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# **INTERFERON-BETA, A POSSIBLE TARGET IN ARTERIOGENESIS**

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# Interferon-Beta, a Possible Target in Arteriogenesis

Sylvia Nieuwenhuis 3323749

## Abstract

In patients with coronary or peripheral artery disease, restoration of perfusion can be achieved via the growth of collateral arteries, known as arteriogenesis. Circulating monocytes play a pivotal role as cellular modulators in the development of arteriogenesis. After monocyte adhesion to the endothelium of the collateral vessel, transmigrated monocytes secrete chemokines, proteases and growth factors. These secreted molecules are essential for arteriogenesis, through the induction and proliferation of smooth muscle cells (SMCs) and endothelial cells. In this thesis we consider body's own cytokine interferon-beta (IFN $\beta$ ) as a possible target in arteriogenesis, therefore it is interesting how to translate this statement into the field of research subsequently in a therapeutic treatment. Previously, in literature it has been described that IFN $\beta$  plays a pivotal role in the inhibition of arteriogenesis. This will be further discussed into detail by using comparable angiogenesis research performed in animal models. Finally, the discussion hypothesizes at least seven different IFN $\beta$  mediated mechanisms to promote arteriogenesis.

## INTRODUCTION

Cardiovascular disease claims approximately 17.1 million lives each year in the industrialized world. The numbers of patients in developing countries are rising. Therefore, research is essential for understanding the cause and possible therapeutic approaches for patients suffering from cardiovascular disease.<sup>1,2</sup> Cardiovascular disease is a class of several diseases involving the heart or the blood vessels e.g. atherosclerosis. Plaque formation in the arteries, blocks the blood flow in the artery, known as atherosclerosis obstructive disease. Atherosclerosis obstructive disease is mainly caused by high cholesterol levels. According to the World Health Organization (WHO) high cholesterol levels leads to approximately 4.4 million deaths each year. Atherosclerosis obstructive disease can lead to deficient oxygen and nutrients supply to the heart tissues leading to impaired heart function, caused by damaged perfusion. Since perfusion is an important factor for preventing further heart tissue damage, it is essential to restore this perfusion function. This can be achieved through the formation of collateral vessels, known as arteriogenesis. From literature two processes for blood vessel development are known, arteriogenesis and angiogenesis. Arteriogenesis describes the development of mature arteries from preexisting arterioles after arterial lumen narrowing or arterial obstruction, whereas angiogenesis is the sprouting of a new vasculature from settled arteries in an ischemic area.<sup>1,3-6</sup> From clinical research is

known that the body's own cytokine interferon-beta (IFN $\beta$ ) has an inhibitory effect on arteriogenesis in patients with impaired arteriogenesis formation<sup>7-9</sup>. Therefore it is interesting to appoint IFN $\beta$  as a possible target in arteriogenesis.

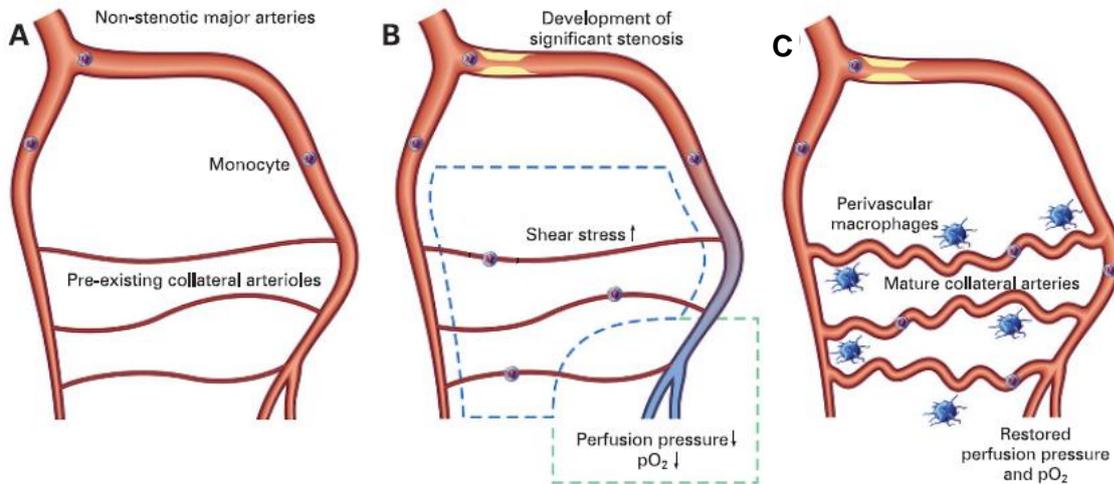
From literature more research is performed for angiogenesis compared to arteriogenesis in relation to IFN $\beta$ . Therefore this thesis will discuss the interplay between angiogenesis and IFN $\beta$ . Subsequently, a translation will be made from angiogenesis to arteriogenesis in relation to IFN $\beta$ . First of all, IFN $\beta$  will be described in relation to IFN $\beta$  production by receptor based intracellular signalling and IFN $\beta$  signalling. Finally, this thesis will discuss at least seven different hypothesis for IFN $\beta$  mediated mechanisms to promote arteriogenesis. Followed by the conclusion that local inhibition of IFN $\beta$  downstream signalling molecules could lead to less side-effects for the promotion of arteriogenesis.

## Interferons

More than fifty years ago, Interferon (IFN) was discovered as an interfering agent of viral replication *in vitro*.<sup>10-12</sup> Soon after, it became clear that IFNs are not only antiviral cytokines, but display pleiotropic biologic properties, including antiproliferative, antitumor and immunomodulatory characteristics. Subsequently, upregulated IFN induction, production, and action were related to clinical diseases, e.g. multiple sclerosis (MS), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), cancer and atherosclerosis. This led to studies using IFNs as a target to treat several of these diseases.<sup>13-15</sup> Patients with MS are nowadays treated by administration of human recombinant IFN $\beta$ <sup>16</sup> and the anti-angiogenesis effects of type I IFNs resulted in tumor growth regression in experimental models.<sup>12,17-20</sup> We reported recently that elevated IFN $\beta$  levels are associated with a defective arteriogenic response.<sup>7,9,21</sup> Thus, IFNs constitute a promising new target in cardiovascular disease, especially since they also play a role in the underlying disease of arterial obstruction, namely atherosclerosis.

