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## **Modulation of vascular remodeling : a role for the immune system, growth factors, and transcriptional regulation**

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## **Bone marrow derived cells in arteriogenesis: a crucial role for leukocytes**

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## **Abstract**

In recent years cell therapeutic approaches for induction of arteriogenesis, including injection of autologous bone marrow, have gained popularity for experimental treatment of patients with coronary or peripheral arterial ischemic disease. However, beneficial results of these cell therapies in patients remain inconsistent to date. Evidence points towards a supportive paracrine role of bone marrow derived cells by secretion of various pro-arteriogenic cytokines and growth factors. Moreover, inflammatory cells, originating from the bone marrow, are recently pointed out as the key regulators of arteriogenesis. This chapter focuses on the role of leukocytes in arteriogenesis. In particular, the role of monocytes, natural killer cells and T-cells, including various subpopulations of T-cells, are addressed. Activation (or inhibition) of selective leukocyte subpopulations may ameliorate pro-arteriogenic cell therapy in the future.

## **Introduction**

In the past ten years, therapeutic arteriogenesis using bone marrow derived cells has become a promising treatment for patients with either coronary or peripheral ischemic disease [1-6]. Although initial reports of autologous bone marrow transplantation for patients with limb ischemia were very promising [7], beneficial results of cell therapy in patients with peripheral arterial disease (PAD) are not very consistent to date [8]. Although the concept of the development of adult collateral arteries from a pre-existing arteriole network is indisputable for therapeutic intervention, the exact cellular mechanism underlying arteriogenesis remains unclear. There are different hypotheses on the role of bone marrow derived cells in arteriogenesis. Nowadays, evidence points towards a more supportive paracrine role of bone marrow derived cells by secretion of various factors instead of the initially proposed role in promoting arteriogenesis by incorporating into the vessel walls [9]. Also the role of different inflammatory cells, originating from the bone marrow, has extensively been studied in order to evolve a more efficient cell therapeutic approach than infusion of the total bone marrow derived cell fraction. Previously, numerous animal studies have established that ischemia induced angiogenesis and shear stress induced arteriogenesis can be enhanced with different types of leukocytes, cytokines and growth factors. The molecular and cellular mechanisms that play a role in collateral artery formation start to become unravelled more recently. Increased shear stress induces arteriogenesis by stimulating the attraction, adhesion and invasion of circulating inflammatory cells [10] such as monocytes, T-lymphocytes and NK-cells. Growth factors that are already present in the ischemic tissue, as well as those that are produced by the invading cells play an important role too. Recently, also subpopulations of T-lymphocytes, such as CD4+ T cells, CD8+ T cells and CD4+CD25+FoxP3+ T cells and their role in arteriogenesis have been studied in more detail. This chapter will focus on the role of leukocytes in collateral artery formation.

### **Monocytes in arteriogenesis**

Monocytes play a crucial role in mediating neovascularization in response to increased shear stress and ischemia. Already in the 1970s monocytes were found to invade the vessel wall of developing coronary collateral arteries [11,12]. Later on, functional proof for the participation of monocytes in arteriogenesis has been reported by the results that after increasing blood monocyte concentration in a hind limb ischemia mouse model, arteriogenesis is enhanced [11-13]. In contrast, after depletion of monocytes, collateral artery formation is much attenuated [12]. Increased shear stress, due to the arterial occlusion, activates the vascular endothelium. The activated endothelial cells upregulate the monocyte chemoattractant MCP-1 and adhesion molecules (eg. ICAM-1 and VCAM-

