

# Role of Inflammatory Mediators in Angiogenesis

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**Abstract:** The angiogenic process involves several cell types and mediators, which interact to establish a specific microenvironment suitable for the formation of new capillaries from pre-existing vessels. Angiogenesis occurs in several physiological and pathological conditions, such as embryo development and wound healing, diabetic retinopathy and tumours. Inflammatory cells, namely monocytes/macrophages, T lymphocytes and neutrophils, fully participate in the angiogenic process by secreting cytokines that may affect endothelial cell (EC) functions, including EC proliferation, migration and activation. Angiogenesis is the result of a net balance between the activities exerted by positive and negative regulators. With regards to inflammatory cells and endothelium cross-talk, such balance is conceptually very similar to that of pro-inflammatory and anti-inflammatory mediators that modulate an appropriate inflammatory response. In this review we will mainly discuss the relevance of both physiological and pathological inflammatory processes in angiogenesis, with particular regards to microenvironmental contribution. We will also describe some of the most relevant pro-inflammatory cytokines in the modulation of the angiogenic process. Furthermore, we will concentrate on what has been recently reported about the mechanism by which some of these cytokines are induced during inflammation to promote a suitable microenvironment for angiogenesis and tumour progression. Pro-angiogenic cytokines, such as IL-1 and TNF, and anti-angiogenic cytokines such as IFN- $\gamma$  and IL-12, will be briefly described. We will try to provide a rationale for the use of both cytokines and cytokine blockades as novel potential pharmaceutical targets to modulate angiogenesis in chronic inflammation as well as in cancer.

**Keywords:** Angiogenesis, inflammation, cytokine, monocyte, macrophage, T lymphocyte, tumour microenvironment.

## INTRODUCTION

Angiogenesis is a complex process, which involves the interactions of several cell types and mediators to establish a specific microenvironment suitable for the formation of new capillaries from pre-existing vessels [1]. Such biological processes occur in either physiological or pathological conditions, such as embryo development and wound healing, diabetic retinopathy and tumours [2].

Inflammatory cells, namely monocytes/macrophages, T lymphocytes and monocytes, fully participate in the angiogenic process by secreting pro- and anti-inflammatory cytokines, that could control endothelial cell (EC) proliferation, their survival and apoptosis, as well as their migration and activation [3]. Pro-inflammatory cytokines released by monocytes have been extensively studied in that context. In previous reports, interleukin (IL)-1 has been shown to promote angiogenesis by modulating EC [4] and more recent reports have shown that IL-1 is a strong promoter of angiogenesis *in vivo* and that IL-1 is required for the angiogenic process [5]. The increased expression of IL-1 has been described in monocytes exposed to hypoxic condition [6,7] and to pro-angiogenic factors, such as thrombin [8]. In addition, a variety of studies support the hypothesis that other pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- $\alpha$ , and inflammatory cells may modulated angiogenesis by direct and indirect effects on EC, resulting in the promotion of the angiogenic process.

It is widely accepted that angiogenesis is the result of a net balance between the activities exerted by positive and negative regulators. This balance is conceptually very similar to that of the pro- and anti-inflammatory mediators that modulate an appropriate and specific inflammatory response. Generally speaking, pro-inflammatory mediators promote angiogenesis and the pro-angiogenic effects mediated by IL-1 and TNF- $\alpha$  support such hypothesis [9]. However, there are exceptions: indeed, it has been extensively reported that other pro-inflammatory cytokines, such as interferon (IFN)- $\gamma$  and IL-12, are associated with an anti-angiogenic programme [10]. In this review we will mainly discuss the relevance of both physiological and pathological inflammatory processes in angiogenesis, with particular regards to microenvironmental contribution. We will also describe some of the most relevant pro-inflammatory cytokines in the modulation of the angiogenic process. Furthermore, we will concentrate on what has been recently reported about the mechanism by which some of these cytokines are induced during inflammation to promote a suitable microenvironment for angiogenesis and tumour progression. Pro-angiogenic cytokines, such as IL-1 and TNF, and anti-angiogenic cytokines such as IFN- $\gamma$  and IL-12, will be briefly described. We will try to provide a rationale for the use of both cytokines and cytokine blockades as novel potential pharmaceutical targets to modulate angiogenesis in chronic inflammation as well as in cancer.

## PHYSIOLOGICAL INFLAMMATION AND THE WOUND HEALING PROCESS

To better understand the role of inflammation in the angiogenic process, it is important to understand what inflammation is and how it contributes to physiological processes, such as wound healing. In response to tissue injury multiple chemical signals are activated to initiate and maintain a host response suitable for healing the injured tissue [11].