## Cardiovascular disease

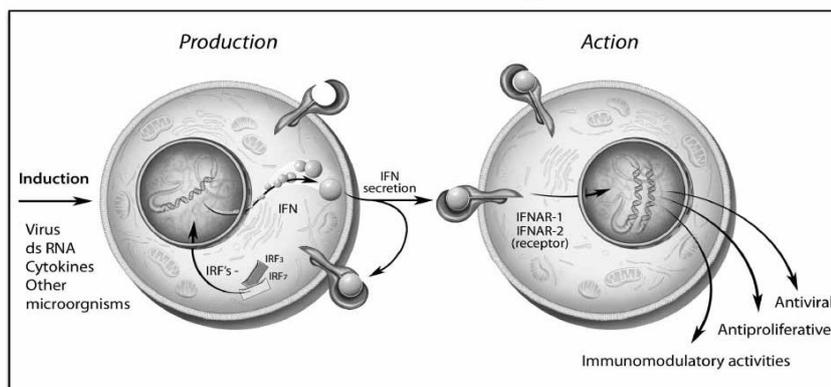
According to the World Health Organization (WHO), atherosclerosis obstructive disease is mainly caused by high cholesterol levels, claiming approximately 4.4 million deaths each year in the industrialized world. The numbers of patients in developing countries are rising. Therefore, research is essential for understanding the cause and possible therapeutic approaches for patients suffering from arterial obstructive disease.<sup>1,2</sup> One therapeutic approach would be the stimulation of collateral arteries, also referred to as arteriogenesis. Arteriogenesis is the development of preexistent arterioles into natural bypasses to circumvent arterial lumen narrowing or arterial obstruction, known as stenosis, this is illustrated in Fig. 1. This process involves a complex interplay of several factors, which will be discussed later into further detail.<sup>21,7</sup> A well developed collateral circulation can be life-saving after e.g. acute myocardial infarction or atherosclerotic lumen narrowing.<sup>8</sup>



**Fig. 1A.** Healthy arteries without obstruction and normal blood flow, the small pre-existing collateral arterioles barely carry blood. **B.** Development of stenosis (yellow color), this obstruction results into lowering of blood pressure and less oxygen supply distal from the obstruction (blue color). Increased pressure over the pre-existing collateral arterioles leads to increased shear stress. **C.** Prolonged shear stress over the pre-existing collateral arterioles restores the perfusion over the affected distal part of the artery, whereby oxygen can be supplied to the distal part of the heart tissue (from Schirmer, *et al.*).

## Interferon production by receptor based intracellular signalling, and IFN $\beta$ signalling

Interferons are glycoproteins, known as cytokines, which are produced and released by specific cells of the immune system, *e.g.* monocytes/macrophages or dendritic cells. There are three types of IFNs (IFN I, IFN II and IFN III), each type can be divided in several classes.<sup>13</sup> Type I IFNs include the classes IFN $\alpha$ , IFN $\beta$ , IFN $\epsilon$ , IFN $\kappa$  and IFN $\omega$ .<sup>22</sup> By contrast type II IFN consists only of IFN $\gamma$  and type III IFNs can be divided into IFN $\lambda_1$ , IFN $\lambda_2$ , and IFN $\lambda_3$ .<sup>13</sup> After induction with danger signals *e.g.* virus, ds RNA, cytokines or other microorganisms, type I IFNs get released. Released type I IFN interacts with the type I IFN receptor complex, consisting of the interferon- $\alpha/\beta$  receptor IFNAR-1 and IFNAR-2, in a paracrine and/or autocrine manner and leads to antiviral, antiproliferative and immunomodulatory activities, as illustrated in Fig. 2. In this review we mainly discuss the IFN $\beta$  class from the type I IFNs. Human IFN $\beta$  is a pleiotropic cytokine that consists of 166-amino acids and is approximately 20 kDa in weight.



**Fig. 2.** Production and action of human type I interferons (from Bekisz *et al.*).

## Production of type I Interferon by different receptors and ligands

Type I IFN production is initiated via different receptor pathways in host cells in the context of inflammation, which is dependent on the type of pathogens, such as PAMPs. PAMPs are pathogen-associated molecular patterns, such as viral ligands and LPS, which can be sensed by one group of receptors expressed on sentinel cells. These receptors are the first line of defense in innate immunity in mammalian species, among which are the group of Toll-like receptors (TLRs).<sup>23,24</sup> A total of ten functional TLRs (TLR1-10) are identified in humans. Most TLRs are expressed on the cell surface, with exception of TLR3, 7, 8 and 9, which are expressed in the endolysosomes of the cell. The TLR2 subfamily exists of TLR1, TLR2, and TLR6 and are able to form TLR2-TLR1 and TLR2-TLR6 heterodimers, which recognize a broader spectrum of ligands, such as viruses, gram-positive bacteria, protozoa and yeast.<sup>23</sup> Dietrich *et al.* showed that both heterodimers TLR2-TLR1 and TLR2-TLR6 induce type I IFN responses, activating NF- $\kappa$ B dependent transcription of proinflammatory cytokines.<sup>13,25</sup> Additionally, TLR4 is able to induce the production of type I IFNs.<sup>23,24</sup>

Nucleic acids derived from viruses are recognized by two families of the pathogen-recognition receptors, TLRs and RIG-I-like receptors (RLRs). Binding initiates downstream signalling for innate antiviral responses provided by type I IFNs.<sup>26</sup> TLR3, TLR7/8 and TLR9 are located inside membranes of sentinel cells, *e.g.* monocytes, macrophages and dendritic cells. These receptors are able to sense double-stranded (ds) RNA, single-stranded (ss) RNA and DNA (hypomethylated CpG DNA).<sup>27</sup> According to Kawai *et al.* TLR8 is phylogenetically comparable to TLR7<sup>28</sup> and is therefore at the same time mentioned as TLR7/8 complex. The other family of the pathogen recognition receptors available in the cytosol, such as RLRs, including retinoic acid inducible gene (RIG-I) and the melanoma-differentiation-associated protein 5 (MDA5), are present in conventional dendritic cells (cDCs), macrophages and fibroblast cells, able to sense nucleic acids.<sup>13,29</sup>

Furthermore, other cytosolic sensing nucleic acid receptors are the RNA polymerase III and DAI. Additionally, sensing of cytoplasmic DNA is induced by the RNA polymerase III and the DAI receptor (DNA-dependent activator of (interferon regulatory factors (IRFs))).<sup>26</sup> RNA polymerase III is an enzyme which induces the transcription of the sensed DNA template into RNA ligand, which can subsequently be recognized by the RIG-like receptor.<sup>30</sup> In conclusion, in context of inflammation, interaction of these previously described receptors with their ligands lead to the activation of intracellular signalling pathways. Activated intracellular pathways initiate the production of type I IFN by sentinel cells, *e.g.* monocytes, macrophages and dendritic cells.