1) to which the receptor of monocytes binds [14,15]. This leads to monocyte adhesion and infiltration with the subsequent production of growth factors and proteases. In the next paragraph we will discuss these aspects of monocytes in arteriogenesis into more detail. The local production of cytokines and chemokines within the first 3 days after ischemia mediates the recruitment of monocytes to the ischemic area [16-19]. To stimulate arteriogenesis, monocytes must home and retain around the arterial occlusion. There is evidence that MCP-1, but also VEGF and SDF-1 $\alpha$  contribute to the recruitment and retention of monocytes [19-23]. For example, local infusion of MCP-1 [24] or MCP-1 gene transfer [25] in a hind limb ischemia rabbit model increase the local number of monocytes in the ischemic area and enhance revascularization. Voskuil et al. [26] reported impaired monocyte recruitment after hind limb ischemia in MCP-1 $^{-/-}$  mice. This effect was reversed with treatment of the MCP-1 protein. Furthermore, Heil et al. [27] showed that disruption of the MCP-1 receptor (CCR2) in CCR2 $^{-/-}$  mice leads to an impaired blood flow restoration after femoral artery ligation. This impairment was based on a reduced migration capacity of these monocytes.

The interaction of the monocyte with the activated endothelium is a complex multistep process. Auffray et al. [28] reported recently the importance of the role of monocytes in the initial recruitment of blood monocytes. They observed a subset of monocytes that could crawl on endothelial cells. These resident monocytes patrol healthy blood vessels and allow rapid tissue invasion by monocytes in case of ischemic injury or tissue damage [28]. Two integrins are responsible for the interaction of monocytes with endothelial cells: Mac-1 and LFA. These integrins interact with adhesion molecules on the endothelial surface, like selectins, intercellular adhesion molecules (ICAM-1 and ICAM-2) and vascular adhesion molecules (VCAM-1) [14]. It was demonstrated that factors like MCP-1 and VEGF, released by the activated endothelial cells, can increase the expression of integrins on monocytes and enhance monocyte adhesion [29-31]. After monocyte adhesion, integrins also mediate transmigration of monocytes into the perivascular tissue. Hoefer et al. showed that *in vivo* treatment with antibodies against ICAM-1 blocked collateral artery growth [32].

For invading into deeper vessel wall layers, monocytes can use their capacity to produce proteases such as matrix-metalloproteinases and u-PA. Their proteolytic activity can create gaps by which monocytes can migrate further into the vessel wall [33-35]. After transmigration into the perivascular tissue, monocytes differentiate into macrophages and secrete growth factors and cytokines that attract other inflammatory cells and stimulate smooth muscle cell proliferation and endothelial cell mitosis, both necessary for collateral growth [36]. One of the pro-inflammatory factors secreted by monocytes and macrophages is TNF- $\alpha$ . In a rabbit hind limb ischemia model, it is demonstrated that arteriogenesis is attenuated after treatment with TNF- $\alpha$  inhibitors, most likely due

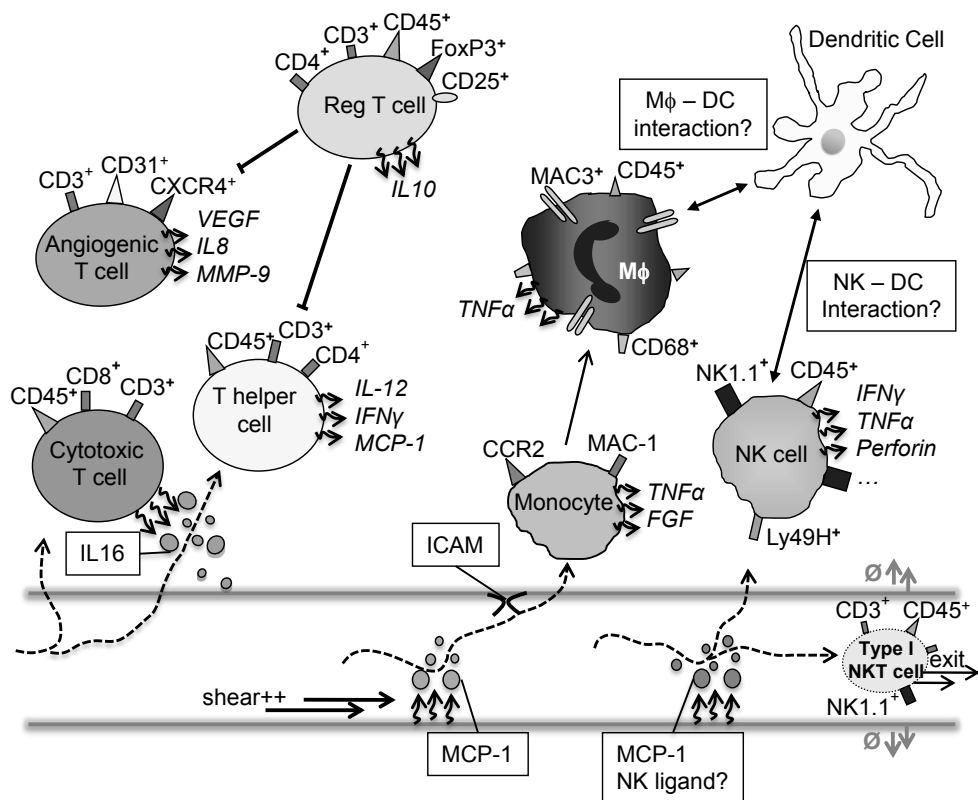
to the inhibition of leukocyte infiltration around collateral arteries and lower vascular smooth muscle cell proliferation [37]. Also fibroblast growth factors (FGF), provided by monocytes in a paracrine way, do have a stimulating effect on arteriogenesis [13].

