXR agonists can inhibit experimental atherosclerosis [297], but LXR activation also induces hypertriglyceridemia through effects on hepatocytes.

Bile acid formation is controlled by the farnesoid X receptor (FXR). When FXRs sense a reduced return of bile from the gut, the enzyme, cholesterol 7 alpha-hydroxylase (CYP7A1) is activated and bile production is increased [298]. On the contrary, retinoid X receptors (RXR) can downregulate *CYP7A1* and bile acid synthesis. RXRs also downregulate the re-absorption of cholesterol in the gut. To make this regulation more intricate, LXRs, FXR, and RXRs form heterodimers to regulate transcription in response to their various ligands.

Fatty acid metabolism is controlled by peroxisome proliferator-activated receptors (PPAR). They form heterodimers with RXRs and regulate gene transcription of key enzymes in fatty acid and triglyceride metabolism, e.g., LPL. Endogenous PPAR ligands are free fatty acids and eicosanoids. Three main forms exist, PPARα, PPARγ, and PPARδ. They have slightly different roles with differences in tissue distribution. PPARα is important in the liver and in muscles. The hypolipidemic fibrate drugs activate PPARα and affect a range of steps in the lipid metabolism through lowering LDL and triglycerides, but mainly by raising HDL by transcriptionally regulating ApoA1 [299]. These drugs are used as a complement to statins and have been shown to lower non-fatal myocardial infarctions, but not all-cause mortality [300].

PPARγ is important in adipocytes and controls lipid storage. Thiazolidinediones, more commonly known as glitazones, target and activate PPARγ, which increases insulin sensitivity and lowers glucose levels. A common polymorphism in the *PPARG* gene decreases the risk of insulin resistance and protects against diabetes [301]. PPARγ is also involved in cellular differentiation. It induces an alternatively activated macrophage phenotype that is anti-inflammatory and is involved in tissue repair. Agonists of PPARα and PPARγ can limit experimental atherosclerosis though inhibition of foam cell formation [302]. PPARδ is expressed in many tissues. Agonists to PPARδ can protect against experimental atherosclerosis through effects in macrophages and anti-inflammatory actions in atherosclerotic plaques [303].

1.3.2.2 Inflammatory-mediated regulation of lipid metabolism

Inflammation is linked to metabolic disorders such as obesity, diabetes, and cardiovascular disease. Combinations of abdominal obesity, elevated blood pressure, raised plasma triglycerides, low HDL, and reduced glucose tolerance is diagnosed as metabolic syndrome,

which is associated with cardiovascular disease [304]. During evolution, immunity has developed to mount strong responses against pathogens. Metabolism has, at the same time, evolved to store surplus energy in adipose tissue for periods of starvation. In modern society, starvation and infections are lesser problems than they were during the early evolution of mankind. This provides grounds for inapt reactions by both the immune system and metabolism.

Interestingly the fruit fly, Drosophila, has a common organ serving as adipose tissue, liver, and hematopoietic system [305]. Thus, there is an evolutionary link between the immune system and metabolism. A lot of immune cells still reside in the human liver, e.g., liver macrophages known as Kupffer cells, and during fetal life, hematopoiesis occurs here. The interconnections between inflammation and metabolism are crucial for the immune system to mobilize energy for its response during infections. All forms of inflammation increase energy expenditure. Conversely, malnutrition or overnutrition can cause aberrant immune responses. Infiltrations by immune cells are seen in the adipose tissue during obesity, and similar to cardiovascular disease, the metabolic syndrome is characterized by a local chronic inflammatory process with elevated systemic inflammation markers such as CRP [75].

Hypertriglyceridemia is observed during acute infections [306]. The cytokine, TNF, mobilizes energy from adipose tissue and muscles, leading to cachexia, along with leukocytosis [307]. Initially, TNF was described to block the activity of LPL [308], but the functionality has been shown to be more complex [309]. Similar to TNF, lipopolysaccharides, and the inflammatory cytokines IL-1β, and IL-6, increase plasma VLDL-triglycerides [309]. This is attributed to increased hepatic lipogenesis and reduced clearance by lower LPL activity. Specific induction of hepatic inflammation by activation of NF-κB in hepatocytes has been shown to increase VLDL production [310].

Mobilization of cholesterol is of less importance in acute inflammation, with accordingly smaller effects of inflammation on plasma cholesterol levels. Nonetheless, the cellular cholesterol content is important for rapidly proliferating cells, such as T cells, with their need to synthesize new cell membranes [311]. In addition to these effects, inflammation downregulates the cellular lipid sensors, RXRs, LXRs, and PPARs, thereby loosening the tight control of lipid metabolism [309].

The effects on lipid metabolism by chronic inflammation are more complex. Dyslipidemia is often seen in rheumatoid arthritis, but several mechanisms are likely to contribute to the elevated cardiovascular risk in these patients [312]. Systemic lupus erythematosus is more distinctly associated with a pro-atherogenic lipid profile and with an elevation of VLDL levels [313]. Various TNF blocking drugs that are commonly used to treat rheumatoid arthritis have been shown to both increase and decrease plasma lipids [314]. The treatments are nonetheless efficient in reducing inflammation and patients with rheumatoid arthritis under treatment with these drugs have a lower incidence of cardiovascular events [315].

Alterations of gut microbiota have been linked to metabolic syndrome, cardiovascular disease, and type 2 diabetes [2]. Gut microbiota promotes obesity through assisting intestinal uptake of nutrients and increasing LPL activity, which in turn cause triglyceride storage in adipose tissue [316]. At the same time, gut microbiota can reduce cholesterol absorption through a reduction in bile acid release [317]. This is achieved through FXR antagonism with subsequent *CYP7A1* downregulation [318].

Short-chain fatty acids, consisting of less than six carbon atoms, are important microbial products in the intestinal tract and can affect immune cells. Interestingly, Th1, Th17, and Treg cell differentiation is influenced by short-chain fatty acids [319, 320]. Moreover, gut microbiota can cause formation of trimethylamine-N-oxide in response to digestion of red meat [321]. This accelerates atherosclerosis and provides an explanation why red meat consumption is associated with cardiovascular disease. The increased atherosclerosis is suggested to be mediated by upregulated scavenger receptors on macrophages and impaired reverse cholesterol transport [321]. Diet, genetic factors, and the immune system can all influence the microbiome [2], and numerous mechanisms exist by which microbiota regulate host metabolism, reflecting its great diversity.

