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Functions of Diverse Myeloid Cells in the Tumor Micro-Environment

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1. Introduction

Myeloid cells are abundant in solid tumors and early infiltrate neoplastic lesions since the first stages of tumourigenesis, usually preceding other leukocytes (e.g. lymphocytes). (Clark et al., 2007) In the last decades there has been growing evidence that infiltrating T lymphocytes (CD3+ CD8+CD45RO+) are associated with favourable prognosis in human colorectal cancer (Laghi et al., 2009; Pages et al., 2005) melanoma, ovarian and breast cancer (Clemente et al., 1996; Mahmoud et al., 2011; Vesely et al., 2011; Zhang et al., 2003) In marked contrast, cells of the innate immunity, like myeloid cells, are most frequently associated with poor clinical outcomes. A number of studies have demonstrated that tumor-associated myeloid cells (TAMCs) have the ability to support tumor cell proliferation and invasion, activate the neo-angiogenic switch, and suppress anti-tumor immune responses. (DeNardo et al., 2009; Mantovani et al., 2004a; Martinez et al., 2009; Pollard, 2004; Qian and Pollard, 2010; Talmadge et al., 2007) Thus, in a simplified scheme, adaptive immunity is usually protective and limit tumour progression, while innate immunity favours disease development. However, research in recent years have added a further level of complexity, as components of the adaptive immunity (e.g. IL-4-producing CD4 T cells and antibody-producing B cells) have been shown to activate innate immune cells in a pro-tumour manner. (DeNardo et al., 2009; Wang and Joyce, 2010) Therefore, the dynamic interplay between tumor-infiltrating cells of the innate and adaptive immunity is of paramount importance for the outcome of tumour progression or regression.

Tumor-associated myeloid cells (TAMCs) include at least four different myeloid populations **(Figure 1)**: 1) tumor-associated macrophages (TAMs), considered crucial orchestrators of cancer-related inflammation (Mantovani et al., 2008), promoting angiogenesis, immunosuppression, tissue remodelling and metastasis (Sica, 2010); 2) the angiogenic monocytes expressing the tunica internal endothelial kinase 2 (Tie2), the angiopoietin receptor, playing a key role in tumor angiogenesis (De Palma et al., 2005); 3) the Ly6G and Ly6C subsets of an heterogeneous population of immature myeloid cells, called myeloid-derived suppressor cells (MDSCs) for their ability to suppress T cells

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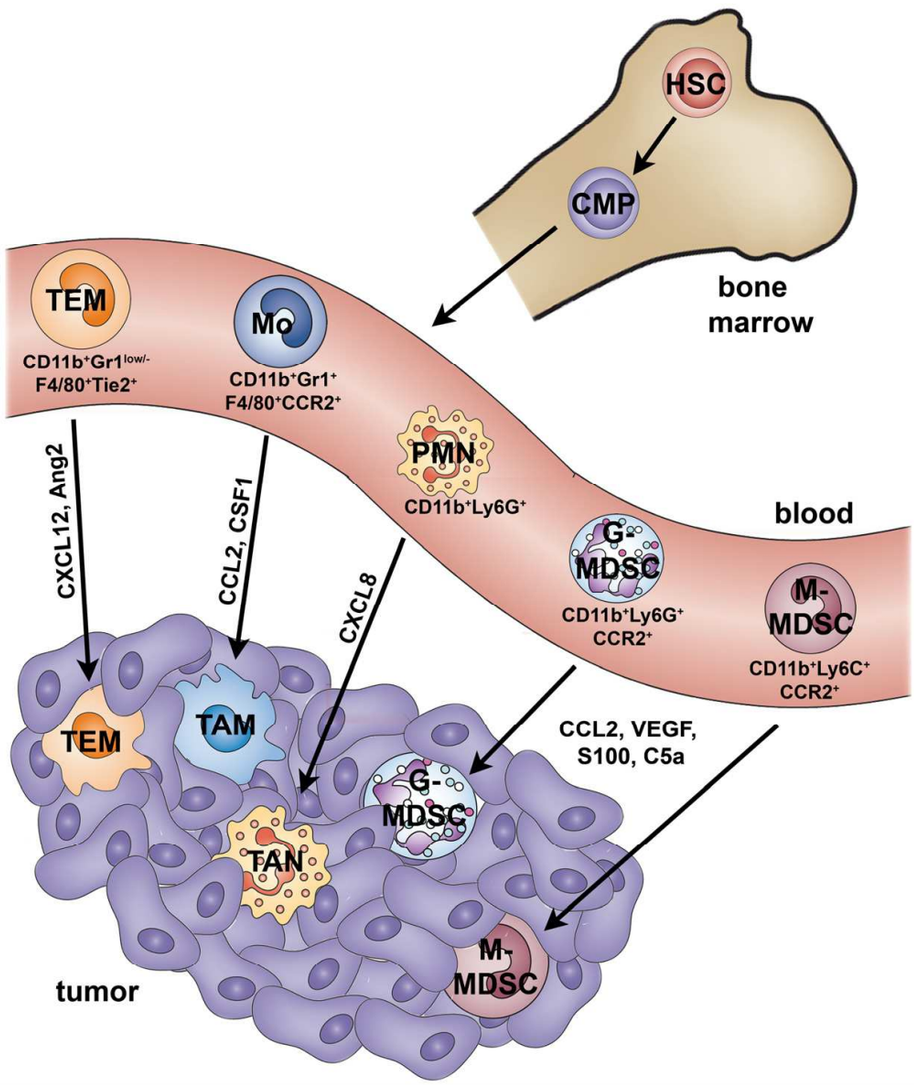


