Chapter 1

General intoduction

Introduction

Atherosclerotic cardiovascular disease

Cardiovascular diseases are the leading cause of death worldwide responsible for an estimated 16.7 million deaths annually which can be primarily attributed to atherosclerosis, a chronic inflammatory disease of the large and medium-sized arteries[1]. Atherosclerosis lies at the basis of coronary heart disease (CHD) and cerebrovascular pathologies, which both do not become manifest until the development of an acute thrombotic vascular occlusion. In the United States alone, atherosclerosis is responsible for 610.000 new and 325.000 recurrent cases of myocardial infarction every year[2]. Fortunately, the mortality of CHD has been steadily declining in the past 20 years, by almost 33% in the Netherlands alone due to improved primary prevention and treatment strategies[3]. Nevertheless, current incidence rates continue to stress the importance of ongoing research into the improvement of prevention and treatment of these atherosclerosis-related pathologies.

An atherosclerotic plaque is comprised of a subendothelial accumulation of lipids and infiltrated leukocytes such as monocyte-derived macrophages, mast cells and B and T lymphocytes, covered by a fibrous cap build up from smooth muscle cells (SMC) and extracellular matrix (ECM) deposition. Lesion formation is initiated by a qualitative change in the endothelial monolayer by irritating stimuli such as dyslipidaemia, hypertension, and pro-inflammatory mediators that lead to the exposure of adhesion molecules[4]. Local leukocyte adhesion and infiltration follows with continuing cellular activation and pro-inflammatory mediator and enzyme production, leading to a localized chronic inflammatory state supported by continuing cholesterol accumulation. Over time, apoptosis and necrosis occur which lead to the formation of a necrotic core containing cellular debris and free cholesterol crystals. Coronary plaque progression causes luminal stenosis and ischemia in distal tissues, evoking clinical symptoms of angina pectoris. Eventual plaque rupture and endothelial erosion can trigger local arterial thrombosis due to the exposure of underlying thrombogenic material to the circulation causing platelet aggregation and humoral coagulation, resulting in occlusive thrombus formation[5]. Such atherothrombosis events elicit brain or myocardial infarction as life-threatening complications, often requiring lesion revascularization through angioplasty, bypass-grafting or thrombolysis.

Target lesion revascularization and post-interventional vascular remodeling

Revascularization strategies are comprised of percutaneous coronary intervention (PCI), coronary artery bypass-grafting (CABG) surgery and thrombolysis. PCI is primary choice of revascularization strategy in patients presenting with objective myocardial ischemia due to occlusive CHD, diagnosed when presented with two out of the following three markers: clinical symptomatic presentation, typical changes on electrocardiography (ECG) and of circulating cardiac markers[6].

Transluminal angioplasty was invented by the American Charles Dotter in 1964, who applied this technique to re-open blocked arteries in the lower extremities of patients and improve blood flow. PCI for the treatment of obstructive coronary artery disease was first performed by the cardiologist Andreas Guenztig and colleagues in Zurich in 1977, who used Dotter catheters modified by adding an inflatable balloon to allow dilation of occluded coronaries[7]. Despite initial benefits, renewed obstruction termed

'restenosis' developed in 30-60% of all cases due to elastic recoil and negative remodeling of the injured artery[8]. Restenosis following angioplasty has been the major problem limiting the success rate of coronary interventions and tremendous efforts have been made to target this problem[9].
The introduction of bare-metal stents (BMS), which are deployed in the lesion segment during balloon inflation, prevented elastic recoil and have reduced the incidence of restenosis to 16-44%[10], but also led to the development of neointimal hyperplasia and in-stent restenosis (ISR) (figure 1).

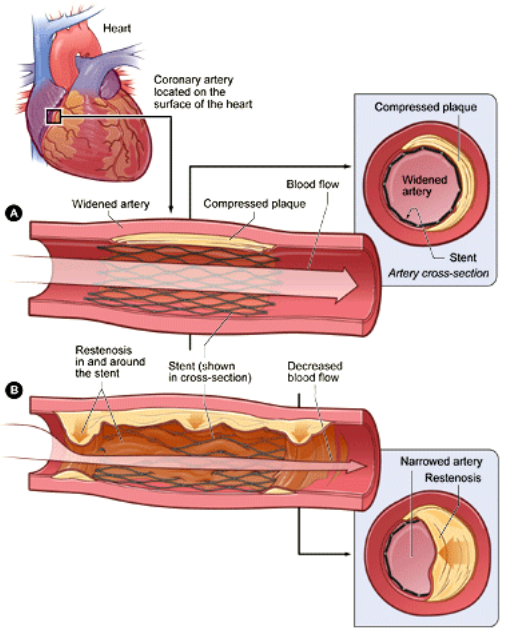

Figure 1.1 In-stent restenosis development
Stent expansion during therapeutic angioplasty compresses the atherosclerotic plaque into the arterial wall, re-esthablishing blood flow (panel A). The inset displays a cross-section of the compressed plaque and stent-widened artery. Over time, inflammation and accelerated atherosclerosis support fibrous tissue deposition, SMC migration and proliferation and foam cell formation. This can cause renewed (partial) blockage of the artery and distal ischemia (panel B). The inset shows a cross-section of the tissue growth around the stent.

This results from SMC migration and proliferation at the site of injury and extracellular matrix (ECM) formation by these cells. BMS struts serve as scaffolds to keep injured intimal and medial flaps from protruding into the lumen. Drug-eluting stents (DES) and drug-coated balloons have been developed to counter this phenomenon[11]. DES struts are coated with polymers which can release anti-proliferative drugs such as sirolimus and paclitaxel to prevent SMC proliferation. These significantly reduced ISR incidence, although rates of 5-10% are still reported, responsible for over 200.000 revascularizations annually in the United States alone[12]. Advantages of drug-coated balloons over stents are high drug concentration delivery per square millimeter of balloon surface and application possibilities in locations anatomically unfavorable for stent placement, such as bifurcations, small vessels and coronary

ostia. However, the lack of continued presence of both drug and polymer is the most important difference with stents. They reduce the chance of local hypersensitivity reactions, but also run the risk of an insufficient sustained period of drug-release to be fully effective[11].

Primary PCI has been shown to be clinically superior to thrombolysis with less major adverse events and with increased preservation of myocardium, particularly when applied in the period 3-12h after onset of symptoms. Thrombolysis however remains a viable alternative to primary PCI if it can be delivered within 3h after symptom onset[6].

PCI is less invasive and generally preferable above CABG surgery except in cases such as chronic total occlusion, three-vessel or left main stem disease with distal vessel disease or ostial stenosis, provided patients are presented in a hospital with PCI facility and an experienced team. PCI only treats a spot, whereas CABG surgery into the distal third or the artery treats the entire vessel[13]. Nevertheless, PCI and CABG surgery both provide good symptom relief and clinical trials have so far been unable to show a significant difference in mortality 1-8 years after revascularization. The original trend favoring CABG disappeared, despite a reduction in mortality, due to the introduction of stenting to PCI. Recommendation of either PCI or CABG surgery will be guided by technical improvements in cardiology or surgery, hospital expertise and patients' preference[6].