Thus, a number of inflammatory cells, namely neutrophils, monocytes and lymphocytes, are recruited at different and subsequent steps. Neutrophils initiate wound healing by releasing early-response pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1- $\alpha$  and IL-1- $\beta$ . Such cytokines promote leukocyte adhesion to vascular endothelium and repair is initiated. As a consequence of tissue injury, monocytes migrate from venous system to the site of tissue injury, being guided by a number of chemotactic factors, including chemokines and the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ . Once present, they differentiate into mature macrophages or immature dendritic cells. After activation macrophages release growth factors and cytokines, namely platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)-2, transforming growth factor (TGF)- $\beta$ 1, as well as TNF- $\alpha$  and IL-1, that modulate tissue repair [12].

Macrophage products affect the local microenvironment, where EC, epithelial and mesenchymal cells are present, and regulate local tissue remodelling by modulating the extracellular matrix components and modulating angiogenesis. Fibroblasts migrate at the site of wound and secrete collagen type III, being stimulated by factors including PDGF, TGF- $\beta$ 1, IL-1 $\alpha$  and IL-1 $\beta$ . During wound repair, production as well as degradation of collagen is under precise and special and temporal control.

Finally, as inflammatory cells are activated, the expression of their TGF- $\beta$  receptors changes, and this results in differential susceptibility to TGF- $\beta$  and enhanced sensitivity to suppression by TGF- $\beta$  [13]. Such event is critical to resolving inflammation. Thus, it is obvious that, with regards to inflammation, wound healing is a self-limiting event.

## PATHOLOGICAL INFLAMMATION AND TUMOUR STROMA FORMATION

It is well recognised that wound healing and tumour stroma formation share many properties [14]. However, while wound healing is a self-limiting event, tumours are not [15]. Tumour cells themselves release pro-inflammatory cytokines as well as vascular endothelial growth factors (VEGF) [16]. Thus, with regards to inflammatory responses, tumour stroma formation is not a self-limiting event. In addition, neoplastic tissues present an inflammatory infiltrates characterized by the presence of tumour-associated macrophages (TAM), derived from monocytes recruited mainly by monocyte chemotactic proteins (MCP) or chemokines [17]. TAM, when activated by IFN- $\gamma$  and IL-12, kill neoplastic cells, however they produce several pro-angiogenic cytokines that promote tumour progression. Along with tumour cells, TAM produce IL-10, which abrogate the antitumour effects mediated by cytotoxic T cells [18].

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During tumour development, the cross-talk between activated macrophages and surrounding cells is crucial. For example, during melanoma development activated macrophages release IL-1 $\alpha$ , TNF- $\alpha$  and TGF- $\beta$ , while melanocytes release IL-8 and VEGF-A [16]. Through such cellular cross-talk, a local angiogenic process is induced and this underlines the association between macrophage infiltration and the invasiveness of primary melanoma. The alteration of the local balance between pro- and anti-angiogenic factors is not unique to the melanoma model. TAM release pro-angiogenic factors, such as VEGF-C and VEGF-D, during human cervical carcinogenesis, and the presence of activated macrophages may promote tumour spread [19]. All these reports confirm the association between cancer and chronic inflammation, association that is evident in colon carcinogenesis in patients affected by inflammatory bowel disease (e.g. Crohn's disease) [20].

In addition there is growing body of evidence that many malignancies are initiated by infections, characterized by chronic inflammation (e.g. hepatitis C infection and liver carcinoma, *Helicobacter pylori* and stomach cancer) [21]. It is now clear that inflammatory cells are recruited into the neoplastic process, through a mechanism mediated by chemokines (such as IL-8), released by tumour cells [15]. In summary, we may consider cytokines and chemokines as soluble factors that modulate the cross-talk between stromal and neoplastic cells. In a recent past, it was widely accepted that pro-inflammatory cytokines may promote angiogenesis, while anti-inflammatory cytokines act as anti-angiogenic mediators. However, the scenario is much more complex and in this review we will try to identify the role of pro-inflammatory cytokines the angiogenic process. A summary of the complex roles played by such cytokines in angiogenesis is given in Table 1.

**Table 1. Pro-inflammatory Cytokines in Angiogenesis**

Cytokine	Group	Angiogenesis	EC proliferation
IL-1	Pro-inflammatory	+	+
TNF	Pro-inflammatory	+	-
IL-12	Pro-inflammatory Th1	-	-
IFN- $\gamma$	Pro-inflammatory Th1	-	-

+ (stimulatory); - (inhibitory).

## PRO-INFLAMMATORY AND PRO-ANGIOGENIC CYTOKINES

### IL-1

IL-1 is a cytokine well known for its pleiotropic activities on a variety of cell [22]. There are three molecules associated with IL-1: IL-1 $\alpha$ , IL-1 $\beta$  and IL-1 receptor antagonist (Ra); IL-1Ra blocks the activities of IL-1 $\alpha$  and IL-1 $\beta$ , by binding to IL-1 receptors. IL-1 related molecules are present in several physiological and physio-pathological situations, where angiogenesis is also involved, and IL-1 is considered a highly inflammatory cytokine. With regards to inflammatory cells, IL-1-related molecules are produced mainly by activated monocytes and macrophages.

