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REVIEW PAPER

The Tumor-Promoting Flow of Cells Into, Within and Out of the Tumor Site: Regulation by the Inflammatory Axis of TNFα and Chemokines

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Abstract Tumors are dynamic organs, in which active processes of cell motility affect disease course by regulating the composition of cells at the tumor site. While sub-populations of tumor-promoting leukocytes are recruited inward and endothelial cell migration stands in the basis of vascular branching throughout the tumor, cancer cells make their way out of the primary site towards specific metastatic sites. This review describes the independent and cross-regulatory roles of inflammatory chemokines and of the inflammatory cytokine tumor necrosis factor α (TNFα) in determining cell motility processes that eventually have profound effects on tumor growth and metastasis. First, the effects of inflammatory chemokines such as CCL2 (MCP-1), CCL5 (RANTES) and CXCL8 (IL-8) are described, regulating the inward flow of leukocyte sub-populations with pro-tumoral activities, such as tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), tumor-associated neutrophils (TAN), Th17 cells and Tregs. Then, the ability of inflammatory chemokines to induce endothelial cell migration, sprouting and tube formation is discussed, with its implications on tumor angiogenesis. This part is followed by an in depth description of the manners by which TNFα potentiates the above activities of the inflammatory chemokines, alongside with its ability to directly induce migratory processes in the tumor cells thus promoting metastasis. Note worthy is the ability of TNFα to induce in the tumor cells the important process of epithelial-to-mesenchymal transition (EMT). Emphasis is given to the ability of TNFα to establish an inflammatory network with the chemokines, and in parallel to form a cell re-modeling network together with transforming growth factor β (TGFβ). The review concludes by discussing the implications of such networks on disease course, and on the future design of therapeutic measures in cancer.

Keywords Inflammation · Chemokines · Cytokines · Tumor necrosis factor α · Angiogenesis · Leukocytes

Abbreviations

CAF	Cancer-Associated Fibroblasts
DCIS	Ductal Carcinoma In Situ
EMT	Epithelial-to-Mesenchymal Transition
IDC	Invasive Ductal Carcinoma
IFNγ	Interferon γ
IL-1β	Interleukin 1β
IMPC	Invasive Micropapillary Carcinoma
MDSC	Myeloid-Derived Suppressor Cells
MMP	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cells
TAM	Tumor-Associated Macrophages
TAN	Tumor-Associated Neutrophils
TGFβ	Transforming Growth Factor β
TNFα	Tumor Necrosis Factor α
VEGF	Vascular Endothelial Growth Factor

Introduction

Studies of the last several years have put much emphasis on the tumor microenvironment and its contribution to tumor growth and progression. It is now known that the composition of the tumor milieu, along with genetic instability and epigenetic modifications in the tumor cells, dictate disease course and metastasis. Accordingly, emphasis was put

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recently on the contribution of inflammatory components to the microenvironmental setup of many tumor types. Inflammatory cells and soluble mediators were shown to have tumor-promoting consequences in a large number of malignancies, facilitating the establishment of primary tumors and driving metastatic processes. Such activities of immunological elements may very well reflect attempts of the immune system to combat the developing tumor; however, these efforts inflict selective pressures on the tumor cells, eventually leading to survival of tumor cells that are able to exploit the immune system for their own benefit [1–8].

Within the tumor site there is often persistence of soluble inflammatory mediators, including chemokines and cytokines. The tumor-supporting activities of these factors are diverse as they affect all the steps required for tumor growth and progression, including proliferation and motility of the tumor cells, matrix degradation, angiogenesis and seeding of the tumor cells at selected metastatic sites [9–24].

Between others, inflammatory chemokines and cytokines regulate dynamic motility processes that take place at the tumor microenvironment. The tumor is an ever-changing organ in which active and dynamic processes of cell motility are taking place. Sub-populations of leukocytes with tumor-promoting roles are recruited inwards, endothelial cells are mobilized within the tumor and form the required vascular infrastructure, and cancer cells are migrating out of the tumor bed, making their way to metastatic sites.

These migratory processes—into, within and out of the tumor site—eventually contribute to "successful" processes of malignancy. The present review describes the roles of inflammatory mediators in governing this intensive flow of leukocytes, stroma cells and tumor cells at the tumor microenvironment. Specifically, this review concentrates on the axis that is established between inflammatory chemokines and the inflammatory cytokine Tumor Necrosis Factor α (TNFα), and their independent and cross-regulatory roles in dictating motile processes at tumor sites. The effects of these factors on cell migration are many, and in the limits of this review emphasis will be given to selected aspects only. To keep this review within reasonable limits, these aspects will be illustrated mainly in the representative case of breast cancer.

The theme of this review is that inflammatory mediators control the inward migration of pro-malignancy leukocytes to the tumor site (see Scheme 1). The inflammatory chemokines also regulate migratory processes in endothelial cells leading to formation of new blood vessels. These migratory activities of the inflammatory chemokines are potentiated by TNFα, a key and most powerful inducer of chemokine release at the tumor site. In parallel, TNFα is directly responsible for the exit of the tumor cells out of the tumor site and facilitates metastasis formation. These activities of TNFα reflect its being a seminal inducer of tumor cell motility and of epithelial-to-mesenchymal transition (EMT) in the tumor cells (references below).