Restenosis risk factors

The underlying causes of post-PCI restenosis can be divided into four general categories, namely biological, arterial, stent and implantation factors[14, 15]. Biological factors are comprised of the natural vascular wall resistance to antiproliferative drugs, the development of a sustained hypersensitivity reaction directed towards the polymer or metallic stent platform and local concentration of proteinases that stimulate SMC proliferation and migration. Arterial factors that can influence restenosis development are unfavorable (low) coronary artery wall shear stress levels, the progression of primary atherosclerotic lesion growth within a segment treated with angioplasty, as well as previous positive vascular remodeling[15]. Stent factors that determine ISR risk are the antiproliferative drug concentration and duration of sustained drug release. The stent gap, strut thickness and polymer disruptions, cracking and fractures are all major risk factors for ISR[10]. Above all, carefully-conducted technical implantation is highly important for adequate therapeutic effectiveness and factors such as barotrauma, inadequate stent expansion and geographical misses, where the stent is deployed proximally or distally from the lesion and deployment of a DES in a clot-laden arterial segment are all factors that contribute to ISR risk[10, 14].

Animal models for restenosis

Over the past decade, positive results have been obtained with sirolimus and paclitaxel-eluting stents (SES and PES respectively), although studies investigating other anti-restenotic drug were unable to provide similar results. Animal models that mimic the pathophysiology of post-interventional vascular remodeling allow for testing of new potential anti-restenotic drugs and evaluation of their therapeutic effectiveness, as well as effects of arterial wall integrity, atherosclerotic lesion initiation and progression and plaque stability[16]. Genetically-modified mouse strains allow for

screening of genes of interest concerning their role in lesion development, even in a dyslipidemic setting[17]. One well-defined model is the femoral arterial cuff model for post-interventional vascular remodeling and, during hypercholesterolemia, accelerated atherosclerosis development in which a non-constrictive cuff is surgically placed around the mouse femoral artery. This model allows for drug-eluting cuff placement to mimic DES deployment. This and other animal models to investigate restenosis and effects of (local) drug therapy are described in detail in chapter 3.

Arterial thrombosis

Arterial thrombosis lies at the basis of myocardial infarction in patients and their ischemia-related symptoms. In addition, catheter insertion into the thrombotic blocked coronary artery during PCI, balloon dilatation and stent deployment all favor the development of progressive and renewed arterial thrombosis. For this reason, antiplatelet drugs are the cornerstone of adjunctive medication[6].

Thrombocyte adhesion, activating and aggregation result from contact to tissue other than the intact endothelium containing von Willebrand factor, but also from thrombin and epinephrine. Upon activation, platelets release prothrombotic mediators such as serotonin, tromboxane A2 and ADP which cause vasoconstriction and platelet aggregation. Signaling through intracellular transduction pathways leads to the extracellular expression of the GPIIb/IIIa-complex which enables fibrinogen binding and clot formation. Currently often applied anti-platelet therapy consists of acetylsalicylic acid, clopidogrel and abciximab, also used in combination. Acetylsalicylic acid irreversibly acetylates cyclo-oxygenase, necessary for the formation of prostaglandin thromboxane A2 from arachidonic acid, whilst the thienopyridine clopidogrel irreversibly blocks the adenosine phosphate (ADP) receptor on platelets necessary for GPIIb/IIIa-complex activation[18] and the monoclonal antibody abciximab binds the GPIIb/IIIa-complex[19]. Despite the increased risk for bleeding complications, anticoagulant therapy has been shown to be very effective in reducing myocardial infarction, PCI and CABG-surgery-related arterial thrombosis and mortality[18], also in combination with oral anticoagulant therapy (e.g. vitamin K antagonists) when sustained for at least 2 weeks following intervention[6]. Platelet aggregates facilitate leukocyte tethering and rolling[20], supporting the eventual leukocyte infiltration and local inflammatory response responsible for arterial inflammation and remodeling.

Annexin A5

The cellular membrane consists of a phospholipid bilayer that contains positively- and negatively charges phospholipids. Viable and healthy (endothelial) cells and platelets actively express the negatively charged phosphatidylserine (PS) on the inner cytosolic cellular membrane leaflet. Certain circumstances lead to a loss of membrane symmetry and translocation of PS to the outer membrane side, for example platelet activation and collagen adherence, but also erythrocyte aging, microparticle shedding and apoptosis[21, 22]. Platelet-expression of PS enables the assembly of the prothrombinase complex comprised of factors Va, Xa and II (prothrombin). This complex promotes the conversion of prothrombin into the proteolytically active thrombin which cleaves fibrinogen to form fibrin polymers that lead to thrombus formation[23].

Annexin A5 is a member of the annexin family, a group of highly-conserved proteins that are able to bind to negatively-charged phospholipid membranes in the presence

of Ca^{2+} ions. Annexin A5 resides intracellularly and is released upon injury and binds reversibly, specifically and with high affinity to PS[24]. For this reason annexin A5 has been used diagnostically world-wide for the detection of apoptosis and atherosclerosis both in vitro and in vivo. PS serves as an 'eat-me' signal on apoptotic cells for circulating phagocytes and annexin A5 binds PS forming two-dimensional crystals and may thereby act as a lattice shielding PS from phagocytes and from interacting in phospholipid-dependent coagulation reactions[22, 25]. The binding between annexin A5 and PS is uncompromised by circulating heparin, although annexin A5 can bind to the heparin oligosaccharide complex[26]. Annexin A5 was originally discovered as an anticoagulant and antithrombotic protein and has been shown to be essential in the maintenance of placental integrity, where the pro-coagulant apical surfaces of syncytiotrophoblasts possess many binding sites for annexin A5. Its binding is crucial for the continuous blood flow and fetal viability[27]. Annexin A5 has since also been shown to associate with the interferon γ receptor and prevent inflammatory cellular responses to secreted interferon γ[28]. This inflammatory cytokine is produced by natural killer (NK) and T-cells and is involved in monocyte recruitment and activation, central in pro-atherogenic cellular responses and inflammation[29].
In addition to its strong anti-coagulant properties, annexin A5 binds with high affinity to oxidized low-density lipoprotein (oxLDL) cholesterol particles and is therefore present in high concentrations in atherosclerotic lesions[30, 31]. Circulating annexin A5 plasma levels are inversely related to the severity of coronary stenosis and are indicative of atherosclerotic plaque extent[32], but are also elevated in hypertensive patients with systolic dysfunction[33] and following acute myocardial infarction[34]. The binding of annexin A5 to oxLDL cholesterol particles, to apoptotic cells and the inhibitory effects on inflammation and coagulation have identified annexin A5 as a protein with high clinical anti-atherosclerotic and anti-restenotic potential.

The innate immune system in atherosclerosis

Immune responses against circulating and local immunogenic antigens in the arterial wall play a critical role in the initiation of inflammatory processes that characterize accelerated atherosclerosis development. The immune system can be divided into the innate and adaptive systems which are closely linked together and are tightly regulated. Innate immunity forms the first line of defense and exerts a fast although unspecific immune response to invading micro-organisms, whilst adaptive immunity reacts more slowly, but targets highly specific antigen-bearing targets on foreign intruders[35].
The innate immune system is comprised of the complement system, various toll-like and scavenger receptors and natural antibodies that target pathogenic antigens. Their activation evokes responses that are comprised of both internalizing and signaling pathogen recognition receptors (PRRs) that recognize pathogen- and damage-associated molecular patterns (PAMPs and DAMPs respectively) leading to inflammation (figure 2)[36].

