Tumor Immune Microenvironment during Epithelial-Mesenchymal Transition

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Abstract

Epithelial-mesenchymal transition (EMT) has been shown to play a critical role in tumor development from initiation to metastasis. EMT could be regarded as a continuum, with intermediate hybrid epithelial and mesenchymal phenotypes having high plasticity. Classical EMT is characterized by the phenotype change of epithelial cells to cells with mesenchymal properties, but EMT is also associated with multiple other molecular processes, including tumor immune evasion. Some previous studies have shown that EMT is associated with the cell number of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), and the expression of immune checkpoints, such as programmed cell death-ligand 1, in several cancer types. At the molecular level, EMT transcriptional factors, including Snail, Zeb1, and Twist1, produce or attract immunosuppressive cells or promote the expression of immunosuppressive checkpoint molecules via chemokine production, leading to a tumor immunosuppressive microenvironment. In turn, immunosuppressive factors induce EMT in tumor cells. This feedback loop between EMT and immunosuppression promotes tumor progression. For therapy directly targeting EMT has been challenging, the elucidation of the interactive regulation of EMT and immunosuppression is desirable for developing new therapeutic approaches in cancer. The combination of immune checkpoint inhibitors (ICIs) and immunotherapy targeting immunosuppressive cells could be a promising therapy for EMT.
Introduction

Epithelial-mesenchymal transition (EMT) is a pleiotropic and flexible change in cellular phenotypes during which epithelial cells acquire mesenchymal properties. It is triggered when cells receive signals, such as TGF-β or hypoxia, from their microenvironment during embryonic development and in certain disease states, including cancer and fibrosis(1,2). In cancer pathogenesis, EMT plays a role in tumorigenesis, invasion, metastasis, tumor stemness, and resistance to therapy. EMT is also associated with immunosuppression in the tumor microenvironment, causing resistance to immunotherapy using immune checkpoint inhibitors (monoclonal antibodies targeting programmed cell death 1 [PD-1], programmed cell death-ligand 1 [PD-L1], or cytotoxic T lymphocyte antigen 4 [CTLA-4]). The relationship between EMT and the tumor immune system has been a hot topic in recent years and could be helpful for devising strategies to treat tumors with high EMT properties (3,4).

In this review, we summarize the recent findings on the molecular mechanisms involved in EMT in the tumor immune microenvironment. In addition, we will discuss the future strategy for cancer treatment from the viewpoint of EMT-associated immune evasion.

The definition of EMT

The EMT International Association (TEMTIA) pronounced the guidelines and definitions of EMT in 2020 to unify the concept of the EMT program because an increasing number of articles on EMT have been published over the past few years and
the definition of EMT is becoming vague and controversial(2). The EMT program was previously considered a binary switch from fully epithelial (E) to fully mesenchymal (M) phenotypes; however, it has recently been proposed that EMT is a sequential process involving intermediate hybrid epithelial and mesenchymal (E/M) phenotypes (a partial EMT) because many recent in vitro and in vivo studies have shown that the transition is often incomplete, resulting in cells in intermediate states that have both epithelial and mesenchymal characteristics. TEMTIA recommends the use of the term “epithelial-mesenchymal plasticity” (EMP) to describe the ability of cells to adopt such intermediate phenotypes and move rapidly through various intermediate states. Importantly, these intermediate states can be diverse in plasticity and metastatic potential, and are also more efficient in reaching the circulation, colonizing, and forming metastases (Figure 1)(5,6). Single-cell transcriptomic analysis of primary and metastatic head and neck cancer showed that tumor cells undergoing the partial EMT program spatially localized to the leading edge of the primary tumors. This predicts metastasis and adverse pathological features such as extracapsular extension and lymphovascular invasion(7). The diverse EMT program generates spacious heterogeneity within tumors and may provide tumor cells with increased adaptability and resistance, enabling them to survive under harsh circumstances(8,9).

