TITLE:

Tumor-associated macrophages, multi-tasking cells in the cancer landscape

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Abstract

It is now well recognized that myeloid cells of the innate immunity infiltrating the tumor micro-environment, instead of halting tumor progression, favour the proliferation of tumor cells and their invasive ability. In particular, macrophages represent the most abundant leukocyte population recruited at tumor sites, from early stages till the occurrence of metastasis. Tumor-Associated Macrophages (TAMs) are crucial determinants of cancer cell survival and proliferation; they efficiently trigger neo-angiogenesis and matrix degradation and suppress potential anti-tumor adaptive immune responses. Established evidence demonstrated that high density of infiltrating TAMs is usually associated with fast tumor progression and resistance to anti-cancer therapies. Targeting of TAMs or modulation of their functions is now actively pursued. In this review we discuss recent knowledge and current therapeutic approaches behind TAMs. A better understanding of their features, heterogeneity in particular, and of their tumor-promoting functions is essential to better design TAM-centered therapeutic interventions. Understanding of how best to combine TAM-targeted approaches and conventional chemotherapy or immunotherapy, holds promise for successful anti-cancer treatments.

Keywords: Tumor, macrophages, TAMs, Cancer-related inflammation.
Introduction

Tumor tissues are composed by a heterogeneous mixture of normal and neoplastic cells, whose dynamic interactions are crucial elements for tumor progression. Among normal cells, stromal fibroblasts, blood vessels and immune cells have raised much interest. Leukocytes, and in particular myeloid cells, are key determinants of the reactive tumor micro-environment, which is characterized by a condition of persistent and non-resolving inflammation (cancer-related inflammation) (1, 2). Immune cells populate the tumor micro-environment from the early stages and are profoundly affected by the presence of neoplastic cells, which in turn are influenced by immune effectors in different ways (3, 4).

Innate and adaptive immunity cells have an ambiguous relationship with cancer cells and can either restrain or promote tumor growth. Current knowledge considers that in emerging tumors the immune system has a defensive role and actively eliminates antigen-expressing tumor cells, but over time, those cancer cells that escape the immune surveillance and are antigenically “silent”, overgrow, eventually giving rise to clinically evident tumors (5). At this stage, proliferating cancer cells have sufficiently grown to a critically big mass and have the ability to affect the immune system and to turn immune cells into harmless effectors, devoid of cytotoxic ability and with immunosuppressive functions. The outstanding clinical results obtained with therapeutic antibodies blocking the immune checkpoints demonstrate, in different neoplasia, that an immune response does initially occur in cancer patients, but is subsequently put to silence via different immunoregulatory mechanisms.

Cells of the monocytic-macrophage lineage are abundantly present in established tumors and their presence is usually associated with disease progression and poor patient prognosis (6-8). Thus, in the cancer context, cells of the innate immunity favour tumor growth, instead of fighting against it.
Macrophages are plastic cells which are able to generate distinct functional programs in response to various stimuli. When exposed to signals present in the tumor-microenvironment, differentiating macrophages generally acquire pro-tumoral functions (9).

In this review, we will describe the origin and phenotype of Tumor-associated Macrophages (TAMs), their contribution in tumor progression and their role in the effectiveness of different anti-tumor therapeutic approaches.

**Origin and phenotype of tumor-associated macrophages (TAMs)**

Tissue macrophages can be divided into resident macrophages, characterized in mice by the expression of the chemokine receptor CX3CR1, which protect tissues and maintain homeostasis, and inflammatory macrophages, characterized by the expression of CCR2, which are recruited at inflammatory sites and contribute to the inflammatory response. It is nowadays accepted that resident macrophages (Kupffer cells in liver, microglia in brain, Langerhans cells in the skin and alveolar macrophages in lung) develop in the yolk sac at embryonic stage (10). During this process, monocyte progenitors colonize peripheral tissues and differentiate into resident macrophages that will self-maintain throughout life (11). On the other hand, inflammatory macrophages exclusively derive from bone marrow-derived monocytes. However some exceptions are, for example, resident macrophages in the gut, heart and dermis which originally derive from the yolk sac, but during adult life are replenished by bone marrow progenitors (12-15).