## Intracellular signalling through TLR2, TLR3 and TLR4

Subfamily TLR2 (TLR2-1, TLR2-6) and TLR4 localized on cell membranes of cDCs or macrophages activate downstream effectors containing Toll/interleukin-1 receptor (TIR) domains and both TLR2 and TLR4 utilize the TIR-containing adaptor protein (TIRAP) bound to TLR in combination with Myeloid differentiation primary response gene (88) (MyD88), as illustrated in Fig. 3. This pathway is known as the MyD88-dependent signalling pathway.<sup>23,31</sup>

This MyD88-dependent pathway results in the recruitment of Interleukin-1 receptor-associated kinase 1 (IRAK1), IRAK2, and IRAK4 and TNF-receptor-associated factor 6 (TRAF6). TRAF6 initiates the transforming-growth-factor- $\beta$ -activated kinase 1 (TAK1). TAK1 is then able to activate the NEMO/IKK $\alpha$ /IKK $\beta$ -complex (IKK $\alpha$ , IKK $\beta$  and NEMO (NF $\kappa$ B essential modifier)/IKK $\gamma$ ) and to phosphorylate the blocking molecule of I $\kappa$ B. Then NF $\kappa$ B

translocates to the nucleus and transcription of NF $\kappa$ B dependent proinflammatory genes is initiated. An alternatively route for TAK1 is via mitogen-activated protein kinase MAPK, which initiates directly the transcription of proinflammatory cytokines as presented in Fig. 3.

The MyD88-independent signalling pathway, proceeds via the following route: TRAM connects TLR4 with TIR-containing adaptor inducing IFN $\beta$  (TRIF) and TLR3 is only coupled to the TIR domain of TLR3. A highly conserved intrinsic membrane protein, known as UNC93B1 also plays an important role. Kim *et al.* showed that TLR3, TLR8 and TLR9 are all UNC93B1-dependent to traffic from the ER to the endolysosome for sensing nucleic acids.<sup>32</sup> The downstream pathways from TLR3 and TLR4 follow the same path via TRIF, this is in literature known as the TRIF-dependent signalling pathway. From TRIF three different downstream pathways are described.<sup>23,31</sup> The first is directly linked with TRAF6, the second is indirectly linked via the retinoic acid-inducible gene-I (RIP1) with TAK1 leading to the transcription of late NF $\kappa$ B proinflammatory cytokines. The last interaction route is from TRIF via TRAF3 thereby activating the TBK1/IKKi kinases which phosphorylate the serine and threonine sites on the non-active Interferon regulatory factor 3 (IRF3) and IRF7 in the cytoplasm.<sup>13,26</sup> Phosphorylated IRF3 and IRF7 are able to translocate to the nucleus where the type I IFN genes get transcribed, which are mediating the production of chemokines *e.g.* CXCL-family. In total 10 different vertebral IRFs (IRF-1 to IRF-10) were identified based on their unique DNA-binding domain (DBD).<sup>33</sup> The transcription factors IRF-3 and IRF-7 play a pivotal role in the expression of type I IFN.<sup>29,33-35</sup> Fig. 3 illustrates the production of human type I Interferons *e.g.* IFN $\beta$ .

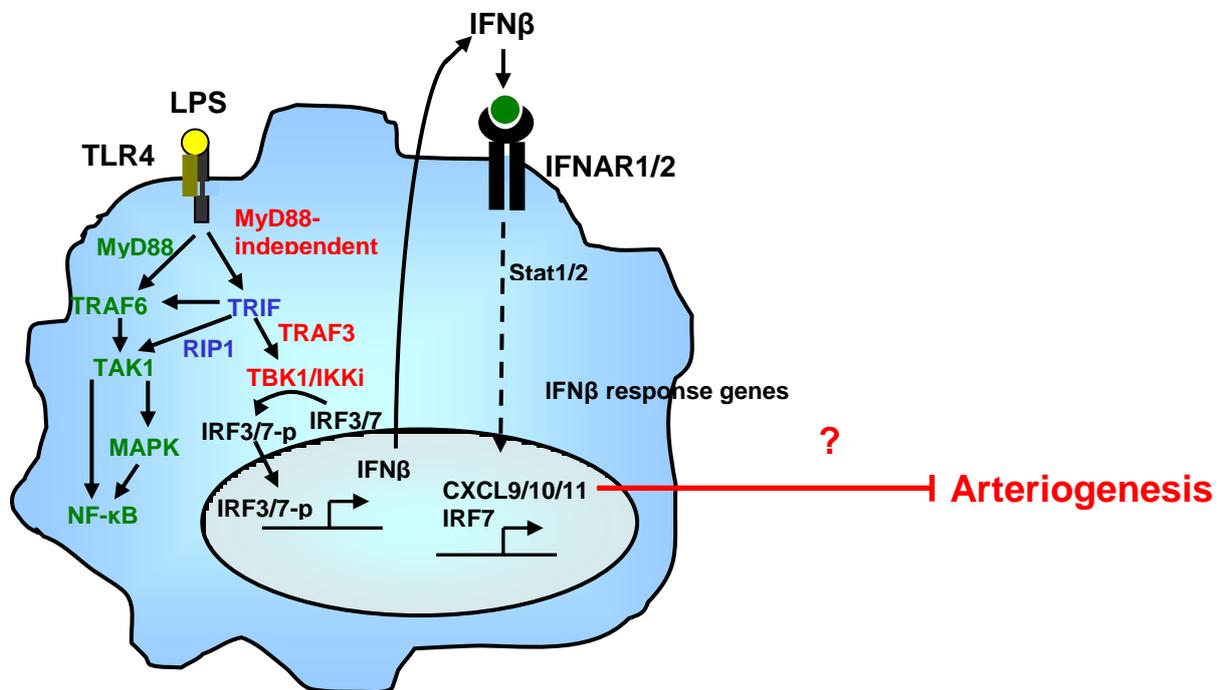
### Intracellular signalling through TLR7/8, and TLR9

TLR7/8 and TLR9 are transported from the ER by UNC93B1, into the endolysosomes, in order to fulfill their roles for sensing nucleic acids in the context of infection.<sup>32,36</sup> Upon ligand interaction, TLR7 and TLR9 recruit MyD88, leading to interaction with adaptor molecules IRAK4 and IRAK1. This interaction leads to the phosphorylation of IRF7 through the IKK $\alpha$ -complex, which activates the transcription of type I IFN genes in the nucleus. Moreover, IRF7 gets phosphorylated by TRAF3 after interaction with IRAK1. Another downstream pathway from IRAK4 is via TRAF6, which activates TAK1, responsible for the activation of IKK-complex to phosphorylate the NF $\kappa$ B inhibitor I $\kappa$ B. This facilitates the translocation of NF $\kappa$ B (p50/p65) to the nucleus, where NF $\kappa$ B genes are transcribed. Another pathway leading to the NF $\kappa$ B gene transcription is initiated by TAK1 through the MAPK.<sup>23</sup>

### Intracellular signalling through MDA5 and RIG-I

Upon viral recognition, MDA5 is linked to the caspase recruitment domain (CARD), whereas RIG-I is linked to ubiquitinated CARD, both types of CARDS bind to the CARD of IFN $\beta$  promoter stimulator-1 (IPS-1) positioned on the external membrane of mitochondria.<sup>23</sup> TRIM25 initiates ubiquitination of the RIG-I CARD, IPS-1 gets activated and interacts with TRADD forming a complex with FADD and caspase-8/10. This IPS-1/TRADD/FADD/caspase 8/10 complex is able to induce phosphorylation of I $\kappa$ Bs leading to translocation of NF $\kappa$ B to transcribe proinflammatory cytokines. The other intracellular signalling pathway from IPS-1 linking TRAF3 through TRADD is capable to activate the TBK1/IKKi complex and

phosphorylate IRF3 and IRF7. Phosphorylated IRF3 and IRF7 play both an essential role for the initiation in the transcription of IFN $\beta$  and IFN $\alpha$  in the nucleus.

