Monocyte/macrophage infiltration in tumors: modulators of angiogenesis

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Abstract: The role of a tumor immune infiltrate in cancer progression and metastasis has been debated frequently. Although often considered to be associated with improved prognosis and leading to the enhanced survival of cancer patients, inflammatory cells have also been described to assist the tumor's capabilities to progress, proliferate, and metastasize. Tumor-associated macrophages (TAMs), for example, have been shown to be symbiotically related to tumor cells: Tumor cells recruit TAMs and provide them with survival factors, and TAMs in turn produce a variety of angiogenic factors in response to the tumor microenvironment. This review will describe the composition of an immune infiltrate in tumors and the angiogenic and angiostatic properties of the cells present. Special emphasis will be on the angiogenesis-associated activities of TAMs. The development of immunotherapy and gene therapy using TAMs to mediate tumor cytotoxicity or to deliver gene constructs will be discussed as well. As immunotherapy has so far not been as effective as anticipated, a combination therapy in which angiostatic agents are used as well is put forward as a novel strategy to treat cancer. *J. Leukoc. Biol.* 80: 1183–1196; 2006.

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INTRODUCTION

Without the formation of new blood vessels (angiogenesis), expansion of a tumor mass is limited to 1–2 mm in size because of insufficient supply of oxygen and nutrients, a concept first proposed by Dr. Judah Folkman in 1971 [1]. Many tumors remain dormant at this size and therefore, stay clinically undetectable for years [2]. A dysequilibrium between positive and negative angiogenesis regulators eventually results in the progression of these in situ tumors to an angiogenic phenotype, the so-called angiogenic switch [3, 4]. Tumor angiogenesis is promoted by hypoxia, resulting in enhanced expression of angiogenic factors, such as vascular endothelial growth factor (VEGF), IL-8, and basic fibroblast growth factor (bFGF), and in decreased expression of angiogenesis inhibitors, such as platelet factor-4, thrombospondin, and angiostatin [5].

Once a tumor is vascularized, it is infiltrated by leukocytes, a phenomenon observed in all solid cancers. The first observation of leukocyte infiltration in tumors was done by Rudolf Virchow in 1863 [6] and was thought to be the result of chronic inflammation, which was already present before tumor development. Today, however, it is known that the presence of leukocytes is a consequence of an immune reaction to the tumor itself—first, innate, and later, specific immunity—as the immune system is able to recognize tumor-associated antigens [7]. In cancer patients, specific cytotoxic T lymphocytes recognizing tumor antigens have been reported, and the presence of these cells is related to a better prognosis. In addition, antibodies to tumor-associated antigens produced by B cells may also play a role in limiting tumor growth [8, 9]. Leukocyte infiltration in tumors is, therefore, often associated with better prognosis and overall survival [7]. Moreover, the ability of the immune system to recognize antigens expressed by tumors or tumor microvasculature can be used to identify molecules to define targets for cancer immunotherapy [8].

Under normal physiological circumstances, leukocytes are recruited in response to inflammation by the local synthesis of chemokines and other cytokines and by products that are associated with tissue breakdown. These processes are all part of a complex signaling system including recognition of the pathological state, organization of a proper cellular response, and subsequent suppression of this response once the situation is resolved. In wound healing, these processes include proliferation and migration of epithelial cells, angiogenesis, and tissue remodeling [10]. In tumors, similar chemoattractive factors are thought to play a role in leukocyte recruitment. Tumor cells, however, continuously send out signals that are associated with growth and migration processes, which recruit additional leukocytes to the tumor site [11]. This concept has contributed to the characterization of tumors as "wounds that fail to heal" [12].

Although associated with better prognosis, immune responses to a tumor are often weak and not able to destroy a tumor completely [7], suggesting that tumors have developed

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mechanisms to escape immune surveillance. Among a series of mechanisms that can explain this escape, including tolerance induction and activation of regulatory immune functions [13], one mechanism to escape tumor-directed immunity is inhibition of leukocyte-vessel wall interactions in tumor vessels [14–17] as a result of suppressed expression of endothelial adhesion molecules [18–21] and induction of endothelial cell anergy [22]. As a result, tumors frequently show only limited leukocyte infiltration, predominantly by cells of the innate immune system [19]. Leukocytes that do reach the tumor often remain localized in the tumor periphery or stroma and are often not able to exert strong antitumor effects [12]. Moreover, it is known that infiltrated leukocytes show impaired maturation as a result of exposure to the tumor microenvironment [7, 23]. Therefore, infiltrated leukocytes are often not capable of destroying the tumor completely. By contrast, some leukocyte subsets have been described to promote proliferation and metastasis and hence, tumor development. Tumor-associated macrophages (TAMs), for example, can exert protumor effects through the secretion of immunosuppressive cytokines, the release of free radicals such as NO and hydrogen peroxide (rendering immune cells hyporesponsive or even apoptotic), and last but not least, the secretion of angiogenic cytokines and enzymes (**Fig. 1**) [24]. This review will focus on the angiogenic effects of leukocytes that are infiltrated in a tumor. It will discuss the composition of a leukocyte infiltrate in tumors and the angiogenic and angiostatic properties of the immune cells present. Special emphasis will be on the role of TAMs, their capability to reach a tumor in large quantities, and hence, their possible role in immunotherapy.

IDENTITY AND ANGIOGENESIS-RELATED EFFECTS OF CELLS IN A TUMOR-IMMUNE INFILTRATE

In tumor-immune infiltrates, practically all cells involved in inflammation can be found. Leukocytes are known to accumulate in a tumor through a series of interactions with tumor endothelial cells, including leukocyte tethering, rolling, firm adhesion, and eventually, diapedesis [25]. The notion that tumor infiltration by leukocytes is always a prognostically positive phenomenon has changed in recent years; the finding that angiogenic factors cannot only be produced by tumor cells but also by infiltrating leukocytes indicates possible stimulating effects on tumor angiogenesis of the latter [26].