The role of the EMT program in cancer is debatable(10). Although classical EMT is just a process in which epithelial cells lose cell-cell adhesion and acquire mesenchymal characteristics, a variety of other EMT-associated changes, including cell proliferation, apoptosis, stemness, and immunosuppression, occur during EMT. These EMT-associated changes are mostly triggered and regulated by EMT transcription factors (EMT-TFs) including Snail, Slug, Twist1, Zeb1, and Zeb2, which play major roles in the execution
of EMT. EMT-TFs regulate cell-cell adhesion, cell migration, and extracellular degradation, as described in classical EMT; furthermore, these transcriptional factors are also involved in other cellular functions, such as cell proliferation, apoptosis, stemness, and immunosuppression, showing that EMT-TFs play pleiotropic roles in cancer initiation, metastasis, and therapy resistance and are associated with poor prognosis in both epithelial and nonepithelial tumors (Figure 2)(10-13). Extracellular signals (TGF-β, EGF, PDGF, VEGF, WNT, and Notch), external agents (UV light or alcohol), and pathological states such as hypoxia in the tumor microenvironment can activate EMT-TFs(14). Micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) have also been reported to regulate EMT, and some of them control expression of EMT-TFs(15,16). The miR-200 family members (miR-200a, miR-200b, miR-200c, miR-429, and miR-141) are well-known inhibitors of EMT(17,18). The MiR-200 family interacts with Zeb1 and Zeb2 to repress EMT and promote tumor progression in various types of human tumors. They also regulate other signaling pathways, including the Notch and WNT/β-catenin pathways, which leads to cell apoptosis and cancer stemness(19,20). Zeb2-AS1 lncRNA prevents the splicing of the 5’UTR intron in Zeb2 mRNA to encourage Zeb2 expression(21). Another lncRNA, UCA1 lncRNA, upregulates Zeb1 and Zeb2, which downregulates ATG7 to impair autophagy(22). Taken together, EMT covers a wide range of cellular functions involved in tumor progression.

Heterogeneous tumor immune microenvironment

In recent years, cancer immunotherapy such as immune checkpoint inhibitors (ICIs) or chimeric antigen receptor (CAR)-T cell therapy, has caused a revolution in
cancer treatment and prolonged patient survival in melanoma, non-small cell lung carcinoma (NSCLC), renal cell carcinoma, and hematological malignancies (23). CD8+ cytotoxic T cells (CTLs) play a central role in engaging anti-tumor immunity and have been the focus of the development of immunotherapy. Tumor cells and other components of the tumor microenvironment try to avoid the attack of CTLs for survival by producing immunosuppressive factors that block the anti-tumor activity of CTLs, which contributes to immune evasion. These immunosuppressive factors are mainly classified as immune checkpoint molecules and immunosuppressive cells (24). The immune status of the tumor microenvironment is diverse and heterogeneous within patients, and within tumors. This is because tumor heterogeneity is closely linked to immune status (24-26); therefore, we need to identify the immune status of cancer patients for stratified treatment. We describe the immunosuppressive factors in the tumor microenvironment below.

**Immune checkpoint molecules**

There are two major immune checkpoint pathways for cancer immunotherapy: CTLA4 and PD-1 (23, 27-29). These two pathways have an inhibitory effect on T cell function. CTLA4 is a negative costimulatory molecule expressed on activated T cells, which interferes with the binding of CD28 to B7 ligands (CD80 and CD86), inhibits T cell activation, and induces T cell anergy. PD-1, a receptor expressed on T cells after TCR stimulation as well as on B cells and myeloid cells, promotes T cell exhaustion and inhibits T cell activation and proliferation. PD-L1 is a ligand for PD-1 expressed on antigen-presenting cells (APCs) and tumor cells.
ICIs targeting immune checkpoint molecules, such as anti-CTLA-4 and anti-PD-L1/PD-1 antibodies, have been widely used in the last decade and are expected to be novel cancer treatments(27,30). Ipilimumab, a human CTLA-4-blocking antibody, was first approved by the FDA for unresectable or metastatic melanoma in 2011(31). The FDA has approved several monoclonal antibodies targeting PD-1 (pembrolizumab, nivolumab, and cemiplimab) and PD-L1 (atezolizumab, avelumab, and durvalumab), which have been used as monotherapy or in combination with chemotherapies or other ICIs in several cancer types(27). However, their efficacy is limited to a minority of patients when used as monotherapy or combination therapy. The response rate to PD-1 blocking antibodies ranges from 40% to 70% in cancers such as melanoma or Hodgkin’s lymphoma, while the response rates in most other cancer types are limited to 10%-25%(32). It is necessary to identify predictable biomarkers for therapeutic response and to combine ICIs with other drugs, including other immunotherapies, that can improve the efficacy of ICIs(23).

Immunosuppressive cells

In the tumor microenvironment, immunosuppressive cells, including regulatory T cells (T\textsubscript{reg} cells), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), repress the activity of T cells and promote tumor growth. These immunosuppressive cells exist within the tumor microenvironment and suppress anti-tumor immunity.

T\textsubscript{reg} cells are a special subset of CD4\textsuperscript{+} T cells that are essential for maintaining immune tolerance by keeping other immune cells from attacking self-tissues(33,34). They are identified by the expression of CD4 and CD25. FOXP3 is also expressed in T\textsubscript{reg} cells,
which is essential for their development and function. These cells inhibit the expression of costimulatory signals CD80/CD86 on antigen-presenting cells (APCs), consume IL-2, and produce immune inhibitory cytokines (TGF-β and IL-10), and immune checkpoint molecules (TIM3, CTLA4, LAG3, TIGIT, and PD-1) to inhibit T cell activation(35). T_{reg} cells in cancer have a high number of immune suppressive checkpoint molecules, which are associated with stable FOXP3 activity and strong immunosuppressive function.

