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What is the function of B cells in experimental atherosclerosis?

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The B lymphocyte in atherosclerosis: promotion or protection?

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cardiovascular disease is one of the leading causes of death worldwide. A major cause is atherosclerosis, which is a chronic inflammatory disease of medium and large size arteries characterized by the accumulation of cholesterol rich low-density lipoprotein (LDL) particles in the sub-endothelium layer of the vessel.

Retention and modification of LDL triggers a systemic inflammatory response sustained by the humoral and cellular branches of the immune system. Early atherosclerosis initiation is often in the form of fatty streaks whilst necrotic core formation, inflammation, fibrosis and stenosis are characteristics of advanced atherosclerotic plaques.

A protective humoral immune response is induced upon vaccination against oxidized LDL in experimental models of atherosclerosis, whilst studies on follicular B cells report an opposite pathogenic function of this subset. Furthermore, association studies on cardiovascular disease risk and antibody titers against oxidized LDL provide inconsistent results. Given these seemingly contradictory observations, the role of B cells and antibodies in atherosclerosis is still poorly understood.

Atherosclerosis can develop as a silent disease over decades, and asymptomatic plaques usually have a robust fibrotic cap consisting of collagen and extracellular matrix deposited by smooth muscle cells. However, sudden clinical symptoms can occur in terms of myocardial infarction and stroke when the stability of the cap is impaired and the content of the plaque is released into the bloodstream.

In this thesis we identify a potential mechanism involved in regulating the growth and the stability of atherosclerotic lesions. We first demonstrate that a germinal center response occurs promptly upon acute hypercholesterolemia, and is associated with an autoimmune-like deposition of immune complexes and systemic autoantibody production. Secondly, we characterize the effects of B cells in late stage atherosclerosis and we show that antibodies promote increased lesion size. Furthermore, we illustrate how these effects are depending on germinal center-derived antibodies. Thirdly, we observe that antibodies originating from the germinal center reaction induce a stable atherosclerotic plaque phenotype, in part through promoting smooth muscle cell proliferation. Finally, we report that antibodies regulate transcriptional pathways involved in maintaining the integrity, function and stability of the aorta.

Collectively, the findings presented in this thesis identify germinal center B cells as an important factor affecting the composition, size and stability of the atherosclerotic plaque.

LIST OF SCIENTIFIC PAPERS

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LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette
AID (*Aicda*)	Activation induced cytidine deaminase
APC	Antigen presenting cell
Apo	Apolipoprotein
BAFF	B cell activating factor
Bcl6	B-cell lymphoma 6
Blimp-1 (*Prdm-1*)	B lymphocyte–induced maturation protein 1
CRP	C reactive protein
CSR	Class switch recombination
CVD	Cardiovascular disease
CXCR	CXC-motif chemokine receptor
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cell
FcγR	Fcγ receptor
GC	Germinal center
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LpL	Lipoprotein lipase
LPS	Lipopolysaccharide
MCP1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloprotease
MS	Multiple sclerosis

NAbs	Natural antibodies
NLRP3	NOD-like receptor, pyrin domain containing 3
oxLDL	Oxidized lipoprotein
PAX5 (BSAP)	B cell specific activator protein
PC	Phosphocholine
PLA2	Phospholipase A2
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
Rag	Recombination activating enzyme
ROS	Reactive oxygen species
Scid	Severe combined immunodeficiency
SHM	Somatic hypermutation
SMA	Smooth muscle actin
SMase	Sphingomyelinase
SMC	Smooth muscle cell
TGF	Transforming growth factor
Th	T helper
TLO	Tertiary lymphoid organ
TLR	Toll like receptor
TNF	Tumor necrosis factor
T reg	T regulatory
XBP1	X-binding protein 1

1 INTRODUCTION

Atherosclerosis is thought to be an inflammatory disease that is triggered by accumulation and modification of lipoproteins within the intima layer of the artery. Atherosclerosis is a major cause for the development of acute cardiovascular events such as myocardial infarction and stroke and is the main cause of death worldwide. Rather than being simply the passive accumulation of cholesterol in the vessel wall, atherosclerosis is a complex chronic inflammatory disease which is thought to be triggered by the accumulation of apolipoproteinB containing lipoproteins in the sub-endothelial spaces of medium and large-sized arteries, with involvement of both innate and adaptive immunity in disease progression [1].

The molecular series of events leading from infiltration of cholesterol-loaded lipoprotein particles to chronic inflammation and thrombosis are still not fully understood. In particular, the interrelationship of perturbed lipid metabolism with immune responses remains largely unknown. Wild-type mice do not develop spontaneous atherosclerosis as a result of their high circulating levels of 'anti-atherosclerotic' high-density lipoproteins (HDL) and low levels of 'pro-atherogenic' low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL). Consequently, mouse models for atherosclerosis need either genetic manipulation or diet intervention in order to achieve perturbations of the lipoprotein metabolism. The most commonly used mouse models are Apolipoprotein E–deficient mice (*Apoe*$^{-/-}$ mice), in which loss of the *Apoe* gene leads to hypercholesterolemia and spontaneous atherosclerosis, and LDL receptor–deficient mice (*Ldlr*$^{-/-}$ mice) that develop atherosclerosis upon a lipid-rich diet.

Combining these models with strains defective in immune system components has provided overwhelming evidence for both autoimmune and immune-protective mechanisms in atherosclerosis.

1.1 Atherosclerosis

Arteries are composed of three layers. The inner layer, facing the vessel lumen and in contact with the blood, is called the tunica intima and is composed of a monolayer of endothelial cells overlying a basal membrane. Next is the tunica media, the middle or media layer, which consists largely of smooth muscle cells. The outer lining is called tunica adventitia with connective tissue, fibroblasts, macrophages and other immune cell. Occasionally lymphoid structures called tertiary lymphoid organs (TLOs) are also seen in different arteries [2].

Atherosclerotic lesion development is first visualized as fatty streaks, an early accumulation of lipid-containing cells in the sub-intimal space and, according to some observations, may physiologically regress during childhood [3].

Atherosclerotic lesions are preferentially located in areas where the blood flow is disturbed or turbulent, such as in proximity to the branching sites of the arterial tree. Cholesterol-rich low density lipoprotein (C-LDL) is believed to play a major role in the establishment of atherosclerosis. Atherosclerosis initiation is driven by localized adhesion of ApoB-rich lipoproteins on sub-endothelial matrix proteoglycans. Retention of these particles is aided by lipoprotein lipase, sphingomyelinase, and phospholipase-A2. The modification, such as oxidation, of retained LDL triggers a cascade of immune reactions that eventually lead to an adaptive inflammatory response [4-7].

1.1.1 Lipoproteins

Lipoproteins are complex molecular entities consisting of a hydrophobic internal core made of lipids (cholesterol esters and triglycerides) and an outer shell made of apolipoproteins which allow transportation through the circulation between peripheral tissues and lipid deposits. The protein moiety also serves other functions such as providing structural support during the assembly of the lipoprotein, interacting with lipoprotein receptors and through exerting functional roles in enzymatic pathways.

Lipoproteins are classified in categories depending on size, lipid content and density: increasing in density there are first chylomicron and chylomicron remnants (diameter > 80 nm with density < 0,9 g/ml), VLDL (30-80 nm), IDL (25-35 nm), LDL (18-25 nm), HDL (5-12 nm) and finally Lipoprotein(a) (~ 30 nm). Chylomicrons, VLDL and LDL are associated with a pro-atherogenic role, whilst HDL is considered to have protective effects, although this is debated.

ApoB100 containing VLDL are formed in the liver as a result of metabolism of dietary lipids that are rich in triglycerides. In the muscle and adipose tissues, VLDL deposit triglycerides and are consequently enriched in cholesterol cargo. Because of this chemical change the lipoprotein species is now termed LDL.

LDL, which also contains one ApoB100 molecule, interacts with the LDL receptor (LDLR) on several tissues, predominately the liver and intestine. Non-physiological conditions such as type 2 diabetes, metabolic syndrome, hypertriglyceridemia and obesity, are associated with increased LDL concentration. LDL levels are the result of a balance between the rate of LDL production and clearance, both of which depend on the LDLR and also other receptors to a lesser extent.

HDL contains ApoA-1 and its function is the reverse transport of cholesterol. HDL uptakes cholesterol from peripheral storages via the ATP-binding cassette transporters (ABCA1, ABCG1) and transports it to the liver where it will be metabolized into bile acids [8].