TAMs mainly originate from bone marrow monocytes (16-18) although local proliferation has been observed in some mouse tumors (19, 20) and a splenic contribution has also been suggested (21).

Chemokines (e.g. CCL2, CCL5, CXCL12) and the growth factor CSF-1 (M-CSF) are major determinants of monocyte infiltration in tumors. Indeed, chemokines have served as a paradigm for the recruitment of leukocytes in peripheral tissues, including tumors (22, 23). Recently, also products of the complement cascade have been described to have a role in macrophage recruitment
Incoming blood monocytes preferentially localize in hypoxic or necrotic areas within tumor stroma; they are profoundly influenced by the tumor environment and rapidly differentiate into tumor-conditioned macrophages.

Macrophages are very versatile cells showing a vast spectrum of heterogeneity (25). A useful (although highly schematic) way to classify macrophages in functionally different subsets is the binary distinction into M1 and M2 macrophages. They can be “classically activated” (M1) by Th1 cytokines (e.g. IFNγ) or bacterial products, or “alternatively activated” (M2) in response to Th2 cytokines (e.g. IL-4, IL-13) and immunoregulatory stimuli (e.g. IL-10, TGFβ) (26, 27). As a matter of fact, M1 and M2 polarized macrophages are extremes of a continuum, comprising different phenotypic features, which are dictated by the context and the availability of specific signals modulating their characteristics.

M1-polarized macrophages produce pro-inflammatory mediators like IL-1, TNF and iNOS, via NFκB and Stat1 activation, have bactericidal and tumoricidal activity, and sustain Th1 responses. Instead, M2 macrophages activate different transcriptional factors, among which IRF4, Stat6, Myc and sustain Th2 responses; they have immunoregulatory functions via the production of TGFβ, IL-10 and arginase; they are importantly involved in resistance to parasites, in the maintenance of homeostasis, by suppressing Th1-type inflammation, in the remodelling of tissues and in wound healing (26, 28-32). Most frequently, and especially in advanced metastatic tumors, TAMs display features similar to M2-polarized macrophages; however, heterogeneity of TAMs is increasingly emerging and it is not unusual the presence of M1-like TAMs, especially in the early stages of tumorigenesis or in regressing tumors. (33-35). In the cancer-initiating phase, a variety of mechanisms has been implicated in the potential anti-tumor activity of macrophages: for example, TNFα and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce apoptosis in tumor cells, inhibiting the NF-κB survival pathway (36-38). When tumor cell death is somehow initiated, danger signals such as heat-shock proteins, uric acid or high-mobility group protein 1 (HMBG1) are released by necrotic cells and can induce macrophage activation, which in turn may...
contribute to NK cells activation (39, 40). Recent studies revealed that host production of soluble factors like E-FABP or HRG can induce M1 polarization in macrophages, promoting anti-tumor immune responses (recruitment of CD8+ effector T cells and NK lymphocytes), vessel flow normalization, and reducing tumor growth and metastatic spread (41-43).

Over time, however, with tumor progression, the microenvironment enriches of growth factors and inflammatory mediators that cause a bias from a type 1-like inflammatory response to a type 2 phenotype (2, 3, 44-46). These changes strongly condition tumor macrophages and newly recruited monocytes, so that they switch to different functional programmes and generally acquire pro-tumoral functions. Among signals derived from tumor or host cells that importantly impact on TAM differentiation are growth factors (CSF-1, VEGF), cytokines (IL-10, TGFβ, IL-4, IL-13 and IL-6), metabolic products of cancer cells (lactic acid) and hypoxia (1, 17, 47-53).

As mentioned above, macrophages are very versatile cells and heterogeneity is also found in TAMs in the tumoral milieu. In general, TAMs are categorized by the expression of CD11b^+ Ly6G^low Ly6C^low F4/80^high MHC-II^low, and high scavenger and mannose receptors, but TAMs with MHC-II^high, and M1-like features have also been described, in particular in normoxic areas.