TAMs are one of the most abundant infiltrating immune cells in several cancers(36). Monocytes and monocytic-MDSCs are recruited from blood to local tumor sites by CCL2, CCL5, and CSF-1 secreted from tumors and differentiate into TAMs. TAMs produce immunosuppressive cytokines such as TGF-β and IL-10, which promote T_{reg} cell activity, and inhibit T cell activity and dendritic cell maturation. They decrease arginine and produce indoleamine 2,3-dioxygenase-1 (IDO-1) to inhibit T cell activity and proliferation. TAMs can also have the surface expression of PD-L1, which leads to T cell exhaustion via the PD-L1/PD-1 inhibitory pathway. They are a significant factor, not only as immunosuppressive cells, but also as angiogenic drivers because they produce angiogenic factors, such as VEGFA and MMP9, which promote angiogenesis and lymphangiogenesis for tumor progression(37).

MDSCs are a heterogeneous population of immature myeloid cells that are generated during chronic inflammation and cancer(38-40). Myeloid cells generated under these pathological conditions are poorly phagocytic, produce reactive oxygen species (ROS), nitrogen species (NO), and anti-inflammatory cytokines(41). As a result, they are different from mature well-differentiated myeloid cells, such as macrophages or neutrophils, and acquire immunosuppressive potential that inhibits T cell activity.
MDSCs circulate in the bloodstream and are attracted to inflamed sites and local tumors by chemokines. MDSCs are classified into two types: monocytic-MDSCs (M-MDSCs) and polymorphonuclear-MDSCs (PMN-MDSCs). In mice, M-MDSCs are defined as CD11b+Ly6C\(^{hi}\)Ly6G\(^{-}\) and PMN-MDSCs as CD11b+Ly6C\(^{lo}\)Ly6G\(^{+}\). In human peripheral blood mononuclear cells (PBMCs), M-MDSCs are defined as CD11b+CD14\(^{-}\)HLA-DR\(^{-}\)/loCD15\(^{-}\) and PMN-MDSCs as CD11b+CD14\(^{-}\)CD15\(^{+}\) or CD11b+CD14\(^{-}\)CD66b\(^{+}\). Inhibition of T cell activity is required to identify MDSCs(38). They inhibit T cell activity in different ways. M-MDSCs have more suppressive functions than PMN-MDSCs, because M-MDSCs suppress both antigen-specific and non-specific T cell responses whereas PMN-MDSCs can suppress only antigen-specific T cell responses. The leading mechanisms implicated in their suppressive functions include depletion of l-arginine via arginase 1 (Arg1) and production of NO, IDO-1 and ROS(42,43). While PMN-MDSCs mainly produce ROS, M-MDSCs produce high levels of NO and Arg1. MDSCs also express PD-L1 on their surface, which leads to T cell exhaustion(44). Recent studies have shown that the transfer of the metabolite dicarbonyl methylglyoxal from MDSC to nearby CD8\(^{+}\) T cell paralyses T cell effector functions(45).

EMT and immune evasion

In several cancer types, EMT has been clinically reported to be associated with immunosuppression in the tumor microenvironment. In non-small cell lung cancer, EMT status is strongly associated with an increased population of CD4\(^{+}\) Foxp3\(^{+}\) T\(_{reg}\) cells and an inflammatory tumor microenvironment with elevation of immune checkpoint molecules, including PD-L1, PD-L2, PD-1, TIM-3, B7-H3, BTLA, and CTLA-4(46). In
addition, EMT status of circulating tumor cells is linked to PD-L1 upregulation (47). A comprehensive genomic analysis of sarcomatoid urothelial bladder cancer shows dysregulation of the EMT network and a heavy infiltration of immune phenotype with upregulation of PD-L1 (48). In breast cancer, tumor cells arising from mesenchymal carcinoma cell lines expressed low levels of MHC-1 and high levels of PD-L1 and had T\textsubscript{reg} cells, M2 type macrophages, and exhausted CD8\textsuperscript{+} T cells within their stromal areas (49). In ovarian cancer, we reported fewer intraepithelial CD8\textsuperscript{+} tumor-infiltrating T lymphocytes in the mesenchymal subtype that enriched EMT-related gene signatures and showed the worst prognosis (50). Based on these results, we can infer that EMT is associated with immunosuppression in the tumor microenvironment, and we summarize recent studies on the molecular mechanism of EMT-associated immunosuppression (Figure 1).

The tumor microenvironment consists of cancer-associated fibroblasts, endothelial cells, and several types of immune cells. Most of these cells, including immune cells, secrete cytokines and chemokines to regulate the EMT program for tumor progression. Conversely, tumor cells undergoing EMT can also produce immunosuppressive cytokines or chemokines to aggravate the immunosuppressive condition of the tumor microenvironment, which contributes to cancer development. We focused on the molecular mechanism by which EMT promotes immunosuppression in the tumor microenvironment and vice versa.