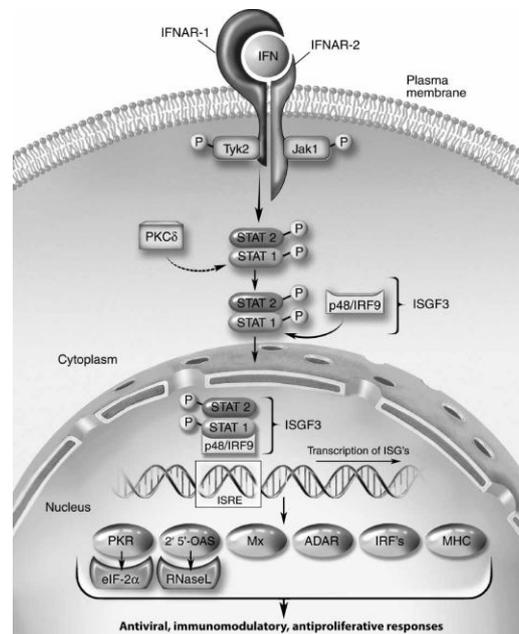


**Fig. 3.** Scheme of TLR- and IFN $\beta$ -signaling pathways involved in the production of IFN $\beta$  and IFN $\beta$  response genes. Stimulation of TLR4 by LPS activates the MyD88-dependent and -independent pathways. Activation of MyD88 leads to the activation of TRAF6, and subsequently TAK1. TAK1 activates NF- $\kappa$ B directly or indirectly via MAPK. Activation of MyD88-independent pathway activates the TRIF molecule which links the MyD88-independent pathway with the MyD88 pathway via TRAF6 or RIP1. Stimulation of TRIF leads to the induction of TRAF3 which activates TBK1/IKK1, kinases that phosphorylate the transcription factors IRF-3 and 7. These transcription factors induces the expression of IFN $\beta$ . IFN $\beta$  exits the cell and binds to its receptor IFNAR1/2 and activates the IFN $\beta$  responsive genes through the STAT1/2 complex.

### Interferon- $\beta$ signalling

IFN $\alpha$  or IFN $\beta$  can interact with the type I IFN receptor complex (IFNAR), composed of the two subunits, Interferon Alpha Receptor-1 (IFNAR-1) and IFNAR-2, which leads to the attachment of the Janus kinases (JAKs) family of tyrosine kinases in the cytoplasm, known as TYK2 (Tyrosine kinase 2) and JAK1 (Janus kinase 1), respectively. These two kinases phosphorylate the Signal Transducers and Activator of Transcription 1 (STAT1) and STAT2, which facilitates the binding of DNA-binding protein p48 (IRF-9, IFN-regulatory factor 9) forming the IFN-stimulated gene (ISG) factor 3 transcription factor complex (ISGF3). The activated ISGF3-complex, translocates to the nucleus and under influence of bound IRF-9 (p48), it interacts with the IFN-stimulated response element (ISRE) on the DNA.<sup>22,34,37</sup> This pathway activates the desired antiviral, immunomodulatory and antiproliferative response, through the transcription of IFN-stimulated genes (ISGs).<sup>37</sup> Furthermore, during type I IFN signalling STAT1, STAT3, STAT4, STAT5 and STAT6 can form homodimers or heterodimers binding IFN $\gamma$ -activated site (GAS) elements to initiate gene transcription. Consequently, type

I IFN can initiate transcription of genes by binding the ISRE or GAS elements, whereas type II IFN only could bind the GAS element to induce gene expression.<sup>22</sup> Fig. 4 presents the signal transduction mechanism after type I IFN binding.



**Fig. 4.** The signal transduction mechanism (from Bekisz, *et al.*). After IFN binding to the type I IFN receptor both subunits (IFNAR1 and IFNAR2) phosphorylate the bound kinases TYK2 and JAK1, leading to the phosphorylation of both signal transducers and activator of transcription factors STAT1 and STAT2. After P48 (IRF9) binding to the complex the formed ISGF3 complex (IFN-stimulated gene factor-3) translocates to the nucleus and binds on the DNA bearing ISRE (IFN-stimulated response element) genes to initiate the transcription of antiviral, immunomodulatory and antiproliferative responses.

## Angiogenesis versus arteriogenesis

Arteriogenesis describes the development of mature arteries from preexisting arterioles after arterial lumen narrowing or arterial obstruction, whereas angiogenesis is the sprouting of a new vasculature from settled arteries in an ischemic area.<sup>1,3-6</sup>

Remarkable, are the two contrary effects of detrimental angiogenesis in tumors versus the desirable arteriogenesis effects in patients with an impaired coronary or peripheral artery system. In oncology, research is performed to inhibit angiogenesis for tumor regression, including therapies to stimulate endogenous IFN $\alpha$  and/or IFN $\beta$  production or administration.<sup>17-20</sup> Comparable less research is performed for arteriogenesis stimulation where blocking of both IFNs is preferable. Therefore this review describes short the successes and knowledge obtained for inhibition of angiogenesis in the oncology and subsequently angiogenesis stimulation as well as arteriogenesis stimulation in cardiovascular diseases. Angiogenesis can be divided into three main classes. The first class is during embryogenesis; *de novo* angiogenesis and during female reproduction, which will not be discussed further in detail in this review. The second class can be described as regenerative angiogenesis in wound repair, whereas the last class describes angiogenesis in diseases e.g. cancer.<sup>38,39</sup>

