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BRIDGING INNATE AND ADAPTIVE IMMUNITY IN CARDIOVASCULAR DISEASE

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BRIDGING INNATE AND ADAPTIVE IMMUNITY IN CARDIOVASCULAR DISEASE
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cardiovascular disease is the leading cause of death and morbidity in the world. Myocardial infarction and stroke constitute the main manifestations of atherosclerosis that lead to the majority of cardiovascular events. Calcific aortic valve stenosis is the most common valve pathology. Atherosclerosis and aortic valve stenosis share common risk factors such as hypercholesterolemia. Lipid lowering treatment has ameliorated the incidence of fatal events; however, residual risk remains indicating the need to address the inflammatory component in cardiovascular disease.

Many inflammatory mediators, such as cytokines and receptors have been implicated to play important role in the pathogenesis and the endpoints caused by an atherosclerotic plaque rupture. The role of pattern recognition receptors has been highlighted in several experimental studies. Both protective and detrimental effects have been described for the members of Toll-like receptor (TLR) family, a class of pattern recognition receptors. Several studies have focused on the role of the cell surface TLRs. The aim of the current thesis is to investigate the role of the intracellular pattern recognition receptor, TLR7. To gain information of the pathophysiological mechanisms that TLR7 is involved, both human cohorts of atherosclerosis and aortic valve stenosis as well as experimental models of atherosclerosis have been utilized.

In Paper I, mRNA expression of TLR7 in human carotid plaques was associated with patients´ outcome. Patients that expressed higher levels of TLR7 in their removed plaque had fewer future adverse cardio- and cerebrovascular events. Macrophages and T cells were co-localized with TLR7 in carotid plaques. Furthermore, carotid plaque tissue responded with increased cytokine secretion upon *ex vivo* stimulation with a synthetic TLR7 ligand.

Paper II showed TLR7 mRNA expression in calcified aortic valves. TLR7 mRNA was increased in calcified areas of the aortic valves compare to intermediate and healthy areas. In addition, TLR7 expression was associated with M2 macrophage markers in all parts of the aortic valve. Stimulation of calcified aortic valves *ex vivo* with a synthetic TLR7 ligand elicited cytokine response that was possibly derived directly or indirectly by macrophages.

In Paper III, we investigated the in vivo effects of a synthetic TLR7 ligand in experimental atherosclerosis. Locally, treatment with the synthetic TLR7 ligand led to decrease in lesion size and changes in plaque composition. The lesions of the treated mice presented lesions with smaller necrotic core and fewer apoptotic cells compare to the control. The treatment had effect in the spleen, leading to marginal zone B and regulatory T cell expansion. In the plasma, we observed decrease in cholesterol levels and increase in IgM antibodies against oxidized low-density lipoprotein.

The three studies presented in this thesis illustrate the protective role of TLR7 in atherosclerosis and aortic valve stenosis. TLR7 was expressed in both myeloid cells and lymphocytes

indicating a role of the receptor in bridging innate and adaptive immune. The current results can encourage the investigation of TLR7 ligands as therapeutic intervention in cardiovascular disease.

LIST OF SCIENTIFIC PAPERS

I. Karadimou G, Folkersen L, Berg M, Perisic L, Discacciati A, Roy J, Hansson GK, Persson J and Paulsson-Berne G.
Low TLR7 gene expression in atherosclerotic plaques is associated with major adverse cardio- and cerebrovascular events.
Cardiovasc Res. 2017;113:30-39

II. Karadimou G, Persson O, Carracedo M, Eriksson P, Franco-Cereceda A, Paulsson-Berne G and Bäck M.
TLR7 expression is associated with M2 macrophage subset in calcific aortic valve stenosis.
(Manuscript)

III. Karadimou G, Gisterå A, Gallina AL, Caravaca AS, Centa M, Salagianni M, Andreakos E, Hansson GK, Malin S, Olofsson PS, Paulsson-Berne G.
Treatment with a Toll-like Receptor 7 ligand evokes protective immunity against atherosclerosis in hypercholesterolemic mice.
J Intern Med 2020; 00: 1– 14

* In Paper I, the two first authors contributed equally.

OTHER RELATED PUBLICATIONS

Cederström S, Lundman P, Folkersen L, Paulsson-Berne G, Karadimou G, Eriksson P, Caidahl K, Gabrielsen A, Jernberg T, Persson J, Tornvall P.
New candidate genes for ST -elevation myocardial infarction.
J Intern Med 2020;287:66–77

CONTENTS

LIST OF ABBREVIATIONS

ABCA1	ATP Binding Cassette Subfamily A Member 1
ALP	Alkaline phosphatase
APCs	Antigen presenting cells
Apoe$^{-/-}$	Apolipoprotein E-deficient mice
AVS	Aortic valve stenosis
BCRs	B cell receptors
BiKE	Biobank of Karolinska Endarterectomies
BMP-2	Bone morphogenetic protein-2
CaMKII	$Ca2^{+}$/calmodulin-dependent protein kinase II ()
CEA	Carotid endarterectomy
CETP	Cholesteryl ester transfer protein
CLRs	C-type Lectin Receptors
CRP	C-reactive protein
CSFs	Colony stimulating factors
CTLs	Cytotoxic T lymphocytes
CVD	Cardiovascular diseases
DAMPs	Danger Associated Molecular Patterns
DCs	Dendritic cells
ECM	Extracellular matrix
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HDL	High-density lipoprotein
HMGB1	High mobility group box 1
HSPGs	Heparan Sulphate
ICAM	Intercellular adhesion molecule
IDL	Intermediate-density lipoprotein
IFN	Interferons
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin

IMQ	Imiquimod
IRF	Interferon response factor
IRF5	Interferon factor 5
LCAT	Cholesterol acyl transferase
LDL	Low-density lipoprotein
LDLR	LDL receptor
Ldlr$^{-/-}$	LDL-receptor-deficient mice
LPS	Lipoprotein polysaccharides
LRP	LDL receptor-like protein
LRP1	LDLR-related protein 1
MACCE	Major adverse cardio- and cerebrovascular events
MHC	Major histocompatibility complex
MI	Myocardial infarction
mmLDL	Minimally modified low density lipoproteins ()
MMP-2	Matrix metalloproteinase 2
MyD88	Myeloid differentiation protein 88
MZ	Marginal zone
NF-κB	Nuclear factor kappa B
NLRs	NOD-like receptors
OSEs	Oxidation specific epitopes
oxLDL	Oxidized LDL
PAMPs	Pathogen Associated Molecular Patterns
PC	Phosphatidylcholine group
PPR	Pattern Recognition receptors
qPCR	Quantitative Polymerase Chain Reaction
RIG	Retinoic acid-inducible gene
RLRs	RIG-like receptors
RMA	Robust multi-array average
RNAseq	RNA sequencing
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism

SR-B1	Scavenger receptor B1
ssRNA	Single stranded RNA
TCR	T cell receptor
TFH	T follicular helper cells
T_H	T helper
TLR	Toll-like receptors
TNF-R	Tumor necrosis factor receptor
TRAF	TNF-R-associated factor
Tregs	Regulatory T cells
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VICs	Valve interstitial cells
VLDL	Very low-density lipoprotein

1 INTRODUCTION

1.1 CARDIOVASCULAR DISEASE BURDEN

Cardiovascular diseases (CVD) are the leading cause of death worldwide [1]. One third of mortality in the world is due to cardiovascular disease endpoints. Between 1990 and 2015, approximately a 5 million increase in cardiovascular deaths occurred worldwide [2]. The prevalence of cardiovascular disease is associated with the sociodemographic status of the population. Increase in the sociodemographic status resulted in decrease in the number of deaths in a course of 25 years. However, this decrease seems to have reached a plateau. Ischemic heart disease is the first cardiovascular disease responsible for premature death and morbidity in the population [2]. In addition, morbidity caused by CVD has great impact in the economic costs for the healthcare system.

Most cardiovascular events are a result of the manifestations of atherosclerosis, such as ischemic heart disease and ischemic stroke. Collectively these manifestations are classified as major adverse cardio- and cerebrovascular events (MACCE). MACCE is defined as the incidence of myocardial infarction (MI), stroke, coronary and peripheral artery interventions [3, 4]. The main risk factors for developing atherosclerosis are established, including hypercholesterolemia, hypertension, smoking and obesity [5]. Aortic valve stenosis (AVS) is another cardiovascular disease that shares common risk factors with atherosclerosis with high prevalence in the population. AVS is ranked third in the most-common cardiovascular diseases in the developed countries [6]. The prevalence of the disease increases by age with 25% in individuals >65 years old.

Lipid lowering drugs have successfully reduced atherosclerotic disease mortality. However, 70% of the events are still not prevented [7]. Regarding AVS the only therapeutical approach is surgical or transcutaneous valve replacement. There is no pharmacological treatment that reduces the progression of AVS and in contrast to atherosclerosis, statins have no effect on the clinical outcome of the disease [8]. Taken together, the above indicate the emerging need for developing new therapeutic approach combined with the established.

1.2 INFLAMMATION AND CARDIOVASCULAR DISEASE

Early evidence for the causative factor for atherosclerosis development was generated by the experimental studies of Anitschkow and Chalatow [9]. They have showed that rabbits fed a high cholesterol diet formed fatty streaks. For several decades, the pathogenesis of atherosclerosis was attributed to cholesterol infiltration in the vessel wall, accumulation and eventually formation of lesions. However, lipid loaded foam cells were later identified in the lesions of rabbits fed with high cholesterol diet. These cells were having characteristics similar to macrophages [10]. Furthermore, Jonasson and Hansson demonstrated infiltration of other immune cells in atherosclerotic lesions [11-13]. Specifically, T cells and monocytes/macrophages were detected both in early stage fibrous plaques and in advanced lesions. Macrophages and smooth muscle cells expressed major histocompatibility complex

(MHC) II cell surface receptor (HLA-DR) indicating ongoing antigen presentation to T cells starting at early stage in the lesions [12]. That finding indicated involvement of immune system in the pathogenesis and progression of atherosclerosis [5].

More evidence was added later to the inflammatory hypothesis as a pathogenic factor in atherosclerosis by clinical studies and trials. High levels of C-reactive protein (CRP) was shown to be elevated in patients with stable angina and myocardial infarction that had a history of stable angina. This study indicated that the elevation in CRP was prior to myocardial infarction and thus was not attributed to myocardial necrosis [14]. Although, there was an increasing body of evidence regarding the involvement of the immune system in atherosclerosis, the inflammatory hypothesis was validated in 2017 with the results of the CANTOS trial. In CANTOS trial, patients with prior myocardial infarction and high CRP levels were treated with canakinumab, an interleukin (IL)-1β inhibitor. At the end of the treatment, the patients that received canakinumab presented lower levels of CRP compared to baseline and fewer future cardiovascular events compared to the placebo group. The decrease in CRP levels and reoccurring cardiovascular events was independent of cholesterol levels, which remained unchanged, showing the importance of immune contribution in the pathogenesis of cardiovascular disease [15].

1.3 CHOLESTEROL METABOLISM

Cholesterol is an established cause of cardiovascular disease. Familial hypercholesterolemia (FH) is an autosomal dominant disorder. Patients with FH present elevated blood cholesterol levels and cardiovascular symptoms from the third decade of life. Homozygous individuals have up to six-fold increase of cholesterol levels that lead to atherosclerotic plaque formation during childhood and often to fatal cardiovascular event before the age of thirty [16]. The studies of Goldstein and Brown revealed that FH disorder was the result of mutations involved in cholesterol metabolism. They discovered that low-density lipoprotein (LDL), where most of blood cholesterol is found, enters the cells through binding to a specific receptor called LDL receptor (LDLR) in cultured human cells from healthy donors and FH patients. FH patients had decreased number of LDLR, which were totally absent in homozygous individuals [17]. For their discoveries in the regulation of cholesterol metabolism and the underlying mechanisms causing FH Goldstein and Brown were awarded the Nobel Prize in Physiology or Medicine in 1985 [18].

Cholesterol is a crucial component of the cell membranes that is available by diet and de novo biosynthesis in the body. Furthermore, the synthesis of vitamin D, steroid hormones and bile salts require cholesterol as a precursor. The cholesterol that is absorbed by the intestine derives from diet and enters the intestine in bile. Approximately, half of the total amount of cholesterol is absorbed in the intestine while the rest is excreted in feces, bile salts and sebum [19].

The liver plays central role in the biosynthesis and the distribution of cholesterol around the body. Enterocytes in the gut are assembling cholesterol and triglycerides with $ApoB_{48}$ into chylomicrons [20]. Subsequently, chylomicrons transfer to the blood stream through the

lymphatic system. In the circulation, the triglycerides of the chylomicrons are hydrolyzed by lipoprotein lipase that is located in the vascular endothelium. This process generates cholesterol rich chylomicrons of reduced size. Monocytes, adipocytes or the liver take up the fatty acids that are released from the chylomicrons. Similarly, the cholesterol rich chylomicron remnants are removed from the circulation from the liver by binding to both heparin sulphate and LDL receptor-like protein (LRP).

Cholesterol is exported from the liver to the tissues in very low-density lipoprotein (VLDL). VLDL export by the liver involves the packaging of triglyceride with Apo B_{100} in the endoplasmic reticulum. The newly synthesized VLDL is assembled together with unesterified cholesterol and triglycerides into secretory vesicles in the Golgi. In pathological conditions such as obesity and insulin resistance, VLDL is overloaded with triglycerides. VLDL is removed in the liver by the LDLR, heparan sulphate (HSPGs), LDLR-related protein 1 (LRP1) and scavenger receptor B1 (SR-B1) [21, 22]. In the presence of cholesteryl ester transfer protein (CETP) cholesteryl ester from high-density lipoprotein (HDL) and LDL is transferred to VLDL. Esterification of cholesterol by cholesterol acyl transferase (LCAT) allows the accommodation of larger amounts of cholesterol into the lipoprotein core.

Triglyceride removal from VLDL by lipoprotein lipase leads to formation of LDL. LDL can due to its small size cross the vascular endothelium and supply tissues with cholesterol. Excess of cholesterol arriving at the tissues in LDL is reversely transported back to the liver via HDL or the ATP Binding Cassette Subfamily A Member 1 (ABCA1) receptors of extrahepatic tissues. In the liver LDL is removed by LDL receptors or enters hepatocytes after leaving HDL [19].

1.4 TOOLS FOR STUDYING CARDIOVASCULAR DISEASE

Different approaches and methodologies have been developed in order to uncover the pathogenesis of human disease, to identify biomarkers for improved diagnosis methods and to provide therapeutic approaches. Important tools to achieve the above are the combination of *in vitro* cultures, mouse model and studies of human material either population based or through molecular analysis.

1.4.1 Biobanks

Biobanks are organized repositories of biological samples, responsible for appropriate long-term storage of samples combined with several clinical and epidemiological patient data [23]. Biobanks follow standardized operating protocols that facilitate the comparability between samples in the same biobank and between biobanks worldwide. Usually, biobanks are built based on a disease-oriented model and are connected to a specific study. Biobank samples and data can provide several advantages such as better disease stratification, development of personalized medicine and establishment of worldwide health policies [24].

Today functional genomic studies use large-scale analysis to get information from gene to RNA to protein on pathological processes. Blood samples that are easy to collect can be used in large

cohorts of patients for the identification of genetic markers and other parameters [25]. Genetic predisposition for certain diseases have been investigated through genome-wide association studies (GWAS). Several GWAS have associated single nucleotide polymorphisms (SNPs) to disease outcomes such as myocardial infarction [26, 27]. Furthermore, polymorphisms in a regulator gene of MHC II expression was correlated with autoimmune diseases and myocardial infarction [28].

A blood sample cannot provide detailed information regarding the ongoing disease processes in the affected tissue. Valuable information is obtained regarding pathogenic or protective processes by the analysis of the infected tissue. Expression and regulation of genes can be affected not only by genetic factors but also by the microenvironment of the lesions in a specific disease. Transcript profiling provided the possibility to analyze mRNA from 20 000 genes in one sample and get information about the activation state of the genes in each sample. Nowadays, microarrays and sequencing provide information about the transcript profile of tissues or cells of interest in a specific disease.

1.4.2 Animal models

Several animal models are used for the study of atherosclerosis and aortic valve stenosis such as hypercholisterolaemic mice, pigs, rabbits and more seldom non-human primates. Mouse is the most favorable experimental animal for the study of cardiovascular diseases [29, 30]. The advantages of the use of mice in research include the relatively small cost, ease in breeding and genetic manipulation and short life cycle.

Apolipoprotein E-deficient mice (*Apoe*$^{-/-}$) [31, 32] and LDL-receptor-deficient mice (*Ldlr*$^{-/-}$) [33] are the most commonly used animal models in atherosclerotic research [34-37]. *Apoe*$^{-/-}$ mice lack apoE, an apolipoprotein necessary for the removal of lipoprotein particles in circulation. In addition, the mice present high levels of plasma cholesterol and develop atherosclerosis spontaneously. Importantly, in *Apoe*$^{-/-}$ mice plasma cholesterol is located mainly in VLDL, chylomicrons and intermediate-density lipoprotein (IDL) particles. The atherosclerotic lesions develop particularly in the aortic branch and in branch points throughout the aorta. The lesions have similar morphology to the human plaques with presentation of all stages of plaque development, fatty streak, fibrous plaque and advanced lesions. In contrast to *Apoe*$^{-/-}$ mice that develop spontaneously atherosclerotic lesions, *Ldlr*$^{-/-}$ mice require high fat diet (HFD). Another difference between *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice is that in *Ldlr*$^{-/-}$ mice plasma cholesterol is mainly located in the IDL and LDL fractions. *Ldlr*$^{-/-}$ mice on a high fat, high-cholesterol diet have increased plasma cholesterol levels and develop lesions that resemble fatty streaks. HFD can also be used to accelerate atherogenesis in *Apoe*$^{-/-}$.

Nowadays, several methodologies have been developed that allow in depth study of the different processes from the initiation of atherosclerosis to advanced lesions. Genetic manipulation including generation of transgenic animals, temporal and conditional gene knockout and knock-in provided the opportunity to manipulate either the level or function of specific immune component. These genetic manipulations were of major importance in

dissecting the cellular and molecular mechanisms of the role of the immune system on cardiovascular diseases [29]. Furthermore, the study of the initiation of atherosclerosis has been possible by the generation of inducible models. Pharmacological depletion of ApoE leads to increase in plasma cholesterol levels at the chosen time point [38].

Apoe$^{-/-}$ and *Ldlr*$^{-/-}$ mice are also used for the study of calcific aortic valve stenosis. *Apoe*$^{-/-}$ on a chow diet develop aortic valve stenosis phenotype with immune cell infiltration and calcification of aortic valve at the advanced age of 2.5 years [39], while dietary interventions can accelerate the phenotype [40]. In addition, hypercholesterolemic *Ldlr*$^{-/-}$ ApoB (100/100) mice develop valve calcification without dietary intervention at an age of 17 to 22 months [41].

Experimental studies in mice can provide information about the *in vivo* pathways in cardiovascular diseases, although they have several differences with the humans such as size, lifespan, lipid profile, heart rate and also disease endpoint [29]. Combination of *in vivo* experimental animal studies with data generated in human disease set up can lead to advances in the understanding of the pathogenesis of cardiovascular diseases and open possibilities for future therapeutic approaches.