1.1.2 The immune system in atherosclerosis

Endothelial cells (ECs) can initiate inflammatory responses that rise in response to blood borne invaders, following engagement of their scavenger receptors and TLRs. ECs exposed to oxidized LDL (oxLDL) produce chemoattractant factors to recruit immune cells such as MCP-1, which binds the CCR2 receptor on monocytes, and produce M-CSF that triggers self-sustained macrophage differentiation. Moreover ECs control vascular tone via the inducible nitric oxide synthase 2 enzyme, interleukin-1 and endothelin, and the endothelium activation into an inflammatory phenotype marks the initial stages of early atherosclerosis [9, 10].

Macrophages are abundant in the atherosclerotic plaque. Activated macrophages show a pro-inflammatory phenotype due to induction of the NF-kB cascade and upregulate their pattern recognition receptors such as surface scavenger receptors and toll like receptors (TLRs). Within the atherosclerotic plaque extensive macrophage proliferation amplifies the inflammatory environment, whereupon TNF-α, IFNγ, IL-6 and IL-1β, amongst other pro-inflammatory cytokines and growth factors, are secreted [11].

Plaque degradation and remodeling occurs as a consequence of macrophage and smooth muscle cell derived proteases. Collagenases such as MMP-1, MMP-8, MMP-9, MMP-13 and cathepsins S, K, L and B are found in human and experimental lesions [11].

Nucleotide-binding domain, leucine-rich repeat-containing proteins (NLRP) represent sensors which function as pattern recognition receptors of the innate immune system (PRRs). The most studied member is NLRP3 due to its role in forming the inflammasome. The inflammasome is a multimeric protein complex which aggregates to its mature form in response to microbial molecules or “danger” signals. The assembly of the inflammasome initiates pro-caspase-1 self-cleavage to generate active caspase-1, which in turn processes by proteolysis pro-interleukin1β to generate mature IL-1β. A variety of stimuli can activate the NLRP3 inflammasome: LPS, extracellular ATP, monosodium urate (MSU) crystals, cholesterol crystals and oxLDL. The role of NLRP3 in atherosclerosis is contradictory: a study using *Nlrp3*$^{-/-}$ bone marrow transplanted into *Ldlr*$^{-/-}$ and fed high-fat diet revealed a pathogenic role of NLRP3 whilst a double knock-out *Apoe*$^{-/-}$ *Nlrp3*$^{-/-}$ fed high fat diet showed no effect at all [12, 13].

Circulating mast cells derive from a progenitor originating from the hematopoietic compartment. The cytoplasmic granules contain a variety of inflammatory mediators which have pro-atherogenic effector functions. Present in the granules are vasoactive amines such as tryptases, and specific mast cell subclasses contain chymases. Human data and experimental models demonstrate a pro-atherogenic role of mast cells in atherosclerosis, despite their minor abundance in atherosclerotic plaque. The *Ldlr*$^{-/-}$ *Kit (W-sh/W-sh*) mouse lacks mast cells and shows reduced plaque burden together with reduced serum lipid levels and vascular inflammation [14]. Tryptases and chymases are reported to act both in chronic and acute manner on plaque progression and destabilization. Released granule contents drive leukocyte recruitment *in situ* and foam cell accumulation during plaque growth. At later stages they can activate MMPs to degrade extracellular matrix, induce SMCs apoptosis and promote vessel wall permeabilization and microhemorrhage, which are events linked with atherosclerotic plaque destabilization [15].

Natural Killer (NK) cells are scarce in plaques and localize in the shoulder regions. NK cells possibly promote atherosclerosis via IFNγ, GM-CSF and TNF-α production, and a lack of NK function in Ly49A transgenic bone marrow transplanted into *Ldlr*$^{-/-}$ recipients resulted in a protective phenotype [16].

Neutrophils are a minor cell population in established plaques. In their cytoplasmic granules are present large amount of reactive oxygen species (ROS) and matrix degrading proteases which are secreted extracellularly and possibly recruit monocytes, promote further LDL oxidation and enhance the pro-inflammatory milieu. Hypercholesterolemia triggers bone marrow-derived leukocytosis and neutrophilia which stimulate early atherosclerosis formation [17]. Aside from their proposed function in initiation of arterial inflammation, neutrophils might also play a role in destabilization of established plaques [18].

The cross-talk between humoral, cellular immunity and lipid metabolism plays an important role in atherosclerosis and adaptive immune responses are detected during the phases of atherosclerotic plaque development and progression. The B-T cell interactions result in pro- or anti-inflammatory functions with production of antibody specificities against modified LDL. *Rag1*$^{-/-}$ and *Scid*$^{-/-}$ mice, two models lacking T and B cells, crossed into the atherosclerosis prone *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice show a reduction in early atherosclerotic lesion formation, but upon western diet feeding *Rag1*$^{-/-}$ mice can still develop atherosclerosis [19, 20]. T lymphocytes in the plaque are mainly of the CD4+ T helper 1 memory/effector phenotype, compared with the CD8+ T cells within the plaque and in peripheral blood, at least in mice, and elicit a pro-atherogenic response.

Apoe$^{-/-}$ mice lacking CD4 cells display reduced atherosclerosis [21], although subsets of the CD4 lineage are proposed to play different roles in atherosclerosis.

Th1 cells are induced by the IL-12-IL-18 axis, that is established by activated pro-inflammatory macrophages. The required signature transcription factor for this lineage is TBET and the characteristic Th1 cytokine is the inflammatory IFN-γ. *IFN-γ receptor*$^{-/-}$ *Apoe*$^{-/-}$ mice show less atherosclerosis, and Th1 cells are associated with inflammation, increased disease burden and plaque instability with reduction in collagen fiber formation, increased expression of MHC II, enhanced protease and chemokine secretion, upregulation of adhesion molecules and pro-inflammatory cytokines and activation of macrophages and endothelial cells [22, 23]. The Th2 subset is reported to have opposite effects in atherosclerosis studies compared to Th1 cells. GATA3 transcription factor drives Th2 cell differentiation and IL-4, IL-5, IL-13 are signature Th2 cytokines. IL-5 possibly mediates protective functions by promoting B1 cell expansion and natural antibodies (NAbs) secretion [24]. IL-4 has complex effects on multiple cell types and was reported to promote atherosclerosis, whilst IL-13 might confer protection [25, 26]. Th17 cells are identified by the signature cytokine IL-17A and their role in atherosclerosis is controversial. In *Apoe*$^{-/-}$ mice IL-17A was associated both with protection and promotion [27, 28]. T regulatory cells are characterized by the production of IL-10 and TGF-β. This subset is strongly associated with dampening inflammation and reducing atherosclerotic lesion size, possibly by decreasing cholesterol levels [29-31].

1.1.3 Atherogenesis

Endothelial cells (ECs) in healthy arteries are normally resistant to adhesion of circulating leukocytes and molecules. Upon dyslipidemia, hypertension, hyperglycemia, smoking, and other pro-inflammatory stimuli, in the first phases of the response to retention of sub-intimal oxLDL the endothelium is activated. In this process the expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), and chemoattractants (i.e. MCP-1) which in turn mediate leucocyte attachment, is enhanced. The endothelial permeabilization and chemokine release allow monocytes to migrate into the sub-endothelium, differentiate into macrophages in response to endothelial M-CSF, upregulate their pattern recognition receptors (PRRs) and phagocyte oxLDL to eventually become cholesterol-laden foam cells [10].

Next, immune cells such as activated IFN-γ releasing T cells, mast cells, monocytes

differentiating into inflammatory macrophages, antigen presenting cells (APCs), mast cells and NKT cells infiltrate from the shoulder region of the plaque and contribute to amplify and worsen the ongoing inflammatory response. The process is accelerated by continuous LDL accumulation in established lesions whilst a few smooth muscle cells (SMCs) migrate from the media to the sub-endothelial space and promote the formation of a fibrous cap of variable thickness composed of smooth muscle cells and secreted collagen. However, as the lesion progresses and becomes an atheroma, macrophages die and become necrotic areas (necrotic cores) consisting of cellular debris, cholesterol crystals, proteases, and thrombogenic material. Over decades these advanced plaques grow and give rise to clinical manifestations such as narrowing the lumen (stenosis) which impair the blood flow, or becoming susceptible to fibrous cap thinning, plaque rupture or erosion. If the cap stability is impaired this can ultimately lead to acute thrombotic vascular events such as myocardial infarction and stroke [7]. At this stage biomarkers such as C-reactive protein and IL-6 can be found in the circulation [32, 33].

