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Stromal-dependent tumor promotion by MIF family members

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Abstract

Solid tumors are composed of a heterogeneous population of cells that interact with each other and with soluble and insoluble factors that, when combined, strongly influence the relative proliferation, differentiation, motility, matrix remodeling, metabolism and microvessel density of malignant lesions. One family of soluble factors that is becoming increasingly associated with pro-tumoral phenotypes within tumor microenvironments is that of the migration inhibitory factor family which includes its namesake, MIF, and its only known family member, D-dopachrome tautomerase (D-DT). This review seeks to highlight our current understanding of the relative contributions of a variety of immune and non-immune tumor stromal cell populations and, within those contexts, will summarize the literature associated with MIF and/or D-DT.

Keywords

AMPK; CAF; NK cell; T lymphocyte; MIF; D-DT; Hypoxia; HIF-1 α ; Monocyte; Macrophage; TAM; MDSC; CTL; MSC; Stroma; Cancer; Solid tumor; Tumor microenvironment

1. Introduction

Intercellular communication between malignant cells and stromal cells in tumor microenvironments is essential for maintaining neovascularization, stromal remodeling, immune evasion and metabolic adaptation. Despite significant advances in our understanding of stromal cell contributions - and the factors involved - to solid tumor progression, there is still much to be learned regarding individual stromal cell phenotypes, effectors and, perhaps more importantly, how to therapeutically target each one. Potentially complicating these efforts is the fact that each stromal cell type may be represented by several independent subtypes due to differences in activation, differentiation and/or reversible polarization. For example, late stage tumors may contain up to five different monocytic lineage subpopulations: M2 tumor-associated macrophages (TAMs), M1 TAMs, Tie-2-expressing TAMs, myeloid-derived suppressor cells (MDSCs) and bone marrow-derived monocytic cells (BMDCs).

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Both tumor cell-derived and stromal cell-derived factors dictate stromal cell mobilization, recruitment, differentiation and polarization. One of these effectors, macrophage migration inhibitory factor (MIF), has been centrally implicated as a tumor cell- and stromal cell-derived mediator of stromal cell recruitment, polarization and differentiation. Unlike prototypical cytokines and chemokines, MIF does not contain a secretion signal peptide sequence and is non-classically secreted through an ABCA1 transporter mechanism [1]. Three-dimensional X-ray crystallographic studies reveal that human MIF exists as a homotrimer and is structurally related to the bacterial enzymes 4-oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase [2, 3]. MIF possesses the unusual ability to catalyze the tautomerization of the non-physiological substrates D-dopachrome and L-dopachrome methyl ester into their corresponding indole derivatives [4]. More recently, phenylpyruvic acid, p-hydroxyphenylpyruvic acid (HPP), 3,4-dihydroxyphenylaminechrome, and norepinephrinechrome also have been found to be MIF substrates [5]. However, high Michaelis constant (K_m) values suggest that these also are unlikely natural substrates for MIF [5, 6]. The N-terminal proline of MIF (Pro-1) appears to be a critical residue for tautomerase enzymatic activity, as site-directed mutagenesis that substitutes a serine for this proline (P1S) is devoid of D-dopachrome tautomerase activity [7]. Similarly, a proline to glycine (P1G) MIF mutant is also catalytically null for both D-dopachrome and HPP tautomerase activities [8, 9]. Interestingly, a knock-in mouse expressing this catalytically inactive MIF exhibits a phenotype that is intermediate between mice with MIF wildtype alleles and those that are genetically deficient in MIF [10].

Despite being one of the oldest cytokine activities ever identified [11, 12], a primary cell surface receptor for MIF was not identified until more than 35 years after MIF's initial characterization [13]. Using expression cloning and functional analyses, CD74 was identified by the Bucala group as a high affinity cell surface binding protein for MIF and was found to be responsible for extracellular MIF-dependent activation of the Erk1/2 MAP kinase cascade, cell proliferation and prostaglandin E₂ (PGE₂) production. CD74 is a Type II integral transmembrane protein that is expressed on monocytes/macrophages, B cells and mesenchymal, epithelial and endothelial cells. In antigen presenting cells, CD74 functions as the invariant chain of the MHC class II receptor and serves to ferry class II proteins from the endoplasmic reticulum to the Golgi [14]. Extracellular MIF binds CD74's extracellular, C-terminal domain which initiates CD74 signaling by intramembrane cleavage, co-activating CD44 [13, 15, 16] or co-activating chemokine receptors CXCR2, CXCR4 and/or CXCR7 [17–20].

The purpose of this review is to discuss in detail the array of stromal phenotypic contributions made by MIF and MIF's only other known family member, D-dopachrome tautomerase (D-DT), within the tumor microenvironment which broadly serves to facilitate solid tumor progression.

2. D-DT

Several studies indicate that gene targeting, immunoneutralization or small molecule antagonism of MIF generally phenocopies loss or inhibition of CD74 but the effect is ~ 2-fold more pronounced in CD74 targeted cells [17, 21, 22]. These seemingly incongruous

observations have now been rectified by studies that identified D-dopachrome tautomerase (D-DT) – the only known homolog of MIF – as cooperatively signaling with MIF in a CD74-dependent manner [21, 23–25]. Although human D-DT shares only 34% amino acid identity with human MIF, X-ray crystallography reveals that D-DT also exists as a homotrimer and retains significant structural conservation with MIF, especially in their substrate binding pockets [2, 21]. As its name implies, D-DT also catalyzes a tautomerization of D-dopachrome but, unlike MIF, D-DT also decarboxylates D-dopachrome to give a final product of 5,6-dihydroxyindole [26].