Fig. 1. **Pathways of differentiation and accumulation of TAMCs.** In the bone marrow hematopoietic stem cell (HSC) differentiate into common myeloid progenitors (CMPs), which can subsequently differentiate into different subsets of circulating myeloid cells: monocytes (Mo), Tie2-expressing monocytes (TEM), neutrophils (PMN), and granulocytic and monocytic myeloid-suppressor cells (G-MDSC and M-MDSC). Tumors secrete factors which sustain myelopoiesis, and promote both the recruitment and pro-tumor differentiation of circulating myeloid cells. TAMs are recruited into the tumor site by chemotactic factors (eg. CCL2, CSF-1) and represent the prominent phagocytes population orchestrating cancer-related inflammation. TEMs derive from circulating $Tie2^+$ monocytes and are recruited in tumors by hypoxia-inducible chemoattractants, such as Ang2 and CXCL12. Tumor-associated neutrophils (TANs) stem from circulating neutrophils and are recruited in tumors by chemokines (e.g. CXCL8). TANs participate in tumor promotion by the expression of crucial pro-angiogenic factors. During tumour progression an heterogeneous population of myeloid cells (G-MDSC and M-MDSC) accumulate in blood and lymphoid organs. MDSCs may be recruited by selected chemoattractants (CCL2, S-100, VEGF, C5a) into the tumor microenvironment, where they contribute to suppression of the adaptive immunity.

functions, which accumulate mainly in blood and lymphoid organs during tumor progression, but may also be recruited to the tumor site (Sica and Bronte, 2007); 4) tumor-associated neutrophils (TANs) that, despite their short half-life, have been recently proven to participate in tumor promotion by the expression of crucial pro-angiogenic factors (Fridlender et al., 2009).

TAMCs originate in the bone marrow where hematopoietic stem cells (HSCs) differentiate into common myeloid precursors (CMPs), which subsequently give rise to different subsets of circulating cells: immature myeloid cells (IMCs) that can be further subdivided in a granulocytic ($CD11b^+/Ly6G^+$) and a monocytic ($CD11b^+/Ly6C^+$) subpopulation, monocytes ($CD11b^+/Gr1^+/F4/80^+/CCR2^+$), Tie2-expressing monocytes ($CD11b^+/Gr1^{low/-}/Tie2^+$) and neutrophils ($CD11b^+Ly6G^+$) (Mantovani et al., 2009). Tumors secrete factors which sustain myelopoiesis, promote the recruitment of circulating cells into the tumor mass, and orientate their functional differentiation to their own advantage (Mantovani et al., 2009; Sica and Bronte, 2007). In addition, Dendritic cells (DCs) also belong to the family of myeloid cells stemming from CMPs. Cells with dendritic characteristics are scarcely present in neoplastic tissues (Murdoch et al., 2008). Tumor-associated DCs generally show an immature phenotype and are poor inducers of effective responses to tumor antigens. The properties of these cells have been extensively reviewed elsewhere (Ma et al., 2011; Palucka et al., 2010) and are not discussed here.

2. Pro-tumour functions of tumor-associated myeloid cells

2.1 Tumor-associated macrophages

TAMs derive from circulating monocytes which are recruited at tumor sites by a number of diverse chemoattractants secreted by tumour and stromal cells. For instance the chemokine CCL2 was discovered as a tumour-derived factor inducing chemotaxis in monocytes.(Bottazzi et al., 1983; Zachariae et al., 1990) Other chemokines these include : CCL3, CCL4, CCL5, CXCL12 (Balkwill, 2004; Konishi et al., 1996; Schioppa et al., 2003). Non-chemokine chemotactic factors are also important, for instance: urokinase plasminogen activator (uPa) (Zhang et al., 2011), M-CSF, TGFβ; fibroblast growth factor, FGF; vascular endothelial growth factor, VEGF) (Joyce and Pollard, 2009; Lin et al., 2002; Sica and Bronte, 2007) and antimicrobial peptides (β-defensin-3, BD-3) (Jin et al., 2010). Many of these molecules correlate with TAMs infiltration in different types of tumor, while other (eg. uPa, BD-3) are specifically associated with certain types of cancer, prostate and gastric cancer respectively (Jin et al., 2010; Zhang et al., 2011).

Once in tumours, monocytes differentiate to macrophages, primarily because of the presence of M-CSF produced by tumour cells, and polarize to tumour-educated macrophages by exposure to the local milieu rich in immune-suppressive mediators such as IL-10, TGFβ and VEGF.

Macrophages are versatile cells that are capable of displaying different functional activities, some of which are antagonistic: they can be immuno-stimulatory or immune suppressive, and either promote or restrain inflammation. (Auffray et al., 2009; Gordon and Taylor, 2005; Hamilton, 2008; Mantovani et al., 2004b; Martinez et al., 2009) Macrophage heterogeneity has been simplified in the macrophage polarization concept where the two extreme phenotypes, the M1 and M2 macrophages, have distinct features. (Allavena et al., 2008; Goerdt and

Orfanos, 1999; Gordon and Taylor, 2005; Mantovani et al., 1992; Pollard, 2009; Stein et al., 1992) M1 or classically–activated macrophages are stimulated by bacterial products and Th1 cytokines (e.g. IFNγ); they are potent effectors that produce inflammatory and immuno-stimulating cytokines to elicit the adaptive immune response, secrete reactive oxygen species (ROS) and nitrogen intermediates and may have cytotoxic activity to transformed cells. M2 or alternatively activated macrophages differentiate in micro-environments rich in Th2 cytokines (e.g. IL-4, IL-13); they have high scavenging activity, produce several growth factors that activate the process of tissue repair and suppress adaptive immune responses. (Gordon and Martinez, 2010; Mantovani et al., 2005; Qian and Pollard, 2010)

While this M1 vs M2 dual subsets simplification offers a mechanistic model of the functional polarization of macrophages, tissue microenvironments are likely to elicit simultaneous activation of different signalling pathways with opposite influence on macrophage functions, contributing to the extensive heterogeneity in patterns of gene expression seen in macrophages (Gratchev et al., 2008; Murray and Wynn, 2011; Ravasi et al., 2002; Riches, 1995; Stout et al., 2005; Tannenbaum et al., 1988). This in vivo functional skewing of myeloid populations is an emerging paradigm of tumor-mediated immunosuppression, where myeloid cell plasticity plays as a double-edged sword (Mantovani and Sica, 2010; Sica and Bronte, 2007). In early phases, high production of M1 inflammatory mediators (e.g. tumor necrosis factor, TNF; reactive oxygen species, ROS) appears to support neoplastic transformation (Sica and Bronte, 2007), whereas in established cancers the expression of M2-like phenotypes with immunosuppressive, pro-angiogenic and tissue remodelling activities promotes immune escape, tumor growth and malignancy (Dinapoli et al., 1996; Mantovani and Sica, 2010; Movahedi et al., 2010; Pollard, 2004; Saccani et al., 2006; Sica and Bronte, 2007; Sica et al., 2008; Sica et al., 2000).