## Angiogenesis stimulation in wound healing

Angiogenesis is critical in regenerative processes after cutaneous wound damaging. An impaired angiogenic process can lead to prolonged wound healing, characterized by necrotic tissue. Therefore it is necessary to promote blood vessel formation during wound healing. Different stages of wound healing are described in literature, such as the inflammatory phase, proliferative phase, and remodeling phase.<sup>40</sup> Angiogenesis takes place in the proliferation phase of granular tissue formation, in this phase vascular endothelial growth factors (VEGFs) play an important role and are mainly secreted by epidermal keratinocytes.<sup>41</sup> The role of VEGF during vessel formation in wound healing has been reviewed in detail elsewhere.<sup>41,42</sup> In a wound healing research with HUVEC cells was found that IFN $\alpha$ 2b suppressed its proliferation. Additionally, IFN $\alpha$ 2b inhibited VEGF-induced proliferation and tube formation by HUVECs. These effects are caused by inhibiting the expression of the VEGF receptor on endothelial cells. This is interesting since IFN $\alpha$  and IFN $\beta$  both act on the same IFNAR, moreover both are induced by the same transcription factors.<sup>43</sup>

## Angiogenesis inhibition in oncology

Several studies have focused on strategies to induce apoptosis in tumor cells or to induce an anti-angiogenesis effect by stimulating specific ISGs.<sup>12,18,44</sup> Angiogenesis is required for tumor growth to receive oxygen, and nutrients.<sup>18,45,46</sup> Research performed by McCarty and collaborators in 2002, found evidence that endogenous IFN $\alpha$ /IFN $\beta$  is involved in the regulation of angiogenesis. This was proven in a IFN $\alpha$ / $\beta$  receptor *-/-* mice model with an *in vivo* angiogenesis assay, in which gelfoam sponges are filled with the proangiogenic molecules bFGF, VEGF and TGF- $\alpha$  and are then implanted in IFN $\alpha$ / $\beta$  receptor *-/-* mice. After two weeks these sponges contained significant increased vasculature compared to sponges implanted in control mice, indicating that IFN $\alpha$ / $\beta$  signalling is involved in the inhibition of developmental angiogenesis.<sup>19</sup>

In another study, an adeno-associated virus (AAV) vector with the human IFN $\beta$  transgene was used to deliver continuously IFN $\beta$  in tumors. It was shown that endothelial cell density was significantly reduced in these tumors. Based on this study they suggested that the anti-angiogenic effects of IFN $\beta$  were not caused by the direct downregulation of pro-angiogenic factors VEGF and bFGF, but were caused by the direct toxic effects of IFN $\beta$  on tumor endothelial cells.<sup>47</sup> In contrast, Jablonska and collaborators claimed that endogenous IFN $\beta$  inhibits tumor angiogenesis through tumor-infiltrating neutrophils instead of direct IFN $\beta$  toxicity effects on tumor endothelial cells. This research was performed in a transplantable mouse tumor model wherein tumor-infiltrating neutrophils, reacting on endogenous IFN $\beta$ , reduced the expression levels of VEGF, MMP9 and CXCR4, the receptor specific for stromal-derived-factor-1 (SDF-1). Moreover, in IFN $\beta$ -deficient mice these tumors grew faster and were bigger in size compared with controls, implying improved tumor angiogenesis. Furthermore, they found elevated levels of tumor-infiltrated CD11b+Gr1+ neutrophils, which are able to express high levels of proangiogenic genes and homing factors. Supporting this fact; they injected tumors with WT neutrophils and saw faster tumor growth compared with untreated tumors. Subsequently, tumors injected with neutrophils that were unable to react to endogenous IFN $\beta$ , due to their deficiency in IFNAR, grew much faster as compared to WT. Therefore they concluded that neutrophils are the main cell population for tumor angiogenic activity and are likely controlled by endogenous IFN $\beta$ .<sup>46</sup>

Paradoxically, in another study by Dickson *et al.* performed on the vasculature of human xenografts in immunodeficient mice, it was shown that continuous delivery of IFN $\beta$ , via the AAV vector with the human IFN $\beta$  transgene, increased angiogenesis in tumors. This was due to increased expression levels of angiopoietin-1 which resulted in increased activation of the Tie2-receptor signalling leading to the recruitment of perivascular cells to the tumor vessels. Remarkably, this recruitment of perivascular cells resulted in sustained maturation of intratumoral vasculature. Additionally, angiopoietin-1 reduced the vessel permeability in tumors. The role of angiopoietin-1 in vessel stabilization was also reported elsewhere.<sup>48</sup> However, the study from Dickson *et al.* is not in line with most literature, the difference with this study compared to the other studies is mainly the low dose of IFN $\beta$ .<sup>49</sup> Furthermore, in their study, the well developed tumor vasculature could serve as an improved intratumoral administration pathway for systemic chemotherapy to induce tumor regression. This is in contradiction with the preferred anti-angiogenic effect in classic tumor therapy as further described in this review.

In a research performed by Cao *et al.* tumors were injected with adenoviral vector encoding the murine IFN $\beta$  gene (AdIFN $\beta$ ) to suppress tumor angiogenesis in a dose-dependent manner. These injected tumors showed elevated levels of IFN $\beta$  and upregulated inducible nitric oxide synthase (iNOS), combined with reduced levels of basic fibroblast growth factor (bFGF) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1). The reduced tumor angiogenesis was suggested to be caused by NO-dependent mechanisms.<sup>50</sup> NO blocks bFGF-induced proliferation of endothelial cells *in vitro*, which may be essential for angiogenesis inhibition. Altogether, these studies indicate that IFN $\beta$  indirectly inhibits tumor angiogenesis through a cascade which upregulates NO, leading to reduced bFGF expression and subsequently reduced proliferation of endothelial cells. The lowest effective concentration of NO that could inhibit endothelial cell proliferation is comparable to the amounts of NO released by smooth muscle cells in context of inflammation.<sup>51</sup>

Interestingly, it is described that bFGF plays a role in angiogenesis stimulation through the attenuation of iNOS expression levels<sup>52</sup>, whereas in the article from Cao *et al.* it was stated that AdIFN $\beta$  injection in tumors induced iNOS expression and suppressed the proangiogenic molecule bFGF in tumors.<sup>50</sup>

