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Role of tumor-associated macrophages in tumor progression and invasion

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Abstract Tumor-Associated Macrophages (TAM) represent the major inflammatory component of the stroma of many tumors, able to affect different aspects of the neoplastic tissue. Many observations indicate that TAM express several M2-associated protumoral functions, including promotion of angiogenesis, matrix remodelling and suppression of adaptive immunity. The protumoral role of TAM in cancer is further supported by clinical studies that found a correlation between the high macrophage content of tumors and poor patient prognosis and by evidence showing that long-term use of non-steroidal anti-inflammatory drugs reduces the risk of several cancers. Here, we discuss evidence supporting the view that TAM represent a unique and distinct M2-skewed myeloid population and a potential target of anti-cancer therapy.

Keywords Tumor-associated macrophages · Tumor · Inflammation · NF-κB · Metastasis · Hypoxia

1 Introduction

Epidemiological studies have revealed that chronic inflammation predisposes to different forms of cancer and that usage of non-steroidal anti-inflammatory agents is associated with protection against various tumors. An inflammatory component is present in the microenvironment of most neoplastic tissues, including those not causally related to an obvious inflammatory process. Hallmarks of cancer-associated inflammation include: the infiltration of white blood cells; the presence of polypeptide messengers of inflammation (cytokines and chemokines); the occurrence of tissue remodeling and angiogenesis.

Already in the late 1970s it was found that a major leukocyte population present in tumors, the so-called tumor-associated macrophages (TAM), promote tumor growth [1, 2]. Accordingly, in many but not all human tumors, a high frequency of infiltrating TAM is associated to poor prognosis. Interestingly, this pathological finding has re-emerged in the post-genomic era: genes associated to leukocyte or macrophage infiltration (e.g., CD68) are part of molecular signatures which herald poor prognosis in lymphomas and breast carcinoma [3].

Gene modified mice, including some with cell-specific targeted gene inactivation, allowed dissection of molecular pathways of inflammation leading to tumor promotion, as well as the initial analysis of the role of distinct elements of the inflammatory process in different steps of tumor progression. TNF, IL-1, the macrophage growth and attractant factor CSF-1, CCL2, a chemokine originally described as a tumor-derived macrophage attractant, the prostaglandin producing enzyme cyclooxygenase 2, the master inflammatory transcription factor NF-κB, enzymes involved in tissue remodeling, all are essential elements for carcinogenesis and/or for acquisition of a metastatic phenotype in diverse organs including skin, liver, mammary gland, intestine [4–9].

Here we will review available information on the role of myelomonocytic cells, tumor-associated macrophages (TAM) in particular, in tumor invasion and metastasis (Fig. 1).

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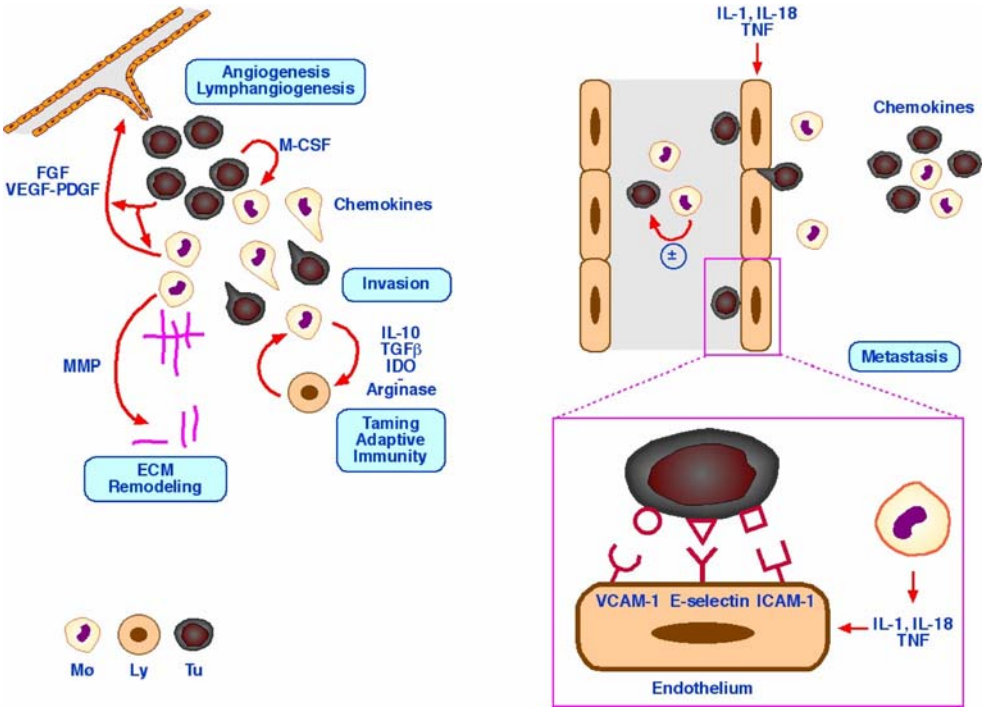