The independent activity of the inflammatory chemokines and of TNFα on cell migration, joined by a regulatory axis that is established between them, may dictate the flow of cells at the tumor site and the spread of the cancer cells to remote organs. Eventually, such processes of cell motility have major influence on the rate of tumor growth and progression, and on the efficiency of tumor cell spreading.

The Flow of Leukocytes and Stroma Cells into the Tumor Site and Within it: Direct Roles for Inflammatory Chemokines

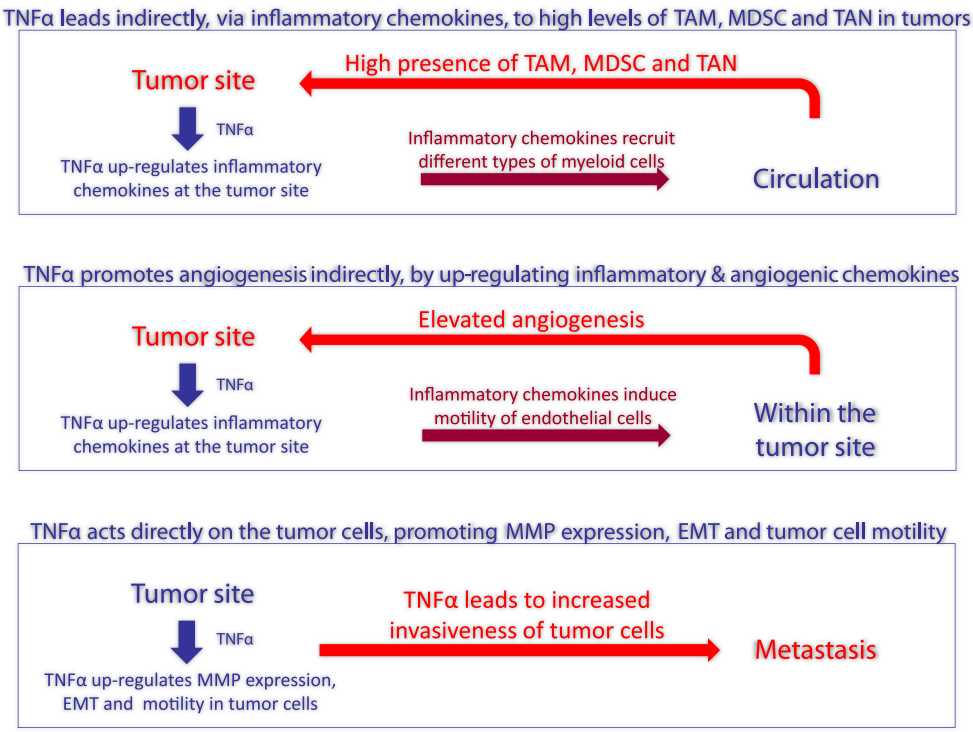
General Outline of Inflammatory Chemokines and their Roles in Malignancy

Members of the chemokine superfamily have been identified long ago as fundamental regulators of host defense and immune homeostasis. As such, they are sub-categorized to chemokines with major impact on inflammatory processes and to others that mainly control leukocyte migration to primary and secondary lymphoid organs. Although this sub-categorization is not rigid, it does reflect the general physiological consequences emerging from the activities of chemokines belonging to the two different subtypes [25–27].

Acting in the inflammatory and immune context, all chemokines are potent chemoattractants of leukocytes; however, each chemokine has its own flavor of preferred target cells. The inflammatory chemokines are usually not constitutively expressed by normal cells, but their expression is strongly induced following exposure of tissues to inflammatory insults. Tissue damage or infection lead through bacterial products and inflammatory cytokines, e.g. interleukin β (IL-β) and tumor necrosis factor α (TNFα) to increased release of inflammatory chemokines recruiting inflammatory/immune cells with protective and tissue-repair properties [25–33].

In parallel to their roles in immunity, some of the inflammatory chemokines have been identified as regulators of malignant processes. Chemokines that belong to the inflammatory subtype were described as having many different effects on tumor growth and progression, most of which are mediated by their chemotactic properties. In general, at the malignancy context the inflammatory chemokines act on many types of target cells. The target cells include primarily leukocytes and endothelial cells, but in parallel, several of the inflammatory chemokines can also act directly on the tumor cells and promote their invasive properties, along with elevated proliferation [9–18].

This part of the review is devoted to the impact of inflammatory chemokines on motility of cells into the tumor site and within it, focusing on specific subtypes of leukocytes that



Scheme 1 The interplay between TNFα and inflammatory chemokines at the tumor site, and the effects of these mediators on the malignancy cascade. The Scheme presents the dynamic motility processes taking place in cancer, and their regulation by inflammatory mediators. The inflammatory chemokines—including CCL2, CCL5 and CXCL8—were shown to have active pro-cancerous effects in a large variety of malignancies. In parallel, the inflammatory cytokine TNFα is abundant in tumors, and was recently shown to have diverse tumor-promoting roles in many cancer types. Also, TNFα has been found to up-regulate the expression of the inflammatory chemokines by tumor cells and by cells of the tumor microenvironment, agreeing with the coordinated expression of these factors in malignancy, as shown for example in breast cancer. In this Scheme, a suggestive model is presented on the manners by which inflammatory chemokines, TNFα and the interplay between them may promote pro-malignancy activities at the tumor site and metastasis formation. **Upper Panel**: Inflammatory chemokines recruit leukocytes with pro-cancerous activities from the blood stream into the tumor site. These may include primarily tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC) and tumor-associated neutrophils (TAN), but also Th17 cells and Tregs. By virtue of its ability to promote the release of inflammatory chemokines, TNFα may potentiate the leukocyte-recruiting activities of the chemokines. **Middle Panel**: Many of the inflammatory chemokines have potent angiogenic activities, promoting the motility of endothelial cells within the tumor, sprouting and branching. By elevating the expression of these chemokines, TNFα may indirectly increase neovascularization at the tumor site. **Lower Panel**: TNFα increases the release of matrix metalloproteinases (MMP), and directly induces epithelial-to-mesenchymal transition (EMT) and motility processes in the cancer cells. These activities of TNFα, often potentiated by TGFβ, increase tumor cell invasiveness and may thus contribute to elevated metastasis