Complement

The complement system consists of a group of liver-synthesized proteins, membrane-bound receptors and regulatory enzymes designed to increase antibody-mediated clearance of pathogens from the body and complement component C3 has a

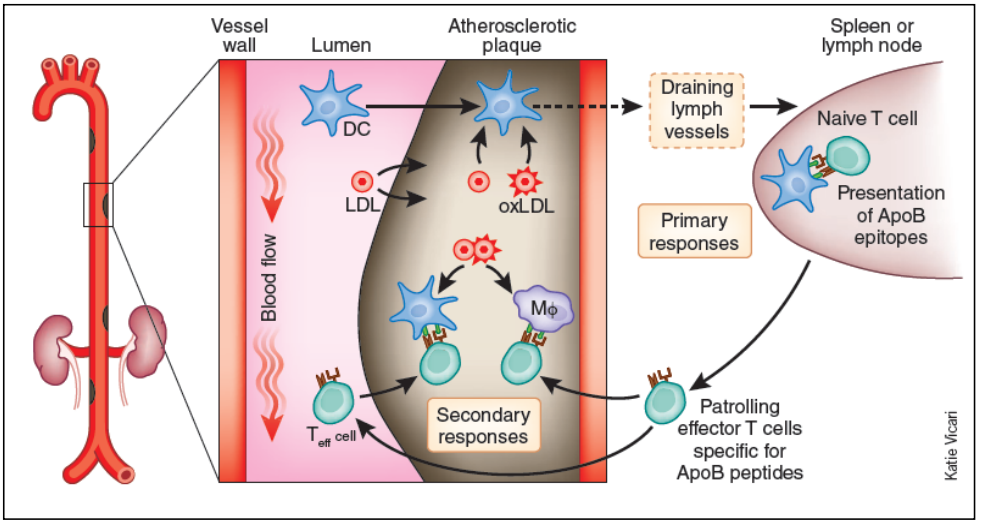

Figure 1.2 Innate and adaptive immunity in (accelerated) atherosclerosis development[53]
Endothelial damage at sites of arterial atherosclerosis or interventional injury can cause platelet adherence and expression of adhesion molecules that enable recruimtnet and infiltration of leukocytes bearing pathogen recognition receptors such as denritic cells. These can ingeste immunigenic antigens such as oxLDL particles and TLR-ligands and travel to draining lymph nodes where antigens are presented to naive T-cells in the presence of co-stimulatory factors. Activated effector T-cells enter the blood stream and travel to the site of inflammation, where they engage in secondary inflammatory reponses in co-operation with residing macrophages.

central function in this process. C3 cleavage by convertases into C3a and C3b is induced by specific substrates through either the classic, alternative or lectin pathways. C3b binds to the surface of pathogenic cells, enabling increased phagocytosis through opsonization[37, 38]. The classic pathway is activated by binding of the C1 complex to antibodies bound to antigen-presenting bacterial cells. The three pathways initiate a cascade that results in C5 activation and formation of terminal complement component C5b-9, the membrane attack-complex that causes bacterial and cell lysis and the chemotactic factor C5a that supports leukocyte recruitment[39]. Many complement triggers reside in the tunica intima in atherosclerotic segments and complement inhibition has been shown to result in reduced accelerated atherosclerotic lesion formation[40].

Toll-like receptors

Toll-like receptors (TLRs) form a major component of innate immunity, innate-adaptive crosstalk and reactions towards infectious and immunogenic auto-agents and are localized on both the internal and external sides of the cellular membrane. Situated on the cellular outer membrane leaflet, TLRs 2, 4 and 5 recognize exogenous PAMPs that originate from bacteria or viruses such as lipopolysaccharide (LPS) or flagellae (TLR5), whilst TLRs 7 and 9 on the inner cellular membrane recognize both pathogenic and endogenous ligands released after tissue damage or cell stress[41]. Myd88-dependent signaling is the dominant activation pathway of TLR signaling leading to nuclear factor kappa B (NFκB) transcription and expression of pro-inflammatory chemokines and cytokines[42, 43]. Endothelial TLR4 activation by LPS on

circulating Gram-negative bacteria during bacteremia leads to nitric oxide production and vasodilation that can initiate hypotensive septic shock[44]. DAMPs recognized by TLR4 include heat shock proteins[45], fibronectin extra-domain A[46], tenascin C and high mobility group box (HMGB) 1[47]. TLR2 and/or 4 deficiency and inhibition are associated with reduced atherosclerosis and vascular remodeling, whilst increased signaling aggravates inflammation and atherogenesis, highlighting the importance of these receptors in the innate immune response.
Endosomal TLRs like TLRs 3, 7 and 9 recognize viral and bacterial DNA and (double-stranded) RNA (TLR3) fragments and possibly also damaged self DNA/RNA that might result from during interventional procedures including PCI and CABG-surgery. Arterial presence and activation of TLR7 and TLR9[48] leads to upregulation of interferon γ, interleukin (IL) 6, IL-12 or tumor necrosis factor (TNF) α by innate immune cells such as macrophages[49]. These TLRs also recognize immune complexes containing self nucleic acids in autoimmune diseases and are localized in human arteries. Development of atherosclerotic plaques and PCI-induced injury cause a release of self RNA/DNA or proteins that enhance the recognition of nucleic acids by intracellular TLRs and can activate TLR7 and 9 signaling, by which these important parts of the innate immune system contribute strongly to arterial inflammation[50, 51]. Indeed, septic shock can also develop when bacterial unmethylated CpG DNA binds TLR9, highlighting TLR7 and 9 as therapeutic targets to prevent restenosis. Surprisingly, TLR3 is suggested to have a protective role in atherosclerosis development.

Scavenger receptors
LDL cholesterol is the most important risk factor for cardiovascular disease (CVD) and cholesterol lowering therapy alone such as HMG-CoA reductase inhibitors (statins) can reduce CVD-risk by 30-40%. Once trapped in the arterial wall, LDL oxidation by enzymes such as lipooxygenases occurs. Oxidative modification of phospholipid fatty acids, degradation of apoB-100 into peptide fragments and modification of these structures by aldehydes derived from oxidized fatty acids leads to development of immunogenic neo-antigens[52]. Intramural oxLDL particle accumulation stimulates oxLDL-uptake by scavenger receptors such as scavenger receptor (SR) A-1, SRA-2, SR-B1 and cluster of designation (CD)36 on monocyte-derived macrophages. Scavenger receptors are multifunctional PRRs that clear the environment of cellular debris and microbes and are responsible for oxLDL particle ingestion by macrophages. Cholesterol efflux, termed efferocytosis, is controlled by ABC-type cassette transporters that mobilize cholesterol into high-density lipoprotein (HDL) particle for transport to the liver[53]. A defective balance between cholesterol influx and efflux in these cells leads to excessive intracellular cholesterol accumulation which is stored in lipid droplets and promotes foam cell formation and lesion progression[54]. Continuous influx of monocytes is observed throughout lesion progression and they are referred to as being signature cells in atherogenesis that are both central and detrimental in lesion progression.

Unfolded protein response
Many exogenous and endogenous sources of cellular stress have been identified, including stress that arises from the accumulation of unfolded protein in the endoplasmatic reticulum (ER). In response to this stress, eukaryotic cells posses a

three-pronged signal transduction pathway collectively known as the unfolded protein response (UPR). This is made up of the IRE-1, PERK and ATF-6 axes[55]. Sustained intracellular UPR is designed to relieve cell stress and reduce amino-acid biosynthesis, but UPR itself can lead to cell pathology and tissue dysfunction. In the arterial wall, saturated fatty acids, oxidative stress and oxysterols, but above all intracellular cholesterol accumulation, all lead to chronic UPR and inflammation in intimal macrophages and endothelial cells[56]. This supports plaque progression and CHOP-induced foam cell apoptosis and necrotic core development[57, 58], but occurs in all stages of atherosclerotic lesion formation[59]. Overall, it is clear that the UPR is fundamental in the pathogenesis of inflammatory diseases and could serve as a target of therapy for modulating cellular stress and inflammation[60].