In conclusion, the connections between immunity and metabolism are vast. Acute inflammation induces hypertriglyceridemia, but the effects of chronic inflammation on metabolism are unclear with several mechanistic links missing. The papers in this thesis illustrate how such links can be methodically explored in well-controlled *in vivo* models in conjunction with isolated *in vitro* systems. Translational approaches can further consolidate experimental findings. In this way, important crosslinks between immunity and metabolism could be identified. T cells orchestrate the immune system, but notions also suggest them to control metabolism [322]. Detailed mechanistic information on how various T cells affect lipid metabolism remains to be discovered, which in turn may unravel drug targets. A novel treatment that both decreases chronic vascular inflammation and corrects a perturbed lipid metabolism is likely to be useful to limit atherosclerosis and its devastating symptoms.

Subendothelial retention of lipoproteins triggers the inflammatory process that leads to atherosclerotic plaque formation. T cells in the plaques perpetuate inflammation, but several questions regarding the pathogenesis of atherosclerosis remain to be clarified. The autoimmune component in the disease development is poorly understood, antibodies connected to the disease have unresolved actions, and the actual role for LDL-reactive T cells in atherogenesis needs to be defined. The investigation of these aspects possibly harbors unexpected therapy targets. For instance, T-helper cell subsets are suggested to have detrimental roles for plaque stability. Th1 cells promote lesion development and destabilize plaques. Circumstantial evidence in the literature suggests opposite effects by both Tregs and Th17 cells. A mechanistic understanding of these processes is needed to draw correct conclusions from such reports. In the future, this could hopefully lead to a specific treatment for stabilization of vulnerable plaques prone to rupture.

This thesis attempts to answer how different T-helper cell subsets affect atherosclerosis development and plaque composition. The papers included illustrate the roles that three separate T cell subsets — Th17, Treg, and Tfh cells — play in atherosclerosis development. All three subsets were surprisingly shown to have major impacts on lipid metabolism by separate mechanisms.

2 AIMS

The studies included in this thesis aimed to investigate T-cell specificity and regulation in atherosclerosis.

The specific aims were to:

I. Investigate the effects of increased TGF-β signaling in T cells on atherosclerosis.

II. Define the role that Foxp3+ Tregs play in atherosclerosis.

III. Examine the role that LDL-reactive T cells play in atherosclerosis.

3 METHODOLOGICAL CONSIDERATIONS

3.1 MOUSE MODELS

C57BL/6 is the most commonly used inbred mouse strain to study atherosclerosis, and it is used as the genetic background for the majority of genetically modified strains [323]. This strain was established in the 1920's [324], and its genome was published in 2002 [325], only one year after the human genome. Experiments in mouse models have several benefits. The colonies reproduce fast, housing is space-efficient, and inbreeding reduces experimental variability. The model also allows for genetic manipulations, such as targeted gene deletions and insertion of transgenes.

C57BL/6 mice develop a Th1-biased immune response, while other mouse strains such as BALB/c are more Th2-prone. BALB/c mice are resistant to atherosclerosis, but if their immune response is Th1-skewed through genetic manipulation, atherosclerosis could be induced [326]. Wild-type mice, such as C57BL/6, are nonetheless relatively resistant to atherosclerosis. They have most of the circulating cholesterol in HDL, and complex atherosclerotic lesions are not formed even when fed a high-fat and high-cholesterol diet. Hyperlipidemic mice, such as *Apoe*$^{-/-}$ on the C57BL/6-background, are therefore used as experimental models of atherosclerosis [327]. The ApoE protein is mainly produced by hepatocytes and macrophages and is taken up by chylomicrons. The lack of ApoE impairs hepatic uptake of remnant particles and severely perturbs lipid metabolism. In *Apoe*$^{-/-}$ mice, circulating cholesterol is predominantly in VLDL particles, and the mice develop complex atherosclerotic plaques on a standard chow diet. ApoE is also involved in the development of myeloid cells [328], and since this may influence immune reactions, we chose to use *Ldlr*$^{-/-}$ mice for studies of atherosclerosis included in this thesis.

Ldlr$^{-/-}$ mice mimic the development of atherosclerosis seen in humans with familial hypercholesterolemia (FH) [329]. The *Ldlr*$^{-/-}$ mice need a Western type diet to develop atherosclerosis [330]. The LDL receptor recognizes ApoB100 or ApoE and mediates uptake of LDL particles through endocytosis. As mentioned before, LDL receptors are fundamental for liver clearance of LDL from circulation. This makes bone marrow transplantation into *Ldlr*$^{-/-}$ recipients a useful tool in experimental atherosclerosis. The effect of the transplanted hematopoietic cells on atherosclerosis development can be assessed in the hyperlipidemic *Ldlr*$^{-/-}$ chimeras.

Human APOB100-transgenic Ldlr$^{-/-}$ (*HuBL*) mice carry the full-length human *APOB100* gene [78, 331]. Codon 2153 of the *APOB100* gene inserted in these mice has been converted from leucine to glutamine to prevent the formation of ApoB48 in the liver and gut [332]. Mice producing such humanized lipoproteins develop hypercholesterolemia and atherosclerosis on a standard chow diet, but the development of complex atherosclerotic plaques takes at least six months [333]. The development of hypercholesterolemia on a low-fat diet is likely due to overexpression of human ApoB or decreased receptor-mediated uptake of the humanized particles. A large fraction of circulating plasma cholesterol is held in

LDL particles. This gives *HuBL* mice a more human-like dyslipidemic lipoprotein profile compared to *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. In addition, *HuBL* mice allow studies of human ApoB as an autoantigen, which may facilitate translation aspects of findings into human disease.