Fig. 1 Role of myelomonocytic cells in tumor invasion and metastasis. TAM promote tumor progression by favoring angiogenesis and lymphoangiogenesis, ECM remodelling and the taming of adaptive immunity. The latter function is prominent in myeloid suppressor cells. Mononuclear phagocytes have also the potential to kill tumor cells (the "macrophage balance") and there is evidence for clearance of circulating tumor cells (± in the figure). Strong evidence shows promotion of metastasis by inflammatory cytokines (IL-1, TNF) produced by macrophages

2 Functional program of TAM: role of NF-κB and HIF-1

The capability to express distinct functional programs in response to different microenvironmental signals is a biological feature of macrophages, which is typically manifested in pathological conditions such as infections and cancer [10, 11]. Chronic infections can tightly regulate the immune responses, being able to trigger highly polarized type I or type II inflammation and immunity. Central to the development of type I or type II polarization is the specificity of the host-pathogen interaction. While intracellular protozoa induce a type I polarized inflammation, with strong neutrophils, macrophage infiltrate, typical in granulomas, parasites such helmints trigger strong type II inflammation, characterized by extensive eosinophilia, mastocytosis and tissue remodeling [12].

Classical or M1 macrophage activation in response to microbial products or Interferon-γ are characterized by: high capacity to present antigen; high interleukin-12 (IL-12) and IL-23 production [10] and consequent activation of a polarized type I response; and high production of toxic intermediates [nitric oxide (NO), reactive oxygen intermediates (ROI)]. Thus, M1 macrophages are generally considered as potent effector cells which kill microorganisms and tumor cells and produce copious amounts of proinflammatory cytokines.

Various signals elicit different M2 forms, able to tune inflammatory responses and adaptive Th2 immunity, scavenge debris, promote angiogenesis, tissue remodelling and repair [2, 10, 13–15] which share selected functional properties (e.g., low IL-12).

Microenvironmental signals expressed at the tumor microenvironment have the capacity to pilot recruitment, maturation and differentiation of infiltrating leukocytes and play a central role in the activation of specific transcriptional programmes expressed by tumor-associated leukocytes, either mediating pro- or anti-tumoral functions [2, 16].

To the extent that they have been investigated, differentiated mature TAM have phenotype and function similar to type II macrophages. In the attempt to clarify the molecular basis of the TAM phenotype, biochemical studies have identified the transcriptional factors NF-κB and HIF-1 as master regulators of their transcriptional programmes and indicated these factors as central regulators of tumor progression and metastasis.

3 NF-κB

Clinical evidence has long suggested that cancers arise at sites of chronic inflammation and this hypothesis has recently received molecular confirmations in inflammation-associated cancer models [7, 8], which provide in vivo evidences supporting a causal relationship between NF-κB-mediated inflammation and tumorigenesis. NF-κB induces several cellular alterations associated with tumorigenesis and more aggressive phenotypes, including: self-sufficiency in growth signals; insensitivity to growth inhibition;

resistance to apoptotic signals; immortalization; angiogenesis; tissue invasion and metastasis [17].

Constitutive NF-κB activation often observed in cancer cells may be promoted by either microenvironmental signals, including cytokines, hypoxia and Reactive Oxygen Intermediates (ROI), or by genetic alterations [18]. In particular, proinflammatory cytokines (e.g., IL-1 and TNF), expressed by infiltrating leukocytes, can activate NF-κB in cancer cells and contribute to their proliferation and survival [7, 8, 19].