The effect of EMT on the immune system
Some studies have reported that activation of EMT-TFs leads to accumulation of immunosuppressive cells in the tumor microenvironment. Snail induces CD4+ Foxp3+ Treg cells and MHC-IIlo IDO-expressing regulatory dendritic cells partly through TSP1 production in melanoma(51). Acetylation of Snail leads to production of CCL2 and CCL5, which promote the recruitment of TAMs into the tumor microenvironment(52). Another EMT-TF, Twist1, also induces the expression of macrophage chemoattractant CCL2 to recruit TAMs(53). We demonstrated that Snail upregulates CXCL1 and CXCL2, ligands of CXCR2, which recruit MDSCs into the tumor microenvironment in ovarian cancer(54). We demonstrated that Snail induces CXCL1 and CXCL2 via the NF-κβ pathway and possibly via direct binding to their promoters(54). The correlation between Snail and CXCL1 or CXCL2 has also been reported in gastric cancer, head and neck squamous cell carcinoma, and lung cancer (55-57). Snail also upregulates other CXCR2 ligands, CXCL5 and IL-8, which also attract MDSCs, in non-small cell lung cancer(58). Another major EMT-TF, Zeb1, accumulates MDSCs into tumors by upregulating IL-6 and IL-8 in breast cancer(59). EMT-TFs also increase the expression of immune checkpoint ligands such as PD-L1. The Zeb1/miR-200 feedback loop regulates PD-L1 expression, tumor invasion, and metastasis in non-small cell lung cancer and breast cancer(60,61). The Zeb1/miR-200 feedback loop also promotes cell plasticity and controls cancer stemness and is important for tumor progression(62-64).

Other factors that induce EMT also contribute to immunosuppression in the tumor microenvironment. A major EMT inducer, TGF-β, promotes the differentiation of non-activated macrophages into a TAM-like phenotype by downregulating TNF-α and IL-12 via TGF-β/Snail signaling(65). TGF-β also increases PD-L1 expression, which is dependent mainly on the activation of PI3K/Akt pathway(66). TGF-β-induced EMT
confers resistance to complement-dependent cytotoxicity by upregulating CD59 expression, which inhibits the membrane attack complex as a complement regulation factor(67). However, TGF-β-induced EMT increases susceptibility to NK cell-mediated cytotoxicity with increased expression of CADM1, which activates NK ligand(68).

Pancreatic ductal adenocarcinoma cells were shown to promote PD-L1 expression and vimentin expression in the presence of IFNγ(69). Hypoxia-induced EMT increases the expression of CCL20 in hepatoma cells, which induces indoleamine 2, 3-dioxygenase (IDO) in monocyte-derived macrophages to suppress T cell proliferation and expand T$_{reg}$ cells via pSTAT1 signaling(70). Hypoxia also regulates the various activities of MDSCs.

Hypoxia stimulates recruitment of MDSCs to local tumors via CCL26 or CD39L1 (40,71,72). We showed that hypoxia induced by anti-VEGF therapy promoted MDSC intratumoral infiltration via the production of GM-CSF(73). Hypoxia promotes glycolysis in MDSCs via the mTOR/HIF-1α pathway, which facilitates MDSC proliferation and T cell inhibition activity. Hypoxia-inducible factor 1α (HIF-1α) also upregulates PD-L1 expression in MDSCs(44). Taken together, EMT inducers, including EMT-TFs, contribute to the immunosuppression in the tumor microenvironment, which could contribute to resistance to immunotherapy.

The effect of the immune system on EMT

CD4$^+$ CD25$^+$ T$_{reg}$ cells produce higher TGF-β levels and induce EMT in hepatocellular cell carcinoma(74). As described above, TGF-β is a dominant EMT inducer. TGF-β is also a cytokine produced by T$_{reg}$ cells. It has been reported that a higher population of CD25$^+$ T$_{reg}$ cells in NSCLC and ovarian cancer are correlated within high levels of TGF-β(75). It is natural that T$_{reg}$ cells promote EMT by producing TFG-β.
TAM secretes TGF-β as described above, which plays an important role in promoting EMT(76-78). M2-polarized TAMs facilitate EMT via the TLR4/IL-10 signaling pathway in pancreatic cancer cells(79). Coculture with TAM promotes EMT via the production of forkhead box Q1 (FOXQ1) in gastric cancer cells(80). TAM also induces EMT through activation of the EGFR/ERK1/2 signaling pathway in head and neck squamous cell carcinoma(81).

