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

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General summery and future perspectives

Vascular remodeling is an active process of structural changes in the vasculature due to changes in the blood flow[1]. It comprises all diseases described in this thesis such as occlusive arterial disease (OAD), peripheral artery disease (PAD), coronary artery disease (CAD), neovascularization (angiogenesis and arteriogenesis) and vein graft disease (VGD), that are covered by cardiovascular diseases (CVD). The main underlying pathology of CVD is atherosclerosis which can narrow arteries due to atherosclerotic lesion formation and can cause ischemia distal to the occlusions[2]. Initially when atherosclerosis occurs, the body naturally prevents ischemia via neovascularization. Via the maturation of arterioles into functional arteries (arteriogenesis)[3-6] a natural bypass develops, and together with the sprouting of new capillaries from a pre-existing vasculature (angiogenesis)[7], the blood flow to the tissue distal of the occlusion is restored. However, when the neovascularization capability of the body is not sufficient and if left untreated, this can lead to limb amputation or death. Symptomatic OAD patients can be treated with revascularization interventions such as angioplasty, using a balloon catheter which can be inflated at the site of occlusion to relieve the narrowing of the artery. In case of severe atherosclerotic lesions, when angioplasty in no longer possible, bypass surgery is indicated. However, due to e.g. VGD the patency rates of vein grafts after 10 years are low.

The aim of this thesis was to elucidate the function of the innate and adaptive immune system in vascular remodeling. Currently there is no textbook treatment to improve neovascularization, or inhibit or prevent VGD. Therefore, investigating the underlying mechanism of vascular remodeling is essential to find targets to treat CVD. We found several targets of the innate and adaptive immune system that can give rise to the development of new therapeutic strategies.

In **chapter 2** we describe the cellular and molecular mechanisms behind the pathophysiology of vein graft failure (VGF) and review therapeutic options to improve the patency of both coronary and peripheral bypasses. Due to the serious consequences of vein graft failure, this needs appropriate attention. We described that to prevent early VGF due to thrombosis, antiplatelet and antithrombotic medication is recommended. For long term prevention of VGF statin treatment has been proven beneficial. New therapies like gene therapy and external stenting are promising but the positive effect on vein graft patency still needs to be proven. In addition to medication prevention strategies, vein graft harvesting, duration of the surgery, handling, size and condition of conduit and target vessel also influence the vein graft patency rates after surgery. Pre-clinical and clinical studies have gained

insight in the cellular and molecular processes of the mechanisms contributing to VGF, which can be interesting for future studies to find a treatment to prevent VGF.

Both the innate and adaptive immune system play an important role in the development of VGD, but an unequivocally effective treatment to target this has not been found yet. However, in the recent years several treatments to stimulate or inhibit T cells of the adaptive immune system have been developed. Modulation of the immune response towards malignant cells has been successfully introduced in cancer treatment and showed beneficial effects with the use of Immune Checkpoint Inhibitors (ICIs). ICIs can block co-inhibitory pathways on T cells, causing T cell activation which can results in tumor cell lysis. However, the role of co-stimulation and inhibitory pathways in CVD are poorly described, so the outcomes of the use of ICI in CVD are unknown. In the review in **chapter 3**, we provided an overview of the major co-stimulatory and inhibitory pathways in the pathogenesis of a number of CVD. Blocking of co-stimulation pathways, resulting in T cell anergy, might be interesting therapeutic targets for CVD. On the other hand, ICIs and stimulation of co-stimulation receptors, resulting in activated T cells, might poses serious cardiovascular risks. Thus, although ICIs are revolutionary in cancer treatment, the risk of cardiovascular toxicity should be taken into consideration. Subsequently, focussing on the inhibition of co-stimulation pathways in the future could be world-shattering for the treatment of CVD.

As neovascularization is an essential natural mechanism to prevention ischemia, we focussed on contributing mechanistic factors of neovascularization in **chapter 4** that might be suitable targets to stimulate neovascularization in symptomatic patients. We observed that interferon regulatory factor (IRF)3 and IRF7 of the innate immune system are inevitable involved in neovascularization. In mice deficient of IRF3 and IRF7 we found a decreased inflammatory respond distal to induced occlusions. In line with this, we observed a decrease in angiogenesis and arteriogenesis resulting in an impaired post-ischemic blood flow recovery. This confirmed the contribution of IRF3 and IRF7 to neovascularization. Furthermore, since IRFs regulated the inflammatory response, this indicates that there might be a linkage between the TRIF-IRF and TRIF-NFκB pathway.