The potential of transplantation of monocytes to stimulate collateral artery formation in the clinical situation has now been studied by using the hind limb ischemia animal model. Herold et al. [15] showed that although autologous monocytes only demonstrated a marginal increase in arteriogenesis, their transduction with GM-CSF before transfusion into the hind limb ischemia model resulted in a robust stimulation of arteriogenesis. Also Urbich et al. [38] provided evidence that transplantation of engineered monocytes represents a highly effective therapeutic approach to stimulate collateral artery formation. In contrast, monocytes injected directly after isolation, without any stimulation, did not show any stimulating effect on arteriogenesis. Isolation of monocytes can easily be done in humans by leukapheresis and these cells have been safely administered for patients with malignancies. The strategy of transplantation of pro-arteriogenic monocytes seems feasible, but further research towards the side effects (e.g. pro-atherogenic effects) is warranted.

### **T cells in arteriogenesis**

More recently, it was found that other leukocytes than monocytes also contribute to collateral artery formation. T lymphocytes also play a role in many vascular diseases ranging from a pro-atherogenic role in atherosclerosis towards a beneficial role in arteriogenesis. Couffinhal [39] showed that nude mice, which are deficient for all types of mature T lymphocytes, did have a hampered capacity to form collateral arteries after induction of hind limb ischemia. The role of T lymphocytes in arteriogenesis has recently been studied more extensively, with focus on arteriogenic function of specific subpopulations of T lymphocytes. Although the exact role is not completely understood yet, we will discuss the contribution of CD4<sup>+</sup> T cells (Helper T cells), CD8<sup>+</sup> T cells (Cytotoxic T cells), CD3<sup>+</sup>CD31<sup>+</sup>CXCR4<sup>+</sup> T cells (angiogenic T cells) and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (regulatory T cells). See Figure 1 for a schematic representation.

The importance of CD4<sup>+</sup> T cells in collateral artery formation has been demonstrated recently. CD4<sup>-/-</sup> mice, which do have a normal development of CD8<sup>+</sup> T cells and myeloid cells showed a markedly reduction in ischemia-induced collateral artery formation. Compared to controls, there was a 25% decrease in blood flow restoration after femoral artery ligation during 28 days in these CD4<sup>-/-</sup> mice [40]. In a rescue experiment, CD4<sup>-/-</sup> mice received spleen-derived purified CD4<sup>+</sup> T cells and restored blood flow recovery after ischemia to the levels of wild type mice [40]. Additionally, CD4 depleted mice, show significantly impaired blood flow restoration after femoral artery ligation [41]. Furthermore, MHC class II deficient mice, which are characterized by the selective lack of the maturation of CD4<sup>+</sup> T cells, display impaired arteriogenesis too [41].