1.5 THE IMMUNE SYSTEM

The primary role of the immune system is to stop entrance of pathogens in the organism and prevent possible infection in the case of breach. However, the immune system has other crucial homeostatic roles that include the clearance of apoptotic cells and tissue healing. In addition, the immune system fights against the establishments of several kind of tumors in the body. The immune system is divided in two branches; the innate and the adaptive. The innate immune system responds rapidly to eliminate any possible threat such as pathogens. The adaptive response needs longer time to develop but offers the advantage of a specific response and long term immunological memory [42]. The main cells of innate and adaptive immune system are depicted in **figure 1**.

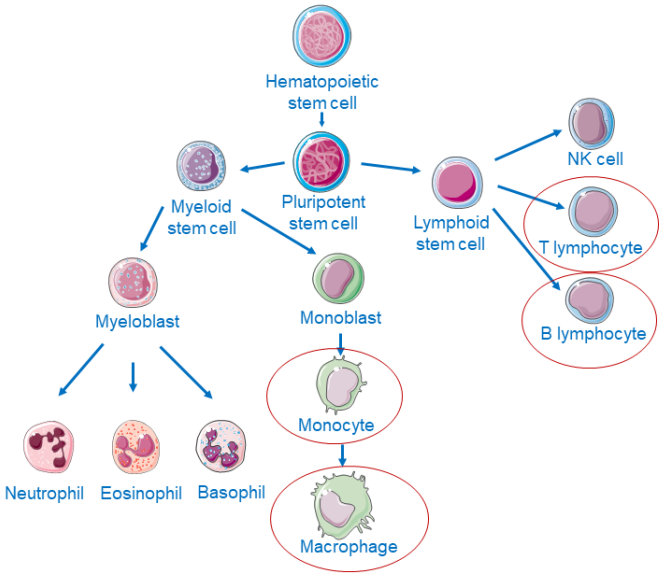

Figure 1. Immune cell types. Hematopoiesis takes place in the bone marrow, where the different stem cell progenitors are generated. Myeloid stem cell progenitors give rise to polymorphonuclear cells and monocytes/macrophages. Lymphocytes derive by the lymphoid stem cell progenitors. The red circles indicate the innate and adaptive immune cells that are the focus of the current thesis. The schematic art pieces used in this figure were provided by Servier Medical art (https://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

1.5.1 The innate immune system

Important roles of innate immunity are host defense against pathogens and healing after injury. The first innate immune barrier for infectious agents is the epithelial layer. In the case of breach in the epithelial layer, innate immune cells are attracted to the site for the elimination of the pathogen. The process of uptake of infectious agents is called phagocytosis. In order to recognize rapidly pathogens the innate immune system has conserved receptors for common pathogen structures. These receptors are called Pattern Recognition receptors (PPR) and are highly conserved through the species, found from the fruit fly Drosophila to humans. They recognize molecular sequences conserved in pathogens, the Pathogen Associated Molecular Patterns (PAMPs). In addition, the innate immune system can recognize endogenous danger signals as response to injury and cell death; the so-called Danger Associated Molecular Patterns (DAMPs). PPR have high specificity for the conserved sequences they recognize, however it differs compared to the specificity of the adaptive immune receptors. All innate immune cells produce the same PPR while the adaptive receptors are specific for each antigen [42].

Neutrophils and monocytes are circulating innate immune cells that are recruited to the sites of infection, where they recognize and eliminate infiltrating pathogens. In case of infection, neutrophils are the first cells to respond and proliferate rapidly. The proliferation and maturation of the neutrophil progenitor cells is stimulated by colony stimulating factors (CSFs) [43]. Neutrophils are short-lived cells with a lifespan of few hours to few days in humans. Fewer numbers of monocytes are circulating in blood compared to neutrophils. Monocytes survive longer periods and circulate in the blood, bone marrow and spleen. Monocytes derive from hematopoietic stem cells from the bone marrow (Figure 1). The suggested role of monocytes in homeostasis include scavenging of dead cells and toxic molecules, and/or renewal of 'resident' tissue macrophages and dendritic cells (DCs). During inflammation due to tissue damage or infection, blood monocytes migrate from blood to lymphoid and nonlymphoid tissues in response to tissue-derived signals. They clear apoptotic cells and modified immunogenic molecules (such as oxidized lipoproteins), produce inflammatory cytokines, and can differentiate into DCs or macrophages in the infiltrated tissues [44].

1.5.1.1 Macrophages

Macrophages are tissue resident cells involved in tissue surveillance, clearance of apoptotic cells, tissue repair and immune modulation. Tissue resident macrophages have embryonic origins. Renewal of tissue macrophages after birth is achieved by proliferation and infiltrating monocytes that subsequently differentiate to macrophages [45]. Macrophages “sample” the surrounding space and distinguish self from non-self/pathogenic. Nowadays, it is believed that macrophages are divided in several subtypes with different functions that is shown by differential expression of group of markers. Most commonly macrophages are divided in two main subtypes; the M1 which are the one taking part in host defense by inhibitory action while the M2 are more involved in healing after tissue injury processes [46]. Furthermore, M2 macrophages are divided in four subclasses; M2a, M2b, M2c, M2d [47]. M2a macrophages are responsible for wound-healing. M2a macrophages express high levels of mannose receptor

(CD206) and contribute to tissue repair by secretion pro-fibrotic factors such as TGF-β [47, 48]. M2b macrophages are involved in inflammatory regulation by secretion of large amounts of IL-10. Similar to M2b, M2c macrophages exert anti-inflammatory functions by secretion of IL-10 and TGF-β and have high capacity of apoptotic cell clearance. Last, M2d macrophages, also called tumor associated macrophages, are characterized by high production of anti-inflammatory cytokines and vascular endothelial growth factor (VEGF) connected to tumor progression and metastasis [47, 49]. Division of macrophages into M1 and M2 subtypes is a simplistic view, since macrophages exhibit great plasticity depending on changes on their microenvironment.

Macrophages are the main phagocytic cells. One of their main roles is to facilitate the clearance of apoptotic cells during development and adult life. The process of removal of apoptotic cells from tissues is named efferocytosis. Apoptotic cells express “find me” and “eat me” signals, attracting macrophages in the site and facilitating the clearance [50]. They are equipped with several receptors that recognize these “find me” and “eat me” signals.

Macrophages recognize and respond to different pathogens through a series of receptors such as the highly conserved PPRs and scavenger receptors. In order to sense PAMPs and DAMPs the innate immune system is equipped with 4 major classes of PPR such as the Toll-like receptors (TLR), C-type Lectin Receptors (CLRs), the Retinoic acid-inducible gene (RIG)-like receptors (RLRs) and the NOD-like receptors (NLRs). PPRs are found both on surface or intracellularly to cover all entries in the cell [51].

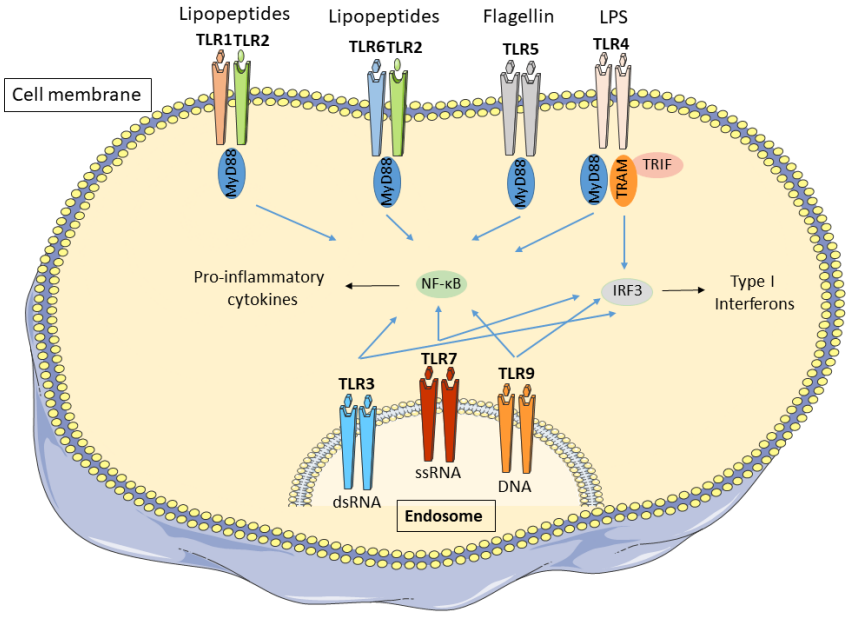

Figure 2. Toll-like receptor signaling pathways and ligands. TLRs are forming hetero- and homodimers. Some of them are located in the cell surface membranes while the intracellular members of the family are spanning the endosomal membranes. Common ligands for extracellular TLRs include molecules of bacterial wall origin, while intracellular TLRs recognize mainly nucleic acids. Most TLRs require the adaptor molecule MyD88 and the downstream translocation of NF-κB for the production of pro-inflammatory cytokines. Activation of intracellular TLRs leads to production of pro-inflammatory cytokines through NF-κB and type I interferons through TRIF and IRF3 pathway. The schematic art pieces used in this figure were provided by Servier Medical art

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1.5.1.2 Toll-like receptors and signalling pathways

TLR family is an important key in all innate response. Today 11 different TLRs have been recognized in humans and 13 in mouse. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the cytoplasmic membrane whilst TLR3, TLR7, TLR8, TLR9 are intracellular receptors and located in the endolysosomes and the endoplasmic reticulum (Figure 2). TLRs can also form homo- and heterodimers like TLR2/TLR6 [52].

Almost all TLRs, with the exception of TLR3, require the adaptor myeloid differentiation protein 88 (MyD88) for downstream signaling. After TLRs encounter a specific ligand there is activation of two major signaling pathways. Pro-inflammatory cytokines are produced through nuclear factor kappa B (NF-κB) activation and type I interferons (IFN) through activation of interferon response factor (IRF) pathway (Figure 2) [53].

1.5.1.3 Toll-like receptor ligands

TLRs recognize PAMPs and DAMPs. The PAMPs include lipid based ligands of bacterial origin like lipoprotein polysaccharides (LPS) for TLR4, bacterial proteins like flagellin that is recognized by TLR5 and viral origin nucleic acids that are binding TLR3, TLR7, TLR8 and TLR9. TLR7 is recognizing single stranded RNA (ssRNA). Furthermore, DAMPs that are released during injury or cell death can also bind to TLRs [52, 54] and elicit response.

1.5.2 The adaptive immune system

In contrast to the innate immune system adaptive immunity offers a less rapid response but with increased specificity and diversity. In addition, upon encounter of a lymphocyte with an antigen for the first time, a primary response is formed and in parallel, there is generation of memory cells. The second time that a lymphocyte will encounter the same antigen, the secondary response as it is called, will progress more rapid, with increased magnitude and more effective in eliminating the antigen. The lymphocytes consist of the different T and B cell subsets (**Figure 1**) [42].

1.5.2.1 T lymphocytes

T lymphocytes are generated in the bone marrow and transfer to the thymus for maturation. They recognize mainly peptide antigens bound to Major histocompatibility complex (MHC) through a membrane bound T cell receptor (TCR). All T cells express the cell surface marker CD3, a protein that is part of the T cell receptor complex. The most usual division of T cells is to T helper (T_H) subsets that express in addition to CD3, the CD4 and cytotoxic T lymphocytes (CTLs) that express CD8 in the cell surface. The role of the T_H subsets is to activate the effector functions of macrophages and B cells, while the CTLs recognize and eliminate cells that have been infected or damaged and cancer cells. T cells are equipped with TCR for antigen recognition. TCR is a heterodimeric receptor that consists of two chains with a constant and

variable region. The variable region of the TCR is responsible for the recognition of the antigen-MHC complex [42].

$CD4^+$ T_H cells are divided to at least three subsets; the T_H1, T_H2 and T_H17. Each subset recognizes different type of antigen and produce distinct set of cytokines. The differentiation into the different subsets it is determined by the microenvironment in combination with the invading pathogen [42].T_H1 cells are mainly responsible for the stimulation of phagocytes for the elimination of intracellular parasites such as bacteria or viruses. The signature cytokine that is secreted by the T_H1 cells is interferon-γ (IFN-γ). In addition, differentiation of naïve T cells to T_H1 subset is induced by IL-12 secreted by dendritic cells [42].

T_H2 cells react against helminth parasites through activation of eosinophils. Differentiation of naïve T cells to T_H2 is induced by the cytokine IL-4. T_H2 cells secrete IL-4 that stimulates antibody class switching and immunoglobulin (Ig) E antibody production, IL-5 that stimulates the secretion of granule content by eosinophils and IL-13 responsible for mucus secretion and intestinal peristalsis [42].

T_H17 cells recruit neutrophils and monocytes to facilitate the killing of extracellular bacteria and fungi. T_H17 cells produce IL-17 and IL-22. IL-1, IL-6, IL-23 and TGF-β lead to differentiation towards the T_H17 subset [55].

Regulatory T cells (Tregs) are a T cell subpopulation with important role in immune homeostasis and suppression of autoimmune responses. Tregs are produced in the thymus or differentiate from naïve T cells in the periphery. Traditionally, they are characterized as $CD4^+CD25^+FoxP3^+$ cells; however, other Treg subpopulations also exist. Tregs exert their immunosuppressive functions by several mechanisms. They suppress pro-inflammatory responses by production of anti-inflammatory cytokines such as IL-10 and TGF-β. In addition, they can downregulate inflammatory processes indirectly by depletion of extracellular ATP and IL-2 and thus making them unavailable for pro-inflammatory cells [56].

1.5.2.2 B lymphocytes

B lymphocytes are produced in the bone marrow and the final maturation stages occur in spleen. B lymphocytes recognize different types of antigens including proteins, polysaccharides, lipids, nucleic acids and small chemicals [42]. They express B cell receptors (BCRs) with unique antigen binding epitopes. Structurally, BCRs are membrane bound immunoglobulins. The development of a specific BCR is achieved by recombination of the available variable (V), diversity (D), and joining (J) genes. Assembling of the recombined heavy and light chain polypeptides form the mature BCR [57, 58].

Upon recognition of an antigen, naïve B cells differentiate into plasma cell and produce the antibodies with the specific epitopes as the one expressed on B cells as surface bound receptors. Antibodies are divided in five main classes, which are IgA, IgD, IgE, IgG and IgM. In addition, the immunoglobulin classes differ structurally and in capacity of secretion and undergo

posttranslational modifications [59]. Naïve B cells bear in their membranes antibody bound receptors IgM and IgG.

B lymphocytes are divided into B1 and B2 cells. The B1 cells are responsible for the surveillance of the peritoneal and mucosal cavities. B1 cells recognize mainly non-protein antigens and produce naturally occurring antibodies in a T cell independent manner. B2 cells are located in the spleen and are divided in follicular and marginal zone (MZ) B cells. Developmental and environmental factors lead to differentiation of B2 into the different subsets [60]. MZ and follicular B cells remain in the spleen in mice [61], while in humans MZ-like cells have been also found in circulation [62]. Follicular B cells interact with T follicular helper cells (TFH), become germinal center (GC) cells and produce high affinity antibodies against protein antigens [63]. MZ B cells are located in the periphery and recognize blood born antigens. Upon encounter with an antigen MZ B cells produce fast T cell independent antibodies. MZ and B1 derived plasma cells are short lived and produce fast, low affinity antibodies. MZ and B1 cells are able to produce mainly IgM antibodies. On the other hand, plasma cells derived by follicular B cells and GC formation, are long lived and keep antibody titers steady for several years. Follicular B cells have the capacity to produce all immunoglobulin isotype classes through class-switching [59, 64].

1.5.3 Tissues of the immune system

The tissues of the immune system consist of the thymus and bone marrow, the generative lymphoid organs, and the lymph nodes, the spleen, the mucosal and cutaneous immune systems, known as peripheral lymphoid organs. The role of the peripheral lymphoid organs is to promote the development of immune responses. In the peripheral lymphoid organs, T cells and B cells reside closely with the antigen presenting cells (APCs). APCs concentrate antigens in the peripheral lymphoid organs and enable responses from T and B cells [42].

The peripheral lymphoid organs filter the lymph, the blood, the skin, the gastrointestinal and respiratory tracts for antigens. The lymph nodes are located along the lymphatic channels and their role is to filter and capture antigens that circulate in the lymphatic vessels. The substances that arrive in the lymphatic system derive from the epithelia and several tissues. The spleen is a highly vascularized organ that filters the blood for blood born antigens. Dendritic cells and macrophages capture blood born antigens. The abundant phagocytes in the spleen eliminate many of the antigens circulating in the blood. The cutaneous and mucosal immune systems, such as the tonsils and intestinal Peyer´s patches recognize pathogens that breach the epithelium [42].

Most of the peripheral lymphoid organs have defined morphology divided into different anatomical compartments. The spleen consists of the red and white pulp. These two regions are separated by the marginal zone (MZ). The red pulp is responsible for removing from the circulation dead, opsonized cells and aged red blood cells. Furthermore, red pulp has the role to filter the blood for pathogens and molecules connected to tissue damage. Several innate immune cells such as macrophages and dendritic cells are located in the red pulp, although the

white pulp is primarily responsible for the generation of immune responses. Upon recognition of a pathogen in the red pulp, several cells such as T cells and plasma cells will migrate in the red pulp for the elimination of the pathogen. The white pulp consists of distinct zones where the B and T cells are located. In the white pulp B cells reside in the follicles while T cells are located in the periarteriolar lymphoid sheath. The marginal zone that acts as a bridge between the white and red pulp is populated by MZ B cells and two macrophage subsets; the marginal zone macrophages and the marginal metallophilic macrophages. Lymph nodes have similar morphology with the spleen. However, in contrast to the spleen, lymph nodes have a capsule that separates the different compartments and also pathogens are entering the lymph nodes through the lymphatic vessels that are absent in the spleen [65].

1.6 BRIDGING INNATE AND ADAPTIVE IMMUNITY

For a long time it was believed that innate and adaptive immune system act individually. Since the innate immune system is highly evolutionarily conserved, it was perceived as more primitive and the interest in immunological research was shifted towards the adaptive immune system. However, long time after the innate and adaptive immune system was described the effects of innate immune system on adaptive response were highlighted [66].

Key players in the bridging of innate and adaptive immunity are the dendritic cells and pattern recognition receptors. Successful adaptive immune activation requires three signals. The first signal is the antigen presentation from DCs to T cells [67]. The second signal is the upregulation of costimulatory molecules due to activation of PPRs in the DCs by PAMPs. The third is the production of innate cytokines by DCs that facilitate T cell differentiation [68, 69].

TLR activation plays a central role in innate modulation of the adaptive immunity. Activation of TLRs enhanced antigen presentation by increasing phagocytic processes. Studies have shown that TLR4 activation increased antigen cross presentation to CD4 T cells [70], while activation of the intracellular TLRs by nucleic acids enchanted antigen presentation and led to activation of CD8 T cells [68, 71].

More evidence is accumulating regarding the expression of TLR in cells of the adaptive immune system. Studies have shown that both T and B cells express TLRs [72, 73]. In addition, TLR ligands have been shown to activate T cells and B cells *in vitro*. Stimulation of human purified T cells *in vitro* stimulated proliferation and cytokine secretion from $CD4^+$ T cells [72]. MZ B cells express high levels of TLRs [74]. It has been shown, that LPS leads to dual stimulation of BCR and TLR that results in fast production of high affinity antibodies [75].

In conclusion, cells such as DCs and MZ B cells facilitate the bridging of innate and adaptive immunity. In addition, expression of pattern recognition in DCs and adaptive immune cells is required for a successful adaptive immune response.