Natural (auto-) antibodies

Natural antibodies are immunoglobulines that arise spontaneously without prior immune exposure or infection and even occur in specific-pathogen-free mouse strains. Natural antibodies occur predominately of the IgM isotype and are produced by B-1 cells in the spleen and act as the humoral equivalent of PRRs[61]. They are suggested to aid the homeostasis of the internal milieu by binding to protein determinants of dying cells, such as PS and to facilitate C3b deposition on pathogens aiding their elimination and that of immunogenic auto-antigens[62].

In both mouse and man, natural IgM and IgG antibodies occur towards malondialdehyde (MDA) and copper-oxidized LDL cholesterol. To investigate their origin, B cell lines were isolated from spleens from non-immunized ApoE$^{-/-}$ mice on high-fat diets[63]. The resulting monoclonal antibodies, termed E0 antibodies were tested for their ability to recognize oxLDL. Gene sequence analysis unexpectedly identified that the genes encoding for the IgM anti-Cu oxLDL E06 antibody were completely similar to a classic B cell clone expressing T15 antibodies directed towards phosphorylcholine (PC) present on Streptococcus pneumoniae bacteria[64]. Of all reported anti-PC antibodies, T15 antibodies are most effective in clearance of pneumococci pathogen infections[65] and they occur frequently in inbred mouse strains[66]. This discovery identified complete molecular mimicry between oxLDL particles and Streptococcus pneumoniae bacteria that express the same PC moiety (figure 3)[67].

Anti-PC IgM T15 and E06 antibodies were found to be functionally equal in their ability to recognize PC on oxidized phospholipids but not on native LDL[68]. This was confirmed when immunization of hypercholesterolemic LDL-receptor (r)$^{-/-}$ mice against Streptococcus pneumoniae raised plasma titers of anti-PC IgM antibodies and prevent native atherosclerosis formation[69]. They are suggested to act by passing through the endothelial barrier and binding to PC on oxLDL particles, thus blocking their uptake by scavenger receptor bearing macrophages and foam cells, preventing their expression of pro-inflammatory cytokines, matrix metalloproteinases (MMPs) and co-stimulatory molecules for T cell activation. In addition, anti-PC IgM may bind and lead to clearance of circulating oxLDL particles, rendering them unavailable for plaque formation and protect endothelial cells from the harmful oxidative properties of oxidized phospholipids (figure 4)[70]. Immunization studies to prevent native atherosclerosis formation in mice have thus far been performed through active vaccination using Streptococcus pneumoniae[69], with PC bound to keyhole limpet hemocyanin (KLH)[71] and through oral administration with oxLDL and MDA-LDL[72], but also through

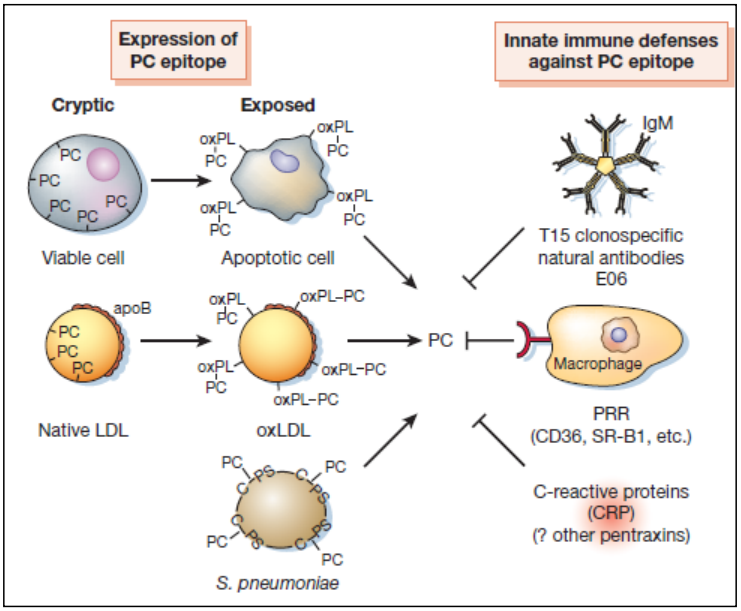

Figure 1.3 Molecular mimicry of pathogen associated molecular patterns[36]
The immunogenic phosphorylcholine (PC) epitope is expressed by apototic cells, by oxidized LDL particles and by Streptococcus pneumoniae bacteria, which share molecular mimicry. These are recognized by various parts of the innate immune defenses such as natural antibodies of the T15/E06 type, macrophage scavenger receptors CD36 and SR-B1 and by C-reactive protein.

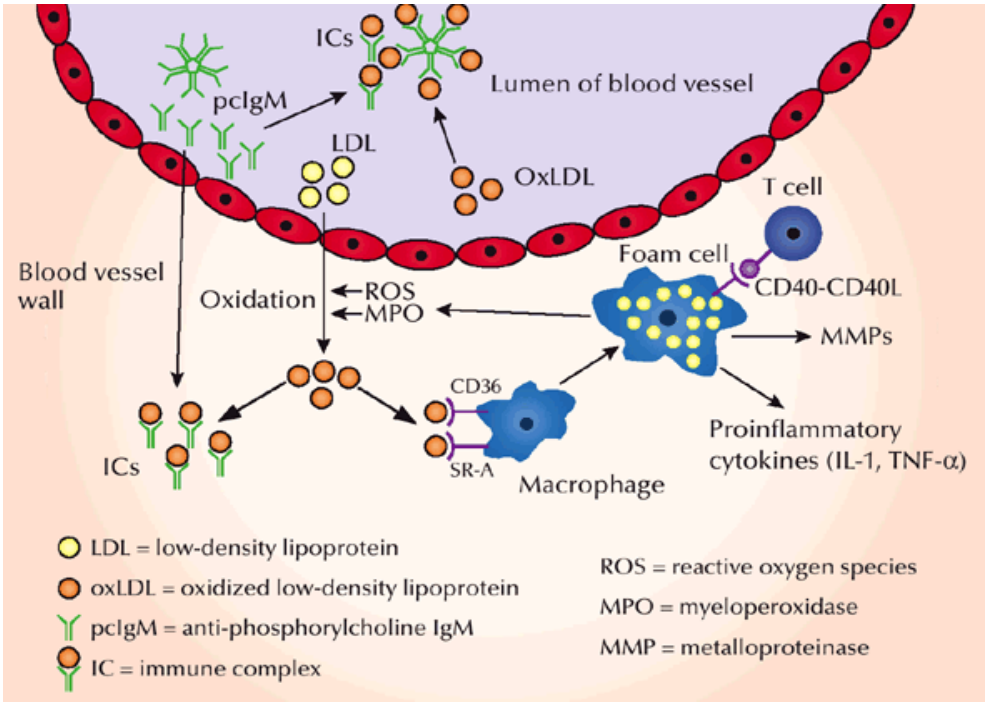

Figure 1.4 Proposed role of anti-PC IgM T15/E06 antibodies in preventing atherosclerosis[70]
Atherosclerosis develops due to scavenger-mediated uptake of LDL cholesterol oxidized by reactive oxygen species leading to lipid-laden foam cell formation. These produce proinflammatory cytokines, MMPs and myeloperoxidase and express co-stimulatory molecules for T-cell activation. Pneumococcal vaccination (in mice) enhances anti-phosphorylcholine (PC) IgM antibodies that cross the endothelium where they bind to oxLDL particles to form immune complexes and prevent oxLDL uptake and foam cell formation. Additionally, anti-PC IgM can bind circulating oxLDL, facilitating its clearance and rendering it unavailable for plaque formation.