**Figure 1.** Involved leukocyte subsets in outward remodeling of collateral arteries. Cytotoxic CD8<sup>+</sup> T cells invade the vessel wall early and secrete IL-16, which in turn attracts CD4<sup>+</sup> T helper lymphocytes. These CD4<sup>+</sup> T helper lymphocytes secrete a variety of pro-arteriogenic cytokines IL-12, IFN $\gamma$  and the chemoattractant MCP-1. The latter induces of perivascular monocyte recruitment. Monocytes adhere to activated endothelium, which expresses increased levels of ICAM-1 and VCAM-1 molecules. Next, monocytes invade in the vessel wall, excrete growth factors such as Fibroblast Growth Factor (FGF) and cytokines such as TNF $\alpha$ . Furthermore, monocytes can differentiate towards macrophages. NK cells are also attracted by MCP-1, and probably via NK ligands that are produced upon endothelial cell stress. In the vessel wall NK cells are supposed to act as cytokine factories by secreting an array of pro-arteriogenic cytokines; IFN $\gamma$ , TNF $\alpha$  and the cytolytic perforin. Furthermore, they might have relevant interactions with dendritic cells. Recent experiments did not demonstrate involvement of type I NK-T cells in arteriogenesis. Regulating T lymphocytes inhibit the pro-arteriogenic action of CD4<sup>+</sup> T helper lymphocytes and of the angiogenic CD3<sup>+</sup>, CD31<sup>+</sup>, CXCR4<sup>+</sup> T lymphocytes. The latter are attracted by hypoxia derived factors such as SDF-1 and have the ability to secrete VEGF, IL8 and MMP-9. The subtle balance of this inflammatory cascade should optimally lead to enlargement of the collateral diameter. See color figure on page 213.

The most likely mechanisms contributing to the arteriogenic-enhancing effects of CD4<sup>+</sup> T cells appears to be that CD4<sup>+</sup> T cells play an important role in the classic immune response in the ischemic area. In wildtype C57BL/6 mice, CD4<sup>+</sup> T cells accumulate around collateral arteries within 3 to 7 days after ligation of the femoral artery [41]. It is believed that perivascular presence of T cells is necessary to destroy cells to create space for collateral growth [42]. Also exogenous CD4<sup>+</sup> T cells home to the inflammatory cell infiltration in the ischemic hind limb within 24 hours after transfusion. In contrast, CD4 negative cells do not have selective homing to the ischemic hind limb [40]. CD4<sup>+</sup> T cells secrete various cytokines for example to induce monocytes/macrophage accumulation in the ischemic muscle [43]. These monocytes and macrophages secrete large arrays of cytokines and growth factors which further facilitate arteriogenesis [43]. Recently, van Beem et al. [44] described the role of CD4<sup>+</sup> T cells in the stimulation of CD14<sup>+</sup> monocytes to differentiate into a pro-angiogenic cell type, namely endothelial progenitor cell colonies (CFU-ECs). Paracrine factors produced by activated CD4<sup>+</sup> T cells present in the ischemic tissue facilitate CFU-EC formation. The exact cocktail of soluble factors stimulating CD14<sup>+</sup> monocytes and the role of these CD4<sup>+</sup> T cell - stimulated monocytes need to be further investigated.

The role of CD8<sup>+</sup> T cells in arteriogenesis is demonstrated by an impaired blood flow restoration in CD8<sup>-/-</sup> mice after hind limb ischemia. An attenuated blood flow persisted from day 3 till day 28 after femoral artery ligation compared to controls. After transfusion of exogenous CD8<sup>+</sup> T cells into CD8<sup>-/-</sup> mice, the impaired blood flow recovery after ligation was rescued [45]. CD8<sup>+</sup> T cells are one of the first cells involved in collateral artery formation [45]. When CD8<sup>+</sup> T cells infiltrate into the ischemic muscle, they express IL-16, which is an important chemo-attractant for several immune cells such as monocytes, eosinophils and dendritic cells [46-48]. Similarly, IL-16 participate in the recruitment of CD4<sup>+</sup> T cells since it has been shown that IL-16 is a natural ligand for CD4 [48].

