Epithelial-Mesenchymal Transition Phenotype and Immune System

Carcinoma cells that undergo an epithelial-mesenchymal transition (EMT) and display a predominantly mesenchymal phenotype (hereafter EMT tumor cells) are associated with immune exclusion and immune deviation in the tumor microenvironment (TME). A large body of evidence has shown that EMT tumor cells and immune cells can reciprocally influence each other, with EMT cells promoting immune exclusion and deviation and immune cells promoting, under certain circumstances, the induction of EMT in tumor cells. This cross-talk between EMT tumor cells and immune cells can occur both between EMT tumor cells and cells of either the native or adaptive immune system.

1. Association of EMT and an Immunosuppressive Tumor Microenvironment TME in Patients

There is now considerable evidence, derived mainly from immunohistochemical and gene expression studies, suggesting that tumors enriched in EMT markers are associated with an immunosuppressive TME and a negative prognosis.

Gene expression clustering studies in ovarian cancer have shown that the mesenchymal subtype, enriched in an EMT-related gene signature, has a worse prognosis and survival compared to other subtypes [1]. In this mesenchymal subtype, a decreased number of $CD8^+$ tumor-infiltrating lymphocytes (TILs) were detected, suggesting an association between EMT tumor cells and exclusion of these immune cells from the TME. Similarly, in a study conducted in non-small cell lung cancer (NSCLC), EMT markers were associated with reduced tumor infiltration of $CD4^+$ and $CD8^+$ T cells, increased expression of immunosuppressive cytokines such as interleukin (IL)-10 and TGF-β, as well as overexpression of inhibitory immune checkpoint molecules such as cytotoxic T-lymphocyte antigen (CTLA)-4 and T-cell immunoglobulin and mucin domain-containing (TIM)-3 [2].

In an immunohistochemistry study performed in patients with gastric cancer, a high expression of EMT traits, the infiltration of TAMs, and the expression of TGF-β1 were associated with a negative prognosis [3]. In a study of lung adenocarcinoma [4], EMT markers were associated with enhanced tumor infiltration of $CD4^+Foxp3^+$ Tregs and upregulation of inhibitory immune checkpoint molecules such as programmed cell death (PD)-ligand (L) 1, PD-L2, TIM-3, B7-H3, and CTLA-4. B7-H3 was identified as a negative prognostic marker for NSCLC. Similarly, a genomic and proteomic analysis based on a tumor cell EMT signature conducted across almost 2000 different tumors [5] revealed a strong association between EMT and markers of inhibited or exhausted immune responses. Thus, a high expression of inhibitory immune checkpoint molecules such as PD-1, PD-L1, CTLA-4, OX40L, and PD-L2 was observed in tumors with the most mesenchymal EMT scores. An association between EMT tumor cells (mesenchymal and hybrid epithelial-mesenchymal phenotypes) and increased numbers of infiltrating $PD-1^+$ cells was also observed in another study in patients with adenocarcinoma of the lung [6]. A very strong association was found between PD-L1 expression and the claudin-low subset of breast cancer, which is characterized by a high EMT score [7].

Overall, these studies allowed for the identification of two different scenarios: first, an association between EMT and a reduced infiltration of immune cells (i.e., a predominantly immune-excluded TME); and second, an association between EMT and the infiltration of suppressive or exhausted immune cells (i.e., a predominantly immune-deviated TME). In both cases, however, the conclusions are similar: EMT markers associate with a TME that has inhibitory effects (through immune exclusion or deviation) on antitumor immune responses.

2. EMT-Associated Changes in the Immunological Profile of Tumor Cells

EMT tumor cells undergo phenotypic alterations that have significant consequences for the recognition by cells of the native and adaptive immune systems. Both down- and upregulation of cell surface molecules of immunological significance have been described. In general, these changes are accompanied by immune resistance and evasion, but there are exceptions to this rule.