1.7 PATHOGENESIS OF ATHEROSCLEROSIS AND AORTIC VALVE STENOSIS

1.7.1 Atherosclerosis

The initiation of atherosclerotic lesions is already happening early in life with the development of fatty-streaks during adolescence, which progressively leads to the formation of advanced lesions with the accumulation of lipids, immune cell infiltration and structural reorganization. Atherosclerotic lesions are formed in the intima layer of the artery [5, 76]. Arteries structurally consist of three layers; the intima, media and adventitia. High LDL cholesterol levels in the circulation lead to infiltration and accumulation of LDL in the intimal layer. The infiltrated LDL particles are no longer protected by antioxidant factors that are present in the plasma and undergo several modifications including oxidation. The modified LDL particles gain pro-inflammatory properties that lead to attraction of monocytes in the site. Monocytes enter the lesions by binding to adhesion molecules like vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) expressed by the activated endothelium. In the intima, the infiltrated monocytes differentiate into macrophages and express scavenger receptors that will facilitate the uptake of LDL particles. Uptake of large amount of LDL particles by macrophages eventually leads to foam cells formation (**Figure 3**). Increased apoptosis of foam cells in the plaques leads to inflammatory responses and the formation of a big necrotic plaque. In the hyperlipidimic environment within the plaque, other macrophage functions such as cholesterol efflux and efferocytosis are also dysregulated [77]. Subsequently, there is infiltration of T cells in the intima that play immunomodulatory role, regulating the functions of the neighboring cells, such as macrophages, endothelial and smooth muscle cells. Secreted mediators of the accumulating leukocytes lead to smooth muscle cell (SMC) migration from the media layer to the intima, in the site of the progressing lesion [78].

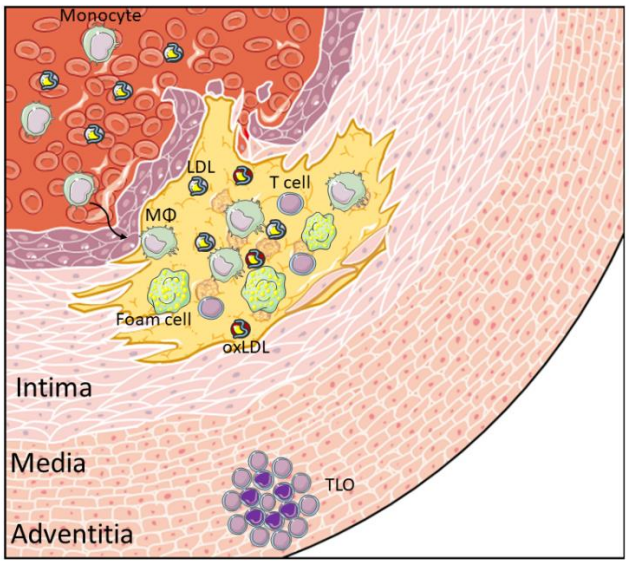

Figure 3. Pathogenesis of atherosclerosis. Infiltration and modification of LDL in the intima, leads to recruitment of monocytes that enter the site through adhesion molecules expressed by the activated endothelium. Macrophages in the lesions uptake LDL and become lipid loaded foam cells. Attraction of T cells in the lesion promote a pro-inflammatory phenotype. In addition, advanced lesions are characterized by smooth muscle cell differentiation,

migration, and changes of the extracellular matrix. For long time the focus was primarily influx of leukocytes from circulation but nowadays more evidence exist that secondary and tertiary lymphoid organs formed in the adventitia are involved in the progression of atherosclerosis. The schematic art pieces used in this figure were provided by Servier Medical art (https://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

Endothelial cells are mostly quiescent however; they can be activated in response to pathological stimuli such as disturbed blood flow. Low laminal shear stress in vessel areas such as bifurcations, prone to develop atherosclerotic lesions, results in activation of pro-inflammatory pathways in endothelial cells [79]. In the intima cytokines, chemokines and growth factors secreted by the activated endothelium induces the proliferation and extracellular matrix synthesis by smooth muscle cells, hence initiating generation and remodeling of the developing atherosclerotic lesions [80]. A study has shown that endothelial endoplasmic reticulum stress and apoptosis results in endothelial erosion and subsequently in thrombus formation [81].

Smooth muscle cells are the main cell type constructing the vessel wall. During atherogenesis vascular remodeling takes place. Proliferation of smooth muscle cells has been connected to protective effects by increasing plaque stability [82]. In contrast, SMC migration and apoptosis due to inflammation and enzymatic activity contribute to plaque rupture [83]. Investigation of the Biobank of Karolinska Endarterectomies (BiKE) (see below, 3.1, Methodological Considerations) led to identification of a convertase involved in processes of plaque instability. The proprotein convertase, PCSK6 was shown to be increased in symptomatic compared to asymptomatic patients and was expressed mainly in SMC located in the fibrous cup [84]. A recent study of the same group showed that PCSK6 is a key regulator of SMC processes such as proliferation and migration [85]. Furthermore, the role of SMC cells in atherosclerosis depends on the origin of the cells. One general view suggests that SMC are atheroprotective whilst macrophages promote the disease [86] but the picture is more complex. Plaques with high macrophage numbers and fewer SMC are vulnerable and prone to rupture. However, several human and mice studies have shown that SMC within the plaque positive for traditional SMC markers express also macrophage markers like CD68, indicating transition into macrophage–like cells [87-89]. These cells have lower capacity of lipid and apoptotic cell clearing compared to macrophages thereby leading to increased inflammation. Stress induced apoptosis of SMC can lead to fibrous cap thinning and rupture, commonly in the shoulder region of the plaques. SMC cells can be beneficial by the protection of fibrous cap and plaque repairing but macrophage –like SMCs have a pro-atherogenic role [86].

Extracellular matrix (ECM) remodeling and degradation is involved in the pathogenesis of several cardiovascular diseases. ECM facilitates the adaptation of the vascular wall to mechanical forces. ECM includes 3 components, the proteoglycans that are located in the subendothelial space and the elastic fibers and collagens that are located in the tunica media together with vascular smooth muscle cells [90]. Single nucleotide polymorphism at the COL4A2 locus was associated with decreased atherosclerotic plaque stability and increased coronary heart disease risk [91]. Versican, the main proteoglycan in the vessel wall, is

associated with pro-atherogenic processes. Versican has been connected with LDL retention in the subendothelial space [92]. The retained LDL is subsequently removed by macrophages that progress to foam cells and thus enhancing plaque development. In another study, depletion of the perlecan heparin sulfate proteoglycan increased experimental atherosclerosis. The mice presented increased smooth muscle cell content. These results indicate a detrimental role for perlecan in atherosclerosis [93].

Especially sensitive sites for atherosclerosis development are coronary and carotid arteries. The atherosclerotic lesion grows slowly and silently, starting with fatty streak formation before the age of 30 and finally reaching an advanced fibrotic plaque 30-40 years later. The lesions are complex in their structures, and show a high degree of infiltrating immune cells such as macrophages and T cells. The actual event leading to an infarction, is the rupture of a plaque followed by thrombus formation that either occlude the vessel on site or embolises. Plaques that are prone to rupture are called vulnerable or unstable plaques [94]. Inflammatory components are often greatly involved in the dangerous atherosclerotic plaque rupture [94]. Several studies from the last years have put the spotlight on specific subtypes of macrophages as being a driving force in plaque rupture [95].

1.7.2 Aortic valve stenosis

Calcific aortic valve stenosis (CAVS) develops due to the gradual remodeling of the aortic valve. The normal aortic valve consists of three leaflets and called tricuspid aortic valve. However, 0.5-1% of individuals have a congenital abnormal bicuspid aortic valve due to incomplete separation during embryogenesis [96, 97]. Individuals with bicuspid valves develop some type of aortic pathology, in which mineralization of the aortic valve occurs in response to several factors such as genetic, hemodynamic and mechanical forces [98]. The most common aortic pathology in individuals with bicuspid valve is CAVS that develops approximately two decades earlier compared to individuals with tricuspid valves [99]. Differences between tricuspid and bicuspid valve patients in the pathogenesis of the disease have been also shown by differential expression of gene profiles with upregulation of inflammatory genes only in tricuspid valve patients [100]. The most important risk factors for developing CAVS is age and bicuspid valve. Other factors include smoking, hypertension, hypercholesterolemia, obesity and renal failure [99, 101-103]. In addition, genetic risk factors have been implicated in the pathogenesis of CAVS [104].

Lipoprotein deposition, immune cell infiltration and differentiation of cells towards an osteoblastic phenotype lead to active leaflet calcification. The human aortic valve is constructed by three leaflets. Each leaflet is composed by the fibriosa facing the aorta, the spongiosa and the ventricularis located in the left ventricular tract. The main cell type composing the aortic valve are the valve interstitial cells (VICs). Aortic valves also contain few smooth muscle cells and endothelial cells that cover the ventricular and aortic surfaces [99].

Endothelial damage due to mechanical stress and pro-inflammatory mediators allows the infiltration of LDL to the subendothelial space. LDL retention in the valve leaflets attracts

immune cells at the site. Macrophages, T and B cells are the main immune cells that infiltrate aortic valves [105, 106]. Reactive oxygen species and enzymes produce lysophospholipid derivatives that activate pro-inflammatory pathways and osteogenic differentiation of VICs. Imbalance of matrix metalloproteinases and their inhibitors in the valve lead to disorganization of fibrous tissue. Macrophages and VICs actively promote calcification by deposition of macrovesicles. Furthermore, production of leukotrienes and prostaglandins promote expression of osteogenic genes and valve mineralization of VICs *in vitro* [107]. Taken together, lipid modification leads to immune cell and activation of pro-inflammatory and osteogenic pathways that actively contribute to the progression of calcific aortic valve stenosis.

1.7.3 Innate immunity in cardiovascular disease

Several studies have shown that innate immune cells and receptors play important role in the pathogenesis but also in the regression of atherosclerosis. Macrophages and TLR activation are highly involved in these processes. In addition, macrophage activation and innate immune signaling is implicated in the pathogenesis of CAVS.

Macrophages

High numbers of macrophages used to indicate plaque instability. However, nowadays the macrophage phenotype is more important than the number. Both M1 and M2 macrophages are present in human lesions [108]. Pro-inflammatory M1 macrophages are located in higher numbers in the shoulder regions than are prone to rupture while M2 anti-inflammatory macrophages are mostly found in the fibrous cap or the adventitia [109, 110]. This suggest that M2 macrophages could balance the detrimental effects of M1 through their healing/reparative capacities. M1 and M2 macrophages that have been identified in the plaque differ in their lipid content. M1 macrophages contain more intracellular lipids than M2 [111]. As previously discussed the M1 versus M2 classification is an oversimplification. Macrophage phenotype depends on tissue microenvironment. Mox is a macrophage phenotype that has been identified in atherosclerotic lesions and is activated by the presence of oxidized phospholipids [112]. Furthermore, a recent study has shown that recruitment of $Ly6C^{hi}$ monocytes to the lesions and differentiation towards M2 macrophage phenotype resulted in atherosclerosis regression [113].

Several macrophage functions such as efferocytosis, are defective in atherogenic environment [114]. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), that is responsible for the regulation of Ca^{2+} related processes, inhibited efferocytosis pathways in macrophages. Depletion of CaMKII in atherosclerotic mice improved plaque stability with smaller necrotic cores [115]. In addition, arginine metabolism through arginase 1 in macrophages enhanced clearance of apoptotic cells. Lack of arginase 1 in atherosclerotic mice was accompanied with defective efferocytosis *in vivo* [116].

Similar effect in disease outcome for the different macrophage subsets was observed in calcific aortic valve stenosis. M1 macrophages were associated with enhanced calcification of VICs in an *in vitro* co-culture model of human VICs and M1 macrophages stimulated with LPS [117]. M1 macrophages were significantly increased in calcified compared to non-calcified human

aortic valves. In the same study, transfer of conditioned media from M1 macrophages to human VICs *in vitro* increased osteogenic gene expression [118].

1.7.4 TLR effect in cardiovascular disease

The role of most TLRs has been investigated in atherosclerosis. The cell surface TLRs have been mainly associated with detrimental effect when it comes to atherosclerosis. In contrast, the intracellular receptors are connected to protective effects.

TLR ligands in cardiovascular disease

Both exogenous and endogenous TLR ligands have been associated with development and exacerbation of atherosclerosis. Most of the exogenous TLR ligands are of bacterial origin, like Tri-acyl lipopeptides, peptidoglycans and liposaccharide [119]. *Tlr2*$^{-/-}$*xLdlr*$^{-/-}$mice presented decreased atherosclerosis indicating an endogenous ligand. In the same study, administration of the synthetic TLR2 ligand Pam3CSK4 exacerbated atherosclerosis in *Ldlr*$^{-/-}$ mice [120]. Peptidoglycans, a gram-positive bacterial component, was detected in human atherosclerotic plaques and correlated with instability [121]. In addition, atherosclerosis was accelerated after LPS administration to hypercholesteraemic mice [122].

Established endogenous ligands in atherosclerosis are derived from lipoprotein modifications, vascular injury and cell death. Minimally modified low density lipoproteins (mmLDL), oxidized LDL (oxLDL) and ApoCIII (very-low-density LDL component) serve as ligands for CD14/MD-2/TLR4 complex, TLR4/TLR6 heterodimer and TLR2 respectively. Another endogenous atherosclerotic ligand is the high mobility group box 1 (HMGB1) that signals directly through TLR2 and TLR4 ligands. One described indirect effect of HMGB1 is activation of the intracellular TLR9 through DNA-immune complexes [123].

TLR2 / TLR4

Several TLRs have detrimental role in atherosclerosis. TLR2 and TLR4 ApoE knockout mice presented decreased atherosclerosis [124]. In addition, activation of TLR2 in the human plaque endothelium lead to up-regulation of adhesion molecules and attraction of neutrophils that elevated inflammation and loosened the endothelial layer causing superficial erosion. This type of erosions release debris in the vessel lumen accompanied with a dangerous thrombus formation [81]. Studies have shown that TLR2 and TLR4 are expressed in VICs and their activation contributes to the pathogenesis of CAVS. *In vitro* activation of TLR2 and TLR4 in human derived aortic VICs resulted in increased expression of pro-inflammatory cytokines and osteogenic genes [125, 126].

TLR3

Surprisingly, *Apoe*$^{-/-}$*xTlr3*$^{-/-}$ indicated a protective role of the receptor against atherosclerosis, shown by increased atherosclerotic burden in *Apoe*$^{-/-}$*xTlr3*$^{-/-}$ and by mechanical arterial injury [54]. However, there are other murine studies showing that TLR3 facilitates extracellular matrix degradation through the control of matrix metalloproteinase 2 (MMP-2) [127]. Its role

in hematopoietic knockout of the downstream regulators TRIM and tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) decreased atherosclerosis [128]. Furthermore, the synthetic ligand Poly (I:C) promoted calcification in human AVICs. The effect was mediated by stimulation of pro-inflammatory and osteogenic factors such as the bone morphogenetic protein-2 (BMP-2) and alkaline phosphatase (ALP) [129].

TLR7

Several studies have been performed for the elucidation of the role of TLR7 in atherosclerosis. In a murine study in collaboration with our group *Apoe*$^{-/-}$*xTlr7*$^{-/-}$ mice presented increased atherosclerosis, with big necrotic core and M1 macrophage infiltration in the lesions. In the same study, TLR7 transcript in human carotid plaques, part of the Biobank of Karolinska Endarterectomies was correlated with M2 anti-inflammatory markers, indicating that TLR7 is important for the switch of macrophages towards a more healing, anti-inflammatory phenotype [130]. In another study, depletion of Interferon factor 5 (IRF5) a downstream mediator upon TLR7 and TLR9 activation lead to aggravated atherosclerosis in lupus mouse model. The effect was mediated by decrease in anti-inflammatory cytokine IL-10. IRF5 is a necessary mediator for the production of IL-10 downstream of TLR7 [131]. Furthermore, stimulation with a TLR7 ligand led to increase in patrolling monocytes in circulation, similar to atherosclerotic conditions. Patrolling monocytes have been shown to have protective role against endothelial damage. These data indicate a protective role for TLR7 activation in atherosclerosis through effects in circulating immune cells [132].

In contrast to the previous data, two recent studies have shown detrimental effects for TLR7 in atherosclerosis. *Apoe*$^{-/-}$*xTlr7*$^{-/-}$ were protected against diet induced atherosclerosis [133]. In the same line stimulation of atherosclerotic mice with a TLR7 ligand promoted lesion formation [134]. The role of several TLRs in atherosclerosis is not clarified yet. Contradictive data have been presented in some studies. This discrepancy may be explained through differences in species, ligand and stage of the disease and prompts further investigations.

Currently there is not so much evidence regarding the role of TLR7 in calcific aortic valve stenosis. The study of TLRs in CAVS has been mainly studied in isolated human VICs *in vitro*. TLR7 is expressed in VICs in very low levels compared to the highly expressed TLR2 and 4. In addition, stimulation of human VICs with the synthetic TLR7 ligand imiquimod *in vitro* did not yield NF-κB activation indicating inactivity of TLR7 in human VICs [135]. However, stenotic aortic valves are infiltrated by several immune cells that have been shown to express the receptor. More studies are required for the investigation of the role of TLR7 in CAVS.

1.7.5 Adaptive immune cells in cardiovascular disease

T lymphocytes

Several studies have been conducted in order to clarify the role of each T cell subset in the athersoclerostic lesions. T cells have been localized mainly in the fibrous cup of atherosclerotic lesions. $CD4^+$ T cells constitute the predominant subtype in atherosclerotic plaques [13]. In

addition, T cells have been shown to recognize ApoB100 through antigen presentation from APCs. Blocking of this response by immunization in atherosclerotic mice decreased disease burden [136].

$CD4^+$ T_H cell subsets (T_H1, T_H2, T_H17 and Treg) are extensively studied with controversial results for some of the subsets. T_H1 are the main producers of INF-γ, an abundant pro-atherogenic cytokine present in lesions [137]. Secretion of IFN-γ in the plaque inhibited antigen presentation processes, differentiation and proliferation of smooth muscle cells [138, 139]. In addition, INF-γ can inhibit the maturation of collagen fibers and thus affect the stability of the fibrous cap [140]. The role of T_H2 cells is not clarified yet. The atherosclerotic environment, specifically severe increase in cholesterol levels resulted in a shift of T_H1 cells towards T_H2 [141]. Furthermore, depletion of T_H2 related cytokines such as IL-4 and IL-13 resulted in contradictory effects with both promoting and inhibiting the progression of atherosclerosis respectively [142, 143]. Decreased intima/media thickness was associated with increased numbers of T_H2 cells in blood and lower risk for women of having an MI [144]. T_H17 cells is another T_H subset with conflicting effects in atherosclerosis. Depletion of IL-17A, the signature cytokine of T_H17 cells resulted in contradictory results in atherosclerosis with both acceleration [145] and reduction [146] in lesion size. IL-17A promoted plaque stability through stimulation of collagen production by smooth muscle cells [147].

