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Epithelial-mesenchymal plasticity in tumor immune evasion

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Abstract

Epithelial-mesenchymal transition (EMT) is a fundamental process that occurs during embryogenesis and tissue repair. However, EMT can be hijacked by malignant cells, where it may promote immune evasion and metastasis. Classically considered a dichotomous transition, EMT in cancer has recently been considered a plastic process whereby malignant cells display and interconvert among hybrid epithelial/mesenchymal (E/M) states. Epithelial-mesenchymal plasticity (EMP) and associated hybrid E/M states are divergent from classical EMT with unique immunomodulatory effects. Here, we review recent insights into the EMP-immune crosstalk, highlighting possible mechanisms of immune evasion conferred by hybrid E/M states and roles of immune cells in EMP.

Introduction

Approximately 90% of cancer-associated deaths are due to metastatic disease (1), and accordingly, 5-year disease-free survival in patients with metastatic disease compared to locoregional disease is greatly reduced across most cancer types (2). Metastatic cancer often displays resistance to systemic therapies including chemotherapy, immunotherapy, and targeted biologics (3). The advent of immunotherapy, namely immune checkpoint blockade (ICB) has dramatically advanced treatment of metastatic disease for a fraction of patients in select cancer types, including melanoma and lung cancer (4). Unfortunately, of the 43% of cancer patients eligible for ICB, only 14% are estimated to respond (5). Thus, a major focus of cancer research is addressing the low response rates of ICB by elucidating mechanisms underlying ICB resistance and identifying opportunities for combination therapies that may overcome resistance (1,4).

Epithelial-mesenchymal transition (EMT) has emerged as a potential contributor to immunotherapy resistance (6). EMT describes the transitional process of an epithelial cell becoming a mesenchymal cell and occurs in embryogenesis and development. However, cancer cells may hijack EMT to drive therapy resistance, invasion, metastasis, and thus, poor

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outcomes in cancer (7). Although classically described as a binary switch between epithelial and mesenchymal states, EMT in cancer is now understood to be a plastic process in which cells interconvert between hybrid epithelial/mesenchymal (E/M) states with varying degrees of both epithelial and mesenchymal features, referred to as epithelial–mesenchymal plasticity (EMP) (8). The hybrid E/M state has recently been found to modulate the immune system to support cancer invasion and metastasis (1,7), and attention has been given to the role of hybrid E/M states in ICB resistance (7,9). Understanding the hybrid E/M-immune crosstalk in cancer is now proving useful in the development of prognostic markers, therapeutic indications, and novel therapy combinations (7,9–11). Here, we review the relevant literature to highlight mechanisms of immune evasion conferred by hybrid E/M states, emphasize the role of the immune system in promoting the hybrid E/M phenotype, and propose areas of investigation that may advance our understanding of EMP in tumor immune evasion.

The Plasticity of EMT

Initial hypotheses addressing EMT were generated in the 1960s by Elizabeth Hay, and EMT has since been attributed important roles in embryogenesis, wound healing, and cancer (8,12–16). The plasticity of EMT, or EMP, and its *intra*-tumoral and *inter*-tumoral heterogeneity is a recent major discovery in oncology with clinical relevance (7–9,17–21). Indeed, hybrid E/M states are associated with poor prognosis in several cancers (22–25) and have been shown to promote therapy resistance (26) and local and distant metastases (27). Metastable hybrid E/M states exhibiting plasticity were predicted through computational modeling of EMT (9,28–33). RNA sequencing and histological staining of primary tumors corroborated these *in silico* findings, demonstrating hybrid E/M states in several cancers (10,11,17,22,23,27,28,34–46). Moreover, recent pan-cancer studies using single cell RNA sequencing (scRNA-seq) have highlighted that the EMP gene program is conserved across cancer types, although their constituent genes are variable and correlate with the tissue of origin (10,34,45,46).