In molecular profiling studies, murine TAMs from fibrosarcoma showed several features of M2 macrophages: arginase-I, YM1, FIZZ1, MGL2, VEGF, osteopontin and MMPs, as well as an immunosuppressive phenotype : high IL-10, TGFβ and low IL-12, RNI and MHC II, which correlate functionally to reduced cytotoxicity and antigen-presenting capacity. (Biswas et al., 2006; Hagemann et al., 2009; Ojalvo et al., 2010) Similar findings were found in human TAMs from ovarian cancer patients.(Allavena et al., 2010) We compared the expression of upregulated genes in human TAMs with the profiling of in vitro-polarized M1 and M2 macrophages. Several genes (e.g. osteopontin, fibronectin, scavenger and mannose receptors) were similarly upregulated in TAMs and in M2 macrophages. By the Principal Component Analysis, the global profiling of TAMs fell much closer to that of M2-polarized macrophages. (Solinas et al., 2010)

However, TAMs heterogeneity is starting to emerge, likely depending on the tumour type and micro-environmental cues. (Lewis and Pollard, 2006; Movahedi et al., 2010) Notably, murine TAMs showed also the expression of typical M1 factors such as IFN-inducible chemokines (CCL5, CXCL9, CXCL10, CXCL16). (Biswas et al., 2006; Stout and Suttles, 2005)

TAMs influence fundamental aspects of tumour biology, as shown in **figure 2**. Among the well documented pro-tumour functions of TAMs is the production of trophic and activating factors for tumour and stromal cells (e.g.EGF, FGF, VEGF, PDGF, TGFβ). These growth factors directly promote the proliferation of tumour cells and increase the resistance to apoptotic stimuli (Ingman et al., 2006; Kalluri and Zeisberg, 2006; Mantovani et al., 2008; Moussai et al., 2011) The cytokine IL-6, released by TAMs, is important to sustain the

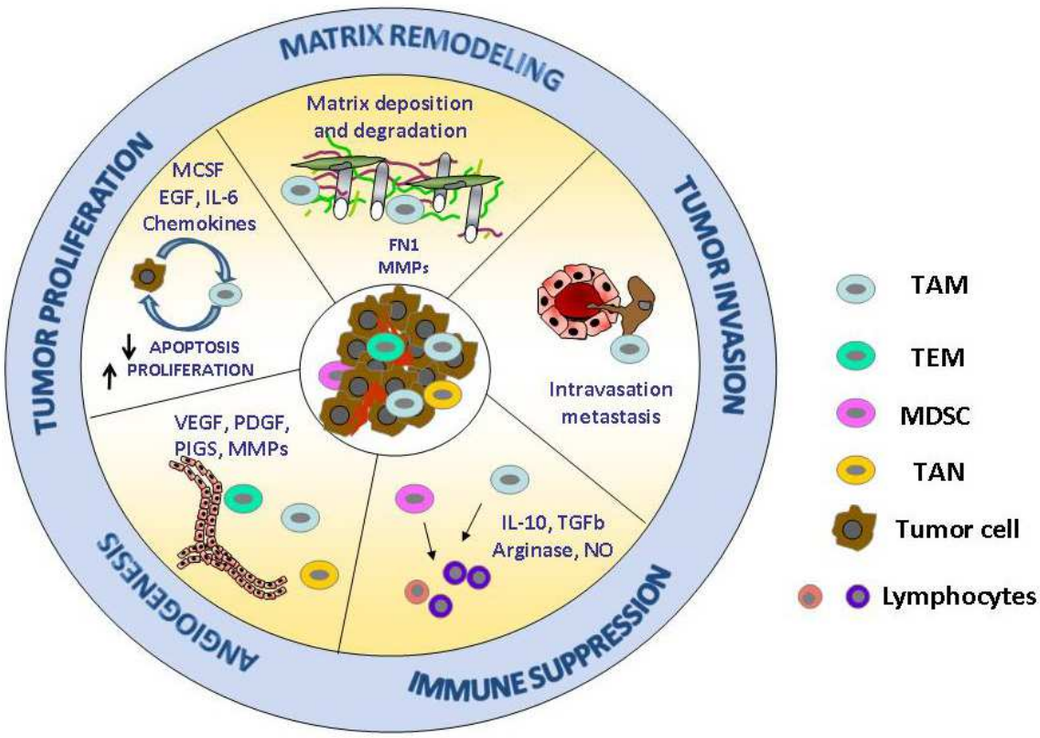


Fig. 2. **Pro-tumour functions of Tumour-Associated Myeloid Cells (TAMCs)**. Different types of TAMCs promote the progression of tumors. TAMs rescue neoplastic cells from apoptotic stimuli and stimulate their proliferation, by producing several growth factors and cytokines (e.g.EGF, IL-6). TAMs, TEMs and TAN activate angiogenesis, via VEGF, MMPs and other angiogenic factors. TAMs have an intense proteolityic activity and degrade the extra-cellular matrix, but also produce matrix proteins, such fibronectin (FN1). TAMs favour tumour cell intravasation and dissemination to distant sites. TAMs and MDSC induce immune suppression by producing suppressive mediators such as IL-10 and TGFβ, arginase 1 and nitric oxide (NO).