Regulatory T cells

Multiple studies indicate that Tregs have anti-inflammatory properties and decrease atherosclerosis. Depletion of Tregs with anti-CD25 antibodies increased disease burden in atherosclerotic mice [148]. In addition, depletion of Tregs resulted in aggravation of atherosclerosis and increase in plasma cholesterol levels. These data indicated that Tregs protect against atherosclerosis by modifications on lipoprotein metabolism [149]. Dendritic cell vaccination recognizing FoxP3, resulted in reduction of $Foxp3^+$ T cells in several organs and had detrimental effect in atherosclerosis with increased immune cell infiltration in the lesions [150]. Transfer of Tregs is protective [151]. In addition, expansion of Tregs by pharmacological intervention such as anti-CD3 or Vitamin D3 administration, decreases atherosclerosis [152, 153]. Another study has shown that Treg expansion led to regress of atherosclerotic lesions [154]. Furthermore, Tregs play different role in the stages of atherosclerosis with decreased atherosclerosis in the initiation and plaque stabilization in advanced lesions [155].

B lymphocytes

The first indication that B cells play important role in atherosclerosis was shown by the presence of immunoglobulins in the atherosclerotic plaques [156-158]. *Caliguiri et al* have shown a functional role for B cells in atherosclerosis. In this study, *Apoe*$^{-/-}$ mice that were splenectomized presented increased atherosclerosis. When the investigators adoptively transferred B cells back to splenectomized mice the effect on atherosclerosis was abrogated [159]. Furthermore, hypercholesterolemic mice injected with apoptotic cells induced protective B cell response within the spleen. The treatment led to expansion of B1a and MZ cells [160].

Transfer of bone marrow cells from B cell deficient mice to irradiated *Ldlr*$^{-/-}$ mice led to increased atherosclerotic burden [161]. Another recent study has shown that high cholesterol diet activated an anti-inflammatory program in the spleen where marginal zone (MZ) B cells controlled T follicular helper cells [162]. In addition, memory B cells have been correlated to better outcome with fewer reoccurring cardiovascular events in a human cohort of patients undergoing carotid endarterectomy [163].

Humoral immunity

The role of immunoglobulins in atherosclerosis have been studied both in human disease and through experimental animal models. As previously described, during atherogenesis LDL undergoes several modifications such as oxidation, acetylation and methylation. It has been shown that antibodies against oxidation specific epitopes (OSEs) are infiltrated in atherosclerotic lesions [164]. Except for LDL molecule, OSEs have been discovered in apoptotic cells, damaged proteins, microvessels and bacteria [165]. Specifically, antibodies recognize the phosphatidylcholine group (PC) in both oxLDL and apoptotic cells.

Contradictory effects have been described by several studies regarding the role of IgG antibodies in cardiovascular disease. In a human cohort anti-OxLDL IgG levels were associated with increased cardiovascular events in participants. The effect was more profound for individuals of black ethnicity [166]. Furthermore, meta-analysis of seven studies have shown association of anti-oxLDL IgG with increased risk for atherosclerosis related cardiovascular events [167]. However, *in vivo* experimental studies have indicated a protective role for IgG antibodies in atherosclerosis. Intravenous administration of polyreactive IgG to *Apoe*$^{-/-}$ mice inhibited early and advanced atherosclerosis [168]. In addition, injection of IgG antibodies against malondialdehyde-modified apoB-100 resulted in regression of atherosclerotic lesions [169]. Adoptive transfer of LDL-reactive T cells promoted the production of T cell dependent anti-LDL antibodies that mediated plasma cholesterol decrease in the *human APOB100-transgenic Ldlr*$^{-/-}$ mice [170]. Furthermore, IgG antibodies promoted smooth muscle cell proliferation and thus atherosclerotic plaque stability [171]. More studies are necessary in order to elucidate the role of IgG antibodies in cardiovascular disease.

In contrast, IgM is associated with protective pathways in cardiovascular disease. Several experimental model studies and human cohorts have demonstrated a protective role of IgM antibodies in atherosclerosis. Human cohorts have negatively associated high levels of IgM antibodies against OxLDL and MDA-LDL with the risk for cardiovascular events [166, 172]. Lack of the soluble IgM due to genetic depletion resulted in increased disease burden and IgM reconstitution conveyed protection against atherosclerosis [173, 174]. Initially, the protective role of anti-oxLDL IgM antibodies was demonstrated by cloning the E06 IgM recognizing oxidized phospholipids from the spleens of atherosclerotic mice. *In vitro* assays have demonstrated the capability of E06 IgM to inhibit OxLDL uptake by macrophages [175, 176]. In addition, passive immunization of *Apoe*$^{-/-}$ mice with T15/E06 IgM antibodies against PC epitope ameliorated vein graft atherosclerotic lesion size [177]. However, depletion of the V1 (VHS107.1.42) immunoglobulin heavy chain gene that is required for the production of T15

IgM antibodies in atherosclerotic mice did not affect the disease burden [178]. Another protective pathway in atherosclerosis that IgM against OSEs are involved is the clearance of apoptotic cells through epitope mimicry. As it was discussed above, apoptotic cells share common epitopes with oxidized phospholipids such as PC. The involvement of IgM antibodies in apoptotic cell clearance has been shown in several studies. Transfer of apoptotic cells in hypercholesterolaemic mice resulted in increase of OSEs IgM antibodies and decreased atherosclerosis. The effect was abrogated in B cell deficient mice [160]. Furthermore, in an experimental set up where apoptotic cells were mimicked by transfer of PC liposomes in atherosclerotic mice, B1a cell numbers and total IgM levels were increased with an accompanied decrease in disease burden [179].

1.8 MEDIATORS IN CARDIOVASCULAR DISEASE

Both pro- and anti-inflammatory mediators play important role in all stages of atherosclerosis. Several groups of mediators are involved in the atherosclerotic processes; cytokines of different classes including interleukins, tumor necrosis factors, interferons, colony-stimulating factors and transforming growth factors.

Cytokines

TNF-α and IFN-γ are two pro-atherogenic cytokines with multiple effects on several cell types within the plaque. They have been shown to alter endothelial cell function like distribution, junction disruption and up-regulate the expression of chemokines and adhesion molecules. These result in increased transmigration of immune cells in the lesion [180]. In addition, IFN-γ facilitates the formation of foam cells by increasing the expression of scavenger receptors and inhibits the cholesterol efflux by decreasing the expression of ATP-binding membrane cassette transporter 1. Furthermore, both IFN-γ and TNF-α are responsible for macrophage and smooth muscle cell death. TNF-α is inducing broad production of MMPs and decreases the antithrombotic functions of endothelial cells. It was shown that *Apoe*$^{-/-}$ mice TNF-α deficient had reduction in their lesion size [180]. IL-12 is another cytokine with pro-atherogenic role. IL-12 induced T_H1 cell proliferation due to the increase of the transcription factor Tbet [181].

IL-10 is the most potent anti-inflammatory cytokine in the plaque, produced by M2 macrophages, Treg, T_H2 and some T_H1 cells in the lesions. The double knock out model *Apoe*$^{-/-}$ deficient for IL-10 had pronounced phenotype with increased lesion formation, inflammatory cell infiltration and pro-inflammatory cytokine production. On the contrary, increased IL-10 serum levels in patients of acute coronary syndromes were correlated with better prognosis [182]. IL-10 modulates several macrophage functions that promote atherosclerosis [183].These functions include inhibition of the secretion of pro-inflammatory cytokines, the expression of adhesion molecules and MMPs. That results in less infiltration of monocytes in the lesions and increases stability. Furthermore, a recent study showed that IL-10 polarizes macrophages towards the anti-inflammatory type M2. IL-10 has anti-apoptotic effects by increasing the expression of genes like Bfl-1 and Mcl-1. Another protective modulation of IL-10 is the

increase in cholesterol uptake from macrophages in combination to increased cholesterol influx that prevents foam cell formation [183].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a growth factor that promotes the maturation of dendritic cells and monocytes from bone marrow. The role of GM-CSF in atherosclerosis has not been well clarified yet. Atherosclerosis was increased in the *Apoe*$^{-/-}$*xGm-csf*$^{-/-}$ mice indicating protective effect [184]. However, the opposite effect was observed in *Ldlr*$^{-/-}$*xGm-csf*$^{-/-}$ mice [185].

Type I IFN

Type I interferons are mainly produced by dendritic cells and macrophages after the activation of intracellular TLRs. Their role in atherosclerosis seems to be detrimental. IFN-α increases the expression of scavenger receptors and upregulates the uptake of ox-LDL leading to formation of foam cells [186]. Treatment of *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice with IFN-β increased the secretion of chemokine CCR5 that lead to increased monocyte infiltration in the lesions [187].

2 AIMS

The present thesis aimed to investigate the role of the Pattern Recognition Receptor TLR7 in atherosclerosis and calcific aortic valve stenosis.

The specific aims were to:

I. Investigate the role of TLR7 in advance human carotid plaques
II. Explore the effect of TLR7 in calcific aortic valve stenosis in the different macrophage subsets
III. Identify activated pathways upon *in vivo* stimulation with a TLR7 ligand in experimental atherosclerosis

3 METHODOLOGICAL CONSIDERATIONS

3.1 BIOBANK OF HUMAN CAROTID ATHEROSCLEROTIC PLAQUES

The Biobank of Karolinska Carotid Endarterectomies was established in 2001 in collaboration between the Experimental Cardiovascular Research Unit at Karolinska Institutet and the Department of Vascular Surgery at Karolinska University Hospital. The biobank consists of samples and data from approximately 1000 patients. Carotid plaques removed under carotid endarterectomy (CEA) are collected, also including from each patient plasma, RNA from PBMC, and DNA. All operated patients presented more than 70% occlusion according to the North American Symptomatic Carotid Endarterectomy Trial, NASCET criteria [188]. Several of the patients experienced symptoms such as minor stroke, amaurosis fugax and transient ischemic attacks. In addition, BiKE includes transcript profile data of carotid plaques and clinical data. Non-atherosclerotic normal arteries were obtained from 10 macroscopically disease-free iliac arteries or aorta from organ donors without a history of cardiovascular disease at Karolinska University Hospital.

Specific aim for our group in the BiKE cohort has been to study inflammation and immune response in patients with severe atherosclerosis by using transcript profiling. In addition, we aimed to investigate whether clinical parameters and patients´ outcome can be correlated to transcriptional profiles. The above types of analyses has led to identification of several key molecules in atherosclerosis [85, 189-192]. The publicly available BiKE dataset has been also utilized by external users, such as in a recent publication of immune cell atlas for atherosclerotic plaque [193].

3.2 FOLLOW UP AND DEFINITION OF EVENTS

In order to investigate the effect of TLR7 in patients´ outcome in **Paper I**, BiKE database was merged with the Swedish Hospital Discharge Register and the Swedish Cause of Death Register. Follow-up data of adverse cardiovascular, cerebrovascular, and vascular events for all patients included in the cohort were collected from the above registers. MACCE were defined as the combined incidence of myocardial infarction, stroke, percutaneous coronary intervention, coronary artery bypass grafting, endovascular intervention of peripheral arteries, and open surgery on peripheral arteries, as reported to the registers listed above. Ischemic events were defined as fatal or nonfatal myocardial infarction or ischaemic stroke. All causes of death other than MACCE were excluded. The retrieval of myocardial infarction and stroke incidence data from the Swedish Hospital Discharge Register and the Swedish Cause of Death Register is a reliable, validated alternative to the use of revised hospital discharge and death certificates. The total number of MACCE and MI events in the microarray discovery cohort was 34 and 24, respectively. In the qPCR validation cohort, 47 MACCE and 33 MI events occurred.

3.3 BIOBANK OF HUMAN AORTIC VALVES

Tricuspid aortic valves were collected from patients undergoing aortic valve replacement surgery at Karolinska University Hospital, Solna. All patients have given written consent according to the Declaration of Helsinki and the local ethics committee. The valves used for microarray analysis were collected in RNAlater. Each valve was macroscopically divided in healthy, intermediate and calcified part. For *ex vivo* and *in vitro* assays and histological analysis the valves were collected in Dulbecco's Modified Eagle Medium (DMEM).

3.4 *EX VIVO* CULTURE OF HUMAN CAROTID ATHEROSCLEROTIC PLAQUES AND AORTIC VALVES

Fresh carotid plaques were obtained from patients undergoing CEA at Karolinska University Hospital, Solna. At the operation day, the plaque tissue was cut into small pieces (2 mm^3), distributed into the wells of a 48-well plate and thereafter pre-incubated for 1 h in RPMI 1640 medium supplemented with 10% human serum at 37 ºC in 5% CO_2 before stimulation with TLR7 agonist imiquimod (IMQ) (**Figure 4**). In the case of carotid plaques 2.5, 5, or 12.5 µg/mL IMQ was used in a dose–response challenge. Each dose was run in duplicates and the reported values are the average for each concentration. As a control, 100 ng/mL LPS was added to assure that live cells were present in the tissue after the mincing and that innate response was intact. After 20 h of stimulation, supernatants from the plaques were collected and stored for cytokine analysis.

In **Paper II** the *ex vivo* set up with tissue from aortic valve replacement was performed in the same way as previously described for carotid plaque tissue in **Paper I**. Depending on the results from the *ex vivo* carotid tissue stimulation with the TLR7 ligand IMQ, in **Paper II** only the highest dose of IMQ was used in the *ex vivo* aortic valve culture.

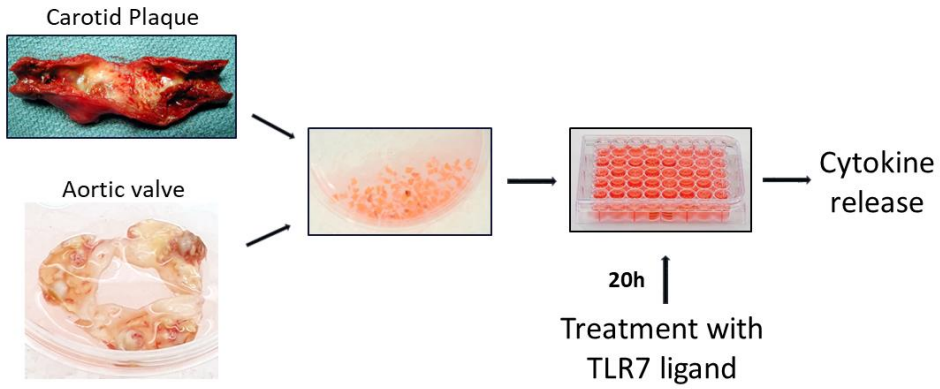

Figure 4. *Ex vivo* tissue stimulation assay. Human carotid plaque (**Paper I**) and tricuspid aortic valve tissue (**Paper II**) was cut into ~2mm^2 pieces and distributed to 48 well plate. The tissue was stimulated with the TLR7 ligand IMQ. After 20h supernatant was collected and cytokine measurements were performed.

The *ex vivo* assay can provide information on how diseased human tissues response to a ligand binding to TLR7, although there are several parts to consider. One consideration is the possibility the homogenizing procedure of the tissue to result in an uneven cell distribution in the different wells/conditions. The tissue was minced in small pieces and all parts were

thoroughly mixed before the distribution in the different conditions. Each condition was performed in duplicates. Furthermore, in our assay we cannot discriminate which cell subsets respond to *ex vivo* tissue stimulation with the TLR7 ligand. Cell isolation, sorting of the different cell populations and subsequent stimulation with a TLR7 ligand can provide additional information for the responding cells. However, this experimental approach comes with several disadvantages. Disassociation of immune cells and structural components from human carotid plaque and aortic valve requires strong enzymatic treatment that could modulate the activation state of the cells and thus can alter the response. In future set-ups more complex stimulation strategies could be used with testing of different TLR7 ligands and also combining with other mediators involved in atherosclerosis, *eg* oxLDL, TLR2 or TLR4 ligands.

3.5 TRANSCRIPT ANALYSIS

The most commonly used techniques for gene expression analysis are quantitative Polymerase Chain Reaction (qPCR), microarrays and RNA sequencing (RNAseq). In **Paper I** mRNA expression analysis of TLR7 in human carotid plaques was obtain from microarrays and qPCR. Microarrays, as previously discussed (above 1.4.1), offer the advantage of creating a transcript profile for a diseased tissue and thus providing information for the ongoing processes in the specific disease. However, microarrays demand robust normalization for comparison and background issues can occur for low expressing probes and similar sequences [194]. In **Paper I and II** microarray data were normalized with the Robust multi-array average method (RMA). This normalization is broadly used as a standardized method to normalize array data. In **Paper I** microarray analysis identified an association of TLR7 mRNA expression that was validated with qPCR. qPCR offers high specificity and sensitivity.

Technological advances have led to the discovery of newer methods such as RNAseq for transcript analysis. RNAseq has offered several advantages including the dynamic range, high resolution with detection of changes in a single base and accuracy [194]. However, the high cost and the generation of a big complex dataset have earlier created difficulties in data handling. Still microarrays are used to some extent in new projects since they offer lower cost and the established standardized analysis procedures facilitate the interpretation of the data but their usage will probably decrease.

3.6 MOUSE MODELS OF ATHEROSCLEROSIS

The most common mouse models used for the study of experimental atherosclerosis are *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. In **Paper III** we choose to use 22 weeks old female *Apoe*$^{-/-}$ mice. *Apoe*$^{-/-}$ mice spontaneously develop atherosclerosis without the demand of additional high fat diet. We choose to use mice that have already advanced atherosclerotic lesions in order to have the same disease stage as in **Paper I** where our results were generated in late stage advanced human lesions. Furthermore, our group and others have shown that mice around 22 weeks old have established lesions. In addition, the use of high fat diet was avoided due to possible effect on immune activation. Several members of the TLR family, such as TLR4 and TLR2 can be strongly activated by PAMPs present in high cholesterol atherogenic diets [195].

4 RESULTS AND DISCUSSION

4.1 TLR7 EXPRESSION IN HUMAN CAROTID PLAQUE IS ASSOCIATED WITH FEWER ADVERSE CARDIO- AND CEREBROVASCULAR EVENTS

In **Paper I** we investigate the role of TLR7 in human carotid advance atherosclerosis using data and samples included in our biobank of carotid endarterectomies, BiKE. Most of the studies exploring the role of TLR7 in atherosclerosis have been performed in animal models. The receptor has been previously connected with protective effect in an *Apoe*$^{-/-}$*xTlr7*$^{-/-}$ mouse model [130]. The double deficient mice presented increased atherosclerosis, accompanied by accumulation of pro-inflammatory macrophages in plaques in the aortic root. In addition, mRNA levels of TLR7 were increased in the human atherosclerotic plaque compared to normal arteries and correlated with anti-inflammatory M2 macrophages in BiKE [130]. In our study, TLR7 mRNA levels in patients´ removed carotid plaque were divided in tertiles and analyzed for the re-occurrence of cardio- and cerebrovascular events. The cox regression analysis revealed that the patients in the highest and middle tertiles were associated with better outcome with fewer cardio and cerebrovascular events compared to the patients that expressed low TLR7 mRNA levels in their removed plaque (**Figure 5**). These data indicated that TLR7 could be involved in protective pathways that lead to stabilization of advanced carotid plaques. The association of high TLR7 mRNA levels with better outcome was validated by Real time (RT)-PCR. In addition, in our analysis we adjusted for common cofounders in atherosclerosis; age, sex and LDL levels. Adjustment for these cofounders did not affect the outcome; however, there are other factors to be considered that could be addressed in larger cohorts.