The first class of phenotypic alterations consists of the regulation of factors directly or indirectly involved in immune recognition. Thus, EMT-like alterations in melanoma cells have been reported to reduce the expression of multiple tumor antigens, with a consequent escape from being killed by T cells specific for these antigens [8]. However, when targeting antigens whose expression was unaltered during EMT, the capacity of T cells to kill melanoma cell lines in vitro was unaltered [9]. Similarly, T cell-driven immunoediting of breast tumors in neu-transgenic mice led to the emergence of antigen-loss variants that had undergone an EMT [10]. Reduced antigen presentation may depend on the downregulation of components of the antigen processing machinery. Thus, EMT NSCLC cells showed a significantly reduced expression of immunoproteasome components and their regulators [11]. The immunoproteasome generates antigenic peptides that bind to human leukocyte antigen (HLA)-I molecules for recognition by CD8$^+$ T cells. Consequently, reduced expression of the immunoproteasome leads to reduced presentation of antigenic peptides. EMT-associated downregulation of molecules involved in the presentation of antigenic peptides can also lead to tumor cells hiding from immune recognition. This has been shown for HLA-I molecules, which were downregulated in epithelial cell lines of different tumors as a result of EMT [12][13], with a consequent reduction in antigen presentation and recognition by CD8$^+$ cytotoxic T lymphocytes (CTLs) [12].

Upregulation in tumor cells of inhibitory immune checkpoint molecules of native and adaptive immunity can also occur in tumor cells in response to EMT. These molecules inhibit the onset or the continuation of ongoing antitumor immune responses. EMT has been associated with the upregulation of several inhibitory immune checkpoint molecules, such as PD-L1 [14][15], TIM-3 [16], B7-H3 [17], B7-H1 [18], or CD47 [19]. Upregulation of PD-L1 induced resistance to CTL-mediated killing [20]. Interestingly, some of these immune checkpoint molecules have been shown to act by themselves as EMT inducers and promote the acquisition of tumor-initiating potential [7][12][17][18][21], thereby contributing to the amplification of the EMT process and its associated functionalities [22].

Another broad class of EMT-associated tumor cell alterations consists of the acquisition of resistance to killing by cytotoxic effector cells independently of antigen display on the tumor cell surface. This has been shown for tumor cells overexpressing the EMT transcription factor (TF) brachyury and the consequent upregulation of the transmembrane glycoprotein mucin-1 (MUC-1). Overexpression of MUC-1 led to reduced susceptibility to killing by tumor necrosis-related apoptosis-inducing ligand (TRAIL) and to CTL lysis [23]. Brachyury has also been shown to reduce the susceptibility of tumor cells to cytotoxic lymphocytes (CD8$^+$ T lymphocytes and NK cells) due to inefficient caspase-dependent apoptosis, but in the presence of normal levels of HLA-I molecules, antigenic peptides, or the various components of the antigen presentation machinery [24]. In fact, increased resistance to apoptosis is recognized as a hallmark of EMT [25]. Mechanisms other than resistance to apoptosis appear to operate in some other situations. Thus, it has recently been reported that hypoxia-exposed lung adenocarcinoma subclones with a predominant mesenchymal phenotype displayed resistance toward CTLs and NK cells through a mechanism involving defective immune synapse signaling [26]. In human breast adenocarcinoma cells, reduced susceptibility to CTL-mediated lysis depended on upregulation of the stem cell marker Kruppel-like factor-4 (KLF-4) and miR-7 downregulation [27]. Hypoxia-inducible miR-210 inhibited the susceptibility of lung carcinoma and melanoma cells to lysis by CTLs [28]. An interesting study has reported that acquisition of an EMT phenotype by breast cancer cells was associated with morphologic changes, actin cytoskeleton remodeling, and increased resistance to CTL-mediated lysis [29]. Resistant cells exhibited attenuation in the formation of an immunologic synapse with CTLs along with induction of autophagy. Autophagy appeared to be critical to resistance to CTL-mediated lysis because silencing of beclin1 to inhibit autophagy restored susceptibility to CTL-induced lysis. Thus, this entry introduces autophagy as another player in EMT-associated resistance to immune effector mechanisms. EMT and autophagy are, in many instances, mutually exclusive mechanisms of tumor cell resistance, but in addition to the present article, other examples of coexistence or sequential acquisition of markers of EMT and autophagy have been described (discussed in Reference [30]).