survival and proliferation of malignant cells in tumours of epithelial and hematopoietic origin. (Bollrath et al., 2009; Fukuda et al., 2011; Grivennikov et al., 2009; Lesina et al., ; Ribatti and Vacca, 2009) TAMs are also a major source of proteolytic enzymes that degrade the ECM, thus favouring the release of matrix-bound growth factors. (Joyce and Pollard, 2009; Mantovani et al., 2008)

TAMs a key effectors of the “angiogenic switch" where the balance between pro- and anti-angiogenic factors, commonly present in tissues, tilts towards a pro-angiogenic outcome. (Baeriswyl and Christofori, 2009; Du et al., 2008; Murdoch et al., 2008; Zumsteg et al., 2009) In hypoxic conditions the transcription factor HIF-1alpha induces in TAMs the production of VEGF and of the angiogenic chemokine CXCL8. (Lewis et al., 2000)

TAMs are probably the most active contributors to the incessant matrix remodelling present within tumours, as they produce several MMPs and other proteolytic enzymes. (Mason and Joyce, 2011) Tumour cells exploit the ECM degradation mediated by TAMs to invade locally, penetrate into vessels and disseminate to give distant metastasis. (Wyckoff et al.,

2007) TAMs aiding cancer cell invasion have been directly visualized in experimental tumours in vivo by multiphoton microscopy: by using fluorescently labelled cells Wyckoff and colleagues showed that tumour cell intravasation occurs next to perivascular macrophages in mammary tumours. (Pollard, 2008; Wyckoff et al., 2007) Further, it has been recently shown that cathepsin protease activity, by IL-4-stimulated TAMs, promotes tumour invasion.(Gocheva et al., 2010) IL-4 is produced by tumour-infiltrating CD4 T cells and there is mounting evidence of its relevance in the polarization of macrophages with pro-tumour functions. (DeNardo et al., 2009; Wang and Joyce, 2010) The chemokine CCL18 produced by TAMs has been recently shown to play a critical role in promoting breast cancer invasiveness by activating tumour cell adherence to ECM. (Chen et al., 2011)

We recently found that human TAMs and in vitro tumour-conditioned macrophages express high levels of the Migration Stimulation Factor (MSF), (Solinas et al., 2010) a truncated isoform of Fibronectin. (Schor et al., 2003) Macrophage-secreted MSF displays potent chemotactic activity to tumour cells in vitro,(Solinas et al., 2010) confirming that the pro-invasive phenotype of cancer cells is modulated by macrophage products released in the tumour-micro-environment.

Further support to the concept of a reciprocal interaction between tumour cells and TAMs was provided by a recent paper where SNAIL-expressing keratinocytes became locally invasive after macrophage recruitment elicited by M-CSF. (Du et al., 2010)

In line with the above experimental evidence, high numbers of infiltrating TAMs have been significantly associated with advanced tumours and poor patient prognosis, in the majority of human tumours.(Bingle et al., 2002; Mantovani et al., 2008; Pollard, 2004; Qian and Pollard, 2010) There are, however, notable exceptions to this pro-tumour phenotype, probably dictated by their functional polarization. One such exception is human colorectal cancer, where some studies reported that TAMs density is associated with better prognosis.(Forssell et al., 2007; Ohno et al., 2003; Sconocchia et al., 2011) The localization of TAMs within colorectal cancers appears of primary importance: the number of peritumoural macrophages with high expression of costimulatory molecules (CD80 and CD86), but not of those within the cancer stroma, was associated with improved disease-free survival.(Ohtani et al., 1997; Sugita et al., 2002)

Specific TAMs subsets identified by surface markers may have predictive values: in lung adenocarcinoma, the number of TAMs CD204+ (scavenger receptor) showed a strong association with poor outcome while the total CD68+ population did not. (Ohtaki et al., 2010)

Macrophage-related gene signatures have been identified in human tumours such as ovarian and breast cancer, soft tissue sarcoma and follicular B lymphoma; (Beck et al., 2009; Finak et al., 2008; Ghassabeh et al., 2006; Lenz et al., 2008) in classic Hodgkin's lymphoma, tumours with increased number of CD68+ TAMs were significantly associated with shortened progression-free survival. (Steidl et al., 2010)

Recent addition to the molecular repertoire of TAMs includes semaphorin 4D (Sema4D) (Sierra et al., 2008) and growth arrest-specific 6 (Gas6) (Loges et al., 2010), which are respectively involved in promoting tumor angiogenesis and cancer cell proliferation.

2.2 Tie2-expressing onocytes/macrophages (TEMs)

Tie2-expressing monocytes/macrophages (TEMs) are a small subset of myeloid cells characterized by the expression of the angiopoietin receptor Tie2 and powerful pro-angiogenic activity (De Palma and Naldini, 2009; De Palma et al., 2005; Murdoch et al., 2007; Venneri et al., 2007). They derive from circulating Tie2-expressing monocytes which are recruited in tumors by hypoxia-induced endothelial-derived chemotactic factors, such as Ang-2 and CXCL12 (Coffelt et al., 2011; Murdoch et al., 2007; Venneri et al., 2007; Welford et al., 2011b) The CXCL12-CXCR4 axis is a well known circuit driving accumulation of TAMs in hypoxic areas of solid tumors (Schioppa et al., 2003). In addition, it has been demonstrated that pharmacological inhibition of CXCR4 is associated with a significant reduction of TEM recruitment into mammary tumors (Welford et al., 2011b). Both ablation and adoptive transfer studies have demonstrated that TEMs are crucial promoters of tumor angiogenesis (De Palma et al., 2005; De Palma et al., 2003; Venneri et al., 2007). In two models of mammary tumours and orthotopic human gliomas, Ganciclovir-driven ablation of $Tie2^+$ monocytes induced a significant reduction of both tumour mass and vasculature, demonstrating their importance in tumour angiogenesis and growth (De Palma et al., 2005; De Palma et al., 2003; Venneri et al., 2007). In line, adoptive transfer studies demonstrated that subcutaneous co-injection of tumor cells with TEMs increases tumor vascularization (De Palma et al., 2005).