In humans, IL-1 $\alpha$ , IL-1 $\beta$  and IL-1 Ra genes are located on chromosome 2. IL-1 $\alpha$  and IL-1 $\beta$  are synthesized as precursors and processing of IL-1 $\alpha$  and IL-1 $\beta$  to mature forms requires specific cellular proteases. Although several similarities between IL-1 $\alpha$  and IL-1 $\beta$  exist, in many respects IL-1 $\beta$  is a different molecule from IL-1 $\alpha$ . IL-1 $\beta$  may appear as a systemic hormone-like mediator, which is released by cells, while IL-1 $\alpha$  is primarily involved in regulating intracellular events and is a mediator of local inflammation. These characteristics are crucial for the involvement of IL-1 related molecules in tumour angiogenesis. At present, two primary IL-1 receptors (IL-1RI and IL-1RII) and one receptor accessory protein (IL-1RAcP) have been identified [23]. As for IL-1 $\alpha$ , IL-1 $\beta$  and IL-1 Ra genes, IL-1RI and IL-1RII genes are located on chromosome 2. IL-1RI is found mainly on cells involved in inflammatory response and in tissue repair, such as EC, smooth muscle cells, epithelial cells, fibroblasts, keratinocytes, epidermal dendritic cells and T lymphocytes. However such receptors are present also on hepatocytes and in hippocampal areas of normal brain [24,25]. The type II receptors appear to act as decoy molecules, particularly for IL-1 $\beta$  and when such receptors bind IL-1 $\beta$ , this prevents the binding of IL-1 $\beta$  to the type I receptors [26].

Among the variegated biological activities exerted by IL-1 $\beta$ , its controversial role in tumour growth deserves great attention. It is well known that IL-1 $\beta$  exhibits direct cytotoxic effects and increases non-specific host responses, resulting in tumour regression in particular tumours *in vitro* [22]. However, and in contrast to the growth-inhibitory

effects, administration of IL-1 $\beta$  to mice with a subcutaneous melanoma increases tumour size and pulmonary metastasis [27,28]. With regards to the relationship between chronic inflammation and cancer susceptibility, we must underline the upregulation of IL-1 $\beta$  during *Helicobacter pylori* infection [29]. It is well recognized that patients with extensive gastritis, hypochlorhydria and gastric atrophy, as a result of *Helicobacter pylori* infection, have the greatest risk of gastric malignancy. IL-1 $\beta$  is a potent inhibitor of gastric acid secretion and, since a decreased flow of gastric secretions may allow accumulation of bacterial toxins and inflammatory mediators, this may result in an increase damage. IL-1 $\beta$  production enhancement may be related to IL-1 gene cluster polymorphism [29]. Similarly, higher IL-1 $\beta$  production is observed in pancreatic cancer patients homozygous for allele 2 of the IL-1 $\beta$  gene: such enhancement is accompanied by a significantly shorter survival [30]. In addition, IL-1 $\beta$  has growth stimulating activity for gastric carcinoma and for myeloid leukaemia [31,32].

With regards to the cross-talk between inflammatory cells and EC present in the tumour microenvironment, IL-1 $\beta$  stimulates the proliferation of EC, adhesion molecule expression and production of cytokines and inflammatory molecules *in vitro*, suggesting that IL-1-related molecules may be deeply involved in the control of the angiogenic process [22,33]. Indeed, in the past two years IL-1 has been shown to be a strong promoter of angiogenesis *in vivo* [34-36]. One of the first reports, showing that IL-1 $\beta$  promotes tumour growth by induction of angiogenic factors, underlines the relevance of IL-1 $\beta$  secreted into the tumour milieu [37]. In this study, a retroviral vector coding human IL-1 $\beta$  gene was transduced into mouse Lewis lung carcinoma (LLC) cells and then the transformant (LCC/IL-1 $\beta$ ) was inoculated to syngeneic C57BL/6 mice. Interestingly, the transformant cells did not show any difference in the growth *in vitro*, when compared with parental cells or with cells transduced by control retroviral vector. In contrast, LCC/IL-1 $\beta$  cells grew much more rapidly in mice and the rapid growth was associated with hyperneovascularization. Such hyperneovascularization was induced by several angiogenic factors, secreted by both tumour and stromal cells in the tumour milieu.

In another attempt to prove the involvement of IL-1-related molecules in angiogenesis, it has been reported that also IL-1 $\alpha$  promotes angiogenesis *in vivo*. Again, similarly to IL-1 $\beta$ , IL-1 $\alpha$  promotes angiogenesis through the release of pro-angiogenic factors in the tumour microenvironment [38]. In this report, IL-1 $\alpha$  induces VEGF synthesis and secretion in human peripheral blood mononuclear cells (PBMC) and enhances angiogenesis *via* VEGFR-2 pathway *in vivo*. This could explain why in the past IL-1 $\alpha$  has failed in enhancing angiogenic responses on EC *in vitro*: the presence of inflammatory cells is crucial for IL-1 $\alpha$  pro-angiogenic activities. The relevance of microenvironmental IL-1 in angiogenesis and in the invasiveness of different tumour cells has been recently demonstrated using IL-1 $\beta$  knockout (KO) mice [5]. In such mice, local tumour or lung metastases of B16 melanoma cells were not observed when compared with wild type mice. In the same study, angiogenesis was assessed by the recruitment of blood vessel networks into Matrigel plugs containing B16 melanoma cells and vascularization of the plugs was present in wild type mice, but was absent in IL-1 $\beta$  KO mice. Moreover, the addition of exogenous IL-1 into B16-containing Matrigel plugs in IL-1 $\beta$  KO mice partially restored the angiogenic response. Finally, the addition of IL-1 receptor antagonist to B16-containing plugs in wild type mice inhibited the vascularization of the Matrigel plugs. Thus, IL-1 is required for tumour invasiveness and angiogenesis.