While the EMT-associated changes of the immunological profile of tumor cells that have been discussed so far lead to the inhibition of antitumor immune responses, there are some significant exceptions. Thus, colon cancer cells undergoing EMT have been shown to undergo upregulation of ligands activating NK cells, as well as downregulation of HLA-I molecules [12][31]. These changes led to reduced recognition of the tumor cells by CD8$^+$ CTLs and enhanced recognition by NK cells. Expression of the NK cell ligands, however, was very low in vivo in advanced tumors with invasive properties,

and, concomitantly, enhanced infiltration of NK cells was observed [12]. These results suggested that NK cells had eliminated NK cell ligand-expressing tumor cells and had selected variants that were not recognized by NK cells.

Recent results have shown that EMT can directly increase tumor cell susceptibility to NK cells, thereby contributing, at least in part, to the inefficiency of the metastatic process [32]. The depletion of NK cells allowed spontaneous metastasis without affecting primary tumor growth. EMT-induced modulation of E-cadherin and cell adhesion molecule 1 (CADM1) mediated increased susceptibility to NK cytotoxicity. These results are of considerable interest because, contrary to most results published on this issue, they showed that tumor cell EMT could exert antimetastatic effects through enhanced susceptibility to NK cells. At odds with these results, a recent study showed that EMT-like changes in melanoma cells were induced by NK cells and were dependent on the engagement of the activating NK receptors NKp30 or NKG2D and the release of cytokines [33]. Moreover, an EMT-associated upregulation of HLA-I molecules was observed. This favored an escape from NK cell attack given the protective role of HLA-I molecules toward NK cell cytotoxicity and because of the contemporary downregulation of tumor-recognizing activating receptors on NK cells. On the other hand, upregulation of HLA-I molecules was expected to enhance CTL-mediated cytotoxicity toward tumor cells. How these results can be reconciled with the previous ones remains to be established, but may depend, among other things, on the timing of the tumor progression at which the experiments were performed [12]. **Table 1** summarizes the findings that have demonstrated EMT-associated changes in the immunological

Table 1. Epithelial-mesenchymal transition (EMT)-associated changes in the immunological profile of tumor cells.

Type of Alteration	Consequences	References
Reduced Expression of Tumor Antigens		
Reduced expression of tumor antigens in melanoma cells	Escape from antigen-specific killing by CTLs	[8]
Emergence of antigen-loss variants in EMT tumor cells from neu-transgenic mice	Escape from antigen-specific killing by CTLs	[10]
Reduced Expression of Antigen-Presenting Molecules		
Downregulation of HLA class I molecules	Reduced antigen presentation and escape from antigen-specific killing by CTLs.	[12][13]
Enhanced Expression of Inhibitory Immune Checkpoint Molecules		
Upregulation of PD-L1, TIM-3, B7-H1, B7-H3, CD47	Downregulation of antitumor immune responses, resistance to killing by CTLs, amplification of tumor cell EMT	[7][12][14][15][16][17][18][19][20][21][22]
Enhanced Resistance of EMT Tumor Cells to Killing by T Cells		
Overexpression of MUC-1	Reduced susceptibility to killing by TRAIL and CTLs	[23]
Expression of the EMT TF brachyury, leading to inefficient apoptosis, with normal levels of HLA class I, antigenic peptides, and components of antigen presentation machinery	Reduced susceptibility to killing by CTLs and NK cells	[24]
EMT tumor cells showing defective immune synapse signaling	Resistance to killing by CTLs and NK cells	[26]