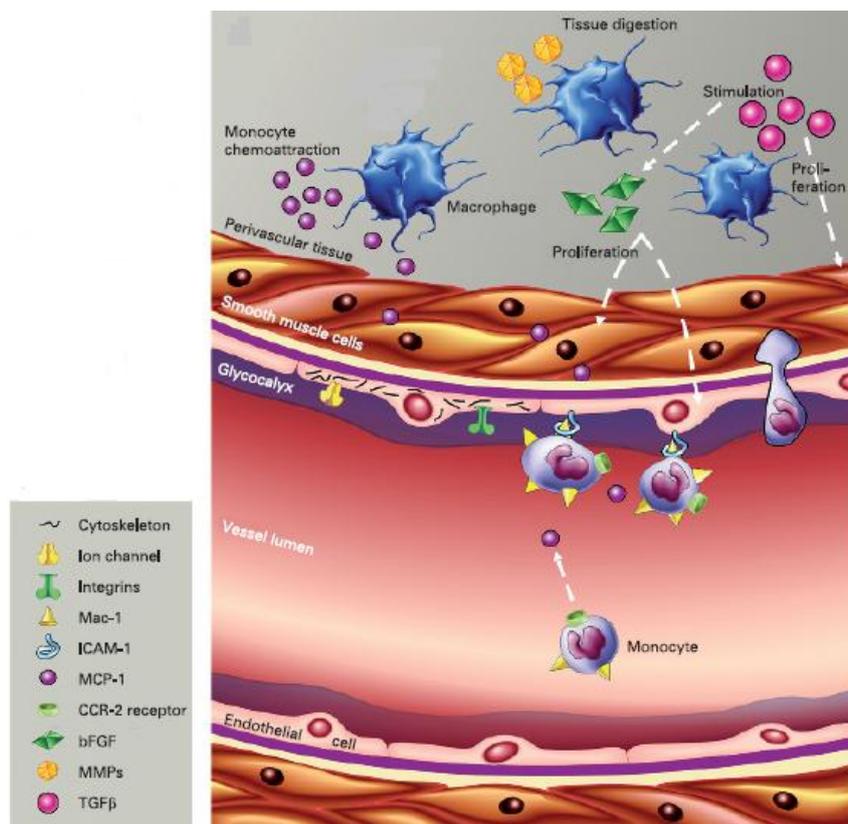
According to Cao *et al.* IFN $\beta$  can also inhibit tumor growth directly by induction of apoptosis or indirectly such as by activation of NO-dependent tumor inhibitory properties of macrophages, stimulation of natural killer cells, modulation of T-cell-dependent responses or by inhibition of proangiogenic factors.<sup>50</sup>

## Arteriogenesis

During coronary artery disease (CAD), obstruction of the coronary arteries leads to a difference in the pressure distal and proximal from the obstruction. Comparable to all fluids, blood flows from the highest pressure to the lowest pressure, thereby circumventing the obstructed artery. During the early phase of arteriogenesis, the blood flows from the blocked artery into preexisting arterioles.<sup>1,6,53</sup> The increased blood flow over the pre-existing arterioles increases shear stress which is the initial trigger of arteriogenesis. See Fig.1 for an overview illustration.

This directly activates the endothelium, thereby increasing the expression levels of adhesion molecules such as ICAM-1, VCAM-1 and PECAM-1, and monocyte-chemoattractant protein-1 (MCP-1) known as a chemokine for monocytes. MCP-1 interacts with CCR2-receptor on the monocytes, as illustrated in Fig. 5. The monocytes are recruited to the intima (site of inflammation), where they differentiate into macrophages enabling the production of growth factors and cytokines, including TGF- $\beta$ 1, bFGF, VEGF and MCP-1. It is known that these factors enhance the remodeling by inducing the proliferation of SMCs and endothelial cells, and attraction of more monocytes.<sup>5,54</sup> Once inside the intima, monocytes degrade the internal elastic lamina by the excretion of matrix metalloproteinases (MMPs), enabling cell movement and general tissue reshaping for artery growth.<sup>55</sup>

Monocyte recruitment plays a major role in the mediation of arteriogenesis as was proved in an experiment from Hofer *et al.* They showed in a rabbit hind limb model after femoral artery ligation, that after MCP-1 stimulation, elevated levels of chemoattracted monocytes were found.<sup>54,56</sup> Finally, a prolonged state of shear stress along the preexisting arteriole results into matured vessel able to transfer blood to circumvent the obstructed occlusion in the artery, which could be life-saving.<sup>1,53,57</sup>



**Fig. 5.** Fluid shear stress sensing by the endothelium, leads to the upregulation of adhesion molecules, like ICAM-1. ICAM-1 interaction with MAC-1 receptor on monocytes, leads to the transmigration into the perivascular tissues and monocytes differentiate to macrophages. Macrophages are now able to release growth factors and cytokines, which are needed to stimulate SMCs and endothelial cells for collateral formation (from Schirmer, *et al.*).

# Role of IFN $\beta$ in arteriogenesis

## Shear stress

Increased shear stress along the coronary endothelial cells of the preexisting arterioles is crucially involved in the development of collateral arteries, as previously discussed.<sup>5,6,58-60</sup> Therefore it seems logical that increased shear stress leads to improved collateral vessel growth, which was elegantly demonstrated by Schaper *et al.* They shunted the collateral flow into the vein, and observed that collateral conductance was improved by more than 100% after 7 days.<sup>60</sup>

Collateral vessels exist of endothelial cells lining the inside of the vessels, consisting of glycocalyx, which is composed of proteoglycans, glycoproteins (integrins) and ion channels, see Fig 5.<sup>61</sup> However, the endothelial cells lining the glycocalyx, contains cytoskeleton proteins, which are essential factors for cell shape, see Fig. 5. The glycocalyx includes the mechanosensor platelet endothelial cell adhesion molecule-1 (PECAM-1), which is able to sense shear stress.<sup>62</sup> PECAM-1, known as CD31, is a member of the immunoglobulin super family (such as VCAM-1 and ICAM-1) and predominantly expressed at the lateral borders between endothelial cells. However in context of inflammation, PECAM-1 plays also a role in the interaction between leukocytes (platelets, monocytes, and neutrophils) and endothelial cells leading to migration through the endothelium to the site of inflammation.<sup>58</sup> There are some studies that suggests that PECAM-1 acts in close cooperation with CD99. CD99 is a glycoprotein found on endothelial membranes and leukocytes and play an important role in monocyte transmigration.<sup>63</sup> However, *in vitro* research investigating the cooperation between PECAM-1 with CD99 or CD99L2, concluded that CD99 and CD99L2 operate independently from PECAM-1 at the same site during leukocyte transmigration.<sup>64</sup>

Chen *et al.* have demonstrated that PECAM-1 deficient mice show impaired collateral artery formation after femoral artery ligation. This was associated with reduced shear-stress induced signalling, resulting in impaired NF $\kappa$ B activation and diminished inflammatory cell accumulation.<sup>62</sup> Another experiment performed *in vitro* showed that blocking of PECAM-1, blocked tube formation in rat endothelial cells. Furthermore, this group found hampered vessel growth after PECAM-1 blocking in a angiogenesis assay model using bFGF. From these results they proved that PECAM-1 is involved in angiogenesis.<sup>65</sup> In line with previously discussed experiment, recently another study showed evidence that PECAM-1 plays a pivotal role in angiogenesis, in which a lack of endothelial PECAM-1 in mice inhibited angiogenesis.<sup>66</sup> This experiment could be one possible clarification for impaired collateral artery development for patients with coronary or peripheral artery disease.