Lesions that rupture typically have a thin, collagen-poor fibrotic cap with few SMCs and abundant macrophages, and often the rupture events occur in the shoulder area of vulnerable plaques [34]. Smooth muscle cells are the source of arterial collagen. The inflammatory cells may enhance plaque disruption by producing collagenolytic enzymes that can degrade collagen. The matrix metalloproteinase family (MMP) is the most studied and held responsible for the loss of collagen. The MMP member MMP-13 is associated with plaque collagen degradation in mouse models of atherosclerosis. Neutrophils produce proteases that further promote degradation of the extracellular matrix, thus weakening the fibrotic cap and destabilizing the plaque [35, 36]. When pro-coagulant necrotic material from the plaque core is exposed to the blood stream, it can react with the plasma coagulation components to form a thrombus. Main components of the thrombus are platelets, fibrin and erythrocytes. A thrombus can either interrupt blood flow locally or detach to become an embolus that can block the blood flow in distal arteries [37-40].
Occurrence of intra-plaque hemorrhage may also contribute to plaque instability.

LDL is the most studied antigen in atherogenesis: LDL contains several epitopes recognized by B and T cells, and thereby induces both humoral and cellular immunity via a cross-talk between T and B cells. When an antigen presenting cell such as a dendritic cell first encounters the antigen, it processes and presents it on MHC II and then migrates through the lymphoid organs where interactions with T cells can take place. After a T cell bearing the specific TCR sequence for the epitope is recognized and activated, the CD4+ T helper cell migrates towards the B cell zone

where it will stimulate a cognate B cell. The activated B cell will rapidly secrete a first wave of IgM antibodies, then may move into the follicle to initiate a germinal center reaction. This can result in production of higher affinity antibodies against the antigen. Vaccination against modified LDL confers protective effects in experimental models of atherosclerosis and auto-antibodies against these modified LDL are found in the circulation of experimental models and humans [41-44].

Natural antibodies perform an innate-like immune response in atherosclerosis, in the form of germline encoded antibodies produced by B1 cells that recognize phosphocholine (PC) exposed on the surface of oxLDL, on the cell membrane of apoptotic cells and on *Streptococcus pneumoniae* surface, and eventually are thought to prevent atherosclerosis via different mechanisms. First, natural antibodies to a variety of oxidation-specific epitopes enhance clearance of apoptotic blebs released from dying cells. Secondly, they can inhibit scavenger-receptor-mediated uptake of oxLDL by macrophages, thereby decreasing foam cell formation and macrophage derived inflammation which has pro-atherosclerotic effects. Finally, by forming immune complexes with various oxidized lipids, they may not only enhance clearance, but also provoke further immunogenic responses [1, 45-47].

1.2 B lymphocytes and antibodies in atherosclerosis

In order to provide an efficient, full range protection against a very large number of different epitopes the B cell compartment needs to produce a broad and diverse spectrum of immunoglobulins (Igs).

Few B lymphocytes are found in human and murine plaques, but immunoglobulins are present in the plaque [48-50]. Both immunoglobulins and B lymphocytes can also be found in the tertiary lymphoid organs in the artery adventitia of old *Apoe*$^{-/-}$ mice [51].

The role of antibodies and B cells in atherosclerosis has been investigated in a number of studies using mouse models. The data from these reports point towards a protective role of IgM antibodies against atherosclerosis. In fact, *Ldlr-/-* mice deficient also in serum IgM, *Apoe*$^{-/-}$ mice treated with polyclonal IgM or polyclonal IgG develop less atherosclerosis than the controls [52-54]. Splenectomized *Apoe*$^{-/-}$ mice develop less atherosclerosis after receiving total splenic B cell extracts (B2 and B1), which contributed to the hypothesis that the spleen resident B cell pool confers protection against atherosclerosis [55].

μMT mice lacking the μ chain of the BCR have no B cells, and when their bone marrow is transplanted into *Ldlr-/-* mice this results in increased atherosclerosis [56]. On the contrary, ablation of follicular B2 cells but not B1 cells, via anti-CD20 therapy, reduces atherosclerosis which would indicate an opposite function [57]. Kyaw and colleagues also reported of an atherogenic role of B2 cells by using *Tnfrsf13c*$^{-/-}$ *Apoe*$^{-/-}$ mice and an antibody depletion of BAFFR strategy [58, 59]. However, soluble BAFF might be atheroprotective per se beyond its effects on B cells, and accumulates in the BAFFR deficient models, as recently reported by Tsiantoulas et al. In this study the hypothesis was that soluble BAFF signalling dampens the rise of inflammatory atherogenic M1 polarized macrophages [60].
As mentioned earlier natural IgM antibodies, mostly derived from B1 cells, have been reported to mediate atheroprotective roles, although the V heavy chain used in the prototypic T15 E06 natural antibody is not responsible for the atheroprotective effects of this antibody [61] but the functional role of other immunoglobulin classes, in particular IgG, still remains unclear [62].

1.2.1 B cell development

In the hematopoietic stem cell pool, Pax5 or B cell specific activator protein (BSAP), is a B cell lineage restricted transcription factor whose expression is detected starting from the pro-B cell stage until the mature B cell stage [63, 64].
Pax5 is one of the 9 members of the Pax5 family of transcriptional regulators, is considered a master regulator for commitment to the B cell fate [65] and its expression is down-regulated upon terminal differentiation to antibody secreting plasma cells [65, 66].

BLIMP1 is the B-lymphocyte induced maturation protein. It is encoded by the *Prdm1* gene and functions as the regulator of B and T cell terminal differentiation [67].
BLIMP1 acts as a transcriptional repressor by associating with deacetylases and histone-modifying enzymes with repressor activities [68-70] and its targets are, between others, Pax5, Bcl6, Myc [65, 66, 71-73]. In particular, BLIMP1 repression of Pax5 is required for the differentiation of plasma cells [66].
BLIMP1 is expressed in plasma cells produced upon T-I and (primary and secondary) T-D responses, and in long lived bone marrow plasma cells but not in memory B cells [74].
Bcl6 maintains repression of BLIMP1 in germinal center B cells to allow the GC reaction to continue [75].

AID is the B cell–specific activation-induced cytidine deaminase (AID) enzyme, and is required for both class switching and somatic hypermutation of the immunoglobulin genes occurring in the germinal center. Upon AID over-expression there is an increase in IgA production over IgM, whilst AID deficiency is associated with aberrant class switch and hyper IgM syndrome [76].
In class switched and hypermutated B cells, AID is then repressed by the activation of BLIMP1 which also inhibits the proliferative phenotype and drives the expression of X-box-binding protein 1 (Xbp-1), which in turn induces the immunoglobulin secreting pathway genes [77].

Genetic rearrangements of the immunoglobulin heavy (H) and light (L) chain loci known as V(D)J recombination are designed to assemble a functional BCR complex on the cell surface. At the common lymphoid progenitor (CLP) and pre-pro-B cell stage a D_H gene is recombined with a J_H gene segment by the RAG-1/RAG-2 recombination machinery. In the pro-B cell stage a V_H segment is coupled to the pre-formed D_HJ_H fragment to produce a rearranged $V_HD_HJ_H$ heavy chain gene, which also contains the IgM constant region in form of a μ polypeptide (Cμ). During the H chain recombination an enzyme called terminal deoxynucleotidyl transferase (TdT) includes *N additions* in the form of short nucleotide sequences at the V_HD_H and D_HJ_H junctions. These recombined transcripts then associate with surrogate light chains and with the Igα/Igβ dimer on the cell surface to form the pre-BCR complex. In the following pre-B cell stage the L chain is rearranged into a V_LJ_L segment similarly to the H chain by RAG-1/RAG-2, however no *N additions* are included. At the end of the pre-B stage, the newly produced L transcript is paired with the previously recombined μ H chain to form a surface bond IgM immunoglobulin. This IgM associates with the Igα/Igβ dimer to assemble a BCR complex. The maturation then continues with repertoire selection of the BCR pool. The final aim is to increase the affinity and specificity of the existing BCRs in order to neutralize the sample of autoreactive BCRs from the immature B cell compartment by employing one of the following mechanisms: editing, anergy or deletion. During this negative selection, cross-linked BCRs reactive against self are inactivated or removed. BCR editing is performed by the RAG-1/RAG-2 enzyme complex which introduces further genetic rearrangements into the L chains. Anergy is induced when the BCR is modestly engaged and in the absence of T-cell help, which turns the B cell into a non-responsive state in which there is no downstream signalling following the BCR cross-linking. Deletion is achieved via apoptosis, which is induced in two ways, via either the intrinsic or extrinsic pathway.

The surviving B cells eventually break away from the bone marrow stromal cells and move into the circulation to reach the spleen to complete the maturation. This stage is called transitional 1

(T1), characterized by expression of CD24 (HSA) and CD95. At first the T1 cell is still expressing surface bound IgM whilst the latter transitional 2 (T2) stage expresses increasing levels of IgD and decreasing IgM. The surviving T2 cells constitute the mature, circulating, naive B cell pool which migrates to the periphery into the secondary lymphoid organs. The viability of the transitional B cell pool is strongly dependent upon the survival factor BAFF/BAFF-Receptor signalling [10, 11].