Polymorphonuclear granulocytes (PMNs) and more specifically, neutrophilic granulocytes, are the first line of defense against infections, and as they do not show any specificity for antigens, their predominant roles are cell killing and phagocytosis [27]. Furthermore, PMNs are known to infiltrate tumors and constitute an important part of the immune infiltrate. They may be considered as potential antitumor cells, but clinical studies have been contradictory with respect to the function of tumor-infiltrated PMNs. Whereas in patients with adenocarcinoma, the presence of increased numbers of tumor-infiltrating neutrophils was linked to poorer outcome [28], studies of gastric carcinoma suggested a correlation of neutrophil infiltration with good prognosis [29]. To improve PMN recruitment for immunotherapy, bispecific antibodies (with one specificity for FcγRI and another for a tumor-associated antigen) can be used. Results from a Phase I clinical trial with a bispecific anti-FcγRI/anti-human epidermal growth factor receptor 2/neu show that although the clinical course of the disease was not altered by the treatment, immunohistochemical analyses of tumor biopsies in individual patients documented infiltration of monocytes and PMN after antibody infusion [30]. It is interesting that PMNs can be induced to produce substances that stimulate or inhibit the angiogenic process. Activated PMNs contain granules that harbor matrix metalloproteinases (MMPs), capable of degrading the extracellular matrix (ECM) [27, 31] and hence, stimulate angiogenesis. In addition, they have been reported to produce a number of other angiogenic factors (VEGF, IL-8). On the contrary, PMNs are also known to be involved in the generation of endogenous angiogenesis inhibitors {e.g., angiostatin and other angiostatic factors, such as IL-12-inducible protein 10 (IP-10) and IFN-γ [7]}. Despite this body of information, the actual role of infiltrated PMNs in tumors is poorly investigated and remains unclear. Next to these angiogenesis-related effects, PMNs are strong producers of chemotactic proteins to recruit other PMNs, monocytes, immature dendritic cells (DC), and T lymphocyte subsets in a self-sustained process [7, 27].

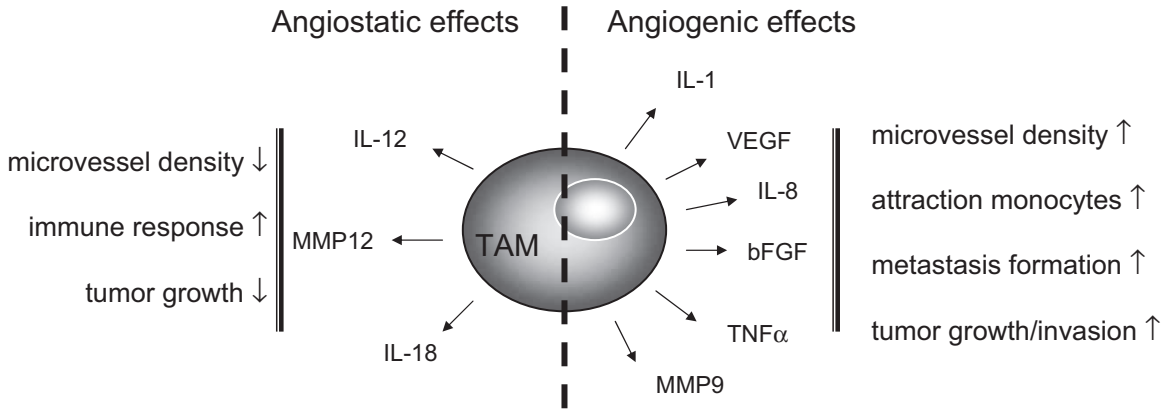

Fig. 1. Angiostatic effects versus angiogenic effects of tumor-associated macrophages (TAM).

Like PMNs, NK cells can recognize and lyse tumor cells without having to be primed and without the need for MHC recognition, and they are thought to be involved in immune surveillance against cancer [7, 21, 32]. Although not frequently seen in tumors [33, 34], there is some evidence that NK cells can play a critical role in angiogenesis inhibition by IL-12-induced IFN-γ secretion [35]. In a study by Yao et al. [35], IL-12 was inoculated in a human Burkitt tumor in mice; subsequently, extensive vascular damage and tumor tissue necrosis were observed as well as an accumulation of activated NK cells in necrotic areas surrounding blood vessels. In another study by Hayakawa and co-workers [36], using three different mouse tumor models, it was shown that IFN-γ secreted by NK cells is involved in inhibition of tumor angiogenesis by α-galactosylceramide, as presented by the MHC Class I-like molecule CD1d, a ligand for NK cells. Depletion of NK cell activity markedly reduced angiogenesis inhibition [35] and enhanced tumor growth [36], providing evidence that NK cells can play a critical role in the regulation of angiogenesis. Activated NK cells have also been reported to effectively kill tumor cells and endothelial cell targets and hence, inhibit angiogenesis [37, 38]. Therefore, NK cells can be considered to have a central role in inhibiting tumor angiogenesis at the interplay between host immunity and tumor cell biology.

T lymphocytes secrete a repertoire of molecules (e.g., IL-2, TNF-α, and IFN-γ), which regulate intercellular signaling events involved in host responses to foreign antigens, such as recruitment of immune effector cells (IL-2, IFN-γ) and regulation of expression of cell surface proteins on endothelial cells (TNF-α) [39]. T cell infiltration is commonly seen in tumors (60% of an infiltrate can be composed of lymphocytes, of which a large majority is T cells [34]) and is often associated with improved prognosis and survival [33, 40–42]. IFN-γ secretion by CD4+ T cells of the Th1 subset has been shown to be important in vivo in inhibiting tumor cell proliferation, activating macrophages, and enhancing the secretion of angiostatic chemokines [43]. IL-4 secretion by Th2 cells might be involved in tumor development as well by inhibiting VEGF- and bFGF-induced angiogenesis in vitro [44]. Although tumor-infiltrating lymphocytes have been reported to show cytolytic activity induced by tumor cells [39], tumors have evolved mechanisms to escape their effects in vivo. Some tumors contain anergic lymphocytes, probably as a result of local secretion of immunosuppressive factors (i.e., TGF-β [45]) in the tumor microenvironment [39, 46] or the loss of MHC expression by tumor cells [7]. Alternatively, a decreased activity of the T cells may be a result of the presence of CD25-positive regulatory T cells antagonizing a specific T cell response. For this reason, current clinical immunotherapy approaches are combined with simultaneous infusion with anti-CD25 antibodies to eliminate these regulatory T cells [47, 48]. Although T cell infiltrates in tumors are mostly seen as evidence of a protective immune response and hence, associated with better prognosis, it has been demonstrated that CD4+ and CD8+ lymphocytes are capable of promoting angiogenesis in vivo as well [39]. Inside and outside a tumor, T lymphocytes can produce the angiogenic compounds bFGF and VEGF in response to hypoxia, suggesting an important role for these cells in tumor proliferation and metastasis [39, 49, 50]. Although B lymphocytes [51, 52] are also described to be present in a tumor immune infiltrate, no specific information about angiogenesis-related effects of these cells has been found. However, as B lymphocytes are a known source of cytokines such as IL-1 and IL-6, some positive effects of this lymphocyte subset on the process of angiogenesis may be speculated.