promote malignancy, and on endothelial cells, respectively. These aspects have been reviewed elsewhere (references provided below), and in the framework of this review they will be briefly summarized in order to provide a general overview of the subject.

Inflammatory Chemokines Promote the Presence of Tumor-Supporting Leukocyte Sub-populations at the Tumor Site

At the tumor microenvironment, the chemotactic activities of the inflammatory chemokines have major impact on the types and amounts of leukocytes that are present at the tumor site, and accordingly they can dictate the way that the inflammatory nature of the tumor affects the malignancy process. Chemokines can induce the recruitment of leukocyte subsets with beneficial properties or with devastating implications on the cancerous process. The final leukocyte composition at the tumor site is governed by the types of chemokines and their levels at the tumor milieu. For example, chemokines that belong to the CXCR3-binding subfamily of non-ELR CXC chemokines chemoattract Th1 and NK cells to the tumor locus, and are usually considered potential anti-tumor factors [16–18, 34]. However, the attraction of Th1 cells by CXCR3 chemokine ligands, as well as by other inflammatory chemokines that act alike (such as CCL5 [35]), can be negated by the opposing effects of the same chemokines or other chemokines that promote the migration of leukocyte sub-populations supporting the malignancy cascade.

Typical representatives of the tumor-supporting arm of inflammatory chemokines are the chemokines CCL2, CCL5 and CXCL8. Their pro-cancerous activities are many and

diverse (reviewed for example in [12–14, 36–38], and are well manifested by studies in animal model systems in which inhibition of each of these chemokines has led to reduced tumor growth and metastasis [39–45]. The chemokines CCL2 and CCL5 act at the tumor site in similar manners to the way they function in immune-related inflammatory processes, where they serve as powerful chemoattractants of monocytes [25–27]. Likewise within tumors, CCL2 and CCL5 recruit circulating monocytes to tumor sites. Under the influence of the tumor microenvironment and of tumor-derived factors, the infiltrating monocytes and possibly also resident macrophages may differentiate to tumor-associated macrophages (TAM), with deleterious consequences ensued due to the release by TAM of a large variety of factors that promote tumor growth and motility, as well as angiogenesis [46–48].

Actually, chemokines such as CCL2 and CCL5 may shift the balance between leukocyte sub-populations at tumor sites, favoring immune and inflammatory cells with tumor-promoting activities. This is because in addition to monocytes, they may give rise to high presence of myeloid-derived suppressor cells (MDSC) in tumors, and it was postulated that they also play active roles in recruiting Tregs and inflammatory Th17 cells to the tumor site [11–13, 36, 49–52].

In addition to these cell sub-populations, neutrophils recently have gained increased attention with respect to their roles in malignancy. Tumor-associated neutrophils (TAN) produce substances that promote the inflammatory nature of the tumor microenvironment and the invasive properties of the tumor cells [53–56]. The migration of neutrophils to tumor sites is expected to be induced mainly by CXCL8 (Interleukin 8), the chemokine with most powerful chemoattracting effects on these cells [25–27]. This chemokine was recently identified as an important pro-tumoral factor that is expressed by many tumors, including of the breast [14, 43–45, 57–60]. Accordingly, recent studies by Strell et al. have demonstrated that in response to tumor-derived CXCL8, neutrophils migrate towards breast tumor cells, thereafter linking the tumor cells to endothelial cells [61, 62].

By virtue of its neutrophil-chemoattracting activities, CXCL8 joins the inflammatory chemokines CCL2 and CCL5 in dictating the inward flow of leukocytes, therefore together they have considerable effects on the inflammatory nature of the tumor surroundings.

Inflammatory Chemokines Promote Endothelial Cell Migration and Sprouting, thus Increasing Angiogenesis

The chemotactic activities of inflammatory chemokines are not limited to leukocytes, and many of them are known to induce migration of endothelial cells, therefore promoting angiogenicity. Angiogenesis, the formation of new blood vessels from pre-existing capillaries, provides essential blood supply to the growing tumor and is required for metastasis. Vascular expansion can proceed by several processes, one of which is sprouting that requires endothelial cell migration and branching throughout the tumor. The sprouting process is controlled by a large variety of angiogenic and angiostatic factors, including chemokines from the inflammatory and homeostatic branches [16, 17, 34, 63, 64].