Like MIF and CD74 [27, 28], D-DT is highly expressed in a number of human cancers [23–25]. MIF and D-DT overexpression allows for cooperative, additive and compensatory signaling in a CD74-dependent manner. The first example of MIF and D-DT cooperative signaling came from an investigation of MIF family members in human non-small cell lung carcinoma (NSCLC) [23]. Both MIF and D-DT were found to be necessary for maximal expression and activity of pro-angiogenic growth factors in human lung adenocarcinoma cell lines. Importantly, MIF and D-DT were able to fully compensate for each other in providing maximal signaling – in a CD74-dependent manner – to CXCL8 and VEGF expression [23]. More recently, MIF and D-DT were found to cooperatively antagonize the lung adenocarcinoma tumor suppressor pathways associated with activated AMP-activated protein kinase (AMPK) and tumor suppressor p53 [29, 30]. In the case of AMPK maintenance, MIF and D-DT additively signal through CD74 to maintain glycolytic flux resulting in efficient steady state ATP generation, NADPH reduction, ensuing control of oxidative stress that cumulatively serves to reduce steady state AMPK activation [29]. Importantly, D-DT, like MIF, is a well-documented regulator of AMPK activity in a variety of cell types [31–33]. The central importance of AMPK in dictating balances between energy homeostasis and inflammation [34] may provide an important clue as to potential effectors of MIF and/or D-DT-dependent monocyte/macrophage stromal contributions to tumorigenesis [35] (to be discussed below).

3. Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) within the tumor stroma have long been known to promote several different aspects of tumorigenesis. CAFs are distinguished from normal tissue mesenchymal cells by an activated phenotype that is acquired due to tumor-derived soluble factors such as TGF- β and/or PDGF [36]. These activated CAFs, which exhibit traits similar to those of myofibroblasts [37], are responsible for producing chemokines that can directly promote tumor cell proliferation as well as recruit endothelial cell progenitors into the tumor stroma which then leads to enhanced neovascularization processes [37]. In addition to chemokines, activated stromal fibroblasts produce copious amounts of both extracellular matrices as well as matrix metalloproteases that, combined, broadly serve to remodel tumor/matrix interactions and allow for metastatic cell egress from primary lesions [38, 39]. Towards this, several reports indicate a significant potential for both autocrine and paracrine-derived MIF in mesenchymal cell activation and the upregulation of matrix metalloproteases (MMPs) 1, 2 and 3 from synovial fibroblasts [40, 41]. Additionally, both extracellular and autocrine-derived MIF promote ERK MAP kinase, Rho GTPase,

arachidonic acid metabolism, cell cycle progression and oncogene-induced malignant transformation in fibroblasts [42–45].

Hypoxia is a critically important byproduct and determinant of tumor stromal microenvironments generally [46] and CAFs specifically [47, 48]. Arising as a consequence of rapid tumor growth in the absence of accompanying increased blood supply, low oxygen tension: 1) promotes incomplete vascular formation leading to intermittent hypoxia that further exacerbates the cycle, 2) decreases the effectiveness of redox-requiring chemotherapeutics [49], 3) stabilizes hypoxia-inducible factor-1 alpha (HIF-1 α) and/or HIF-2 α (HIF-2 α) transcription factors in malignant tumor cells that, in turn, increases the expression of gene products that promote stemness [50, 51], anaerobic metabolism [52] and anti-oxidant defense [53] and, 4) initiates differentiation/polarization of both circulating and intratumoral pro-tumorigenic monocytic cell types [54, 55].

Arguably, one of the most compelling functional roles for MIF in fibroblast-associated phenotypes is its unique ability to counter-regulate hypoxia-induced cell senescence [56]. Welford and colleagues identified that MIF is a direct hypoxia-inducible factor-1 α (HIF-1 α) transcriptional target and MIF up-regulation by HIF-1 α in fibroblasts serves to prevent hypoxia-induced cell senescence. MIF-deficiency was found to phenocopy, almost exactly, HIF-1 α -deficiency in the aberrant induction of cell senescence induced by low oxygen tensions. It is tempting to speculate, as the authors of this study do, that HIF-dependent MIF transcription prevents hypoxia-induced senescence by inhibiting tumor-suppressor p53 [56]. This potential mechanism for MIF-dependent senescence evasion is both feasible and likely as p53 is a necessary determinant of hypoxia-induced senescence and/or apoptosis [57, 58]. This, coupled with the fact that MIF functionally inhibits p53 in a variety of cell types, including mesenchymal, monocytic and lung adenocarcinoma cells [30, 59, 60], provides compelling evidence that MIF provides important survival/activation signals to cancer-associated fibroblasts in hypoxic tumor microenvironments.

Recent studies demonstrate that like MIF, D-DT is also transcriptionally regulated by hypoxia-induced HIF-1 α [25] and, intriguingly, MIF family members promote HIF-1 α stability and/or mTOR-dependent translation [29, 61–63]. These findings suggest an intriguing and potentially very important paradigm for MIF family members in amplifying CAF-associated hypoxic signaling nodes in solid tumor microenvironments. Given the importance of hypoxia-induced HIF in CAF-dependent neovascularization [48], ECM deposition [64] and, intriguingly, lactate generation and secretion that provides aerobic respiration substrates to surrounding tumor cells [65], this MIF/HIF amplification loop likely represents a centrally important determinant of CAF-dependent tumor progression.

All things combined, it is becoming increasingly evident that both tumor cell-derived *and* CAF-derived MIF family members serve to promote stromal fibroblast activation, survival and associated tumor progression within solid tumor microenvironments. That being said, a recent study identified an unexpected *anti*-tumorigenic function for tumor-derived MIF in rhabdomyosarcoma (RMS)-mediated CAF recruitment [20]. Tarnowski and colleagues discovered that MIF can initiate signal transduction through the chemokine receptor CXCR7 and that MIF-deficient RMS cells transplanted into immune-deficient mice develop larger

tumors than MIF-competent RMS cells. MIF-deficient RMS tumors paradoxically displayed reduced neovascularization but had dramatically higher numbers of CAFs within the tumor stroma [20]. While there was no determination as to how much, if any, direct tumor support the increased numbers of CAFs provided to the MIF-deficient RMS tumors, it is likely that these CAFs were at least partially responsible for the observed increased tumor burden. However, several questions remain unresolved – not the least of which is whether the CAFs present in MIF-deficient tumors are as functionally active as those found in MIF-competent tumors and what is the mechanism by which tumor-derived MIF acts to impede CAF intratumoral accumulation. An even more pressing question is whether loss or inhibition of MIF in CAFs versus loss or inhibition in tumor cells would provide an alternative phenotype. This will be especially important in any rigorous examination of relative MIF contributions to CAFs going forward as loss of *stromal* MIF has been observed to play a dominant phenotypic role in promoting stromal-dependent tumor progression when compared directly to loss of tumor-derived MIF [35, 66].

4. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are pluripotent cells that that can be induced to differentiate into a variety of cell types. These cell types can be of either mesenchymal or non-mesenchymal lineages and include: adipocytes, osteoblasts, chondrocytes, tenocytes, myocytes, neurons and endothelial cells [67]. Mesenchymal stem cells have been found in the tumor stroma of: melanoma, colorectal carcinoma, pancreatic ductal adenocarcinoma, lung adenocarcinoma and glioblastoma, to name a few [67]. MSCs are highly tropic towards tumor microenvironments, in part because bone marrow-derived MSCs migrate towards gradients of cytokines and/or chemokines that are expressed and released by inflammatory cells during wound repair processes [68]. Once in the tumor stroma, MSCs can differentiate into a variety of tumor-supportive cell types including pericytes - which serve as endothelial progenitor cells – and cancer-associated fibroblasts (described above). In addition to the contributions to tumor-associated angiogenesis and stromal remodeling stemming from these MSC differentiated cell types, MSCs may directly promote metastases of adjacent tumor cells within the stroma. A landmark study from the Weinberg lab demonstrated that bone-marrow-derived MSCs (BM-MSCs), co-implanted with weakly metastatic mammary adenocarcinoma cells results in a profound increase in metastatic potential and tumor aggressiveness [69]. MSC-secreted CCL5 paracrine activation of surrounding mammary adenocarcinoma cells was found to be responsible for the bulk of the metastatic induction by MSC. These findings highlight the potential importance of MSC-derived cytokines and chemokines in malignant disease progression. Intriguingly, MIF is consistently one of the highest expressed cytokines/chemokines found in human bone marrow-, cord blood- and placental-derived MSCs [70] and hypoxia induces MIF expression and secretion beyond its already high steady state levels [71]. Hypoxia-induced MIF reportedly provides similar evasion from cell senescence in MSCs [71] as that which is observed in fibroblasts [56] although it utilizes an Akt-dependent pro-survival pathway to accomplish this as opposed to an inhibitory effect on tumor suppressor p53 [56].

In separate studies, extracellular MIF or mAb-mediated CD74 activation serves to inhibit the motility of MSCs consistent with MIF's original activity as a "migration inhibitory factor"

[72, 73]. Although it's not clear what functional contribution, if any, MIF provides to MSC-mediated tumor progression, one could speculate that tumor- and/or MSC-derived MIF may actively promote MSC survival while antagonizing MSC motility out of the tumor microenvironment. Beyond that, it is tempting to speculate that MIF – and/or D-DT – may provide some functional contribution(s) to MSC differentiation processes in normal and/or malignant disease processes. Given that MIF has been found to contribute to differentiation processes in other cell types [74–76] and, in fact, regulates the expression of MSC lineage specifying transcription factors Oct3/4 and Sox2 in MSCs [71], it is not unlikely that MIF family members may participate in MSC differentiation. It should be noted that MIF participates in both epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) [77, 78] – two differentiation-like processes that serve to coordinate metastatic dissemination and distal secondary tumor growth, respectively [79]. Despite the significant, albeit largely anecdotal, evidence to support a role for MIF and/or D-DT in MSC-dependent malignant disease progression, a great deal of study is still needed to clarify whether: 1) MSC vs. tumor-derived MIF/D-DT provide functional contributions to disease progression, 2) whether MIF/D-DT participate in MSC differentiation processes and, if so, which ones, 3) whether MIF/D-DT are functionally involved in hypoxia/HIF-dependent MSC stromal processes, and 4) if therapeutic targeting of MSC-associated MIF and/or D-DT provides clinically efficacious responses.

5. Myeloid-derived Suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells with suppressive properties that preferentially expand in cancer. MDSCs suppress T-cell proliferation and cytotoxicity, inhibit NK cell activation, and induce the differentiation and expansion of regulatory T cells (Treg). There is also evidence that this cell subset is involved in an array of non-immunological functions, such as promotion of angiogenesis, tumor invasion and metastases [80, 81]. MDSCs accumulate in peripheral blood, lymphoid tissues as well as draining tumor sites of cancer-bearing hosts [82].

Mouse MDSCs are characterized by the expression of Gr-1 and CD11b. CD11b⁺Gr-1⁺ cells represent approximately 2 to 4% of all nucleated splenocytes, but can increase to up to 50% in tumor-bearing mice [83, 84]. These cells are a mixture of immature myeloid cells, immature granulocytes, monocytes-macrophages, dendritic cells (DCs) and myeloid progenitor cells. Murine MDSCs are further subdivided into two major groups: CD11b⁺Gr-1^{high} granulocytic MDSC (also identified as CD11b⁺Ly6-G⁺/Ly6C^{low} MDSC) and CD11b⁺Gr-1^{low} monocytic MDSC (also identified as CD11b⁺Ly6-G⁻/Ly6C⁺MDSC) [84]. Recently, murine MDSCs have been further sub-divided into 5 different classes dependent on the relative expression of CD11b and Gr-1 [85].

Murine MDSCs suppress T cell responses by multiple mechanisms. L-arginine represents an important molecule central to the immune suppressive function of mouse MDSCs. L-arginine serves as a substrate for both inducible nitric oxide synthase (iNOS) and Arginase-1 (ARG1), both of which are highly expressed in MDSCs isolated from tumor-bearing mice. While iNOS utilizes L-arginine as a substrate for nitric oxide (NO) generation, L-arginine catabolic pathways serve to suppress T cell function in several ways [86]. For example,

depletion of L-arginine (and L-cysteine, in some cases) causes the downregulation of the ζ -chain in the T cell receptor (TCR) complex resulting in proliferative arrest of antigen-activated T cells [87]. Reactive oxygen species (ROS) represent another T cell suppressive mechanism. Nitric oxide, superoxide and peroxynitrite – formed from the cooperative activities of iNOS, NADPH oxidase and ARG1 overexpressed in MDSCs – prevent lymphocyte responses in several ways including via T cell receptor and CD8 nitrosylation [88].