passive vaccination with two types of recombinant IgG1 against MDA-modified apoB 100[73] (including regression of existing lesions[74]), with anti-PC IgM antibodies[75] and using oxLDL-pulsed mature dendritic cells[76].
Interestingly, recent investigations into the mechanisms of recognition that govern T-cell responses to LDL particles yielded surprising results. Research into T-cell hybridomas from human apoB100 transgenic mice immunized with oxLDL revealed they no longer recognized oxLDL, but solely LDL particles. Nevertheless, serum from these animals did contain anti-oxLDL IgG, indicating that T-cell responses to LDL lead to anti-oxLDL antibody production by plasma cells. The hypothesis behind these findings states that ingested and degraded oxLDL particles by scavenger receptor-expressing cells are co-expressed with MHC class II on the cell surface where they subsequently be recognized by CD4+ (TRBV31+ T-cell receptor bearing) T-cells. These CD4+ T-cells can stimulate macrophages, but also plasma cells to produce simultaneously anti-LDL and anti-oxLDL and related (PC) atheroprotective antibodies[77], emphasizing that such an antibody response is not just confined to oxLDL, but also extent towards native LDL particles.
Advantages of active vaccination are life-long protection and the need for only relative few immunizations, whilst passive immunization allows the induction of a fast and controlled immune response against the desired antigen. Fully human monoclonal antibodies are ideal for such passive immunization strategies and are preferred above re-engineered, de-immunized or rodent monoclonal antibodies. They can be generated using phage display platforms, which allow for identification of clinical candidates selected for their optimal desired functions[78]. Previously, such recombinant antibody libraries have proven to serve as an excellent source of active and well tolerated experimental therapeutics[79].

Natural antibodies in CHD
Over the past twenty years, many basic and epidemiological studies have suggested a role for indolent infections such as Chlamydia pneumoniae in atherosclerosis progression, although clinical trials using antibiotics failed to reduce CHD incidence[80]. Thus far, a clinical study in healthy human volunteers (both smokers and non-smokers) was unable to show an increase in serum anti-PC or anti-oxLDL immunoglobulines following the application of wildly-used adult pneumococcal polysaccharide vaccine, despite an increase in antibodies directed towards surface capsular polysaccharides[81]. Another study identified that hospital-administered patients suffering from myocardial infarction were less likely to have received a pneumococcal polysaccharide vaccine than patients admitted for other causes, suggesting vaccination might protect from myocardial infarction. However, effects are likely to result from a reduced incidence of pneumonia, which has been shown to trigger myocardial infarction[82], and thus acute events triggering infarction rather than through reduced

atherogenesis. Indeed, in contrast to chronic infections, prevention and adequate treatment of acute infections may prevent abrupt and severe inflammatory changes in high-risk patients groups and reduce myocardial infarction incidence[83]. Nevertheless, a save immunization protocol that can elicit a proper and sustained immune response or the development of fully human antibodies towards PC, preferably of the IgG isotype, could prove very effective in the prevention of both native and post-interventional accelerated atherosclerosis formation in clinical setting.

The adaptive immune system in atherosclerosis

The adaptive immune system can recognize an almost infinite number of molecular structures and depends on the vast variety of B and T cell receptors and immunoglobulines, generated by somatic rearrangement processes in blast cells. In contrast to PRRs, an almost infinite number of receptors exists and is capable of interacting with infiltrating pathogens.

Effector T-cells

Most T-cells in the atherosclerotic lesion are CD3+ and CD4+ T-helper (Th) cells that express the αβ T-cell antigen receptor (TCR) and have a Th1 phenotype. These cells are identified by their production of interferon (IFN) γ and IL-2 and are derived from native major histocompatibility complex (MHC) class-II CD4+ T-cells following presentation with specific antigens and co-stimulatory signals in the presence of cytokines such as IL-12 and IFN γ[84]. Cytotoxic MHC class I-recognizing CD8+ T-cells are also present in the lesions, although their function remains unclear. CD4+ T-cells recognize class-II presented peptides by antigen-presenting cells (APCs) such as oxLDL particles, heat shock proteins 60 and 65, but also exogenous pathogens such as Chlamydia pneumoniae. An extensive body of evidence exists identifying the direct role of pro-inflammatory Th1 T-cells in atherosclerosis development[77, 85, 86]. IgG2a antibodies in plasma dominate early atherosclerotic lesion progression, indicating predominant Th1 involvement. The two most important effector molecules produced by Th1 cells are membrane-bound CD40 ligand and the pleiotropic cytokine IFN γ that contribute to monocytes-differentiation and activation. IFN γ is highly produced in atherosclerotic lesions by not only Th1 cells, but also macrophages and NKT cells[87]. These molecules in turn stimulate pro-inflammatory IL-1, TNF α and MMP expression by macrophages and contribute substantially to local inflammation (IL-6 and C-reactive protein), lesion growth and fibrous cap thinning[88]. Indeed, strong atherosclerotic lesion reduction was found in $ApoE^{-/-}$ mice with genetic CD40 ligand disruption[89] and IFN γ deficiency[90] as well as following anti-CD40 ligand antibody treatment[91]. Finally, daily IFN γ administration significantly augmented lesion growth in hypercholesterolemic $ApoE^{-/-}$ mice[92].

Th2 cells primarily produce IL-4, IL-5, IL-10 and IL-13 and stimulate B-cell antibody production, but are not detected in large quantities in atherosclerotic lesions. IL-10 itself has strong anti-inflammatory and anti-atherosclerotic and restenotic effects[93]. Nevertheless, Th2 specific IgG1 antibodies are only detected in plasma at the advanced stages of atherosclerotic lesion progression. Studies involving Th2 cells have shown that Th2 cells in general inhibit atherogenesis[29].

Regulatory T-cells

Regulatory T-cells (Tregs) have a T-cell suppressive function and contribute effectively to maintenance of immunological response and auto-reactivity. They comprise approximately 5-10% of all peripheral CD4+ T-cells and are defined as the cell population expressing CD4+CD25+CD127^{low} markers on their extracellular membrane surface and intracellular forkhead box (Fox) P3, vital for their suppressive function[94]. Mice genetically deficient for FoxP3 develop an autoimmune-like lymphoproliferative disease, emphasizing the suppressive function of Tregs in maintaining peripheral tolerance[95], whilst humans lacking FoxP3 suffer from severe autoimmune disease that develops in infancy.
Tregs exert suppressive function through inhibition by cytokines, cytolysis, metabolic disruption and intervention in dendritic cell maturation. Thymus-derived Tregs express inhibitory cytokines IL-10, IL-35 and transforming growth factor (TGF) β, which stimulates collagen synthesis and is fibrogenic[96]. They migrate towards an atherosclerotic lesion similarly to Th1 cells, but the local pro-inflammatory microenvironment favors Th1 cell survival, causing an imbalance in T-cell adaptive immunity[97]. Tregs constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA-4)[98], acting as physiological dendritic-cell-mediated co-stimulation inhibitor and have been shown to control the development of atherosclerotic lesions through regulation of Th1 and Th2 responses[99] and is vital for their suppressive function[100]. Indeed, loss of adequate CTLA-4 function with blocking monoclonal antibodies produces auto-immune disease in mice similar to CD4+CD25+ Treg depletion and abolishes their protective function both in vitro and in vivo, since these cells can no longer block TCR and CD28-mediated co-stimulatory signals leading to Th1 activation, even to immunogenic self auto-antigens[98]. CTLA-4 on Tregs has also been shown to induce the enzyme indolamine 2, 3-dioxygenase (IDO) through CD80 and CD86 interaction on dendritic cells, which catalyzes the conversion of tryptophan to kynurenine[101] that has potent immunosuppressive effects on dendritic cells, and thus T-cell activation[102].