MDSCs have also been reported to induce EMT. Human linCD45+CD33+ MDSCs triggered miRNA101 expression in ovarian cancer cells, which enhanced the EMT gene signature(82). CD11b*Gr1+ mouse MDSCs in primary tumors and in metastatic organs secrete IL-6 and soluble IL-6Rα(83). IL-6 signaling has been linked to EMT phenotypes via the JAK/STAT3/Snail signaling pathway(84). The secreted protein acidic and rich in cysteine (SPARC) derived from MDSCs has immune suppressive activity and supports the induction of EMT in breast tumors(85). TGF-β is also a well-known cytokine secreted from MDSCs. Deletion of Tgfbr2 (encoding TGF-β receptor II) specifically in CD11b*Gr1+ myeloid cells suppresses tumor metastasis, which shows that the myeloid-specific TGF-β signaling pathway could be associated with EMT(86). When focused on the MDSC subtype, CD11b*Gr1hiF4/80- PMN-MDSCs actively promote cancer cell dissemination by inducing EMT via TGF-β, EGF, and HGF signaling pathways(87). CD11b*Ly6C+Ly6G- M-MDSCs increase the EMT/CSC phenotype depending on the activation of the STAT3 signaling pathway(88). Ouzounova et al. reported that tumor-infiltrating CD11b*Ly6C+Ly6G- M-MDSCs localize at the invasive edge of the primary tumor and induce the EMT/CSC phenotype, whereas CD11b*Ly6C-Ly6G+ PMN-MDSCs in pulmonary metastatic sites promote metastatic tumor growth by reverting to the EMT/CSC phenotype(89). However, no other study has
reported separate infiltration of the MDSC subtype, and further investigation is required.

IL6, a major EMT inducer, is mainly expressed in CD11b^+Ly6G^+Ly6C^- PMN-MDSCs in colon carcinoma(90).

In summary, the malicious loop between EMT inducers and immunosuppressive cells and/or immune checkpoint molecules maintains a harmful tumor microenvironment that promotes tumor progression.

Therapeutic strategies targeting EMT and EMT-induced immunosuppression

As summarized in this review, EMT reveals tumor plasticity and plays an important role in tumor development and heterogeneity, including the promotion of the immunosuppressive tumor microenvironment. Although EMT-TFs have pleiotropic functions during cancer progression and could be potent therapeutic targets for heterogeneous cancer, few therapies directly targeting EMT-TFs have been developed. As novel therapeutic approaches for EMT, therapy targeting EMT inducers as well as EMT-TFs has been pharmaceutically developed. Furthermore, targeting EMT-induced immunosuppression could partially suppress EMT due to the loop between EMT and immunosuppression. We classified the current therapeutic strategies targeting EMT into three groups: therapy directly targeting EMT, therapy targeting EMT-induced immunosuppression and comprehensive therapy targeting both EMT and immunosuppression (Figure 1).

Therapy targeting EMT
Brachyury, an EMT-TF, is a good candidate that many clinical trials have adopted as a therapeutic target in cancer (91-93). It is expressed in many tumor types such as chordoma, breast cancer, NSCLC and colorectal cancer, but not in normal tissues. GI-6301, a yeast-based vaccine to express brachyury, showed T cell responses in a phase I clinical trial (NCT01519817), whereas a randomized phase II clinical trial of GI-6301 with radiation showed no significant difference compared to placebo in chordoma (NCT02383498) (94). MVA-BN-Brachyury with FPV-BN-Brachyury, poxvirus-based vaccines to express brachyury, also increased T cell response in advanced solid tumors and have now been tried in some clinical trials (Table 1) (95).

EMT inducers could also be good therapeutic candidates targeting EMT. First, TGF-β could be the most useful target because TGF-β induces EMT and immunosuppressive cells as well. Galunisertib, a small molecule inhibitor of TGF-β receptor type I (TGF-βRI), recovered the function of CTL and NK cells, and the proliferation of naïve T cell suppressed by CD4+CD25+ Treg cells (96, 97). Vacosertib, another small molecule TGF-β receptor I kinase inhibitor, inhibited EMT and enhanced CTL activity in breast cancer orthotopic-grafted mice model and has been utilized in clinical trials (98). Secreted clusterin (sCLU) is involved in TGF-β-induced EMT and lies downstream of the EMT-promoting transcriptional cascade of TGF-β (99). AB-16B5 is a humanized IgG2 antibody against sCLU, which showed its safety in a phase I clinical trial (NCT02412462). A phase II clinical trial (NCT04364620) of AB-16B5 in combination with docetaxel in NSCLC has been planned.