Very much like the induction of leukocyte infiltration, the inflammatory chemokines can have opposing effects on angiogenicity. While ELR-expressing CXC chemokines and some of the CC chemokines are categorized as angiogenic chemokines, the CXCR3-binding non-ELR CXC chemokines are characterized as potent angiogenic factors. This characteristic of the CXCR3-binding chemokines, together with their potentiating effects on Th1 and NK recruitment to tumors, has led to their coining “immunoangiostatic” chemokines [16, 17]. In parenthetical clause, it should be noted that under specific conditions these CXCR3 chemokine ligands gain tumor-promoting functions that raise fundamental questions as to the true nature of their activity at tumor sites, whether anti- or pro-tumoral [16–18, 34].

One of the most potent angiogenic chemokines is the ELR-expressing CXC chemokine CXCL8. CXCL8 acts on endothelial cells to promote their migration, invasion and proliferation, eventually giving rise to formation of capillary-like structures that can support tumor growth and metastasis [16, 17, 44, 65–67]. The CXCL8 receptor CXCR2 was shown to be critical for this activity, although contribution of CXCR1 was also described [16, 17, 65] . By virtue of its angiogenic activities that lead to neovascularization, tumor growth and metastasis, CXCL8 is categorized as a powerful tumor-promoting factor in many malignancies.

For example in breast cancer, the expression of CXCR1 and CXCR2 was denoted on endothelial cells in breast tumors [68], supporting a role for CXCL8 in regulating neovascularization and tumorigenesis thereof. Indeed, breast tumor-derived CXCL8 was shown to induce the formation of tube-like structures, endothelial cell migration through matrigel and blood vessel formation in vivo [69, 70]. Furthermore, it was recently shown that CXCL8 which is produced by monocytic cells that have been exposed to breast tumor cell supernatants, is active in microvessel formation [71]. A direct role for CXCL8 in angiogenesis in breast cancer was provided by the targeting of CXCL8 expression in breast tumor cells by shRNA [44]. This measure has led to reduced angiogenicity and as a consequence to inhibition of metastasis in mice [44]. Also, endothelial cell proliferation induced by cancer cell-produced CXCL8 was inhibited by neutralizing antibodies against the chemokine, and tumors with low CXCL8 levels have shown reduced angiogenesis [70].

CXCL8 is one of several ELR-expressing CXC chemokines that positively regulate angiogenesis [16, 17]. In parallel,

members of the CC branch also promote vascularization, as demonstrated for CCL2 whose activities on endothelial cells are mediated by its CCR2 receptor [17, 59, 67, 72–74]. The angiogenic activities of CCL2 can be indirect through induction of an inflammatory microenvironment. Following CCL2-induced recruitment of monocytes to tumors, these monocytes differentiate to TAM that release a large variety of angiogenic factors [17, 75–78]. For instance, the recent study by Pollard and his team suggested that following the CCL2-induced recruitment of inflammatory monocytes to breast tumors, angiogenesis is promoted by vascular endothelial growth factor (VEGF) [78]. These indirect effects of CCL2 on neovascularization may be further potentiated by direct signaling induced by CCL2 on endothelial cells, leading to their migration and to sprouting and tube formation [39, 67, 72]. Such direct angiogenic activities of CCL2 may stand in the basis of the roles found for CCL2 in elevating metastatic lesions of breast tumor cells in mice [39].

To conclude, inflammatory chemokines acting potently as angiogenic factors have cardinal influence on disease course. Particularly, the chemokines CXCL8 (and other ELR-expressing CXC chemokines) and CCL2 promote the migration of endothelial cells within the tumor site, the result being sprouting and tube formation. Eventually, these events give rise to increased angiogenesis that supports not only tumor growth, but also metastasis.

The Effects of the TNFα-Chemokine Axis on Migratory Processes at Tumor Sites

The above discussion has illustrated the direct roles of inflammatory chemokines in controlling the migration of leukocytes and endothelial cells into the tumor site and within it. Such processes position leukocyte sub-populations with tumor-promoting activities in proximity of the tumor cells, and they lead to extended blood supply to the tumor cells.

The major source of the inflammatory chemokines at breast tumor sites is in the cancer cells themselves, although expression in stroma cells and leukocytes was also denoted. In the standard immunological setting the inflammatory chemokines are rarely expressed by normal tissue cells, rather they are up-regulated by the different mediators of the inflammatory set-up, between others by inflammatory cytokines such as IL-1β and TNFα. Accordingly, many of the inflammatory chemokines including for example CCL2 and CCL5, are minimally detected in normal breast epithelial cells; In contrast, breast tumor cells in cancer biopsies and in culture show highly detectable levels of these chemokines [68, 70, 76, 77, 79–106]. These observations raise the possibility that in line with the mechanisms regulating the inflammatory chemokines in immunity, a major mechanism leading to chemokine expression by tumor cells in situ is exposure of these cells to inflammatory mediators that up-regulate chemokine transcription and release.

Indeed, the surrounding milieu of many tumors, particularly in breast cancer is enriched with inflammatory cytokines. IL-1β and TNFα are prevalent in breast tumors, where they are produced by breast tumor cells and also by cells of the tumor microenvironment, at their vicinity [90, 101, 107–117]. Along the years, these two cytokines were shown to up-regulate the release of CCL2, CCL5 and CXCL8 by breast tumor cells, as well as by adjacent stroma cells and leukocytes [85, 86, 90, 117–125]. Therefore, indirectly by promoting the inflammatory chemokines, IL-1β and TNFα may potentiate the tumor-promoting activities of the inflammatory chemokines at the tumor setting.