The pro-inflammatory components of tumour microenvironment are crucial for angiogenesis and tumour progression [39,40]. This is supported by a recent report describing another link between inflammation, inflammatory cytokines and cancer development: the hypoxia-inducible factor (HIF)-1 $\alpha$  [41]. Indeed, IL-1 $\beta$  upregulates HIF-1 $\alpha$  in a human lung cancer cell line (A549). Such upregulation involves an NF $\kappa$ B/COX-2 pathway activation and identifies HIF-1 $\alpha$  as a critical link between inflammation and oncogenesis. In addition, this report points out an additional role played by hypoxia, which normally occurs in tumour microenvironment. Hypoxia itself affects inflammatory cells by inducing pro-inflammatory mediators, which turn out to be relevant in tumour progression.

### TNF

TNF- $\alpha$  is a 17 kDa polypeptide and is a pleiotropic cytokine, initially shown to be secreted mainly by macrophages. However, it is now known that TNF- $\alpha$  is produced by many cell types, including stromal and malignant cells [9]. TNF- $\alpha$  acts biologically as a trimer and binds to two distinct cell surface receptors; the p55 TNF receptor (TNFR-1) is

ubiquitously expressed on mammalian cells, while the p75 receptor (TNFR-2) expression is more restricted, being most commonly found in haemopoietic cells, in cells of the immune system and in EC. TNF- $\alpha$  is well recognized as an inflammatory cytokine and, interestingly, the inflammatory response induced by TNF- $\alpha$  is modulated by feedback inhibition, up- and downregulation of receptor expression, soluble processing of the membrane tethered ligand and cleavage of receptors from the membrane [42].

In inflammation and angiogenesis, TNF- $\alpha$  plays a dual role: it controls both tissue destruction and recovery, and these roles are determined by the context in which this cytokine acts [43]. With regards to cancer development and progression, high doses of exogenous TNF- $\alpha$  induce death of tumour cells [44]. However, low concentrations of endogenous TNF- $\alpha$  promote tumour growth and spread [45]. Over the past 15 years the reports regarding the effects of TNF- $\alpha$  in angiogenesis have been contradictory and certainly intriguing. Back to 1987, TNF- $\alpha$  was described as a strong anti-angiogenic mediator *in vitro* [46]. However, in the same year, experiments conducted *in vivo* led to the opposite conclusion: TNF- $\alpha$  was angiogenic *in vivo* [47]. A few months later, other authors reported that TNF- $\alpha$  was the pivotal mediator of macrophage-induced angiogenesis, suggesting that inflammatory cells and mediators present in tumour microenvironment should play a concerted role [48]. This is certainly in agreement with the most recent hypothesized attempt in anticancer treatment: the stromal therapy, which addresses an early but dynamic target and can therefore be applied at multiple points in the treatment cascade, from primary chemoprevention to treatment for relapsed and disseminated disease [49].

It is now widely accepted that TNF- $\alpha$  is an angiogenic factor [15]. However, it should be pointed out that in inflammation and wound healing TNF- $\alpha$  could promote repair by enhancing angiogenic processes; in tumours, TNF- $\alpha$  might stimulate tumour development by promoting vessel growth and participate in tumour and endothelium destruction by direct cytotoxicity. In agreement with such hypothesis, it has been reported that TNF- $\alpha$  promotes angiogenesis *via* ephrin A1 expression in EC [50]. In contrast, other authors reported that TNF- $\alpha$  induces EC growth arrest, because TNF- $\alpha$  markedly reduced cyclin A mRNA stability [51].

With regards to tumour angiogenesis, TNF- $\alpha$  induces angiogenic factor upregulation in malignant glioma cells [52] and is involved in melanoma tumour growth and progression [53]. In addition, TNF- $\alpha$  can be detected in malignant and/or stromal cells in human ovarian, breast, prostate, bladder, and colorectal cancer, lymphomas, and leukaemias, suggesting possibilities for both paracrine and autocrine actions [9]. TNF- $\alpha$  is also implicated in the induction of chemokines, which are essential for macrophage and lymphocyte infiltrate. The latter effect is critical in developing a potential strategy that may block the ability of tumour cells to recruit macrophages into the tumour microenvironment [15,17].