Schirmer *et al.* found in patients with coronary artery disease and poor developed collateral arteries elevated expression levels of IFN $\beta$  in LPS-stimulated monocytes. These findings were substantiated in the mouse hind limb model, where exogenous application of IFN $\beta$  was associated with reduced collateral artery formation. Additionally, an enhanced collateral artery formation was seen in IFNAR1  $-/-$  mice.<sup>9</sup>

In another study from Schirmer *et al.* they found significant elevated expression levels of IFN $\beta$  downstream genes, such as CXCL11, CCL8, IL27 and IL15RA in LPS-stimulated monocytes from patients with chronic total coronary occlusion (CTO) and a poor collateral circulation.<sup>7</sup> The role of CXCL10 in context of PECAM-1 downregulation requires further study. As previously discussed, PECAM-1 downregulation can result in impaired arteriogenesis. This was observed by Glaser *et al.*, showing that CXCL10 neutralization resulted in elevated PECAM-1 expression levels in a spinal cord injured mice model.<sup>67</sup> This

suggests that elevated levels of IFN $\beta$  induce CXCL10 gene expression, resulting in reduced PECAM-1 levels but not directly via IFN $\beta$ .<sup>68</sup>

In conclusion Schirmer *et al.* found that the anti-arteriogenic effect of IFN $\beta$ , and not CXCL10, is mediated by inhibition of SMC proliferation. However, in previously described study from Glaser *et al.* they associated CXCL10 with reduced PECAM-1.<sup>67</sup> Thus, we speculate that CXCL10 can also be approached in a way of downregulating PECAM-1 which may lead to impaired arteriogenesis. Downregulation of PECAM-1 at the lateral endothelial sites can result in i) impaired monocyte transmigration to the site of inflammation and ii) lack of shear stress sensing.

The observed upregulated gene expression levels of CXCL10 is correlated with impaired collateral formation, which could be linked to the significantly increased gene expression levels of CCL8. CCL8 is also known as monocyte chemoattractant protein 2 (MCP-2). This can be explained by the fact that CXCL10 can inhibit PECAM-1, preventing monocyte transmigration, leading to impaired monocyte count in the collateral vessels. To compensate the reduced number of transmigrating monocytes, local monocytes start to produce more CCL8. This was also found in the study of Schirmer *et al.*<sup>7</sup>

## CXCL family

The chemokine CXCL superfamily are defined as small proteins and able to induce chemotaxis, tissue extravasation and could modulate leukocyte behavior in context of inflammation. Chemokines are divided into four families, based on their cysteine residue structures. Among these four families are the CXC chemokines, which could be separated in 17 different CXC chemokine classes. Upon interaction with dsRNA or LPS, TLR3 or TLR4 are both able to phosphorylate IRF3, which translocates to the nucleus and induces the expression of CXCL10 gene. CXCL10 is a chemokine ligand which regulates important biological processes and interacts with chemokine receptor CXCR3, such as CXCL9 and CXCL11 (known as I-TAC). Interestingly, CXCL9 was also upregulated in the study from Schirmer *et al.*, although not significantly.<sup>7</sup> In an inflammatory context, CXCL10 is induced by diverse stimuli such as IFN $\alpha$ , IFN $\beta$ , and IFN $\gamma$ , while CXCL9 and CXCL11 are only induced by IFN $\gamma$ .<sup>69</sup> Moreover it has been demonstrated that direct after myocardial infarction, during the healing process, elevated CXCL10 levels are measured in the affected tissue region, preventing premature angiogenesis and fibroblast migration. After sufficient cleaning of the wound from debris, the myocardial infarct area receives its fibrin-based matrix for tissue granulation. After 24 hours CXCL10 is downregulated and CXCL8 (IL8) upregulated, stimulating leukocyte infiltration and enhancing angiogenesis.<sup>70</sup> Earlier performed research suggested that CXCL10 is an angiogenic inhibitor, however in HUVEC cells the proangiogenic proteins, including bFGF, VEGF and IL8 were not downregulated after type I IFN treatment. In endothelial cells this implies that not the proangiogenic proteins were downregulated, but the antiangiogenic proteins were upregulated after type I IFN treatment.

<sup>18</sup>

Another research in accordance with an anti-angiogenesis effect through IFN $\beta$  is associated with the inhibition of osteoclastogenesis. Type I IFNs are important for osteoclastogenesis, in this research primary monocytes (undergoing osteoclastic differentiation) from 5 to 9 independent blood donors were monitored for differentiation behavior after IFN $\beta$  administration. Quantification techniques measured upregulated chemokine CXCL11 genes induced by IFN $\beta$ . They suggest that elevated IFN $\beta$  expression levels upregulate chemokine CXCL11

through inhibition of c-Fos. Interestingly, they established the difference between IFN $\beta$  and IFN $\alpha$ 2 and found a 100-fold more potent activity for IFN $\beta$  compared to IFN $\alpha$ 2 in inhibition of osteoclastogenesis. This could mean that IFN $\beta$  is a more potent inhibitor of monocyte differentiation.<sup>71</sup>

Interestingly, in patients who underwent transplantation and developed severe Transplant Coronary Artery Disease (TCAD) it was found that CXCL11 levels were upregulated in both serum and on endothelial cells from TCAD lesions. Additionally, immunohistochemical stainings showed the infiltration of CXCR3+ mononuclear cells within the TCAD lesions which can clarify the pathogenesis of the disease. In this article the researchers even suggested CXCL11 as a possible marker to diagnose patients with a high risk for TCAD.<sup>72</sup>

Remarkably, these elevated levels of CXCL11 gene were also found in patients with insufficient collateral formation.<sup>7</sup> Both studies combine elevated levels of CXCL11 in the two different research questions which can be distinguished in two different biological processes. In a review from Lacotte *et al.* it was suggested that the CXCR3 receptor-ligand plays a major role in context of inflammation in chemotaxis of immune cells and in the inhibition of angiogenesis.<sup>73</sup> Since it is known that CXCL11 is the ligand for CXCR3 this makes sense in the inhibition of angiogenesis caused by infiltration of CXCR3+ cells. From research performed by Schirmer *et al.* it is known that CXCL11 upregulation is associated with the inhibition of arteriogenesis.<sup>7</sup> To test previous hypothesis it is more convincing if indeed CXCR3 positive cells were upregulated in patients with impaired collateral formation.