Using an implantable syngeneic mouse metastatic breast cancer model, Simpson *et al.* showed that tumors derived from MIF shRNA-expressing 4T1 cells contain significantly fewer monocytic MDSCs than control tumors [89]. Importantly, reconstitution of MIF-depleted cells with wild-type MIF restores MDSC tumor infiltration and increases the metastatic potential of the tumors. In contrast, a tautomerase-inactive MIF variant fails to reconstitute MDSC tumor infiltration and metastatic tumor burden. Based on these findings, the authors conclude that the tautomerase activity of tumor-derived MIF is important for its effects on MDSCs and tumor metastasis [89].

We recently identified that splenic MDSCs isolated from melanoma-bearing MIF-deficient mice are less immunosuppressive than those isolated from MIF wildtype mice and these phenotypes correspond to significantly reduced primary and metastatic melanoma growth and progression [35]. Importantly, 4-iodo-6-phenylpyrimidine (4-IPP – our previously discovered small molecule MIF tautomerase inhibitor) fully recapitulates MIF-deficiency *in vitro* and *in vivo* and serves to attenuate MDSC immunosuppression and melanoma disease progression in mice [35]. Our current efforts are focused on delineating the molecular mechanisms driving the MDSC-dependent tumor-promoting effects of MIF. Recent studies demonstrate, for example, that *in vitro* differentiated bone marrow-derived MDSCs require MIF for maximal ARG1 expression and MIF-deficient bone marrow-derived monocytic MDSCs possess reduced MDSC immunosuppressive activity (*Unpublished Results*).

The importance of a hypoxic tumor microenvironment in dictating the differentiation and functional properties of tumor-infiltrating MDSCs has recently been established [55]. Hypoxia-induced HIF-1 α dramatically alters the function of MDSC in the tumor microenvironment and serves to redirect MDSC differentiation toward tumor-associated macrophages (TAMs), providing a mechanistic link between different myeloid suppressive cells in the tumor stroma [55]. Functionally, hypoxia-induced HIF1- α in MDSC promotes T lymphocyte immune suppression via ARG1 and iNOS transcription [55]. One critical question that remains unanswered is what role – if any – does MIF play in influencing the hypoxia/HIF1- α -dependent MDSC differentiation/function [56].

In contrast to murine MDSCs, which are well defined, human MDSCs are inadequately characterized. The best marker for human MDSCs remains their suppressor function, which can be either direct or indirect through the induction of Treg. Human MDSCs are defined as cells that express the common myeloid markers such as CD14⁺, CD11b⁺ and CD33⁺, but are usually negative/low for HLA-DR and lack expression of lineage specific antigens (Lin) such as CD3, CD57, CD19. Monocytic MDSCs are usually characterized by HLA-DR^{low/-}, CD11b⁺, CD33⁺ and CD14⁺ phenotype in humans (represented by CD11b⁺Ly6-G⁻/Ly6C⁺

in mice) whereas mature monocytes express high HLA-DR. Human granulocytic MDSCs are generally characterized by HLADR^{low/-}, CD11b⁺, CD33⁺, and CD15⁺ phenotype in humans (CD11b⁺Ly6-G⁺/Ly6C^{low} in mice). Monocytic or granulocytic MDSCs are present in patients with melanoma [90], multiple myeloma [91], hepatocarcinoma [92], NSCLC [93], renal cell carcinoma [94], prostate cancer [95] among others. Despite ample evidence supporting the superior immune suppressive activity of tumor-infiltrating MDSCs in murine tumor models [96], most human studies of MDSCs have focused on peripheral blood. The accumulation of MDSCs in the peripheral blood correlates with tumor burden, stage and grade in a variety of cancers. For example, among stage IV solid-tumor patients, those with extensive metastatic tumor burden have the highest percent and absolute number of MDSCs [97].

The molecular mechanisms governing the immune-regulatory role of human tumor-infiltrating and circulating myeloid cells are largely unexplored. Human MDSCs in some cancers are shown to have elevated arginase activity, which is associated with a decreased CD3 ζ chain expression on T cells [98, 99]. In addition to impairing T cell proliferation in response to TCR triggering, MDSCs can impair the migratory properties of activated T lymphocytes, as reported in patients with head and neck, lung and urinary cancers [100]. Depletion of L-arginine and L-cysteine, increased nitric oxide, superoxide, peroxynitrates and a variety of cytokines have been shown to mediate human MDSC T cell-suppressive function. In head and neck squamous cell carcinoma patients, both tumor infiltrating and circulating MDSC-suppressive activity is associated with activated STAT3-mediated events [101]. Mao *et al.* have shown that monocytic MDSCs from melanoma patients suppress autologous T cell proliferation via COX-2/PGE₂ production. In fact, PGE₂ is sufficient to induce monocytes to independently suppress proliferation and IFN- γ production in autologous T cells *ex vivo* [102].

In melanoma circulating MDSCs expressing myeloid markers are quantitatively predominant, while granulocytic MDSCs are rarely detected [103]. In an attempt to assess if MIF participates in human melanoma-induced MDSC differentiation and/or immune suppression, we studied the CD14⁺CD11b⁺HLA-DR^{low/-} monocytic MDSCs – a population that is significantly expanded in the periphery in all advanced melanoma patients [90]. Our findings indicate that small molecule MIF inhibitors dramatically attenuate the suppressive properties of circulating CD14⁺HLADR^{low/-} MDSCs isolated from late stage metastatic melanoma patients confirming MIF as a critical mediator of MDSC-dependent immune suppression in patients with advanced stage melanoma (*Manuscript in preparation*).