Strikingly, gene expression analysis highlighted that TEMs are highly related to TAMs, but express a more pronounced M2-skewed gene signature, with higher expression of M2 genes, including arginase 1 (Arg1), scavenger receptors (CD163; Mannose receptor 1, Mrc1; Macrophage scavenger receptor 2, Msr2; stabilin-1) and lower levels of pro-inflammatory molecules (IL-1β; prostaglandin endoperoxide synthase 2/cyclooxygenase 2, PTGS2/COX2; IL-12; TNF; inducible nitric oxide synthase, iNOS; CCL5; CXCL10; CXCL11) (Pucci et al., 2009). These results suggested that $Tie2^+$ monocytes could be a distinct lineage of myeloid cells, committed to execute physiologic pro-angiogenic and tissue-remodeling programs, which can be co-opted by tumors (Andreu et al., 2010). Noteworthy, human $Tie2^+$ circulating monocytes express high levels of pro-angiogenic genes (e.g. VEGF-A; Matrix metallopeptidase 9, MMP9; COX2; wingless-related MMTV integration site 5A, WNT5A) and are powerful inducers of endothelial cells activation (Coffelt et al., 2010). In agreement, sub-cutaneous tumors growing in Ang-2-overexpressing mice showed increased number of TEMs associated with enhanced microvessels density (Coffelt et al., 2010). Tie2 engagement by Ang-2 in both mouse and human TEMs not only elicits a chemotactic response but also enhances their pro-tumoral activities (Coffelt et al., 2010). It was also recently demonstrated that Ang-2 levels in 4T1 mammary tumors correlates with both TEM-derived IL-10 and Treg infiltration, resulting in suppression of T cells proliferation (Coffelt et al., 2011). In contrast, Ang-2 inhibited the expression of M1 cytokines (IL-12 and TNFα) in TEMs exposed to hypoxia (Murdoch et al., 2007).

2.3 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs represent an heterogenous population of cells whose common characteristics are an immature state and the ability to suppress T-cell responses both *in vitro* and *in vivo* (Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009).

MDSC recruitment and expansion are regulated by several cytokines, chemokines and transcription factors (Sica and Bronte, 2007). It has been demonstrated that among chemokine receptors, CCR2 plays a pivotal role in the recruitment and turnover of MDSC to the tumour site (Sawanobori et al., 2008). Furthermore, the C5a complement component, which interacts with a G protein-coupled receptor, has been shown to play a role in MDSC recruitment and activation in a cervix cancer model (Markiewski et al., 2008). Some factors which are found in the tumour microenvironment, such as pro-inflammatory S-100 proteins, are also crucial for MDSC recruitment. Sinha and co-workers demonstrated that MDSCs can produce S-100 proteins by themselves, providing evidence for an autocrine loop that promotes MDSC recruitment (Cheng et al., 2008; Sinha et al., 2008).

MDSCs possess several mechanisms for immune suppression: 1) depletion of arginine, mediated by Arg1 and iNOS; 2) production of ROS; 3) post-translational modifications of T cell receptor (TCR) mediated by peroxynitrite generation; 4) depletion of cysteine; 5) production of TGFβ; 6) induction of Tregs (Bronte et al., 2005; Huang et al., 2006; Movahedi et al., 2008; Nagaraj et al., 2007; Srivastava et al., 2010; Terabe et al., 2003; Yang et al., 2006; Youn et al., 2008). In healthy individuals, IMCs differentiate in mature granulocytes, macrophages or dendritic cells, whereas in pathological conditions they expand into MDSCs. MDSCs have been observed in cancer, chronic infectious diseases, and autoimmunity. In tumor-bearing mice, MDSCs accumulate within primary and metastatic tumors, in the bone marrow, spleen and peripheral blood. In cancer patients, MDSCs have been identified in the blood.

Recent studies have contributed to partially clarify the biology of MDSCs. In mice, two major subsets were identified on the basis of their morphology and the expression of Ly6 family glycoproteins: monocytic MDSCs (M-MDSCs) and granulocytic MDSCs (G-MDSCs). M-MDSCs are $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{high}$ cells with monocyte-like morphology, while G-MDSCs are $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$ with granulocyte-like morphology (Ostrand-Rosenberg and Sinha, 2009). Cells with similar phenotype, precursors of myeloid cells, are present in physiological conditions, but they are devoid of immunosuppressive activity. These cells, therefore, should not be named MDSCs (Youn and Gabrilovich, 2010). Other markers of MDSC subsets are: IL-4Rα (CD124), F4/80, CD80, and CSF-1R (CD115) (Sica and Bronte, 2007). The characterization of MDSCs deeply suffers from the lack of specific markers. However, recent characterizations have identified human MDSCs as $CD34^{+}$ $CD33^{+}$ $CD11b^{+}$ HLA-DR^{-} cells (Ostrand-Rosenberg and Sinha, 2009). The ability to differentiate into mature DCs and macrophages *in vitro* has been shown to be restricted to M-MDSCs (Youn et al., 2008). M-MDSC-mediated immune suppression does not require cell-cell contact, but utilizes up-regulation of iNOS and Arg1, as well as production of immunosuppressive cytokines (Gabrilovich and Nagaraj, 2009). On the contrary, G-MDSCs suppress antigen-specific responses using mechanisms, including the release of ROS, that require prolonged cell-cell contact between MDSC and T cell (Gabrilovich and Nagaraj, 2009). The C5a subunit of the complement system appears a key regulator of MDSC functions, by modulating their migration and ROS production (Markiewski et al., 2008).