1.2.2 B cell subsets

The origin of the B1 and B2 lineages is unclear. There are two hyphothesis for their development. According to the "dual lineage model", early on in the HSC compartment there is a differentiation into a B1-precursor and a B2-precursor. However in the alternative theory, the "activation model", a common B1/B2 precursor matures into the transitional stage: the BCR signalling will trigger either the B1 or B2 development.

1.2.2.1 B1 cells

Depending on the presence of the CD5 molecule on the cell surface, B1 cells can be further classified into B1a and B1b cells. It is unclear whether B1a and B1b cells develop from a common progenitor or they differentiated early on from two different hematopoietic precursors. B1 cells are derived from the fetal liver and the adult bone marrow, and the adult B1 cell compartment is able to maintain a homeostatic expansion called self-replenishment. After birth they migrate to populate the spleen and the serosal cavities of the body such as peritoneum and pleura. Upon activation they migrate into the spleen to produce most of the circulating natural antibodies, mainly of the IgM isotype. Natural antibody production by B1 cells begins already at one week of age and occurs in an innate, fast, T-independent manner. It confers humoral protection against bacterial pathogens. Consequently, natural antibodies show germline VDJ recombination, poly-reactivity and low affinity.

1.2.2.2 B2 cells

B2 cells are the conventional mature B cells found in the peripheral secondary lymphoid organs and constitute most of the B cells in spleen, lymphnodes, blood and the recirculating population

in bone marrow. During adult life, B2 cells are continuously replenished from bone marrow precursors. Within the B2 cell pool there are two subsets: the follicular B (FoB) and the marginal zone B (MZB) cells.

Follicular B cells are found in the lymphoid follicles in spleen and lymph nodes. Their life span is months, are resting at steady conditions and react usually within days after exposure to a T-dependent antigen. FoB can produce class switched, high affinity and highly specific antibodies in T-dependent responses. In fact, FoB that have undergone class-switch recombination (CSR) and somatic hypermutation (SHM) processes are responsible for secreting most of the circulating IgA, IgG and IgE antibodies.

The marginal zone B cell pool colonize the splenic marginal zone from the marginal sinus and patrol the interface between red and white pulp, therefore constituting a first line of defense against blood-borne TI (T-independent) antigens. They can, however, shift towards the follicle zone for epitope presentation to the T cells in the T zone. MZ B cells react to lipid antigens by presenting them on CD1d, have a low threshold for activation and mount a fast, innate-like, antibody response consisting mainly of IgM and IgG3 antibodies.

B cells migration from the bone marrow to the periphery is driven by chemokine and cytokine expression. Classic B cell chemokines are CXCR4, which binds to CXCL12, and CXCR5, which recognizes CXCL13. Naive B cells are attracted to the splenic red pulp, where CXCL12 is strongly induced, and are driven to the white pulp follicles via CXCL13 recruitment. Other immunologically active sites express different receptors for other B cell chemokines [78, 79].

1.2.2.3 Germinal centers

The goal of the germinal center reaction is to produce a significant amount of highly specific antibodies against the invading pathogen during the course of an infection or against neo-self, sterile epitopes, such as during atherosclerosis [17, 80].
The germinal center is the result of the clonal expansion of B cells consequent to a T-dependent immune response in a secondary lymphoid organ. The pathogen is first captured in the external area of the follicle called the T cell zone by a B cell or another antigen presenting cell (APC): then the microbe is engulfed, processed and presented in MHC II to a responding CD4+ naive T cell. If the peptide presented in MHC is recognized by the TCR this provides the so called signal

1, which must be also accompanied by a second co-stimulatory interaction, such as the CD40-CD40 ligand interaction, between many others.

According to this "two-signal" model of T cell activation, upon both positive signals the primed T cell migrates towards the B-T cell border to interact with the naive cognate B cell. If a TCR reacts with the MHC-epitope complex on the B cell, the T cell differentiates into a T follicular helper cell (Tfh). A Tfh population is usually noticeable from day 2 of the GC reaction and is identified by the expression of the surface markers CXCR5, PD1 and GL7 and production of IL-21 [17, 81].

The genetic rearrangements that can take place in the GC are the class switch recombination (CSR) and the somatic hypermutation (SHM). The GC is supplemented by the cytokine and chemokine milieu, which will trigger positive selection of the B cells whose V chains have gained the highest affinity after affinity maturation.

The initial expansion that occurs within the early GC is not of a mono-clonal kind, in fact more than a single clone is found, that originate from non related B progenitors [64, 70, 76].

Within the developing GC two structurally different zones can be identified: the dark and the light zone. GC B cells migrate rapidly between the two zones in an iterative manner many times during the GC activity. The dark zone of the GC is composed by rapidly dividing CXCR4hi CD83lo CD86lo centroblasts undergoing SHM of their immunoglobulin genes. When they express the rearranged BCR with the highest affinity they shuttle towards the light zone of the GC as CXCR4lo CD83hi CD86hi centrocytes, where antigen-driven selection takes place upon interaction with the follicular dendritic cell pool [17, 68]. Both GC B cells and Tfh cells can migrate not only between the different zones of the GC but also between different GCs.

Two responses rise from the germinal center: a plasma cell and a memory reaction, and both will migrate into the blood as the GC reaction extinguishes [69].

The post-GC plasma cells are the immediate result of the GC reaction. They secrete a high amount of recombined and mutated immunoglobulins, mainly IgG but IgA and IgE as well.

The post-GC memory cells are long lived (years), resting B cells that survive latent in bone marrow niches. This compartment can be readily activated upon a second encounter with the same pathogen and will begin a specific, high affinity humoral defense.

Memory B cells are not only produced by GCs, in fact they can be generated either by T-independent or T-dependent B-cell responses [101]. Memory B cells deriving from T-independent or extra-follicular T-dependent activation are generally short lived, although this notion has been challenged [102,103]. On the other hand, memory B cells derived from T-

dependent reactions (either from GCs or extra-follicular clusters) can live for months in mice and for years or decades in humans.

1.2.3 Effector functions of B cells and antibodies

As antibody producing cells, B lymphocytes are key elements of the immune system in physiologic defense mechanisms against invading antigens and non physiologic deleterious responses towards self and modified self components via autoantibody production. But their function is not limited to antibody related effects, in fact B cells can also participate in autoimmune diseases through cytokine production, which can be beneficial or deleterious [82].
B cell derived IL-10 and IL-35 promoted recovery in EAE models [32, 83] and production of IL-21 by B cells can drive B regulatory cells differentiation which reversed established EAE [33].
IL-6 producing B cells worsen disease progression of EAE models and at least partly drive disease in MS patients [84]. Lymphotoxin, granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor TNF-α are also reportedly increased in MS patients [85, 86].
B cell production of IFN-γ promotes autoimmune experimental arthritis possibly via differentiation and accumulation of Tfh cells [62]. Class-switched activated memory B cells found in synovial fluid of RA patients express RANKL, a cytokine driving osteoclast differentiation and function, which can exacerbate bone loss in the inflamed joint [87].
B1a derived IRA B cells produce GM-CSF in sepsis models and aggravate atherosclerosis in irradiated *Ldlr*$^{-/-}$ mice following reconstitution with *Csf2*$^{-/-}$ bone marrow [88, 89].

Immunoglobulins exhert their protective functions in order to inhibit a pathogen infection or its spreading via different mechanisms such as opsonization, complement activation, phagocytosis, antibody-dependent cellular cytotoxicity (ADCC). It is possible that several of these mechanisms occur in parallel or sequentially. Opsonization is the process by which antibodies recognize and coat the surface of the pathogen. An opsonized microorganism or infected cell is the classic example of clearance via phagocytosis, being the target of Fc receptor-bearing phagocytes. Neutralization can occur in different ways. In one, immunoglobulins neutralize the pathogen by inhibiting the attachment to the host tissues via a process called agglutination, mostly known as dependent on the polymeric IgM and IgA antibodies, by which the pathogen is trapped in the mucous surfaces. Another neutralization strategy is targeting immunoglobulins against the adhesins on pili or fimbriae of the microbe which are normally used to phisically attach to the

host cells. Neutralization can also take place at later stages during the infection, intracellularly, when antibodies interfere with the pathogen`s replication steps in the infected cell.

Furthermore immunoglobulins, both natural antibodies and IgG, can bind and activate the complement, through which the infected cell or pathogen is cleared by phagocytes or removed via cell lysis.

The biological function of antibodies is the response generated by the interaction of the Fc fragment with an Fc receptor expressed on the surface of the target cell. The nature of such response depends on a number of variables which are, among others, the identity of the target cell, of the immunoglobulin (or immune-complex), and the cytokine environment.