An accumulation of phenotypic MDSCs is associated with the decreased number of DCs in the peripheral blood of patients with head and neck, lung, or breast cancer [104]. In functional testing, MDSCs isolated from peripheral blood of HLA-A2-positive cancer patients inhibit production of IFN- γ by CD8⁺ T cells re-stimulated with specific peptide-pulsed DCs [105]. Thus, accumulation of MDSCs could be one of the mechanisms by which a growing tumor may induce antigen-specific CD8⁺ T-cell unresponsiveness. It seems logical that elimination of these immune suppressive cells may help to enhance the anti-tumor immune mechanisms in patients. A promising, clinically relevant approach to reduce

the proportion of MDSCs in tumor-bearing hosts may be the use of agents that promote the differentiation of MDSC into DC. Retinoic acids, ligands of the retinoic acid receptors [RAR; retinoid X receptor (RXR)], are among the compounds that have been shown to stimulate differentiation of myeloid progenitors into myeloid DCs [106, 107]. *In vivo*, parenteral or oral administration of *all-trans* retinoic acid (ATRA) significantly reduces the presence of MDSCs, and effectively serves to differentiate MDSCs *in vivo* into CD11c⁺MHC class II⁺ myeloid DCs, macrophages, and granulocytes [108]. A reduction in the number of Lin[−] HLA-DR[−] CD33⁺ cells accompanied by an improvement of tetanus toxoid-specific T-cell response is observed in metastatic renal cell carcinoma patients treated with ATRA [109]. A randomized phase II trial is currently testing whether ATRA can enhance the efficacy of DC-based vaccine in patients with late-stage small cell lung cancer (NCT00618891).

Initial studies performed by our group using human melanoma MDSC models lend strong support to the rationale that therapeutic inhibition of MIF in human melanoma MDSCs may represent a clinically viable approach to enhancing anti-tumor T cell immunity. Two questions that are currently being explored are: 1) how to use MIF antagonists therapeutically to eliminate MDSCs in cancer patients, and 2) whether MIF inhibition can be used to effectively induce differentiation of MDSCs into DCs with concomitant improvement in myeloid/lymphoid DC ratio, DC function, and antigen-specific T-cell-mediated immune responses in cancer patients.

6. Tumor-associated macrophages

Solid tumors have long been known to be infiltrated by inflammatory leukocytes, and evidence clearly demonstrates a strong correlation between increased numbers of tumor-infiltrating macrophages and poor prognosis in a variety of human malignancies [110–112]. As such, both the recruitment and activation of tumor-associated macrophage (TAMs) are regarded as pivotal to solid tumor progression, and are therefore considered critically important targets for therapeutic intervention. TAMs are derived from circulating monocytes that are recruited to tumors by chemotactic factors such as CCL2, CCL5, CCL7, CCL8, and CXCL12. Cytokines, including VEGF, platelet-derived growth factor (PDGF), and IL-10, are also reported to promote macrophage recruitment [110, 113]. Additionally, several lines of evidence indicate that some proportion of circulating MDSCs is recruited into the tumor stroma where they can differentiate into mature TAMs [114, 115]. Once incorporated into the tumor stroma, TAMs secrete a variety of paracrine acting factors that functionally promote tumor-associated angiogenesis, tumor cell division, metastatic dissemination, immunosuppression, matrix deposition and matrix remodeling.

Macrophages can be activated by a variety of stimuli and polarized to functionally different phenotypes. Two distinct subsets of macrophages have been proposed, including classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages express pro-inflammatory cytokines, chemokines, and effector molecules, such as IL-12, IL-23, TNF- α , iNOS, IFN- γ , IL-1 β , IRF5, and MHC class I/II [110, 116, 117]. In contrast, M2, alternatively activated macrophages express a wide array of anti-inflammatory molecules including: IL-10, TGF- β , Fizz1, Mrc2 and ARG-1 [111, 118]. In most solid cancers,

infiltrated macrophages are polarized into an M2 phenotype that functionally provides an immunosuppressive, pro-angiogenic, pro-metastatic tumor microenvironment [112, 116, 119, 120]. M2 TAMs promote intratumoral neoangiogenesis through the coordinated expression of VEGF, CCL2, FGF2, CXCL8, CXCL1, and CXCL2 [110, 121–123]. TAM-derived proteases, such as matrix metalloproteases (MMP-2 and MMP-9), plasmin, and urokinase plasminogen activator promote matrix remodeling, tumor metastatic dissemination and colonization [124, 125]. TAM-derived cytokines and proteases, such as TGF- β , IL-10, and ARG 1, induce antigen-specific lymphocyte non-responsiveness [110, 117, 126, 127] and skew T cell responses from a pro-tumoral, Th1 phenotype, to an anti-tumoral, Th2 phenotype, through the production of CCL17 [110], CCL18 [128], and CCL22 [129].

Intratumoral TAMs can be re-educated to potentiate anti-tumor immunity by various immune-regulatory cues [130–132]. This has spurred significant interest in developing therapies aimed at skewing TAMs from a pro-tumoral M2 phenotype towards an anti-tumoral M1-like phenotype [133]. However, to date, very few target molecules have been identified that can orchestrate this process *and* be therapeutically targeted. Studies performed by our group indicate that stromal macrophage-derived MIF (as opposed to tumor cell-derived MIF) polarizes TAMs towards an M2 phenotype that – in turn – promotes an immunosuppressive, pro-angiogenic microenvironment within malignant melanoma lesions [35]. Implantation of high MIF-expressing melanoma cell lines into syngeneic MIF-deficient mice results in significant reductions in both subcutaneous melanoma outgrowth and metastatic melanoma lung colonization compared to MIF wildtype mice. Corresponding with the reduced melanoma disease phenotypes in MIF-deficient mice is an attenuation of TAM alternative activation markers and immunosuppressive activities. Moreover, MIF-deficient TAMs exhibit significant reductions in pro-angiogenic growth factor expression and angiogenic potential consistent with a reduced alternative activation phenotype. Importantly, 4-IPP [134], recapitulates MIF-deficiency *in vitro* and *in vivo* and can rapidly re-polarize TAMs from an M2, alternative activation phenotype toward a pro-inflammatory, reduced angiogenic, M1 classically activated phenotype [35]. Consistent with a role for MIF in contributing to the angiogenic phenotype of alternatively activated TAMs, lung adenocarcinoma-derived MIF promotes CXCL8 (IL-8) and VEGF expression in human monocytes [135, 136]. More recently, stromal macrophage MIF was found to be an important contributor to intratumoral angiogenesis required for murine teratoma formation [137]. Moreover, a study by the Dranoff group revealed that melanoma patients showing durable anti-melanoma immune responses to an experimental therapeutic had high levels of anti-MIF auto-antibodies that specifically neutralized MIF-dependent Tie-2 and MMP-9 expression in TAMs leading to disrupted tumoral vasculature, lymphocyte/granulocyte infiltrates and, by extension, a significantly improved prognosis [138]. Despite these advances, the precise melanoma-promoting angiogenic and adaptive immune effector cell requirements – and the respective roles that tumor-derived MIF *vs.* TAM-derived MIF plays in them – are still largely unresolved.