While several in vitro and in vivo evidence suggest that NF-κB-induced proliferation and cell survival are two major mechanisms of NF-κB-mediated tumorigenesis, a direct role of NF-κB in metastasis formation has been recently confirmed in vivo by Luo et al., in a murine cancer metastasis model of colon adenocarcinoma, which demonstrated that the LPS-induced metastatic growth is dependent on both TNF-α production by hematopoietic cells and NF-κB activation by tumor cells [20]. In addition, defective NF-κB signaling by retroviral delivery of a dominant negative inhibitor of NF-κB resulted in the downregulation of prometastatic metalloproteinase, a urokinase-like plasminogen activator, and heparanase and reciprocal up-regulation of antimetastatic tissue inhibitor of metalloproteinases 1 and 2 and plasminogen activator 2 [21]. These observations are in line with several reports indicating that NF-κB regulates a myriad of genes playing a role in invasion and metastasis, such as cytokines and chemokines, adhesion molecules, matrix metalloproteinases, stress response genes, growth and angiogenic factors [22].

Other evidence suggest that inhibition of NF-κB suppressed angiogenesis along with vascular endothelial growth factor (VEGF) and IL-8 [23], thus preventing initial steps of tumor cells spread. NF-κB regulates expression of adhesion molecules [24, 25] and cell-surface metalloproteases, such as MMP-9 and MMP-2 [26]. Consistent with the observed association between inflammation and cancer, it was shown that upregulation of NF-κB in head and neck squamous cell carcinoma promotes inflammatory cytokines production and metastasis [27].

These findings propose NF-κB as a possible target for development of anti-cancer treatments and clinical trials with drugs that block NF-κB are currently in progress with promising results [28, 29].

To the extent they have been investigated TAM display defective NF-κB activation in response to different pro-inflammatory signals [30]. Detective NF-κB activation in TAM correlates with impaired expression of NF-κB-dependent inflammatory functions (e.g., expression of cytotoxic mediators, such as NO, and cytokines, TNFα, IL-1 and IL-12) [2, 30, 31] observed in these cells. However, these observations were obtained in TAM isolated from tumors characterized by advanced stages [30] and are in contrast with a protumor function of inflammatory reactions and TAM in particular [7, 8]. This apparent discrepancy may reflect a dynamic change of the tumor microenvironment during the transition from early neoplastic events toward advanced tumor stages, which would result in progressive modulation of NF-κB activity expressed by infiltrating inflammatory cells. While full activation of NF-κB in inflammatory leukocytes resident in preneoplastic sites may exacerbate local inflammation, thus favouring tumorigenesis, tumor growth may results in the progressive inhibition of NF-κB in infiltrating leukocytes, as observed in both myeloid [30, 31] and lymphoid [32] cells associated with solid tumors.

4 HIF-1

Hypoxia is a common feature of solid tumors that has been associated with decreased therapeutic response, malignant progression, local invasion and distant metastasis. The transcription factor hypoxia-inducible factor-1 (HIF-1) is a major regulator of cell adaptation to hypoxic stress [33] and therefore a potential target for anticancer therapies [34]. HIF-1 mediates switch from aerobic to anaerobic metabolism thus conferring a glycolitic phenotype to cancer cells and ensuring their energy requirements, thereby allowing their survival in a hostile environment. It was also proposed that the glycolitic phenotype of cancer cells is required for invasive tumor growth and observed that persistent increase in glycolysis results in chronic acidification of the local microenvironment, a condition which stimulates in vitro invasion and in vivo metastasis [35]. Increased glycolysis was associated with enhanced incidence of metastasis in cervical and head neck cancers [36–38]. HIF-1 activation in tumor cells activates several mechanisms leading to angiogenesis, glycolysis, inhibition of apoptosis, upregulation of growth factors (e.g., PDGF, TGF-β, IGF-2, EGF, VEGF) and protein involved in tumor invasion (e.g., urokinase-type plasminogen activator). Moreover, hypoxia downregulate adhesion molecules thus contributing to cancer cell detachment [39, 40].