Antibody-dependent cytotoxicity occurs when an antibody bound to either a pathogen or an infected cell also engages an interaction with an Fc receptor on the effector cell, which eventually leads to lysis or apoptosis of the target [90].

As a final note on the activity of antibodies, a recent study in the context of atherosclerosis illustrates the generation of an *Ldlr*$^{-/-}$ *E06-scFv*$^{-/-}$ murine model deficient for the effector function of the stereotypical E06 natural antibody. *E06-scFv*$^{-/-}$ is a single chain variable fragment of E06 lacking the Fc fragment, therefore the E06-scFv antibody can not mediate Fc-dependent functions and is only able to bind oxidized phospholipids. This clearance of oxidized phospholipids prevents macrophage uptake and activation, which results in reduced inflammation and atherosclerosis [91].

1.3 Smooth muscle cells

In healthy arteries smooth muscle cells compose the media layer of the vessel wall and control the tone and diameter of the vessel by using their repertoire of contractile proteins. At a steady state SMCs proliferate slowly with low extracellular matrix secreting activity. However, it has been proposed that in the atherosclerotic vessel the smooth muscle cells may migrate from the arterial media to the sub-intimal space. Here SMCs undergo phenotypic changes that promote their proliferation and deposition of extracellular matrix, collagen and elastic fibers, which eventually form the fibrotic cap that contains the atherosclerotic plaque. Some reports have introduced the term "SMC transdifferentiation" to describe this process [92, 93]. Detailed analysis of the origin of intra-plaque and fibrous cap SMCs revealed how, in early atherosclerosis, few medial SMCs might migrate across the shoulder region and clonally proliferate into the lesion. The expansion of few selected clones could give rise to a variety of phenotypes, with lipid-loaded SMCs found in the body of the plaque and a layered structure in the SMCs forming the fibrous cap [94].

There is no unique lineage transcription factor for the identification of mature differentiated SMCs but rather a series of them, which are also expressed by other cell types, at certain developmental stages or in not healthy conditions. Such markers are smooth muscle actin alpha (α-SMA) [95], SM22 [96], smooth muscle myosin heavy chain (SMHC) [97] , h1-calponin [96] and smoothelin [78].

Moreover, a number of reports have shown that SMCs can start expressing myeloid markers such as CD68 [79] whilst other studies reported the presence of α-SMA positive cells of myeloid origin extracted from the atherosclerotic plaque [98] and hematopoietic stem cells can originate vascular SMCs in vivo and in vitro [99].

In conclusion, it is still not possible to unequivocally identify and track SMCs by using a lineage marker or the classical α-SMA marker, and this is therefore the major limitation in understanding the phenotype switch and migration activity of SMCs in atherosclerosis.

2 AIMS

The aim of my PhD is to discover the functions of B cell subsets and antibodies in atherosclerosis, in particular by elucidating:

I. The function of V1 heavy chain encoded innate-like natural antibodies in atherosclerosis (*paper I*).
II. The adaptive immune response to acute hypercholesterolemia in an inducible model of atherosclerosis (*paper II*).
III. The role of germinal center derived immunoglobulins in atherosclerotic plaque size and stability (*paper III*).

3 METHODOLOGICAL CONSIDERATIONS

3.1 Mouse models

The mouse models presented in this thesis were crossed into the *Apoe*$^{-/-}$background in order to develop spontaneously atherosclerosis or on the *Ldlr*$^{-/-}$ strain, which is susceptible to lesion formation upon western diet. In order to deplete plasma cell formation we used the *Blimp1*^{Flox} *CD19*^{Cre} *Apoe*$^{-/-}$ model, where plasma cell depletion is conditionally achieved in a B cell restricted way. The *Pax5*^{Flox} *CD23*^{Cre} *Apoe*$^{-/-}$ model has a follicular and marginal zone B cell defect. To achieve a germinal center B cell specific depletion we crossed the *Pax5*^{Flox} *Aicda*^{Cre} *Apoe*$^{-/-}$ mouse. Furthermore, we created a novel inducible atherosclerotic *Apoe*$^{-/-}$ mouse, the *Rosacre26ERT2 Apoe*^{Flox} which is a model of acute inducible hypercholesterolemia and atherosclerosis. Finally, we assessed the role of the $V_HS107.1.42$ heavy chain gene in atherosclerosis development in both the *VhS107.1.42*$^{-/-}$*Apoe*$^{-/-}$ and *VhS107.1.42*$^{-/-}$ *Ldlr*$^{-/-}$.

3.2 Experimental methodology

A single dose of 9 mg tamoxifen was administered by oral gavage and, in order to monitor in time the rise in hypercholesterolemia, the mice were routinely bled several days after tamoxifen administration.

For the atherosclerotic plaque stability studies we performed a plaque rupture model [100]. 10 to 12 week-old mice were maintained on a normal chow diet during the course of the study. We performed incomplete ligation of the right common carotid artery below the bifurcation. In the following four weeks after surgery the arterial intima remodeling processes formed a neointima and a carotid atherosclerotic plaque. To induce plaque rupture we placed a cuff around the common carotid artery, next to the ligation, to induce collagen degradation. The mice were sacrificed 4 days after cuff placement and both right, injured, and control left carotid arteries were analyzed. A ruptured plaque was identified by the presence of a thrombus in the carotid lumen which is microscopically confirmed with a positive fibrin staining.

For the carotid artery ligation model, **s**imilarly to the plaque stability model, we performed right carotid artery complete ligation at the bifurcation on 10 to 12 week-old mice on chow diet, and 28 days later we sacrificed the animals and the carotid arteries were processed for thrombus evaluation.

Bone marrow was harvested from tibia and femur of donor mice. Each recipient mouse received Gy irradiation. Following irradiation they received the donor bone marrow by tail vein injection. Recipient mice were then maintained in a pathogen-free facility for approximately a 3 months recovery time.

Aortic smooth muscle cells from 6 to 8 week old *Apoe*$^{-/-}$ mice were isolated from the aorta after removal of the adventitia. The media was dissociated to a single cell suspension via enzymatic digestion with collagenase and elastase, then SMCs were cultured and expanded. For experiments, fetal calf serum was replaced in the culture media with 2% serum either from control or experimental mice. Cell proliferation was calculated as average confluence/area using live cell imaging.

Splenectomy was performed on 10 weeks old mice. The animals were anaesthetized and the spleen was extracted from a 1cm incision made on the left side of the abdomen. After, the vessels and nerves were slowly cauterized, the peritoneum and skin were sutured. For sham operation the mice were surgically cut as the splenectomized but re-sutured thereafter.

4 RESULTS AND DISCUSSION

4.1 V_HS107.1.42 does not protect against atherosclerosis

Germline encoded natural IgM antibodies are implicated in the recognition and clearance of oxLDL, processes associated with protection against atherosclerosis. At the same time natural antibodies also confer protection against *Streptococcus pneumoniae* infection. The archetypal natural antibody T15 or E06 recognises phosphatidylcholine in the bacterial cell wall or in the POVPC headgroup exposed on oxidized LDL or in the apoptotic blebs found on the membranes of dying cells [101]. T15/E06 is encoded by the *V_HS107.1.42* immunoglobulin heavy chain gene which has not been directly linked to protection against atherosclerosis, therefore in **paper I** we crossed *V_HS107.1.42*-deficient mice with the atherosclerosis-prone *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ strains. These mice lost early immune responses against phosphocholine after immunization, confirming the specificity of V_HS107.1.42 towards PC, but the T15 titers as well as other antibodies reacting to oxidized LDL were unaffected. Assessment of atherosclerosis development showed no protection. In summary, we concluded that natural IgM antibodies using the *V_HS107.1.42* chain are not likely involved in conferring protection against atherosclerosis, despite driving the humoral response to PC and protecting against *S. pneumoniae* infection [102] (Figure 1).

