The role of the innate and adaptive immune system on vascular remodeling

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Chapter 1

General introduction and outline of the thesis

Cardiovascular disease

Cardiovascular diseases (CVD) include all heart and blood vessel diseases and are the major cause of mortality worldwide. The number of individuals that decease globally of CVD still increases due to the aging of population, population growth and shifting demographics[1, 2]. Stroke, myocardial infarction, heart failure and cardiomyopathy are all types of CVD, but the main underlying pathology of CVD is atherosclerosis[3]. Atherosclerosis is a chronic inflammatory disease in which plaque formation occurs in arteries. Plaques consist of lipids, cholesterol, debris, calcium, fibrin and inflammatory cells, which accumulate in the intimal layer of mainly large and medium sized arteries[4]. Atherosclerotic narrowing of an artery resulting in impaired blood flow and ischemia distal to the obstruction is entitled occlusive arterial disease (OAD). When narrowing in coronary arteries occurs, it is called coronary artery disease (CAD)[5] and can result in myocardial infarction and (un) stable angina. Peripheral artery disease (PAD) is the most common OAD affecting the lower legs[6]. Vascular remodeling is an active process of structural changes in the vasculature due to e.g. physiologic alterations in blood flow or vessel wall injury, which comprises all CVD described in this thesis[7].

Neovascularization

To resolve ischemia distal to the atherosclerotic obstruction, neovascularization naturally occurs in the body to form new blood vessels and restore blood flow. Neovascularization consists of angiogenesis and arteriogenesis.

Arteriogenesis is mainly driven by shear stress and is the maturation of pre-existing arterioles into functional arteries[8-11]. In addition, inflammation, elevated flow and circumferential stretch on the vessel wall induced by atherosclerotic obstructions also cause arteriogenesis. More specifically, vessel wall damage will lead to an up regulation of monocyte chemoattractant protein-1 (MCP-1) molecules expressed on ECs resulting in adherence and invasion of inflammatory cells such as monocytes, CD4+ and CD8+ T cells. Subsequently inflammatory cytokine levels such as TNFα increase as well as chemokine and growth factor levels, which causes further maturation of pre-existing arterioles[10, 12-14]. The mature collateral arterioles can naturally bypass the occluded vessel.

Angiogenesis, the sprouting of new capillaries from a pre-existing vasculature[15], is driven by hypoxia and mainly occurs far distal to the occlusion in the most hypoxic area. Upon ischemia, hypoxia inducible factor 1a (HIF1a) is up regulated to induce transcription of growth factors such as vascular endothelial growth factor (VEGF). Nearby vessels that express VEGF receptors, particularly VEGFR2 on endothelium, begin to grow toward the

hypoxic area. VEGFR2 directs endothelial cells (EC) from the pre-existing vasculature to migrate, grow and differentiate to shape new capillaries[16, 17].

Vasculogenesis involves de novo formation of blood vessels from embryonic precursors through differentiation of angioblasts into endothelial cells followed by the recruitment of vascular smooth muscle cells (VSMC), which can shape new blood vessels[18]. Vasculogenesis differs from angiogenesis since there is no pre-existing vasculature. For optimal neovascularisation in OAD patients, a proper vascular bed of pre-existing arterioles is essential, which are initially formed by vasculogenesis during embryologic development of the circulatory system.

Treatment options

If neovascularisation in the body is not sufficient to prevent ischemia after OAD, therapeutic treatment options are available. In asymptomatic OAD patients, risk factors are addressed including smoking, medication and in case of PAD physical exercise, to confine the development of symptomatic or severe OAD[19]. Other therapeutic options for patients with established OAD are pharmaceutical interventions including statins, antihypertensive drugs, or aspirin. Symptomatic patients can benefit from revascularization interventions such as angioplasty, also known as balloon angioplasty, with or without stent placement.