TAMs preferentially localize to hypoxic areas of tumors [139, 140]. Hypoxia has profound effects on TAM functions including their migration into tumors and patterns of gene

expression, especially those encoding pro-angiogenic cytokines and enzymes [141]. Hypoxia induces gene expression in TAMs through up-regulation of HIF-1 α and HIF-2 α and subsequently a wide array of HIF target genes in hypoxic/necrotic areas of human tumors [141]. Importantly, hypoxia is a potent inducer of both VEGF and MMP7 in TAMs, both of which are known to support tumor angiogenesis, invasion, and metastasis. In addition, hypoxia up-regulates the expression of M2 macrophage markers like IL-10, arginase, and PGE₂ [142]. It is possible that the expression/function of MIF in M2 polarized TAMs is regulated through this hypoxia-HIF1- α /HIF2- α circuitry.

In addition to HIF-1 α , NF- κ B has been identified as master regulator of TAM transcriptional programs, and evidence suggests that modulation of NF- κ B activity in these cells is an important mechanism by which their pro-tumoral functions can be controlled [110]. TAMs from advanced tumors show defective NF- κ B activation in response to different pro-inflammatory signals [111, 117, 126]. This defective NF- κ B activation in TAMs correlates with impaired expression of NF- κ B-dependent inflammatory mediators including TNF- α , IL-1 β , and IL-12 [110]. Importantly, restoration of NF- κ B activity in TAMs from advanced tumors results in increased expression of inflammatory cytokines (e.g., TNF- α) and is associated with a delay in tumor growth [117].

Emerging results indicate that signaling through the energy homeostatic AMPK pathway may inhibit the inflammatory responses induced by NF- κ B in alternatively activated M2 TAMs [118]. AMPK- α is the catalytic AMPK subunit of a kinase complex consisting of 3 AMPK subunits and its activity is tightly regulated by phosphorylation on a conserved threonine residue at position 172 within the activation loop. Several recent studies demonstrate an important and central regulatory role for MIF in promoting stress-response AMPK activation [143–145]. Interestingly, activated AMPK antagonizes NF- κ B pro-inflammatory signaling [146] while activating Foxo3 [147], likely resulting in immunosuppressive signaling [148]. Preliminary results from our group indicate that MIF-deficient and 4-IPP-treated melanoma TAMs have significantly reduced AMPK pathway activation compared to control macrophages, which suggests that MIF may control TAM M2 polarization by promoting AMPK activity. Studies are ongoing to identify the precise mechanisms of action for TAM alternative activation in the context of MIF and AMPK focusing on both upstream and downstream MIF and AMPK effectors.

7. NK Cells

Natural killer (NK) cells are lymphocytes that are part of the innate immune system. They are an important component of the first line of defense that protects the body from pathogen invasion and malignant transformation. NK cells comprise ~ 5–10% of peripheral blood lymphocytes and are also found in the liver, spleen, bone marrow, and lymph nodes in humans. Strikingly, high activity of peripheral blood NK cells is associated with a 10% lower incidence of tumors for men and 4% for women [149], and their infiltration into certain tumor tissues is an indicator for better disease prognosis [150]. NK cells are characterized by strong cytolytic activity against susceptible target cells and by the ability to release several cytokines. Unlike cytotoxic T lymphocytes (CTLs), NK cells kill without prior sensitization via the polarized release of cytotoxic granules, which are loaded with

perforin and granzymes [151]. Cytolysis requires the formation of a complex immunological synapse between the target cell and the NK cell, in a highly organized manner [152].

Over the past two decades, major advances have been made in the definition of NK cell function including the molecular mechanisms enabling NK cells to selectively kill tumor or virus-infected cells while sparing normal cells. A conceptually important advance was proposed in 1990 by Ljunggren and Kärre [153] in their 'missing-self' hypothesis. According to this hypothesis, one of the functions of NK cells is to recognize and eliminate cells that fail to express self major histocompatibility complex (MHC) class I molecules or human leukocyte antigen (HLA) class I – that is to say, when the cells are missing expression of self-molecules, which are usually expressed on healthy tissue. The finding implied that NK cells worked like T cells by recognizing foreign antigens on the target cell, and are strongly influenced by the expression of MHC/HLA class I molecules on the target cell. Two models were proposed to explain the role of class I molecules controlling target cell resistance/susceptibility to NK cell lysis. The first model, the receptor inhibition model, states that a putative receptor specific for MHC/HLA class I molecules on the NK cell will transmit an inhibitory signal that will turn off NK cell activation. The second model – the target interference model – postulates that ligands on target cells for activating NK cell receptors will be masked by the expression of MHC class I molecules, making them unable to trigger NK cell activation. The parallel identification, in the early 1990s, of MHC class I-specific inhibitory and activating receptors in mice [154] and humans [155] provided the molecular basis underlying the missing-self hypothesis. The balance of activating and inhibitory receptor stimulation determines NK cell activation.

The inhibitory receptors on NK cells (iNKR) comprise receptors that mostly recognize MHC/HLA class I molecules on the surface of target cells. Inhibitory receptors on human NK cells include the promiscuous immunoglobulin-like transcript (ILT)2 receptors, the killer immunoglobulin-like receptors (KIRs), which recognize different allelic groups of HLA-A, HLA-B, and mainly HLA-C molecules and the CD94–NKG2A receptor, which recognizes HLA-E [156]. The activating receptors of human NK cells trigger cytolytic activity mainly against tumor cells and virus-infected cells [150, 157]. NK cells express several activating receptors, including natural-killer group 2, member D (NKG2D), DNAX accessory molecule (DNAM)-1 and 2B4 [158]. As it is necessary for NK cells to be turned 'off' to prevent the NK-mediated killing of normal MHC/HLA class I⁺ autologous cells, an 'on' signal must occur when NK cells interact with target cells. This 'on' signal can be readily detected whenever NK cells interact with MHC/HLA class I⁺ target cells. The receptors involved in NK-cell activation during this process of natural cytotoxicity are collectively termed 'natural cytotoxicity receptors' (NCRs), they are represented by NKp46, NKp44 and NKp30. The importance of these activating receptors is underscored by the fact that the surface density of NCRs is correlated with the degree of NK cell-mediated cytotoxicity towards tumor cells [159, 160]. Moreover, blocking of NCRs results in significantly decreased killing of tumor cells *in vitro* [161].