DC are not often found in tumor infiltrates [33, 34], and if found, they are mostly in an immature state and incapable of inducing an effective immune response [53, 54]. Normally, DC are the most efficient APC, with the unique ability to present tumor-specific antigens and subsequently, activate a specific antitumor T cell response in vivo. Immature DC are capable of antigen ingestion and processing, but they must mature to activated DC to elicit a productive immune response. Conflicting data have been published about the effect of infiltrated DC on tumor development. Immature DC have been shown to silence immunity and induce tolerance by deleting T cells or by expanding regulatory T cells [53, 55]. This phenomenon appeared to be mediated by tumor-derived factors such as VEGF, IL-6, and M-CSF (or CSF-1) [23, 54]. Conversely, it was also shown that DC infiltration in tumor lesions is associated with improved survival rates and reduced incidence of recurrent disease in different types of malignancies [56]. In addition, immunotherapy using DC has been investigated thoroughly and has proven to be feasible, nontoxic, and effective in some patients, particularly when DC are mature and active before administration [53, 57–59]. It is unknown, however, whether DC themselves serve as angiogenesis mediators.

Mast cells are bone marrow-derived accessory cells, which are not found in circulation (and do not migrate into tumor areas) but associated with certain structures as blood vessels or nerves [60]. They are often associated with allergic reactions to certain allergens but are also known to stimulate immunity by enhancing the inflammatory reaction. Mast cells have been found in a variety of human cancers (such as melanoma and breast and colorectal cancer), and their accumulation at tumor sites is associated with enhanced tumor growth and invasion [11, 60]. Infiltration by mast cells and activation of MMP-9 have been shown to coincide with the angiogenic switch in premalignant lesions [61]. They have been reported to support tumor proliferation and progression by their ability to induce tumor angiogenesis [60]. Activated mast cells are known to produce a number of angiogenic factors, such as VEGF, bFGF, IL-8, TNF-α, and MMP-9, which is involved in degrading the ECM [11, 60]. Furthermore, they are able to produce the angiogenic mediators histamine and heparin, which can stimulate endothelial cell proliferation (directly or indirectly by the stabilization of growth factors) and may have a role in the hyperpermeable nature of newly formed vessels in tumor angiogenesis [62]. Reduction of tumor mast cell density by transfection of a vector containing antisense stem cell factor cDNA (stem cell factor, which stimulates mast cell migration, proliferation, and degranulation) has been shown to inhibit angiogenesis and therefore, tumor growth [63]. Therefore, interfering with mast cell recruitment to tumor sites might be a possible therapy for cancer.

When monocytes are released from the bone marrow into the blood, they are still immature. They only differentiate into macrophages once they are infiltrated into tissues [64]. The

majority of malignant tumors appears to be infiltrated by macrophages, which can comprise more than 50% of the total tumor mass [65]. These TAMs are capable of killing tumor cells and releasing angiostatic compounds, but they can also stimulate tumor growth by producing angiogenic factors and metalloproteinases [7, 65, 66]. During tumor development, the tumor-stimulating effects of these infiltrated macrophages often over-rule the tumor-inhibiting ones [7]. Overexpression of macrophage chemoattractants within tumors has been shown to correlate with poor prognosis [67], and the level of macrophage infiltration in tumors is known to be positively correlated with microvessel density, tumor stage, and angiogenesis in human tumors [68]. TAM presence is, therefore, associated with reduced, relapse-free and overall survival [65, 68–71] in several tumor types (i.e., melanoma [68] and cervical [69, 70] and breast [65, 71] cancer). Recently, Naldini and co-workers [72] showed that Tie-2-expressing monocytes are a distinct, hemopoietic lineage of proangiogenic cells that account for most of the angiogenic activity of myeloid cells in mouse tumors. Another group identified the population of tumor-infiltrating macrophages as being of the polarized M2 phenotype, distinguishable by specific profiles of chemokines and chemokine receptors [73, 74]. Conversely, however, in other tumor types (i.e., prostate [75] and stomach [76] tumors), TAM presence appears to be associated with improved prognosis. It is suggested that extensive monocyte invasion in a tumor results in elimination of tumor mass, and a low level of monocyte recruitment provides an angiogenic stimulus, which permits tumor proliferation [77].

Altogether, a tumor infiltrate stimulates and inhibits angiogenesis, depending on the type, timing, number, and activation state of all the cells present [7]. In the next part of this review, we will focus more explicitly on the recruitment and the possible angiogenic and angiostatic activities of TAMs.

RECRUITMENT OF TAMs BY TUMORS

Chemokines and cytokines

Monocytes, like other leukocytes, are actively attracted to the tumor site as a result of the production of cytokines and chemokines by tumor cells. Chemokines are divided in major subfamilies, such as CXC and CC, depending on the arrangement of the two N-terminal cysteine residues. They play major roles in all aspects of tumor biology, including recruitment of leukocytes, regulation of angiogenesis and tumor growth (mainly CXC chemokines [78]), and control of the movement of tumor cells during metastasis [79]. Several CC chemokines, such as MCP-1 (or CCL2) and RANTES (or CCL5), produced by tumor cells, endothelial cells, fibroblasts, and also by TAMs, are known to attract monocytes [79, 80]. MCP-1 is expressed highly in several tumor types and is associated with infiltration of monocytes. It is interesting that MCP-1 expression has been shown to correlate significantly with levels of VEGF, TNF-α, and IL-8, suggesting a possible role for MCP-1 as an angiogenesis regulator as well [64, 81, 82]. The expression of another CC chemokine, RANTES, in breast tumor cells is known to be elevated synergistically by IFN-γ and TNF-α, regulating monocyte migration into tumor sites and promoting cancer progression. Moreover, RANTES has been found to stimulate monocytes to secrete MMP-9 and MMP-19, indicating its indirect involvement in degrading the basement membrane and therefore, in angiogenesis [83, 84]. Although the involvement of CXC chemokines in regulating angiogenesis is well studied [78], their role in directing tumor infiltration by TAMs is less well defined. The angiogenic factor IL-8 (CXCL8), however, has been shown to cause rolling monocytes to adhere firmly onto endothelial cell monolayers expressing E-selectin, indicating that IL-8 is not only acting as a chemoattractant but is also involved in translating initial monocyte tethering into firm adhesion through activation of leukocyte integrin [85].