In patients with severe symptomatic OAD, bypass graft surgery is commonly performed. Coronary artery bypass graft (CABG) surgery is the type of surgery that improves blood flow to the heart in CAD patients, whereas peripheral artery bypass grafting (PABG) surgery is performed in patients with severe PAD[20-23]. The internal mammary artery is the preferred graft for CABG surgery of the left anterior descending coronary artery[24]. However, the great saphenous vein remains the most commonly used and preferred conduit for PABG surgery, owing to availability, structure and length[25]. However, surgical treatment is the last therapeutic option for OAD patients. Therefore, it is important to identify new therapeutic targets to prevent the development of OAD, stimulate natural neovascularization and prevent vein graft failure.

Vein graft disease

Vein graft failure can have several causes. Early after engraftment, acute thrombosis is a dominant cause of vein graft failure, usually as a result of technical failures of the procedure. Size mismatch of the graft and target vessel as well as rough surgical handling of the vein graft during harvesting and the engraftment resulting in endothelial cell damage, can cause acute thrombosis[26].

From one month after engraftment, intimal hyperplasia is the main etiology of vein graft failure. After PABG or CABG surgery, arterialization of the engrafted vein graft starts, to adapt to the high-pressure arterial environment. This is characterized by structural vessel wall remodeling and intimal thickening, which is essential for long-term graft patency. However, continuing arterialization can lead to narrowed and occluded vein grafts, called vein graft disease (VGD)[26, 27]. Smooth muscle cells (SMC) migrate into the intima and proliferate and subsequently an inflammatory cell influx of e.g. macrophages occurs. This inflammatory response plays an important role in the development of VGD. Immediately after surgery, inflammatory cells are activated which can produce pro-inflammatory cytokines and growth factors. This causes extracellular matrix (ECM) deposition and fibrotic changes leading to the development of intimal hyperplasia. Late vein graft failure is usually due to progressive atherosclerosis. However, if VGD is prevented, late atherosclerosis can hardly develop. Therefore, we focussed on targets to prevent the development of VGD.

Murine models for vein graft disease and neovascularization

To study neovascularization and VGD in vivo several murine models are developed. In brief we will discuss the murine models that are used in this thesis.

A hind limb ischemia (HLI) mouse model was developed to study neovascularization. We used a unilateral hind limb ischemia model with single and double ligation. In brief, a skin incision was made in the left inguinal region and the femoral artery was prepared. In the single ligation model, ischemia was induced by electrocoagulation of the left common femoral artery proximal to the bifurcation of the popliteal and saphenous artery[28-30]. In the double ligation model, unilateral HLI was induced by electrocoagulation of the left common femoral artery proximal to the superficial epigastric artery and proximal to the bifurcation of the popliteal and saphenous artery[28]. To investigate neovascularization, post-ischemic blood flow recovery was measured in the left ischemic and right non-ischemic paw with the use of Laser Doppler Perfusion Imaging (LDPI) before and after surgery until sacrifice 28 days after surgery. At sacrifice the adductor and soleus muscle were harvested and cross sections of embedded muscles were made. Adductor muscle sections were stained with alpha smooth muscle cell actin to visualize vascular smooth muscle cells (VSMC) and quantify arteriogenesis by measuring the diameter of collateral arterioles. Soleus muscle sections were stained with CD31 to visualise endothelial cells and quantify angiogenesis by counting the number of angiogenic capillaries.

To study VGD, a murine vein graft model was used. Vein graft surgery was performed by donor caval vein interpositioning in the carotid artery of recipient mice as

introduced by Zou et al[31]. In brief, in this model the right common carotid artery was dissected free from its surrounding from the bifurcation at the distal end toward the proximal end. After cutting the carotid artery central, cuffs were placed at both ends of the carotid artery. Subsequently, the free ends of the carotid artery were everted over the cuffs and ligated. The vena cava was harvested of a donor mice and used as a graft by sleeving the ends of the vena cava over the carotid artery cuffs and fixing them with sutures[32]. At sacrifice, 28 days after surgery, the vein grafts were harvested and embedded. Cross sections were made and vein graft sections were stained with Hematoxylin, Phloxin 0.25%, and Saffron 0.3% to quantify the vessel wall thickening, total vessel area and lumen area.