In line with the above, it was shown that CCL2 and CCL5 are co-expressed with IL-1β and TNFα in breast tumors, and furthermore that the expression of the four factors is coordinated along different stages of breast cancer progression [65, 67, 76, 90, 117]. The expression of all four factors was minimally detected in normal breast epithelial cells, but was predominantly elevated in the tumor cells, starting from the Ductal Carcinoma In Situ (DCIS) stage on towards Invasive Ductal Carcinoma (IDC). The incidence of CCL2, CCL5, IL-1β and TNFα expression was beyond 50% in IDC patients, with or without disease recurrence [90].

The coordinated expression of the inflammatory chemokines CCL2 and CCL5 with the inflammatory cytokines IL-1β and TNFα along disease course in breast cancer supports the existence of an inflammatory network between chemokines and cytokines in breast tumors. Importantly, the incidence of IL-1β and TNFα expression was further elevated in patients who relapsed [90]. Disease relapse was characterized by return of local disease or appearance of new metastases, processes that involve EMT in the tumor cells [126–131]. The exacerbated expression of IL-1β and TNFα in patients with relapse suggested that these two cytokines not only induce the expression of inflammatory chemokines at the tumor site, but have additional levels of activity whereby they act directly on the tumor cells to induce EMT, tumor cell migration and invasion.

Taken together, the above findings suggest that IL-1β and TNFα have two complementary effects at breast tumor sites: (1) They potentiate the expression of inflammatory chemokines at the tumor locus, by that promoting indirectly the inward flux of tumor-supporting leukocytes and endothelial cell migration within the tumor. (2) They induce EMT and tumor cell invasiveness, thus facilitating the outward flow of the tumor cells towards metastatic sites.

Studies of the last several years indicate that of the two inflammatory cytokines, it is mainly TNFα that exerts both these two functions. Below, this review summarizes some of the observations related to TNFα effects as a potent inducer

of inflammatory chemokines, and as a powerful cell remodeling cytokine that leads to EMT in breast tumor cells.

General Outline of TNFα and its Roles in Malignancy

TNFα is a pleiotropic cytokine whose activities are fundamental to immune protection and homeostasis. Its naming "tumor necrosis factor" reflects early reports on its tumor-inhibiting capabilities, showing that high doses of locally-administrated TNFα caused destruction of blood vessels and promoted anti-tumor effects. However, emerging studies have provided evidence to opposite regulatory roles for TNFα, suggesting that its chronic expression at relatively low amounts may have pro-cancerous effects. Indeed, based on extensive research, TNFα is known to be expressed by many types of tumor cells and by cells of the tumor microenvironment, and to exert a large variety of pro-tumoral activities [19–22, 132].

The regulation of malignancy by TNFα is complex and has been discussed in depth in several reviews (e.g. [19–22, 132]). Breast cancer is one of the malignancies in which TNFα was found to have a large variety of pro-cancerous roles (see below); however, in the limits of this review emphasis will be given to the regulation of cell motility by this cytokine, and its roles in dictating indirectly or directly the flow of cells at the tumor site.

mRNA and protein analyses of TNFα, including of biopsies of breast cancer patients, detected the expression of this cytokine in breast tumor cells, in macrophages and in endothelial cells [90, 101, 111–117, 133, 134]. Some of these studies have analyzed the correlation between TNFα expression, disease stage and/or clinicopathological parameters. The analyses differed considerably in the parameters they were using, therefore no concrete conclusions could be made [90, 111, 114, 116, 133]. However, a general tendency was found for correlation between increased levels of TNFα expression, either in incidence or intensity, and more advanced/progressed stages of disease [90, 111, 114, 116]. Between others, the study by Cui et al. has shown that TNFα expression was significantly higher in the aggressive form of invasive micropapillary carcinoma (IMPC) than in IDC, and it was correlated with the rate of tumor cell proliferation, histological grade, lymph node metastasis and angiogenesis [116].

In parallel to the expression of TNFα, its receptors were also detected in breast tumors, where they were found to be expressed by the tumor cells, by endothelial/stroma cells, as well as by leukocytes, primarily macrophages [111, 112, 114, 116]. Of the two receptors, it was TNFRII that was correlated with TNFα expression, with increased proliferation of the tumor cells and with the histological grade of disease [114, 116]. Together, these results suggest that TNFα may act by autocrine as well as paracrine manners, leading to detrimental consequences on the malignant process.

The pro-tumoral roles of TNFα in breast cancer are supported by studies showing direct roles for the cytokine in breast malignancy, as well as indicating that it is correlated with a more aggressive malignant phenotype [135–142]. Animal systems of the neu/erbB2 model have shown that tumorigenesis was increased in TNF+/+ mice compared to TNF-/- mice, and that bone marrow transplantation from TNFα knockout mice into NeuT recipients significantly impaired tumor growth [140, 141]. Furthermore, reduced tumor growth and metastasis formation were obtained by inhibiting TNFα with neutralizing antibodies, particularly by infliximab [140–142], the chimeric antibody that is used in the clinic for TNFα inhibition in inflammatory diseases (such as rheumatoid arthritis).

Overall, emerging studies on TNFα and its roles in breast cancer strongly suggest that under experimental conditions this cytokine is skewed to a pro-tumoral phenotype, and evidence supporting its connection to the malignancy process in breast cancer patients is increasing. Accordingly, it was shown that TNFα contributes to malignant processes in breast cancer and in other types of tumors by many different manners. Two of these pathways that are related to the regulation of cell flow at the tumor microenvironment, are discussed below.