Type of Alteration	Consequences	References
Upregulation of KLF-4 and downregulation of miR-7	Reduced susceptibility to killing by CTLs	[27]
Expression of hypoxia-inducible miR-210	Reduced susceptibility to killing by CTLs	[28]
Actin cytoskeleton remodeling, autophagy, and attenuation of an immunological synapse	Autophagy-dependent reduced susceptibility to killing by CTLs	[29]
Downregulation of HL -I and upregulation of ligands activating NK cells	Reduced recognition by CTLs and enhanced recognition by NK cells	[12][31]
Modulation of E-cadherin and CADM1	Increased susceptibility to killing by NK cells and reduced metastasis formation	[32]
EMT-like changes in melanoma cells accompanied by upregulation of HLA class I and downregulation of activating receptors on NK cells	Escape from killing by NK cells	[33]

Abbreviations: CADM, cell adhesion molecule; CTL, cytotoxic T lymphocyte; EMT, epithelial-mesenchymal transition; HLA, human leukocyte antigen; KLF, Kruppel-like factor; MUC, mucin; NK, natural killer; PD-L, programmed cell death ligand; TF, transcription factor; TIM, T-cell immunoglobulin and mucin domain-containing.

3. EMT-Induced Effects on Cells of the Immune System

An EMT in tumor cells, however, can also have direct effects on cells of the immune system. These effects can be twofold. The first is immune exclusion, i.e., reduced tumor infiltration of immune cells that mediate effective antitumor immune responses. The second is immune deviation, i.e., deviation from an effective antitumor immune response (suppressed and/or inefficient) without necessarily being accompanied by reduced tumor infiltration of cells of the immune system. The different effects can be present at the same time, albeit at varying degrees, with one predominating over the other. Of note, many EMT-induced effects on cells of the immune system are similar to those observed upon activation in tumor cells of oncogenic pathways that are also involved in the induction of EMT [34]. It appears obvious that these two events, i.e., the induction of EMT and immunosuppressive effects in the TME, are intimately linked in a causal relationship through the activation of oncogenic and EMT-promoting pathways in tumor cells.

With regard to immune exclusion, EMT is associated with immune exclusion in human cancers [2][4]. A more direct demonstration has been brought in experimental systems showing that EMT tumor cells cause reduced infiltration of immune cells into the TME [14]. On the other hand, a large number of studies have shown that EMT tumor cells can induce enhanced tumor infiltration of deviated immune cells, such as polymorphonuclear MDSCs [35], myeloid cells [36], mast cells [37][38], or natural $CD4^+CD25^-$ Tregs [39]. Pancreatic cancer cells resistant to anti-vascular endothelial growth factor (VEGF) treatment secreted increased levels of proinflammatory factors, which stimulated the recruitment of $CD11b^+$ proangiogenic myeloid cells and acted also in an autocrine manner to induce and amplify tumor cell EMT [40]. In other situations, both the exclusion of effector $CD8^+$ T lymphocytes and enhanced infiltration of immunosuppressive MDSCs have been demonstrated [41]. Immune deviation through EMT-mediated induction of an immunosuppressive phenotype has been reported for the M2 polarization of macrophages by bladder cancer cell lines [42] and the induction of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) in monocyte-derived macrophages. These IDO-expressing macrophages suppressed T cell proliferation and promoted the expansion of immunosuppressive Tregs [43]. Similarly, mesenchymal-like breast cancer cells were shown to induce activation of macrophages to acquire phenotype and functionalities of immunosuppressive TAMs [44]. EMT tumor cells also induced the generation of immunosuppressive regulatory DCs (DCreg), which induced immunosuppressive $CD4^+Foxp3^+$ Tregs and eventually impaired the induction of antitumor CTLs [45][46]. In an entirely in vitro system where lung, breast, or hepatocellular carcinoma cells were cocultured with T lymphocytes, B lymphocytes, and NK cells, EMT induction in tumor cells led to NK- and T-lymphocyte apoptosis,

the inhibition of lymphocyte proliferation, and the stimulation of regulatory T and B cells. IDO was involved at least in part in these effects [47].