In addition to the innate immune system, an involvement of T cells of the adaptive immune system was suggested in neovascularization. The contribution of co-stimulation in T cell activation in neovascularization was studies in **chapter 5**. Both the CD28-CD80/86 and CD27-CD70 T cell co-stimulation pathways were investigated in a hind limb ischemia model with the use of $CD70^{-/-}$, $CD80/CD86^{-/-}$ and $CD70/80/86^{-}$

$^{/-}$ mice. Post-ischemic blood flow recovery was only impaired in mice lacking CD70. In mice lacking CD70, the embryologic development of the circulatory system was already compromised, since we observed an impaired vasculogenesis, as the number of pre-existing collaterals was reduced in the pia mater and in the skeletal muscle. In addition, angiogenesis in soleus muscles 10 days after ligation was also impaired in mice lacking CD70, which all contributes to a diminished post-ischemic blood flow recovery. Interestingly, the CD27-CD70 T cell co-stimulation pathway, and not the CD28-CD80/86 T cell co-stimulation pathway, validated their purpose in neovascularization

As mentioned before, if the body is incapable of sufficient neovascularization to prevent ischemia after arterial occlusions, venous bypass grafting is a surgical option. However, VGD halts high patency rates. In **chapter 6** we focused on the innate immune system in the development of VGD. In the pathophysiological processes of VGD, Toll like receptors (TLR) are considered to be important accelerating factors via the activation of an inflammatory response. We hypothesised that especially TLR3 is essential in the development of VGD as these TLRs are activated via dsRNA derived from damaged cells. During harvesting and preparation of the vein graft, during and after the surgical procedure, vascular cells are damaged and can release dsRNA. Indeed, TLR3 was involved in VGD development and we even showed a prominent protective role of TLR3 compared to control, TLR2 and TLR4 deficient mice. An increased vein graft thickening in TLR3 deficient mice was accompanied by an increase in macrophages in the vessel wall. We provide evidence that the molecular mechanism of the TLR3-induced protective effect is dependent on the production of type-I IFNs. A regulation of the mRNA expression of type-I IFN regulated genes via TLR3 in vein grafts and *in vitro* in BMM was observed. To validate that this protective effect in VGD development was regulated via type-I IFNs, the role of IRF3 and IRF7 in VGD development was investigated in **chapter 7**. IRF3 and IRF7 are downstream factors of TLRs and regulate the transcription of type-I IFNs; IFNα and IFNβ. We showed that IRF3 and IRF7 are both activated downstream TLR3 and able to produce type-I IFNs, but IRF3 can also be activated downstream TLR4 and regulate the production of inflammatory cytokines *in vitro*, in VSMCs and macrophages. It was observed that IRF3 and IRF7 deficient mice have similar protective effects in the development of VGD as TLR3 deficient mice, but IRF3 and IRF7 have different effects on VGD development in time. IRF3 is an early regulator of type-I IFNs, whereas IRF7 regulated late type-I IFN production. Thus, the data in chapter 6 and 7 suggest that the TLR3-IRF3/IRF7-type-I IFN signaling pathway can be a novel mechanism via which VGD development can be prevented.

In **chapter 8** we investigated the role of the adaptive immune system, in addition to the innate immune system. CD4+ and CD8+ T cell and their activation pathways via the T cell receptor (TCR), co-stimulation and bystander cytokines were investigated. Among the top 35 of significantly up-regulated gene sets found in vein grafts were TCR and CD8+ T cell pathways. We demonstrated *in vivo* in a vein graft mice model that both CD4+ and CD8+ T cells are abundantly present in vein grafts, with a higher percentage of CD8+ T cells. Interestingly, depletion of CD8+ T cells resulted in occluded vein grafts after 28 days, showing that CD8+ T cells mediate a robust protective role in VGD. We questioned whether antigenic triggering via the TCR is required for CD8+ T cells activation in vein grafts. Interestingly, after vein graft surgery was performed in TCR transgenic OT-I mice and control mice, we found similar vein graft patency rates and CD8+ T cell activation, showing that CD8+ T cell activation in vein grafts is TCR independent. Furthermore, bystander T cell activation-inducing cytokines are present in the vein grafts and these cytokines are collectively able to activate CD8+ T cells. A synergistic effect of bystander cytokines to activated CD8+ T cells was observed with co-stimulation signals. Knocking out co-stimulation exhibited a modulating role of co-stimulation in VGD. Thus, T cells are modulators of the development of VGD with a specific and local protective role of CD8+ T cells. Activation of the CD8+ T cells in vein grafts is TCR independent and bystander signals via cytokines and additionally co-stimulation are imperative.