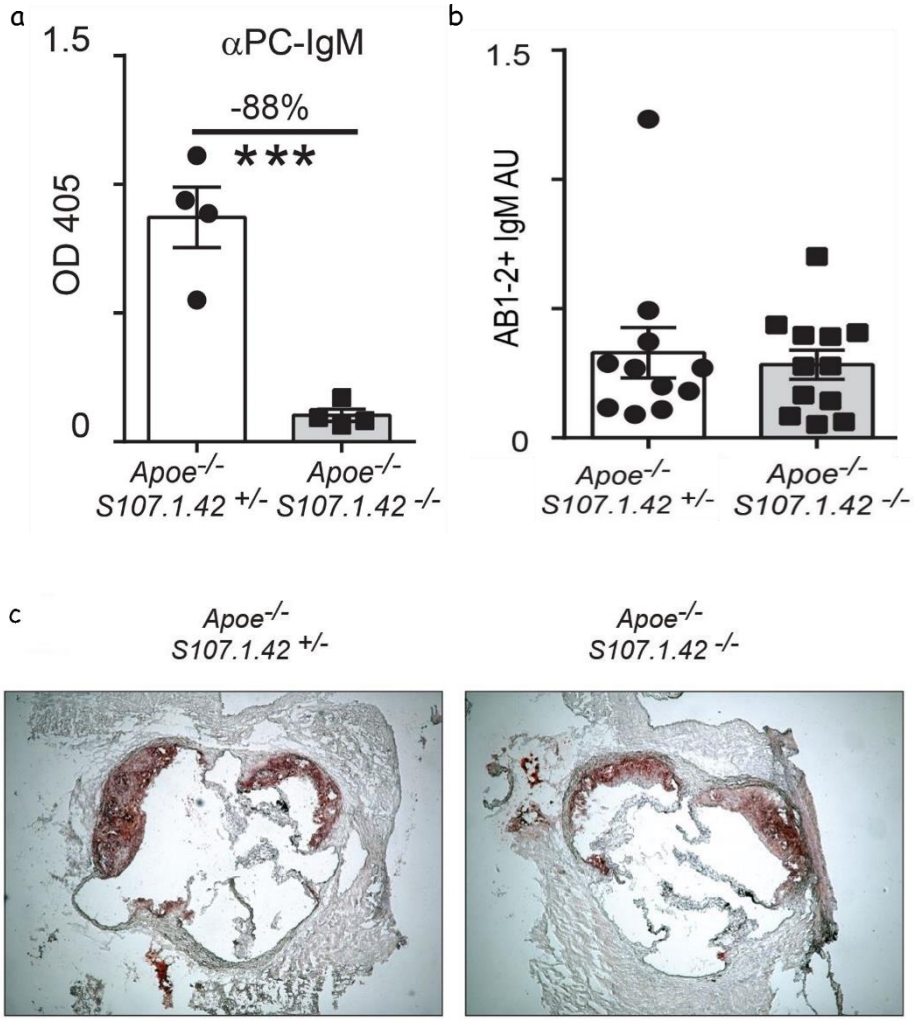


Figure 1

A. Plasma anti-PC IgM antibody titers in the response to PC-KLH immunization in *V_HS107.1.42$^{-/-}$ Apoe$^{-/-}$* mice.

B. T15 IgM antibody measured in plasma of *V_HS107.1.42$^{-/-}$ Apoe$^{-/-}$* mice.

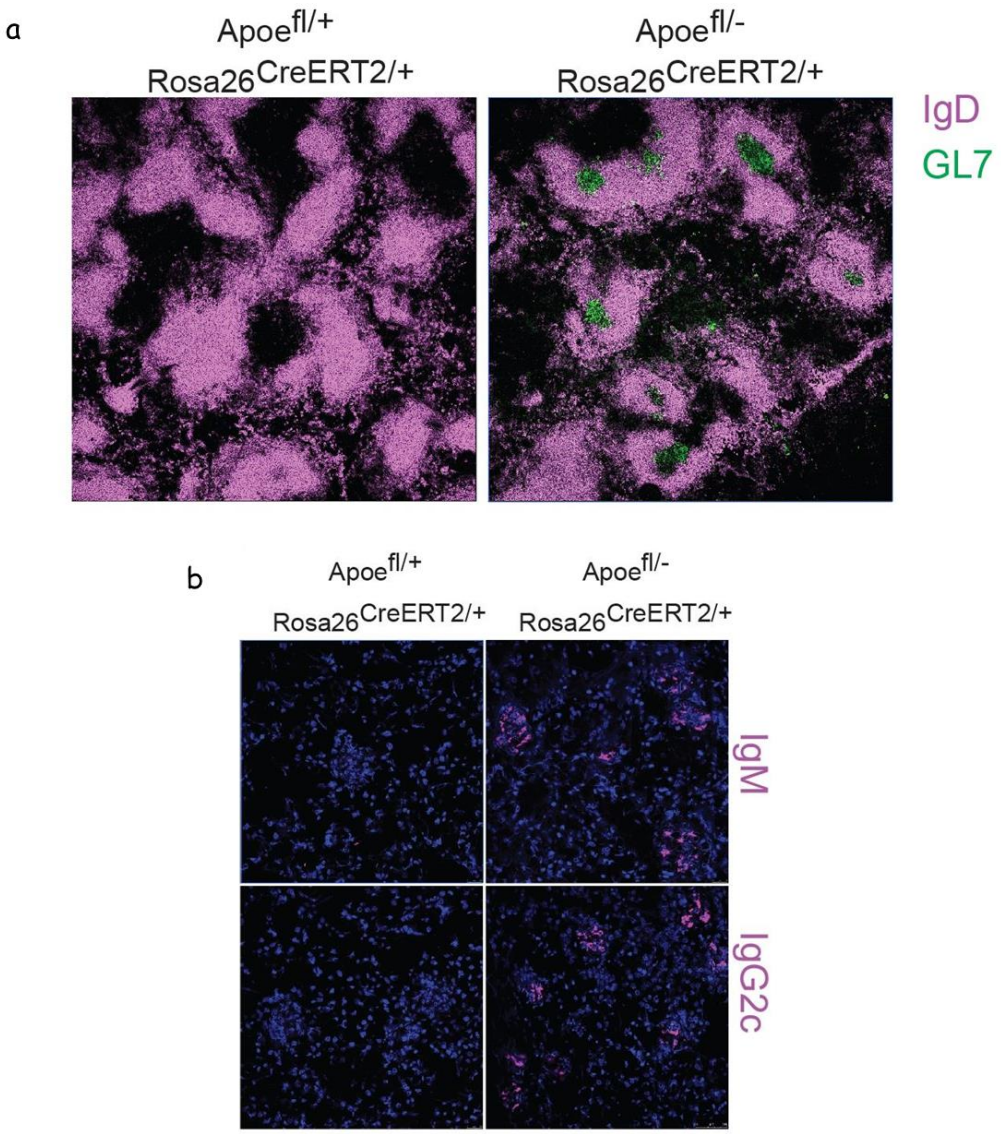
C. Development of atherosclerosis in the aortic root of *Apoe$^{-/-}$* mice deficient for the V_HS107.1.42 antibody.

4.2 Autoimmune-like response to acute hypercholesterolemia

Hypercholesterolemia is associated with vascular inflammation and hence susceptibility to atherosclerosis. However, the causality between hyperlipidemia, inflammation and early atherosclerosis is not yet clear.

Therefore, in **paper II** we hypothesized that a rapid conversion to an Apolipoprotein E deficient state would reveal whether the immune system also responds during the transition to hypercholesterolemia.

We created a tamoxifen inducible *Apoe*$^{-/-}$ mouse, in which loss of APOE induced in the adult mouse resulted in rapid and maintained hypercholesterolemia and subsequent spontaneous atherosclerosis. This transition to hypercholesterolemia induced local adaptive immune responses in the spleen. We detected two waves of germinal centers formation, first at the transition to hypercholesterolemia which occurred with Th1 and Tregs activation and preceded the appearance of atherosclerosis. At this early time point there was also a systemic autoimmune reaction to hypercholesterolemia, with immune complexes deposition and plasma autoantibodies production. The second wave of germinal centers was detected in aged mice in which atherosclerosis was established and resembled the chronic late stage inflammatory disease. Therefore, we concluded that germinal centers are an indicator of early atherosclerotic plaque formation and acute hypercholesterolemia induces an autoimmune response (Figure 2).


a
Apoe^fl/+
Rosa26^CreERT2/+
Apoe^fl/-
Rosa26^CreERT2/+
IgD
GL7
b
Apoe^fl/+
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Apoe^fl/-
Rosa26^CreERT2/+
IgM
IgG2c
c
Pax5^fl/+ Aicda-Cre Apoe^-/- →
Apoe^fl/- Rosa26^CreERT2/+
Pax5^fl/- Aicda-Cre Apoe^-/- →
Apoe^fl/- Rosa26^CreERT2/+

Figure 2

A. Germinal centers development upon transition to hypercholesterolemia (tamoxifen induction) in the spleen of inducible *Apoe* mice.

B. Immune complexes deposition in the kidney after transition to hypercholesterolemia in inducible *Apoe* mice.

C. Atherosclerosis development in the aortic root of inducible *Apoe* recipient mice transplanted with bone marrow from germinal center deficient mice.

