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Macrophages and cancer

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TABLE OF CONTENT

1. ABSTRACT

Macrophages are ubiquitous cells physiologically involved in a variety of processes including pathogen destruction, inflammation, tissue repair and remodeling. They have a highly plastic phenotype and their functional polarization is determined by cytokines and factors found within local microenvironments. The role of macrophages during tumor development is ambiguous. At late stages, tumor-associated macrophages are known to produce molecules directly promoting tumor growth, invasion, and metastasis; the so called "myeloid-derived suppressor cells" also suppress the adaptive anti-tumor immune response. However, if properly activated, macrophages may control initial tumor development, and pilot studies in cancer patients suggest that adoptive transfers could be beneficial as adjuvant treatment in patients with minimal residual disease. Indeed, a limited tumor mass will probably be insufficient to educate macrophages into a suppressive phenotype. Thus, the macrophage effect *in vivo* may be determined by a variety of factors including the tumor type and stage, the degree of macrophage infiltration and their functional polarization. Unfortunately, the *in vivo* mechanisms responsible for the anti-tumor activity of macrophages are still unclear. Current promising strategies to target tumor macrophages *in vivo* include pharmacological agents capable to re-polarize them towards a classically activated phenotype or to inhibit their suppressive properties.

2. INTRODUCTION: MACROPHAGE DIFFERENTIATION AND POLARIZATION

Macrophages were discovered in 1882 by Ilya Metchnikov, who first described cells with phagocytic properties in starfish larvae; he proposed that the function of these cells is the defense of the organism from intruders. In mammals, macrophages derive from monocyte precursors that migrate and differentiate into tissues as the bone marrow, the liver (Kupffer cells), the lung (alveolar macrophages), the connective tissues (histiocytes), the gut, the spleen, the lymph nodes, the thymus, the brain (microglia), the bone (osteoclasts) and various serous cavities (pleural and peritoneal macrophages) (1, 2). Although macrophages from different organs share some general functions, described below, the cytokine and growth factor milieu within the tissue of residence imprints them with specific characteristics and polarization (2, 3).

Blood monocytes have been classified into subsets based on the level of expression of the markers CD14 (lipopolysaccharide co-receptor) and CD16 (a low affinity receptor for the Fc portion of IgG) (2, 4). In humans, the CD14high/CD16neg subset, accounting for approximately 90% of the circulating monocytes, has been proposed to specifically home to inflamed tissues. The CD14low/CD16pos cells, on the other hand, may give rise to tissue macrophages during steady-state conditions. The development and function of these different monocyte

lineages are still however ill-defined. The mechanisms by which monocyte recruitment occurs are also not completely understood, but the homeostatic cytokine macrophage colony-stimulating factor (M-CSF or CSF-1) is probably critical for this process (5, 6).

The tissue macrophage pool may also be maintained by local proliferation of tissue-resident precursors. This is a mechanism that may have been so far underestimated. Macrophage precursors within ovary have recently been reported to undergo local proliferation prior to terminal differentiation (7).

Macrophages are involved in two major processes: 1) tissue trophism and remodeling, and 2) phagocytosis and destruction of pathogens. During these events, macrophages release mediators affecting differentiation, activation, proliferation of immune, stromal, and parenchymal cells.

In the steady-state, resident macrophages participate in tissue trophism and remodeling activities such as removal of apoptotic cells, promotion of angiogenesis and of cell differentiation. Apoptotic cells are recognized by scavenger receptors and integrins, phagocytosed and cleared without generation of an inflammatory reaction. Secretion of matrix metalloproteinases (MMP) contributes to extracellular matrix degradation, while production of angiogenic factors as VEGF induces neovessel formation. These activities are essential during processes as embryonic development and wound healing. Thus, macrophage-deficient CSF-$1^{-/-}$ *op/op* mice exhibit infertility, neuronal disfunctions, defective development of mammary glands and of insulin-secreting cells, as well as osteopetrosis for the absence of osteoclasts (5, 8).

In the presence of an infection, macrophages phagocytose and destroy viruses and bacteria. Pathogen recognition occurs via lectins and scavenger receptors specific for bacterial glycoproteins or lipids, or via Fc and complement receptors, which bind very efficiently to opsonized microorganisms. Upon pathogen destruction in the phagolysosomes, macrophages process and present their antigens on MHC class II and contribute to the activation of helper T cell responses. The ability of macrophages to cross-present exogenous antigens on MHC class I for activation of CD8 T cells is still controversial. Although some cross-presentation activity by macrophages was detected *in vitro*, only dendritic cells were unambiguously shown to cross-present antigens *in vivo* (9).