Cells of the vessel wall

It is known that immune cells of the innate and adaptive immune system play a pivotal role in the vascular remodeling as well as other cells such as endothelial cells (EC) and VSMC[33, 34]. ECs covering the tunica intima layer of the vascular wall are the first cells involved in vascular remodeling. Due to changes in blood flow or pressure ECs can be activated or damaged. As a result, ECM components underneath the EC layer are uncovered. The ECM containing elastin, collagens, proteoglycans and structural glycoproteins are essential to provide a structural network in the vessel wall and maintain a full functional vessel wall. Exposure of the subendothelial ECM can trigger a coagulation process. VSMC, present in the tunica media, can also trigger ECM remodeling processes due to their ability to synthesize ECM molecules and protease inhibitors. In addition, CVD resulting in ischemia can trigger VSMC which causes VSMC to secrete inflammatory and/or growth factors, VSMC apoptosis or can differentiate VSMC with a contractile phenotype to a proliferating synthetic phenotype. VSMC reside in the tunica media, but VMSC with a synthetic phenotype are able to migrate toward the intimal layer and contribute to the development vascular remodeling. Other cells primarily reside in the tunica adventitia e.g. dendritic cells (DCs), mast cells, natural killer (NK) cells and T and B cells[35], but mainly fibroblasts are present in the tunica adventitia.

Cells of the immune system

Cells in the tunica adventitia can either migrate towards the affected site or contribute to vascular remodeling from the perivascular region. Leucocytes, or white blood cells, are the main cell type in the tunica adventitia, besides fibroblasts, and can be divided in granulocytes (mast cells, neutrophils, basophils and eosinophils), monocytes (macrophages and dendritic cells) and lymphocytes (NK cells, T cells and B cells)(figure 1). Mast cells contain granules with histamine, heparin and cytokines which are released following mast cell degranulation after IgE activation,

and mediate inflammatory responses such as hypersensitivity and allergic reactions and in addition, vascular remodeling[36]. It is observed that activated mast cells can produce chemokines that are capable of neutrophil recruitment to affected areas. Neutrophils, abundantly present granulocytes (60% of all leucocytes), are able to phagocyte foreign cells and subsequently contribute to vascular remodeling together with mast cells[33]. Eosinophils and basophils are mainly involved in allergic reactions via the release of respectively IgE and histamine, however, in addition to mast cells they can increase vascular leakage and permeability[37].

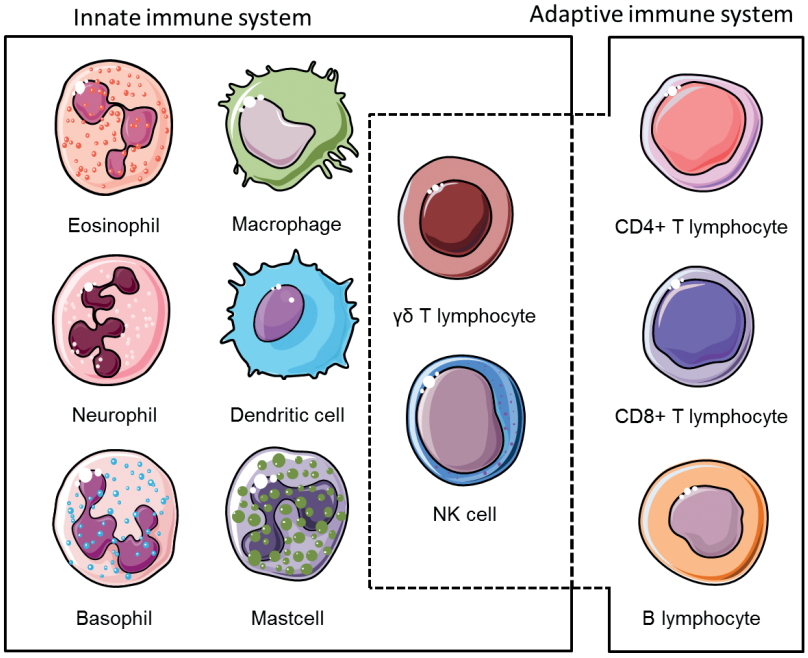

Figure 1. Cells of the innate and adaptive immune system. Natural killer cells and γδ T cells overlap both innate and adaptive immunity.