The atherosclerosis-prone mouse strains mentioned above develop complex atherosclerotic plaques with shared features of human disease. However, the mouse models are fairly resistant to plaque rupture with ensuing myocardial infarction. Atherothrombosis is sporadically detected and experimentally challenging to assess [334-336]. Special models of plaque rupture have been developed, but the experimental field lacks a reliable and reproducible model for assessment of plaque stabilizing agents.

To study T cells influenced by increased TGF-β signaling, a mouse model that targeted the TGF-β-inhibitory molecule Smad7 was used. Through the Cre-lox technique, conditional gene deletions are possible. Specific recombination at LoxP-sites is induced by a Cre recombinase expressed under a tissue-specific promoter [337]. In the Smad7-flox mice, created for this project, the promoter, exon 1, and exon 2 of the *Smad7* gene are flanked by LoxP-sites. Crossing the Smad7-flox strain with a CD4-Cre expressing mouse conditionally deletes the *Smad7* gene in T cells. Bone marrow transplantation into *Ldlr*$^{-/-}$ mice was used to study atherosclerosis development [338]. The results are presented in **Paper I**.

To study the effects of Foxp3+ Treg depletion, the DEREG (depletion of regulatory T cells) mouse model was employed. It expresses a fusion protein of the human diphtheria toxin receptor and enhanced green fluorescent protein (eGFP) under the control of the *Foxp3* promoter [339]. Bone marrow was transplanted into *Ldlr*$^{-/-}$ mice to study atherosclerosis development. Mice are naturally unaffected by diphtheria toxin [340]. A selective depletion of the human diphtheria toxin receptor-expressing Foxp3+ T cells was achieved through administration of diphtheria toxin. This does not affect CD25+ T effector cells, as CD25-depleting antibodies do. CD25-depletions, which have been used to study Treg function [244], are therefore not optimal and do not reproduce the autoimmune phenotype seen in scurfy and DEREG mice [63, 339]. The results from the Foxp3+ Treg depletion in atherosclerosis are presented in **Paper II**.

To investigate T-cell specificity in atherosclerosis, three new TCR transgenic strains were generated that were reactive to human LDL. Previously, a panel of T-cell hybridomas had been created from mice immunized with human LDL particles [211]. TCR cDNA was cloned from three of the hybridomas reactive to human LDL and ApoB100 protein. These TCRs were subcloned under the *CD2* promoter and used for production of transgenic mice. The mice strains from these three hybridomas were named: *BT1*, *BT2*, and *BT3* and all expressed the β-chain TRBV31 together with the α-chain TRAV12, 4, and 14, respectively. A population of around 8% TRBV31+ T-helper cells exists in wild-type mice. This should be compared to the population of over 90% TRBV31+ T-helper cells in the *BT* strains. Exposing the transgenic T cells to their antigen, human LDL, evoked a strong response with proliferation and IFN-γ secretion. The effect on atherosclerosis was assessed in **Paper III**.

Different diets were used to study atherosclerosis in the mouse models. Table 2 describes the main differences, with the "Standard American diet" used as an example of food consumption in the Western world [341]. The main dietary component that dictates development of atherosclerosis in *Ldlr*$^{-/-}$ mice is cholesterol content [342]. A 1.25% cholesterol diet was used to rapidly induce atherosclerosis in the bone marrow transplanted *Ldlr*$^{-/-}$ mice. In *HuBL* mice, 0.15% cholesterol diet was used to study atherosclerosis development. Mice used for breeding or used as cell donors were fed standard chow, a low-fat diet that does not correspond to food intake by humans (Table 2).

Table 2. Comparison of different diet compositions.

Diet	Energy (kcal/g)	Carbohydrates (kcal %)	Protein (kcal %)	Fat (kcal %)	Cholesterol (w/w)
Nordic nutrition recommendations	-	45-60	10-20	25-40	-
Standard American diet	4.8	50	15	35	0.07%
Standard chow	3.0	72	17	11	0.02%
Western diet	3.7	41	16	44	0.15%
High fat diet	4.5	40	20	40	1.25%

3.2 EXPERIMENTAL METHODOLOGY

Standard methods were used to assess T-cell phenotypes. T-cell proliferation was assessed as ^{3}H-thymidine incorporation and cytokine secretion was analyzed in supernatants. Flow cytometry analysis was used to discriminate between cell populations. Intracellular staining of transcription factors and cytokines was performed to separate T-helper cell lineages.

Quantification of atherosclerosis was done at several locations in the vascular tree. Cross-sections from the first millimeter of the aortic root were stained and used for quantification of atherosclerotic burden [343]. This method leaves several sections available for immunohistochemical evaluation of plaque composition [344]. *En face* preparations allow quantification of lipid-laden plaques in the rest of the aorta. Disease burden in the innominate artery can also be quantified by the use of carefully pinned specimens. Typically, only the aortic arch with branches was used for *en face* preparation, and the thoracic and abdominal parts were saved for RNA purification.

Blood sampling was done through tail vein bleeds or *post-mortem* cardiac puncture. Since CRP is expressed at very low levels in mice and does not act as an acute phase reactant [345], serum amyloid A was used as an inflammation marker in the mice studies. Other cytokines were analyzed with enzyme-linked immunosorbent assay [346] or cytometric bead array.

LDL was prepared from plasma through a two-step ultracentrifuge purification, which isolates plasma lipoproteins with a density between 1.019 g/ml and 1.063 g/ml [347]. LPL activity was measured in post-heparin plasma. Heparin releases LPL to the blood through interference of LPL's interaction with GPIHBP1. Lipoprotein production was assessed after inhibition of LPL with tyloxapol and subsequent measurements of plasma triglycerides. To distinguish chylomicron- from VLDL production, ^{3}H-glycerol was injected intravenously simultaneously with an olive oil gavage with ^{14}C-trioleate. Radioactivity from tritium in plasma was assessed as a measurement of VLDL particles assembled in the liver. Radioactivity from radiocarbon in plasma was assessed as a measurement of chylomicrons produced in the gut.