Future perspectives

In this thesis, we reviewed that the therapeutic options to improve the patency of both coronary and peripheral bypasses and stimulate neovascularization, are insufficient. Therefore, we investigated the role of several components of the innate and adaptive immune system on vascular remodeling. TLRs, IRFs, type-I FNs of the innate immune system, as well as T cells and T cell co-stimulation of the adaptive immune system were identified as essential regulators of vascular remodeling. This gives rise to potential new strategies to use these targets in a therapeutic approach to prevent or treat CVD.

Most components that we investigated show a wide variety of interactions due to their serried signaling pathways as shown in figure 1. TLR3 stimulation can activate a TRIF dependent pathway, leading to the phosphorylation of IRF3 and IRF7. Subsequently IRF3 and IRF7 produce type-I IFNs, IFNα and IFNβ. TLR2 stimulation can activate a Myd88 dependent pathway, while TLR4 signaling can be via either the MyD88 or TRIF pathway. The Myd88 dependent pathway mainly activates NFκB which results in pro-inflammatory cytokine production, such as the innate immune related TNFα and CCL2, but also IL12 and IL2, which are potent inducers of the adaptive

immune response. Pro-inflammatory cytokines IL12, IL2 and type-I IFNs, IFNα and IFNβ can bind to their receptor present on T cells, respectively IL12R, IL2R and IFNAR. Together with the TCR and/or co-stimulation molecules, these bystander cytokines can induce T cell activation. As we showed, this can be independent of the TCR in CD8+ T cells. The signaling pathways downstream TLR2, TLR3 and TLR4 connect all components of the innate and adaptive immune system. Interestingly, we showed beneficial effects of TLR3, IRF3, IRF7 and CD8+ T cells on vascular remodeling, which are all in sequentially involved in this signaling pathway.

With regard to IRF it might be interesting to see the effect of type-I IFNs directly on vascular remodeling. We show that they are produced via the innate immune system and they can induce T cell activation, however they might also have a direct effect on vascular remodeling. This might be therapeutically interesting, since IFNs are already therapeutically available. IFNs are used to treat renal cell cancer, malignant melanoma, multiple myeloma, multiple sclerosis and some types of leukaemia[8, 9]. The therapeutic use of IFNs is based on their antiviral and anti-proliferative effect via the activation of cytotoxic CD8+ T cells and can be injected easily subcutaneously or intraperitoneal[9, 10]. IFNs have been shown to increase class I MHC expression on tumor cells, activating CD8+ T cells that subsequently attack tumor cells. If IFN can also protect arteries to occlude or stimulates neovascularization, might be interesting to investigate. However, as we showed in chapter 7, the IFN treatment period after surgery should also be determined, since IRF3 and IRF7 are differently functional in time.

In addition to type-I IFNs, we showed that bystander cytokines IL2, IL12, IL18 and IL33 are cooperatively capable to activate CD8+ T cells in a bystander-mediated fashion and in synergy with co-stimulation. In chapter 8 a protective effect of CD8+ T cells in VGD was suggested, since CD8+ T cell depletion caused fully occluded vein grafts. Clinically it would be interesting to investigate the effect on VGD after local CD8+ T cell activation. This could be established via a CD8+ T cell expansion in vein grafts via a cocktail of bystander cytokines and co-stimulation, or monoclonal antibodies that can induce a clonal CD8+ T cell expansion. However, a clonal CD8+ T cell expansion should only be induced locally, since systemic clonal CD8+ T cell expansion could result in serious adverse effects. For future experiments it would be interesting to test several local delivery strategies, to create a clonal expansion of CD8+ T cells via bystander cytokines. Local delivery is mainly interesting for VGD, since (open) harvesting of the saphenous vein gives therapeutic opportunities without systemic side effect. For local delivery of e.g. IL2-IL12-IL18, l*entiviral vectors or adeno-associated viral (AAV) vector could be used. In addition to viral vectors, monoclonal* antibodies that induce a clonal CD8+ T cell expansion could be tested. Previously it was demonstrated that anti-4-1BB mAb is a

successful rheumatoid arthritis treatment. Anti-4-1BB mAb induced a clonal expansion of $CD8^+CD11c^+$ T cells which produced IFNγ and subsequently suppressed CD4+ T cells through an indoleamine 2,3-dioxygenase-dependent mechanism[11]. Therefore, anti-4-1BB might also be beneficial in vascular remodeling.