Dendritic cells are central to the initiation of primary immune responses and are antigen presenting cells (APC) which are able to process antigen material and present it to lymphocytes. Antigen presentation can be in draining lymph nodes, but also within the vessel wall. DCs are classical APCs, but in the vessel wall non-classical APC such as VSMC and EC, can also be functionally active[38]. Macrophages are phagocytic cells and also able to act as classical APC. Macrophages play a pivotal role in vascular remodeling. Monocytes can migrate towards inflamed vessels and adhere to (damaged) EC, mainly dependent on chemokines CCL2 and CCL5 and intercellular

adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). After extravasation monocytes accumulate and differentiate into macrophages. In the vessel wall lipid uptake causes macrophages to turn into foam cells and macrophage apoptosis can lead to necrotic core formation, both detrimental in vascular remodeling and atherosclerosis[39].

Lymphocytes of the adaptive immune system can be activated via (non)-classical APC. NK cells can be classified as a member of the innate immune system but due to the cytotoxic function, which resembles the cytotoxic T cell function, it is also a member of the adaptive immune system. NK cells can act rapidly after an infection by releasing perforins and granzymes which induce apoptosis of the intruding cell. This cytotoxic function makes NK cells critical regulators of vascular remodeling[40, 41].

T and B cells have been identified in the vessel wall and are differentially involved in vascular remodeling. Similar to B cells, T cells also demonstrated diverse functions in CVD due to the differential functions of CD4+ and CD8+ T cells and T cell subtypes.

Innate immunes system;

The innate immune system is a first line host defense mechanism against many common microorganisms[42]. Cells of the innate immune system, such as macrophages, mast cells and NK cells, detect microorganisms and subsequently release cytokines and inflammatory mediators to regulate inflammation. Microorganisms are recognized by pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs)[43]. TLRs are transmembrane PRRs and play a crucial roles in the innate immune system by recognizing pathogen-associated molecular patterns (PAMPs), which are molecular components of micro-organisms[44]. In addition, TLR recognise danger-associated molecular patterns (DAMPs) such as endogenous danger signals to alert other cells of the innate immune system. Multiple TLRs showed a role in CVD; a role for TLR4 in VGD and accelerated atherosclerosis has been established[45], TLR2 promotes atherogenesis[46], and blocking TLR7 and TLR9 showed reduced post interventional remodeling[47].

In humans, 10 different TLRs have been identified, whereas 12 distinct TLRs exist in mice. TLRs are either expressed on the cell surfaces recognizing mainly microbial membrane components or expressed in intracellular vesicles where they recognize microbial nucleic acids[44, 48]. After activation, TLRs signaling is either dependent on the TIR domain-containing adaptor molecule MyD88 or TRIF dependent/Myd88 independent [49] (figure 2). It is demonstrated that TLR3 signals exclusively via a TRIF

dependent pathway and TLR2 only via the Myd88 dependent pathway, while TLR4 signaling can be via either the MyD88 or TRIF pathway[44, 50]. It is assumed that the Myd88 dependent pathway mainly activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) which results in pro-inflammatory cytokine production. Whereas the TRIF pathway phosphorylates interferon regulatory factor (IRF) 3 and IRF7 which results in the production of type-I interferons (IFNs); IFNα and IFNβ. Although these are the most common signaling pathways, activation of pro-inflammatory transcription factors via IRF3 and IRF7 is not excluded[51, 52]. Previous studies showed a protective effect of IRF3 and IRF7 through inhibition of VSMC proliferation and neointima formation in a mouse carotid artery wire injury model and carotid artery balloon injury model[53, 54]. In addition, IRF3 and IRF7 were demonstrated to have angiogenic properties[55, 56]