3.3 BIOBANK OF HUMAN ATHEROSCLEROTIC PLAQUES

The Biobank of Karolinska Endarterectomies (BiKE) was established in 2001 as a collaborative research effort between the vascular surgeons at Karolinska University Hospital and the Experimental cardiovascular research unit at Karolinska Institutet. The biobank consists of carotid plaques from patients undergoing carotid endarterectomies at Karolinska University Hospital [348]. Patients usually present themselves with symptoms such as minor stroke, transient ischemic attacks or amaurosis fugax, but also asymptomatic patients are operated. Carotid endarterectomy is recommended in cases with more than 70% stenosis as determined by ultrasonography. The atherosclerotic specimens were snap frozen, followed by RNA purification, and subsequent gene transcription analysis with chip arrays [349]. This biobank was interrogated to correlate mRNA levels in **Paper I**.

4 RESULTS AND DISCUSSION

4.1 IL-17 STABILIZES ATHEROSCLEROTIC PLAQUES

Paper I investigates the effect of increased TGF-β signaling in T cells on experimental atherosclerosis using a genetically modified mouse model. The increased TGF-β signaling was achieved through T-cell specific gene deletion of the signaling molecule Smad7, an intracellular inhibitor of TGF-β signal transduction. This led to differentiation of naïve T-helper cells into Th17 cells in the pro-inflammatory context of atherosclerosis (Fig. 2). IL-6 mRNA was expressed in the aorta as a response to hyperlipidemia and innate immune activation. IL-6 protein was also found in the draining lymph nodes where the conversion to Th17 cells occurred. Since no upregulation of IL-6 mRNA was observed in these lymph nodes, a plausible explanation is that IL-6 is drained to this location from the atherosclerotic plaques. TGF-β is abundantly available in the aorta [258], and TGF-β and IL-6 jointly differentiated the naïve T cells into Th17 cells.

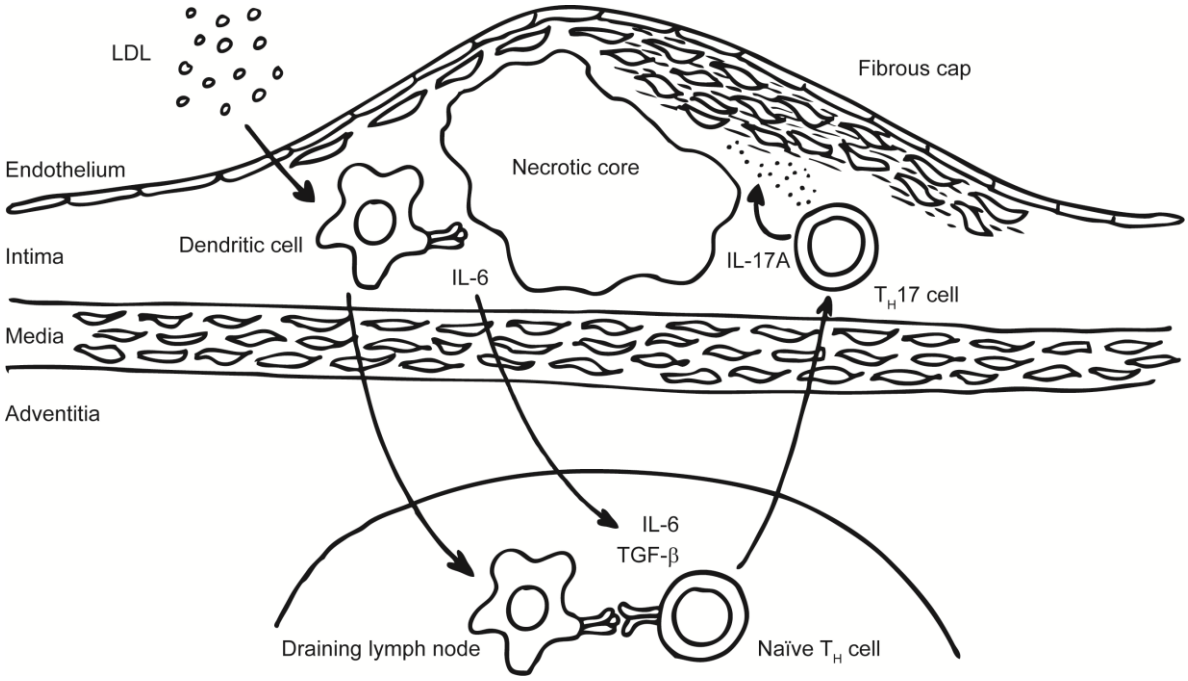

Figure 2. Schematic illustration of an atherosclerotic plaque. Lipoproteins infiltrate the arterial wall and initiate an inflammatory response with IL-6 production. Dendritic cells take up antigens in the plaque and migrate to draining lymph nodes where they present antigens to naïve T cells. In the present study, the T cells had a genetic predisposition to increased TGF-β signaling by the lack of the negative feedback molecule Smad7. Together with IL-6 present in draining lymph nodes of the aorta, the T cells differentiated into Th17 cells. These cells migrate back into the lesion where they secrete IL-17A that stimulates smooth muscle cells to produce collagen, thereby stabilizing the fibrous cap, which could prevent ruptures.

A stable fibrous cap was formed on top of the atherosclerotic lesions, and this effect was linked to IL-17A. In human carotid plaques, associations between IL-17A and markers for stable plaques were found. We could further show that IL-17A directly stimulates collagen production in vascular smooth muscle cells. Low levels of IL-17A in serum are associated with recurrent myocardial infarctions [241]. This corroborates our findings and suggests that

IL-17A is important for plaque stability in clinical disease. New treatments that neutralize IL-17A in conditions such as psoriasis might therefore be hazardous.

