Chapter 12

Summary and general discussion

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Cardiovascular diseases remain the major cause of death throughout the world and can be primarily attributed to atherosclerotic vascular disease leading to stroke and coronary heart disease (CHD). Improved primary prevention and the introduction and subsequent optimization of percutaneous coronary interventions (PCI) for myocardial ischemia due to obstructive CHD have significantly improved patient outcome and reduced morbidity and mortality. The insight into disease pathology has however expanded tremendously over the past decade and continuing research has shifted the focus of interest towards post-interventional accelerated atherosclerosis development due to a dysfunctional (auto) immune inflammatory response, responsible for vascular remodeling, re-occlusion and recurrence of symptoms.

The aim of this thesis therefore was to investigate the role of the immune system in this pathophysiological process that ultimately results in post-interventional atherosclerotic vascular remodeling and apply this insight for the development of new immune-modulatory therapies in a preclinical setting. The important contribution of the various parts of the immune system involved in both clinical and preclinical vascular remodeling are described in **chapter 1**. Preclinical screening and testing of immune-modulatory therapy effectiveness is a vital step towards clinical application of such interventions at the time of PCI or CABG-surgical procedures to prevent vascular re-occlusion and the necessity for re-interventions. Insight into the evoked immune responses, both in mouse and man, during these procedures lies at the basis for the discovery and application of new therapies with ultimate clinical potential.
Chapter 2 provides an overview of the immune reactions of the innate and adaptive immune systems that develop during native atherogenesis, as well as those which are evoked by vascular injury during revascularization strategies. Specific leukocyte receptors, ligands, co-stimulatory molecules and inflammatory cytokines are highlighted and are shown to be involved in disease initiation and lesion progression during inflammation. In addition, their association with disease severity is independent of traditional risk factors such as smoking and hypertension and they could serve as helpful tools for biomarker-based risk stratification or and diagnosis. This includes diagnostic assessment of patients eligible for intensified treatment evaluated clinicians performing target lesion revascularization interventions. Moreover, future biomarkers could be helpful in assessing treatment effectiveness in a way that traditional makers such as plasma lipoprotein levels or electrocardiographic do not provide adequate insight. Plasma measurements are above all strongly preferred over diagnostic angioplasty procedures and local biomarkers in the arterial segments of interest could improve optimal disease severity assessment. The search for new biomarkers is therefore essential and markers of inflammation that are causally linked to post-interventional vascular remodeling could prove to be the most valuable makers available to clinicians.
Chapter 3 illustrates that investigational screening of the therapeutic effects of drug therapy on vascular remodeling and accelerated atherosclerosis development requires preclinical models that optimally mimic the clinical situation of the vessel after intervention, not only in vascular anatomical aspects such as size, diameter and wall thickness, but also in features of disease stage aspects such as hypercholesterole-

mia and other conventional risk factors for atherosclerosis. To this end, humanized animal models are discussed that have the best predictive value for the pathophysiological process in the development of restenosis, intimal hyperplasia and accelerated atherosclerotic lesions. Various vascular interventions in transgenic mouse models are mentioned, with a strong focus on the mouse femoral artery cuff model. To study effects of (local) drug therapy, animals should be susceptible to the treatment of interest, have similar metabolic levels, coagulatory phenotype and react in a human-like fashion. The use of humanized (transgenic) animal models has extensively increased the similarity between human and animal lesions and the translation of new therapies into the clinical setting. Furthermore, mechanistic and pathophysiological studies have shown that local vessel wall inflammation, proliferation and proteolysis are central in post-interventional vascular remodeling. It was therefore concluded that highly-reproducible animal models for post-interventional vascular remodeling are essential for studying the process of restenosis and the development of future anti-restenotic therapies.

Specific stages of the immune response and their usefulness as target of immune-directed interventions are discussed below. Vascular remodeling is originally initiated by endothelial damage and injury to the underlying plaque by balloon inflation, stent deployment or surgical harvesting and engraftment procedures during revascularization interventions. Exposure of thrombogenic tissue will evoke platelet adherence, activation and thrombosis formation and support the recruitment of leukocytes to the site of vascular injury. Platelet activation and binding is supported by the presence of phosphatidylserine (PS) on activated and apoptotic cells. **Chapter 4** shows a therapeutic role for the PS-binding annexin A5 protein against vascular inflammation, remodeling and dysfunction in mouse models for accelerated atherosclerosis development. It was demonstrated that annexin A5 injection resulted in a marked reduction in circulating plasma cytokine concentrations and early inflammatory cell recruitment to the vessel wall after injury, eventually leading to decreased intimal hyperplasia with less plaque instability features. Although the exact role through which annexin A5 led to these effects was not completely clarified, annexin A5 was shown to act through local platelet-supported leukocyte binding and prevention of the subsequent immune response, thus extending the role of annexin A5 as a regulator of inflammatory processes and demonstrated its potential therapeutic use in inflammation-associated vascular disease.