The recently published CANTOS trial regained attention to the immune system as a potential target for CVD, by using IL1β inhibitor canakinumab as a treatment for atherosclerosis[12]. If the effect of canakinumab is specifically due to the inhibition of IL1β, a different approach to block IL1β may also be used. Targeting the inflammasome may be beneficial in vascular remodeling. The inflammasome is a component of the innate immune system and is a protein complex that regulates the activation of the caspase-1. Activated caspase-1 functions to cleave the pro-inflammatory IL-1 family of cytokines into their bioactive forms, IL-1β and IL18[13]. Targeting the inflammasome may reduce both IL-1β and IL18, which we observed as a key cytokine in addition to IL12 and IL2 to activate CD8+ T cells, and thereby reduce or even prevent CVD. Monocytes, VSMC and EC express Il-1β and IL18 receptors and can proliferate and migrate after activation and thereby contribute to vascular remodeling. In addition, inflammasome activation causes pyroptosis, a rapid type of inflammatory cell death. There are different forms of inflammasomes containing a Nod-like receptor, of these NLRP3 is the most well know and unique receptor[14]. An inflammasome inhibitor targeting NLRP3 may be an interesting new target for CVD via the inhibition of vascular remodeling.

Clinical application of our knowledge obtained in the pre-clinical studies described in this thesis, could be via stimulation or inhibition of TLRs, IRFs, type-I IFNs or T cells. TLR stimulation might be a useful and realistic therapeutic target, since this receptor is constantly expressed on APCs, whereas phosphorylated IRFs translocate to the nucleus and activated the transcription of IFNα and IFNβ, which makes IRFs harder to target. TLR signaling can be induced via endogenous or exogenous ligands, however, this is not clinically applicable yet. TLR treatments should focus on differential methods due to the variety in CVD, e.g. the pathophysiology of neovascularization is different than VGD. For VGD, local stimulation or inhibition of TLRs is optional, since (open) harvesting of the saphenous vein gives therapeutic opportunities without systemic side effect. For neovascularization, soluble, injectable treatments should be investigated, to stimulate neovascularization in the lower legs.

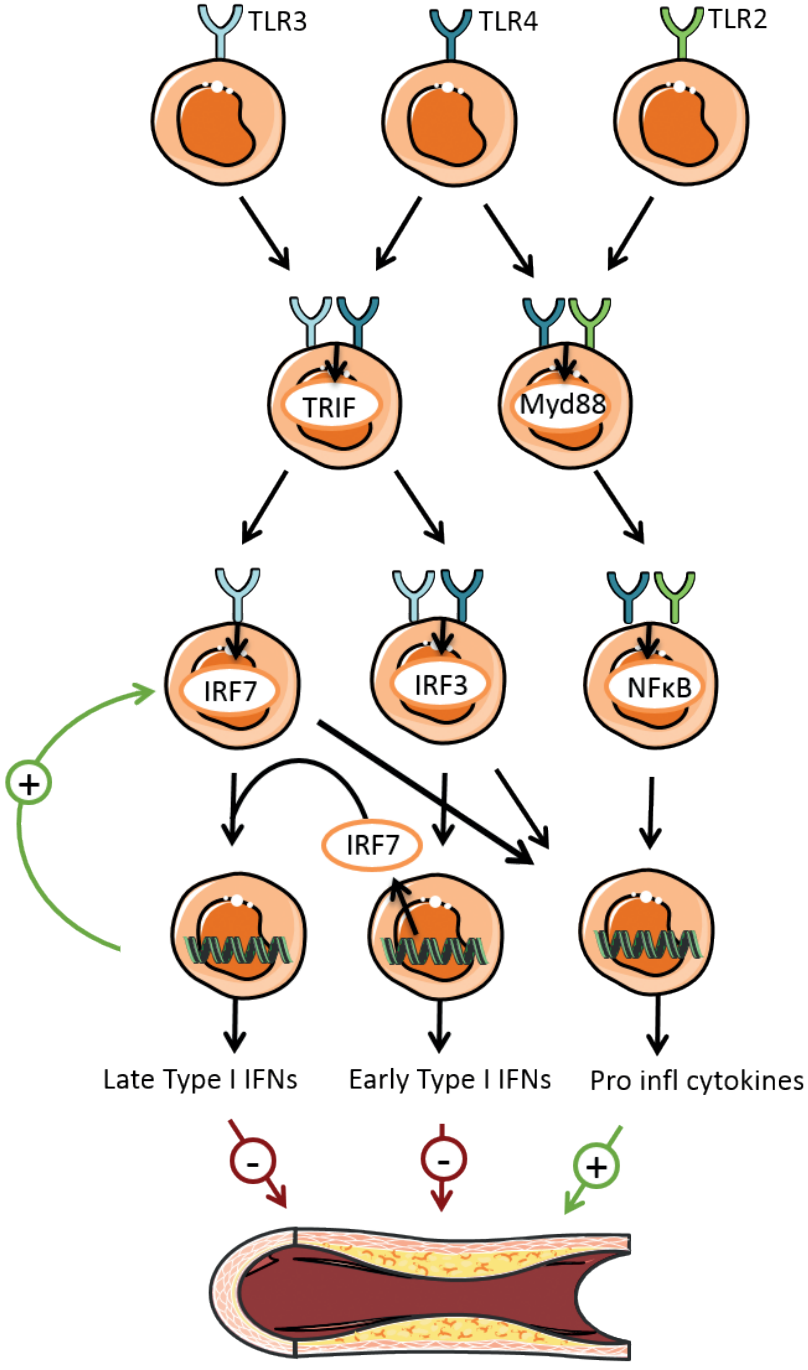
Recently nanoparticles were introduced as a promising new strategy for co-delivery of genes and therapeutic agents[15]. Drugs, e.g. targeting TLRs, IRFs or T cells, conjugated to nanoparticles could aid targeted delivery[16]. Seijkens et al. already successfully used