While EMP induction is heterogeneous across tumors (10), some signals appear conserved. These include extracellular ligands such as transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), and vascular endothelial growth factor (VEGF), environmental factors like hypoxia, dysregulated intracellular signaling in MAPK or p53 pathways, biophysical properties of the extracellular matrix (ECM), genetic mutations, and epigenetic alterations (10,47–50) (Figure 1). Downstream canonical transcription factors (TFs) that mediate EMP include SNAIL1/2, TWIST1/2, and ZEB1/2 (51). However, others TFs such as JUNB, FOSB, and KLF4/6 are also associated with EMP (10). The inducing signals of EMP interface with a dynamic balance of intracellular inducing and inhibiting signals occurring epigenetically, transcriptionally, post-transcriptionally by microRNA (miRNA) and long non-coding RNA (lncRNA), and post-translationally by regulatory circuits and signaling pathways that dictate the relative expression of epithelial and mesenchymal features (9,17,32,47,52,53). A classic example of post-translational regulation is the balance between the interconnected miR-34/SNAIL negative feedback loop and miR-200/ZEB negative feedback loop, in which SNAIL and ZEB promote EMP while the miRNAs inhibit EMP (30,54,55).

EMP in Tumor Immunology and Cancer Immunoediting

The dynamic relationship between the immune system and tumors is directly influenced by the cancer immunoediting hypothesis (56). Classically, cancer immunoediting is a three-stage model including elimination, equilibrium, and escape. In the elimination phase, cancer cells are recognized by the immune system as foreign due to expression of tumor-associated antigens (TAAs). TAAs are released to the extracellular environment following cancer cell death, and these antigens are taken up by antigen presenting cells (APCs) that present these neoantigens to a cytotoxic T lymphocyte (CTL) with a T cell receptor (TCR) specific to the TAA, triggering CTL activation. Although dendritic cells (DCs) are the prototypical APC, all nucleated cells – including cancer cells – express major histocompatibility complex (MHC) class I that serves to primarily present endogenous antigens to the immune system and activate CTLs. Activated CTLs home to the tumor, bind to a TAA presented on MHC class I, and lyse the cancer cell in combination with initiating a local immune response via inflammatory cytokines and chemokines (57). The cycle of immune recognition of cancer cells via MHC class I and their subsequent lysis results in either tumor elimination or an equilibrium phase. In the equilibrium phase, the persistent immune response acts as a selective pressure that, in combination with tumor heterogeneity and cancer cells' DNA repair malfunctions, enriches tumor clones with an immune escape phenotype. The immune escape tumor phenotype is characterized by enrichment of immunosuppressive cell types such as regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), expression of immune checkpoint proteins including programmed death-ligand 1 (PD-L1), defective antigen presentation, and/or dysregulated cytokine and metabolism regulation that promotes immune suppression (58). Tumor clones that enter the escape phase resist immune cell cytotoxicity and grow despite the anti-tumor immune response.

Spatial and Temporal Features of EMP-Tumor Immune Modulation

Advances in single cell technologies and the use of genetically engineered animal models have allowed for increased granularity in evaluating the roles of malignant, stromal, and immune cells of the tumor microenvironment (TME) in promoting EMP (23,27,38). Single cell sequencing has also helped to elucidate the heterogeneity in EMP, defining commonalities and differences in the EMT gene signature from tumor to tumor *within* and *across* primary cancer sites (10,34,45,59). Pastuschenoko et al. (2018) were among the first to demonstrate that malignant cells with varying expression of epithelial and mesenchymal markers existed within the same tumor and formed neighborhoods with the most mesenchymal cells in close proximity to a fibrovascular niche composed of endothelial cells and cancer-associated fibroblasts (CAFs) (23). Our group used scRNA-seq in head and neck squamous cell carcinoma (HSNCC) patient samples and found that cells enriched for a hybrid E/M signature localized to the leading edge of tumors adjacent to immune cells of the stroma and engaged with CAFs via TGFβ signaling (27). More importantly, we found that tumors enriched for this hybrid E/M signature were associated with worse clinical outcomes (22). Additionally, histological evidence of expression of classical EMT markers at the tumor-stroma interface has been associated with poor prognosis in several cancer types, such as breast, lung, prostate, pancreatic, colorectal, and HNSCC (27,40,60–63). The mechanism underlying this spatial localization is unresolved. However, a selective advantage of the

hybrid E/M state in resisting stromal cytotoxic immune cells while coopting other stromal cells to promote EMP via soluble and contact signaling at the tumor-stroma interface likely contributes.