When the inflammation resolves, wound healing is initiated. Re-organization of the tissue occurs by replacement of the immune cells with tissue-resident cells and extracellular matrix. Trapped immune cells, with a low migratory capacity, characterize the atherosclerotic plaque [88]. The resolution of inflammation is compromised, and wound healing is restricted to the build-up of a stable cap. Smooth muscle cells migrate to form a layer underneath the endothelium where they proliferate and produce collagen, mainly of types I and III. IL-17A stimulates this process with induction of collagen type I synthesis. This process reflects IL-17A's function to form granulomas around pathogens and confine infections spatially [350]. However, in some organs such as the liver, the fibrogenic properties of IL-17A could be detrimental [58]. Local fibrogenic effects are beneficial for the stability of atherosclerotic plaques, but therapeutic stabilization would need a highly specific drug target, since off-target effects, such as liver fibrosis, are not acceptable. The option of local delivery to vulnerable plaques could be a futuristic possibility. The currently used local delivery method with drug-coated stents is not suitable. A major problem is restenosis after a percutaneous coronary intervention with placement of a stent. The desired effect is to inhibit smooth muscle cell proliferation in these cases, and IL-17A would probably do the opposite.

The IL-17 signaling pathway is operative in several tissues, and many cell types express IL-17 receptors. Downstream signaling includes general inflammation pathways such as mitogen-activated protein kinases [351]. Indeed, we demonstrated that extracellular signal-regulated kinase inhibition led to reduced collagen synthesis. The transcriptional regulation is mainly mediated through CCAAT/enhancer-binding proteins. The potential for IL-17 as a plaque stabilizing therapy, with such broad actions, is small, sharing limitations with TGF-β [352], even though the fibrogenic signal strength is less.

In **Paper I**, an interesting difference in lipoprotein metabolism was observed (Fig. 3). Increased amounts of chylomicron remnants were detected in postprandial plasma. LPL and hepatic lipase had normal activity, and the hypertriglyceridemia was lessened in the fasted state. This indicates that synthesis, rather than catabolism of triglyceride-rich lipoproteins, was mediating the effect. The phenotype presented itself only during the last weeks of the experimental period and was not a result of the increased IL-17A signaling, as neutralization of IL-17A indicated. Thus, the stabilization of the plaques with a thicker cap and more collagen was independent of the effect on chylomicrons. The phenotype is nonetheless interesting in regards to the selective deficiency of Smad7 in T cells. To evaluate the effect, further studies of cholesterol turnover, with a closer examination of bile-mediated triglyceride uptake, are needed.

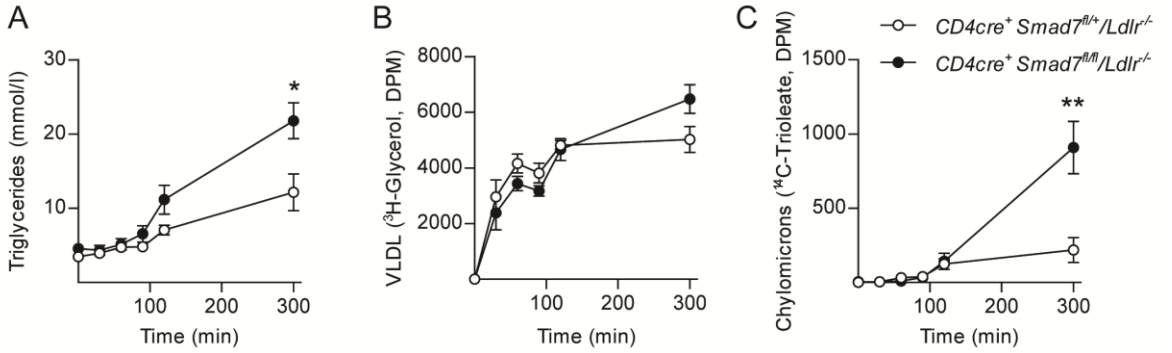

Figure 3. Assessment of Chylomicron and VLDL production. In this experiment, LPL was inhibited by tyloxapol injected intraperitoneally, then the mice were injected with ^{3}H-Glycerol intravenously and received a 100 µl olive oil gavage with ^{14}C-trioleate. (**A**) Total triglycerides were measured in plasma at baseline, and after 30, 60, 90, 120, and 300 minutes. Open circles represent *Ldlr*$^{-/-}$ controls and closed circles *Smad7*-deficient bone marrow chimeras (n=5). (**B**) ^{3}H radioactivity in plasma as a measurement of VLDL production by the liver (DPM=Disintegrations per minute). (**C**) Chylomicron accumulation in the plasma, measured as ^{14}C radioactivity, derived from the trioleate in the olive oil gavage mixture.

The initial hypothesis of the project postulated that Smad7-deficient T cells would increase Treg differentiation. However, raised Treg levels were not observed in the spleen of these animals. Smad-dependent signaling is not of major importance for thymic differentiation of natural Tregs [36]. Increased Foxp3 mRNA levels in inguinal lymph nodes suggested a minor induction of peripheral Tregs. This effect was not seen in the para-aortic lymph nodes. In this location, the augmented TGF-β signaling in T cells led to Th17 cell differentiation. Th1 cell differentiation was at the same time impaired. A previous study has investigated the opposite conditions. Disruption of TGF-β signaling in T cells induces Th1 cell differentiation and promotes atherosclerosis [257]. This experiment contrasts with the present study and sheds light over a possible equilibrium existing between Th17 cells and Th1 cells in regard to plaque stability. Th17 cells stabilize plaques, while Th1 cells do the opposite.

4.2 REGULATORY T CELLS CONTROL LIPID METABOLISM

In **Paper II** the role of Foxp3+ Tregs in atherosclerosis was assessed by depleting this cell type specifically (Fig. 4). This led to the development of large atherosclerotic plaques, but the plaques did not contain signs of increased inflammation and expansions of uncontrolled effector T cells as expected. The number of T cells per lesion area was similar to controls and both macrophages and APCs were decreased in the plaques in spite of an observed monocytosis in the blood. The large plaque size was linked to elevated plasma cholesterol in VLDL particles. This was further associated with a downregulation of sortilin in the liver. *Sort1* expression, encoding sortilin protein, is negatively regulated by Atf3 [353], a transcription factor under inflammatory control. In the liver, a concomitant increase of TNF and IFN-γ, pro-inflammatory cytokines, was observed on the mRNA level. Sortilin acts as a lipoprotein-regulating receptor in the liver [289]. The decreased sortilin levels led to delayed clearance of VLDL particles that instead were deposited in the vessel wall. Thus, depletion of Tregs both contributes to hypercholesterolemia and atherosclerosis through its effects on VLDL turnover. The *SORT1* locus has the strongest identified association with LDL-

cholesterol in humans [354]. Pathways that increase sortilin expression in the liver could potentially be targeted as treatments for dyslipidemia. Possibly, an anti-inflammatory drug that affects the sortilin pathway in the liver would lower plasma VLDL-cholesterol, which may lead to decreased atherosclerosis.