During pathogen recognition and destruction, macrophages are activated by microbial-associated molecular patterns (MAMPs) like lipopolysaccharide (LPS), proteoglycan, lipotheicoic acid, double-stranded RNA, etc. Activation generally occurs via toll-like receptors (TLR) expressed on the membrane or in endosomes, or via members of the NOD family as NOD1 and NOD2, which are located in the cytoplasm. Macrophage activation can also be triggered by endogenous stress and danger signals (HMGB1, uric acid, heat-shock proteins) as well as cytokines like TNF-alpha and IFN-gamma. Upon activation, macrophages increase their phagocytic activity and production of cytotoxic factors and mediators as cytokines and chemokines. These molecules can have either pro-inflammatory (TNF-alpha, IL-1) or suppressive (TGF-beta, PGE_2, IL-10) functions.

Macrophage polarization is dictated by the type and combination of the activation stimuli they come into contact with. The groups of Gordon and Mantovani have extensively characterized macrophage polarization and classified them as M1 and M2, for classically or alternatively activated macrophages, respectively (10-13). Classical activation is induced by IFN-gamma priming in combination with LPS or other TLR ligands. It results in cells able to secrete pro-inflammatory and Th1-polarizing cytokines as TNF-alpha, IL-12 and IL-23, as well as effector molecules as reactive nitrogen and oxygen intermediates (RNI and ROI); these macrophages have been described as having anti-parasitic and anti-tumor properties. Macrophages exposed to cytokines as IL-4 or IL-13, to IL-10, glycocorticoids or immune-complexes undergo instead alternative programs of activation and are collectively referred to as M2 macrophages. These are the cells that are physiologically involved in tissue remodeling and repair. Martinez *et al.* have recently proposed that steady-state macrophages physiologically found in tissues may be *bona fide* alternatively activated (14). The classification into M1 and M2 macrophages is nowadays recognized as a useful but oversimplified descriptive tool, given the high plasticity of this cell type and frequent occurrence of "intermediate" or "mixed" phenotypes (12).

Macrophages often infiltrate tumors and can promote tumor growth and metastasis. On the other hand, properly activated macrophages have shown anti-tumor cytotoxic activity *in vitro* and *in vivo*. In this review, we will discuss the evidence for this dichotomy and how some of the macrophage activities within tumors can be exploited or targeted to improve therapeutic interventions in cancer.

3. PRO-TUMOR MACROPHAGES

3.1. Prevalence and epidemiology *in vivo*: findings from clinical studies and animal models

Tumor growth, invasion, metastasis, and escape from immune recognition can all be supported by its stromal compartment. Myeloid cells contribute to all these activities. Macrophage accumulation has been observed in many human tumors, although it varies according to the tumor types (15-20). Recruitment in tumors is mediated by CCL2, CSF-1 and/or endothelin-2 (21-24). Hypoxic conditions within the tumor core downregulate the CCL2 receptor CCR2 and thus inhibit further macrophage motility (25, 26).

A body of evidence indicates that high numbers of tumor-associated macrophages (TAM) correlate with poor prognosis in breast, prostate, ovarian, cervical, lung cancer, follicular lymphoma as well as uveal and cutaneous melanoma (15, 16, 18, 27, 28). These epidemiological studies have indirectly suggested that a macrophage-rich

Table 1. Molecules produced by tumor-associated macrophages or myeloid-derived suppressor cells that directly or indirectly promote tumor development