In fact, there can be micro-anatomical diversity within the cancer tissue with accumulation of TAM with M2-like phenotype and pro-tumor properties preferentially in hypoxic areas (54). Recently, Casazza and colleagues demonstrated that macrophages are recruited in hypoxic regions via Sema3A/Nrp1 signaling: blocking this recruitment inhibited the pro-tumoral features of macrophages that were no longer able to sustain neo-angiogenesis and tumor growth (55). In colorectal cancer, TAMs localized within the tumor mass and those at the tumor margin seem to have different impact on patients’ survival: while TAM infiltrating tumor cells promote tumor progression, TAM accumulating outside tumor nests, are associated with good prognosis, (56-58).

Thus, TAMs localized in different compartments of the same tumor may have considerably different functional properties.
Overall, in the vast majority of tumors (especially in advanced tumor stages), TAMs share functional features with M2 macrophages (8, 30): TAMs are not cytotoxic, have tissue trophic functions, promote angiogenesis and matrix remodelling and suppress Th1 adaptive immune responses (17, 59).

**Pro-tumoral functions of TAM**

TAMs are key promoters of the cancer-related inflammation, now a recognized hallmark of cancer, eventually promoting tumor progression (1, 7, 35, 60-62). Experimental and clinical observations strongly support correlative data between poor patient prognosis and high macrophage infiltration of tumor tissues (6, 63, 64). TAMs affect every aspect of tumor cell behaviour, including protection from apoptosis/necrosis, proliferation, invasion and metastasis in an intense and reciprocal cross-talk (Figure 1).

**Tumor cell survival and proliferation:** A first level of interaction between TAM and tumor cells is the ability of TAM to produce mediators that directly support tumor growth or enhance their resistance to death-inducing stimuli, for instance epidermal growth factor (EGF) and IL-6. IL-6 activates the signal transducer and activator of transcription 3 (STAT3) pathway, a crucial mediator of the cancer-related inflammation. In tumors, STAT3 controls cell proliferation and apoptosis, through the modulation of specific genes, such as cyclin D, proliferating cell nuclear antigen (PCNA), Bcl-XL, Bcl-2 and Mcl-1 (65). In pancreatic cancer, STAT3 is mainly activated by TAM-derived IL-6: ablation of IL-6 expression or of STAT3 activation results in reduced tumor-progression and decreased inflammatory cell infiltration (66, 67). TNF is a primary inflammatory cytokine produced by several cell types. While M1-polarized macrophages secrete high levels of TNF that can induce tumor necrosis, TAMs produce low concentrations, which are known to stimulate tumor growth and angiogenesis (68). Evidence from recent studies indicates that TAMs protect cancer cells, including cancer-initiating cells, against chemotherapy-induced toxicity (69,
Furthermore TAMs indirectly support tumor growth by triggering the angiogenic switch and by inhibiting the cytotoxic activity of T lymphocytes (see below). TAMs also shape the type of the leukocyte infiltrate: by releasing chemokines (e.g., CCL17, CCL18, CCL22) that mostly recruit immune suppressive Treg or Th2 cells devoid of cytotoxic function, they facilitate mechanisms of tumor immune escape (51).

**Tumor cell invasion:** Macrophages have been shown to play a pivotal role in the process of tumor cell invasion and metastasis. As mentioned above, tumor cells produce CSF1, which stimulates TAMs to secrete EGF, which in turn contributes to tumor cell migration (60, 71). The importance of the CSF1-EGF pathway in trans-endothelial migration of cancer cells and metastasis formation has been recently confirmed by the use of CSF1R antagonists, which inhibited the migration of both cell types (72).