Other studies have associated mediators to cardiovascular disease outcome [196-198]. Our group has previously identified that high levels of adiponectin in the plasma of patients undergoing carotid endarterectomy were associated with all cause mortality [196]. In addition, increased expression of TLR2 and TLR4 in monocytes of patients admitted in the hospital with stroke were associated with worse outcome regarding neurological impairment [197, 198]. These studies were performed in material derived by blood. In our study, we associated the expression levels of a pattern recognition receptor in carotid atherosclerotic plaques with patients´ secondary cardiovascular events. Although, blood biomarkers are involved in disease pathogenesis, how well the microenvironment and the ongoing processes is reflected in blood is not clear. In **Paper I** we have identified TLR7 as part of possible protective pathways in atherosclerotic lesions.

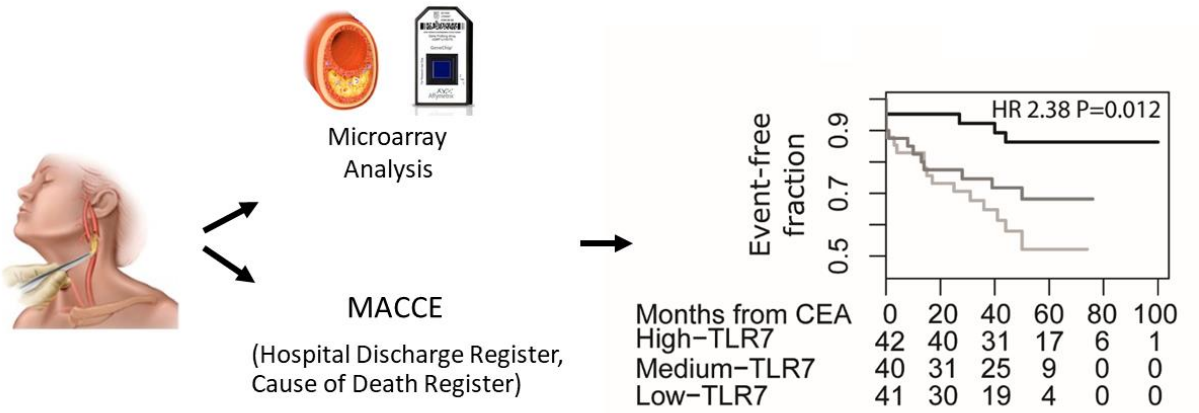

Figure 5. Better outcome was found for patients with higher levels of TLR 7 transcript in their removed carotid plaques. Kaplan–Meier curve illustrating MACCE-free survival of patients with TLR7 expression of highest tertile (black), the middle tertile (dark grey) and the lowest tertile (light grey). TLR7 mRNA levels were derived by microarray analysis of carotid plaques. Below the Kaplan Meier plot, the count of event-free patients is indicated. The x-axis indicates the time since CEA in months. In the upper-right corner is reported the Hazard ratio (HR) generated by Cox regression for the lowest tertile of TLR7 expression vs. the middle and highest tertiles combined [199].

In order to explore the expression of TLR7 in advanced atherosclerosis, human carotid plaques were co-stained for TLR7 and cellular markers. The double immunofluorescence staining revealed that infiltrated leukocytes but not carotid smooth muscle cells expressed the receptor. Specifically, both $CD4^+$ and $CD8^+$ T cells expressed TLR7 as well as $FoxP3^+$ Tregs. $CD4^+$ cells (T_H subtypes) are mainly thought to be pro-atherogenic [200]. However, it has been shown that TLR7 activation can lead to anergy in human $CD4^+$ T cells [201]. In addition, Tregs have been shown to be highly atheroprotective in several experimental studies by promoting resolution of inflammation [202]. The role of $CD8^+$ T cells is not fully understood yet. A recent study has shown that depletion of $CD8^+$ cells resulted in plaques with bigger necrotic cores and decreased collagen. In addition, skewing towards $CD4^+$ T helper cell phenotype was observed. These data indicate that $CD8^+$ T cells might control proatherogenic $CD4^+$ T cells and increase plaque stability [203]. Another study has shown that $CD8^+$ regulatory T cells control T follicular helper cells and the formation of germinal centers and thus limiting the progression of atherosclerosis [204]. In contrast, activation of the CD137 receptor, a member of the TNF family that is expressed on $CD8^+$ T cells and endothelial cells, promoted the progression of atherosclerosis [205].

Regarding plaque macrophages, TLR7 was co-expressed with the pan-macrophage marker CD68 and CD163 a marker expressed by M2 macrophage. M2 macrophages have been shown to promote resolution of inflammation and plaque regression [113, 206]. In our study, we have investigated the main immune cell types involved in the development of atherosclerosis. In addition to macrophages and T cells we have attempt to investigate the expression of TLR7 in dendritic cells in the plaque. However, due to technical challenges these data are not available.

Further understanding of the expression and role of TLR7 in human carotid plaques could be addressed in future studies.

Staining with the endothelial cell marker von Willebrand factor (vWF) revealed differential expression for TLR7 in endothelial cells in the human carotid plaque. TLR7 staining was observed in capillary endothelium but not in lumen endothelial cells. Although, the mechanism for differential expression of TLR7 in plaque endothelial cells is not clear, expression in the newly formed capillaries could be attributed to the atherogenic environment.

Thereafter, the responsiveness of TLR7 in the human atherosclerotic plaques was evaluated in an *ex vivo* tissue model using a synthetic TLR7 ligand. Stimulation with the TLR7 ligand imiquimod (IMQ) elicited cytokine secretion in the culture media. A total of 20 cytokines were screened. Interestingly, IL-10 was significantly increased in response to TLR7 stimulation (**Figure 6**). IL-10 has been shown to mediate several atheroprotective processes, such as dampening of pro-inflammatory responses and promoting cholesterol efflux [207]. Some cytokines connected to pro-inflammatory phenotypes were also significantly increased. TNF-α is traditionally considered as a mediator that promotes inflammatory responses [208]. However, a recent study has connected TNF-α to cardioprotective effect in an experimental model of heart failure [209]. Notably, established pro-atherogenic cytokines such as IL-6 were not significantly affected upon stimulation with the TLR7 ligand (Supplemental material).

Although the natural ligand for TLR7 in atherosclerosis have not been established yet, DAMPs derived from apoptotic and necrotic cells such as RNA and HMGB1 have been suggested to activate intracellular TLRs [210]. For the stimulation of TLR7 in the *ex vivo* carotid plaque culture model, the synthetic ligand IMQ was used. IMQ has been shown to specifically stimulate TLR7 without co-stimulation of other TLRs [211]. Moreover, this ligand is already approved for the treatment of basal cell carcinoma and genital warts in humans [212].

To further identify which cells respond to TLR7 ligand, we stimulated *in vitro* $CD4^+$ and $CD8^+$ T cells and $CD14^+$ monocytes isolated from PBMC of patients undergoing carotid endarterectomy with IMQ. The stimulated immune cells elicited a weaker cytokine response compared to the response generated by stimulation of carotid plaque tissue *ex vivo*. Immune cells isolated from blood do not have the same differentiation stage compared to the cells present in the plaques. In addition, the plaque microenvironment and cell interactions can affect the magnitude of the response. Thus, to identify the cells that produce IL-10 upon stimulation with TLR7 ligand, we performed immunofluorescence staining following the *ex vivo* stimulation of carotid plaque tissue with IMQ. The staining showed that macrophages, T cells and smooth muscle cells produce IL-10 (**Figure 6**). It has been previously shown that all the above cell types have the capacity to produce Il-10 [213, 214]. Although we did not observe co-staining of TLR7 with the smooth muscle cell marker a-actin, smooth muscle cells were expressing IL-10. It has been suggested that smooth muscle cells have a basal level of IL-10 production in the plaque [214]. In addition, expression of IL-10 from smooth muscle cells upon TLR7 stimulation might be the result of cell interactions.

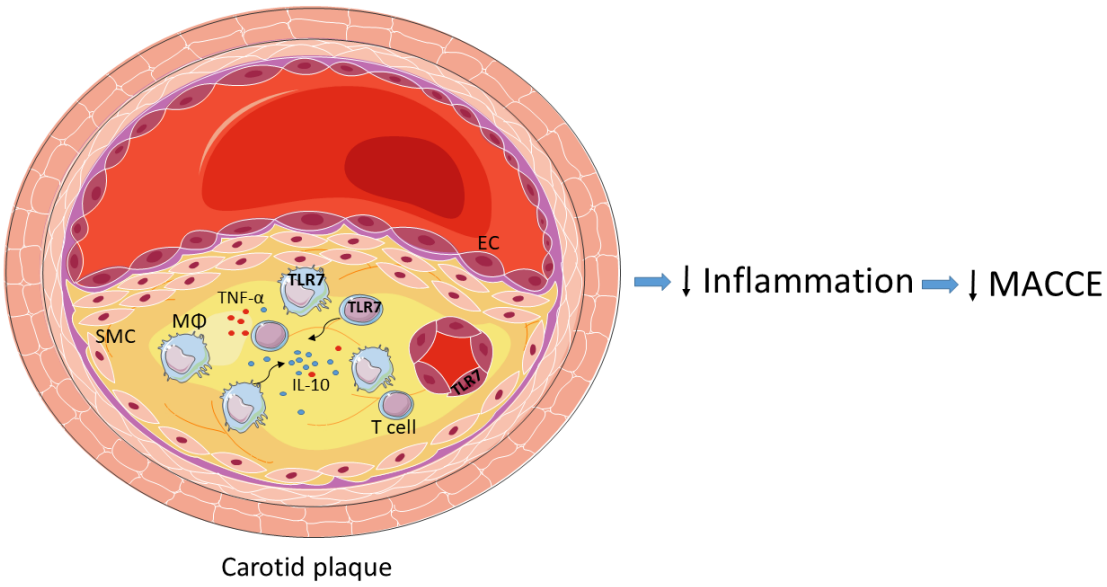

Figure 6. Summary of Paper I. High expression of TLR7 in the removed carotid plaque was correlated with better outcome for the patient with fewer reoccurring MACCE. TLR7 was expressed in macrophages, T cells and capillary endothelial cells in the human carotid plaque. Addition of TLR7 ligand in *ex vivo* cultures of carotid plaque tissue elicited the secretion of IL-10, TNF-α and GM-CSF. Both macrophages and T cells upon stimulation of carotid plaque with a TLR7 ligand produced IL-10. In **Paper I** we suggest that activation of TLR7 with endogenous or exogenous ligand in the plaque would lead to immunomodulatory effects by macrophages and T cells and eventually to a more stable plaque phenotype. Stabilization of the plaque would protect patients with high TLR7 expression in their lesions from future myocardial infarction or stroke. The schematic art pieces used in this figure were provided by Servier Medical art (https://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

4.2 ASSOCIATION OF TLR7 EXPRESSION WITH ANTI-INFLAMMATORY MACROPHAGE SUBTYPE IN AORTIC VALVE STENOSIS

In **Paper II** we explore the expression and role of TLR7 in calcific aortic valve stenosis (CAVS). CAVS shares some risk factors and mechanisms of pathogenesis with atherosclerosis. In **Paper I** we have associated TLR7 with protection against atherosclerosis. Thus, we were interested to explore whether TLR7 is associated with similar effects in CAVS. Macrophages are present in atherosclerotic plaques and calcified aortic valves [99, 215]. In addition, macrophages have shown to be involved in all stages of atherosclerosis [215]. Hence, in **Paper II** we focus on expression of TLR7 in the different macrophage subsets in CAVS. In order to shed more light on the role of TLR7 in macrophage subtypes in CAVS, human calcified aortic valve samples and microarray data were obtained. The valves were macroscopically divided in a healthy, intermediate and calcified part, representing different stages of the disease (**Figure 7a**).

Transcript analysis revealed that the expression level of TLR7 was significantly increased in the calcified part of the aortic valve compared to the healthy and intermediate parts of the valve. The role of TLRs has been addressed mainly in VICs, where TLR7 expression was low to absent compared to TLR2 and TLR4 [125, 216]. In CAVS the research has been primarily

focused on VIC calcification processes. VICs are the most abundant cell type in the valve. However, immune cells, such as macrophages, T and B cells infiltrate aortic valves [99]. In contrast to other published studies, in our approach we include both infiltrating leukocytes and VICs.

In order to investigate which macrophage subtype express TLR7, we correlated TLR7 mRNA levels with a set of macrophage markers associated with either M1 and M2 macrophages in the calcified aortic valves. TLR7 mRNA levels were significantly correlated with M2 macrophage subset genes in all parts of the valve (**Figure 7b**). Highest correlation was detected between TLR7 and the scavenger receptors MSR1, CD206 and CD163. No correlation with M1 macrophage subset genes or vimentin (VIM) expressed by VICs, was observed. Co-expression of TLR7 with M2 macrophage markers was validated by double immunofluorescence staining of TLR7 and two of the high correlated M2 macrophage markers CD163 and CD206. A recent study indicated that M1 macrophages were increased in calcified compared to non calcified aortic valves, while M2 macrophage subtype presented the opposite trend. These data in combination with *in vitro* transfer of conditioned media from M1 macrophage to VIC, suggest that M1 macrophage promote calcification processes through secretion of pro-inflammatory cytokines [118]. As previously described, M2 macrophages have been associated with resolution of inflammation and tissue healing [217]. TLR7 was associated with scavenger receptors expressed by M2 macrophages, which could indicate capacity to clear apoptotic cells. Further studies are needed to show whether TLR7 is active in this process of clearance of apoptotic cells and calcification nodules.

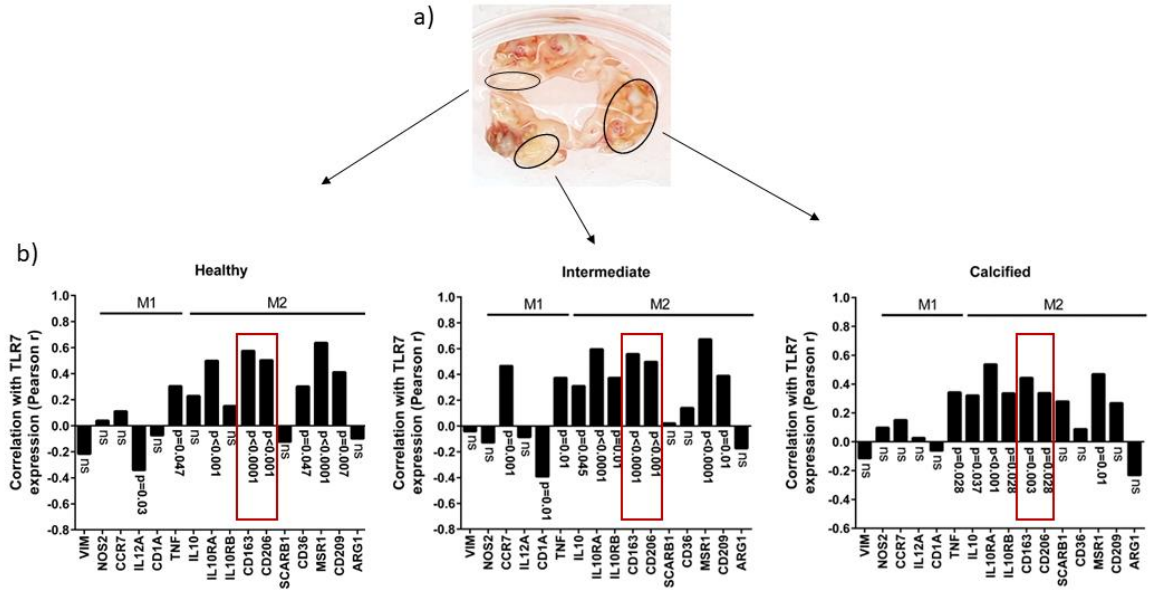

Figure 7. Association of TLR7 with M2 macrophage markers in calcified aortic valves. a) Image of calcified tricuspid valve. The circled areas indicate the different parts of the valve. **b)** Correlation of TLR7 mRNA levels with M2 macrophage markers in the healthy, intermediate and calcified part of the aortic valves. The red squares are highlighting the markers that were used to validate co-expression of TLR7 with M2 macrophages by double immunofluorescence staining.

In **Paper II,** we focused on the expression of TLR7 in macrophages in CAVS. However, it has been shown that human calcified aortic valves are infiltrated by other leukocytes such as T and B cells [218]. The expression and role of TLR7 in other immune cells could be addressed in future studies.

Similarly, to **Paper I** we investigated the ability of human calcified aortic valve to respond by stimulating *ex vivo* with the TLR7 ligand IMQ. The dose of IMQ used in this study and the selection of measured cytokines in the culture media was based on our results in **Paper I**. Stimulation of aortic valve tissue with IMQ increased significantly the secretion of IL-10, TNF-α and GM-CSF in the culture media. The effect of IL-10 in calcific aortic valves has not been studied yet. The proposed beneficial effects are supported by studies performed in atherosclerosis and other inflammatory diseases. TNF-α increased calcification in VIC derived from human calcified aortic valves in an *in vitro* calcification assay [219]. However, as it was previously discussed TNF-α have been connected to atheroprotection in another cardiovascular disease. Fine-tuning of the amount of TNF-α could lead to beneficial effects in cardiovascular disease.

The *ex vivo* aortic valve tissue model provided information for the secretion of selected mediators in the culture media, yet we could not identify the responding cell types. In order to discriminate which cells respond to stimulation with TLR7 ligand, primary macrophages derived from blood monocytes and VICs extracted from calcified aortic valves were stimulated with IMQ. Upon stimulation with TLR7 ligand macrophages significantly increased the secretion of IL-10 while no change was observed in VICs. Unresponsiveness of VICs to stimulation with a TLR7 ligand was previously shown *in vitro*. In addition, TNF-α was undetectable in VICs. These results indicate that the main producer of IL-10 and TNF-α in calcified aortic valves are the macrophages. In another study, immunofluorescence staining in human calcific aortic valve reveal co-expression of TNF-α with the macrophage marker CD68 [220]. Nevertheless, response to TLR7 ligand by other immune cells cannot be excluded.

In our study, we investigate the expression and role of TLR7 activation mainly in macrophages. However, we did not assess the effects of TLR7 activation in calcification, the hallmark of calcific aortic valve stenosis [218]. VICs seem to not respond or being low responders upon *in vitro* stimulation with a TLR7 ligand, in contrast to macrophages. Experiments such as conditioned media transfer from macrophages stimulated with TLR7 ligand to VICs or co-culture systems of macrophages and VICs in the presence of a TLR7 ligand would provide more insight on calcification processes.

4.3 *IN VIVO* TREATMENT WITH A TLR7 LIGAND INDUCED PROTECTIVE RESPONSES IN SPLEEN AND DECREASED EXPERIMENTAL ATHEROSCLEROSIS

Paper III investigates the *in vivo* effects upon activation of TLR7 in experimental atherosclerosis, connecting to the two studies in biobanks. We were interested to evaluate both

local and system effects of the treatment. The primary focus of the study was the effect of treatment with a TLR7 ligand on atherosclerotic lesion burden. In order to investigate the effects of the treatment in atherosclerotic lesions, we treated atherosclerotic mice with established lesions. Several studies and experience with the mouse model in the group indicate that mice in the age of 20 weeks present complex atherosclerotic lesions that are still progressing. The age selection of the mice at the initiation of the treatment was chosen to connect to our results in **Paper I**, where we investigated the role of TLR7 in advanced atherosclerotic plaques.

Our treatment of 22 weeks old *Apoe*$^{-/-}$ mice with a TLR7 ligand resulted in decreased atherosclerotic burden compared to control group injected with PBS. Between treated mice and a baseline group that was euthanized at the initiation of the treatment, we detected a tendency towards smaller lesions, however the change was not statistically signinficant. These data indicate that stimulation with TLR7 ligand activated atheroprotective processes that arrested lesion progression.