Molecule/ Product	Mechanism of pro-tumor activity	References
Arginase	Depletion of arginine: T cell anergy or apoptosis	19, 59, 61, 62, 68, 69, 71-73
iNOS	Depletion of arginine, production of NO: T cell anergy or apoptosis NO promoting tumor proliferation, angiogenesis	56, 69, 71, 73, 74 55
H_2O_2	Induction of T cell anergy or apoptosis	63, 70
IDO	Depletion of tryptophan: T cell anergy or apoptosis	64
TNF-alpha	DNA mutagenesis, stimulation of tumor cell survival and proliferation, angiogenesis	48, 50, 51, 75-77
CCL2	Macrophage attraction positive feedback loop	40
IL-1beta	Promotion of angiogenesis, tumor growth and metastasis	37, 48, 78
IL-6	Stimulation of tumor cell survival and proliferation	48, 77
IL-8	Promoting inflammation via attraction of neutrophils and monocytes, stimulation of angiogenesis	48
IL-10	Suppression of IL-12 production and Th1 responses	38, 77
TGF-beta	Immunosuppressive, promoting T_{reg} and Th_{17} development	77
IL-23	Promoting inflammation via induction of Th_{17}, suppression of Th1 responses	77, 79
MMP	Facilitate invasion and metastasis via degradation of the basement membrane, increase of VEGF bioavailability	48
COX-2	Production of PGE2 which stimulates angiogenesis, increases cell survival, suppresses the immune response.	48, 65, 80
VEGF	Stimulation of angiogenesis, inhibition of DC and T cell development	81, 82
HMGB1	Stimulation of tumor proliferation and metastasis	83

microenvironment will shape aggressive tumors with a high metastatic potential. It is however important to note that in several reports macrophage infiltration was found to be unrelated (29) or even positively associated (30-32) with patient survival. The reason for these discrepancies is not clear; macrophages may have a different impact on tumor progression depending on their polarization, on their interaction with other infiltrating immune cells, and on the tumor stage and grade. Unfortunately, epidemiological studies have merely enumerated the number of $CD68^+$ cells within tumors and rarely looked into their function.

Animal models have shed some light on this question. Macrophage infiltration has been reported in a variety of tumor models, whether spontaneous (33, 34), syngeneic (35-38), or xenografts in immune-deficient mice (39-41). Their role in tumor progression has been determined using $CSF\text{-}1^{-/-}$ mice and macrophage-specific depletion protocols (42-45). Results are discussed in the following chapters.

3.2. Mechanisms favoring tumor progression

The impact of macrophage during the various stages of tumor progression – initiation, growth, invasion and metastasis - has been mostly studied *in vivo* in animal models. However, murine models may have a limited predictive power due to the differences between the mouse and the human immune systems, and to the fact that transplanted tumor models do not always recapitulate the normal process of tumor development. Models of spontaneous tumors are nowadays available that allow to at least address the latter concern (33, 34, 46, 47).

It is quite established that chronic inflammation, whether due to persistent viral or bacterial infection, or to genetic polymorphisms, predisposes to cancer initiation (48). Inflammation alone is however not sufficient to cause cancer, at least in animal models (49). Macrophages are key players in inflammatory processes. In a spontaneous model of mammary carcinoma, the absence of CSF-1 did not affect primary tumor incidence and growth, but it delayed tumor invasion and metastasis (43). Still, several molecules potentially produced by macrophages during chronic inflammation are known to induce DNA mutagenesis. TNF-alpha is a transforming agent for carcinogen-treated fibroblasts. It markedly stimulated transformation of BALB/3T3 cells initiated with 3-methylcholanthrene (50). Nitric oxide (NO) produced by macrophages can directly oxidize DNA, resulting in mutagenic changes. Inflammatory cytokines may also affect genome integrity via inhibition of cytochrome p450 or glutathione S-transferase isoenzymes (51, 52). There is indeed some indirect evidence that macrophages may participate in the initial phase of tumor development, i.e. appearance of preneoplastic lesions and malignant transformation (34).

Once malignant transformation has been triggered, and under the influence of cytokines and hypoxic conditions in the developing tumor, TAM secrete several mediators facilitating tumor progression (reviewed in (15, 18), summarized in Table 1). Macrophages contribute to stroma formation, angiogenesis and tumor proliferation via release of a variety of cytokines and growth factors, including TGF-beta, VEGF, EGF, PDGF, IL-8, TNF-alpha (18, 24, 53). Metalloproteinases production (e.g. MMP-2 and MMP-9) results in break-down of extracellular matrix and basement membrane, and promote tumor invasion. It also increases the bioavailability of VEGF (39, 54). Angiogenesis can also be stimulated by low concentrations of nitric oxide produced by macrophages (55, 56). Overall, these factors support tumor cell proliferation and provide access to systemic circulation thus increasing metastatic potential.