More recently, the role of a specific subset of CD4<sup>+</sup> T cells, the regulatory T cells, has been studied. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells constitute 10% of CD4<sup>+</sup> T cells in the peripheral blood. This T cell population is specialized to suppress immune responses and contributes to the maintenance of immunological self-tolerance and immune homeostasis. In addition, these regulatory T cells do have an important function in the T cell homeostasis and suppress T cell responses against self-antigens or foreign antigens. Interestingly, it has been shown that regulatory T cells participate in the control of the development of atherosclerotic lesions. The role of regulatory T cells in ischemia-induced collateral artery formation has been recently studied by Zougari et al. [49]. They modulated regulatory T cell levels by intervening in the B7/CD28 interaction, which is required for regulatory T cell generation and homeostasis. In this regard, hind limb ischemia was induced in CD28<sup>-/-</sup> and B7-1/2<sup>-/-</sup> mice, which are deficient for



regulatory T cells. In these mice, a 1.2 to 2.0 fold increase in neovascularization after hind limb ischemia induction was found. In line with this, collateral artery formation was also increased in anti-CD25-treated mice compared to controls [49]. Taken together, regulatory T cell reduction with B7- or CD28 deficiency or anti-CD25 treatment, increases post-ischemic neovascularization. So, regulatory T cells play an important role in arteriogenesis, most probably by controlling the effector immune cell response [49]. Modulation of the regulatory component of post-ischemic inflammatory cell response, could provide a novel level of therapeutic intervention for pro-arteriogenic strategies for the treatment of PAD.

Another subpopulation of T cells, CD3+CD31+CXCR4+ T cells, has been suggested as a potential target for ischemic cardiovascular diseases. This cell is also referred as angiogenic T cell in literature. Hur et al. [50] report that the central cluster of endothelial progenitor cells (EPCs), which play an important role in neovascularization [51,52], is composed of CD3+CD31+CXCR4+ T cells. These angiogenic T cells are required for colony formation and early EPC differentiation as was investigated by depletion and adding these T cells during EPC-culture.

Furthermore, CD3+CD31+CXCR4+ T cells secrete various pro-angiogenic cytokines such as VEGF, IL-8, IL-17 and granulocyte colony-stimulating factor. Moreover, these cells also secrete MMP-9 which is known to play an important role in angiogenesis related extracellular matrix degradation. *In vitro* experiments as tube formation assays, as well as *in vivo* experiments further illustrate the pro-angiogenic capacity of CD3+CD31+CXCR4+ T cells. Infusion of CD3+CD31+CXCR4+ T cells in a hind limb ischemia mouse model improved blood flow recovery significantly. Clinical studies showed that the level of angiogenic T cells in peripheral blood MNCs is decreased when risk scores for cardiovascular disease are increased. This stresses the clinical relevance of this subpopulation T cells and further research towards this cell as target for cell therapy or as biomarker of cardiovascular disease is necessary [50].

### **Natural killer cells in arteriogenesis**

Natural Killer (NK) cells were recently proven to contribute to arteriogenesis as well [41]. As explained previously, collateral growth is initiated by shear stress induced release of chemo-attractants, such as MCP-1. Not only is MCP-1 a potent chemo-attractant for monocytes [24,53] but also for NK cells [54].

The role of NK cells in processes of vascular remodeling, e.g. atherosclerosis [55] but also remodeling of spiral arteries in the placenta [56] was already described. Mice deficient for functional NK cells have reduced atherosclerotic lesions and mice deficient for uterine NK (uNK) fail to modulate spiral arteries feeding the placenta.