The hybrid E/M state is associated with immune *evasion* and *suppression* that may mediate such a selective advantage at the tumor leading edge. Immune evasion mechanisms include decreased expression of MHC class I and other antigen presentation proteins, which may be directly regulated by the hybrid E/M TF SNAIL2 and prevent recognition of hybrid E/M cells by CTLs (64–66) (Figure 1). Additionally, autophagy induction and the activity of EMT TFs brachyury and mucin 1 (MUC1) mediate resistance to natural killer (NK) and CTL-meditated cytotoxicity (67–69) (Figure 1). EMP is also associated with increased expression of immunosuppressive proteins, such as PD-L1, CD73, and CD276 (35,66) (Figure 1). The microRNA miR-200 has a dual role in both inhibiting EMP and PD-L1 expression; however, the EMT TF ZEB1 is activated in EMP, suppresses miR-200, and thereby increases PD-L1 expression (33,70–72). Interestingly, compared to epithelial cells, hybrid E/M cells and full mesenchymal cells upregulate PD-L1 to a similar extent, suggesting that hybrid E/M states adopt immunosuppressive features of a mesenchymal cell without losing the plasticity advantageous for metastasis (33). Due to increased immune checkpoint protein expression and alterations in adhesion proteins in hybrid E/M states, CTL activation is attenuated in the immunological synapse with an EMP-induced cell compared to a non-EMP-induced cell (73–75) (Figure 1).

In addition to immune *evasion*, hybrid E/M malignant cells trigger immune *suppression* through secreted factors upregulated in this state, such as TGFβ, IL-8, colony stimulating factor 1 (CSF1), and CCL18, and support immunosuppressive cell recruitment and polarization of Tregs, tumor associated macrophages (TAMs), and MDSCs (76–78) (Figure 2). Leveraging a multi-omics approach incorporating scRNA-seq, spatial transcriptomics, and multiplex ion beam imaging, Ji et al. (2020) identified a population of “tumor-specific keratinocytes” (TSKs) in human cutaneous squamous cell carcinoma with striking overlap with the hybrid E/M signature identified in HNSCC (27,35). These TSKs co-localized with CAFs, endothelial cells, macrophages, and Tregs. In addition, both TSKs and CAFs were found to express CD276 (B7-H3), an immune checkpoint protein that dampens the anti-tumor immune response (79). Notably, TSK cells expressed integrins ITGA3 and ITGB1, which serve as receptors for ligands expressed by TAMs and MDSCs (35). Hybrid E/M cells’ secreted factors can polarize TAMs and MDSCs to a pro-tumor phenotype that promotes immunosuppressive polarization of other immune cells as well as EMP induction, tumor growth, and invasion (62,80–83) (Figure 2). Tumor immune evasion and suppression mechanisms conferred by the hybrid E/M state are consistent with the observation that non-small cell lung cancer (NSCLC) and breast cancer tumors with more mesenchymal-like features show decreased infiltration of CTLs and increased immunosuppressive cell infiltration (66,84–86).

The spatial localization of hybrid E/M cells to the tumor invasive front may also arise due to modulation of non-immune cells, including CAFs and normal epithelium. Indeed, CAFs have been shown to contribute to induction and maintenance of EMP in malignant cells via their secretion of latent TGFβ binding proteins (LTBPs), stromal cell-derived

factor 1 (SDF1, or CXCL12), fibroblast growth factor (FGF), and TGFβ (27,85–87) (Figure 2). Additionally, hybrid E/M cells in HNSCC have been shown to promote lateral tumor invasion by secreting TGFβ and TNFα that acts on adjacent normal epithelium to induce EMT, which represents a selective advantage of hybrid E/M cells to invade that is permitted by their localization at the tumor-stroma interface (88).