TAM accumulate preferentially in the poorly vascularized region of tumors which are characterized by low oxygen tension. Such environment promotes TAM adaptation to hypoxia, which is achieved by the increased expression of hypoxia inducible and pro-angiogenic genes, such as VEGF, bFGF and CXCL8, as well as glycolitic enzymes, whose transcription is controlled by the transcription factors HIF-1 and HIF-2 [41, 42]. The in vivo relevance of this metabolic adaptation to hypoxia by macrophages was recently demonstrated by Cramer et al. [43]. Ablation of the hypoxia responsive transcription factor HIF-1α resulted in impaired macrophage motility

and citotoxicity, in low oxygen conditions. This evidence highlights the relevance that the hypoxia-HIF-1 pathway may play in the recruitment and activation of TAM into solid tumors and may be instrumental for TAM-mediated angiogenesis and tumor metastasis. In support of this, we have recently described that hypoxia can influence the positioning and function of cancer and stromal cells, including TAM, by selectively upregulating expression of the chemokine receptor CXCR4 [44]. Moreover, a recent work has shown that HIF-1 activation may play a role in the induction of the CXCR4 ligand, CXCL12 [45], a chemokine involved in cancer metastasis [46].

The Met tyrosine kinase, a high affinity receptor for hepatocyte growth factor (HGF), plays a crucial role in controlling invasive growth and is often overexpressed in cancer. It was shown that hypoxia activates transcription of the met protoncogene through HIF-1 activation, and that inhibition of Met expression prevents hypoxia-induced invasive growth. These data show that hypoxia promotes tumor invasion by sensitizing cells to HGF stimulation, providing a molecular basis to explain Met overexpression in cancer [47].

Similarly to NF-κB, inhibition of HIF-1α is considered a promising therapeutic approach against cancer [48] and in fact some of its inhibitors (e.g., farnesyl transferase inhibitors, PI3K inhibitors) are now in clinical trials as antitumor drugs [49].

5 Angiogenesis

Vascular and lymphatic endothelium are the major routes of metastatic spread of tumor cells. Tumor angiogenesis is often activated during the early, preneoplastic stages of tumor development [49, 50] and is controlled by a number of positive or negative regulators produced by cancer cells and tumor associated leukocytes.

Macrophages can exert a dual influence on blood vessel formation and function. On the one hand macrophages produce molecules that are proangiogenic, on the other hand they can express anti-angiogenic molecules and damage the integrity of blood vessels. On the anti-angiogenic side, in a murine model, CSF-induced, TAM-derived metalloelastase generates angiostatin. In general, as for interaction with neoplastic cells, the pro-angiogenic functions of TAM prevail. In several studies, in human cancer TAM accumulation has been associated with angiogenesis and with the production of angiogenic factors such as VEGF and platelet-derived endothelial cell growth factor [1]. TAM accumulate in hypoxic regions of tumors and hypoxia triggers a pro-angiogenic program in these cells. A number of molecules with possible impact on angiogenesis have been shown to be expressed by macrophage in low oxygen conditions, such as VEGF, TNF-α, bFGF and CXCL8 [51]. Therefore, macrophages recruited in situ represent an indirect pathway of amplification of angiogenesis, in concert with angiogenic molecules directly produced by tumor cells. Strikingly, it was recently reported the HIF-1-dependent chemokine CXCL12 [52] acts as a potent chemoattractant for endothelial cells of different origins bearing CXCR4 and is a participant in angiogenesis that is regulated at the receptor level by VEGF and bFGF. In agreement with these observations, our data suggest that the angiogenic program established by hypoxia relays also on the increased expression of CXCR4 by endothelial cells [44].

Lymphoangiogenesis is mediated by the action of VEGF-C and VEGF-D acting on the receptor VEGFR3. More recently VEGF-A, a chemotactic factor for monocytes, was shown to increase lymphoangiogenesis, via the recruitment of circulating monocytes [53]. In human cervical cancer, VEGF-C production by TAMs was proposed to play a role in peritumoral lymphoangiogenesis and subsequent dissemination of cancer cells with formation of lymphatic metastasis [54]. Additionally, TAM participate to the proangiogenic process by producing the angiogenic factor thymidine phosphorylase (TP), which promotes endothelial cell migration in vitro and whose levels of expression are associated with tumor neovascularization [55].

The contribution of chemokines toward angiogenesis has been the object of intensive investigation. A variety of chemokines, including CCL2, CXCL12, CXCL8, CXCL1, CXCL13, CCL5 [56] CCL17 and CCL22 [1] have been detected in neoplastic tissues as products of either tumor cells or stromal elements. CXCL1 and related molecules (CXCL2, CXCL3, CXCL8 or IL-8) have an important role in melanoma progression by stimulating neoplastic growth, promoting inflammation and inducing angiogenesis [57]. Strong evidence demonstrates that levels of CCL2 are associated with TAM accumulation [2] and that CCL2 may play an important role in the regulation of angiogenesis [58].