In several instances, a bidirectional cross-talk has been demonstrated, with EMT tumor cells inducing immunosuppressive changes in the TME and immune cells inducing further amplification of tumor cell EMT [44]. An interesting example of such a bidirectional cross-talk between EMT tumor cells and cells of the immune system has been brought recently [48]. Overexpression of the extracellular matrix protein secreted protein acidic and rich in cysteine (SPARC) in breast cancer cells reduced their growth rate and induced EMT. This led to the formation of an immunosuppressive TME with increased infiltration of Tregs, mast cells, and MDSCs. On the other hand, inhibition of the suppressive function of MDSCs could revert tumor cell EMT, thereby showing that MDSCs contributed to the induction and/or amplification of tumor cell EMT. In a KRAS^{G12D}-driven mouse model of lung cancer [49], tumor cells expressing the EMT transcription factor Snail secreted a soluble mediator, which increased Gr1$^+$ neutrophil infiltration and secretion of the chemokine C-X-C motif chemokine ligand (CXCL) 2 by the neutrophils themselves. The neutrophils, on the other hand, favored tumor growth, reduced T cell homing in the tumor, prevented successful anti-PD-1 immunotherapy, and altered angiogenesis, leading to hypoxia and sustained Snail expression in the tumor cells [49]. Bladder cancer cells have been shown to recruit mast cells to the tumor [37]. Recruited mast cells could then enhance bladder cancer cell invasion. Thyroid cancer cells recruited and activated mast cells in the TME [50], which in turn released IL-8, which induced EMT and tumor-initiating features in the thyroid cancer cells [50].

Recently, the consequences of the immunosuppressive effects of EMT tumor cells on epithelial tumor cells have also been described [51]. Mammary tumor cells arising from epithelial tumor cell lines expressed high levels of HLA-I, low levels of PD-L1, and were infiltrated by CD8$^+$ T lymphocytes and M1-polarized (antitumor) macrophages. On the other hand, tumors arising from EMT carcinoma cell lines expressed low levels of HLA-I, high levels of PD-L1, and were infiltrated by Tregs, M2-polarized (protumor) macrophages, and exhausted CD8$^+$ T cells. Importantly, the EMT tumor cells protected their epithelial counterparts from immune attack.

4. The Mediators of EMT-Induced Effects on Immune Cells

The effects of tumor cell EMT on cells of the immune system are largely mediated by soluble molecules such as cytokines or chemokines rather than by cell-to-cell contact. Recently, a new player, extracellular vesicles, or exosomes, has joined soluble molecules as a mediator of EMT effects on cells of the immune system.

Several molecules have been shown to directly mediate the effects of EMT tumor cells on cells of the immune system. Examples of these factors are the following: chemokines such as IL-8/CXCL8 [35][52], CXCL1, and CXCL2 [41]; C-C motif chemokine ligand (CCL) 2 [46]; CCL20 [43]; cytokines such as IL-6 [53]; TGF-β [39]; the other member of the TGF-β superfamily bone morphogenetic protein (BMP) 4 [42]; granulocyte–macrophage (GM) colony-stimulating factor (CSF) [44]; other soluble mediators such as thrombospondin-1 [45]; and lipocalin 2 [46]. Exosomes have been shown to promote the polarization of macrophages toward the immunosuppressive M2 phenotype upon engulfment by the cells [54]. **Table 2** gives a synoptic view of the mediators of the EMT-induced effects on immune cells and the experimental or clinical settings in which the effects were observed.

Table 2. Mediators (soluble mediators or exosomes) of the effects of EMT tumor cells on cells of the immune system.