The Role of EMP in Immune Evasion by Circulating Tumor Cells

Along with disrupting the basement membrane and local invasion of tissue, EMP is believed to confer circulating tumor cells (CTCs) with increased potential for distant metastasis (1). CTCs are considered a metastatic precursor that disseminate from the primary tumor mass, travel through the circulatory system, and seed distant sites to establish a metastatic niche that supports secondary tumors. EMP is implicated in CTC-mediated metastasis in several cancer types including breast cancer (89), NSCLC (90), prostate cancer (91), gastric cancer (92), and colorectal cancer (CRC) (93). Hybrid E/M cells travel as CTC clusters and decrease expression of NK cell activating ligands, which may shield CTC clusters from NK cell-mediated lysis when in circulation (94) (Figure 1). While in transit, CTCs of colon and breast cancer cell lines *in vivo* were shown to be coated in platelets that release TGFβ thereby promoting a mesenchymal phenotype, decreasing NK cell recognition, and increasing survival during transit to a metastatic site (1,95) (Figure 1). Upon seeding a metastatic site, a mesenchymal shift within the hybrid E/M state, or a mesenchymal-epithelial transition (MET), is essential for cancer cell seeding and outgrowth (47,96–101). The epithelial shift has been shown to be regulated by key TFs. For instance, Prrx1 promotes EMT and is suppressed to enable metastatic colonization (100), and the TFs OVOL1 and OVOL2 inhibit EMT and promote MET (102,103). The molecular mechanism of an epithelial shift at a metastatic site is likely distinct from the mechanism of a mesenchymal shift at the primary tumor site. Indeed, hysteresis regulation of EMP, in which the paths of epithelial and mesenchymal shifts are distinct, was found to have increased metastatic potential compared to non-hysteresis regulation in a breast cancer mouse model (104). For disseminated hybrid E/M malignant cells that seed a distant site, cell extrinsic factors likely contribute to the epithelial shift. For example, evidence in mouse models of breast cancer suggests that MDSCs facilitate a reversion to a more epithelial state, enhance survival of metastatic cells, and promote outgrowth (105). Thus, understanding the EMP-immune crosstalk in metastatic colonization may reveal immune interactions that are uniquely associated with MET and not observed at the primary tumor.

Cancer Stem Cells and Hybrid E/M Cells Similarly Modulate the Immune System

Cancer stem cells (CSCs) comprise a fraction of hybrid E/M cells and have the ability to differentiate into diverse cell types, self-renew, and both evade and suppress the immune system (106–108). Strikingly, cancer stemness is molecularly and functionally associated with EMP. For instance, the EMT TFs TWIST1 and ZEB1 increase cancer cell stemness, which has been shown to be associated with the enhanced metastatic potential of the hybrid E/M state (24,25,33,109–112). Indeed, a full EMT state in which a cell fully differentiates into a mesenchymal cell is unable to colonize a metastatic site due to loss of stemness (99,100). Thus, EMP, in which cells do not fully differentiate, is proposed to be compatible with maintenance of stemness, tumor initiating capacity, and metastatic seeding (24,98,108).

Similar to hybrid E/M states, CSCs also display immune evasion and suppression tactics (107,113,114). For instance, CSCs display MHC class I downregulation (115), and CSCs may evade NK cells through downregulation of the NK cell-activating ligand NKG2D (116). In addition, through secreted cytokines such as TGFβ, IL-10, and periostin, CSCs suppress and polarize local immune cells into a pro-tumor phenotype (117–119). For example, CSCs induce macrophages into a pro-tumor phenotype associated with secretion of cytokines that further promote stemness (120,121). However, in contrast to EMP, the transcriptional profiles characteristic of cancer stemness negatively correlate with PD-L1 expression in several cancer types (122). Although CSCs and hybrid E/M cells appear to share mechanisms of immune evasion and suppression, their similarity in underlying signaling remains incompletely understood; however, a more complete characterization of these cell states has the potential to reveal converging hubs suitable for pharmacologic inhibition.

Direct and Indirect EMP-Immune Crosstalk

EMP is involved in immunosuppression as result of soluble mediators that recruit and promote immunosuppressive immune cells as well as direct inhibition of immune cells (Figure 2). Many malignant tumors with an immunosuppressive phenotype also demonstrate an enrichment of EMP markers (84,123). These tumors feature decreased CTL infiltration and increased expression of immune checkpoint molecules, often portending a poor prognosis (124). In a study of lung cancer patients, a strong correlation between a “tumor EMT score” (based on the expression of 76 classical EMT signature genes) and expression of the immune checkpoint PD-L1 expression was identified (125). A similar result was found using a pan-cancer EMT signature spanning 11 different cancer types: Tumors with a high EMT score also had high expression of immune checkpoint proteins, including programmed cell death protein 1 (PD-1), PD-L1, cytotoxic T-Lymphocyte associated protein 4 (CTLA-4), and PD-L2. In patients with metastatic melanoma, an upregulation of E/M-related genes in the primary tumor was associated with primary resistance to anti-PD-1 therapy (126). A similar observation has been noted in CTCs, where classical EMT markers N-cadherin and vimentin were enriched in the CTCs of NSCLC patients with recurrence following anti-PD-L1 therapy (127). While these studies strongly suggest an association between EMP and markers of immunosuppression, mechanistic studies underlying the EMP-immune signaling axes are needed to better understand the link between hybrid E/M states and immune resistance.