6 TAM induce an invasive phenotype

Several lines of evidence indicate that inflammatory cells and cytokines found in tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective host antitumor response [1].

Macrophages play an important role in this scenario as these cells produce large quantity of pro- and anti-inflammatory cytokines, which can promote cancer dissemination and metastasis. TNF-α, is a proinflammatory cytokine generally produced by macrophages in response to pro-inflammatory signals [59, 60]. Direct evidence for the involvement of TNF in malignancy comes from the

observation that mice lacking the gene for TNF are resistant to skin carcinogenesis [61]. A recent report showed that co-cultivation of tumour cells with macrophages leads to enhanced invasiveness of the malignant cells due to TNF-α dependent MMP induction in the macrophages [62].

Ablation of IL-1β in mice resulted in absence of metastasis development, either with melanoma cell models or with mammary and prostate cancer cells, suggesting the importance of microenvironmental IL-1β. Both, IL-1β and to a minor extent IL-1α, were required for in vivo angiogenesis and invasiveness of tumors in vivo [63].

The colony stimulating factor-1 (CSF-1) is a potent chemoattractant of macrophages into solid tumors [3]. The intercross of transgenic mice susceptible to mammary cancer (PyMT) with mice containing a recessive null mutation in the (CSF-1) gene (Csf^{op}) [64] demonstrated that TAM recruitment is an absolute requirement for productive metastatic growth. Hiratsuka et al. [65] provided evidence suggesting that primary tumors induce endothelial cell MMP9 through an interaction between endothelial cells and lung macrophages, via a VEGFR-1-dependent mechanism. MMP-9 expression in alveolar endothelial cells as well as in TAM, thus render the pulmonary metastatic site fertile for secondary malignant cell growth, dependent upon the presence and activation of the VEGF-VEGFR signaling cascade.

Macrophages produce endothelins, a family of small related, vasoactive peptides that have a great number of physiological roles in many tissues. TAM contribute to endothelins production in the breast tumor microenvironment [66], thus promoting an invasive phenotype of breast cancer cells, which is the result of an interplay with other factors, including cytokines, matrix metalloproteases and activation of TAM [67]. Interestingly the endothelin-1 (ET-1) was reported to activate inflammatory pathways in human monocyte, through the activation of NF-κB [68], while binding of the ET-1 to ovarian tumor cell lines triggers the activation and stabilization of HIF-1α, which than increases VEGF mRNA and protein levels in these cells [69].

7 Remodelling of ECM, tumor dissemination and implantation

Local growth and invasion of solid tumors as well as metastasis depend on the controlled degradation of components of the extracellular matrix (ECM). TAM are recognized as important player in cancer metastasis and clinical evidence showed a strong correlation between the number of TAM and poor prognosis [70, 71]. In turn, genetic studies in mice have shown decreased rates of metastasis to be associated with decreased TAM number [64, 72]. A model by which macrophages promote tumor invasion and metastasis includes expression of their proteolitic activity and subsequent break down of the basement membrane around the pre-invasive tumors, thereby enhancing the ability of tumor cells to escape into the surrounding stroma [63].

TAM express molecules which affect tumor cell proliferation, angiogenesis and dissolution of connective tissues. These include epidermal growth factor (EGF), members of the FGF family, TGFβ, VEGF, chemokines. In lung cancer, TAM may favour tumor progression by contributing to stroma formation and angiogenesis through their release of PDGF, in conjunction with TGF-β1 production by cancer cells [2]. TAM produce several matrix-metalloproteases (e.g., MMP2, MMP9) which degrade proteins of the extracellular matrix, and also produce activators of MMPs, such as chemokines. TAM also produce factors, such as transforming growth factor-β (TGFβ), platelet-derived growth factor, interleukin-6 (IL-6), urokinase plasminogen activator and Tissue-type Plasminogen Activator (t-PA) that may cause degradation of extracellular matrix to facilitate the tumor cell invasion and migration [73–76].