Mediator	Summary of Experimental Observations.	References
	Cytokines	
IL-2	IL-2 from cholangiocarcinoma cells with EMT-like features induced generation of CD4$^+$ CD25$^+$ natural Tregs.	[39]
IL-6	IL-6 induced macrophages to differentiate into M2-polarized macrophages.	[53]
TGF-β	TGF-β from cholangiocarcinoma cells with EMT-like features induced generation of CD4$^+$ CD25$^+$ natural Tregs.	[39]

Mediator	Summary of Experimental Observations.	References
BMP-4	Recombinant BMP4 and BMP4-containing conditioned media from bladder cancer cell lines promoted monocyte/macrophage polarization toward an M2 phenotype.	[42]
GM-CSF	GM-CSF from mesenchymal-like breast cancer cells (BT-549, MDA-MB-436, and MDA-MB-231) promoted the acquisition of a TAM-like phenotype by macrophages.	[44]
Chemokines		
IL-8/CXCL8	IL-8 from claudin-low TNBC cells induced recruitment of PMN-MDSCs in vitro and in vivo, as determined through neutralization experiments with mAb HuMax-IL8.	[35]
CXCL1, CXCL2	CXCL1 and CXCL2 from mouse ovarian cancer cells promoted tumor infiltration of MDSCs, as determined by knockdown of EMT transcription factor Snail.	[41]
CCL2	CCL2 derived from various tumor cell lines induced, in cooperation with Lipocalin-2, DCregs, which in turn induced Tregs, and finally impaired the induction of tumor-specific CTLs.	[46]
CCL20	CCL20 derived from EMT hepatoma cells induced IDO in monocyte-derived macrophages, which in turn suppressed T-cell proliferations and promoted the expansion of Tregs.	[43]
Other Soluble Mediators		
Thrombospondin-1	Snail-transduced melanoma cells with EMT features produced thrombospondin-1, which induced Tregs and impaired DCs in vitro and in vivo.	[45]
Lipocalin-2	CCL2 derived from various tumor cell lines induced, in cooperation with Lipocalin-2, DCregs, which in turn induced Tregs and finally impaired the induction of tumor-specific CTLs.	[46]
Exosomes		
	Snail-expressing EMT human head and neck cancer cells activated the transcription of miR-21 to produce tumor-derived exosomes, which were engulfed by CD14$^+$ human monocytes, suppressing the expression of M1 markers and increasing that of M2 markers.	[54]

Abbreviations: BMP, bone morphogenetic protein; CCL, C-C motif chemokine ligand; CTL, cytotoxic T lymphocyte; CXCL, C-X-C motif chemokine ligand; DCreg, regulatory dendritic cell; GM-CSF, granulocyte–macrophage colony-stimulating factor; ICAM, intercellular adhesion molecule; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; MDSC, myeloid-derived suppressor cell; miR-21, micro-RNA; PAI, plasminogen activator inhibitor; PMN, polymorphonuclear; TAM, tumor-associated macrophage; TGF, transforming growth factor; TNBC, triple-negative breast cancer; Treg, regulatory T cell.

5. Induction of Tumor Cell EMT by Cells of the Immune System

Both the cells of the native and adaptive immune systems have been shown to induce EMT: Cells of the native immune system include NK cells [33][55], MDSCs [48][56][57], lipopolysaccharide (LPS)-activated macrophages [58][59], TAMs [60][61][62][63][64][65][66], neutrophils [67], and mast cells [50]; cells of the adaptive immune system include TILs [68], activated T lymphocytes [69], CD4$^+$ T lymphocytes [70], CD8$^+$ T lymphocytes [71], and B lymphocytes [72]. In some cases, EMT induction

in tumor cells has been shown to be accompanied, as one might expect, by the acquisition of tumor-initiating potential [71]. Moreover, the stimulation of immune cells with cell type-specific activators can enhance their potential for inducing EMT in tumor cells. Thus, M2-polarized TAMs induced EMT in pancreatic cancer cells in a manner dependent on the expression of the LPS coreceptor toll-like receptor (TLR) 4 and IL-10 secretion [66]. This induction was strongly enhanced upon LPS stimulation of TLR4. Eventually, as for EMT-induced effects on immune cells, immune cell-induced EMT in tumor cells can also be part of a bidirectional cross-talk. In addition to the examples cited, in a mouse model of pancreatic cancer, tumor cells converted CD14^{+} peripheral blood monocytes into monocytic MDSCs, which in turn induced tumor cell EMT [73].

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