nanotherapy to target the co-stimulation CD40-TRAF pathway in an atherosclerotic mice model, which resulted in a decrease in atherosclerotic plaque development[17]. In addition, Sokolova et al. incorporated TLR3 ligand PolyI:C into calcium phosphate nanoparticles to treat viral infections[18], which can be interesting for future treatments of VGD and neovascularization via TLR3.

As extensively reviewed in chapter 3, blocking of co-stimulation pathways and stimulating co-inhibition pathways, could be interesting therapeutic targets for CVD. However, opposing therapies, resulting in activated T cell, revolutionized cancer treatments e.g. immune checkpoint inhibitors (ICIs) blocking the inhibition pathways and stimulators of co-stimulation pathways. ICIs may induce cardiovascular toxicity via the activation of T cells, thus the risk of cardiovascular toxicity should be taken into consideration and investigated. Future pre-clinical studies should focus on beneficial treatments for CVD; therapeutic targeting of co-stimulation via e.g. abatacept and belatacept blocking CD80/86, CD40-TRAF6 inhibitor blocking CD40, anti-OX40 blocking the OX40-OX40L interaction or anti-4-1BB antibodies blocking 4-1BB. Clinical studies should teach us whether the cardiovascular outcomes of therapeutic targeting of co-stimulation are as promising as expected.

In conclusion, in this thesis we obtained new knowledge on several contributing factors in vascular remodeling. Described molecules of the innate and adaptive immune system might be used as targets to prevent vein graft failure and stimulate neovascularization and this thesis can serve as a fundamental basis for the development of new medication in the future.

> **Figure 1.** Schematic overview of the discovered signaling pathways of TLR2, TLR3 and TLR4, regulating vascular remodeling in this thesis. In the pathophysiological processes of VGD, TLRs are considered to be important accelerating factors via the activation of an inflammatory response. In chapter 6 we showed that TLR3 was involved in VGD development and we even showed a prominent protective role of TLR3 compared to control, TLR2 and TLR4 deficient mice. TLR3, and partly TLR4, signals via a MyD88-independent pathway with a central role for TRIF and IRF3 and IRF7 resulting in induction of type-I IFNs, such as IFNα and IFNβ. TLR4 can also signal via the MyD88 pathway, similar to TLR2 and most other TLRs, which drives the induction of NFκB, resulting in induction of pro-inflammatory cytokines. We provide evidence that the molecular mechanism of the TLR3-induced protective effect is dependent on the production of type-I IFNs. IRF3 and IRF7 are downstream factors of TLRs and regulate the transcription of type-I IFNs; IFNα and IFNβ. We showed that IRF3 and IRF7 are both activated downstream TLR3 and able to produce type-I IFNs, but IRF3 can also be activated downstream TLR4 and regulate the production of pro-inflammatory cytokines. IRF3 and IRF7 deficient mice have similar protective effects in the development of VGD as TLR3 deficient mice, but IRF3 and IRF7 have different effects on VGD development in time. IRF3 is an early regulator of type-I IFNs, whereas IRF7 regulated late type-I IFN production. 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.


TLR3
TLR4
TLR2
TRIF
Myd88
IRF7
IRF3
NFκB
IRF7
+
Late Type I IFNs
Early Type I IFNs
Pro infl cytokines
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