Chimeric antigen receptor (CAR)-T cell therapy is an adoptive T cell (ATC) therapy, in which T cells isolated from patients are genetically modified to express CARs
on their surface in order to recognize tumor-associated antigens, expanded, and
transferred into patients\(^{(100,101)}\). CAR-T therapy showed tremendous results for
relapsed/refractory B cell hematological malignancies, and CD19-targeted CAR-T
therapies have been approved by the FDA\(^{(102-106)}\). Compared to hematological
malignancies, the efficacy of CAR-T therapy for solid tumors is limited because of tumor-
associated antigen heterogeneity in these, and immunosuppressive factors derived from
the tumor microenvironment. To improve the efficacy of CAR-T therapy for solid tumors,
the use of tumor-associated antigens highly expressed in solid tumors is needed.
Mesothelin has been used as a target antigen for CAR-T therapy in many clinical
trials\(^{(107,108)}\). A phase I study of CAR-modified autologous T cells redirected against
mesothelin (CART-meso) showed CART-meso expansion in the blood and intratumoral
infiltration in 7 out of 10 tumor samples\(^{(109)}\). Mesothelin has also been reported to
promote EMT\(^{(110)}\); therefore, CART-meso could also be an EMT-targeting therapy.
Mucin-1 (MUC1) has also been utilized as a target for CAR-T therapy and has been
reported to promote EMT via Twist1 in triple negative breast cancer\(^{(111)}\). P21-activated
kinase 4 (PAK4) downregulates the expression of cell adhesion molecules such as
claudin-14 and VCAM1 via EMT-TFs, Zeb1, and Slug. The PAK4 inhibitor normalizes
tumor vascularity and improves the efficacy of CAR-T therapy to promote T cell
intratumoral infiltration\(^{(112)}\).

Metabolism-inhibiting drugs approved by the FDA have been reported to inhibit
EMT\(^{(113)}\). Simvastatin (an HMG-CoA inhibitor), Olaparib (a PARP-1/2 inhibitor) and
rolipram (a PDE4 inhibitor) inhibit TGF-β-induced EMT\(^{(114-116)}\). The combination of
these metabolism-specific inhibitors targeting EMT and immunotherapy could be
promising. In particular, olaparib with ICIs is undergoing some clinical trials because
PARP inhibitor-mediated DNA damage is favorable for ICIs(117). However, metabolism-specific inhibitors also exert an influence on tumor immunity. Simvastatin decreases TAM, whereas atorvastatin, another HMG-CoA inhibitor, has been reported to expand MDSCs in murine colitis(118,119). Further investigation of metabolism-specific inhibitors targeting EMT is required.

Therapy targeting EMT-induced immunosuppression

Immunotherapeutic approaches to EMT-induced immunosuppression could also be promising. Some reports have shown that ICIs are effective for tumors with a high number of infiltrating T lymphocytes and checkpoint activation, but not for tumors with immunosuppressive cells or tumors with a low number of infiltrating T lymphocytes(24,120,121). As previously described, EMT promotes the intratumoral infiltration of immunosuppressive cells as well as the expression of immune checkpoint molecules; therefore, EMT-associated tumors cannot be inhibited only by ICIs(120,121). The combination of ICIs with immunotherapies targeting immunosuppressive cells could be effective for tumors with EMT properties.

There are several types of immunotherapies targeting immunosuppressive cells. Anti-CTLA-4 antibody, anti-CD25 antibody, or anti-CCR4 antibody are good candidates for T_{reg} cell depletion(122). An anti-human CD25 antibody (RG6292) monotherapy as well as a combination of RG6292 with anti-PD-L1 antibody showed T_{reg} cell depletion and inhibited tumor growth in mice models(123). Anti-CCL2 or anti-CCR2 antibody has been tested in clinical trials because CCL2 attracts TAM via CCR2 into local tumors and suppresses cytotoxic T cells(76,124,125). Anti-CSF1R antibody could inhibit
polarization into TAMs that are induced via the CSF1/CSF1R axis(126). Regarding therapy targeting MDSCs, all-trans retinoic acid (ATRA) seems to be a promising drug for the normalization of myelopoiesis and differentiation of MDSCs into mature myeloid cells (dendritic cells or macrophages). Chemotherapies such as anti-VEGF antibody, 5-FU, or gemcitabine can also regulate myelopoiesis and deplete MDSCs. CXCR2 blocking is a promising candidate for targeting MDSCs. As we previously described, CXCR2 ligands such as CXCL1, CXCL2, CXCL5, and IL-8 attract MDSCs into tumor sites. CXCR2 blocking inhibits MDSC migration and improves anti-tumor immunity. CXCL1 or CXCL2 depletion using short hairpin RNA decreased breast cancer progression in a mouse model(127). We reported that the CXCR2 antagonist SB265610 suppressed MDSC migration and inhibited Snail-high ovarian tumor progression(54). However, SB265610 did not completely suppress tumor growth induced by Snail because activated CTLs were not increased by SB265610. The main player of anti-tumor immunity is CTLs; hence the reactivation of CTLs by ICIs is required. Some reports have shown that CXCR2 inhibition enhances anti-PD-1 efficacy(128,129). The combination of ICIs and CXCR2 antagonists may be beneficial. SX-682 is an oral CXCR1/2 inhibitor that blocks MDSC recruitment and enhances T cell activation. Combinations of SX-682 and anti-PD-1, or anti-PD-L1 antibodies have been tried in clinical trials (Table 2). Snail has been reported to induce regulatory dendritic cells by TSP1 production(51), Flt3 ligand and IL-12, which promote dendritic cell maturation, and peptide-pulsed dendritic cell vaccines, which is a vaccination with specific tumor antigenic peptide-pulsed dendritic cells, could be a good candidate therapy for tumors with EMT properties(130,131). Although immunotherapy cannot solve all the phenomena induced by EMT-TFs, it is worth trying to treat EMT phenotypic tumors.
Comprehensive therapy targeting both EMT and immunosuppression