The Indirect Effects of TNFα on the Flow of Leukocytes and Endothelial Cells at the Tumor Site

In the immune context, TNFα is a prototype inducer of many of the inflammatory chemokines mainly *via* NF-κB and AP-1 activation, leading to increased chemokine transcription and release thereof [25–30]. Similarly, much evidence was provided to the powerful chemokine-inducing abilities of TNFα in malignancy. By promoting the levels of inflammatory chemokines such as CCL2, CCL5 and CXCL8 at the tumor microenvironment, TNFα may amplify their tumor-supporting activities. Thus, TNFα may indirectly shift the balance in cell flow, facilitating the inward migration of detrimental leukocytes to the tumor site, and migratory processes of endothelial cells.

In the representative case of breast cancer, TNFα is a strong inducer of inflammatory chemokines in the tumor cells and in adjacent cells at the tumor milieu. The receptors for TNFα are abundantly expressed by many cell types, accordingly increased release of CCL2 was denoted upon TNFα exposure in the tumor cells as well as in endothelial cells and leukocytes [85, 86, 90, 117–120]. Such effects could be obtained by TNFα which was produced by the tumor cells or by cells of the microenvironment. For example, it was shown that monocyte-derived TNFα stimulated the release of CCL2 in breast tumor cells, and that the resulting CCL2 in turn up-regulated the secretion of TNFα from

monocytic cells [86]. If such a mechanism takes place in situ, it may lead to a positive feedback loop, whereby the tumor cells and the monocytic cells at tumor site promote each other's ability to express and secrete pro-malignancy factors.

Inducing effects were observed for TNFα also on CCL5 and CXLC8, driving forward the release of these two chemokines by breast tumor cells, endothelial cells and mesenchymal stem cells (MSC) [90, 117, 118, 120–125]. Comparison between breast tumor cell lines has shown that those cells that had a higher metastatic phenotype responded better to TNFα and produced higher levels of CXCL8 [124].

In line with the chemokine-promoting activities of TNFα, its expression was highly correlated with the inflammatory chemokines in analyses performed on biopsies of breast cancer patients [65, 67, 76, 90, 117]. These results strongly support the existence of an inflammatory network in which inflammatory cytokines regulate inflammatory chemokines and are thus coordinated with them. Supporting such a possibility are studies showing that measures inhibiting the tumorigenic potential of breast tumor cells have led to reduced levels of TNFα, which were accompanied with lower expression of inflammatory chemokines [136, 139]. The implications of such an inflammatory network is that the activities of TNFα may lead indirectly, through elevated production of inflammatory and pro-malignancy chemokines, to high presence of detrimental leukocytes at the tumor site (e.g. *via* CCL2 and CCL5), and to high angiogenic processes (by CXCL8 and CCL2).

Moreover, it was recently found that the activities of TNFα are not limited to the chemokines, because it also up-regulated chemokine receptors that are expressed by leukocytes and endothelial cells [73, 118, 143]. The study of Weber et al. has shown that TNFα up-regulated in endothelial cells the expression of CCR2, the receptor through which CCL2 exerts its angiogenic activities [73]. Even more so, TNFα was found to reduce the expression of the scavenger chemokine receptor CCX-CKR [144]. This receptor is known to be of a protective nature because it removes pro-malignancy chemokines from the tumor milieu, thus negating their potential tumor-promoting activities [25].

Therefore, TNFα can shift the balance between chemokine receptors, elevating those that are tumor-promoting and reducing those that are protective. This activity of TNFα complements its powerful effects on the release of the inflammatory chemokines, by that possibly further potentiating the pro-cancerous activities of chemokines and their receptors.

The Direct Impact of TNFα on Cancer Cell Motility and EMT, Possibly Leading to Outward Flow of Tumor Cells Towards Metastatic Organs

The expression of TNFα in tumors deviates from the normal patterns of its expression in normal tissues that are not exposed to pathogenic or inflammatory threat. In those malignant diseases where chronic presence of TNFα is tumor-supporting, the cytokine acquires diverse pro-malignancy activities that extend far beyond those that fit best its roles in inflammation, such as the above-discussed induction of inflammatory chemokines [19–22, 132]. An important manifestation of such unique TNFα activities is its ability to push forward, indirectly or directly, processes of motility and invasion in tumor cells.

When acting in the inflammatory context of immune activities, TNFα prepares the tissue for leukocyte motility by promoting the expression of matrix metalloproteinases (MMP) (e.g. [145, 146]). Based on the above, it came as no surprise that TNFα induced the expression of MMP in macrophages also at the tumor setting [147]. As a consequence of a cross-talk existing between the tumor cells and the macrophages, TNFα activities have led to the release of MMP by the macrophages, than promoting the invasive properties of the tumor cells [147]. The activities of TNFα on MMP production are extended to the tumor cells [85, 86, 119, 120, 123]. Also, tumor-derived TNFα elevated the expression of MMP9 in fibroblasts, and the level of induction correlated with the metastatic potential of the tumor cells [147].