Several factors produced by tumors have been implicated in the differentiation of MDSCs, including granulocyte monocytes-colony stimulating factor (GM-CSF), macrophage-monocytes-colony stimulating factor (M-CSF), IL-6, IL-1β, VEGF and PGE2 (Gabrilovich and Nagaraj, 2009; Marigo et al., 2010). The transcription factor CCAT/enhancer binding protein

β (C/EBPβ) proved to be the key player in the process of MDSC development (Marigo et al., 2010). It has been proposed that two signals are needed for the expansion and function of MDSCs: one factor (e.g. GM-CSF) prevents the differentiation in mature myeloid cells, and a second signal, provided by pro-inflammatory molecules such as IFNγ, activate MDSCs (Condamine and Gabrilovich, 2011).

A remarkable relation exists between MDSCs and TAMs. MDSCs are able to skew TAMs differentiation toward a tumor-promoting type-2 phenotype (Sinha et al., 2007). The cross-talk between MDSCs and macrophages requires cell-cell contact, then MDSCs release IL-10 to reduce IL-12 production by macrophages. MDSCs from an IL-1β enriched tumor microenvironment produce more IL-10 and are more potent down-regulators of macrophage-released IL-12 (Bunt et al., 2009). Circulating MDSCs can differentiate into $Gr1^{-}$ $F4/80^{+}$ TAMs in the tumor site (Kusmartsev and Gabrilovich, 2005) and this conversion is driven by tumor hypoxia (Corzo et al., 2010).

Because of their tumor-promoting activities, MDSCs are associated with type-2 immune responses, however accumulating evidence shows that MDSCs have characteristics of both M1 and M2 macrophages (Sica and Bronte, 2007). As an example, MDSCs express both Arg1 and iNOS, where these enzyme are differentially expressed by M1 (iNOS) and M2 (Arg1) macrophages. A recent study, investigating the molecular mechanisms behind MDSC differentiation, demonstrated an essential role of paired-immunoglobulin receptors (PIRs) in the differentiation of M1 or M2 MDSCs (Ma et al., 2011). The balance between PIR-A and PIR-B modulates MDSC polarization. In support of this, growth of Lewis lung carcinoma was significantly retarded in PIR-B-deficient mice (*Lilrb3*-/-) and PIR-B-deficient M-MDSCs expressed high levels of the M1 molecules iNOS.

MDSCs contribute to tumor growth also by non-immune mechanisms, including the promotion of angiogenesis. MDSCs isolated from murine tumors express high levels of metalloproteases, including MMP9 (Murdoch et al., 2008). MMP9 increases the bioavailability of VEGF sequestered in the extracellular matrix. Further in the tumor microenvironment and in proangiogenic culture conditions, MDSCs acquire endothelial markers such as CD31 and VEGF receptor 2 (VEGFR2) and the ability to directly incorporate into tumor endothelium (Yang et al., 2004). In agreement, tumor refractoriness to anti-VEGF therapy was shown to be mediated by $CD11b^{+}GR1^{+}$ myeloid cells (Shojaei et al., 2007a; Shojaei et al., 2007b).

2.4 Tumor-Associated Neutrophils (TANs)

Tumor-associated neutrophils (TANs) have received little interest by immunologists, also based on their short life span. However, new evidence contradicts this view, in that cytokines like IL-1 or microenvironment conditions such as hypoxia can prolong PMN survival (Sica et al., 2011). TANs are present in various tumors, including kidney, breast, colon, and lung (Houghton, 2010), and are recruited by locally secreted chemotactic factors. As an example, several carcinoma cells produce CXCL8, a prototypic chemoattractant for neutrophils (Bellocq et al., 1998). Furthermore, tumor-derived TGFβ promotes neutrophils migration both directly and indirectly, by regulating the expression of adhesion molecules in the endothelium (Flavell et al., 2010).

Neutrophils are able to produce various cytokines and chemokines that can influence not only immune and antimicrobial responses, but other processes such as hematopoiesis, wound healing, and angiogenesis (Cassatella et al., 2009; Mantovani, 2009; Piccard et al., 2011; Zhang et al., 2009). Despite little attention has been paid to TANs, clinical evidence indicates that their presence is a negative prognostic indicator. A correlation between TANs infiltrate and poor outcome has been described in renal cell carcinoma, bronchoalveolar cell carcinoma, and breast cancer (Jensen et al., 2009; Yang et al., 2005). In agreement, preclinical studies experimenting PMN depletion confirmed the detrimental nature of TANs (Pekarek et al., 1995; Tazawa et al., 2003).

Neutrophils contribute to tumor growth by promoting angiogenesis, cell proliferation, and metastasis (Houghton, 2010). Similarly to macrophages, a recent report described the functional plasticity of neutrophils (Fridlender et al., 2009). The authors investigated the effects of SM16, a TGFβ receptor kinase antagonist in murine lung cancer and mesothelioma models using syngeneic tumor xenografts and the orthotopic LSL-K-ras tumor model. Depletion of neutrophils by a specific anti-Ly6G antibody resulted in a significantly reduced effect of SM16, suggesting that neutrophils participate to the antitumor activity of TGFβ blockade, most likely by the production of oxygen radicals. Also, depletion of neutrophils affected the activation of $CD8^+$ CTLs. Fridlender and colleagues propose a new paradigm in which resident TANs acquire a protumor phenotype, largely driven by TGFβ, to become "N2 neutrophils". If TGFβ is blocked, neutrophils acquire an antitumor phenotype to become "N1 neutrophils" (Fridlender et al., 2009).

It was suggested that N1- and N2-type neutrophils are cells with a different degree of activation (i.e. fully activated or weakly activated neutrophils, respectively) rather than two alternatively activated cell subtypes (Gregory and Houghton, 2011). It is also object of debate the existence of two distinct populations, namely N2-polarized TANs and granulocytic MDSCs, that seem to overlap for many characteristics. In the absence of specific markers, it cannot be determined if N2 neutrophils within the tumors are granulocytic MDSCs recruited from the spleen or whether they are blood-derived neutrophils converted to an N2 phenotype by the tumor microenvironment. In support to the existence of N2-polarized TANs, Fridlender et al. emphasize that TGFβ-blockade does not alter blood neutrophils, splenic myeloid cells ($CD11b^+$), or splenic MDSCs, selectively acting on the intratumor activation of neutrophils. Also, TANs characterized in Fridlender's study have clear features of mature neutrophils, while MDSCs mostly exhibit an immature morphology (Mantovani, 2009).