T-cell co-stimulation

T-cell mediated immune responses are initiated in lymphoid tissues where they are stimulated by APCs and can then interact with B cells to promote an antibody response or migrate to peripheral tissues to engage infiltrated pathogens or immunogenic antigens. A small portion of these cells become memory T-cells, to enable a quick response during re-infection. Their activation is described by the two-signal model which is composed of the presentation of MHC complex-bound peptides, as well as co-stimulatory signals provided by accessory molecules[103]. Activation of the two principal pathways leads to IL-2 regulated T-cell proliferation and their effector function acquisition. Since T-cell activation leads to TCR internalization, native T-cells require prolonged antigen exposure. The dominant co-stimulatory receptor CD28 is constitutively expressed on resting T-cells and binds CD80 (B7-1) and CD86 (B7-2) on dendritic cells, B cells, and monocytes/macrophages. CD80 and CD86 have both been shown to strongly contribute to atherosclerotic lesion formation[104]. Their function is required for an adequate T-cell response to atherosclerotic antigens and their expression of IFN γ. TCR binding in absence of CD28 ligation leads to T-cell anergy or even apoptosis, preventing non-specific T-cell activation and auto-immunity (figure 5)[105].

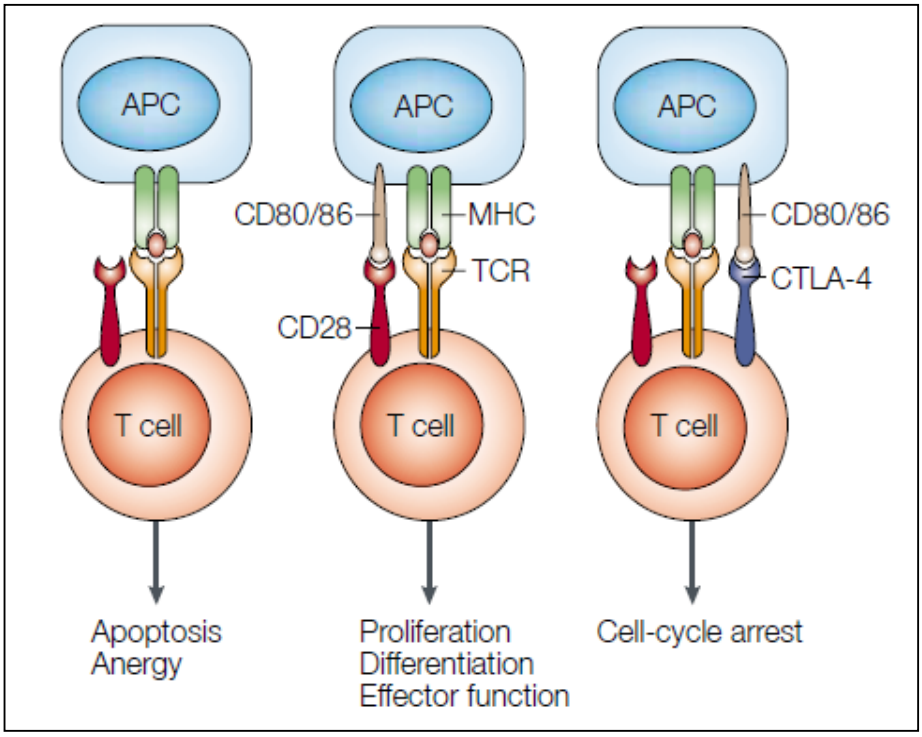

Figure 1.5 T-cell fate under various T-cell receptor engagements[107]
Adequate T-cell stimulation requires simultaneous interaction between a specific major histocompatibility (MHC)-peptide complex and the T-cell receptor and CD28 ligation by the B7-1 (CD80) or B7-2 (CD86) receptors on the antigen-presenting cell (APC). Absence of simultaneous interaction leads to T-cell apoptosis or anergy. After successful activation, T-cells upregulate cytotoxic T-lymphocyte antigen 4 (CTLA-4; CD152) that outcompetes CD28 for CD80/86 binding and results in cell-cycle arrest.

Cytotoxic T-lymphocyte antigen (CTLA)-4 and co-inhibition

CTLA-4 (CD152) is a co-inhibitory receptor expressed on activated T-cells and is homologous to CD28 and binds either a CD80 or CD86 mono- or heterodimer on APCs with approximately 100 times higher affinity than CD28[106]. Most CTLA-4 is expressed intracellularly rather than at the cell surface, thus limiting its engagement to CD80/86 and preventing a premature termination of an immune response[107]. During an ongoing immune response, CTLA-4 is upregulated and outcompetes CD28 for binding to CD80 and CD86, leading to T-cell proliferation inhibition and termination of IL-2 production, IL-2 receptor expression and arresting T-cells in the G1-phase of their cell cycle[105].

The importance of the CD28/CTLA-4 pathways has become evident by generating mice genetically deficient in CTLA-4, which develop fatal lymphoproliferative disease with progressive polyclonal T-cell accumulation in peripheral lymphoid and solid organs such as the heart, liver and pancreas[108].This is prevented by crossing CTLA-4$^{-/-}$ mice with Rag$^{-/-}$ and with CD80$^{-/-}$CD86$^{-/-}$ animals[109]. CTLA-4 has the maximal inhibitory effects in primed rather than naïve T-cells and decreases the proportion of cytokine-secreting T-cells in stead of the response magnitude per T-cell, explaining that more APCs are necessary to generate a detectable cytokine response

during CTLA-4 ligation[107]. In contrast to full-length CTLA-4, transgenic expression of tailless CTLA-4 mutant protein only reduced lymphocytic infiltration in solid, but not lymphoid, organs in CTLA-4$^{-/-}$ mice[110], but transfected mutant CTLA-4 constructs into Jurkat cells indicated scavenging of CD80/86[111]. These findings support the balanced view that CTLA-4 receptors act by both scavenging C80/86 away from CD28 on APCs as well as through direct intracellular pathways and are vital for maintaining T-cell homeostasis and preventing auto-immune reactions.
Unlike activated effector T-cells, Tregs constitutively express CTLA-4 and since blocking CTLA-4 antibodies prevent their inhibitory effects, CTLA-4 is required for their suppressive function[112]. Treg function was shown to be completely inhibited following acute CD80 and CD86 inhibition, further confirming the essential role for co-stimulation and inhibition in controlling adequate Treg function[99].
A second co-inhibitory receptor expressed by T-cells and shown to be involved in atherogenesis is programmed-death (PD)-1. PD-1 is a family member of CD28 which can bind either PD-1-ligand 1 or 2 (PD-L1 and PD-L2 respectively), expressed on either solely hematopoietic cells (PD-L2) or both non- and hematopoietic cells (PD-L1)[106, 113]. PD-1$^{-/-}$ mice also develop lupus-like proliferative arthritis and glomerulonephritis with IgG deposition[114]. Similarly to CTLA-4, PD-1 signaling inhibits T-cell proliferation and activation and their signaling has an important function in downregulating pro-atherogenic T-cell responses[115].