In parallel to our study, another study has been published were treatment with the same TLR7 agonist we used resulted to increased atherosclerosis [134]. The two studies have several differences in experimental design that might explain the different results. We treated 22 week old mice with established lesions while *Krogmann et al* started the treatment of mice at 10 weeks. In addition, the route of injection was different; we performed intraperitoneal while the other group did intravenous injections. Last, as it was previously discussed, in order to avoid cross activation of other TLRs that can affect the disease outcome we used normal diet in contrast to the cholesterol-rich diet used in the other study [134]. Further studies are needed to explore the different effect of TLR7 ligands in atherosclerosis models.

In **Paper I and II** we used the TLR7 ligand IMQ challenging human tissue, while in **Paper III** we used R848. Since R848 has been used in several experimental mouse studies for the activation of TLR7 [221, 222] with clear results we chose to use this ligand. R848 is a ligand that can activate both TLR7 and TLR8 in human cells. Early data has showed that TLR8 is not functional in mice, only in humans [223, 224]. In the KO model of TLR7, the R848 ligand did not elicit any response in spleen cells, a compartment for all leukocytes. A recent study has however showed that TLR8 is active in neuronal tissue of mice [225].

We were interested to investigate systemic effects of TLR7 stimulation in atherosclerotic mice. Previous studies by our group and others have shown an important role for the spleen in the outcome of atherosclerosis [159, 173]. In addition, at time of sacrifice we observed increase in spleen size of the treated mice. We therefore explored changes in the main immune populations in the spleen such as B and T cell subsets. Our analysis revealed an increase of MZ B cells and Tregs (**Figure 9**). Several studies have shown that MZ B cells and Tregs are atheroprotective [149, 162].

In our study, we show expansion of Tregs and MZ B cells in the spleen of treated mice with the TLR7 ligand. However, we did not investigate all steps of TLR7 activation in the respective

cell types. We cannot distinguish whether TLR7 acts directly on Tregs and MZ B cells, or whether the observed effect is the result of cell interactions in the spleen. Previous studies have shown that TLR7 is expressed by both Tregs and MZ B cells [226, 227]. In addition, both cell types have been described to respond to TLR7 ligands *in vitro* [227, 228]. Stimulation of Tregs with a TLR7 ligand enhanced their ability to control the proliferation of $CD4^{+}CD25^{-}$ T cells and inhibited the secretion of pro-inflammatory cytokines [228]. Furthermore, MZ B cells stimulated *in vitro* with R848, were differentiated in plasma cells and produced IgM antibodies [227]. The above studies have shown direct effect of TLR7 in Tregs and MZ B cells. In addition, protective effects of treatment with a TLR7 ligand through interaction between B cells and Tregs have been described. Induction of Tregs as response to stimulation with the TLR7 ligand R848 reduced asthma symptoms in an experimental mouse model in a B cell dependent manner [222].

The main effector function of B cells is the production of antibodies. Since we have observed a two-fold increase of MZ B cells in the spleen, we were interested to investigate changes in antibody levels in circulation. Measurements of IgM and IgG antibodies in the plasma of TLR7 ligand treated mice revealed a significant increase in IgM antibodies. IgM antibodies are polyreactive antibodies secreted by MZ B and B1 cells that bind conserved epitopes through molecular mimicry, such as apoptotic cells and oxLDL epitopes [74]. Several studies have shown that IgM antibodies against oxLDL convey atheroprotection [179, 229].

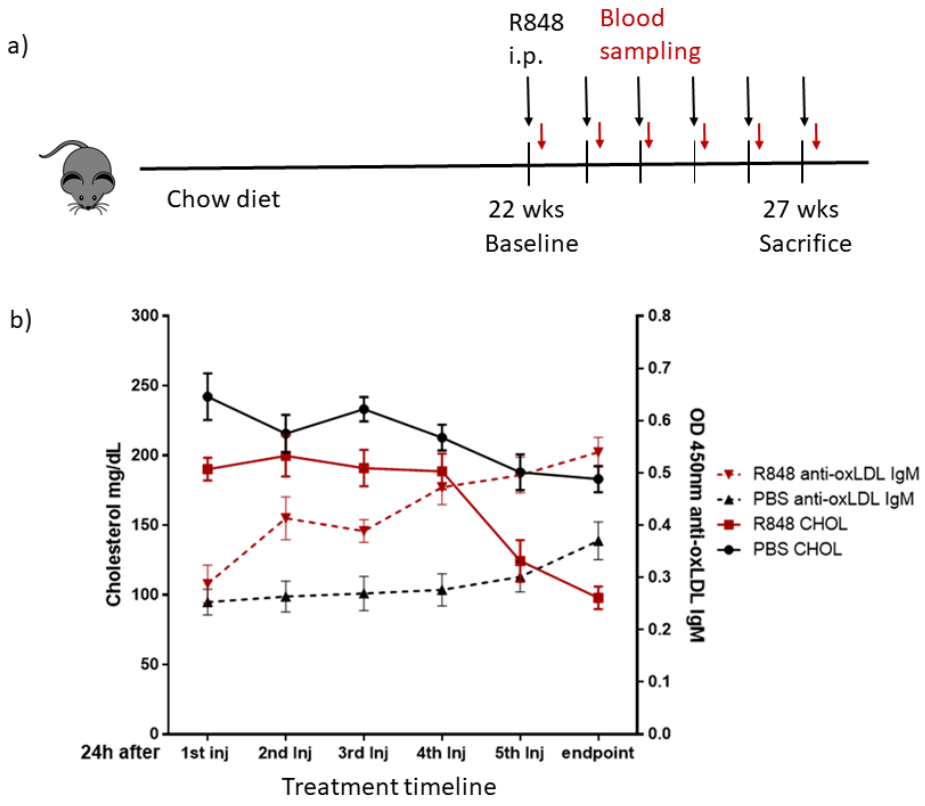

Figure 8. Time course treatment with TLR7 ligand showing inverse trend of cholesterol levels and anti-oxLDL IgM antibodies. a) Schematic representation of the treatment of *Apoe*$^{-/-}$ mice with the TLR7 ligand R848. Blood was collected 24h after injection with R848 or PBS. **b)** Graph depicting cholesterol and anti-oxLDL antibody levels of PBS and R848 injected mice. Cholesterol levels drop significantly after the fourth injection

when we observe significant increase in anti-oxLDL IgM antibody titers. Red lines represent the R848 group and black lines PBS group. Solid lines represent cholesterol levels and dotted lines anti-oxLDL IgM antibodies [230].

MZ B cells and IgM antibodies against LDL oxidation epitopes play important role for the protection against atherosclerosis. Injection of apoptotic cells to *Apoe*$^{-/-}$ mice decreased cholesterol levels through increase of anti-PC antibodies and expansion of MZ B and B1a cells [160]. We have also observed increased levels of anti-oxLDL IgM antibodies upon treatment with a TLR7 ligand. Furthermore, our treated mice presented decrease in plasma cholesterol levels. Taken together, these data indicate that the decrease in cholesterol levels could be related to an increase in anti-oxLDL antibodies in circulation. More evidence has been added in this line by our time course experiment where we treated mice with TLR7 ligand and obtained blood sample after each injection (**Figure 8a**). Measurement of anti-oxLDL IgM antibodies and cholesterol levels indicated that the increase in antibodies preceded the decrease in cholesterol levels (**Figure 8b**). However, we cannot exclude activation of metabolic pathways upon stimulation with a TLR7 ligand. A study of non-alcoholic fatty liver disease (NAFL) revealed that cholesterol accumulation was decreased in the liver of experimental animals through TRL7 induced autophagy. Future studies are necessary to clarify the role of TLR7 in decrease of cholesterol levels [231].

In addition to the systemic effects that were described above, IgM antibodies against oxLDL have been proven to have local atheroprotective effects [173, 175]. There are two suggested mechanisms by which IgM against modified epitopes of LDL exert protective role in atherosclerosis. First, studies have shown that anti-oxLDL IgM antibodies decrease the uptake of oxLDL by macrophages in atherosclerotic lesions [175, 176]. Second, anti-oxLDL antibodies bind to apoptotic cells through molecular mimicry and enhance efferocytosis [232]. Increased foam cell formation and cell death in combination with defective efferocytosis leads to generation of larger necrotic core, one of the characteristics of unstable plaques. We have observed infiltration of IgM antibodies in the plaque of TLR7 ligand treated mice compared to PBS. Staining of the lesions, revealed that treated mice with TLR7 ligand had smaller necrotic core, which was accompanied with fewer apoptotic cells in the lesions. However, in our study we cannot prove whether the decrease in apoptotic cells is due to enhancement of efferocytosis versus promotion of survival signals.

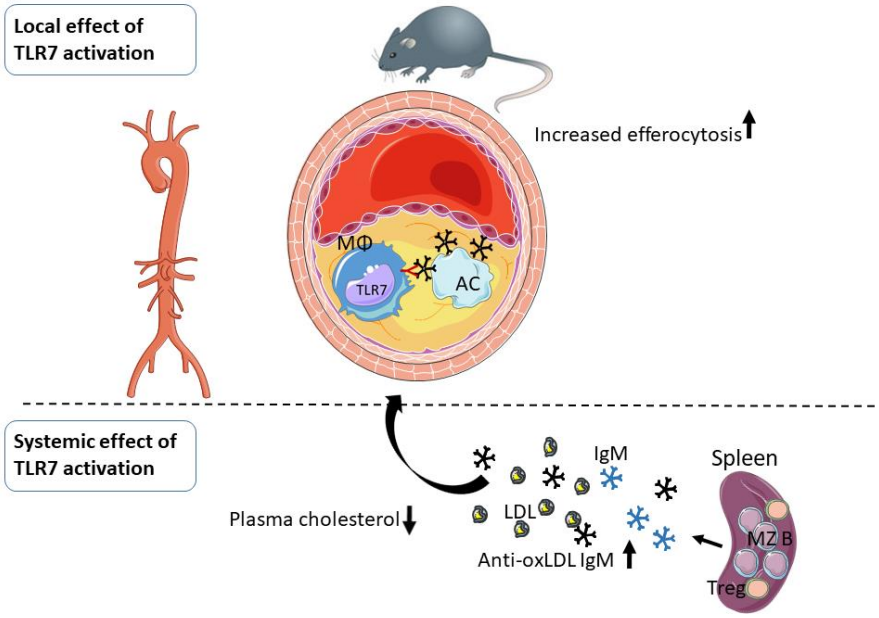

Figure 9. Summary of Paper III. *In vivo* treatment with the TLR7 ligand R848 reduced atherosclerotic lesion size in 22 week old *Apoe*$^{-/-}$ mice. The treatment led to systemic and local lesion effects. The systemic effects included expansion of MZ B cells and Tregs in the spleen. Furthermore, treatment with R848 reduced cholesterol levels and increased anti-oxLDL levels in the plasma of atherosclerotic mice. Locally, in the lesions increased accumulation of IgM antibodies was observed in the treated mice. Furthermore, the lesions presented decrease in necrotic core area and in apoptotic cell numbers. In **Paper III** we suggest that TLR7 induces anti-oxLDL IgM antibodies that decrease plasma cholesterol and reduce plaque size by blocking oxLDL uptake by macrophages and increasing efferocytosis in the plaques. Effective clearance of apoptotic cells would lead in smaller necrotic core and hence a more stable plaque [230]. The schematic art pieces used in this figure were provided by Servier Medical art (https://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

5 CONCLUDING REMARKS

Despite the success in new treatments with focus on the lowering of cholesterol levels, cardiovascular diseases remain the main cause of death worldwide. This indicates that there is the need to address the ongoing inflammatory processes involved in the pathogenesis of cardiovascular disease. The current thesis is focusing on the role of the pattern recognition receptor TLR7 in atherosclerosis and aortic valve stenosis. The expression and stimulation of the receptor has been investigated both in human and *in vivo* in an experimental mouse set up.

In **Paper I** TLR7 expression levels in the atherosclerotic lesion improved patients´ prognosis. The expression of the receptor was revealed and localized mainly in immune cell populations in human atherosclerotic plaques. In addition, stimulation of the plaque with a synthetic exogenous ligand demonstrated the ability of the tissue to react fast and elicit a robust cytokine response. Taken together TLR7 is associated with protective pathways in atherosclerosis. These data could open the possibility for the use of TLR7 expression as a prognostic marker for patients undergoing endarterectomy. Furthermore, activation of TLR7 could be explored as possible therapeutic strategy for the stabilization of atherosclerotic plaque. However, it should be considered that pattern recognition receptors are potent, fast responding receptors that excessive activation could lead to adverse effects. Careful fine-tuning of the degree of activation and the right disease time point is necessary.

In **Paper II** expression of TLR7 was demonstrated in aortic valves. TLR7 was associated with M2 macrophage markers and shown to be expressed in M2 macrophages in human calcified aortic valves. In a similar approach to **Paper I,** in **Paper II** was shown that human aortic valves have the ability to respond to synthetic TLR7 ligand with a mixture of anti-inflammatory and pro-inflammatory cytokines. Macrophages are main responders to stimulation with TLR7 ligand, with the capacity to secrete large amounts of the respective cytokines. In **Paper II** association of TLR7 with M2 macrophages in the aortic valve suggested involvement of the receptor in resolution and tissue repair pathways. Enhancement of these pathways, that are defective in pathological environment, would be beneficial in cardiovascular disease.

In **Paper III** administration of a synthetic TLR7 ligand decreased experimental atherosclerosis. With the *in vivo* treatment approach, we shed light on the systemic and locally activated pathways that TLR7 could be involved. Stimulation of atherosclerotic mice with a synthetic TLR7 ligand led to expansion of MZ B and Tregs and enhanced the secretion of protective antibodies against atherosclerotic epitopes. The treatment reduced cholesterol levels showing a possible immunometabolic effect of TLR7. These antibodies act in circulation by lowering plasma cholesterol and promote a stable plaque phenotype by increasing efferocytosis. A more stable plaque phenotype was observed in the treated mice. Taken together, in **Paper III** we suggest that TLR7 activates several beneficial pathways in atherosclerosis.

As a final conclusion, this thesis highlights the protective role of the pattern recognition receptor TLR7 in cardiovascular disease. Furthermore, TLR7 expression in adaptive immune

cells and expansion of adaptive immune cells upon *in vivo* stimulation with a synthetic ligand displayed the role of the receptor in bridging innate and adaptive immune responses.

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7 REFERENCES

1. Mahmood, S.S., et al., *The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective.* The Lancet, 2014. **383**(9921): p. 999-1008.

2. Roth, G.A., et al., *Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015.* Journal of the American College of Cardiology, 2017. **70**(1): p. 1-25.

3. Merlo, J., et al., *Comparison of different procedures to identify probable cases of myocardial infarction and stroke in two Swedish prospective cohort studies using local and national routine registers.* Eur J Epidemiol, 2000. **16**(3): p. 235-43.

4. Tunstall-Pedoe, H., et al., *Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents.* Circulation, 1994. **90**(1): p. 583-612.

5. Hansson, G.K., *Inflammation, Atherosclerosis, and Coronary Artery Disease.* New England Journal of Medicine, 2005. **352**(16): p. 1685-1695.

6. Go, A.S., et al., *Executive summary: heart disease and stroke statistics--2013 update: a report from the American Heart Association.* Circulation, 2013. **127**(1): p. 143-52.

7. Baigent, C., et al., *Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins.* Lancet, 2005. **366**(9493): p. 1267-78.

8. Zhao, Y., et al., *The effect of statins on valve function and calcification in aortic stenosis: A meta-analysis.* Atherosclerosis, 2016. **246**: p. 318-24.

9. Anitschkow N., C.S., *Classics in arteriosclerosis research: On experimental cholesterin steatosis and its significance in the origin of some pathological processes by N. Anitschkow and S. Chalatow, translated by Mary Z. Pelias, 1913.* Arteriosclerosis: An Official Journal of the American Heart Association, Inc., 1913. **3**(2): p. 178-182.

10. Fowler, S., H. Shio, and N.J. Haley, *Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations.* Lab Invest, 1979. **41**(4): p. 372-8.

11. Hansson, G.K., J. Holm, and L. Jonasson, *Detection of activated T lymphocytes in the human atherosclerotic plaque.* Am J Pathol, 1989. **135**(1): p. 169-75.

12. Hansson, G.K., et al., *Localization of T lymphocytes and macrophages in fibrous and complicated human atherosclerotic plaques.* Atherosclerosis, 1988. **72**(2-3): p. 135-41.

13. Jonasson, L., et al., *Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque.* Arteriosclerosis, 1986. **6**(2): p. 131-8.

14. Liuzzo, G., et al., *The Prognostic Value of C-Reactive Protein and Serum Amyloid A Protein in Severe Unstable Angina.* New England Journal of Medicine, 1994. **331**(7): p. 417-424.

15. Ridker, P.M., et al., *Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease.* New England Journal of Medicine, 2017. **377**(12): p. 1119-1131.

16. Goldstein, J.L. and M.S. Brown, *Familial Hypercholesterolemia: Identification of a Defect in the Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity Associated with Overproduction of Cholesterol.* Proceedings of the National Academy of Sciences, 1973. **70**(10): p. 2804-2808.

17. Goldstein, J.L. and M.S. Brown, *Regulation of low-density lipoprotein receptors: implications for pathogenesis and therapy of hypercholesterolemia and atherosclerosis.* Circulation, 1987. **76**(3): p. 504-507.

18. Nair, P., *Brown and Goldstein: The Cholesterol Chronicles.* Proceedings of the National Academy of Sciences, 2013. **110**(37): p. 14829-14832.

19. Charlton-Menys, V. and P.N. Durrington, *Human cholesterol metabolism and therapeutic molecules.* Experimental Physiology, 2008. **93**(1): p. 27-42.

20. Olofsson, S.-O., P. Stillemark-Billton, and L. Asp, *Intracellular Assembly of VLDL: Two Major Steps in Separate Cell Compartments.* Trends in Cardiovascular Medicine, 2000. **10**(8): p. 338-345.

21. Foley, E.M., et al., *Hepatic Remnant Lipoprotein Clearance by Heparan Sulfate Proteoglycans and Low-Density Lipoprotein Receptors Depend on Dietary Conditions in Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2013. **33**(9): p. 2065-2074.

22. Van Eck, M., et al., *Scavenger receptor BI facilitates the metabolism of VLDL lipoproteins in vivo.* Journal of Lipid Research, 2008. **49**(1): p. 136-146.

23. Paskal, W., et al., *Aspects of Modern Biobank Activity - Comprehensive Review.* Pathology oncology research : POR, 2018. **24**(4): p. 771-785.

24. Bartnik E., V.E., *Biobanks: Balancing Benefits and Risks.*, in *Global Bioethics: The Impact of the UNESCO International Bioethics Committee. Advancing Global Bioethics*. 2016, Springer, Cham.

25. Bäck, M., et al., *Immune-Mediated Mechanisms of Atherosclerosis*, in *Encyclopedia of Cardiovascular Research and Medicine*, R.S. Vasan and D.B. Sawyer, Editors. 2018, Elsevier: Oxford. p. 68-76.

26. Schunkert, H., et al., *Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease.* Nat Genet, 2011. **43**(4): p. 333-8.