TAMs are probably the most active contributors to tissue and matrix remodelling in tumors, being the major source of MMPs and other proteolytic enzymes. Extracellular matrix (ECM) degradation by TAMs favours tumor cells to invade locally, penetrate into vessels and give rise to metastatic dissemination (73). Other TAM mediators are involved in ECM remodelling and contribute to enhanced tumor cell invasion: osteonectin, (also known as SPARC) enhances ECM-tumor cell interaction and migration (74); cathepsin proteases, that degrade the matrix and release sequestered growth factors (2); TGF-β, that promotes epithelial-to-mesenchymal transition in tumor cells (75); chemokines, such as CCL18, that promotes invasiveness by activating tumor cell adherence to ECM (76). Products of matrix degradation also contribute to the formation of a pro-inflammatory microenvironment. For example, versican and hyaluronan fragments trigger different Toll-Like receptors (TLR) on TAM and induce the transcription of several inflammatory genes (77, 78).
Angiogenesis: TAMs are key elements for the acquisition of a vasculature that provides oxygenation as well as nutrition to tumor cells, in a process referred as “angiogenic switch” (62). TAMs favour neo-angiogenesis by providing angiogenic factors such as VEGF, CXCL8, placental growth factor (PIGF) and prokineticin (Bv8). Macrophage depletion in different tumor models results in the reduction of angiogenesis: null mutation in the Csf1 gene reduces macrophage accumulation in mammary tumor and inhibits the angiogenic switch (79). Similarly, other macrophage depletion strategies decrease angiogenesis in different tumor models (80-82). Pro-angiogenic TAMs include a specific population that expresses the angiopoietin (ANG) receptor Tie2 (Tie2-expressing monocytes, TEMs). TEMs are often aligned along the abluminal surface of blood vessels through the interaction of Tie2 with its ligand ANG2. Targeting ANG2 or Tie2, releases macrophages from vessels and reduces angiogenesis (83). Interestingly, CSF-1 upregulates Tie2 expression on TAMs, linking macrophage recruitment to the angiogenic switch (84).

Hypoxia is a major driver of angiogenesis. TAMs preferentially accumulate in necrotic and hypoxic areas of the tumor, due to the hypoxic release of such macrophage chemoattractants as EMAP-II, endothelin 2 and VEGF (85, 86), and up-regulate the transcription factor HIF-1α (87, 88). HIF-1α induces in TAMs the transcription of a large panel of genes associated with angiogenesis, such as VEGF and CXCL8 (89). The proteolytic enzymes (e.g. MMP9) produced by TAMs also play a role, as they mobilize VEGF and other growth factors from extracellular matrix depots (90).

Immunoregulation: TAMs have the capacity to suppress adaptive immune responses by acting as immunoregulatory cells in the tumor microenvironment. Functional studies and transcriptome analyses have shown that TAMs are poor antigen-presenting cells, have defective IL-12 secretion, produce IL-10 and TGFβ, block the proliferation and cytotoxic activity of T-cells (91-95). TAMs may express PD-1L, the ligand of the inhibitory receptor PD-1 (programmed cell death protein 1), as well as B7-1 which engage the receptor CTLA-4 (cytotoxic T-lymphocyte antigen 4). These pathways have been shown to be of major importance in the inhibition of immune effectors.
of the adaptive immunity (96). It remains unclear, however, to what extent these inhibitory molecules contribute to the immunosuppressive activity of TAMs. The over-activation of the transcription factor STAT3 also plays a pivotal role in the immunoregulation by TAMs: in immune cells, STAT3 activation counteracts STAT1-regulated Th1 anti-tumor responses, promoting the differentiation of immature myeloid cells with suppressive activity (97, 98).

TAMs also secrete a variety of cytokines and chemokines able to suppress CD4+ and CD8+ T cell functions directly or indirectly, through the recruitment of natural regulatory T (nTreg) cells to the tumor microenvironment. In human ovarian cancer, TAMs secrete CCL22 that recruit CCR4+ nTreg cells. Similarly, in colorectal cancer, CCL20 secreted by TAM recruits CCR6+ nTreg cells (99). IL-10 and TGFβ produced by TAMs are major players of immune suppression and inhibit cytotoxic CD8+ T lymphocytes and Th1 and Th2 CD4+ T cells (100, 101).

Myeloid derived suppressor cells (MDSC) are another immunosuppressive population that share properties and gene expression profiles with TAMs. MDSC control T-cell responses through the secretion of two enzymes involved in the arginine metabolism: inducible nitric oxide synthase (NOS2) and arginase (Arg1), which metabolizes arginine. By constitutively expressing arginase, with constitutive L-arginine depletion, MDSC can suppress T cell immune activity. IL-4 mediated increased expression of arginase further enhances the immunosuppressive activity of murine MDSC. In addition, murine MDSC can inhibit T cell function through the production of reactive nitrogen or oxygen intermediates, or by producing peroxynitrite from O2− and NO under condition of L-arginine limitation (102-104).