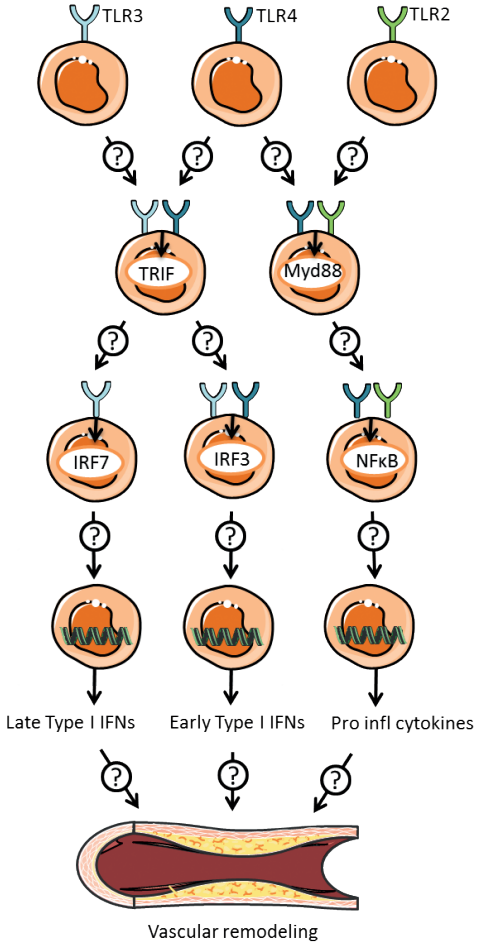

Figure 2. Hypothetic schematic overview of the signaling pathways of TLR2, TLR3 and TLR4, regulating vascular remodeling, that will be discussed in parts in this thesis. TLR = toll like receptor, TRIF = TIR-domain-containing adapter-inducing interferon-β, Myd88 = Myeloid differentiation primary response 88, IRF = Interferon regulatory factor, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, Type I IFN = Type I Interferon. Pro inf cytokines = pro-inflammatory cytokines.

In collaboration with the innate immune system, lymphocytes of the adaptive immune system have a highly important role in the development of CVD. The innate immune system is responsible for controlling pathogens and infections in the first days after contamination. Subsequently after 4–7 days, the adaptive immune response initiates. Some immune cells such as NK T cells and *γδ* T cells even have a function in both the innate and adaptive immune system.

Adaptive immune system; T cells, co-stimulation

The lymphocytes of the adaptive immune system provide increased protection against re-infection with the same pathogen via memory cells. B cell and T cell lymphocytes derive from hematopoietic stem cells in the bone marrow in naive form and develop into CD4+ or CD8+ T cells in the thymus.

In 1970 it was discovered that naive T cells require two signals for activation[57, 58]. The first signal is the interaction of the T cell receptor (TCR) with a cognate peptide-MHC complex on APCs[59, 60]. The second signal is provided via T cell co-stimulation which binds co-stimulation ligands expressed on APCs. Additionally, CD8+ T cells can be activated via bystander cytokines e.g. IL-12 and type-I IFNs[61].