In **chapter 5** it is demonstrated that a strong association exists between genetic polymorphisms in the human annexin A5 gene and increased restenosis-risk in patients undergoing PCI enrolled in the GENDER study, composed of 866 patients of which 295 cases developed restenosis within 1 year following PCI and 571 controls were free of restenosis. Although association exists, this does not implicate a direct causal link between this polymorphism and clinical outcome. To this end, measurement of patient plasma annexin A5 concentration could provide insight, since reduced annexin A5 concentrations are linked to increased coronary stenosis severity, whilst increased concentrations occurs following myocardial infarction. Nevertheless, these results do suggest that annexin A5 genotype could function as a risk marker for restenosis. Together, these date indicate high diagnostic and therapeutic clinical potential for annexin A5 against post-PCI vascular remodeling.

Chapter 6 describes the process of optimization of a natural occurring protective

anti-phosphorylcholine (PC) T15/E06 IgM antibody into a recombinant chimeric IgG which was first evaluated for in vitro anti-inflammatory effectiveness. Remarkably, the inhibitory effects on oxidized low density lipoprotein (oxLDL) uptake by scavenger receptor-bearing macrophages was lost after transition of the constant antibody region from an IgM to an IgG chain. Despite this loss, the recombinant anti-PC T15 IgG was nevertheless effective in preventing monocyte chemotactic protein (MCP)-1 expression by macrophages and passive immunization of cuffed hypercholesterolemic mice with this antibody prevented accelerated atherosclerosis development. These results suggest that although the chimeric antibody did not prevent oxLDL uptake, it did inhibit inflammatory responses towards oxLDL, possibly by masking the immunogenic epitope or by supporting its degradation and clearance from the circulation.

Using the Dyax library, phage display selection allowed identification of phosphorylcholine-specific phages that were converted to full IgGs and could still block oxLDL-uptake by macrophages and subsequent MCP-1 expression. A selection of antibodies was then tested in vivo and yielded potent fully human monoclonal anti-PC T15 IgG (M99-B05) antibodies that were effective in preventing intimal thickening in cuffed hypercholesterolemic animals. Codon optimization of M99-B05 produced the X19-A05 antibody with high specificity for PC and strong anti-inflammatory effects that could inhibit oxLDL uptake in vitro and vascular remodeling in vivo, even in low concentrations. This study, like those performed previously, showed that PC is a promising therapeutic target in the prevention and treatment of accelerated atherosclerosis development in mice and anti-PC IgG antibodies can prevent vascular remodeling. However, the vital new aspect of this study is that it is the first to develop new fully human anti-PC IgG antibodies that are atheroprotective. Others used IgM antibodies passively in mice or induced anti-PC IgM and IgG through active immunization. These techniques are promising, but not applicable in the clinical setting, since active immunization with a pneumococcal polysaccharide vaccine previously did not lead to adequate titers of oxLDL-recognizing anti-PC antibodies. We developed, screened and tested recombinant monoclonal anti-PC IgG antibodies that are suitable for passive therapeutic immunization in humans. By providing immediate and direct control of the patient's immune response with restriction to a single immunogenic epitope, this immunization approach could prove to be an effective treatment modality against post-interventional atherosclerotic vascular remodeling in patients undergoing PCI or CABG-surgery.