The role of TNF- $\alpha$  and of other pro-inflammatory cytokines and chemokines as intercellular mediators on macrophage and EC has been recently emphasized in another article, regarding osteopontin and FGF-2 cross-talk in angiogenesis [54]. Osteopontin is a phosphorylated acidic RGD-containing glycoprotein [55], present as an immobilized extracellular matrix (ECM) component and a soluble molecule. Osteopontin acts as a cytokine that plays important roles in monocyte/macrophage functions [55] and it is implicated in inflammation, cell-mediated immunity, tissue remodelling, and tumour metastases [56]. Interestingly, osteopontin upregulation occurs in EC treated with IL-1, and during angiogenesis *in vitro* [57], suggesting that osteopontin may affect angiogenesis by acting directly on EC and/or indirectly *via* mononuclear phagocyte engagement. Indeed, osteopontin deeply affects the pro-angiogenic potential of human monocytes. With regards to TNF- $\alpha$ , osteopontin enhances its expression in human mononuclear cells. In addition, the conditioned medium of recombinant osteopontin-treated human monocytes induces neovascularization, while no significant angiogenic response was elicited by fresh medium added with the same dose of osteopontin. This report points to a role for mononuclear cell infiltrate in mediating the angiogenic activity exerted *in vivo* by osteopontin and underscores the relevance of pro-inflammatory cytokine, such as TNF- $\alpha$ , in this process.

## PRO-INFLAMMATORY AND ANTI-ANGIOGENIC CYTOKINES

### IL-12

IL-12 is a heterodimeric cytokine and is produced primarily by macrophages, monocytes and dendritic cells. Only IL-12 p70, consisting of a p40 and p35 chain, which are encoded by two separate genes, is

biologically active [58]. The biological activities of IL-12 are mediated through high affinity receptors, where both IL-12R $\beta$ 1 and IL-12R $\beta$ 2 chains are required to generate a functional receptor. IL-12 receptors are present primarily on natural killer (NK) and T cells and IL-12 promotes IFN- $\gamma$  production and enhancement of cytolytic activity in T cells and NK cells [59].

IL-12 is instrumental for the development of a Th1 response and this function requires activation and phosphorylation of the transcription factor signal transducer and activator of transcription (STAT)-4 in T cells [60]. In contrast, in the absence of IL-12, a Th2 response can be developed [61]. This is not unique to T helper lymphocytes, since macrophage TAM are a polarized M2 macrophage population. In such context, IL-10, released by tumour and/or stromal cells, is a strong inducer of Th2 and M2 polarization.

IL-12 is a multifunctional cytokine and can cause tumour regression and reduce metastasis in animal models, due to the promotion of anti-tumour immunity and also to the significant inhibition of angiogenesis [62]. IL-12 is a strong anti-angiogenic cytokine *in vivo* and such suppression is mediated through IFN- $\gamma$  [63]. In fact, reports have shown that IL-12 is not acting *per se* on EC but through IFN- $\gamma$  and that EC respond to IFN- $\gamma$  by induction of the chemokine IFN- $\gamma$ -inducible protein (IP)-10, that is considered the ultimate mediator of the anti-angiogenic activity of IL-12 [64]. Indeed, IP-10 inhibits EC proliferation induced by basic FGF *in vitro* [65].

There is *in vitro* evidence that IL-12 inhibits VEGF produced by breast cancer cells and regulates stromal cell interactions, leading to decreased matrix metalloproteinase (MMP)-9 and increased tissue inhibitor of metalloproteinase (TIMP)-1 production [66]. In addition, other reports suggest that NK cells are required mediators of angiogenesis inhibition by IL-12, and that NK-cell cytotoxicity of EC is a potential mechanism by which IL-12 can suppress neovascularization [67]. It has been also demonstrated that Th cells (CD4+), cytotoxic T cells (CD8+) are needed to mediate the anti-angiogenic effect of IL-12 [68]. The same report shows that IL-12 activates an anti-angiogenic program in activated mouse spleen cells or human PBMC. Such effect does not require direct cell-cell contact, yet result from continuous interaction between activated lymphoid cells and EC.

IL-12-activated lymphocytes influence tumour genetic programs, by releasing higher level of IFN- $\gamma$  and down-modulating VEGF [69]. More recently, it has been suggested the existence of an IL-12-regulated circuit between endothelium and lymphocytes resulting in a MMP homeostasis shift at the site of tissue injury [70]. By showing that in the presence of EC cells it is enhanced the inhibitory effects on MMP-9 expression in activated PBMC, the authors confirm the angiostatic effects exerted by IL-12 through the cross-talk between inflammatory cells and EC. Furthermore, the relevance of inflammatory cells (CD4+T cells) present within the microenvironment of human lung tumours and their mobilization by local and sustained release of IL-12 has been additionally pointed out by other recent reports where CD4+ T cells kill tumours *in situ* by indirect effects of IFN- $\gamma$  [71].