27. Kathiresan, S., et al., *Genome-wide association of early-onset myocardial infarction with single nucleotide polymorphisms and copy number variants.* Nat Genet, 2009. **41**(3): p. 334-41.

28. Swanberg, M., et al., *MHC2TA is associated with differential MHC molecule expression and susceptibility to rheumatoid arthritis, multiple sclerosis and myocardial infarction.* Nature Genetics, 2005. **37**(5): p. 486-494.

29. Getz, G.S. and C.A. Reardon, *Animal Models of Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2012. **32**(5): p. 1104-1115.

30. Sider, K.L., M.C. Blaser, and C.A. Simmons, *Animal models of calcific aortic valve disease.* International journal of inflammation, 2011. **2011**: p. 364310-364310.

31. Plump, A.S., et al., *Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells.* Cell, 1992. **71**(2): p. 343-53.

32. Zhang, S., et al., *Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E.* Science, 1992. **258**(5081): p. 468-471.

33. Ishibashi, S., et al., *Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery.* J Clin Invest, 1993. **92**(2): p. 883-93.

34. Potteaux, S., H. Ait-Oufella, and Z. Mallat, *Mouse models of atherosclerosis.* Drug Discovery Today: Disease Models, 2007. **4**(4): p. 165-170.

35. Agardh, H.E., et al., *Fatty acid binding protein 4 in circulating leucocytes reflects atherosclerotic lesion progression in Apoe(-/-) mice.* J Cell Mol Med, 2013. **17**(2): p. 303-10.

36. Polyzos, K.A., et al., *Inhibition of indoleamine 2,3-dioxygenase promotes vascular inflammation and increases atherosclerosis in Apoe-/- mice.* Cardiovasc Res, 2015. **106**(2): p. 295-302.

37. Petri, M.H., et al., *Aspirin-triggered lipoxin A4 inhibits atherosclerosis progression in apolipoprotein E-/- mice.* Br J Pharmacol, 2017.

38. Centa, M., et al., *Acute Loss of Apolipoprotein E Triggers an Autoimmune Response That Accelerates Atherosclerosis.* Arteriosclerosis, thrombosis, and vascular biology, 2018. **38**(8): p. e145-e158.

39. Tanaka, K., et al., *Age-Associated Aortic Stenosis in Apolipoprotein E-Deficient Mice.* Journal of the American College of Cardiology, 2005. **46**(1): p. 134-141.

40. Aikawa, E., et al., *Multimodality Molecular Imaging Identifies Proteolytic and Osteogenic Activities in Early Aortic Valve Disease.* Circulation, 2007. **115**(3): p. 377-386.

41. Weiss, R.M., et al., *Calcific Aortic Valve Stenosis in Old Hypercholesterolemic Mice.* Circulation, 2006. **114**(19): p. 2065-2069.

42. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Basic Immunology: Functions and disorders of the immune system*. 4th ed. 2014: Philadelphia, PA : Saunders/Elsevier, c2009.

43. Groeneweg, L., A. Hidalgo, and N. A-Gonzalez, *Emerging roles of infiltrating granulocytes and monocytes in homeostasis.* Cellular and Molecular Life Sciences, 2020.

44. Woollard, K.J. and F. Geissmann, *Monocytes in atherosclerosis: subsets and functions.* Nature Reviews Cardiology, 2010. **7**(2): p. 77-86.

45. Röszer, T., *Understanding the Biology of Self-Renewing Macrophages.* Cells, 2018. **7**(8): p. 103.

46. Mills, C.D. and K. Ley, *M1 and M2 macrophages: the chicken and the egg of immunity.* J Innate Immun, 2014. **6**(6): p. 716-26.

47. Wang, L.-x., et al., *M2b macrophage polarization and its roles in diseases.* Journal of Leukocyte Biology, 2019. **106**(2): p. 345-358.

48. Wynn, T.A. and K.M. Vannella, *Macrophages in Tissue Repair, Regeneration, and Fibrosis.* Immunity, 2016. **44**(3): p. 450-462.

49. Wu, H., et al., *Tumor-associated macrophages promote angiogenesis and lymphangiogenesis of gastric cancer.* Journal of Surgical Oncology, 2012. **106**(4): p. 462-468.

50. Brophy, M.L., et al., *Eating the Dead to Keep Atherosclerosis at Bay.* Front Cardiovasc Med, 2017. **4**: p. 2.

51. Netea, M.G. and J.W.M. van der Meer, *Immunodeficiency and Genetic Defects of Pattern-Recognition Receptors.* New England Journal of Medicine, 2011. **364**(1): p. 60-70.

52. Cole, J.E., C. Kassiteridi, and C. Monaco, *Toll-like receptors in atherosclerosis: a 'Pandora's box' of advances and controversies.* Trends Pharmacol Sci, 2013. **34**(11): p. 629-36.

53. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

54. Cole, J.E., et al., *Unexpected protective role for Toll-like receptor 3 in the arterial wall.* Proc Natl Acad Sci U S A, 2011. **108**(6): p. 2372-7.

55. Harrington, L.E., et al., *Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nature Immunology, 2005. **6**(11): p. 1123-1132.

56. Romano, M., et al., *Past, Present, and Future of Regulatory T Cell Therapy in Transplantation and Autoimmunity.* Frontiers in Immunology, 2019. **10**(43).

57. Clark, M.R., et al., *Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling.* Nature Reviews Immunology, 2014. **14**(2): p. 69-80.

58. Schatz, D.G. and Y. Ji, *Recombination centres and the orchestration of V(D)J recombination.* Nature Reviews Immunology, 2011. **11**(4): p. 251-263.

59. Sage, A.P., et al., *The role of B cells in atherosclerosis.* Nature Reviews Cardiology, 2019. **16**(3): p. 180-196.

60. Pillai, S. and A. Cariappa, *The follicular versus marginal zone B lymphocyte cell fate decision.* Nature Reviews Immunology, 2009. **9**(11): p. 767-777.

61. Muppidi, J.R., et al., *Cannabinoid receptor 2 positions and retains marginal zone B cells within the splenic marginal zone.* Journal of Experimental Medicine, 2011. **208**(10): p. 1941-1948.

62. Weller, S., et al., *Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire.* Blood, 2004. **104**(12): p. 3647-3654.

63. Tas, J.M.J., et al., *Visualizing antibody affinity maturation in germinal centers.* Science, 2016. **351**(6277): p. 1048-1054.

64. Corcoran, L.M. and D.M. Tarlinton, *Regulation of germinal center responses, memory B cells and plasma cell formation—an update.* Current Opinion in Immunology, 2016. **39**: p. 59-67.

65. Lewis, S.M., A. Williams, and S.C. Eisenbarth, *Structure and function of the immune system in the spleen.* Science Immunology, 2019. **4**(33): p. eaau6085.

66. Paul, William E., *Bridging Innate and Adaptive Immunity.* Cell, 2011. **147**(6): p. 1212-1215.

67. Bretscher, P. and M. Cohn, *A Theory of Self-Nonself Discrimination.* Paralysis and induction involve the recognition of one and two determinants on an antigen, respectively, 1970. **169**(3950): p. 1042-1049.

68. Jain, A. and C. Pasare, *Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm.* The Journal of Immunology, 2017. **198**(10): p. 3791-3800.

69. Curtsinger, J.M., et al., *Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4+ and CD8+ T Cells.* The Journal of Immunology, 1999. **162**(6): p. 3256-3262.

70. Mantegazza, A.R., et al., *Adaptor protein-3 in dendritic cells facilitates phagosomal toll-like receptor signaling and antigen presentation to CD4(+) T cells.* Immunity, 2012. **36**(5): p. 782-794.

71. de Brito, C., et al., *CpG Promotes Cross-Presentation of Dead Cell-Associated Antigens by Pre-CD8α+ Dendritic Cells.* The Journal of Immunology, 2011. **186**(3): p. 1503-1511.

72. Caron, G., et al., *Direct Stimulation of Human T Cells via TLR5 and TLR7/8: Flagellin and R-848 Up-Regulate Proliferation and IFN-γ Production by Memory CD4+ T Cells.* The Journal of Immunology, 2005. **175**(3): p. 1551-1557.

73. He, B., et al., *The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88.* Nature Immunology, 2010. **11**(9): p. 836-845.

74. Cerutti, A., M. Cols, and I. Puga, *Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes.* Nature Reviews Immunology, 2013. **13**: p. 118.

75. Pone, E.J., et al., *BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF-κB pathway.* Nature Communications, 2012. **3**(1): p. 767.

76. Libby, P. and G.K. Hansson, *From Focal Lipid Storage to Systemic Inflammation: JACC Review Topic of the Week.* Journal of the American College of Cardiology, 2019. **74**(12): p. 1594-1607.

77. Moore, K.J., F.J. Sheedy, and E.A. Fisher, *Macrophages in atherosclerosis: a dynamic balance.* Nature Reviews Immunology, 2013. **13**(10): p. 709-721.

78. Libby, P., et al., *Atherosclerosis.* Nature Reviews Disease Primers, 2019. **5**(1): p. 56.

79. Theodorou, K. and R.A. Boon, *Endothelial Cell Metabolism in Atherosclerosis.* Frontiers in cell and developmental biology, 2018. **6**: p. 82-82.

80. Gimbrone, M.A., Jr. and G. García-Cardeña, *Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis.* Circulation research, 2016. **118**(4): p. 620-636.

81. Quillard, T., et al., *TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion.* Eur Heart J, 2015. **36**(22): p. 1394-404.

82. Davies, M.J., et al., *Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content.* British Heart Journal, 1993. **69**(5): p. 377-381.

83. Libby, P., *Multiple Mechanisms of Thrombosis Complicating Atherosclerotic Plaques.* Clinical Cardiology, 2000. **23**(S6): p. 3-7.

84. Perisic, L., et al., *Profiling of Atherosclerotic Lesions by Gene and Tissue Microarrays Reveals PCSK6 as a Novel Protease in Unstable Carotid Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2013. **33**(10): p. 2432-2443.

85. Rykaczewska, U., et al., *PCSK6 Is a Key Protease in the Control of Smooth Muscle Cell Function in Vascular Remodeling.* Circulation Research, 2020. **126**(5): p. 571-585.

86. Bennett, M.R., S. Sinha, and G.K. Owens, *Vascular Smooth Muscle Cells in Atherosclerosis.* Circ Res, 2016. **118**(4): p. 692-702.

87. Sata, M., et al., *Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis.* Nat Med, 2002. **8**(4): p. 403-9.

88. Caplice, N.M., et al., *Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4754-9.

89. Iwata, H., et al., *Bone marrow-derived cells contribute to vascular inflammation but do not differentiate into smooth muscle cell lineages.* Circulation, 2010. **122**(20): p. 2048-57.

90. Barallobre-Barreiro, J., et al., *Extracellular Matrix in Vascular Disease, Part 2/4: JACC Focus Seminar.* Journal of the American College of Cardiology, 2020. **75**(17): p. 2189-2203.

91. Yang, W., et al., *Coronary-Heart-Disease-Associated Genetic Variant at the COL4A1/COL4A2 Locus Affects COL4A1/COL4A2 Expression, Vascular Cell Survival, Atherosclerotic Plaque Stability and Risk of Myocardial Infarction.* PLOS Genetics, 2016. **12**(7): p. e1006127.

92. Didangelos, A., et al., *Novel Role of ADAMTS-5 Protein in Proteoglycan Turnover and Lipoprotein Retention in Atherosclerosis.* Journal of Biological Chemistry, 2012. **287**(23): p. 19341-19345.

93. Tran-Lundmark, K., et al., *Heparan Sulfate in Perlecan Promotes Mouse Atherosclerosis.* Circulation Research, 2008. **103**(1): p. 43-52.

94. Finn, A.V., et al., *Concept of Vulnerable/Unstable Plaque.* Arterioscler Thromb Vasc Biol, 2010. **30**(7): p. 1282-1292.

95. Johnson, J.L. and A.C. Newby, *Macrophage heterogeneity in atherosclerotic plaques.* Current opinion in lipidology, 2009. **20**(5): p. 370-378.

96. Michelena, H.I., et al., *Bicuspid Aortic Valve.* Circulation, 2014. **129**(25): p. 2691-2704.

97. Roberts, W.C. and J.M. Ko, *Frequency by Decades of Unicuspid, Bicuspid, and Tricuspid Aortic Valves in Adults Having Isolated Aortic Valve Replacement for Aortic Stenosis, With or Without Associated Aortic Regurgitation.* Circulation, 2005. **111**(7): p. 920-925.

98. Mathieu, P., et al., *The pathology and pathobiology of bicuspid aortic valve: State of the art and novel research perspectives.* The Journal of Pathology: Clinical Research, 2015. **1**(4): p. 195-206.

99. Lindman, B.R., et al., *Calcific aortic stenosis.* Nature Reviews Disease Primers, 2016. **2**: p. 16006.

100. Folkersen, L., et al., *Unraveling Divergent Gene Expression Profiles in Bicuspid and Tricuspid Aortic Valve Patients with Thoracic Aortic Dilatation: The ASAP Study.* Molecular Medicine, 2011. **17**(11): p. 1365-1373.

101. Capoulade, R., et al., *Impact of hypertension and renin–angiotensin system inhibitors in aortic stenosis.* European Journal of Clinical Investigation, 2013. **43**(12): p. 1262-1272.

102. Ngo, M.V., et al., *Smoking and Obesity Are Associated with the Progression of Aortic Stenosis.* The American Journal of Geriatric Cardiology, 2001. **10**(2): p. 86-90.

103. Vavilis, G., et al., *Kidney Dysfunction and the Risk of Developing Aortic Stenosis.* Journal of the American College of Cardiology, 2019. **73**(3): p. 305-314.

104. Helgadottir, A., et al., *Genome-wide analysis yields new loci associating with aortic valve stenosis.* Nature Communications, 2018. **9**(1): p. 987.

105. Coté, N., et al., *Inflammation Is Associated with the Remodeling of Calcific Aortic Valve Disease.* Inflammation, 2013. **36**(3): p. 573-581.

106. Mathieu, P., R. Bouchareb, and M.-C. Boulanger, *Innate and Adaptive Immunity in Calcific Aortic Valve Disease.* Journal of Immunology Research, 2015. **2015**: p. 851945.

107. Nagy, E., et al., *Upregulation of the 5-Lipoxygenase Pathway in Human Aortic Valves Correlates With Severity of Stenosis and Leads to Leukotriene-Induced Effects on Valvular Myofibroblasts.* Circulation, 2011. **123**(12): p. 1316-1325.

108. Bouhlel, M.A., et al., *PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties.* Cell Metab, 2007. **6**(2): p. 137-43.

109. Stoger, J.L., et al., *Distribution of macrophage polarization markers in human atherosclerosis.* Atherosclerosis, 2012. **225**(2): p. 461-8.

110. Chinetti-Gbaguidi, G., S. Colin, and B. Staels, *Macrophage subsets in atherosclerosis.* Nat Rev Cardiol, 2015. **12**(1): p. 10-7.

111. Chinetti-Gbaguidi, G., et al., *Human Atherosclerotic Plaque Alternative Macrophages Display Low Cholesterol Handling but High Phagocytosis Because of Distinct Activities of the PPARγ and LXRα Pathways*. Circulation Research, 2011. **108**(8): p. 985-995.

112. Kadl, A., et al., *Identification of a Novel Macrophage Phenotype That Develops in Response to Atherogenic Phospholipids via Nrf2.* Circulation Research, 2010. **107**(6): p. 737-746.

113. Rahman, K., et al., *Inflammatory Ly6Chi monocytes and their conversion to M2 macrophages drive atherosclerosis regression.* The Journal of Clinical Investigation, 2017. **127**(8): p. 2904-2915.

114. Schrijvers, D.M., et al., *Phagocytosis of Apoptotic Cells by Macrophages Is Impaired in Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2005. **25**(6): p. 1256-1261.

115. Doran, A.C., et al., *CAMKIIγ suppresses an efferocytosis pathway in macrophages and promotes atherosclerotic plaque necrosis.* The Journal of Clinical Investigation, 2017. **127**(11): p. 4075-4089.

116. Yurdagul, A., et al., *Macrophage Metabolism of Apoptotic Cell-Derived Arginine Promotes Continual Efferocytosis and Resolution of Injury.* Cell Metabolism, 2020. **31**(3): p. 518-533.e10.

117. Li, X.-F., et al., *M1 macrophages promote aortic valve calcification mediated by microRNA-214/TWIST1 pathway in valvular interstitial cells.* American journal of translational research, 2016. **8**(12): p. 5773-5783.

118. Li, G., et al., *The shift of macrophages toward M1 phenotype promotes aortic valvular calcification.* The Journal of Thoracic and Cardiovascular Surgery, 2017. **153**(6): p. 1318-1327.e1.

119. Claudia, M., *The Tolls and Dangers of Atherosclerotic Disease.* Current Pharmaceutical Biotechnology, 2012. **13**(1): p. 77-87.

120. Mullick, A.E., P.S. Tobias, and L.K. Curtiss, *Modulation of atherosclerosis in mice by Toll-like receptor 2.* J Clin Invest, 2005. **115**(11): p. 3149-56.

121. Laman, J.D., et al., *Significance of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques.* Am J Cardiol, 2002. **90**(2): p. 119-23.

122. Lehr, H.A., et al., *Immunopathogenesis of atherosclerosis: endotoxin accelerates atherosclerosis in rabbits on hypercholesterolemic diet.* Circulation, 2001. **104**(8): p. 914-20.

123. Tian, J., et al., *Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE.* Nat Immunol, 2007. **8**(5): p. 487-496.

124. Michelsen, K.S., et al., *Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(29): p. 10679-10684.

125. Meng, X., et al., *Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: potential roles in aortic valve inflammation and stenosis.* American Journal of Physiology-Cell Physiology, 2008. **294**(1): p. C29-C35.

126. Yang, X., et al., *Pro-osteogenic phenotype of human aortic valve interstitial cells is associated with higher levels of Toll-like receptors 2 and 4 and enhanced expression of bone morphogenetic protein 2.* J Am Coll Cardiol, 2009. **53**(6): p. 491-500.

127. Ishibashi, M., et al., *TLR3 deficiency protects against collagen degradation and medial destruction in murine atherosclerotic plaques.* Atherosclerosis, 2013. **229**(1): p. 52-61.

128. Lundberg, A.M., et al., *Toll-like receptor 3 and 4 signalling through the TRIF and TRAM adaptors in haematopoietic cells promotes atherosclerosis.* Cardiovasc Res, 2013. **99**(2): p. 364-73.

129. Zhan, Q., et al., *Activation of TLR3 induces osteogenic responses in human aortic valve interstitial cells through the NF-κB and ERK1/2 pathways.* International journal of biological sciences, 2015. **11**(4): p. 482-493.

130. Salagianni, M., et al., *Toll-like receptor 7 protects from atherosclerosis by constraining "inflammatory" macrophage activation.* Circulation, 2012. **126**(8): p. 952-62.

131. Watkins, A.A., et al., *IRF5 deficiency ameliorates lupus but promotes atherosclerosis and metabolic dysfunction in a mouse model of lupus-associated atherosclerosis.* Journal of immunology (Baltimore, Md. : 1950), 2015. **194**(4): p. 1467-1479.

132. Quintar, A., et al., *Endothelial Protective Monocyte Patrolling in Large Arteries Intensified by Western Diet and Atherosclerosis.* Circulation research, 2017. **120**(11): p. 1789-1799.