Monocytes are directed to the tumor by other cytokines as well. Although VEGF is a well-known angiogenic cytokine, it is also a chemotactic protein for monocytes via VEGF-receptor-1 (flt-1) [64, 86–88]. It is produced by tumor cells and TAMs and is controlled by oxygen tension [89]. As VEGF is expressed mainly in response to hypoxia, TAMs are often seen to accumulate in poorly vascularized tumor areas [64]. M-CSF (or CSF-1) is another cytokine with chemoattractive properties for monocytes, and it is being produced by tumor cells and TAMs. Elevated expression of M-CSF and its receptor (CSF-1R) have been found in human breast, endometrial, and ovarian tumors [90, 91]. High M-CSF expression is associated with high TAM accumulation in breast carcinomas [92] and poor prognosis [93–95]. It has also been described to be involved in induction of VEGF and IL-8, suggesting a possible role in regulation of angiogenesis for this cytokine as well [81, 96].

Adhesion molecules

Next to chemoattractants, adhesion molecule expression on endothelial cells as well as on monocytes is eminently involved in monocyte recruitment to tumors [19, 20, 97]. To actually infiltrate tissues, monocytes have to interact with the venular vessel wall. This process of monocyte-vessel wall interaction appears to be a multistep cascade in which each step has to be completed for the next step to occur [98]. The first step is the tethering and subsequently, rolling of leukocytes along the vessel wall. Monocyte rolling is mediated mainly by adhesion molecules of the selectin family (L-selectin found on monocytes and P- and E-selectin on activated endothelial cells) binding to their counter-receptors on opposing cells (e.g., CD34 on endothelial cells and P-selectin glycoprotein-1 on monocytes [99]). Rolling monocytes can become activated by chemoattractants or cell contact-mediated signals inducing secondary adhesion molecules [100], by which they can firmly adhere to the endothelium. Monocyte adhesion to the vessel wall is mediated by adhesion molecules of two families: the integrins (e.g., LFA-1 and VLA-4, expressed on monocytes) and the Ig gene superfamily (e.g., ICAM-1 and VCAM-1, expressed on activated endothelial cells and on leukocytes or macrophages) [101]. Adhering monocytes can then transmigrate through intercellular junctions between endothelial cells and migrate into the surrounding (tumor) tissue [102].

Previously, we have shown, in two separate mouse models, that tumor-derived angiogenic factors such as VEGF and bFGF are responsible for down-regulation of endothelial adhesion

molecules such as ICAM-1, VCAM-1, and E-selectin, resulting in diminished leukocyte-vessel wall interactions in tumor vessels [18, 103, 104].

ANGIOSTATIC PROPERTIES OF TAMs

Macrophages are known to accumulate mainly in poorly vascularized, hypoxic areas as a result of specific up-regulation of various chemoattractants (recently reviewed by Murdoch et al. [79]). Once macrophages arrive at the tumor site, they start to produce their own set of proteins to attract more leukocytes and to influence the process of angiogenesis. TAMs are reported to have a number of angiostatic properties (**Table 1**), which will be discussed in this section.

IL-12

IL-12 is produced by monocytes/macrophages and also by DC and B cells; it is known for its antitumor and antimetastatic activity [105]. IL-12 activates a T cell-dependent antitumor immune response that is able to eradicate established, large tumors in a number of immunogenic animal models [121]. Moreover, activated macrophages appear to function as effector cells in this IL-12-induced tumor eradication by inducing apoptosis [121]. IL-12 appears to have angiostatic capacities as well [106, 107], which induce murine tumor necrosis [108]. In human tumors, IL-12-producing TAMs have been demonstrated [122]. Angiogenesis inhibition by IL-12 is not carried out via direct interaction with endothelial cells but merely via initiation of IFN-γ production by Th1 cells and NK cells [64, 109, 110]. The production of IL-12 by macrophages enhances immune function by shifting CD4+ cells toward the Th1 subset, which secrete IL-2 and IFN-γ [112], facilitating in turn the proliferation and activation of CD8+ cytotoxic T cells, NK cells, and macrophages, which may contribute to tumor regression [107]. IFN-γ is, in turn, able to induce the production of IP-10 (or CXCL10) and MIG (CXCL9), which have angiostatic properties [108, 111]. Moreover, IFN-γ is able to induce additional, nonantigen-specific antitumor mechanisms, including a retardation of cellular proliferation and production of NO [107]. In a murine model of breast cancer, IL-12 therapy has shown potent angiostatic effects and subsequent tumor regression. Levels of tumor VEGF and MMP-9 were declined markedly and eventually undetectable [123]. IL-12 is able to accelerate a variety of antitumor mechanisms, some of which are antigen-specific and some of which are not. At the moment, the use of IL-12 is investigated in Phase I and II trials with promising results, including improved, immunological antitumor responses [124–126].

IL-18

IL-18 is another substance known to induce tumor regression via angiogenesis inhibition [107]. It is being produced by (activated) macrophages, DC, and Kupffer cells [105, 107]. Similar to IL-12, IL-18 inhibits angiogenesis through stimulation of IFN-γ production (by Th1 lymphocytes and NK cells), resulting in a systemic, murine antitumor response [107]. It appears to be more potent as an IFN-γ inducer than IL-12, apparently through a separate intracellular signal-transduction pathway [113, 114]. IL-18-treated tumors show a significant decrease of microvessel density [115]. Administration of IL-18 inhibits the growth of fibrosarcoma by hypovascularization [114]. One of the angiostatic mechanisms of IL-18 is its ability to inhibit bFGF-induced endothelial cell proliferation, which was demonstrated in vitro, and IL-12 lacks such an effect [115]. Therefore, the angiostatic pathway of IL-18 may not be completely dependent on activation of IFN-γ. It is interesting that IL-18 and IL-12 have been reported to have synergistic, antitumor effects [114, 116] and synergistically inhibit angiogenesis [107]. Systemic administration of IL-18 was shown to potentiate inhibition of proliferation of carcinoma cells modified to express IL-12 [114]. In a murine sarcoma model, intratumoral injection of DC engineered to produce IL-12 or IL-18 showed to decrease tumor proliferation and even complete tumor rejection more strongly then either one of them [116]. Besides its inhibitory effects on angiogenesis, IL-18 has also proven to be a proinflammatory cytokine and a strong inducer of immune responses [114]. Taken together, IL-18, particularly, in combination with IL-12, may be a promising antitumor agent.