The efficacy of IL-12 as anticancer as well as antivascular agent is still under intensive investigation. Novel data indicate that the therapeutic susceptibility of tumour vasculature to recombinant murine IL-12 and, potentially, to other anti-vascular agents, is limited by its level of maturation [72]. A developmental order, similar to that described during physiological neovascularization, is evident among vessels in growing tumours. This order markedly influenced tumour vessel response to anti-vascular therapy with recombinant IL-12, resulting in a reduced tumour vessel density, due to a decrease in angiogenic sprouts and induction of EC apoptosis in pericyte-negative vessels. Thus, the authors suggest that tumour susceptibility is limited and pericyte coverage of tumour vasculature may be a potential indicator of tumour responsiveness.

IL-12 represents a cytokine with potent anti-tumour effects and may therefore be an attractive agent in anticancer therapy. Its clinical applications are limited, however, by severe side-effects, and investigators are attempting to solve this problem. It has been recently described how to obtain an enhancement of the anti-tumour activity of IL-12 by targeted delivery to neovasculature, using the extra domain (ED)-B of fibronectin, a naturally occurring marker of angiogenesis, expressed in the majority of aggressive solid tumours but is not detectable in normal vessels and tissues [73]. Furthermore, IL-12 has been used in combination with another anti-angiogenic drug, vasostatin, that inhibits EC growth and neovascularization [74]. In this report, the combination of IL-12 and vasostatin halted the growth of human Burkitt lymphoma, colon carcinoma, and ovarian carcinoma.

In a novel therapeutic approach, a linearized, single-chain version of IL-12 has been used [75]. In this report it has been demonstrated that the induction of tumour-protective immunity by IL-12 in a murine neuroblastoma model depends entirely on the CXC chemokine IP-10, suggesting that IP-10 plays a crucial role during the early immunization phase in the induction of immunity against neuroblastoma by single chain IL-12 gene therapy. Finally, IL-12 has been used in a preventive gene therapy approach [76]. In such approach, naked DNA IL12 gene transfer resulted in strong prevention of angiogenesis *in vivo* in mice. Interestingly, angiogenesis inhibition was observed in NK cell-depleted C57BL/6 and nude mice as well as in IFN- $\gamma$ (-/-) and CXCR3(-/-) knockout mice, indicating that NK- and/or T-cell-initiated IFN- $\gamma$ -chemokine cascades were not involved in the inhibition of angiogenesis observed *in vivo*. The authors suggest that a preventive gene therapy approach using antiangiogenic cytokines can effectively inhibit tumour angiogenesis, representing an example of angioimmunoprevention.

### IFN- $\gamma$

IFN- $\gamma$ , a 50kDa protein originally identified for its antiviral capacity, is produced primarily by specific subsets of T lymphocytes and NK cells [77]. IFN- $\gamma$  exerts its cellular effects by interacting with high affinity receptors, consisting of two polypeptides, IFN- $\gamma$  receptor  $\alpha$  chain or IFN $\gamma$ R1, that is responsible for ligand binding, and IFN- $\gamma$  receptor  $\beta$  chain or IFN $\gamma$ R2, that plays a minor role in ligand binding but is required for IFN- $\gamma$  signalling [78]. IFN- $\gamma$  receptors are present on a wide variety of cells and promote the generation of protective cell-mediated immune responses. IFN- $\gamma$  receptor utilizes a specific signal transduction pathway termed the Janus protein-tyrosine kinase (JAK)-STAT pathway, that regulates transcription of specific IFN- $\gamma$ -inducible genes [79]. IFN- $\gamma$  rapidly primes the macrophage *via* JAK1/2-STAT1 pathway so that it can subsequently undergo a slower classical type 1 activation upon exposure to Th1 cytokines such as IFN- $\gamma$  or other activators, including TNF. Indeed, along with IL-12, IFN- $\gamma$  is responsible for driving the development of a Th1 response and promotion of cell-mediated immunity.

Physiological inflammatory processes, such as wound healing, ovulation, embryo implantation, and foetal growth are all associated with suppressed cell-mediated immunity and neovascularization or angiogenesis. All the chronic inflammatory conditions known to be associated with the subsequent development of malignant disease, including chronic obstructive airway disease and ulcerative colitis, are similarly associated with suppressed cell-mediated immunity and a pro-angiogenic microenvironment [80]. Furthermore, chronic immune activation leads to the synthesis and release of factors such as macrophage inflammatory protein (MIP)-1 that inhibit apoptosis through suppression of p53 activity [80]. In keeping with this, cell-mediated immunity-associated cytokines, such as IFN- $\gamma$ , tend to be anti-angiogenic. This hypothesis is supported by a recent report showing that cross-talk between IFN- $\gamma$  and TGF- $\beta$ 1 is essential in the skin wound-healing process [81]. IFN- $\gamma$  KO mice exhibited accelerated wound healing and enhanced angiogenesis when compared with wild type mice. Thus, suppressed cell-mediated immunity, angiogenesis, and reduced apoptosis would provide the ideal microenvironment that is required for the development of malignant disease.