After cell-to-cell contact, NK cells integrate signals from its surface receptors in seconds, resulting in either target-cell attack or no response. It is well known that tumors often express low levels of MHC/HLA class I molecules. Over 85% of human metastatic

carcinomas display deficient HLA class I expression. In the case of downregulation of all MHC/HLA class I molecules, lysis of tumor cells can be mediated by all mature NK cells because of the insufficient engagement of the various iNKR. In the case of downregulation of individual class I alleles (e.g. loss of single alleles or of one full haplotype), only KIR⁺ NK cells would be involved. NK cells also kill malignantly transformed cells after interaction of induced or over-expressed ligands with their activating receptors, the most common one being NKG2D. NKG2D recognizes several well-defined ligands on the target cells including MHC class I chain-related protein (MIC)A, MICB, and UL16-binding proteins (ULBPs) [158]. In healthy adult cells, the expression of NKG2D ligands is restricted to the thymic epithelium and to the gastrointestinal mucosa. However, MICA/B and other ligands are upregulated on the surface of many tumor cell types. Ligand overexpression has been detected in solid tumors of multiple origins and in lymphoproliferative malignancies. Some oncogenes have been reported to directly upregulate the expression of NKG2D ligands. The upregulation of NK cell ligands might be a cell-intrinsic protective mechanism in order to render altered/tumor cells susceptible to killing, and on the contrary reduced ligand expression or shedding of the ligands is beneficial to tumor cells in order to prevent NK cell activation.

Tumor cells employ many tricks to actively bypass detection and elimination by NK cells of the immune system. Persistent expression of activating ligands and sustained triggering of NKG2D leads to hypo-responsiveness and decreased cytotoxicity due to decrease in NKG2D expression and reduced IFN- γ production and also, tumor-released cytokines such as TGF- β and IFN- γ repress MICA/B expression and down-modulate NKG2D expression in NK cells. In this context, ovarian-carcinoma-derived MIF contributes to tumoral immune evasion by directly inhibiting NK cell killing of ovarian cancer cells. MIF is overexpressed in ovarian carcinoma cells and this expression correlates with disease severity and the presence of ascites [162]. Secretion of MIF by the carcinoma cells decreases NKG2D levels in both CD8⁺ T cells and NK cells [162]. Mechanistically, MIF appears to exert its effects on the NK cells in an immediate and profound manner by inhibiting the transcription of NKG2D mRNA, whereas other tumor-derived suppressive mediators such as TGF- β and MMPs appear to inhibit NKG2D expression in a more delayed fashion mainly via post-transcriptional mechanisms [163]. Neutralization of MIF in the ovarian carcinoma cells restores NKG2D expression and anti-tumor cytotoxicity of NK cells exposed MIF-deficient tumor cells *in vitro* [162].

Various studies have revealed that NK cells infused into cancer patients are particularly efficient in the eradication of metastasizing tumor cells and small tumors. Rosenberg's group pioneered NK cell-based immunotherapy by administration of autologous IL-2-activated NK cells to patients with advanced cancer [164]. In fact, allogeneic and haplo-identical NK cells transduced with NKG2D can target human malignancies in a superior way and are attractive for cell-based immunotherapy because of minimal toxicities and negligible interaction with standard cancer treatments. Since MIF is present ubiquitously in the periphery and is highly expressed in most malignancies targeting MIF may represent an attractive combinatorial immunostimulatory approach to render tumors more susceptible to NK cell-based immunotherapy of human cancers.

8. Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTLs), also known as killer T cells, provide a cell-mediated response to specific foreign antigens associated with cells. CTLs (or effector CD8⁺ T cells) respond to foreign antigens presented in the context of MHC-1 expressed on the cell surface. CTLs do not respond to soluble antigens, but induce apoptosis in viral-infected cells and in cancer cells. Most CTLs express T-cell receptors (TCRs) that can recognize a specific antigen. Once released into the periphery, naïve T cells constantly survey and sample antigen-presenting cells (APCs) in secondary lymphoid tissues in search of cognate peptide-MHC-1 molecules. T cell-APC interactions, in the context of infection or inflammation, drive the activation and clonal expansion of naïve T cells to become effector cells that exhibit potent cytolytic function [165]. CTLs induce apoptosis in the target cell primarily by two pathways; one involving perforin-mediated apoptosis and the other involving Fas/Fas-ligand interaction. Activated CTLs release perforin proteins that integrate into the membrane of the target cell and organize to form a membrane pore. This allows the protease granzyme to enter the cell and activate the apoptotic/proteolytic cascade, and also allows other effector molecules to cross the cell membrane and trigger osmotic lysis of the target cell membrane [165].

Although a variety of host immune effector cells participate in tumor cell killing, tumor antigen-specific CTLs are highly effective in mediating tumor destruction. An important goal of current immunotherapy research is to induce durable and long-lasting functional CTLs that can mediate cytotoxic effects on tumor cells. To attain this goal, there are four distinct steps that must be achieved. To initiate an effective CTL-mediated anti-tumor immune response, mature DCs must capture antigens derived from tumors. Next, tumor-antigen-loaded DCs must activate CTLs in lymphoid organs (also called cross-priming). Subsequently, activated CTLs must enter the tumor microenvironment [then called tumor-infiltrating lymphocytes (TILs)] to perform their effector functions, at which point a variety of negative regulatory signals suppress the immune response. Finally, CTL-mediated cytotoxic effects must overcome the tolerance induced by tumor cells. Each step is a complex process that may be disrupted in many ways and the constantly changing tumor/stromal characteristics alongside tumor growth demands a continuous adaptation of the immune system.