A less direct but critical mechanism by which myeloid cells facilitate tumor development is via the regulation of the anti-tumor immune response. Experiments in mice have led to the description of a population of tumor infiltrating immature myeloid cells which have been functionally labeled as myeloid suppressor cells or "myeloid-derived suppressor cells" (MDSC) (57, 58). These cells are characterized by the common ability to promote tumor growth via suppression of the local adaptive immune response. They are phenotypically heterogeneous and have been alternatively designated as TAM, inflammatory monocytes, immature

Table 2. Molecules produced by macrophages with potential inhibiting effect on tumor development

Molecule/Product	Mechanism of anti-tumor activity	References
TNF-alpha	Tumor cell apoptosis, inhibition of proliferation	89, 90, 110
TRAIL	Tumor cell apoptosis	53, 77, 91
iNOS	Production of NO with cytotoxic effect. High level of NO might be produced by murine but not human macrophages	55, 89, 111
H_2O_2	Tumor cell necrosis or apoptosis	112, 113
TGF-beta	Anti-inflammatory activity, inhibits tumor cell growth	77
IL-10	Anti-inflammatory activity, inhibits angiogenesis	77
IL-23	Enhancement of anti-tumor T cell responses	100, 101
HMGB1	Macrophage activation	92, 93
Fcgamma Receptors	ADCC, tumor cell phagocytosis in the presence of antibodies	99, 103, 104, 107, 108

dendritic cells, immature neutrophils. In mice, they generally express the CD11b and Gr-1 markers (58), although in the murine lung 3LL carcinoma they were characterized as mature $CD11b^+Gr\text{-}1^-CD68^+$ myeloid cells (59). In mouse models, MDSC are found in tumors but also in the spleen and in circulation. Although what determines their enrichment in tumor-bearing animals is not yet completely clear, the production of GM-CSF by tumor cells has been shown to increase MDSC (60).

MDSC induce T cell apoptosis or anergy by downregulation of the CD3zeta signaling chain, decreased cytokine production, inhibition of proliferation. The mechanisms implicated in these effects include:

- depletion of L-arginine by arginase and inducible nitric oxide synthase (iNOS) (59, 61, 62)

- production of RNI and peroxynitrites by iNOS (63)

- depletion of tryptophan and production of toxic metabolites by IDO (64)

- suppression of IL-12 secretion via PGE_2 (produced by COX-2 (65)) or IL-10 (38)

- induction of regulatory T cells via secretion of TGF-beta and IL-10 (66).

MDSC are not simply alternatively activated macrophages as they can simultaneously upregulate the activity of arginase and iNOS, which are generally differentially regulated in M2 and M1 macrophages (65); they also express high levels of the IFN-gamma-inducible chemokines as CXCL9 and CXCL10, thought to be characteristics of M1 macrophages (36).

Their characterization in humans has only recently initiated (19, 63, 67-70). Circulating suppressor cells have been identified as $CD14^-CD11b^+CD15^+$ in renal cell carcinoma patients (19, 68), $CD14^+$ in multiple myeloma (69), $CD34^+$ in head and neck carcinoma (67).

4. ANTI-TUMOR MACROPHAGES

4.1. Evidence for anti-tumor effect?

In some animal models, depletion of macrophages resulted in tumor regression and improved survival (42, 43). However, there is also evidence that tumor progression may worsen in the absence of macrophages. Oosterling *et al.* have reported that depletion of macrophages with clodronate liposomes in a rat model of colon cancer results in highly differentiated (that is, less aggressive) tumors, while tumors of control animals have lower differentiation, loss of basement membrane, and more vascularization; however, macrophage-depleted rats developed higher tumor loads and had poorer survival compared to non-treated counterparts (84). These tumors had also lower T cell infiltration. This study and others from the same group (45) exemplify that macrophages may also be important as effector cells in anti-cancer responses, possibly in concert with the adaptive T cell response.

The "macrophage balance" model hypothesizes that macrophages can act as anti-tumor effector cells provided that 1) they are properly activated to a *bona fide* M1 phenotype, and 2) they are present in sufficient numbers within the tumor (85). We also propose that macrophages may have a predominant anti-tumor effect during the initial phase of tumor development (41) or in minimal residual disease (including initial control of metastasis (86)), while in later stages those same cytotoxic properties may be redirected to inhibiting the newly developed adaptive anti-tumor immune response. Indeed, macrophages were originally described as anti-tumorigenic cells. In the early '80s, Fidler and al. showed that macrophages were mediating the anti-metastatic activity of muramyl dipeptide in a B16 melanoma model (87). More recently, high macrophage density in colon and non-small-cell lung cancers was found to be highly correlated with patient survival advantage (32, 88).