The role played by IFN- $\gamma$  in protecting against tumour development has been identified [82]. The mechanisms that have been proposed to explain the anti-tumour activity of IFN- $\gamma$  include IFN- $\gamma$ -dependent direct effects on tumour growth or survival, enhanced cell-mediated immunity against tumours, and angiostatic effects. With regards to angiogenesis, it is widely accepted that IFN- $\gamma$  mediates IL-12 anti-angiogenic effects [63] and that IL-12 is not acting *per se* on EC but through IFN- $\gamma$  [64]. IFN- $\gamma$  was the first identified cytokine shown to affect EC [83] and IFN- $\gamma$  inhibits not only the growth of EC but also the capillary formation in a dose-dependent manner [84]. Exposure of human EC to IFN- $\gamma$  results also in a reduced activation of integrin  $\alpha$ v $\beta$ 3, an adhesion receptor that plays a key role in tumour angiogenesis, leading to a decreased  $\alpha$ v $\beta$ 3-dependent EC adhesion and survival [85]. With regards to cell adhesion, EC respond to IFN- $\gamma$  also by increased expression of CD40 and engagement of CD40 amplifies induction of adhesion molecules [86]. IFN- $\gamma$  affects EC responses by induction of IP-10, which results in inhibition of angiogenesis [87].

Tumour cells respond directly to IFN- $\gamma$  by suppressing the expression of MMP-9 gene, that is implicated in the invasion and angiogenesis process of malignant tumours and in inflammatory diseases of the central nervous system [88]. This is a STAT1-mediated mechanism, since IFN- $\gamma$  fails to suppress MMP-9 expression in STAT-1 $\alpha$ -deficient primary astrocytes and

human fibrosarcoma cells and reconstitution of human STAT-1 $\alpha$  successfully restores the inhibitory effects of IFN- $\gamma$  on MMP-9 gene expression. Further, IFN- $\gamma$  inhibits the expression of MMP-2 [89], an important component of human astrogloma invasion. Thus, IFN- $\gamma$  may have beneficial effects in attenuating astrogloma invasive properties. Such hypothesis, along with the fact that current treatments for malignant gliomas are still largely ineffective in significantly improving prognosis, has led to the development of new therapeutic strategies involving IFN- $\gamma$  and, specifically, treatment of established intracranial tumours by *in situ* retroviral IFN- $\gamma$  transfer [90]. Earlier reports have already described that gene transfer of IFN- $\gamma$  into established brain tumours represses growth by anti-angiogenesis [91], supporting the hypothesis that this therapeutic strategy may provide an effective method of eradicating established intracranial tumours.

IFN- $\gamma$  inhibition of angiogenesis is critical also in colon carcinoma, since IFN- $\gamma$  causes transcriptional silencing of perlecan gene expression in a colon carcinoma cell line [92]. Thus, IFN- $\gamma$ -mediated transcriptional repression of perlecan may represent a novel anti-angiogenic and anti-tumoural effect of this cytokine. Other reports regarding IFN- $\gamma$  involvement in angiogenesis point out that IFN- $\gamma$ -dependent inhibition of tumour angiogenesis by tumour-infiltrating CD4+ T cells requires tumour responsiveness to IFN- $\gamma$  [93]. In this report, the authors show that tumour responsiveness to IFN- $\gamma$  is necessary for IFN- $\gamma$ -dependent inhibition of tumour angiogenesis by CD4+ T cells, demonstrating a critical and pivotal role for CD4+ T cells in restraining initial tumour development through the inhibition of tumour angiogenesis. More recently it has been reported that IFN- $\gamma$  mediates IL-12-induced mobilization of human CD4+ T lymphocytes within tumour microenvironment of human lung cancer [71].

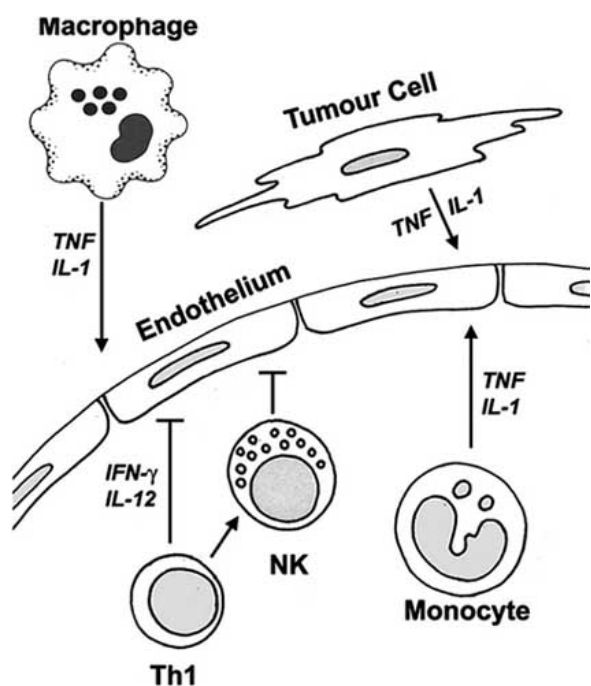
It is widely recognised that tumour rejection by CD8+ T cells is primarily mediated by direct killing. However, it has been recently reported that IFN- $\gamma$ -mediated angiostasis is critical also for tumour rejection by CD8+ T cells [94]. Indeed, rejection of different tumours, such as fibrosarcoma, ras-transformed fibroblasts, colon carcinoma and plasmacytoma, by CD8+ cells is always preceded by inhibition of tumour-induced angiogenesis. Thus, IFN- $\gamma$ -dependent anti-angiogenesis is a general mechanism involved in tumour rejection by T-cell effectors.