Here, it is important to realize that the source of TNFα may vary, and the cytokine may be produced by the tumor cells as well as by other cells that are located at their vicinity. Conditioned medium of macrophages could induce the migration of breast tumor cells in a TNFα-mediated manner [148]. Moreover, macrophage inflammatory products, such as the daintain peptide, were found to increase the release of TNFα by breast tumor cells, followed by elevated migratory properties of the cancer cells [149]. It was also shown that IL-2-stimulated lymphocytes released TNFα, and their conditioned medium induced tumor cell migration [150]. The regulatory roles of TNFα *via* MMP on tumor cell migration was found to involve another interesting host cell sub-population, of bone marrow (BM)-derived MSC. BM-MSC are precursors for cancer-associated fibroblasts (CAF), whose tumor-promoting roles have been realized recently [151–153]. The study by Shin and colleagues has shown that TNFα induced the expression of the inflammatory CXCR3 ligands CXCL9, CXCL10 and CXCL11 by MSC, and those chemokines have promoted the migration of the tumor cells, probably partly *via* MMP9 [118].

While induction of MMP by TNFα facilitates tumor cell migration, questions were raised on the ability of the cytokine to act directly on the tumor cells and regulate intracellular mechanisms that promote their adhesion, motility and invasion. Here, a major breakthrough was made by studies on novel roles for TNFα as a direct inducer of EMT in the tumor cells, with breast cancer being one of the systems in which such effects were strongly reinforced.

EMT is a process in which the tumor cell fate changes from an epithelial type to a mesenchymal phenotype that supports tumor cell motility and metastasis. Cells that

undergo this process show reduced levels of epithelial markers and adhesion molecules responsible for cell-to-cell contacts, enabling them to more easily detach from one another and spread to remote organs. In parallel, mesenchymal markers are increased in the tumor cells undergoing EMT, the cells develop adhesive and invasive protrusions and eventually acquire a high motility and invasive phenotype. As such, tumor cells that have undergone EMT can more easily complete the multistage process of metastasis, where their migratory and metastasizing capabilities come into effect [126–131].

The process of EMT is known to be induced by several mediators, one of which is Transforming Growth Factor β (TGFβ) [154–156], however studies of the last several years indicate that TNFα is also a prominent inducer of this essential step of cellular remodeling. To date, a number of studies have already shown that TNFα acts directly on breast tumor cells to promote typical characteristics of EMT [90, 157–160]. It was found that stimulation by TNFα has led to loss of epithelial markers and transition of the tumor cells to a mesenchymal phenotype. The transition was manifested by reduced expression of E-cadherin and lower surface expression of β-catenin; elevated expression of mesenchymal markers including vimentin; acquisition of cellular protrusions and actin re-organization [90, 157–159].

As expected, the prime result of tumor cells that have undergone TNFα-induced EMT was improved migratory and invasive properties [90, 148, 157, 161, 162], whose consequence may be increased local recurrence and metastasis. Indeed, a recent study has shown that high persistence of TNFα in breast tumors was significantly more prevalent in patients diagnosed with local recurrence and appearance of new metastasis than in patients with less progressed stages of disease (DCIS) [90]. These findings support the possibility that TNFα has an important role in promoting disease relapse by promoting EMT processes in situ, within the tumor.

The active roles taken by TNFα in inducing EMT emphasize the need to identify the mechanisms through which this cytokine acts in this manner. This research direction is only in its beginning, however it is already known that the NF-κB signaling pathway is involved in EMT and migratory effects induced by TNFα [148, 157, 162–164]. As with TGFβ, also in the case of TNFα-induced EMT, the process was correlated with snail, slug and Zeb1 regulation [148, 157, 158], intracellular factors known for their ability to repress E-cadherin expression and to induce EMT.

The similarities between TGFβ and TNFα in terms of EMT induction are of interest, suggesting that different arms of the microenvironment cooperate in elevating metastasizing properties in the tumor cells. For example, the combined stimulation of mouse mammary carcinoma cells by both these cytokines together has induced prominent EMT phenotypes in the cells, leading to increased migration and invasion. In addition, the stimulated cells formed tumors with as low as 100 cells, compared to their non-stimulated ancestors that could only form tumors at higher cell numbers. This observation may have been accounted for by the fact that the combined stimulation by TGFβ and TNFα has led to generation of cells with a stable $CD44^{+}/CD24^{-/low}$ stem cell phenotype. Cells expressing this phenotype were suggested to have self-renewal properties, being in line with the high tumorigenicity yield and resistance to chemotherapy obtained for the TGFβ + TNFα-stimulated cells [165]. Moreover, gene expression analyses found that the TGFβ + TNFα-derived breast tumor stem cells showed a shift to the claudin-low molecular subtype [165], a new breast cancer subtype exhibiting a mesenchymal and stem cell phenotype which is correlated with poor prognosis.

The EMT-inducing properties of TNFα are general, and are not limited to breast cancer. Similar effects of TNFα were described in pancreatic carcinoma cells, colon cancer, alveolar epithelial cells and models of airway wound repair and others, where usually TNFα potentiated the effects of TGFβ on EMT [166–172]. The mechanisms involved in the cooperative effects of TGFβ and TNFα have not been addressed in depth, and much is still to be learnt on the manners by which TNFα complements the well-established EMT-inducing properties of TGFβ.

Discussion and Concluding Remarks

During the last decade extensive information was provided about the contribution of the inflammatory microenvironment to development of tumors, and to the ability of tumor cells to spread and reach remote organs. The composition of the tumor milieu varies between tumors, and at different stages of tumor progression certain elements may dominate, while other factors may dictate the interactions between the tumor and its surroundings at other phases of the process. The inflammatory composition of the tumor obeys similar guidelines, and its components may differ depending on the type and stage of disease. At a certain setup, an inflammatory network may exist in the tumor microenvironment, affecting the type and amount of leukocytes and stroma cells that participate in the process, thus dictating the malignant potential of the tumor cells themselves.