The important role of the innate immune system was confirmed in **chapter 7**, where a novel TLR7/9 dual antagonist was tested in a mouse restenosis model. Toll-like receptors are a vital part of the innate immune system and serve as pattern recognition receptors (PRR) that recognize extracellular ligands that originate from bacteria or viruses (TLR2, 4 and 5), as well as intracellular ligand such as damaged or viral (double stranded) RNA (TLR3, 7 and 9). These endogenous ligands may be released after tissue damage or cell stress, processes that may be initiated by PCI. TLR7 or TLR9 presence occurred in vascular lesions at the location of macrophage accumulation. In vitro, these cells responded to TLR7 or TLR9 activation by increased oxLDL uptake and TNFα production, which could be inhibited by the novel TLR7/9 dual antagonist. These effects led to reduced intimal thickening when applied in vivo. The magnitude of individual TLR signaling remains unknown, but

the therapeutic potential of targeting TLR7 and TLR9 to prevent restenosis and accelerated atherosclerosis has been made clear. Since TLRs are easily accessible for circulating drugs (directly or after cellular infiltration) and form the first line of defense of the innate immune system, they are favorable as therapeutic targets in the acute phase of vascular injury and can be readily silenced or stimulated. Novel inhibitors can be designed to bear desired drug properties, whilst effectively targeting a TLR of great interest. Dual targeting of multiple receptors greatly increases drug effectiveness, clearly demonstrated in this study.

In **chapter 8**, the contribution of the adaptive immune system to post-interventional atherosclerotic vascular remodeling was elucidated by investigating the role of CD4+ T-cells and CD28-CD80/86 co-stimulatory pathways. Post-interventional vascular remodeling was significantly attenuated in $CD4^{-/-}$ and $CD80^{-/-}CD86^{-/-}$ mice compared to controls. To show that CTLA-4 is a key and vital regulator in this process, abatacept was injected in control mice that then failed to develop significant intimal thickening. Next, this was repeated in hypercholesterolemic ApoE3*Leiden mice, producing similar striking results. Flow cytometry analysis of activated lymphocytes in spleen and draining lymph nodes revealed that abatacept prevented effector CD4+ T-cell activation and led to a reduced number of regulatory T-cells in the spleen, although without affecting Teff : Treg ratio. However, abatacept did not affect cellular activation status in draining lymph nodes. These findings were later linked to reduced plasma levels of interferon γ in abatacept-treated animals. The role of CTLA-4 co-inhibition in this process was finally confirmed by treating ApoE3*Leiden mice with hybridoma-derived blocking anti-CTLA-4 IgG antibodies, which developed significantly larger lesions.

Immune-mediated interventions directed towards therapeutically controlling the inflammatory T-cell response such as abatacept are widely applied in other immune (e.g. rheumatoid) disorders and could now be used in an early phase following vascular interventions to prevent subsequent vascular remodeling. Abatacept is a registered drug with an established efficacy and safety profile in patients, which can dramatically shorten the time necessary for bench-to-bedside translation for this specific drug. Although initial beneficial effects of abatacept were observed, these need to be reproduced in the prolonged treatment setting before they can be applied in the clinic. In addition, effects of abatacept treatment on other CD28-expressing cell types such as B-cells should be fully clear before application during vascular revascularization interventions. Nevertheless, this study clearly demonstrates the important role of CD4 T-cells and the CD28/CTLA-4-CD80/CD86 co-stimulatory pathway in the inflammatory reaction as part of the adaptive immune system that occurs after vascular intervention in vivo.

In **chapter 9** it is showed in three large prospective studies that the -2481C allele in the PCAF promoter is associated with a significant survival advantage in elderly patients while also protecting against clinical and angiographic restenosis after PCI. It is suggested that the effect of this allele on these endpoints may be due to the well-known involvement of PCAF in inflammatory and proliferative processes. These results not shed light on a causal role of this polymorphism in the development of restenosis, but do promote the concept that epigenetic processes are under genetic control and that, other than environment, genetic variation in genes encoding KATs may also determine susceptibility to CHD outcomes and mortality. Until this point,