Ahmed et al., described a method to observe the orientation of individual tumor cells as they enter blood vessels, in real time and in a living animal [77]. They found that tumor cells seem to be attracted to macrophages, which line the outside of the vessels. Goswami et al. [78] described a paracrine signaling loop between tumor cells and macrophages, in which tumor cells secrete macrophage colony stimulating factor (M-CSF, also known as Csf1). This, in turn, causes macrophages to secrete epidermal growth factor (EGF), a chemoattractant for the tumor cells. Interrupting either of these signals results in decreased tumor-cell motility. Direct evidence have been presented that MMP-9 derived from hematopoietic cells of host origin contributes to skin carcinogenesis [79]. Chemokines have been shown to induce gene expression of various MMPs and, in particular, MMP-9 production, along with the uPA receptor [80]. Evidence suggests that MMP-9 has complex effects beyond matrix degradation including promotion of the angiogenesis switch and release of growth factors [79].

8 Interplay with adaptive immunity

In established tumors, available information suggests that TAM have a skewed M2 phenotype as discussed above. M2 mononuclear phagocytes are characterized by an $IL\text{-}12^{low}$ $IL\text{-}10^{high}$ phenotype and TAM also produce TGFβ. In addition, tumor-associated dendritic cells (DC) have an immature phenotype, various cytokines (M-CSF, IL-6, IL-10) present in the tumor microenvironment, contribute to blocking DC maturation in tumors. Immature myeloid cells are expanded in chronic infections and cancer and act as potent suppressors of T cell dependent antitumor immunity via unbalanced iNOS and arginase-1 activity [81]. Thus,

tumor-associated myelomonocytic cells favour progression by taming and skewing antitumor T cell responses.

Transgenic mice carrying the early region genes of HPV16 under the control of the human keratin 14 promoter offer a useful model which recapitulates tumor progression of squamous cell carcinoma from hyperplasia to displasia to overt malignancy. Innate immunity cells, most prominently mast cells and granulocytes infiltrate HPV16 premalignant tissues, followed by macrophages in carcinoma. They drive a chronic inflammatory process which promotes epithelial hyperproliferation, tissue remodeling and angiogenesis, followed by displasia and invasive carcinoma [79]. By crossing HPV transgenic mice with severely immunodeficient mice (RAG-1 −/−), it was found that genetic elimination of T and B lymphocytes blocks recruitment of innate immunity cells, tissue remodeling and angiogenesis, with an arrest of the carcinogenesis process at the stage of epithelial hyperplasia [82]. Dissection of cells and molecules involved revealed that B cells, which do not infiltrate the lesions, act as remote orchestrators of the innate immune cells in situ. Circumstantial evidence suggests that this remote control mechanism of cancer promoting inflammation operates via deposition of immunoglobulins, but this was not formally proven.

Interestingly, immune complexes in concert with microbial molecules or inflammatory cytokines have been shown to elicit an M2 form of macrophage activation [14] and TAM are a prototypic M2 population [2, 10, 13]. M2 cells tune inflammation and adaptive immunity and promote cell proliferation, angiogenesis, tissue remodeling and repair. B cells by causing formation of immune complexes may contribute from a distance to the M2 polarization of phagocytes which are set in a tissue remodeling and repair mode and orchestrate the smouldering and polarized chronic inflammation associated to established neoplasia [12, 19].

The IFN-γ-inducible enzyme indoleamine 2,3-dioxygenase is a well known suppressor of T cell activation. It catalyzes the initial rate-limiting step in tryptophan catabolism, which leads to the biosynthesis of nicotinamide adenine dinucleotide. By depleting tryptophan from local microenvironment, IDO blocks activation of T lymphocytes [83]. It was recently reported that the BAR adapter-encoding gene Bin-1 inhibits IDO expression in cancer cells and macrophages and that inhibitors of IDO, such as methyl-thiohydantoin-tryptophan (MTH-trp), cooperate with cytotoxic agents to elicit regression of established tumors [84].

9 Concluding remarks

Macrophages are a key component of cancer promoting inflammatory reactions. Several lines of evidence, ranging from adoptive transfer of cells to genetic manipulations, suggest that myelomonocytic cells can promote tumor invasion and metastasis, although under certain conditions they can express antitumor reactivity. Thus, therapeutic targeting of macrophage derived mediators may provide innovative therapeutic strategies against invasion and metastasis.

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