MMP-12

MMPs are a family of zinc-dependent endopeptidases, which can cleave all major molecules of the ECM [64, 117, 118]. During tumor growth, MMP can enhance angiogenesis and degrade connective tissue and basement membranes to enable tumor growth and metastasis [117]. Next to a tumor-promoting effect, some MMPs, such as metalloelastase (MMP-12), matrilysin (MMP-7), and gelatinase-B (MMP-9), have also been shown to possess angiostatic properties [117, 118]. MMP-12 is generally expressed in macrophages, although tumor cells were

TABLE 1. Angiostatic Factors Secreted by TAMs

Cytokines	Effects	References
IL-12	-IL-12 → ↑ IFN-γ and TNF-α by Th1 cells → ↑ angiostatic factors IP-10 (CXCL10) and MIG (CXCL9)	[64, 105–111]
IL-18	-IL-18 → ↑ IFN-γ by Th1 cells → ↑ angiostatic factors IP-10 (CXCL10) and MIG (CXCL9) - ↓ bFGF-induced endothelial cell proliferation	[105, 107, 112–116]
MMP-12	- ↑ M-CSF/IL-1β/TNF-α/VEGF → ↑ MMP-12 → ↑ cleavage of plasminogen to angiostatin	[27, 64, 117–120]

MIG, Monokine induced by IFN-γ.

shown to express this enzyme as well [117, 127]. Pro-MMP-12 is released by macrophages in response to several factors (such as M-CSF, IL-1β, TNF-α, and VEGF). The amount of macrophage-derived MMP-12 appears to be higher in Grade I and II vulvar tumors compared with the more aggressive Grade III tumors, indicating that the level of MMP-12-positive macrophages correlates with a better prognosis [117]. Moreover, in a study of colorectal carcinoma, patients with high expression of MMP-12 mRNA significantly demonstrated better survival compared with those patients who did not show high MMP-12 expression. Patients with high MMP-12 expression showed lower microvessel density, demonstrating that MMP-12 is able to inhibit angiogenesis in vivo in human tumors [119]. MMP-12 is capable of generating angiostatin [120], a potent angiogenesis inhibitor identified by O'Reilly et al. [128] in Lewis lung carcinoma (LLC). Angiostatin is a cleavage product of plasminogen, which has been shown to inhibit endothelial cell proliferation and impairs primary and metastatic tumor growth through inhibition of angiogenesis [118]. Altogether, MMP-12 and angiostatin appear to play an important role in the inhibition of angiogenesis and tumor progression in patients.

ANGIOGENIC PROPERTIES OF TAMs

In contrast to the limited knowledge concerning angiostatic activities of TAMs, more research has focused on their angiogenic properties (see Table 2). Understanding the angiogenic activity of TAMs in a tumor will hopefully raise opportunities to counteract these activities to treat cancer.

IL-1

The cytokine IL-1 is produced mainly by monocytes/macrophages and appears to be a highly inflammatory cytokine, also possessing various immune, degradative, and growth-promoting properties. There are two IL-1 proteins, IL-1α and IL-1β, and one naturally occurring IL-1 receptor antagonist (IL-1Ra) [129]. All IL-1-associated proteins bind to the same receptor but exert different effects. IL-1α and IL-1β are involved in tumor angiogenesis, but the role of IL-1β appears to be more evident [129]. This is probably a result of the fact that IL-1β is secreted in the tumor microenvironment and hence, activates tumor cells. IL-1α has a less-prominent effect, as this cytokine is largely cell-associated and remains cytosolic [129]. Song et al. [130] demonstrated in mice that IL-1α potentiates the development of an antitumor immune response, thereby limiting tumor growth, whereas IL-1β enhances invasiveness of the tumor and induces angiogenesis and immune suppression in the patient. IL-1β has been shown to selectively induce the expression of hypoxia-inducible factor 1 (HIF-1), thereby promoting VEGF production. Macrophages used from IL-1α or IL-1β knockout mice showed reduced VEGF secretion [129]. It has been demonstrated that IL-1β is able to accelerate tumor growth of LLC in transplanted mice by neovascularization and induction of angiogenic factors such as VEGF, MIP-2, and human growth factor [131]. Another effect of IL-1β on angio-

TABLE 2. Angiogenic Factors Produced by TAMs and Their Effects

Cytokines/chemokines	Effects	References
IL-1β	-HIF → ↑ VEGF -MIP-2/HGF/TNF-α -↑ angiogenin	[64, 129–132]
VEGF	-↑ vascular permeability -↓ endothelial adhesion molecules -Chemotactic for monocytes	[18, 96, 135, 149]
IL-8	-↑ microvessel density -↑ macrophage infiltration	[81, 82, 141, 149, 162]
bFGF	-↑ VEGF -Chemotactic for monocytes -↓ endothelial adhesion molecules	[18, 144, 146, 149]
TNF-α	-↑ TP → ↑ angiogenic factor deoxyribose 1-phosphate -↑ IL-8/VEGF/bFGF/angiogenin -↑ IL-8/VEGF/bFGF receptors	[64, 67, 68, 132]
MMP-9	-↑ VEGF -↑ pericyte recruitment/vessel stabilization	[117, 152, 153, 163]
M-CSF	-↑ VEGF/IL-8 -Chemotactic for monocytes	[81, 96]
MCP-1	-↑ VEGF/IL-8/TNF-α -Chemotactic for monocytes	[81, 82]
MIF	-↑ TNF-α/IL-1β/CXC chemokines	[157, 158]
uPa	-degradation ECM	[64, 67, 156]
PAF	-↑ TNF-α/IL-1/bFGF/VEGF	[159]
TGF-β	-IL-8/TNF-α/VEGF/bFGF/IL-1 -Chemotactic for monocytes	[82]
HB-EGF	-↑ VEGF/MMP-9/MMP-3 -VEGF → ↑ HB-EGF	[160]
PDGF	-Macrophage recruitment and migration -Vessel stabilization	[33, 161]

HGF, Hepatocyte growth factor.

genesis is through its involvement in the production of TNF-α [129] and angiogenin [132]. The properties of IL-1 associated with tumor growth and metastasis make it an attractive target for therapeutic intervention. Bar et al. [133] genetically engineered NIH/3T3 cells to continuously secrete the IL-1Ra and encapsulated these cells within microspheres to provide a barrier for the immune system. The microspheres were injected s.c. in mice with fibrosarcoma cells. It was shown that tumor development and tumor angiogenesis were inhibited and that survival was prolonged. In conclusion, as many tumors and TAMs have been shown to express IL-1, IL-1Ra may be useful in anticancer therapies.