4.2. Direct and indirect mechanisms of anti-tumor activity

A variety of mechanisms has been implicated in the anti-tumor effector activity of macrophages (89). Some of them are summarized in Table 2. Direct tumoricidal effect can be mediated by production of cytotoxic and anti-proliferative molecules. For example, ROI and RNI can induce DNA damage as well as p53 accumulation and apoptosis (55). TNF superfamily members as TNF-alpha and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can mediate apoptosis of cells expressing the respective receptors, provided that NK-kappaB survival pathway is inhibited (53, 90, 91). Macrophages can also indirectly enhance the T cell or NK-mediated anti-tumor immune response by secreting chemokines as CCL5 and CXCL10, and cytokines as IL-12 and IL-23. Most of these mediators are normally upregulated by priming with IFN-gamma or GM-CSF, and activation by TNF-alpha or MAMP like LPS. Although the pathogen-associated molecules required for full macrophage activation may not

necessarily be present within the tumor microenvironment, tumor cells can secrete pro-inflammatory cytokines as TNF-alpha. If some extent of tumor cell death is initiated, danger signals as e.g. heat-shock proteins, uric acid, HMGB1 are released from necrotic cells and play a role in macrophage activation (92), which in turn can contribute to NK cells stimulation (93). In addition, some reports have recently suggested that macrophage cytotoxic activity could be physiologically stimulated by interaction with $CD4^+$ T cells within tissues and tumors (44, 94).

Results from *in vitro* models indicate that activated macrophages may be capable of selectively kill tumor but not normal cells (95, 96). What are the mechanisms of this specificity? Current hypothesis include the expression of the integrin LFA-1 **(**binding to ICAM-1 on tumor cells) and of phosphatidylserine receptor. Phosphatidylserine can be overexpressed on the membrane of tumor cells which could thus be cleared similarly to apoptotic cells (97). Abnormal carbohydrate structures present on tumor cells were also described as being recognized by macrophage-expressed lectins. Whether these interactions are relevant *in vivo* is still unclear.

Destruction of tumor cells by phagocytosis has been proposed as a possible consequence of these macrophage-tumor cell contacts (98). It is indeed possible to measure uptake of tumor-derived material by macrophages both *in vitro* and *in vivo* (98, 99). However, it remains problematic to demonstrate that phagocytosis is a primary mechanism of tumor killing, rather than a scavenging phenomenon secondary to tumor cell death occurring via other pathways (99).

The described mechanisms of macrophage anti-tumor activity pose some dilemmas. The first problem, which we have already partially addressed, is that most of the described mediators of cytotoxic activity, including RNI, ROI, TNF-alpha, IL-6, IL-1beta, and with the possible exception of TRAIL, can also promote tumor growth. Despite the targeting specificity observed *in vitro*, events leading to macrophage activation and release of toxic mediators are likely to have by-stander effects on a variety of cells in the tumor microenvironment, including T cells. Pro-inflammatory factors and cytokines produced by macrophages, as HMGB1 and IL-23, can stimulate the anti-tumor immune response (93, 100, 101). Paradoxically, even IFN-gamma and GM-CSF, considered respectively a strong Th1/M1 polarizing factor and an effective vaccine adjuvant, may be required for the immune suppressive function of MDSC (60, 66, 71). The outcome of this double-edged situation may be determined by a variety of factors including the tumor type, mass and stage, the type and degree of infiltration (M1 or MDSC, T cells or not), and the sheer amount of mediators produced.

The second issue is that several tumors are known to be resistant to apoptosis induced by e.g. TNF-alpha both *in vitro* and *in vivo*. Thus, the effect of some of these mediators may be selective or even induce tumor escape variants.

Finally, anti-tumor mechanisms that have been discovered in murine systems may not be relevant in humans. For example, the role of nitric oxide in the anti-tumor effect of rodent macrophages is well established but, in humans, it remains controversial (102). In addition, it is disturbing to notice that none of the macrophage cytotoxic mechanisms described as having a role *in vitro* have yet been directly and unequivocally validated *in vivo*.