After activation, naive T cells can differentiate into effector T cells that are actively involved in the elimination of a pathogen. After an infection, memory cells are formed that can act rapidly if a second infection occurs. Central memory cells and effector memory cells, called circulating memory T cells, are present in respectively secondary lymphoid organs and tissues. Tissue-resident, non-circulating, memory T cells, can also act rapidly if a second infection occurs. Once activated, T cells can be polarized into either Th1, Th2, Treg or Th17 cells. T cell subsets can induce differential functions in CVD via the production of either pro-or anti-inflammatory cytokines. Th1 cells, producing pro-inflammatory cytokines IFNγ and TNFα, are well-known for their pro-atherogenic effects. In contrast, Tregs, anti-inflammatory producing IL-10 and TGFβ, demonstrated mainly anti-atherosclerotic effects, [62-66] and to mitigate abdominal aortic aneurysms progression[67] and post-interventional restenosis[68, 69]. The functions of Th2 and Th17 cells are less clear. Th2 cells secreting IL-4, IL-5 and IL-13 and Th17 cells secreting IL-17A, IL-17F, IL-22 and IL-21, showed both pro- and anti-atherosclerotic effects are shown[70-76].

T cells are essential in neovascularization and are involved in the development of CVDs. However, CD4+ and CD8+ T cells can also have differential functions in CVD. Both CD4+ and CD8+ T cells have shown a specific role in arteriogenesis via respectively the attraction of monocytes and macrophages and subsequent productions of pro-

inflammatory cytokines[77, 78] or through the expression of IL-16[79]. CD8+ T cells are also involved in angiogenesis[80] and can promote the development of vulnerable atherosclerotic lesions[81] and enhanced atherosclerotic lesions[82].

Outline of the thesis

The aim of this thesis was to identify the role of the innate and adaptive immune system in neovascularization and VGD as well as the underlying mechanism. **Chapter 2** comprises a review on the pathology of VGD. In this review, we evaluate, by discussing both experimental and clinical studies, the pathophysiology behind vein graft failure and the latest therapeutic options to improve patency for both coronary and peripheral bypasses. In the review in **chapter 3,** the importance of co-stimulation and co-inhibition pathways in the pathogenesis of CVD is highlighted in (pre-) clinical studies e.g. VGD, myocarditis, graft arterial disease, post-ischemic neovascularization and atherosclerosis. The potential use of targeting co-stimulation or co-inhibition pathways, with immune checkpoint inhibitors, as a treatment for CVD, as well as the cardiovascular benefits and adverse events after treatment is discussed. Finally, we emphasize cardiovascular monitoring of patients treated with immune checkpoint inhibitors, since there is a close relation between treating cancer with immune checkpoint inhibitors and the risk of cardiovascular toxicity. **Chapter 4** describes the role of IRF3 and IRF7 in neovascularization. The main components of neovascularization, angiogenesis and arteriogenesis, are driven by ischemia, inflammation, cytokines and growth factors, which are thought to be regulated via IRF3 and IRF7. Therefore we hypothesise that IRF3 and IRF7 of the innate immune system are involved in neovascularization. In addition, it is known that T cells have a distinctive role in neovascularization. However, the role of co-stimulation, part of the adaptive immune system, in T cell activation in neovascularization has yet to be established. Therefore, **chapter 5** describes the role of T cell activation via co-stimulation in angiogenesis, arteriogenesis and vasculogenesis.

Chapter 6 focusses on TLRs as contributing factors in the development of VGD. Toll like receptors (TLRs) can be activated in vein grafts by endogenous ligands and activate the innate immune system. Therefore we hypothesise a role of TLRs in VGD. TLR2, TLR3 and TLR4 are described with specific attention to TLR3. **Chapter 7** provides an insight in the role of IRF3 and IRF7 in VGD. IRF3 and IRF7 are thought to be the transcriptional regulators of type-I IFNs and type-I IFN responsive genes and are downstream factors of TLRs. Relatively little is known with regard to the interplay of IRFs and TLRs in VGD development. Therefore, chapter 7 describes the role of IRF3 and IRF7 signaling downstream TLRs and the effect of IRF3 and IRF7 in VGD. **Chapter 8** describes the function of T cells of the adaptive immune system in the development

of VGD with specific attention to CD8+ T cells. In addition, we investigate T cell activation pathways via the TCR, co-stimulation and bystander cytokines *in vitro* and *in vivo*. **Chapter 9** summarizes all results described in this thesis and discusses future perspectives.

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