VEGF

VEGF promotes endothelial cell proliferation and new blood vessel formation and is produced by TAMs and also by tumor cells in a variety of human cancers [64]. Although there are multiple angiogenic factors that promote blood vessel formation in human cancer, VEGF appears to play a dominant role [96]. The VEGF family consists of the prototype member VEGF (also referred to as VEGF-A), placenta growth factor, VEGF-B, VEGF-C, and VEGF-D. Besides VEGF, TAMs also express VEGF-C and VEGF-D, both associated with the formation of lymphatic vessels and lymphatic metastasis [134]. Next to a stimulatory effect on the process of angiogenesis, VEGF increases vascular permeability [135] and prevents the up-regulation of endothelial adhesion molecules induced by inflammatory cytokines [18]. At present, two VEGF receptors, VEGFR1 (or Flt-1) and VEGFR2 (or Flk-1) are known. Although inactivation of both receptors resulted in embryonic lethality, there is now consensus that VEGFR2 is mainly involved in the angiogenic-, mitogenic-, and permeability-enhancing effects of VEGF [135], and VEGFR1 is likely to have important roles in the recruitment of monocytes to tumors [86, 87] and in the induction of MMP-9 [136]. VEGF expression in macrophages [137] and tumors is induced via hypoxia [138] and HIF, regulated by the product of von Hippel-Lindau tumor-suppressor gene [138, 139]. In monocytes, VEGF is also induced via M-CSF and MCP-1, indicating a role for these macrophage chemoattractants in angiogenesis regulation [81, 96]. TAMs preferentially migrate to hypoxic areas within tumors and strongly express HIF-2α, and normal tissue macrophages do not, resulting in expression of HIF-2α-responsive, angiogenic factors such as VEGF and high tumor vascularity. This high macrophage HIF-2α appears to be an independent, prognostic factor for poor outcome. The mechanisms up-regulating HIF-2α in macrophages may include inflammatory cytokines as well as hypoxia [140].

Tumor growth inhibition has been demonstrated by several laboratories using a number of anti-VEGF approaches, including anti-VEGFR-2 antibodies [135]. In vivo treatment of mice with anti-VEGF antibodies was shown to result in higher leukocyte-vessel wall interactions in tumor vessels compared with nontreated animals [18]. Moreover, combining anti-VEGF treatment with chemotherapy or radiation results in greater antitumor effect than either treatment alone, indicating that anti-VEGF approaches for the treatment of cancer are promising.

IL-8

IL-8 is a potent angiogenic factor in several cancers and associated with metastasis [141]. It has been reported to be produced by TAMs and by endothelial cells, fibroblasts, and tumor cells in human malignancies [64, 141]. Monocytes can be induced to up-regulate IL-8 secretion by M-CSF, MCP-1, and TGF-β, suggesting an important role for these chemotactic factors in monocyte/macrophage-mediated tumor angiogenesis [81, 82]. The interaction between infiltrated macrophages and tumor cells is also known to up-regulate IL-8 (and VEGF) expression in both cell types in a paracrine manner [82, 141]. TAMs produce TNF-α and IL-1, which in turn induce tumor cells to produce higher levels of IL8 and VEGF [82]. It is interesting that it was shown by Sparmann and Bar-Sagi [142] that IL-8 is a transcriptional target of Ras oncogene signaling. In a tumor xenograft model, Ras-dependent IL-8 secretion appeared to be required for the initiation of tumor-associated neovascularization. IL-8 expression has been correlated negatively with patient survival and positively with macrophage infiltration in tumors, suggesting that IL-8 might be dominantly derived from TAMs [69]. In contrast to these results, Chen et al. [141] reported that cancer cells were the major source of IL-8 in patients with nonsmall cell lung cancer. There is a significant, positive correlation between IL-8 expression and microvessel density (in patients with uterine endometrial cancers) [143], indicating the angiogenic potential of this IL. The induction of IL-8 mRNA expression involves activation of the NF-κB pathway, which can be suppressed >93% by anti-inflammatory agents [141]. Specific inhibition of tumor angiogenesis via NF-κB and IL-8 using anti-inflammatory agents, such as aspirin, might be a new approach to inhibit angiogenesis in cancer [141].

bFGF

bFGF (or FGF-2) is secreted by macrophages, mast cells, lymphocytes, fibroblasts, and by some malignant cells [144–146]. bFGF belongs to a large family of homologous polypeptide growth factors and promotes every phase of the angiogenic process, including synthesis of proteinases, endothelial cell migration, and differentiated capillary tube formation in vitro [144]. bFGF induces angiogenesis through the high-affinity heparin-binding receptors FGFR-1 and FGFR-2 [80]. The number of bFGF-positive mast cells and macrophages appears to be higher in pancreatic adenocarcinomas with bFGF-positive cancer cells in patients [146], suggesting a cohesion between bFGF expression in these cell types. In various human tumors, bFGF has also been localized to stromal macrophages, and in vitro experiments showed expression of bFGF in macrophages, induced by tumor-derived signals [145]. Increased bFGF expression correlates with poor prognosis, and bFGF expression by cancer cells shows a direct correlation with microvessel density in patients [146]. Stromal bFGF expression, however, did not correlate with microvessel density or the frequency of metastasis in pancreatic carcinoma [144]. bFGF has been found to be chemotactic for macrophages [146], implying that this factor also has indirect stimulatory effects on angiogenesis and negatively affects patient survival.