3. Therapeutic approaches targeting TAMCs

The frequent association of TAMCs with poor prognosis makes these cells reasonable targets of biological anti-cancer therapies. Further, in the last few years there has been increasing evidence that TAMCs are strongly implicated in the failure of conventional chemotherapy and anti-angiogenic therapy.(Ferrara, 2010; Welford et al., 2011a) Accumulation of myeloid CD11b+Gr1+ cells (including TAMs, MDSC and immature cells) in tumours renders them refractory to angiogenic blockade by VEGF antibodies. (Shojaei and Ferrara, 2008) This effect was traced to a VEGF-independent pathway driven by the G-CSF-induced protein Bv8. (Shojaei et al., 2007b) Further, pharmacological inhibition of TEMs in tumour-bearing mice markedly increased the efficacy of therapeutic treatment with a vascular-disrupting agent.

3.1 TAMs and TEMs

Elimination of TAMs at tumor sites, or inhibition of their survival could result in improved prognosis. Earlier and more recent studies of macrophage depletion in experimental settings have been successful to limit tumour growth and metastatic spread (Aharinejad et al., 2009; Lin et al., 2001; Mantovani et al., 1992), and to achieve better therapeutic responses (De Palma et al., 2007; Ferrara, 2010; Gabrilovich and Nagaraj, 2009; Marigo et al., 2008; Welford et al., 2011a)

A number of studies have shown that the bisphosphonate clodronate encapsulated in liposomes is an efficient reagent for the depletion of macrophages in vivo. Clodronate-depletion of TAMs in tumour-bearing mice resulted in reduced angiogenesis and decreased tumour growth and metastatization.(Brown and Holen, 2009; Zeisberger et al., 2006) Moreover, the combination of clodronate with sorafenib, an available inhibitor of tyrosine protein kinases (e.g,VEGFR and PDGFR), significantly increased the efficacy of sorafenib alone in a xenograft model of hepatocellular carcinoma. In clinical practice, bisphosphonates are employed to treat osteoporosis; current applications in cancer therapy include their use to treat skeletal metastases in Multiple Myeloma, prostate and breast cancer. Treatment with zoledronic acid was associated with a significant reduction of skeletal-related events and, possibly, direct apoptotic effects in tumour cells. (Martin et al., 2010; Morgan et al., ; Zhang et al., 2010)

Our group reported that the anti-tumour agent of marine origin, Trabectedin (Yondelis), was unexpectedly found to be highly cytotoxic to mononuclear phagocytes, including TAMs. This cytotoxic effect is remarkably selective, as neutrophils and lymphocytes were not affected. (Allavena et al., 2005; D'Incalci and Galmarini, 2010)

A second approach is to inhibit the recruitment of circulating monocytes in tumour tissues.

The M-CSF receptor (M-CSFR) is exclusively expressed by monocytes-macrophages. In patients with advanced tumours, clinical studies are under way to check the feasibility and possibly clinical efficacy of inhibitors to the CSF-1R. Among the many chemokines expressed in the tumour micro-environment, CCL2 (or Monocyte Chemotactic Protein-1) occupies a prominent role and has been selected for therapeutic purposes. Pre-clinical studies have shown that anti-CCL2 antibodies or antagonists to its receptor CCR2, given in combination with chemotherapy, were able to induce tumour regression and yielded to improved survival in prostate mouse cancer models (Li et al., 2009; Loberg et al., 2007; Popivanova et al., 2009)

In the opposite direction, another approach is to exploit the tumor-homing ability of TAMCs: after all, they are at the right place at the right time. Indeed, delivery of cytokines and cytotoxic proteins to tumors by means of gene modified cells represents a promising strategy to treat cancer. It was recently shown that TEMs could be used to deliver interferon-alpha (IFNα), a potent cytokine with angiostatic and antiproliferative activity (De Palma et al., 2008), thanks to the preferential homing of TEMs to the tumors (De Palma and Naldini, 2009).

A fourth and more recent approach is to 're-educate' TAMs to exert anti-tumour responses protective for the host, ideally by using factors able to revert TAMs into M1-macrophages, with potential anti-tumour activity. It is becoming accepted that macrophages are flexible and able to switch from one polarization state to the other. (Pelegrin and Surprenant, 2009)

This was achieved in experimental mouse tumours, by injecting the TLR9 agonist CpG-oligodeoxynucleotide (CpG-ODN), coupled with anti-IL-10 receptor.(Guiducci et al., 2005) or the chemokine CCL16 (Cappello et al., 2004). CpG-ODN synergized also with an agonist anti-CD40 mAb to revert TAMs displaying anti-tumour activity. (Buhtoiarov et al., 2011) A remarkable anti-tumour effect of re-directed macrophages has been recently reported in human pancreatic cancer with the use of agonist anti-CD40 mAb. (Beatty et al., 2011) Still in the same direction, a recent report showed that the plasma protein histidine-rich glycoprotein (HRG) known for its inhibitory effects on angiogenesis (Juarez et al., 2002; Olsson et al., 2004) is able to skew TAMs polarization into M1-like phenotype by down-regulation of the placental growth factor (PlGF), a member of the VEGF family. In mice, HRG promoted anti-tumour immune responses and normalization of the vessel network. (Rolny et al., 2011)