In line with the above clinical evidence, in the majority of cases TAM infiltration correlates with tumor progression and worst patients’ prognosis. Macrophage-related gene signatures have been observed in a variety of human tumors, such as ovarian and breast cancer, soft tissue sarcoma and various haematological malignancies (63, 64, 105, 106).

However, some exceptions to this pro-tumoral phenotype have been described: studies in human colorectal cancer reported that TAM density is not associated with worst prognosis and can...
actually be related to longer patient survival. In colorectal cancer, TAM localization within tumors appears to be of primary importance: in fact, only those TAMs localized at the tumor invasive front (likely less influenced by cancer cells) are associated with increased patients’ disease-free survival (56-58). In lung adenocarcinoma, only a specific subpopulation TAM expressing CD204 was associated with a poor outcome (107).

**Targeting of TAM in tumors**

The evidence summarized above that tumor macrophages have pro-tumor activities stimulated an extraordinary impetus for scientific research aimed at the targeting of TAMs to improve anti-cancer therapies. In general, two main approaches have been used: direct depletion of macrophages or inhibition of monocyte recruitment, and re-stimulation of their cytotoxic function (re-education of TAMs) (Figure 2). Inhibition of monocyte/macrophage recruitment has been extensively investigated in experimental mouse models and is now under clinical evaluation. Antibodies against CCL2, a prime chemokine in tumors, are being tested in clinical trials and showed anti-tumor activity (108, 109). The CSF1-CSF1R is a major target for anti-macrophage strategies. Several antagonists of the CSF-1R or anti-CSF1 antibodies have been developed and tested in different preclinical models, generally decreasing the number of macrophages (110-113). From the numerous recent studies, some heterogeneous results start to emerge: for instance, inhibition of the CSF-1R in glioblastoma did not reduce TAM numbers but blocked their tumor-promoting functions (113). Chemotherapy (e.g. paclitaxel) may also affect the production of CSF-1 by tumor cells. Combination treatment of chemotherapy and inhibition of the CSF1/CSF1R loop showed higher therapeutic efficacy and also had positive impact on adaptive immune responses, by increasing the recruitment of CD8 T cells in tumors (112). Other studies showed that macrophage inhibition is able to influence the effect of chemotherapy (17, 59, 114). TAM depletion by clodronate treatment in tumor-bearing mice lead to reduced angiogenesis and tumor growth; when clodronate was combined with sorafenib, an inhibitor of tyrosine protein kinase (such as VEGF-R,
PDGFR), the effect of the drug was significantly increased (115). In a mouse model of pancreatic cancer, targeting of TAMs, by blocking CCR2 or CSF1R, resulted in significant inhibition of tumor initiating cells and improved therapeutic effect by gemcitabine (116). In prostate cancer, local irradiation was associated with systemic increase of CSF1 expression; administration of a CSF1 receptor inhibitor, improved the therapeutic effect of radiotherapy (117).

The anti-tumor agent trabectedin induces a rapid apoptosis selectively in cells of the monocyte-macrophage lineage, including TAMs (118). Trabectedin is a recently registered compound currently used in soft tissue sarcoma and ovarian cancer, but under clinical investigations in several other human malignancies. Inhibition of TAMs was shown to be an important determinant of the anti-tumour efficacy of trabectedin in tumor-bearing mice, and was confirmed in tumor tissues of treated patients (118). Furthermore, trabectedin inhibits the production of several bioactive mediators relevant in the tumor context (e.g. CCL2, IL-6, VEGF), overall fading the cancer-related inflammation and angiogenesis in the tumor microenvironment.