Another way for bFGF to induce angiogenesis indirectly is by its capacity to promote VEGF synthesis. In addition, there is a synergistic interaction between bFGF and VEGF in inducing endothelial cell proliferation and angiogenesis [80, 144]. Zhang and Issekutz [80] reported that bFGF significantly reduces monocyte adhesion and migration to resting and stimulated endothelial cells, even when these monocytes were activated with chemotactic factors such as RANTES or MCP-1 [80]. The underlying mechanism appeared to be bFGF-induced down-regulation of the endothelial adhesion molecules ICAM-1, VCAM-1, and E-selectin, which are necessary for leukocyte capture, rolling, firm adhesion, and emigration [147, 148]. This effect of bFGF might interfere with infiltration of monocytes and other leukocyte subtypes in tumors. Overall, TAM-derived bFGF seems to be a less-prominent angiogenic factor as compared with the other factors described in this review. As its expression has been correlated with decreased patient survival, bFGF presumably exerts its tumor growth-stimulating effect via ways other than direct promotion of angiogenesis.

TNF-α

TNF-α is an inflammatory cytokine with angiogenic activity, which is expressed mainly by TAMs [67]. Other sources are mast cells, T lymphocytes, NK cells, neutrophils, and some tumor cells [149]. This cytokine has been found to promote the production of angiogenic factors, such as IL-8, bFGF, and VEGF, through activation of transcription factors in tumor cells and surrounding other cell types [68, 149]. The in vitro tube formation in vascular endothelial cells, which is induced by TNF-α, is inhibited by administering anti-IL-8, anti-VEGF, and anti-bFGF antibodies. When all these antibodies were administered together, an almost complete abrogation of tube formation was seen. In vivo, anti-IL-8 and anti-VEGF antibodies were shown to block angiogenesis induced by TNF-α in rabbit corneas [149]. TNF-α also stimulates tumor cells to express angiogenin, a potent angiogenic cytokine [132]. Angiogenin has found to be up-regulated in pancreatic cancer patients and contributed to the aggressiveness of pancreatic cancer. In colorectal cancer, increased serum angiogenin concentration is correlated with cancer progression [132]. TNF-α is also known to up-regulate the expression of the angiogenic enzyme thymidine phosphorylase (TP) in cancer cells [67]. TAM expression of TNF-α has been correlated positively to up-regulation of TP in breast tumors [150]. TP catalyzes the reversible conversion of thymidine to thymine and the production of the angiogenic factor deoxyribose 1-phosphate, thereby influencing the angiogenic process. TP expression in breast TAMs appears to correlate with increased microvessel density and poor prognosis in patients with various tumor types [67].

TNF-α also up-regulates the expression of endothelial cell adhesion molecules, a necessary step for leukocyte-vessel wall interactions and hence, leukocyte infiltration into the tumor to occur. Previously, we have shown [18] that TNF-α induces a decrease in the number of circulating leukocytes in nude mice bearing a small LS174T colon carcinoma in their ear. This is probably a result of the fact that TNF-α induces leukocyte adhesion systemically. This effect was evident for monomorphonuclear cells (MMNs) and PMNs but was most prominent for MMNs [80% reduction vs. 55% for PMNs ($P<0.01$)]. In contrast to a small ear tumor, the presence of a large flank tumor tended to mask these TNF-α-induced changes, suggesting a systemic effect of tumor-derived signals on endothelial cell adhesion molecule expression and hence, leukocyte-vessel wall interactions. In summary, TNF-α is seen as a potent activator of tumor angiogenesis, which is modulated by various angiogenic factors, and an important factor in recruiting leukocytes to a site of injury or a tumor.

MMP-9

Expression of MMP-9 (or gelatinase-B) is associated directly with increased tumor invasiveness and metastasis as well as poor patient outcome [127, 151]. MMP-9 is expressed by cancer cells, stromal neutrophils, fibroblasts, mast cells, and macrophages [117]. In a variety of invasive human cancers, TAMs have been shown to be a major source of MMP-9 [64], and its expression appears to be significantly higher in Stage III and IV neuroblastoma than in Stage I or II, suggesting an important role for this proteinase in tumor progression. Stromally derived MMP-9 has recently been shown to contribute to angiogenesis by promoting blood vessel morphogenesis and pericyte recruitment and hence, vessel stabilization [152]. In monocytes/macrophages, MMP-9 is induced by inflammatory mediators such as TNF-α and bacterial LPS [153]. VEGFR-1 is also described to be involved in MMP-9 induction in macrophages [154], and MMP-9, in turn, increases availability of VEGF [152]. Furthermore, leukocyte infiltration and subsequent activation of MMP-9 expression have been associated with the "angiogenic switch" in premaligant tissue, and it promotes tumor angiogenesis in mouse models [117]. MMP-9 is known to degrade the vascular basement membrane by cleavage of Type IV collagen [153]. Despite the angiogenic effects of this MMP, MMP-9 has also been described to generate angiostatin, implying its involvement in angiogenesis inhibition as well [117]. The net result, however, of MMP-9 presence in tumors seems to be promotion of angiogenesis. Further evidence that MMP-9 contributes to tumor progression came from studies about the endogenous tissue inhibitors of MMPs (TIMPs). Overexpression or i.p. injection of TIMPs was shown to reduce metastasis in experimental models [155]. However, effects of synthetic metalloproteinase inhibitors in Phase III clinical trials have been disappointing, showing no significant difference in patient survival [155]. It remains to be seen whether these inhibitory substances become a standard cancer treatment.

Other angiogenic factors produced by TAMs

Besides the earlier-mentioned angiogenic substances, TAMs also express a number of other angiogenic factors (see Table 2). Among them are the chemotactic proteins M-CSF and MCP-1, which induce VEGF and IL-8; they have already been described previously in this manuscript. Furthermore, TAMs express urokinase-type plasminogen activator (uPA) and its receptor uPAR (CD87) [64, 67, 156], macrophage-inhibitory factor (MIF) [157, 158], platelet activating factor (PAF) [159], TGF-β [82], heparin-binding epidermal growth factor (HB-EGF) [160], and platelet-derived growth factor (PDGF)

[33, 161], which are described further in **Table 2**. Although these angiogenic factors might be of equal importance, knowledge about them is limited compared with the ones described in this review.

TAMs IN IMMUNOTHERAPY?