The importance of the inflammatory network can be well demonstrated in breast cancer, where many inflammatory elements act together to regulate disease course. This review has focused on the contribution of inflammatory chemokines and of the inflammatory cytokine TNFα to the flow of cells into and within the tumor microenvironment and to the outward flux of cancer cells from the tumor, and has emphasized their detrimental implications.

The inflammatory chemokines can regulate the inward migration of leukocytes of many different types to the tumor site, some may even be of the anti-tumor type. However, in the limits of this review, the discussion has addressed the ability of specific inflammatory chemokines to induce the much more prevalent events in which leukocytes with tumor-enhancing activities are recruited to the tumor site, such as monocytes, MDSC, TAN, Th17 cells and Tregs. These tumor-infiltrating cells would eventually elevate the ability of the tumor cells to successfully establish the primary tumor and later on to metastasize.

In parallel, representative examples were given to the roles of inflammatory chemokines in promoting migration of endothelial cells within the tumor, thus serving as key angiogenic factors. These chemokines can act alongside with other inflammatory chemokines that are angiostatic in their nature, mainly of the non-ELR CXC subgroup. While the latter chemokines are mostly regulated by interferon γ (IFNγ), many of the inflammatory chemokines that belong to the ELR-expressing CXC and CC subtypes, including those that are angiogenic, are powerfully induced by the inflammatory cytokines IL-β and TNFα. By means of tight regulation, IL-β and TNFα can promote the release of pro-cancerous inflammatory & angiogenic chemokines, therefore indirectly amplifying their tumor-supporting activities and their ability to induce an inward flow of damaging host cells and endothelial cell motility. Along these lines, it has been found that the expression of the inflammatory chemokines and the inflammatory cytokines is coordinated in breast cancer, strongly supporting the existence of an inflammatory network in this disease.

In parallel, of the two inflammatory cytokines it is mainly TNFα that can act directly on the tumor cells to promote their migratory and invasive capabilities. Through increased production of MMP by the tumor cells and by cells of the tumor milieu, together with EMT induction in the tumor cells, the activities of TNFα provide the tumor cells with much improved metastasizing capabilities. These effects of TNFα can amplify the impact of the powerful EMT-promoting cytokine TGFβ, thus establishing another network that is characterized by cell-remodeling properties. The cooperative activities of the two cytokines on EMT were found to expand to many systems, suggesting that TNFα is not a mere bystander at the tumor site. Therefore, in addition to potentiating the activities of inflammatory chemokines, TNFα has cell-remodeling properties. These characteristics of TNFα position it as a cancer-regulating cytokine that may stand in the focal point of inflammatory networks and cell-remodeling networks existing at the tumor site.

Taking these findings into account, the complex nature of the tumor microenvironment tells us that it might be difficult to establish concrete rules regarding the manners by which the inflammatory and cell-remodeling networks indeed dictate the fate of the tumor. TNFα provides a good example in this respect. Its roles in malignancy are debated, because it was shown to be a strong anti-tumor agent under specific conditions. However, in other circumstances, TNFα acts as a powerful tumor-promoting factor. It is possible that tumor cells that have resisted the pressures endowed by the cytotoxic activities of TNFα have undergone selection which enabled them to use the cytokine for their own benefit. Those TNFα-resistant cells can now respond at many different levels to the signals transmitted by the cytokine, release pro-tumor inflammatory chemokines and MMPs, and undergo EMT processes that enable them to invade remote organs, as was indeed suggested by several recent studies [158, 159, 161].

Overall, the above findings suggest that care should be taken when targets for therapy are chosen. Different factors of the tumor microenvironment can form networks in which cytokines of many kinds cross-regulate each other's activities. The inhibition of one tumor-promoting factor may not necessarily have dramatic effects on the malignant process because other factors can compensate for its lack, and establish substitute networks of different kinds. The networks established between different pro-malignancy factors stand in the basis of the multifactorial nature of malignant diseases, and they reflect the flexible nature of the tumor microenvironment. Based on the above, combination therapies are now used in the treatment of many malignant diseases, using therapeutic modalities that target simultaneously a variety of tumor-promoting factors.

The same applies for novel approaches that aim at targeting inflammatory mediators at the tumor site. For example, although TNFα is a powerful promoter of malignancy, affecting many cells that are present in vicinity of the tumor cells and the cancer cells themselves, it certainly does not act alone and in its absence other inflammatory mediators may take over and drive the tumor milieu into the devastating phases of tumor-promotion.

Along these lines, therapeutic measures targeting TNFα are making their initial stages to the clinic for the treatment of malignant diseases. While these modalities have been proven to be safe, it is not yet clear whether they can impact disease course [173–175]. The existence of inflammatory and cell-remodeling networks at the tumor site suggest that modalities targeting TNFα should be combined with other measures that inhibit additional factors acting side by side with this cytokine, including chemokines and TGFβ. Such a clinical approach needs to be based on further research, in which the regulatory networks acting at the tumor microenvironment will be better identified, so that we will know which network is acting at each stage of disease, to what extent and whether other networks act in parallel.

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