Clinical significance of CTLA-4

Rheumatoid arthritis (RA) is a chronic inflammatory disease that leads to progressive joint damage and disability and activated T-cells were shown to play a central role in inflammation progression[116].The potential for CTLA-4 to interrupt T-cell co-stimulatory signals was harnessed by fusing the extracellular domain of human CTLA-4 to the modified Fc portion of human IgG1, creating the soluble fusion protein CTLA-4Ig named abatacept[117]. Circulating abatacept has been shown to prevent CD28-CD80/86 co-stimulation of follicular Th-cells[118] and subsequently decreasing T-cell production of TNF α, IFN γ and IL-2 and has been approved for the treatment of mild to severe RA in patients who respond insufficiently to other disease-modifying antirheumatic drugs, including methotrexate, and TNF α inhibitors[117]. Abatacept displays little immunogenicity, with <3% of patients developing an antibody response[119]. Interestingly, soluble CTLA-4Ig has also been shown to effectively prevent T-cell mediated lymphoproliferative disease in CTLA-4$^{-/-}$ mice[120].

B lymphocytes and dendritic cells

Co-stimulatory and -inhibitory receptors are not restricted to T-cells, but are also expressed on B lymphocytes. These cells are present at the base of early fatty streaks and thereafter constitutively in the adventitia of human coronary lesions. At first, they were suggested to have a protective function against atherogenesis, since splenectomy increased atherosclerotic lesion size in hypercholesterolemic mice due to loss of B-cell produced protective anti-oxLDL antibodies[121]. However, acute B-cell depletion using anti-CD20 antibodies was since then shown to reduce atherosclerotic lesion size by reducing pathogenic T-cell responses whilst preserving the production of protective anti-oxLDL IgM, rather than IgG, antibodies. This reduced production of IFN γ and enhanced that of IL-17[122]. Very recently, the important role of CD28 in

this process was elucidated. The half-life of antibodies is days to weeks, whereas long-term immunity against infection through antibody production by plasma cells continues of a life-time. It was shown that CD28-expressing long-lived plasma cells (LLPCs) residing in the bone-marrow, but not splenic short-lived plasma cells, require continuous CD28 signaling through CD80 or CD86 for their function and loss of the CD28-CD80/86 pathway strongly reduces LLPCs half-life[123, 124]. Since these cells are the producers of protective natural antibodies (e.g. against oxLDL particles), CD28 is suggested to be important in maintaining adequate antibody titers associated with atherogenesis risk.
Dendritic cells express co-stimulatory molecules such as CD80 and CD86 and accumulate in the intima of atherosclerotic lesions and two distinctive types have been identified, namely classic myeloid dendritic cells (mDC) which mainly recognize endogenous and exogenous bacterial signatures through TLR2 and 4 and can produce MMPs and IL-12, leading to recruitment of cytotoxic T-cells[125]. Plasmacytoid dendritic cells (pDC) specifically recognize viral fragments through TLR9 and can produce large amount of IFNγ[126], leading to TLR4 upregulation on other APCs and increases atherosclerotic lesion development[127]. pDC presence in the shoulder region of atherosclerotic lesions contributes strongly to plaque instability and rupture[29].

Epigenetic regulation of inflammation
Post-interventional atherosclerotic vascular remodeling results from local arterial inflammatory processes that are tightly regulated by local gene expression profiles immediately after vascular interventions such as PCI and CABG-surgery. Inflammation results from increased transcription of inflammatory genes and is limited by the extent of chromatin accessibility to gene-regulatory proteins and transcriptional factors. Chromatin consists of DNA wrapped around histones (called nucleosomes) and non-histone proteins[128] and can display various degrees of compactness. This is controlled by the amount of histone modifications exerted by epigenetic factors that cause acetylation and methylation of lysine residues[129], but also DNA methylation at CpG dinucleotides. Since they determine gene expression independently of DNA sequence, their actions can even lead to notably different gene expression patterns between monozygotic twins[130].
Low-density chromatin, termed euchromatin, consists of active genes and is associated with hypomethylation of CpG dinucleotides in DNA and acetylated histones[131, 132], whilst compact chromatin (heterochromatin) is a hallmark of silent genes and is associated with hypermethylation of CpG dinucleotides in DNA and non-acetylated histones. Epigenetic factors have counterbalancing and reversible actions and vital epigenetic factors that control the degree of chromatin methylation and acetylation are histone methyltransferases and demethylases and lysine histone acetyltransferases (KATs) and deacetylases (KDACs)[133, 134].
KATs are nuclear enzymes that are recruited to gene promoter regions by gene regulatory proteins such as class II transactivator (CIITA) in leukocytes and can serve as master switches by increasing general inflammatory gene accessibility. Examples of KAT-regulated genes involved in atherosclerosis development include MHC class II and nuclear factor kappa-beta (NFκB), a pivotal transcription factor in inflammation[135]. KATs are considered to act dominantly in the nucleus of injured endothelial and SMCs immediately following vascular jury, such as occurs during PCI or CABG-

surgery. Intervention in the function of these KATs and their accessory molecules could affect the local arterial inflammatory response in the acute setting, affecting the degree of post-interventional vascular remodeling and the need for re-intervention in the long term[136].

P300/CBP-associated factor (PCAF)

P300/CBP-associated factor (PCAF/KAT2B) is a transcriptional co-activator with KAT-activity and regulates histone acetylation of NFκB-regulated inflammatory genes[137]. PCAF can bind the cyclooxygenase (COX)-2 promoter region following cellular exposure to inflammatory mediators and regulates the cellular inflammatory response through prostaglandin H2 formation[138]. Furthermore, PCAF also enhances the p65-mediated increase in TNF α promoter activity[139] and both TNF α and COX-2 regulate the cellular inflammatory responses by leukocytes that lead to atherosclerosis (figure 6)[140, 141].

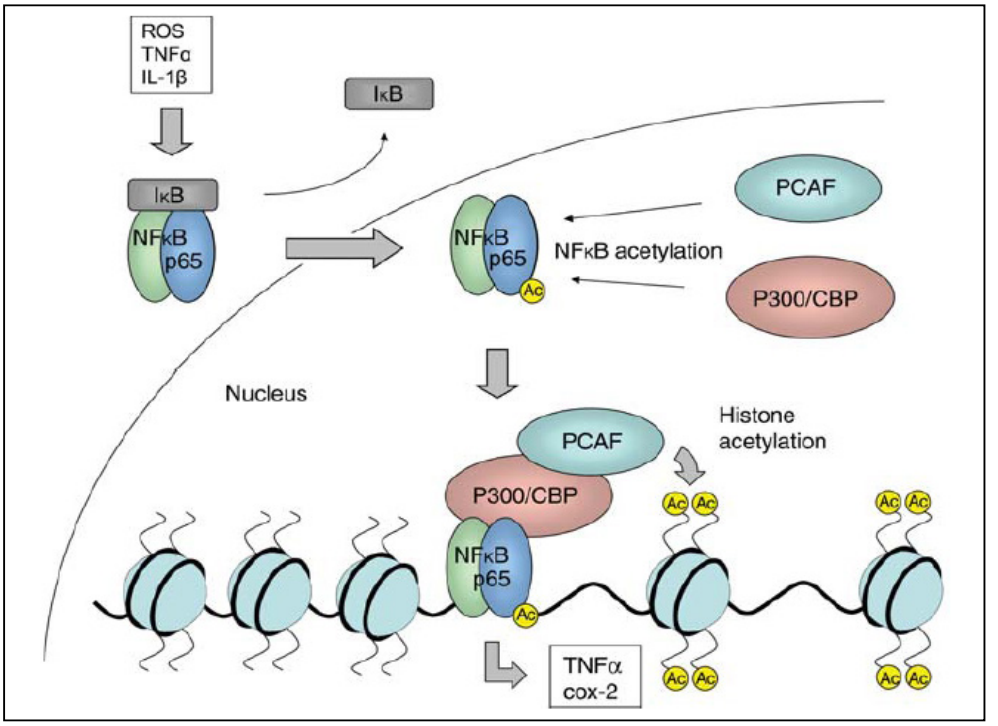

Figure 1.6 The role of PCAF in inflammation[136]

The role of p300/CREB binding protein (CBP)-associated factor (PCAF) in the activation of nuclear factor kappa B (NFkB)-mediated gene transcription is initiated by NFkB stimulators such reactive oxygen species (ROS), tumor necrosis factor (TNF) α and interleukin-1β. PCAF and P300 can act as factor acetyltransferases and acetylate NFkB directly, but also enable NFkB-target gene transcription through histone-acetylation at promoter regions of target genes such as TNF α and cyclo-oxygenase-2 involved in inflammation.