Combined blockade of immune checkpoint molecules and TGF-β has been attempted in several cancer types because it can block both immunosuppression and EMT simultaneously and enhance T cell activity. Bintrafusp alfa (M7824), a bifunctional fusion protein targeting TGF-β and PD-L1, is an ideal drug because it can antagonize the TGF-β pathway and PD-L1/PD-1 immunosuppressive pathway(132). Bintrasfusp alfa is composed of a human IgG1 monoclonal antibody against PD-L1, which is based on avelumab, fused with the extracellular domain of 2 TGF-β receptor II (TGF-βRII) molecules serving as a TGF-β trap(133-135). This drug reverted TGF-β-mediated EMT in vitro and in vivo(133). In a breast cancer mouse model, bintrasfusp alfa increased CTLs, tumor-infiltrating NK cells, and decreased intratumoral MDSCs compared to anti-PD-L1 antibody or trap control(135). Phase I clinical trials have shown manageable safety in patients with solid tumors(132). It has been under further investigation in many clinical trials (Table 3). Combination therapy of TGF inhibitors with anti-PD-1 or anti-PD-L1 antibodies has also been tested in several cancer types (Table 3). In addition, mesothelin-targeted CAR-T cells secreting PD-1 nanobodies have been developed and tested in patients with solid tumors during phase I clinical trials (NCT04503980 and NCT04489862).

Future perspectives
Many recent studies have reported that EMT is associated with anti-tumor immunosuppressive activity. Tumor cells undergoing EMT reprogram the tumor immune microenvironment through the production of signals such as cytokines recruiting or expanding immunosuppressive cells, and in turn, immune cells in the tumor microenvironment induce EMT in tumor cells. The bidirectional regulation between EMT and anti-tumor immunosuppressive activity should be investigated extensively because this malicious crosstalk could aggravate tumor invasion and tumor metastasis. As the tumor grows, epithelial cells undergoing EMT acquire mesenchymal properties that enable tumor cells to invade surrounding tissues and disseminate to distant sites. Although T cell-mediated anti-tumor immunity prevents tumor progression, tumor cells undergoing EMT induce an immunosuppressive environment around the tumor, contribute to T cell dysfunction, and promote the growth of tumor cells at the primary site as well as dissemination to and colonization of metastatic sites. Immunosuppressive tumor microenvironment also promotes EMT in tumor cells, thereby further accelerating the aggressive activity of tumor cells. Besides facilitating invasion and metastasis, EMT also contributes to therapy resistance (8, 9, 14, 136, 137). Therapy resistance is always an important clinical problem as it makes it difficult to decide on the next medical treatment. Immunotherapy, such as immune checkpoint inhibitors, has been recently developed and is widely used as a new cancer treatment. The efficiency of this novel therapeutic strategy is also expected to be limited for tumors with EMT characteristics (138, 139). The failure of this crosstalk could target both EMT and immunosuppression, which would inhibit tumor progression and circumvent treatment resistance.

EMT targeting therapeutic approaches have been investigated, but EMT targeted drugs are still being tested in preclinical and clinical studies (140, 141). The difficulty
faced in targeting EMT can be attributed to the complexity and plasticity of the EMT process. As tumors progress from initiation to metastasis, EMT can be implemented at different sites of tumors and at different timings of cancer development. At different phases of tumor progression, different EMT-TFs could be involved in the EMT program, which leads to the heterogeneity of EMT. As we have described in this review, EMT inducers confer immunosuppressive conditions to tumor microenvironment and in turn tumor microenvironment’s immunosuppressive functions contribute to EMT to promote tumor progression. Targeting of the malicious feedback loop between EMT and the tumor immunosuppressive environment could be a promising approach for tumors undergoing EMT because when tumors with epithelial-mesenchymal plasticity are treated, the current standard therapy and immunotherapy can be selected from the perspective of targeting immunosuppression. In this review, we focused on extracranial solid tumors. Clinical trials of metastatic tumors mentioned (Tables 1-3) may have excluded brain metastasis and further investigation of intracranial tumors is required. Furthermore, current immunotherapies such as anti-PD-1 or anti-CTLA-4 antibodies can work well only for a limited number of patients, and the addition of novel agents against immunosuppressive factors is needed. We suggest that agents targeting EMT could be candidates for improving the efficiency of current immunotherapies. The feedback loop between EMT and immune suppression should be further investigated, which could help in developing an efficient strategy for cancer treatment.
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Author’s contribution