According to Cai *et al.* is the development of arteriogenesis subdivided into three phases, the early phase, growth phase, the active growth phase and the final maturation phase. One of the proteins involved in all three phases include the highly expressed adventitial MMP2 and MMP9, initiating the degradation of extracellular matrix and elastin to initiate the enlargement of collateral vessels.<sup>74</sup> Ma *et al.* found *in vitro* that IFN $\gamma$  or IFN $\beta$  both inhibited MMP9 gene transcription resulting in reduced MMP9 expression levels *in vitro*, in a STAT-1 $\alpha$  -dependent manner.<sup>75</sup> This finding is in accordance with the research performed by Nelissen *et al.*, they also showed *in vitro* that IFN $\beta$  inhibited the production of MMP9.<sup>68</sup> This is interesting, since MMP9 is one of the adventitial proteins to facilitate the growth of collateral vessels, as previously discussed.

## DISCUSSION

Circulating monocytes play a pivotal role as cellular modulators in the development of arteriogenesis.<sup>76</sup> After monocyte adhesion to the endothelium of the collateral vessel, transmigrated monocytes secrete chemokines, proteases and growth factors. These secreted molecules are essential for arteriogenesis, through the induction and proliferation of SMCs and endothelial cells.<sup>77</sup>

Since we consider IFN $\beta$  as a possible target in arteriogenesis, it is interesting how to translate this statement into the field of research subsequently in a therapeutic treatment. Previously, it has been described that IFN $\beta$  plays a pivotal role in the inhibition of arteriogenesis, which may occur by at least seven different IFN $\beta$  mediated mechanisms.

At first, IFN $\beta$  may directly degrade endothelial cells based on its toxicity properties as described in anti-tumor angiogenesis treatment.<sup>47</sup> However, Izawa *et al.*, showed that inhibition of tumorigenicity after human adenoviral IFN $\beta$  gene therapy, resulted from an indirect apoptotic effect of IFN $\beta$  on endothelial cells, through downregulation of specific endothelial cell survival factors.<sup>78</sup> This raises the question which endothelial cell survival factors were ment in this article. Unfortunately this was not further discussed in this article, so this aspect could be unraveled in future research.

Additionally, they found that both proangiogenic factors bFGF and MMP-9 were direct decreased after human IFN $\beta$  gene therapy.<sup>78</sup> Therefore we found it interesting to investigate the direct effects of IFN $\beta$  on such proangiogenic factors, important for arteriogenesis.

Secondly, IFN $\beta$  expression levels were associated with upregulated CXCL10 gene expression levels found in LPS stimulated monocytes from patients with poor collateral vessel formation.<sup>7</sup> High CXCL10 levels may lead to downregulated PECAM-1<sup>67</sup>, preventing monocyte transmigration and/or shear stress sensing, which are both essential processes for collateral vessel formation.

A third potential mechanism involves again the increase of CXCL10 gene expression only now clarified with accompanied increased CCL8 levels in the same monocytes.<sup>7</sup> Since CCL8 levels (monocyte chemo attractant protein-2/MCP-2), were also increased<sup>7</sup>, we suggest that lack of monocyte transmigration by CXCL10 could led to the production of CCL8 by these monocytes. We suggest that this mechanism is to compensate for the decreased monocyte migration to the collateral vessels. Thus more CCL8 production by poor collateral formation could be associated with high CXCL10 levels, and can be investigated in a lentiviral silencing of CXCL10 in monocytes in a hind limb mice model of poor collateral formation. This can address the question if CXCL10 upregulation leads to CCL8 upregulation in monocytes from bad collateral formers.

A fourth potential mechanism involves the increase of CXCL11 expression levels during poor collateral vessel formation<sup>7</sup>. Interestingly, in a research performed by Coelho *et al.* they observed that upregulated gene expression levels of CXCL11 by IFN $\beta$  and recombinant CXCL11 by itself affected the differentiation of primary monocytes during osteoclastogenesis.<sup>71</sup> This suggests that CXCL11 also impairs the differentiation of monocytes during collateral vessel formation.

A fifth potential mechanism involves that IFN $\beta$  inhibited the production of MMP9, which has been previously described to be involved in the degradation of extracellular matrix proteins and elastin important for the growth of collateral vessels.<sup>68</sup> This degradation creates space to allow the proliferation of SMCs and endothelial cells, which is important for arteriogenesis.

A sixth mechanism could be due to the fact that IFN $\beta$  inhibits bFGF production through the production of NO in monocytes, as previously described in tumor angiogenesis.<sup>50</sup> Recently performed research by Wu *et al.* showed that bFGF is an important factor in the growth of collateral vessels.<sup>79</sup> However, in a study from Eitenmüller *et al.* they showed that NO is essential for arteriogenesis.<sup>80</sup>

At last, CXCL11 has been reported to induce the infiltration of CXCR3 positive cells, thereby inhibiting angiogenesis.<sup>73</sup> Since CXCL11 upregulation was also found in monocytes of patients with a poor collateralization<sup>7</sup>, this suggests that these patients could also have an infiltration of CXCR3 positive cells. This can be investigated to provide an answer whether the upregulation of CXCL11 is associated with increased infiltration of CXCR3 positive cells in a murine hind limb model of established collateralization.

In conclusion, these studies show that IFN $\beta$  could be a possible target in arteriogenesis. We must take into account the side-effects of blocking IFN $\beta$ . Since IFN $\beta$  has antiviral properties, it is not recommended to interfere in upstream IFN $\beta$  production, this could exacerbate inflammation. Furthermore, from the clinic it is known that blocking of IFN $\beta$  can exacerbate the symptoms of MS in MS patients.<sup>81</sup> Therefore it makes sense to inhibit local downstream signalling molecules of IFN $\beta$ , to prevent side-effects for the promoting of arteriogenesis. Blocking of downstream targets, which were upregulated in the study from Schirmer *et al.*<sup>7</sup>, such as CXCL9, CXCL10 and CXCL11, can be good targets to promote arteriogenesis. Whereas blocking of CCL8 is not recommendable since this prevents monocyte transmigration. From literature is known that MS patients receiving IFN $\beta$ , develop autoimmune thyroid disease (AITD), through upregulated CXCL10 secretion levels in thyrocytes.<sup>82</sup> This association was also found by Antonelli *et al.*, wherein they blocked CXCL9 and CXCL10 secretion after IFN $\beta$  addition in primary cultures of human thyrocytes with the peroxisome proliferator-activated receptor-gamma (PPARgamma) agonist, known as rosiglitazone.<sup>83</sup> CXCL9 is also interesting since this chemokine was also upregulated in the study from Schirmer *et al.*<sup>7</sup> Unfortunately, in this last study they did not investigate the side-effects after CXCL9 and CXCL10 blocking, therefore it is interesting to investigate these side-effects. The same applies for blocking of CXCL11, which also might be interesting for further investigation.

Altogether, it makes sense that local inhibition of IFN $\beta$  downstream signalling molecules could lead to less side-effects for the promotion of arteriogenesis. At best, this remains speculative, and further studies to test these hypotheses must be performed in the future. If it was to be true, it would offer new insights into future therapeutic approaches.

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