There is one possible exception. A large body of evidence indicates that opsonisation of tumor cells by tumor antigen-specific antibodies can result in specific tumor killing by macrophages via secretion of lytic mediators or phagocytosis (90, 99, 103, 104). Depending on the model, all types of Fc receptors (FcgammaRI, FcgammaRII, FcgammaRIII, FcalphaRI) have been implicated in antibody-dependent cellular cytotoxicity (ADCC) *in vitro* (99, 103, 104). An essential role of FcgammaRIII in the anti-tumor response after anti-CD20 antibody treatment has also been established *in vivo*, in animal models as well as clinical trials (105, 106). The high affinity FcgammaRI is restricted to myeloid effector cells, and upregulated by IFN-gamma in M1 macrophages. FcgammaRI is very efficient at mediating tumor ADCC *in vitro*. However, since it can bind to circulating monomeric IgG, it is generally thought to be saturated and essentially inactive *in vivo*. Still, recent reports demonstrate a role of FcgammaRI expressed on monocyte/macrophages in anti-tumor effector mechanisms upon antibody treatment (107, 108). Spontaneous anti-tumor humoral responses are frequently elicited during tumor development in patients (109). Whether these antibodies have the right specificity (i.d., recognize membrane molecules), sufficient affinity, and can reach adequate local concentration to be permissive for macrophage-mediated ADCC is currently unknown.

4.3. Macrophage-based cancer therapies: adoptive immunotherapy

Based on the hypothesis that infusing large number of properly activated effector cells may be beneficial to cancer patients, several clinical trials of adoptive transfer of macrophages were conducted in the ’90. Autologous macrophages were differentiated *ex vivo* from circulating monocytes, activated (with IFN-gamma, LPS, or muramyl dipeptide), and re-infused to the patients (114-121). Different routes of injection were tested, included intravenous (120, 121), intraperitoneal (116), intrapleural (118), intravesical (119). Biodistribution studies after intravenous administration were controversial, indicating either no specific tropism for tumor tissue (121), or positive tumor homing (122). However, following regional infusion, macrophages were found localized to the sites of the tumor mass (115, 122). Macrophage administration was always well tolerated with minor side effect like low-grade fever, chills, nausea or headache. Biological activity was indicated by increased levels of IL-6, IL-8 and IL-1 following intravenous infusion (120). Evidence for tumor response could not be demonstrated in all trials. Clinical responses were observed in some cases, described below.

Metastatic ovarian cancer patients with minimal residual disease were treated intraperitoneally (locally) with IFN-gamma-activated macrophages together with the bispecific antibody MDX-H210 (anti-FcgammaRI X anti-HER2/Neu); 5 out of 14 treated patients had a complete pathological response, one had a complete clinical response. Responders were among patients who presented micrometastasis (and not macrometastasis) at treatment (116).

Superficial bladder cancer patients underwent tumor resection, then were treated intravesically (locally) with IFN-gamma-activated macrophages. The number of relapses occurring in the year following macrophage treatment was significantly lower compared to the number of relapses that had occurred in the year prior treatment. In addition, 9 out of 17 treated patients had no recurrence during the year following the first instillation (119, 123).

Thus, adoptive immunotherapy with activated macrophages is likely to best work in minimal residual disease and upon local infusion.

This approach could be improved by making the M1 polarization more stable. We recently tested the effect of macrophage transduction with an adenoviral vector coding for a constitutively active form of the interferon regulatory factor 7 (IRF-7), a transcription factor involved in the production of type I IFN (124). *In vitro*, IRF-7 macrophages demonstrated cytotoxic activity for a larger spectrum of tumor cell lines. In addition, IFN-alpha and beta are powerful adjuvants. Thus, macrophages could work as cargo cells and deliver into the tumor cytokines capable to re-polarize the local cytokine milieu and to make the microenvironment conducive to an anti-tumor immune response. *In vivo* studies will be essential to discriminate between direct effector mechanisms and indirect regulation of the local immune response.