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Analysis and interpretation of data: M. Taki, K. Abiko, J. Hamanishi,

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Study supervision: M. Mandai
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inhibitor, promotes anti-tumor immunity leading to durable, complete
responses, as monotherapy and in combination with checkpoint blockade. *J

Preclinical assessment of galunisertib (LY2157299 monohydrate), a first-in-class
transforming growth factor-beta receptor type I inhibitor. *Oncotarget*

ALK-5 kinase inhibitor, potently inhibits breast to lung metastasis. *Mol Cancer

Transcriptome profiling of a TGF-beta-induced epithelial-to-mesenchymal
transition reveals extracellular clusterin as a target for therapeutic antibodies.


Table 1. Therapies targeting EMT

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<tr>
<th>Drug</th>
<th>Additional therapy</th>
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<th>Phase</th>
<th>Status</th>
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Figure legends

Figure 1. Therapeutic approaches to the feedback loop between EMT and immunosuppression

Tumor cells have epithelial-mesenchymal plasticity, which adopt intermediate hybrid cells that display both epithelial and mesenchymal phenotypes (partial EMT) and modify phenotypes through various intermediate states. Partial EMT contributes to tumor heterogeneity and provides the ability to survive and metastasize in diverse tumor microenvironment.

EMT-TFs or EMT-TF inducers initiate production of TSP1, CCL2/5 and CXCR2 ligands to attract immunosuppressive cells (regulatory T cells (T$_{reg}$ cells), tumor-associated macrophages (TAMs) and myeloid-derived suppressive cells (MDSCs)) and induce expression of immune checkpoint molecules on tumor cells with partial EMT, leading to an immunosuppressive tumor microenvironment. Conversely, immunosuppressive cells activate signaling pathways that promote EMT via chemokines such as TGF-β. This malicious feedback loop needs to be blocked by drugs targeting EMT and the immunosuppressive tumor microenvironment.

Figure 2. The multiple cellular mechanisms induced by EMT

EMT transcriptional factors (EMT-TFs) play pleiotropic roles in cancer progression and are involved in cellular processes involved in classical EMT (the loss of cell-to-cell adhesion) as well as other cellular processes (cell apoptosis, cell proliferation, stemness, and immunosuppression) during EMT.
Table 1. A clinical trial list of therapies targeting EMT

Table 2. A clinical trial list of therapies targeting immunosuppression

Table 3. A clinical trial list of comprehensive therapies targeting both EMT and immunosuppression
Figure 1:

- Tight junction
- Adherent junction
- Desmosome

**Epithelial-mesenchymal plasticity**

- Intermediate EMT (partial EMT)

**Targeting EMT (refer to Table 1)**
- Vaccines targeting Brachyury
- TGFβ blockade
- Mesothelin CAR-T
- MUC1 CAR-T

**Targeting EMT-induced immunosuppression (refer to Table 2)**
- CXCL2/5
- IL-6/8
- EGF
- HGF
- CXCR2 blockade
- Anti-VEGF antibody
- Anti-CCR2 antibody
- Anti-CC1 antibody
- Anti-CSF1R antibody
- Anti-VEGF antibody
- Anti-CCR4 antibody
- Anti-CD3 antibody

**Comprehensive therapy targeting both EMT and immunosuppression (refer to Table 3)**
- TGFβ blockade + PD-L1/PD-1 blockade
- CAR-T + PD-L1/PD-1 blockade
- Anti-CD25 antibody
- Anti-CCR4 antibody
- Anti-CTLA4 antibody
- Anti-CCR2 antibody
- Anti-CSF1R antibody
- CXCR2 blockade
- Anti-VEGF antibody
Figure 2:

EMT-TF inducers
- TGFβ
- EGF
- PDGF
- WNT
- Notch
- UV light
- Alcohol
- Hypoxia

miRNAs
- miR-200 family
- miR-34

IncRNAs
- Zeb2-AS1
- UCA1

EMT-TFs
- Snail
- Slug
- Zeb1
- Zeb2
- Twist1
- KLF8
- AP1
- Brachyury
- Six1
- HMG2a

Cell apoptosis

Cell proliferation

Loss of cell-cell adhesion
Acquire invasiveness

Immunosuppression

Stemness
Clinical Cancer Research

Tumor Immune Microenvironment during Epithelial-Mesenchymal Transition

Mana Taki, Kaoru Abiko, Masayo Ukita, et al.

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