Direct activation with IFNγ, a prototypical M1-polarizing cytokine, has been shown to re-educate TAMs (Duluc et al., 2009) and there is evidence for antitumor activity of this molecule in minimal residual disease (Mantovani and Sica, 2010). Inhibition of STAT3 activity, required for IL-10 biological functions and gene transcription, restored production of pro-inflammatory mediators (IL-12 and TNF-α) by infiltrating leukocytes and promoted tumour inhibition (Kortylewski et al., 2005). Recent results suggest that SHIP1 functions in vivo to repress M2 macrophage skewing. Consistent with this, Ship1−/− mice display enhanced tumor implant growth (Rauh et al., 2005). In agreement, inhibition of the M2 polarizing p50 NF-κB activity resulted in restoration of M1 inflammation and tumor inhibition in different cancer mouse models (fibrosarcoma, melanoma)(Saccani A. et al Cancer Res 2006) (Porta et al., 2009)

3.2 MDSC

The translational potential of MDSC research is dual. The immunosuppressive activity of MDSCs could be exploited to inhibit immune responses in autoimmune diseases and organ transplantation. Conversely, elimination of MDSCs could be essential in cancer patients undergoing active (vaccination) or passive (adoptive transfer of *ex-vivo* expanded anti-tumor T cells) immunotherapy. A possible approach to contrast MDSC pro-tumoral activities consists in the promotion of MDSC differentiation into mature cells devoid of suppressive activity. Vitamin A represents an interesting candidate to restore immunosurveillance. In fact, Vitamin A metabolites stimulate the differentiation of myeloid progenitor cells into DCs and macrophages and reduce MDSC accumulation (Gabrilovich et al., 2001; Kusmartsev et al., 2003). A clinical trial testing the effects of all-*trans*-retinoic acid (ATRA) in patients with metastatic renal cell carcinoma showed the efficacy of this compound in reducing MDSCs in peripheral blood. The decrease in MDSC number correlated with improved-antigen-specific T cell responses (Mirza et al., 2006). It has been reported that some chemotherapeutic drugs, such as gemcitabine, are able to eliminate MDSCs, without affecting T cells, B cells, NK cells, and macrophages (Ko et al., 2007; Suzuki et al., 2005). Another strategy is aimed to inhibit MDSC suppressive function. Compounds under investigation for this ability belong to COX2 inhibitors, phosphodiesterase 5 (PDE5) inhibitors, and NO-releasing non-steroidal anti-inflammatory drugs (NSAIDs) (Gabrilovich and Nagaraj, 2009). Preclinical evidence supports the use of IL-1 antagonists in treating

human metastatic disease. Blocking IL-1 activity, mainly IL-1β, reduces both metastasis and tumor growth (Dinarello, 2010). Recently, it was shown that the effect is also mediated by the decrease of MDSC accumulation and suppressive activity (Ostrand-Rosenberg and Sinha, 2009). It has also been reported that $CD11b^+$ $Gr1^+$ cells enhance tumor refractoriness to anti-VEGF antibody (bevacizumab) treatment (Shojaei et al., 2007a). In this situation, MDSCs release the pro-angiogenic protein Bv8 that surrogates VEGF in the stimulation of tumor angiogenesis (Shojaei et al., 2007b). Because Bv8 is also important in MDSC mobilization and homing to the tumor site, this is an interesting candidate for cancer therapy.

3.3 TANs

TAN depletion represents a potential therapeutic approach for cancer cure (Tazzyman et al., 2009). However, since oncologic patients are already immunocompromized individuals, a complete ablation of neutrophils is not desirable. Alternatively, given that activated neutrophils can kill tumor cells through the release of toxic substances, it would be of interest to modulate TAN phenotype, with a switch from N2- towards N1-polarization. Nevertheless, this plan would lead to the generation of highly cytotoxic cells and could result in excessive tissue damage, potentially lethal. A more manageable therapeutic strategy can target neutrophils recruitment to tumors. Inhibition of CXCR2-mediated PMN chemotaxis with a specific antibody or a CXCR2 antagonist has been successfully tested in pre-clinical experimentation (Gregory and Houghton, 2011). The description of the pivotal role of TGFβ in the promotion of a protumor phenotype of TAN suggests that therapies contrasting this cytokine could contribute to re-educate neutrophils in the tumor microenvironment (Flavell et al., 2010). Interestingly, a recent study showed that the CCL2-driven accumulation of TAMs limits the influx of neutrophils in solid tumors by a yet unidentified mechanism. If TAMs accumulation is suppressed, neutrophils are recruited to the tumor providing a secondary source of MMP-9. Therefore, in the absence of TAMs, TANs provide alternative paracrine support for tumor angiogenesis and progression (Pahler et al., 2008). Hence, the elimination of TAMs alone may be insufficient to eradicate myeloid cell support to tumor growth .

4. Conclusions

Recent results indicate that tumour development promotes expansion and functional skewing of different myeloid cell populations, leading to accumulation of protumoral TAMC populations, which include TAMs, TEMs, MDSCs and TANs. These myeloid cell populations display distinct specialized functions, as well as overlapping activities (eg. angiogenesis). It is becoming evident that TAMCs appear to constitute a robust pro-tumour system and the functional elimination of a single myeloid population may be insufficient to eradicate their support to tumor growth. New strategies able to target different myeloid cell populations, simultaneously, are therefore desirable.

New evidence indicates that pathways promoting polarized functions of either macrophages (eg. M1 vs M2) or neutrophils (N1 vs N2) may share common constituents. (Mantovani, 2009) Understanding of this convergent pathways may offer common target/s and strategies to therapeutically affect the pro-tumoral networks established by TAMCs.

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Tumor microenvironment represents an extremely dynamic niche shaped by the interplay of different cell types (e.g. tumor cells, stromal cells), their soluble products (e.g.cytokines, chemokines and growth factors) and varied physico-chemical conditions (e.g low oxygen concentration or hypoxia). Recent studies have identified myelomonocytic cells as key players in regulating the tumor microenvironment and hence, tumor progression in a variety of cancers. In view of these findings, the present book attemps to provide a comprehensive account of the diversity of tumor microenvironment across different cancers and how myelomonocytic cells have taken the center-stage in regulating this niche to direct cancer progression. A better understanding of the myelomonocytic cells and the mechanisms by which they regulate cancer progression will open new vistas in cancer therapeutics.

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