PCAF$^{-/-}$ mice are developmentally normal without a distinct phenotype, although levels of PCAF-B are drastically elevated in lung and liver tissue. In contract, PCAF-B deficiency is embryonically lethal and PCAF-B$^{-/-}$ mice die between 9-12 days of

gestation[142]. However, when PCAF$^{-/-}$ mice reach 6-12 months of age, they develop amyloid toxicity[143, 144] and short-term memory deficits and exaggerated responses to acute stress[145], confirming that PCAF histone acetylase activity is involved in life-long chromatin remodeling processes by post-translational histone modification.

PCAF in clinical CHD

Recently, association between the -2481G→C SNP in the promoter region of the PCAF gene and reduced CHD mortality and restenosis was shown in three independent large prospective studies[146-148]. A meta-analysis of the dataset showed that patients heterozygous for the low-risk allele had approximately a 20% reduction in risk for cardiovascular events, compared with 40% in patients homozygous for the allele[149]. The SNP might affect binding of a nuclear protein, but could similarly be a mere proxy marker for a functional SNP elsewhere in the PCAF locus due to linkage disequilibrium[150]. This finding has nonetheless added to the growing body of evidence that epigenetic regulation of inflammatory gene expression in activated leukocytes is vital for the process of atherosclerosis and restenosis development and could serve as a target of therapy or as diagnostic risk marker to improve patient screening.

Aim of the thesis

The aim of this thesis was to investigate the role of the immune system in the pathophysiological process leading to the development of post-interventional atherosclerotic vascular remodeling. This research enabled the evaluation of the effectiveness of specific immunomodulatory therapies in the prevention of accelerated atherosclerosis development, which could be applied during PCI or CABG-surgery in the clinical setting.

The development of post-interventional atherosclerotic vascular remodeling can be divided into specific phases of chronic tissue inflammation elicited by the activated immune system. First, intervention for established occlusive atherosclerotic disease induces arterial injury which elicits the expression of pro-coagulant factors and arterial thrombosis. This provides the scaffold for cells and mediators from the innate immune system to undergo local adhesion, infiltration and activation into the arterial wall (phase 1). Innate immune responses are directed towards (self) immunogenic molecular patterns that are present in the subendothelial space such as oxLDL particles and TLR ligands and lead to a pathological inflammatory response (phase 2). These activated cells not only stimulate the process of vascular remodeling and ECM deposition through expression of inflammatory mediators such as cytokines, but also travel to draining lymphoid tissues to recruit residing naïve cells that belong to the adaptive immune system. To this end, intercellular communication through specific receptor-ligand interactions and co-stimulatory signals leads to activation of naïve lymphocytes that enter the circulation to engage their specific antigen-bearing targets (phase 3). Their additive effects at the site of vascular injury to local infiltrated cells, antigen-antibody complexes and pro-inflammatory chemo- and cytokines promotes transcription of their inflammatory genes, enabled by tightly-controlled epigenetic regulation processes (throughout phases 2-3). This ultimately leads to accelerated atherosclerosis development and secondary clinical presentation.

Outline of the thesis

Hallmark events of immune system-mediated inflammatory reactions and their derived factors that could serve as future clinical biomarkers of CVD progression are described in **chapter 2**. The underlying factors that are causally related to inflammatory vascular remodeling are discussed and investigated for their possible application as (additive) biomarkers for adequate risk assessment and patient evaluation with the aim of improved patient screening and enabling future tailor-made treatment. Preclinical small animal models to study restenosis and effects of systemic and local drug therapy are reviewed in **chapter 3**, with special focus on the murine femoral arterial cuff model that encompasses a pathophysiology highly-resembling that of (in-stent) restenosis development. This ensures an ideal intervention model for the screening of candidate genes and new (locally applied) anti-restenotic drugs, especially when investigated in a hypercholesterolemic mouse strain such as the ApoE3*Leiden mouse.
The therapeutic effectiveness of the PS-binding protein annexin A5, which is well-known for it anti-thrombotic properties and usefulness as apoptotic marker, is evaluated in **chapter 4** in three different mouse models for vascular inflammation, remodeling and dysfunction. It was found that systemic annexin A5 treatment lead to annexin A5 accumulation at the site of injury and prevented the initiation of a local inflammatory response with major beneficial therapeutic effects. In **chapter 5**, association between polymorphisms in the annexin A5 gene and restenosis risk in patients undergoing PCI is investigated in patients enrolled in the GENDER study, with further investigations into dose-response effectiveness of annexin A5 at the time of revascularization.
Chapter 6 describes the investigations into therapeutic application of optimized natural occurring anti-PC IgM antibodies by harnessing the anti-inflammatory PC-binding properties in a clinically applicable format using recombinant monoclonal chimeric antibodies. This strategy was pursued further with fully human IgG antibodies towards PC that were identified and produced using phage display techniques, which allowed careful selection for the most optimal anti-inflammatory antibody properties. In this line, **chapter 7** shows the therapeutic efficacy of a newly-synthesized dual TLR7/9 antagonist in the prevention of post-interventional atherosclerosis development through prevention of inflammatory cytokine expression and oxLDL particle-uptake by macrophages during TLR7/9 stimulation in vitro. The novel TLR7/9 antagonist also increased secretion of the anti-inflammatory cytokine IL-10, responsible for reduced oxLDL particle-uptake through scavenger receptors expressed by macrophages.
The importance of T-cells and the co-stimulatory and co-inhibitory pathways is displayed in **chapter 8**, where knock-out mouse stains were used to confirm the importance of CD4 T-cells and the CD28-CD80/86 pathway in vascular remodeling. Blocking anti-CTLA-4 antibodies and abatacept, a soluble CTLA-4Ig that prevents CD28-mediated T-cell activation and is registered for treatment of clinical rheumatoid arthritis, were applied to elucidate the role of CTLA-4 signaling in accelerated atherosclerosis development. The powerful inhibitory effects on CD4+ T-cell status are investigated in the T-cell populations in the spleen and draining lymph nodes in hypercholesterolemic mice.

Chapter 9 identifies strong association between genetic variation in PCAF, a key mediator in epigenetics, and vascular morbidity and mortality in three independent patient cohorts is discussed in, which provides evidence for new concepts in the epigenetic regulation of genetics responsible for the processes of inflammation and proliferation during atherogenesis. The causal role of PCAF in this process is investigated in **chapter 10**, in which PCAF knock-out mice are subjected to surgical cuff-induced vascular injury and inhibitory effects of PCAF deficiency on leukocyte activation and functional cytokine expression were investigated. Additionally, it is shown that perivascular delivery of the only potent natural PCAF inhibitor garcinol at the time or surgery can protect against neointimal formation. Finally, **chapter 11** describes the important role of PCAF in inflammation and vascular remodeling in general, with significantly impaired arteriogenesis in PCAF knock-out mice compared to controls allowing careful assessment of its contributory role in neovascularization.

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