5. PERSPECTIVES: MACROPHAGE TARGETING *IN VIVO*

Strategies aimed at targeting TAM or MDSC *in vivo* may complement the current standards of care in cancer treatment. Investigators are currently exploring potential protocols leading to either 1) specific macrophage depletion, 2) activation of macrophage effector functions, or 3) inhibition of their pro-tumoral and immune suppressive activities. Advancements in the field are hindered by the difficulty in translating to the human system experimental protocols developed in mice. In addition, effective methods of tumor targeting need to be devised.

5.1. Selective depletion of TAM

Selective depletion of TAM or MDSC has been extensively performed in mice in proof-of-principle studies which have also demonstrated their role in tumor progression. The most popular depleting approaches have been the infusion of clodronate liposomes (http://www.clodronateliposomes.org/projects/publications.asp) or anti-Gr-1 antibodies (more specific for neutrophil populations, (37)), and the back-cross with CSF-1$^{-/-}$ mice (43). Schreiber and colleagues even reported that a CTL response directed against tumor antigen-presenting TAM present in the stroma could be sufficient to induce tumor rejection (125). Most of these strategies are not easily applicable to human treatment, as they likely result in important side effects.

However, part of the effect of chemotherapy drugs approved for human care or currently in clinical trials may be mediated through their action on macrophages or other myeloid cells. The first example is gemcitabine (126). Gemcitabine was found to markedly reduce the number of CD11b^{+}Gr1^{+} MDSC in the spleen of mice bearing large syngeneic tumors, without affecting other immune cell populations, including T cells, NK cells, B cells, and macrophages. This depletion increased the efficacy of combination immunotherapy with an adenoviral vector expression IFN-beta. Whether gemcitabine also depletes myeloid suppressor cells in human patients is still unclear (127). Yondelis is a natural anti-tumor agent derived from a marine organism currently in clinical trials in ovarian cancer and sarcoma. Therapeutic concentrations of Yondelis were shown to selectively induce monocyte apoptosis and were also cytotoxic for TAM isolated from ovarian cancer patients (128). At subtoxic concentrations, Yondelis inhibited macrophage differentiation as well as secretion of IL-6 and CCL2 (but not TNF-alpha).

5.2. Activation to effector M1, repolarisation

Macrophage activation may be more effective than selective depletion. The rationale for this approach relies on the evidence that M1 macrophages produce cytotoxic mediators and/or chemokines and cytokines that may stimulate the anti-tumor immune response. Also, TAM plasticity and susceptibility to re-polarisation is a prerequisite.

Beevart *et al.* have shown that the TLR4 agonist monophosphoryl lipid A is capable to enhance the anti-tumor effect of therapeutic antibodies in a mouse model of experimental melanoma, an effect that was mediated by the activation of macrophages expressing FcgammaRI (107).

In humans, the systemic delivery of MAMPs or cytokines capable of activating macrophages is known to results in severe side effects, because of the lack of specificity and high systemic concentrations needed to achieve biological effects in the tissue of interest. However, targeted delivery is in some cases possible and already been successfully tested in patients. The preclinical and clinical development of muramyltripeptide phosphatidylethanolamine (MTP-PE) has been extensively reviewed in (129). When delivered intravenously in multilamellar liposomes, MTP-PE is rapidly taken up by macrophages in lung, liver, and spleen, which are then activated via the intracellular receptor NOD2. This biomodifier is being developed for adjuvant treatment of osteosarcoma in combination with chemotherapy. Osteosarcoma is a rare bone disease metastasizing to the lungs. In a phase III clinical study, patients treated with liposomal MTP-PE had significantly longer survival,

possibly due to lung targeting and local activation of the innate immune system by the liposomes, retarding the development of pulmonary tumor metastasis. Unfortunately, the precise mechanisms of anti-metastatic activity in patients are still unknown. In addition, this liposomal delivery is excellent but selective for lung, liver and spleen, and can hardly be generalized for targeting of macrophages in other tissues.

DMXAA (5,6-dimethylxanthenone-4-acetic acid) is a small molecule currently in clinical trials as a vascular disrupting agent which reduces tumor blood flow. Jassar and colleagues observed that treatment of tumor-bearing mice with DMXAA stimulates secretion of TNF-alpha, and possibly other chemokines and cytokines, by TAM; tumor infiltration by macrophages and $CD8^+$ T cells was increased, and tumor growth reduced in a CD8-dependent fashion (35). They suggest that DMXAA enhancement of the anti-tumor immune response is mediated by its primary action on re-polarization of TAM towards an M1 phenotype.