133. Liu, C.-L., et al., *Toll-like receptor 7 deficiency protects apolipoprotein E-deficient mice from diet-induced atherosclerosis.* Scientific reports, 2017. **7**(1): p. 847-847.

134. O. Krogmann, A., et al., *Toll-Like Receptor 7 Stimulation Promotes the Development of Atherosclerosis in Apolipoprotein E-Deficient Mice.* International Heart Journal, 2020. **61**(2): p. 364-372.

135. Parra-Izquierdo, I., et al., *Calcification Induced by Type I Interferon in Human Aortic Valve Interstitial Cells Is Larger in Males and Blunted by a Janus Kinase Inhibitor.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2018. **38**(9): p. 2148-2159.

136. Hermansson, A., et al., *Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis.* Journal of Experimental Medicine, 2010. **207**(5): p. 1081-1093.

137. Frostegård, J., et al., *Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines.* Atherosclerosis, 1999. **145**(1): p. 33-43.

138. Hansson, G.K., et al., *Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells.* Journal of Experimental Medicine, 1989. **170**(5): p. 1595-1608.

139. Hansson, G.K., et al., *Gamma-interferon regulates vascular smooth muscle proliferation and Ia antigen expression in vivo and in vitro.* Circulation Research, 1988. **63**(4): p. 712-719.

140. Ovchinnikova, O., et al., *T-cell Activation Leads to Reduced Collagen Maturation in Atherosclerotic Plaques of Apoe(-/-) Mice.* The American Journal of Pathology, 2009. **174**(2): p. 693-700.

141. Zhou, X., et al., *Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice.* The Journal of Clinical Investigation, 1998. **101**(8): p. 1717-1725.

142. Cardilo-Reis, L., et al., *Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype.* EMBO Molecular Medicine, 2012. **4**(10): p. 1072-1086.

143. King, V.L., S.J. Szilvassy, and A. Daugherty, *Interleukin-4 Deficiency Decreases Atherosclerotic Lesion Formation in a Site-Specific Manner in Female LDL Receptor−/− Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2002. **22**(3): p. 456-461.

144. Engelbertsen, D., et al., *T-Helper 2 Immunity Is Associated With Reduced Risk of Myocardial Infarction and Stroke.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2013. **33**(3): p. 637.

145. Danzaki, K., et al., *Interleukin-17A Deficiency Accelerates Unstable Atherosclerotic Plaque Formation in Apolipoprotein E-Deficient Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2012. **32**(2): p. 273-280.

146. Usui, F., et al., *Interleukin-17 deficiency reduced vascular inflammation and development of atherosclerosis in Western diet-induced apoE-deficient mice.* Biochemical and Biophysical Research Communications, 2012. **420**(1): p. 72-77.

147. Gistera, A., et al., *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): p. 196ra100.

148. Ait-Oufella, H., et al., *Natural regulatory T cells control the development of atherosclerosis in mice.* Nature Medicine, 2006. **12**(2): p. 178-180.

149. Klingenberg, R., et al., *Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis.* The Journal of Clinical Investigation, 2013. **123**(3): p. 1323-1334.

150. van Es, T., et al., *Vaccination against Foxp3+ regulatory T cells aggravates atherosclerosis.* Atherosclerosis, 2010. **209**(1): p. 74-80.

151. Mor, A., et al., *Role of Naturally Occurring CD4+CD25+ Regulatory T Cells in Experimental Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2007. **27**(4): p. 893-900.

152. Sasaki, N., et al., *Oral Anti-CD3 Antibody Treatment Induces Regulatory T Cells and Inhibits the Development of Atherosclerosis in Mice.* Circulation, 2009. **120**(20): p. 1996-2005.

153. Takeda, M., et al., *Oral Administration of an Active Form of Vitamin D3 (Calcitriol) Decreases Atherosclerosis in Mice by Inducing Regulatory T Cells and Immature Dendritic Cells With Tolerogenic Functions.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2010. **30**(12): p. 2495-2503.

154. Kita, T., et al., *Regression of atherosclerosis with anti-CD3 antibody via augmenting a regulatory T-cell response in mice.* Cardiovascular Research, 2014. **102**(1): p. 107-117.

155. Foks, A.C., et al., *Differential effects of regulatory T cells on the initiation and regression of atherosclerosis.* Atherosclerosis, 2011. **218**(1): p. 53-60.

156. Hollander, W., et al., *Soluble proteins in the human atheroschlerotic plaque: With Spectral Reference to Immunoglobulins, C3-Complement Component, α1-Antitrypsin and α2-Macroglobulin.* Atherosclerosis, 1979. **34**(4): p. 391-405.

157. Hansson, G.K., et al., *Ultrastructural studies on the localization of IgG in the aortic endothelium and subendothelial intima of atherosclerotic and nonatherosclerotic rabbits.* Experimental and Molecular Pathology, 1980. **33**(3): p. 302-315.

158. Vlaicu, R., et al., *Immunoglobulins and complement components in human aortic atherosclerotic intima.* Atherosclerosis, 1985. **55**(1): p. 35-50.

159. Caligiuri, G., et al., *Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice.* J Clin Invest, 2002. **109**(6): p. 745-53.

160. Grasset, E.K., et al., *Sterile inflammation in the spleen during atherosclerosis provides oxidation-specific epitopes that induce a protective B-cell response.* Proceedings of the National Academy of Sciences, 2015. **112**(16): p. E2030-E2038.

161. Major, A.S., S. Fazio, and M.F. Linton, *B-lymphocyte Deficiency Increases Atherosclerosis in LDL Receptor-Null Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2002. **22**(11): p. 1892-1898.

162. Nus, M., et al., *Marginal zone B cells control the response of follicular helper T cells to a high-cholesterol diet.* Nature Medicine, 2017. **23**: p. 601.

163. Meeuwsen, J.A.L., et al., *High Levels of (Un)Switched Memory B Cells Are Associated With Better Outcome in Patients With Advanced Atherosclerotic Disease.* Journal of the American Heart Association, 2017. **6**(9): p. e005747.

164. Ylä-Herttuala, S., et al., *Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL*. Vol. 14. 1994. 32-40.

165. Binder, C.J., N. Papac-Milicevic, and J.L. Witztum, *Innate sensing of oxidation-specific epitopes in health and disease.* Nature Reviews Immunology, 2016. **16**(8): p. 485-497.

166. Prasad, A., et al., *Relationship of Autoantibodies to MDA-LDL and ApoB-Immune Complexes to Sex, Ethnicity, Subclinical Atherosclerosis, and Cardiovascular Events.* Arteriosclerosis, thrombosis, and vascular biology, 2017. **37**(6): p. 1213-1221.

167. Iseme, R.A., et al., *A role for autoantibodies in atherogenesis.* Cardiovascular Research, 2017. **113**(10): p. 1102-1112.

168. Nicoletti, A., et al., *Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice.* The Journal of Clinical Investigation, 1998. **102**(5): p. 910-918.

169. Schiopu, A., et al., *Recombinant Antibodies to an Oxidized Low-Density Lipoprotein Epitope Induce Rapid Regression of Atherosclerosis in Apobec-1−/−/Low-Density Lipoprotein Receptor−/−Mice.* Journal of the American College of Cardiology, 2007. **50**(24): p. 2313-2318.

170. Gistera, A., et al., *Low-Density Lipoprotein-Reactive T Cells Regulate Plasma Cholesterol Levels and Development of Atherosclerosis in Humanized Hypercholesterolemic Mice.* Circulation, 2018. **138**(22): p. 2513-2526.

171. Centa, M., et al., *Germinal Center-Derived Antibodies Promote Atherosclerosis Plaque Size and Stability.* Circulation, 2019. **139**(21): p. 2466-2482.

172. Karvonen, J., et al., *Immunoglobulin M Type of Autoantibodies to Oxidized Low-Density Lipoprotein Has an Inverse Relation to Carotid Artery Atherosclerosis.* Circulation, 2003. **108**(17): p. 2107-2112.

173. Kyaw, T., et al., *B1a B Lymphocytes Are Atheroprotective by Secreting Natural IgM That Increases IgM Deposits and Reduces Necrotic Cores in Atherosclerotic Lesions.* Circulation Research, 2011. **109**(8): p. 830-840.

174. Lewis, M.J., et al., *Immunoglobulin M is Required for Protection Against Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice.* Circulation, 2009. **120**(5): p. 417-426.

175. Binder, C.J., et al., *Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL.* Nature Medicine, 2003. **9**(6): p. 736-743.

176. Boullier, A., et al., *The Binding of Oxidized Low Density Lipoprotein to Mouse CD36 Is Mediated in Part by Oxidized Phospholipids That Are Associated with Both the Lipid*

and Protein Moieties of the Lipoprotein. Journal of Biological Chemistry, 2000. **275**(13): p. 9163-9169.

177. Faria-Neto, J.R., et al., *Passive immunization with monoclonal IgM antibodies against phosphorylcholine reduces accelerated vein graft atherosclerosis in apolipoprotein E-null mice.* Atherosclerosis, 2006. **189**(1): p. 83-90.

178. Centa, M., et al., *Atherosclerosis Susceptibility in Mice Is Independent of the V1 Immunoglobulin Heavy Chain Gene.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2016. **36**(1): p. 25-36.

179. Hosseini, H., et al., *Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyreactive IgM producing B1a lymphocytes.* Cardiovasc Res, 2015. **106**(3): p. 443-52.

180. Ait-Oufella, H., et al., *Recent advances on the role of cytokines in atherosclerosis.* Arterioscler Thromb Vasc Biol, 2011. **31**(5): p. 969-79.

181. Buono, C., et al., *T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1596-601.

182. Heeschen, C., et al., *Serum Level of the Antiinflammatory Cytokine Interleukin-10 Is an Important Prognostic Determinant in Patients With Acute Coronary Syndromes.* Circulation, 2003. **107**(16): p. 2109-2114.

183. Han, X. and W.A. Boisvert, *Interleukin-10 protects against atherosclerosis by modulating multiple atherogenic macrophage function.* Thromb Haemost, 2015. **113**(03): p. 505-512.

184. Ditiatkovski, M., B.H. Toh, and A. Bobik, *GM-CSF deficiency reduces macrophage PPAR-gamma expression and aggravates atherosclerosis in ApoE-deficient mice.* Arterioscler Thromb Vasc Biol, 2006. **26**(10): p. 2337-44.

185. Shaposhnik, Z., et al., *Granulocyte macrophage colony-stimulating factor regulates dendritic cell content of atherosclerotic lesions.* Arterioscler Thromb Vasc Biol, 2007. **27**(3): p. 621-7.

186. Li, J., et al., *Interferon-α priming promotes lipid uptake and macrophage-derived foam cell formation: A novel link between interferon-α and atherosclerosis in lupus.* Arthritis & Rheumatism, 2011. **63**(2): p. 492-502.

187. Goossens, P., et al., *Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions.* Cell Metab, 2010. **12**(2): p. 142-53.

188. Rothwell, P.M., et al., *Critical Appraisal of the Design and Reporting of Studies of Imaging and Measurement of Carotid Stenosis.* Stroke, 2000. **31**(6): p. 1444-1450.

189. Folkersen, L., et al., *Endogenous control genes in complex vascular tissue samples.* BMC Genomics, 2009. **10**: p. 516.

190. Folkersen, L., et al., *Prediction of ischemic events on the basis of transcriptomic and genomic profiling in patients undergoing carotid endarterectomy.* Mol Med, 2012. **18**: p. 669-75.

191. Perisic, L., et al., *Gene expression signatures, pathways and networks in carotid atherosclerosis.* Journal of Internal Medicine, 2016. **279**(3): p. 293-308.

192. Karadimou, G., et al., *Low TLR7 gene expression in atherosclerotic plaques is associated with major adverse cardio- and cerebrovascular events.* Cardiovasc Res, 2017. **113**(1): p. 30-39.

193. Winkels, H., et al., *Atlas of the Immune Cell Repertoire in Mouse Atherosclerosis Defined by Single-Cell RNA-Sequencing and Mass Cytometry.* Circulation Research, 2018. **122**(12): p. 1675-1688.

194. San Segundo-Val, I. and C.S. Sanz-Lozano, *Introduction to the Gene Expression Analysis*, in *Molecular Genetics of Asthma*, M. Isidoro García, Editor. 2016, Springer New York: New York, NY. p. 29-43.

195. Erridge, C., *Diet, commensals and the intestine as sources of pathogen-associated molecular patterns in atherosclerosis, type 2 diabetes and non-alcoholic fatty liver disease.* Atherosclerosis, 2011. **216**(1): p. 1-6.

196. Persson, J., et al., *High plasma adiponectin concentration is associated with all-cause mortality in patients with carotid atherosclerosis.* Atherosclerosis, 2012. **225**(2): p. 491-496.

197. Yang, Q.-w., et al., *Upregulated Expression of Toll-Like Receptor 4 in Monocytes Correlates with Severity of Acute Cerebral Infarction.* Journal of Cerebral Blood Flow & Metabolism, 2008. **28**(9): p. 1588-1596.

198. Urra, X., et al., *Monocytes Are Major Players in the Prognosis and Risk of Infection After Acute Stroke.* Stroke, 2009. **40**(4): p. 1262-1268.

199. Karadimou, G., et al., *Low TLR7 gene expression in atherosclerotic plaques is associated with major adverse cardio- and cerebrovascular events.* Cardiovascular Research, 2017. **113**(1): p. 30-39.

200. Ketelhuth, D.F.J. and G.K. Hansson, *Adaptive Response of T and B Cells in Atherosclerosis.* Circulation Research, 2016. **118**(4): p. 668-678.

201. Dominguez-Villar, M., et al., *TLR7 induces anergy in human CD4+ T cells.* Nat Immunol, 2015. **16**(1): p. 118-128.

202. Proto, J.D., et al., *Regulatory T Cells Promote Macrophage Efferocytosis during Inflammation Resolution.* Immunity, 2018. **49**(4): p. 666-677.e6.

203. van Duijn, J., et al., *CD8+ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4+ T-cell responses.* Cardiovascular Research, 2018. **115**(4): p. 729-738.

204. Clement, M., et al., *Control of the T Follicular Helper–Germinal Center B-Cell Axis by CD8+ Regulatory T Cells Limits Atherosclerosis and Tertiary Lymphoid Organ Development.* Circulation, 2015. **131**(6): p. 560-570.

205. Olofsson, P.S., et al., *CD137 Is Expressed in Human Atherosclerosis and Promotes Development of Plaque Inflammation in Hypercholesterolemic Mice.* Circulation, 2008. **117**(10): p. 1292-1301.

206. Viola, A., et al., *The Metabolic Signature of Macrophage Responses.* Frontiers in Immunology, 2019. **10**(1462).

207. Han, X. and W.A. Boisvert, *Interleukin-10 protects against atherosclerosis by modulating multiple atherogenic macrophage function.* Thromb Haemost, 2015. **113**(3): p. 505-12.

208. Ait-Oufella, H., et al., *Recent Advances on the Role of Cytokines in Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2011. **31**(5): p. 969-979.

209. Papathanasiou, S., et al., *Tumor necrosis factor-α confers cardioprotection through ectopic expression of keratins K8 and K18.* Nature Medicine, 2015. **21**(9): p. 1076-1084.

210. Green, N.M., et al., *Activation of Autoreactive B Cells by Endogenous TLR7 and TLR3 RNA Ligands.* Journal of Biological Chemistry, 2012. **287**(47): p. 39789-39799.

211. Lee, J., et al., *Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: Activation of Toll-like receptor 7.* Proceedings of the National Academy of Sciences, 2003. **100**(11): p. 6646-6651.

212. Bichakjian, C., et al., *Guidelines of care for the management of basal cell carcinoma.* Journal of the American Academy of Dermatology, 2018. **78**(3): p. 540-559.

213. Mallat, Z., et al., *Protective Role of Interleukin-10 in Atherosclerosis.* Circulation Research, 1999. **85**(8): p. e17-e24.

214. Mallat, Z., et al., *Expression of Interleukin-10 in Advanced Human Atherosclerotic Plaques.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1999. **19**(3): p. 611-616.

215. Libby, P., P.M. Ridker, and G.K. Hansson, *Progress and challenges in translating the biology of atherosclerosis.* Nature, 2011. **473**(7347): p. 317-325.

216. Kapelouzou, A., et al., *Differential expression patterns of Toll Like Receptors and Interleukin-37 between calcific aortic and mitral valve cusps in humans.* Cytokine, 2019. **116**: p. 150-160.

217. Moore, K.J., F.J. Sheedy, and E.A. Fisher, *Macrophages in atherosclerosis: a dynamic balance.* Nat Rev Immunol, 2013. **13**(10): p. 709-721.

218. Lindman, B.R., et al., *Calcific aortic stenosis.* Nature reviews. Disease primers, 2016. **2**: p. 16006-16006.

219. Yu, Z., et al., *Tumor Necrosis Factor-α Accelerates the Calcification of Human Aortic Valve Interstitial Cells Obtained from Patients with Calcific Aortic Valve Stenosis via the BMP2-Dlx5 Pathway.* Journal of Pharmacology and Experimental Therapeutics, 2011. **337**(1): p. 16-23.

220. Kaden, J.J., et al., *Inflammatory regulation of extracellular matrix remodeling in calcific aortic valve stenosis.* Cardiovascular Pathology, 2005. **14**(2): p. 80-87.

221. Koltsida, O., et al., *Toll-like receptor 7 stimulates production of specialized pro-resolving lipid mediators and promotes resolution of airway inflammation.* EMBO Mol Med, 2013. **5**(5): p. 762-75.

222. Pham Van, L., et al., *Treatment with the TLR7 agonist R848 induces regulatory T-cell-mediated suppression of established asthma symptoms.* European Journal of Immunology, 2011. **41**(7): p. 1992-1999.

223. Hemmi, H., et al., *Small anti-viral compounds activate immune cells via the TLR7 MyD88–dependent signaling pathway.* Nature Immunology, 2002. **3**(2): p. 196-200.

224. Jurk, M., et al., *Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848.* Nature Immunology, 2002. **3**(6): p. 499-499.

225. Zhang, Z.-J., et al., *TLR8 and its endogenous ligand miR-21 contribute to neuropathic pain in murine DRG.* Journal of Experimental Medicine, 2018. **215**(12): p. 3019-3037.

226. Caramalho , I., et al., *Regulatory T Cells Selectively Express Toll-like Receptors and Are Activated by Lipopolysaccharide.* Journal of Experimental Medicine, 2003. **197**(4): p. 403-411.

227. Genestier, L., et al., *TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses.* J Immunol, 2007. **178**(12): p. 7779-86.

228. Forward, N.A., et al., *Signaling through TLR7 enhances the immunosuppressive activity of murine CD4+CD25+ T regulatory cells.* Journal of Leukocyte Biology, 2010. **87**(1): p. 117-125.

229. Cesena, F.H.Y., et al., *Immune-modulation by polyclonal IgM treatment reduces atherosclerosis in hypercholesterolemic apoE−/− mice.* Atherosclerosis, 2012. **220**(1): p. 59-65.

230. Karadimou, G., et al., *Treatment with a Toll-like Receptor 7 ligand evokes protective immunity against atherosclerosis in hypercholesterolaemic mice.* Journal of Internal Medicine, 2020.

231. Kim, S., et al., *Toll-like receptor 7 affects the pathogenesis of non-alcoholic fatty liver disease.* Scientific Reports, 2016. **6**(1): p. 27849.

232. Quartier, P., et al., *Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro.* Eur J Immunol, 2005. **35**(1): p. 252-60.