The role for NK cells in arteriogenesis was established when these cells were found to accumulate around collateral arteries and by the observation of impaired arteriogenesis following femoral artery ligation in mice depleted or deficient for NK cells [41]. Unlike a well established role for NK-T cells in atherosclerosis [57], this could not be proven for type 1 NK-T cells in arteriogenesis. Especially since J-alpha281-knockout mice, that selectively lack V-alpha14 NK-T cells, did not show impaired arteriogenesis after femoral artery ligation [41]. However, the role of type 2 NKT cells is unclear.

NK cells are cytolytic effector cells of the innate immune system primarily involved in the defense against infectious pathogens, especially viruses. Their state of action is determined by dual signaling via inhibitory and activating surface receptors [58]. Many of these receptors, e.g. NKG2D, NKrp1c (NK1.1), CD94/NKG2, and the highly polymorphic Ly49 receptor family, code for in the Natural Killer gene complex (NKC) [58,59]. Ly49 and CD94/NKG2 receptors are expressed in a stochastic and independent fashion, which results in NK cell subsets expressing distinct combinations of these receptors. NK cell function is, in part, based on the interaction of their inhibitory receptors with Major Histocompatibility Complex class I (MHC I) molecules, expressed on the target cell surface in a complex with  $\beta$ 2-microglobulin ( $\beta$ 2m) [60]. Often, activating NK cell receptors recognize inducible molecules on the target cell, which can be induced upon cellular stress such as hypoxia or infection [61].

Upon activation NK cells produce a variety of cytokines, e.g. perforin, TNF- $\alpha$ , TNF- $\beta$ , IL10 and most dominantly IFN- $\gamma$  and sometimes even vascular growth factors [62]. By their IFN- $\gamma$  production, NK cells are an early source important in T-helper 1(Th1) polarization [63] which is associated with profound vascular remodeling, unlike Th2 that leads to less profound vascular remodeling [64].

The Th1 response leads to rapid arteriogenesis in C57BL/6 mice, whereas BALB/c mice respond poorly due to a Th2 response. Introduction of C57BL/6 genes into BALB/c mice by the creation of a C57BL/6 x BALB/c F1 led to significant improved arteriogenesis when compared to the parent BALB/c strain [41]. This indicates that BALB/c mice are lacking a crucial factor for proper arteriogenesis.

The genetic differences between C57BL/6 and BALB/c mice also include a relevant difference in the content of the above-mentioned Natural Killer gene complex (NKC), as BALB/c mice lack a 200 kb region. This region codes for members of the Ly49 receptor family of which the C57BL/6 NKC region possesses 12 Ly49 genes, whereas the BALB/c strain only has 7 Ly49 genes. Importantly, BALB/c mice lack the NK receptor Ly49H [65] which is crucially involved in murine cytomegalovirus (mCMV) resistance, and is therefore specifically targeted against the viral mCMV glycoprotein m157 [66]. There are strong indications that the key NK cell receptor for arteriogenesis lies within this gene locus, which is normally lacking in BALB/c.

Current research now focuses on the identification of this key NK cell receptor in arteriogenesis. This will also give insight in possible therapeutic ligands to specifically stimulate NK cells for therapeutic arteriogenesis. Next, more insight in NK cell dynamics during arteriogenesis is required, including insight in local effector function. The local effector function could include perivascular cytolysis to create space for outward remodeling, but also cytokine production for modulating the local inflammatory response.

### **Summary**

The role of leukocytes in arteriogenesis is evident, but highly complex. To design new arteriogenic treatment strategies using leukocytes, it is necessary to refine our knowledge on which subsets of leukocytes are involved in collateral formation and how. Ex-vivo activation (or inhibition) of specific inflammatory cell subpopulations may prove beneficial for stimulation of arteriogenesis by cell therapy in the future. A major challenge for the development of these therapies remains that pro-arteriogenic leukocytes and growth factors may contribute to adverse effects such as plaque progression and neointima formation [67,68].

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