5.3. Inhibition of pro-tumor activities, repolarisation

Finally, it may be possible to block those functions of macrophages or MDSC that facilitate tumor progression. In advanced disease, in the presence of bulky tumors, of large macrophage infiltration, and of deeper subversion of the immune system, this approach may in fact be the only option. Indeed, activation of macrophages results in the overproduction of factors that in these conditions may not be able to control tumor growth, and will ultimately stimulate disease progression.

Anti-inflammatory and anti-angiogenesis drugs are some of the agents currently tested or used to improve survival in cancer patients. Anti-inflammatory molecules are mostly tested for cancer prevention and include non-steroidal anti-inflammatory drugs (NSAID blocking COX-1 and/or COX-2), the anti-TNF-alpha antibody infliximab, TNF receptor antagonists, histone deacetylase (HDAC) inhibitors, anti-oxidants and many others. Examples of anti-angiogenic agents are the anti-VEGF antibody bevacizumab and VEGF receptor antagonists (130). Extensive studies are being carried out with these classes of compounds, which however are probably targeting tumor cells as well as TAM.

More relevant to the current subject is the finding that the biphosphonate zoledronic acid, a standard of therapy in malignant bone disease, may exert his anti-tumor effect via inhibition of MMP9 expression by TAM (33). In a spontaneous model of cervical carcinoma, zoledronic acid blocked tumor progression by interfering with the pro-angiogenesis activity of MMP9 produced by macrophages.

Much effort is nowadays devoted to characterizing and inhibiting MDSC, i.e. those immature myeloid cells which are conditioned to interfere with the adaptive anti-tumor immune response. Two examples of compounds that may affect these populations are phosphodiesterase-5 (PDE-5) inhibitors (69) and all-trans-retinoic acid (ATRA) (131, 132).

PDE-5 inhibitors, already used in the clinics, induce the accumulation of cGMP which affects a variety of intracellular pathways. Serafini *et al.* recently reported that this class of drugs can retard syngeneic tumor growth in a CD8-dependent manner, by inhibiting both arginase and iNOS activity in $CD11b^+$ MDSC. Interesting, these authors have observed that treating PBMC of cancer patients with a PDE-5 inhibitor also restore the T cell proliferative potential which is blocked by a $CD14^+$ population of circulating MDSC. Although the use of this drug is by itself not sufficient to completely block tumor progression in mice, it could be envisioned as a complement of standard therapies or immunotherapy. Its pro-angiogenetic properties should however be better evaluated as they may counteract a potential anti-tumor effect.

In mouse models, ATRA has been shown to be able to drive the differentiation of immature MDSC into mature dendritic cells, macrophages, and neutrophils, thus eliminating their immune suppressive activities and increasing the T cell mediated anti-tumor response (131). These finding were later confirmed in patients with renal cell carcinoma (132).

In conclusion, macrophages and other myeloid cells within the tumor stroma have emerged as major players in orchestrating tumor progression. They do so by acting on the tumor cells themselves, on the tumor vasculature, or on immune cells within the cancer microenvironment. Therapeutic interventions that may boost their anti-tumor properties and inhibit their pro-tumor activities in cancer patients are still a field of intensive research.

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Abbreviations: ADCC: antibody-dependent cellular cytotoxicity, COX: cyclo-oxigenase, CSF: colony-stimulating factor, CTL: cytotoxic lymphocyte, DMXAA: 5,6-dimethylxanthenone-4-acetic acid, HMGB1: high-mobility group B1, IDO: indoleamine-pyrrole 2,3 dioxygenase, IFN: interferon, IL: interleukin, iNOS: inducible nitric oxide synthase, IRF: interferon regulatory factor, LPS: lipopolysaccharide, MDSC: myeloid-derived suppressor cells, MMP: matrix metalloproteinases, NO: nitric oxide, PDE: phosphodiesterase, PGE: prostaglandin E, TAM: tumor-associated macrophages, TGF: transforming growth factor, TLR: toll-like receptor, TNF: tumor necrosis factor, TRAIL: tumor necrosis factor-related apoptosis-inducing ligand, VEGF: vascular endothelial growth factor.

Key Words: Macrophages, Cancer, Myeloid-Derived Suppressor Cells, Immunotherapy, Tumor Microenvironment, Review

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