In a tumor-bearing host, DCs play an important role in the immune-surveillance by initiating the primary anti-tumor effector T-cell responses. Both tumor cell- and stromal cell-derived factors such as VEGF, TGF- β , and IL-10 induce functional defects in DCs. Defective DCs express substantially lower levels of MHC molecules, adhesion molecules, and co-stimulatory molecules and have impaired capabilities for antigen uptake, diminished cell motility, and an impaired ability to prime naïve T cells. Cumulatively, these defects may ultimately result in CD8⁺ T cell tolerance to tumor antigens [166]. As discussed earlier, hypoxic microenvironments within the tumor stroma stimulate the accumulation of immunosuppressive TAMs. TAM-derived soluble TGF- β , IL-10, and PGE₂ can directly inhibit the effector functions of anti-tumor CTLs [167] while many tumor cells/stromal cells express cell surface-associated programmed death ligand 1 (PD-L1), that serves to directly inhibit CTL activation [167]. Additionally, tumor-derived soluble factors can induce and

attract immunosuppressive cell types such as Treg and MDSCs into the tumor microenvironment. In cancer patients, CD4⁺CD25⁺ Treg induce CD8⁺ T cell tolerance via direct suppressive functions on T cells or via the secretion of immunosuppressive cytokines such as IL-10 and TGF- β [167]. MDSCs inhibit CTL activation directly in an antigen-specific or non-specific manner, or indirectly by (i) altering the peptide presenting ability of MHC class I molecules on tumor cells, (ii) inhibiting DC differentiation, and (iii) expanding the numbers of Treg [167].

MIF is necessary for both *in vitro* and *in vivo* Th2 subset of CD4⁺ T helper responses [168]. Both mitogen- and antigen-induced Th2 lymphocyte activation depend upon autocrine production of MIF. Mitogen- or antigen-activated T cells express significant quantities of MIF mRNA and protein, and neutralization of MIF inhibits IL-2 production and T cell proliferation *in vitro* and decreases the Th2 cell response to soluble antigen *in vivo* [168]. Abe *et al.* reported that splenocyte cultures treated with neutralizing anti-MIF antibody results in a significant increase in CTL activity and a concomitant increase in IFN- γ production. This increase in CTL activity is associated with increased expression of the common γ_c -chain of the IL-2 receptor that is necessary for CD8⁺ T cell survival [169]. These reports suggest that MIF plays an important role in the regulation of anti-tumor T lymphocytes *in vivo*, and may exert its pro-tumorigenic effects by regulating T lymphocyte responses to tumors. Lending further support to this concept are results from Johnson's group showing that MIF-deficient neuroblastoma cells generate a much more robust CD8⁺ T cell-mediated, anti-tumor responses than MIF-abundant cells when injected into syngeneic mice [170]. Vaccination with MIF-deficient cells resulted in a significant increase in the number of IFN- γ -secreting CD8⁺ T cells in the lymphoid tissues of vaccinated mice. Consequently, MIF-deficient neuroblastoma cells could be more effectively rejected in immune-competent mice when compared to MIF-expressing tumor cells [170].

Immunotherapeutic strategies targeting immune tolerance in cancer patients have generally focused on re-activating adaptive, T cell-mediated immune responses by neutralizing lymphocyte inhibitory pathways induced by malignant cells. For example, CTLA-4 [171], programmed death-1 (PD-1) [172] and Treg [173, 174] are all being targeted with varying degrees of success. The penultimate success of these approaches depends upon a robust CD8⁺ T effector cell response following alleviation of the tumor suppressor pathway being targeted. Since tumor antigen-specific IFN- γ -producing CD8⁺ T cells have been shown to be highly effective in mediating anti-tumor immunity, even when antigen density is low on the target cells, the enhancement of CD8⁺ T effector responses through the inhibition of MIF may be an attractive strategy for increasing the efficacy of immunotherapy for MIF-producing tumors. The mechanism by which tumor-derived MIF regulates CD8⁺ T cell immunity is still unclear. Furthermore, how or whether stromal MIF contributes to this phenotype is currently not known. Because MIF-expressing TAMs and MDSCs, within the tumor stroma, can functionally and differentially dictate tumor infiltrating lymphocyte proliferation, and CTL tolerance, it will be important to elucidate the contributions of MIF-dependent MDSC/TAM polarization to the CTL effector functions in the cancer microenvironment.

9. Conclusions

During the last 20 years, studies on MIF have gone from being primarily focused on its role as an innate immune-acting cytokine/chemokine to its evaluation on direct phenotypic effects in malignant cells back to, more recently, its phenotypic contributions to both immune and non-immune tumor stromal cell phenotypes. Figure 1 depicts the complex array of MIF and D-DT expression characteristics and their autocrine- and paracrine-mediated effects on both malignant and stromal cells within solid tumor microenvironments. There remain a number of outstanding questions regarding mechanisms of action, intracellular vs. extracellular phenotypes, the synergy between MIF and D-DT in immune suppressive stromal mechanisms and whether there are distinctions between tumor-derived vs. stromal cell-derived MIF/D-DT phenotypes. Despite these questions, the cumulative current data strongly indicate that therapeutic targeting of MIF and/or D-DT may provide substantial clinical efficacy by neutralizing both malignant and stromal processes involved in dictating disease progression in late stage cancer patients (Figure 1).

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Highlights

- Current literature is reviewed to provide relevant background for various stromal cell types
- We discuss relevant phenotypes associated with each tumor-associated stromal cell type
- We review current literature on MIF and/or D-DT in the context of each stromal cell type
- Key summaries and hypotheses are provided regarding MIF/D-DT stromal cell contributions
- We discuss therapeutic targeting of MIF and D-DT in the context of tumor-stromal interactions

Figure 1. MIF and D-DT contributions to tumor-stromal interactions

MIF and D-DT are highly expressed in most solid cancers and overexpressed in response to hypoxia. Tumor-derived paracrine and stromal cell-derived autocrine MIF and D-DT promote the phenotypes as indicated for each stromal cell within solid tumor microenvironments.