4.3 Germinal centers drive large and stable atherosclerotic plaque

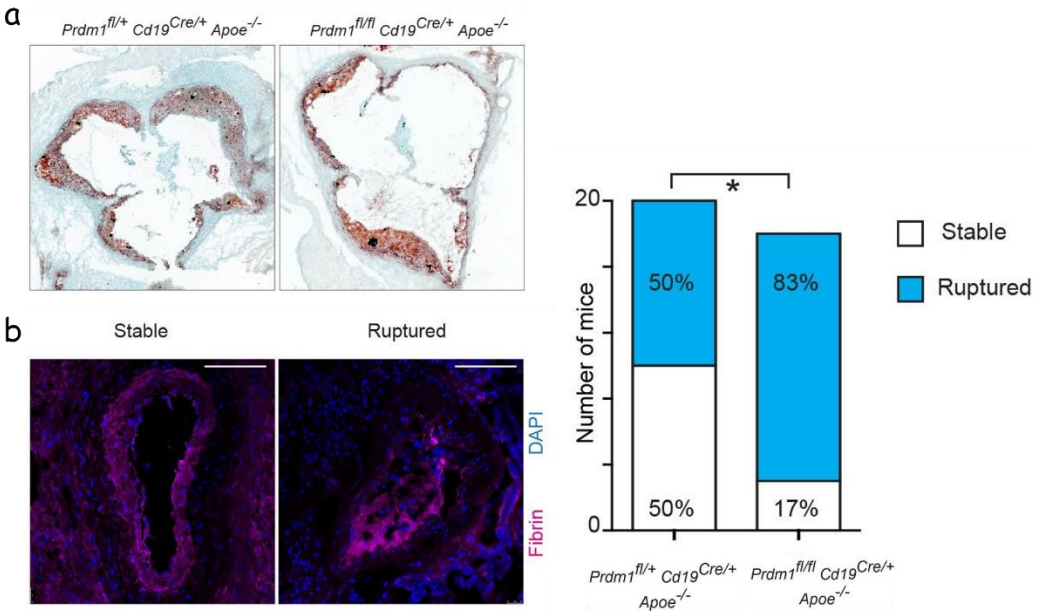
We then asked, how does the loss of immunoglobulins impact upon atherosclerosis burden, without affecting T dependent and independent immune responses nor the function and viability of B cells (such as antigen presentation and cytokine production)?

To do so, in **paper III** we specifically ablated antibody producing plasma cells. Deletion of the *Prdm1* gene results in B cells that are functionally normal but are largely unable to secrete immunoglobulins. Next, we crossed this mouse with a Cre transgenic line. $Cd19^{Cre/+}$ deletes in all mature B cells and hence prevents antibody production. This mouse was crossed into the $Apoe^{-/-}$ background to develop spontaneous atherosclerosis. Therefore, we created a hypercholesterolemic atherosclerotic mouse model unable to mount humoral responses. Interestingly, mice lacking immunoglobulins develop less atherosclerosis than their littermate controls. We could visualize a qualitative difference in the phenotype of the lesions between control and experimental mice, with the latter being richer in lipid-loaded foam cells and with a lower content of smooth muscle actin positive cells, compared with control plaques. Reduced smooth muscle actin and lipid enriched plaques are associated with an unstable plaque phenotype, therefore we assessed the stability of atherosclerotic lesions by using a carotid ligation with cuff placement model [100] to mechanically induce the rupture of the plaque. Our experimental mouse showed a higher rupture rate compared with controls.

We hypothesized that SMCs are affected by the absence of antibodies: we observed that, *in vitro*, $Apoe^{-/-}$ primary aortic vascular SMCs cultured in medium containing plasma from our control mouse proliferate more than those growing in medium containing experimental mouse plasma. Hence, we hypothesized that plasma antibodies act in a FcγR-dependent manner which ultimately can mediate the NF-kB pro-inflammatory pathway [103]. Next, we wanted to investigate what immunoglobulin species is responsible for the smooth muscle cell phenotype we observed. We assessed the growth of $Apoe^{-/-}$ aortic vascular SMCs in the presence or

absence of plasma containing IgG-derived germinal center derived antibodies. We observed that the cells grew significantly faster in presence of IgG antibodies. We were also able to translate this observation *in vivo*, by reconstituting our experimental mouse with purified IgG to the same level as that observed in controls. We observed that IgG reconstitution was able to `rescue` lesion size and that neointima formed upon carotid ligation was also similar to the control group. We then investigated the origin of the IgG antibodies *in vivo* and we used a novel mouse model to assess the role of germinal center-derived IgG antibodies in atherosclerosis. We developed a mouse strain that conditionally ablate the transcription factor *Pax5* using a stage specific *Cre* line in B cells. Pax5 performs an essential function in maintaining B cell identity, and is currently the only known B cell specific factor that is constantly required for correct B cell function throughout all developmental stages [48].

In this model we deleted *Pax5* using $Aicda^{Cre}$. AICDA is specific to the germinal center B cells and loss of Pax5 within this structure results in the loss of germinal centers. Consequently, in this model, somatic hypermutation, memory B cell formation and affinity maturation are lost. We therefore crossed the $Apoe^{-/-}$ mouse with the $Pax5^{f/f}$ $Aicda^{Cre}$ mouse. Examination of atherosclerosis development revealed that loss of the systemic GC formation and GC derived antibodies decreased atherosclerosis burden. Furthermore, loss of germinal center antibodies in the plasma from this mouse can not promote *in vitro* vascular SMCs proliferation suggesting that these antibodies might be responsible for the unstable plaque phenotype. To test this hypothesis we inhibited the germinal center reaction via anti-ICOS ligand therapy in an inducible *Apoe* model and evaluated plaque stability: the loss of germinal center antibodies resulted in unstable atherosclerotic plaques. To understand the effect of antibodies on SMCs we analyzed the transcriptome of the aortic media in our antibody deficient model by mRNA sequencing. Intriguingly, upon loss of antibodies, genes involved in the coagulation, iron metabolism, lipid metabolism and inflammatory pathways were upregulated. Genes involved in viability and function of SMCs were downregulated in the antibody deficient mouse, possibly linking transcriptome to the unstable atherosclerotic plaque phenotype. In summary, in paper III we demonstrated that germinal center derived IgG antibodies affect the composition of the atherosclerotic plaque, increase lesion size and promote plaque stabilization via maintaining smooth muscle cells transcriptome which results in a more stable aorta (Figure 3).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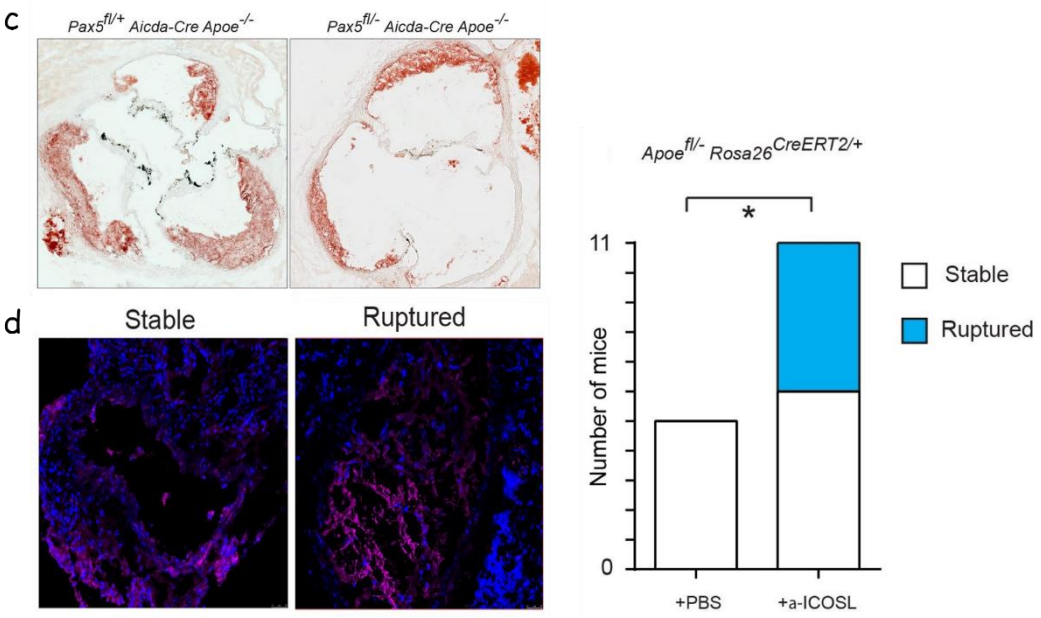
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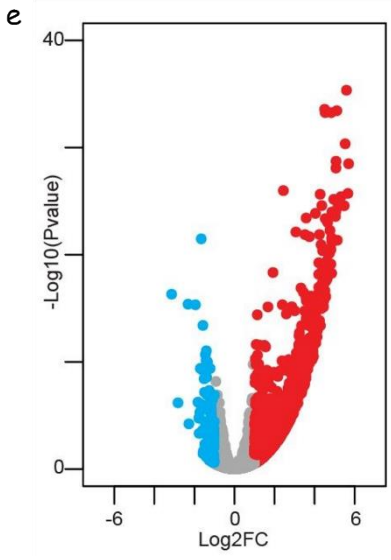
e
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-Log10(Pvalue)
-6
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6
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Figure 3

A. Atherosclerosis development in the aortic root of antibody deficient mice.

B. Immunofluorescence staining for fibrin to detect thrombus formation in the lumen on carotid artery after carotid ligation and cuff protocol, on antibody deficient mice. Quantification of rupture events is shown in the right panel.