it remains to be established whether this SNP functionally affects transcriptional regulation of P300/CBP-associated factor (PCAF). Provided that this SNP influences PCAF transcription and resulting protein levels, this could have a bearing on the cellular portrait of expressed genes and might lead to a dramatic different outcome if the effects accumulate over years. Until this can be established, this SNP could serve as risk marker for both mortality and restenosis risk in patients undergoing PCI. Furthermore, it has provided insight into the association between PCAF and vascular remodeling, the exact role of which was further investigated in the next chapters.
Chapter 10 describes the investigations performed into the causal role of PCAF in controlling the inflammatory response responsible for post-interventional atherosclerotic vascular remodeling. It was found that PCAF regulates MHC class II, but not I, expression in macrophages through CIITA and is upregulated in the arterial wall in the early period following vascular injury at both mRNA and at protein level. It is demonstrated that post-interventional vascular remodeling is reduced in $PCAF^{-/-}$ mice and accelerated atherosclerosis development in hypercholesterolemic mice treated with the natural potent PCAF inhibitor garcinol. Furthermore, it was shown that this reduced remodeling was due to an attenuated inflammatory response, identified by reduced MCP-1 and TNF α-expression in vivo and in cultured SMCs and macrophages in vitro.
Although the role of PCAF in the regulation of inflammation is clear and was shown to affect macrophage recruitment, activation and cytokine expression, the specificity of garcinol for PCAF in this process needs to be further elucidated. Garcinol was shown to be extremely potent and can inactivate PCAF activity within 3 minutes in vitro, but also affects the expression of many other genes and is known to induce apoptosis in high concentrations. Indeed, the anti-inflammatory effects of short-term applied garcinol to inhibit PCAF expression at the time of vascular interventions was lost with prolonged garcinol application in high concentrations in the femoral drug-eluting cuff model (unpublished data). This highlights the necessity of the search towards new and potent PCAF inhibitors that do not display as many or severe side-effects as garcinol does, before effective PCAF inhibition can be applied in the clinical setting. Nevertheless, the results highlight inflammation-regulation by the epigenetic factor in the acute setting as a causal factor in post-interventional vascular remodeling.
The role of PCAF in inflammation and vascular remodeling was further investigated in **chapter 11**, where $PCAF^{-/-}$ mice were shown to display impaired arteriogenesis capability following acute arterial occlusion. This process, a form of collateral artery growth from pre-existing arterioles to arterial occlusion, is initiated by flow and shear-stress-increase and is highly dependent of the activation of circulating inflammatory cells. Using a hind-limb ischemia mouse model based upon surgically-induced acute femoral artery occlusion, PCAF was identified to be vital in this inflammatory process. $PCAF^{-/-}$ animals were shown to display an impaired inflammatory response with functionally less activated dendritic cells, T-cells and reduced inflammatory cytokine expression in vivo and following a LPS-challenge ex vivo. Although exactly which inflammatory genes and to what extent these are regulated by PCAF remains to be further investigated, it has been made clear that PCAF is causally involved in post-interventional vascular remodeling.
PCAF regulation of inflammatory gene transcription was shown to occur in multiple

cell types involved in the innate and adaptive immune responses, including antigen-presenting cells, lymphocytes and smooth muscle cells. By exerting a regulatory function in controlling acute inflammation, PCAF was shown to dominantly affect pro-inflammatory genes and by serving as central-regulating factor with intrinsic histone acetyltransferase activity, it might provide clinicians with an interesting and controllable epigenetic target for anti-inflammatory preventive therapy.
Therapy directed towards expression of such an epigenetic factor as PCAF might be more effective in preventing vascular remodeling than a single gene or single protein treatment approach, since a factor like PCAF can affect chromatin structure throughout the nucleus.

How do we proceed beyond our promising newly discovered therapies?
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 has yielded multiple new therapeutic applications that could be translated into the clinic within a varying range of time. Although statements naming time units are mere predictions, it is wrong not to pursue such goals and leave promising drugs for what they are simply because investigations into their application might take a long time. Abatacept is already a registered drug treatment drug for rheumatoid arthritis and could be relatively quickly (<1 year) tested in a revascularization setting, once therapeutic effectiveness is confirmed in long-term animal studies. Annexin A5, recombinant monoclonal X19-A05 anti-PC IgG antibodies and our novel TLR7/9 antagonist have been tested and found effective in our animal models, but still require additional investigations into their efficacy and safety profiles, before clinical application could be initiated. This should be able to be performed within the foreseeable future (<2 years). Our PCAF results have identified a new factor involved in post-interventional vascular remodeling and still require more work before patients can receive their 'anti-PCAF pill' when undergoing PCI or CABG surgery. Specifically, the target genes other than CIITA and NFκB affected by PCAF modulation need to be fully investigated, in order to provide insight into possible adverse effects. Once this is achieved, new PCAF modulators could be developed (e.g. using phage display selection) with the optimal drug characteristics of our desire (<3-4 years).
Nonetheless, this research was able for a large extent to fulfill the aim of this thesis stated in chapter 1. This has been clearly the result of fruitful collaboration between many departments within the LUMC and pharmaceutical partners and such joint efforts should to be pursued continuously and vigorously to improve patient outcome and reduce morbidity and mortality in clinics worldwide.