A different approach is to re-set TAMs in a cytotoxic mode. Macrophage plasticity provides the basis for strategies aimed to re-educate TAMs into M1-polarized macrophages able to kill tumor cells (29, 119). This changeover can be performed using different agents. Interferons are major cytokines activating the immune effectors: early studies in human ovarian cancer demonstrated partial responses after administration of IFN-γ (120). More recently, the selective expression in monocytes of an IFNα transgene inhibited tumor progression in a mouse model of mammary carcinoma and enhanced cytotoxic T cells (121). Other molecules or compounds having a stimulatory effect on macrophages include TLR agonists, such as CpG oligonucleotides (122) and antibodies activating the CD40 molecule (123). In particular, CD40 agonist antibodies administered in combination with gemcitabine to pancreatic cancer patients, achieved clinical effects (123). In a murine model, anti-CD40 agonists induced the up-regulation of MHC class II and CD86 in macrophages, suggesting an effect on their re-polarization (124). Recently, Klug and colleagues demonstrated that low-dose irradiation shifts macrophage polarization from tumor promoting and
immunosuppressive cells to effectors able to kill tumor cells and to stimulate cytotoxic T cell infiltration, rendering immunotherapy successful in mice (125).

Overall, the enhancement of therapeutic responses to conventional chemotherapy after macrophage depletion or re-education is highly promising and constitutes the rationale for pursuing clinical testing of combined approaches.

Concluding remarks

In the last decade, a huge number of studies investigated the role of tumor macrophages in the promotion of an inflammatory microenvironment, in the context of tumor initiation and progression.

TAMs are abundant cells within tumor tissues. As versatile cells of the innate immunity they react promptly to local stimuli and acquire specific phenotype and functional activities that importantly impact on tumor and other stromal cells and on adaptive immunity. Tumor-educated TAMs produce a wealth of biologically active mediators that sustain the cancer-related smouldering inflammation, eventually favouring tumor cell proliferation and invasion. Biological strategies aimed at depleting or at re-educating tumor macrophages, are at the forefront of cancer research; proof-of-principle experiments have been successful in pre-clinical studies and a number of clinical experimentation are now on going.

In particular, available data show that appropriate targeting of TAMs could be of benefit for the success of conventional anti-cancer therapy. Further definition of macrophage heterogeneity and of their specific roles in different types of cancers and at different stages of disease, will provide the bases for the development of innovative anti-tumor approaches.
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**Fig.1 Pro-tumor functions of TAMs.** In the tumor context, TAMs exert different functions, influencing the cancer-microenvironment from the early stage till the metastatic spread. A first level of interaction between TAMs and tumor cells is the ability of TAMs to directly support tumor growth by the production of cytokines and growth factors (IL-6, low concentrations of TNF and EGF). TAMs can promote tumor development also by the construction of an immune suppressive microenvironment: they counteract the activation of cytotoxic T-cell through the production of immune suppressing cytokines, such as IL-10 and TGFβ; moreover, TAMs produce chemokines, such as CCL20 and CCL22, that stimulate the recruitment of regulatory T cells (Treg) able to switch off the immune reaction against tumor antigens. TAMs mediate matrix remodelling, being active producers of MMPs: the consequent extracellular matrix degradation induces tumor cells to invade locally, penetrate into vessels and give rise to metastatic dissemination. Tumor invasion is also stimulated by the production of EGF, which in turn is induced by CSF1 produced by tumor cells. In addition, TAMs secrete a variety of pro-angiogenic factors, such as VEGF and CXCL8.
Fig.2 Strategies to target TAMs for therapeutic purposes. TAMs can be targeted using two different approaches: through the direct depletion of macrophages and the inhibition of monocyte recruitment (Reduce), or by restoring their anti-tumoral cytotoxic function (Re-activate). The first approach includes antibodies against the chemokine CCL2 and antibodies or antagonists of the CSF1-CSF1R pathway. The anti-tumor compound trabectedin is active against cancer cells and in addition induces the selective apoptosis of monocytes and macrophages. Macrophage plasticity is important to re-educate TAMs from a tumor promoting/immunosuppressive phenotype to anti-tumoral effectors. This changeover could be achieved using different agents, such as interferons, TLR agonists and anti-CD40 agonist antibodies. Low dose irradiation can re-educate TAMs to stimulate CD8+ cytotoxic cells and tumor cell killing.