C. Atherosclerosis development in the aortic root of germinal center deficient mice.

D. Immunofluorescence staining for fibrin to detect thrombus formation in the lumen on carotid artery after carotid ligation and cuff protocol, on germinal center deficient mice. Quantification of rupture events is shown in the right panel.

E. Volcano plot of RNA sequencing data from aorta of antibody deficient mice.

4.4 Concluding remarks

Immunization experiments against oxLDL conferred protection from atherosclerosis due to a humoral response which was directed towards oxidation epitopes on the surface of modified circulating lipoproteins. The germline, unswitched IgM with low specificity and broad spectrum of affinities originate from B1 B cells, which are considered the protective branch of the B cell lineage. In fact, splenectomy experiments resulted in atheroprotection which was accredited to the loss of protective antibodies. The stereotypical natural antibody is the T15 E06 clone, which is constructed from the *V_HS107.1.42* heavy chain gene. This chain was therefore indirectly linked to atheroprotection. We demonstrated, in **paper I,** that such gene is not able to confer protection against atherosclerosis, and probably the T15-id+ positive IgM can be made from alternative immunoglobulin heavy chains, which are however unable to protect against *Streptococcus* infection or provide primary anti-PC responses.

Chronic hypercholesterolemia in atherosclerosis triggers vascular inflammation and contributes to disease progression, but little is known about the effects of acute hyperlipidemia. Therefore, we created an inducible hypercholesterolemic *Apoe* model which we described in **paper II**. Interestingly, we observed that the transition to acute hypercholesterolemia associated with an induced *Apoe* null state triggers splenic activation in terms of GC, Tregs and Tfh expansion and an autoimmune response with systemic autoantibody production and immune-complexes deposition in the kidneys. This autoimmune reaction to acute hypercholesterolemia was dependent on germinal center B cells.

Germinal centers rise from the follicular B2 milieu upon sterile and non-sterile stimuli, such as autoimmune inflammation and pathogen infections, respectively. B2 cells contain the mature follicular and marginal zone B milieu, which includes the germinal centers. In atherosclerosis,

opposite to the role of B1 cells, the more abundant B2 cell pool has a more unclear effect in experimental atherosclerosis, with a possible pathogenic action. The role of germinal center B cells in atherosclerosis has never been directly assessed. For the first time to our knowledge, we demonstrated in **paper III** that germinal center derived antibodies affect lesion content, promote increased atherosclerosis lesion size, but these plaques have a `stable` phenotype.

Such observation raises the debate lesion size *versus* plaque stability in cardiovascular disease. Of note, the carotid ligation with cuff model is an artificial setup. It mimics an acute event, and it is a model for neointima formation and acute athero-thrombosis rather than literally atherosclerotic plaque rupture. From a translational point of view, the vulnerable murine atherosclerotic plaque resembles human late stage lesions for the presence of fibrotic cap, necrotic core and intraplaque hemorrhage. But one limiting factor is that spontaneous rupture events in murine models are hardly ever detected [104]. On a similar note, there is still margin for improvement also of evaluation and quantification strategies of atherosclerosis burden.

It could be of interest to establish if the immune effects we described are directly acting on the plaque itself and to determine the clonal specificity of such germinal center antibodies involved in the stabilization or growth of the lesion. Moreover, a regression study focused on germinal center derived antibodies could shed light on the effects of the adaptive antibodies in atherosclerosis presented in my thesis.
As a final note, in summary this thesis provides the rationale for the germinal center humoral response in promoting initiation of atherosclerosis and progression of lesion size via maintaining transcriptional integrity of the aortic wall (Figure 4).

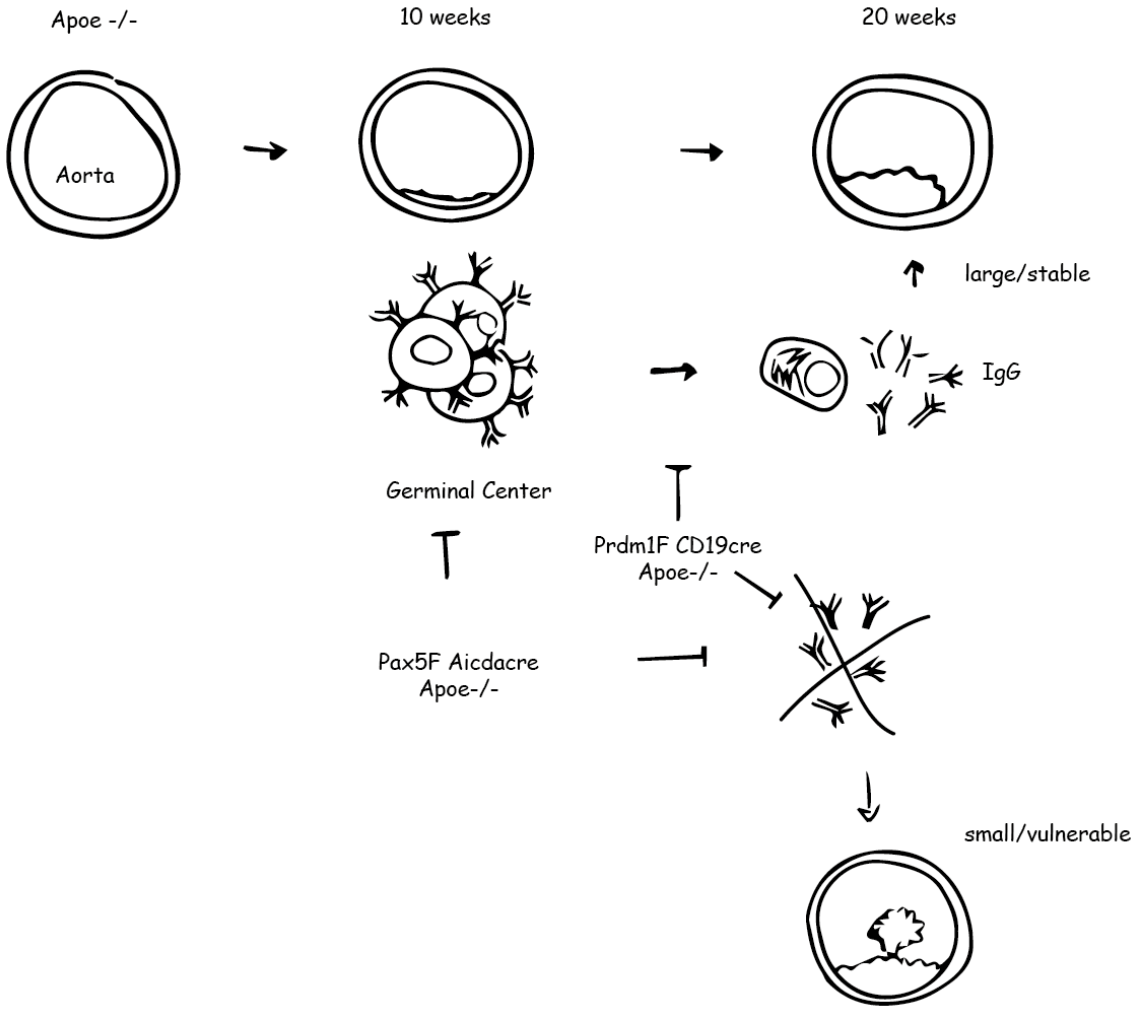

Figure 4

Atherosclerosis formation begins already at 10 weeks of age with the appearance of fatty streaks in the *Apoe*$^{-/-}$ mouse on conventional diet. By the age of 20 weeks, the lesions have grown in size, contain an inflamed necrotic core and a fibrotic cap produced by smooth muscle cells that migrated from the media to the sub-intimal space. Antibodies derived from germinal centers affect the composition and phenotype of atherosclerotic plaques. In particular, these antibodies affect the smooth muscle cell population in the atherosclerotic wall by promoting specific transcriptional pathways. Therefore, in the absence of germinal center-derived antibodies the lesions are smaller in size and possibly contain less smooth muscle cells. Consequently, the lesions have a thinner fibrotic cap which makes them be more susceptible to rupture events.

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Mamma e papi, siete la mia ispirazione, senza l'esempio e la vostra guida niente di tutto questo sarebbe successo.

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