### CONCLUDING REMARKS

In the scenario described above, it is clear that pro-inflammatory cytokines such as IL-1 $\alpha$  and IL-1 $\beta$  behave as positive regulator of angiogenesis through direct (enhanced proliferation) and indirect effects (enhancement of pro-angiogenic factor release by inflammatory cells) mainly on EC. The role played by TNF- $\alpha$  is much more complex: it directly inhibits EC proliferation, but indirectly it enhances angiogenesis, through macrophage recruitment and activation. The net balance for TNF- $\alpha$  activities is to switch a pro-angiogenic programme.

Pro-inflammatory and Th1 cytokines, such as IL-12 and IFN- $\gamma$ , behave as negative regulators of angiogenesis through direct and indirect effects on EC, tumour cells and immune cells. Thus, collectively these observations demonstrate a pivotal role played by pro-inflammatory cytokines in angiogenesis that helps understand physiological and pathological angiogenesis, associated with inflammation, as shown in Fig. (1). However, a comprehensive pathway indicating the interactions between pro-inflammatory cytokines and cells present in tumour microenvironment, including tumour cells, inflammatory cells and EC, remains to be fully elucidated. In addition, only few results have been obtained by mimicking the cellular behaviour and interactions that could be relevant in the microenvironment to switch angiogenesis processes [68-70,95].

Under a therapeutic point of view, such scenario however raises the possibility that cytokines and cells involved may represent novel therapeutic targets. Indeed, IL-12 and IFN- $\gamma$  are being intensively studied for their direct impact on tumour progression, underscored by a number of clinical trials performed by using such cytokines. With regards to TNF- $\alpha$ , two TNF antagonists have been licensed for clinical trials in the treatment of inflammatory disease (rheumatoid arthritis and Chron's disease) [15]. Anti-TNF antibody treatment results in inhibition of cytokine/chemokine production, reduced angiogenesis and prevention of leukocyte infiltration, suggesting that all these actions may be useful in a biological therapy for cancer. Since the recombinant IL-1Ra, anakinra (Kineret; Amgen Inc., Thousand Oaks, CA), has been approved by the US Food and Drug Administration and the European Commission for the treatment of patients with active rheumatoid arthritis [96], observations similar to those derived for TNF- $\alpha$ , could be made for the potential therapeutic effects of IL-1Ra in cancer [36].



**Fig. (1).** Summary of inflammatory cell/ EC / tumour cell network. Inflammatory cells and tumour cells release pro-inflammatory cytokines in the local microenvironment to modulate angiogenesis. Both IL-12 and IFN-γ inhibit angiogenesis, as well as EC proliferation. Both IL-1 and TNF promote angiogenesis, although TNF inhibits EC proliferation. NKs inhibit EC proliferation and angiogenesis. (→ activation; —| inhibition).

However, if it is true that cancer state is the product of its microenvironment [49], we cannot think about the therapeutic implication of only one single cytokine or cytokine blocker. Thus, by resolving the complexity of the pro-inflammatory cytokine and leukocyte/EC network, we may offer fresh approaches to the novel stromal therapy, where early and dynamic targets are addressed [49]. Thus, Th1-cytokines (e.g., IL-12 and IFN-γ) and inflammatory cytokine receptor antagonists (IL-1Ra and TNF antagonists) may be part of more complex and novel biological approach to cancer therapy.

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**ABBREVIATIONS**

- AcP = Accessory protein
- COX = Cyclooxygenase
- EC = Endothelial cell
- ECM = Extracellular matrix
- FGF = Fibroblast growth factor
- IFN = Interferon
- HIF = Hypoxia inducible factor
- IL = Interleukin
- JAK = Janus kinase
- KO = Knockout
- LLC = Lewis lung carcinoma
- MCP = Monocyte chemotactic protein
- MIP = Macrophage inflammatory protein
- MMP = Matrix metalloproteinase
- NK = Natural killer
- PBMC = Peripheral blood mononuclear cells
- PDGF = Platelet-derived growth factor
- Ra = Receptor antagonist

- STAT = Signal transducer and activator of transcription
- Th = T helper
- TAM = Tumour-associated macrophages
- TIMP = Tissue inhibitor of metalloproteinase
- TGF = Transforming growth factor
- TNF = Tumour necrosis factor
- VEGF = Vascular endothelial growth factor

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