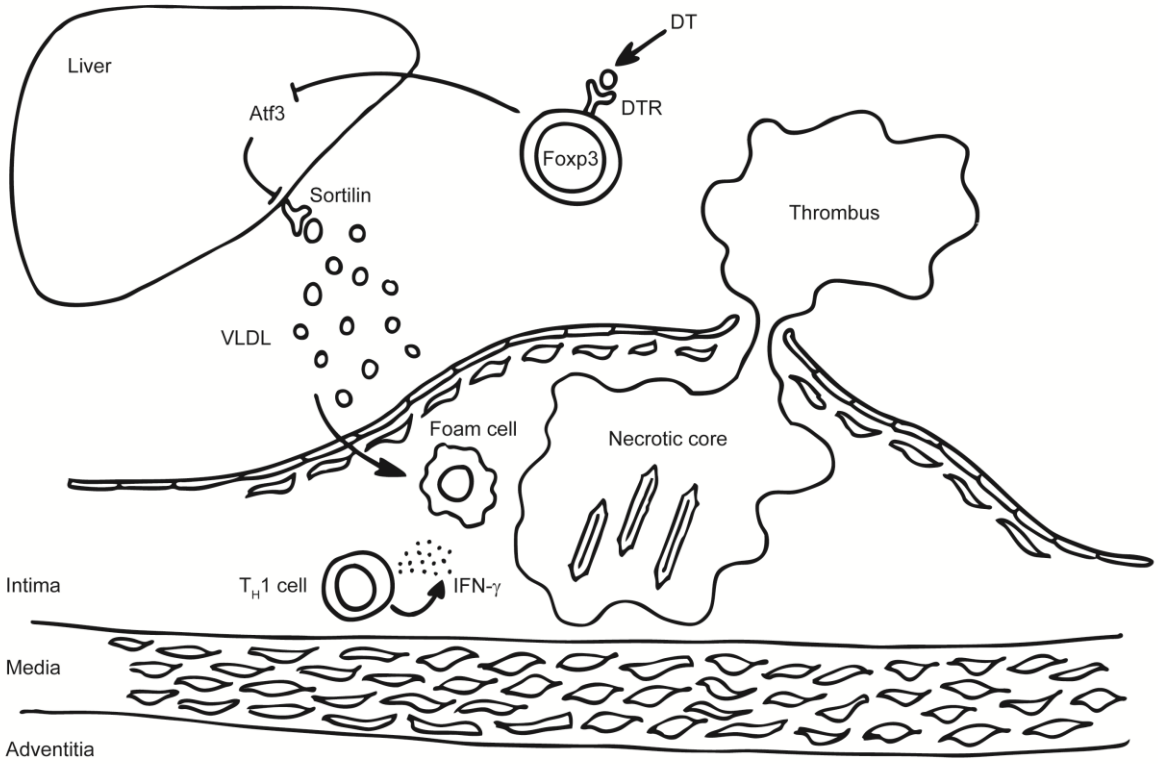

Figure 4. Schematic illustration of a Foxp3+ Treg that expresses a human diphtheria toxin receptor (DTR), which enables specific depletion of this cell type by administration of diphtheria toxin (DT). Tregs normally keep inflammation and autoreactive responses at bay. When depleted, the inflammation-responsive gene *Atf3* was upregulated in the liver causing downregulation of sortilin. Sortilin lowers plasma VLDL by acting as a receptor on the cell surface on hepatocytes and by decreasing release of nascent VLDL particles. The increase in plasma VLDL, mainly caused by delayed VLDL clearance, led to accelerated atherogenesis. The immune activation in the plaques was similar to controls, but the plaques had a large necrotic core with cholesterol crystals. Potentially, these large and lipid filled plaques are more vulnerable to rupture. In case of rupture, the blood comes in contact with thrombogenic material in the core and a thrombus forms that occludes the vessel.

Interestingly, the delayed clearance of VLDL particles led to an increased activity of LPL and hepatic lipase. The lipase activity made the circulating VLDL particles triglyceride poor, which was reflected by a decrease in plasma triglycerides. Increased PLTP activity was also observed. PLTP shuffles phospholipids from VLDL to HDL, and a small increase of triglyceride-containing HDL particles was accordingly seen in the Treg depleted mice. How these factors contribute to atherogenesis in this model is unknown. The elevation of VLDL-cholesterol was the main identified driving factor for atherosclerosis development in this model. This implicates that Tregs have an important role in controlling VLDL during homeostasis. The atherosclerosis process in *Ldlr*$^{-/-}$ mice is greatly influenced by VLDL-cholesterol levels [355]. The use of this mouse model in the present study might exaggerate the impact that VLDL has on atherosclerosis. Additional atheroprotective mechanisms of

Tregs, apart from controlling lipid metabolism, could likely be identified in other models of atherosclerosis. Importantly, this study did not target antigen-specific Tregs that could have anti-inflammatory actions locally in the arterial wall. Overall, the actions of antigen-specific Tregs, such as LDL-reactive ones, remain to be elucidated and enhancements of their specific immune modulatory effects might be potential treatments for human disease.

4.3 LDL-REACTIVE T CELLS LOWER PLASMA CHOLESTEROL

In **Paper III** we could assess T-cell specificity in atherosclerosis by the use of three new TCR transgenic strains reactive to human LDL. Through crossbreeding to *HuBL* mice that express human *APOB100*, we examined the role of LDL-reactive T cells in atherosclerosis. The autoreactive and LDL-specific T cells were not completely deleted in the thymus, and some clones survived (Fig. 5). These cells assisted B cells that were reactive to LDL, which in turn, formed germinal centers, and produced anti-LDL antibodies. The antibodies formed immune complexes with LDL in the blood and increased lipoprotein clearance. This lowered plasma cholesterol levels and protected against atherosclerosis. Correlations between immune complexes, cholesterol, and lesion size suggested that these factors were dependent of each other.