The data presented here about angiogenic and angiostatic activities of TAMs clearly show two faces of the presence of these cells in tumors. It is possible that tumors are eradicated by TAMs in a preclinical stage of cancer development. In most tumors studied, however, the balance between angiogenic and angiostatic effects of TAMs is in favor of the angiogenic ones. The ability of TAMs to infiltrate tumor tissue and their presence at the tumor site can and has been used to counteract their stimulating activities via immunotherapy, as described in the following section.

Although macrophages have the potential to mediate tumor cytotoxicity and to stimulate antitumor lymphocytes, some tumors are apparently capable of escaping these defense mechanisms. This could be a result of the fact that malignant cells often do not express "foreign" surface antigens and are not recognized as such by circulating monocytes. It has also been suggested that tumor-derived signals suppress TAM activity, as neither monocytes isolated from peripheral blood of cancer patients nor TAMs obtained from tumor sites showed any cytotoxicity toward tumor cells ex vivo. Moreover, macrophages isolated from metastatic tumors were shown to suppress tumor-specific T and NK cytotoxicity [64, 112]. Even worse, tumors seem to benefit from the angiogenic capacities of TAMs, as described in this review. Nevertheless, the ability of macrophages to infiltrate tumors can be used to develop immunotherapeutic approaches for the treatment of cancer. In addition, it may be possible to emphasize their angiostatic properties via gene therapy.

Adoptive transfer of ex vivo-generated macrophages in patients with cancer may be a way of overcoming immune silencing effects of the tumor microenvironment. This was performed for the first time by Fidler in 1974 [164], using the B16 melanoma model. The suppression of pulmonary metastases was demonstrated following infusion of ex vivo-activated macrophages. This observation was confirmed by the results of additional rodent studies. However, regression of the primary tumor was seldom seen [165]. In humans, monocytes have been isolated from the blood of cancer patients, matured (using M-CSF), activated (using LPS or IFN-γ), and subsequently, infused back into the patient. Apart from low-grade fever and other flu-like symptoms, no side-effects were observed. There was, however, no clinical evidence of an antitumor effect of these ex vivo-activated macrophages [165]. This may be explained by the observation that the infused macrophages concentrated only partially at sites of metastases, and many cells became trapped in lungs, spleen, liver, or kidneys, being unable to exert a proper antitumor effect [79, 165]. This may be a result of the fact that macrophages instead of monocytes were used in these studies, as monocytes are the cells that are normally recruited from the vasculature into a tumor. The cytokines and chemokines released by tumors may be less effective in recruiting macrophages than their precursor cells [79].

Macrophages have been transfected ex vivo with anticancer genes such as IFN-γ and M-CSF [166] or therapeutic genes [167]; they may also be tagged with chemotherapeutic or angiostatic drugs. Griffiths et al. [167] used an adenoviral vector to transfect human macrophages with a hypoxia-response element-regulated p450 gene construct. The p450 enzyme converts the pro-drug cyclophosphamide to an active, toxic form, which is also known to possess angiostatic properties. Transfected macrophages migrated into the hypoxic center of tumor spheroids. When cyclophosphamide was administered subsequently, significant tumor killing was seen within the tumor mass. It is not yet known, however, whether p450 expression in healthy, normoxic tissues is low enough to avoid killing cells in these areas. Nevertheless, targeting hypoxic tumor tissue may prove to be a successful, innovative form of gene therapy strategy.

Another way to perform gene therapy is to use macrophage function itself as an in vivo target. Except for secreting a variety of angiogenic cytokines and enzymes, macrophages are able to exert tumoricidal functions after stimulation with IFN-γ and LPS, such as phagocytosis of apoptotic tumor cells and presenting tumor-associated antigens to T cells [168]. By transfecting macrophages with genes encoding for tumor antigen-specific mAb, they become able to kill tumor cells specifically, which express the antigens in vitro [169]. Whether this approach will be as effective in vivo remains to be seen.

Despite the fact that nonspecific immunotherapy with cytokines has resulted in some dramatic antitumor responses, it has not been as effective as anticipated [170]. When using nonspecific forms of immunotherapy, it has to be kept in mind that the recruitment of some leukocyte subsets might be detrimental for patient survival. Conversely, failure of specific (T cell-mediated) immunotherapy might be explained by the fact that immune cells are unable to infiltrate the tumor in quantities large enough to effectively destroy it. As endothelial cells are involved in the selection of leukocyte subsets to infiltrate a tumor, tumors have used this endothelial cell property to develop a mechanism to escape immune infiltration. We and others [18, 21, 104] have previously shown that tumor endothelial cells have a suppressed expression of adhesion molecules, thereby reducing leukocyte-vessel wall interactions. This down-regulation has been shown to be a result of exposure to tumor-derived angiogenic factors such as bFGF and VEGF [18, 21, 103]. Recently, we have demonstrated [171] in a mouse tumor model that angiogenesis inhibition can be a way to increase leukocyte-vessel wall interactions in tumors, making them more vulnerable to various immunotherapeutic approaches. However, angiostatic therapy will not result in the attraction of specific leukocyte subsets, such as cytotoxic T lymphocytes, which may be able to destroy the tumor. Macrophages are clearly not the type of immune cells desired in a tumor, because of their tumor-supporting capabilities. However, as macrophages have been shown to produce a variety of angiogenic factors, it is expected that antiangiogenesis therapy will also target and suppress macrophage pathways involving angiogenesis. Moreover, as monocytes/macrophages are known to infiltrate tumors, these cells may be exploited as vehicles or

delivery systems for angiostatic compounds to perform antiangiogenic treatment in situ and possibly avoid negative side-effects of this treatment strategy on other physiological processes associated with angiogenesis.

CONCLUSIONS

TAMs are important regulators of angiogenesis and hence, play a major role in tumor progression and metastasis. A high macrophage density has been associated with poor prognosis for cancer patients and with failure of immune functions, indicating a role for macrophages in assisting the tumor to escape from immune surveillance. Macrophages have been used for immunotherapeutic and gene therapeutic treatments, but until now, the results obtained here were not satisfactory. Another line of research has shown that in tumors, leukocyte-vessel wall interactions are suppressed, resulting in reduced recruitment of inflammatory cells that can be detrimental to the tumor, such as cytotoxic T cells and DC. Angiostatic therapy has been shown to up-regulate leukocyte-vessel wall interactions in tumors and hence, the recruitment of immune cells that can destroy a tumor. We hypothesize that combining immunotherapy with angiostatic therapy will result in improved and possibly synergistic antitumor responses and better prognosis for cancer patients.

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