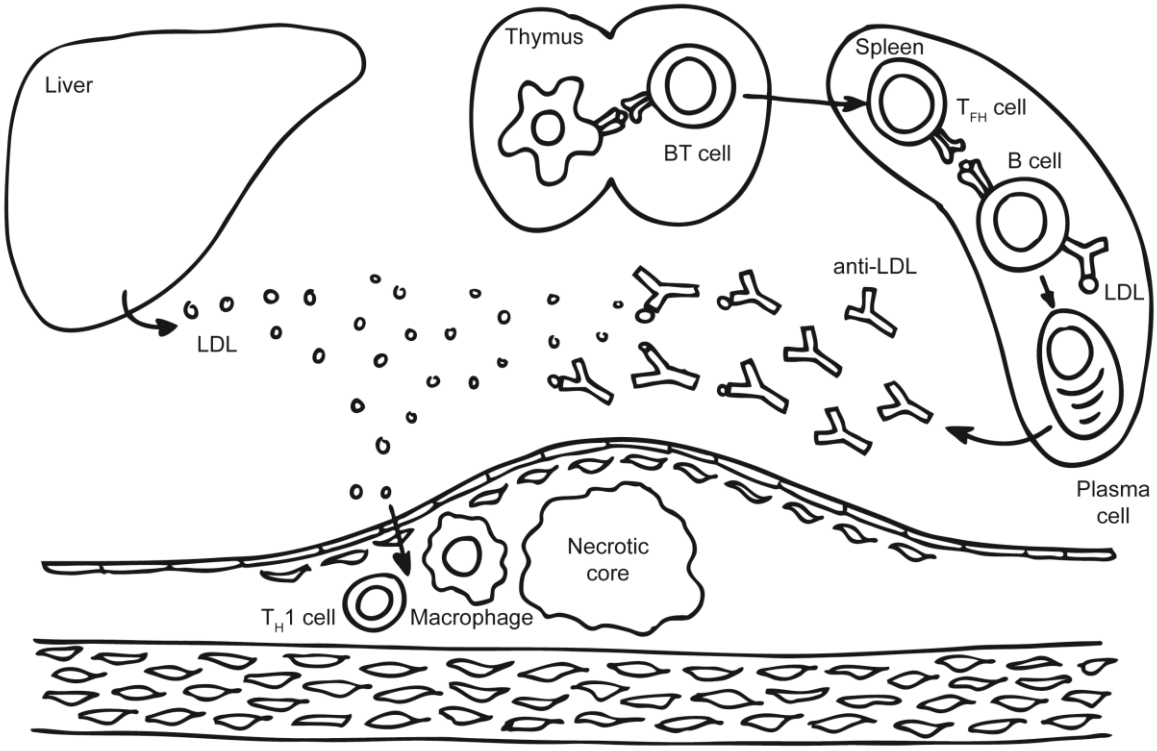

Figure 5. Schematic illustration depicting the thymus with its selection process to delete auto-reactive T-cell clones. Some ApoB-reactive T (BT) cells bypass the selection process and differentiate into Tfh cells in the spleen. The Tfh cells activate B cells reactive to LDL. In response to this, B cells form germinal centers and further differentiate into plasma cells. High-affinity anti-LDL IgG antibodies are secreted in the circulation, where the antibodies bind their antigen, LDL, forming immune complexes. This lowers LDL-cholesterol levels in plasma and protects from atherosclerosis.

Adoptive transfer experiments confirmed the notion of an atheroprotective humoral anti-LDL effect induced by LDL-reactive T cells. The injected CD4+ T cells differentiated into Tfh cells in the spleen. These LDL-specific Tfh cells induced a strong B-cell response with plasma cell differentiation and antibody production. The produced anti-LDL IgG antibodies formed immune complexes with circulating LDL particles and lowered plasma cholesterol levels. Transfer of plasma with high anti-LDL IgG titers from the injected mice could also induce increased LDL clearance in a separate batch of *HuBL* mice.

The initial clearance of IgG-immune complexes is usually driven by Fc-dependent uptake in macrophages in the liver and spleen [356]. Hepatocytes could also directly bind IgG-immune complexes [357] and mediate the clearance of cholesterol seen in the present study, with excretion to the bile and gut. This pathway would be therapeutically interesting to explore. As more antigen-antibody complexes are formed, some of them are deposited in vascular beds, predominately in small arteries and renal glomeruli [1]. This activates the complement system, recruits neutrophils, and, as a consequence, leads to tissue damage. It is currently unknown if anti-LDL immune complexes induce any adverse effects. The main findings in our experiments were reduced plasma cholesterol levels and protection against atherosclerosis.

In **Paper III**, we could show that ApoB is a tissue-restricted antigen with expression in the thymus, possibly controlled by AIRE or other means. To define precise mechanisms involved in the clonal deletion of ApoB-reactive T cells, the regulation of thymic *APOB100* expression needs further evaluation. If ApoB expression were under AIRE-control, an interesting experiment would be to cross *BT1xHuBL* mice to *AIRE*-deficient animals. The negative selection of ApoB-reactive T cells would then be constrained from birth and onwards. Atherosclerosis development would unravel peripheral tolerance mechanisms or the lack thereof. Interestingly, the liver has a special role in peripheral tolerance and expression of proteins in the liver can by itself induce immunological tolerance [358]. Such tolerance to ApoB is seemingly not active in atherosclerosis with LDL-reactive T cells present in the plaques [156]. Speculatively, the local plaque environment breaks the tolerance to ApoB.

Paper III uses TCR-transgenic mice with expression of a re-arranged TCR under control of the *CD2*-promoter. This infers some differences to the physiological clonal selection in the thymus [359]. Nonetheless, the use of this humanized mouse model identified important central tolerance to LDL. In a previous study, which corroborates our findings, the induction of neonatal tolerance to oxLDL led to clonal deletion of LDL-reactive T cells and reduced atherosclerosis development [360]. The central tolerance in the present study did not consist of a substantial induction of natural Tregs, and this might reflect the affinity of the investigated T-cell clone or the transgenic construct. Further studies are needed to clarify this notion. The adoptive transfers clearly suggested that induced LDL-specific Tregs play a minor role, judged by their low frequency. The main tolerance mechanisms identified were instead central clonal deletion and peripheral anergy. These mechanisms acted together with the humoral response to LDL to protect against atherosclerosis.

BCR activation is dependent on cross-linking several receptors on the B-cell surface. How this is achieved with ApoB as antigen is not known. LDL particles contain a single ApoB100 molecule, and there is logically only one epitope per particle. This differs from the oxidation specific epitopes in the lipid moiety of LDL particles. A single epitope per particle would prohibit cross-linking of BCRs and clustering of receptors, important factors for the downstream signaling [361]. Particulate antigens are usually excellent for B-cell mediated antigen presentation, but an avidity threshold tightly regulates the BCR-mediated internalization [362]. Several epitopes per particle are needed to reach this threshold. Speculatively, aggregation and fusion of LDL particles are crucial to exceed the avidity threshold for BCR activation. Two LDL-aggregating enzymes are interesting in this context, phospholipase A2 and sphingomyelinase [79]. These enzymes are implicated in atherosclerosis both with activity in the vessel wall and by enhancing retention of lipoproteins by increasing proteoglycan binding [131, 363]. The present study provides clear *in vivo* observations of B-cell activation against ApoB100. The involvement of aggregated lipoproteins in this process is a highly interesting avenue for further investigations.

4.4 CONCLUDING REMARKS

Atherosclerosis is a chronic inflammatory disease containing both innate and adaptive immune reactions. The autoimmune component includes T cells that react to peptide fragments of ApoB100. During atherogenesis, T-cell activation leads to Th1 differentiation under the influence of pro-inflammatory mediators such as IL-12. Tolerance mechanisms, with induction of Tregs, are at the same time hampered. Under certain circumstances, differentiation into Th17 cells occurs by the influence of TGF-β and IL-6. Effector T cells patrol the body and may undergo reactivation with cytokine production when they encounter their antigen in the plaques. Th1 cells contribute to destabilization of the plaques that make them vulnerable to rupture. On the other hand, Th17 cells promote plaque stability through IL-17A's effects on smooth muscle cells and collagen synthesis. Tregs have an important role to control inflammation and autoimmune responses throughout the body. Uncontrolled inflammation in the liver leads to delayed lipoprotein clearance and dyslipidemia. A protective humoral immunity against LDL can counteract such deviations and lower plasma lipoprotein levels through antibody-mediated clearance of particles. This response requires help from LDL-reactive T cells. The role for these cells is therefore dual, both activating a pro-inflammatory response with perpetuation of the inflammation in the vessel wall as well as evoking a protective B-cell response in the spleen.

LDL particles and inflammatory cells are major components of atherosclerotic plaques. Numerous interactions between lipoproteins and immune cells throughout the body may influence atherosclerosis development. This thesis gives three examples of the intricate interplay between the immune system and lipid metabolism: (**I**) Augmented TGF-β signaling in T cells increases chylomicron levels, (**II**) Tregs regulate sortilin expression in the liver, and (**III**) anti-LDL antibodies mediate clearance of LDL. These interconnections should be interesting to explore further with a therapeutic objective.

Paper III provides unique insights into how LDL-reactive T cells act in atherosclerosis. Adoptive transfers identified differentiation into Tfh cells and unraveled a major atheroprotective humoral response with production of lipid-lowering antibodies. This effect has been observed in previous vaccination studies that induced LDL-reactive T cells, but been dependent on immunization protocols and other factors. When the effect has been observed, it has been downplayed. Importantly, vaccine adjuvants skew T-helper cell differentiation. We could monitor adoptively transferred T cells without such conditions. The *BT1xHuBL* cross in **Paper III** provided an unprecedented model system of LDL-autoimmunity, and the protective anti-LDL humoral response was strong also in this model.

Emerging evidence suggests that non-modified LDL could suffice for both innate and adaptive immune activation in atherosclerosis. The innate immune system could be activated by cholesterol crystals through inflammasome activation [135, 136], and by ApoB-derived peptides [281]. Similarly, the adaptive immune responses to LDL recognize native peptides from ApoB100 [155, 211]. In line with this, treatments that target modifications of LDL has so far been disappointing [132]. Nonetheless, there are factors that remain undetermined such as, how could native LDL activate B cells and why is cellular immune tolerance compromised in the atherosclerotic plaques? Further studies are needed to explore the possibility of local factors in the plaques that may mediate a break of tolerance. A window for oxidative modifications has been suggested in which LDL particles have pro-inflammatory properties, but still contain intact epitopes that activate LDL reactive T cells [80]. However, other LDL modifications might be of importance, e.g., aggregation of LDL for B-cell activation. ApoB-derived peptides are commonly presented by APCs [364], but under which conditions this lead to T-cell activation, pathogenic Th1 cell differentiation, or induction of anti-inflammatory Tregs remain to be determined.

Cardiovascular disease is the main killer in the world, and new preventive measurements are required, e.g., a drug treating cardiovascular inflammation. The pathogenic inflammation in the vessel wall should be specifically targeted without affecting host defense or tumor surveillance. Targeting adaptive immunity with T-cell based treatments or vaccinations are attractive options [365]. The identification of disease-relevant epitopes in ApoB100 could open up possibilities for highly specific vaccine therapies. The properties of the epitopes will decide if therapies could be generalized or need to be personalized in accordance with HLA haplotype and other factors, which are, both genetic and environmental. As a start, the epitopes for the investigated TCR transgenic strains described in this thesis will be exactly mapped within the ApoB100 protein.

As a final summary, this thesis provides mechanistic understanding of how three T-helper cell subsets indirectly counteract the Th1-driven inflammation in atherosclerosis: (**I**) Th17 cells stabilize plaques, (**II**) Tregs control VLDL turnover, and (**III**) LDL-specific Tfh cells induce lipid-lowering antibodies.

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