Factors with Dual Roles in EMP Induction and Immunomodulation

Many of the soluble factors known to induce EMP also contribute to immune suppression in the TME (Figure 2). TGFβ is perhaps the most well-studied EMP-inducing soluble factor with additional immunomodulatory effects (128). TGFβ can be secreted by malignant, immune, and stromal cells. Induction of EMP by TGFβ is mediated by upregulation of key EMT TFs SNAIL (129), SLUG (15), and ZEB1/2 (130,131), as well as other TFs associated with EMP including HMGA2 (132) and ETS1 (133). In addition to modulating malignant cell phenotypes, TGFβ drives immune suppression in the TME by multiple mechanisms. For instance, EMP driven by TGFβ1 in hepatocellular carcinoma (HCC) cells resulted in

increased expression of immune checkpoint proteins PD-L1 and B7-H3 (CD276), and the coordinate expression of TGFβ1 with PD-L1 and B7-H3 correlated with poor prognosis in HCC patients (134). In gastric carcinoma cell lines, EMP triggered by TGFβ1 resulted in increased expression of PD-L1 through activation of nuclear factor kappa B (NF-κB) (135). NF-κB is a known transcriptional regulator of several EMT TFs including TWIST1, SLUG, and ZEB2 in several cancer types, such as NSCLC and pancreatic cancer (136,137). Indeed, inhibition of NF-κB signaling has been shown to decrease the migratory capacity of gastric carcinoma cells and abrogate TGFβ1-mediated expression of PD-L1 (135).

Similar to TGFβ, IL-8 has a dual role in EMP and immune modulation by signaling through its G-protein coupled receptors, CXCR1 and CXCR2. The resulting acquisition of mesenchymal features, namely increased expression of vimentin, fibronectin, and classical EMT TFs, promotes cell migration and invasion (138). Interestingly, in addition to EMP induction, IL-8 has also been shown to drive immunosuppression in the TME by recruiting and enhancing the function of MDSCs (80,81) (Figure 2). MDSCs are known to play a critical role in facilitating tumor immune escape by inhibiting immune cells that are required for the anti-tumor immune response, such as CTLs, NK cells, and DCs (139). However, it is unclear whether the induction of EMP and immunosuppression by IL-8 are parallel pathways or interconnected. Most often, EMP displays a positive feedback loop whereby malignant cells exhibiting EMP produce the very factors that induce a further transition towards this state. In line with this positive feedback loop model, IL-8 has been shown to be upregulated upon induction of EMP by TNFα or TGFβ in colon cancer (140) and by epidermal growth factor (EGF) in breast cancer (141) (Figure 2). In turn, the transcription of IL-8 can be promoted by the EMT TFs SNAIL and brachyury. SNAIL was shown to induce IL-8 production *in vivo* in a NSCLC model (142) and directly regulate IL-8 production by binding to E-box regions within the IL-8 promoter in CRC cells (143). Additionally, IL-8 was one of the most upregulated secretory molecules in human carcinoma cells induced to a mesenchymal state by brachyury expression (144).

The tyrosine receptor kinase AXL is another well studied common inducer of both EMP and tumor immune evasion. In cancer, AXL signaling can become aberrantly activated due to membrane alterations of a mesenchymal-like cell that increase expression of AXL, EGFR, HER2, and Met (65). AXL expression positively correlates with expression of EMT genes in several cancers, including HER2+ breast cancer (145) and esophageal squamous cell carcinoma (146). Furthermore, in pancreatic cancer cells, AXL has been shown to regulate the expression of EMT TFs SNAIL, SLUG, and TWIST (147). AXL was also shown to mediate resistance to NK cell- and CTL-mediated killing through NF-κB and MAPK signaling in lung cancer clones derived from NSCLC patient tumors and induced to a mesenchymal phenotype by hypoxia (148). In keeping with the positive feedback cycle of EMP, the expression of AXL seems to be positively regulated by EMT TFs. For instance, in non-malignant human breast epithelial cells, stable expression of TWIST, ZEB2, SNAIL, or SLUG induced AXL expression along with an EMP phenotype (149). Thus, expression of the very inducers of EMP and tumor immune suppression, such as TGFβ, IL-8, and AXL, by malignant cells exhibiting EMP indicates their capacity to both sustain this EMP phenotype and further promote immune suppression.

Transcriptional Regulation of EMP-Mediated Immune Suppression

The EMT TFs SNAIL and ZEB1 play important roles in promoting tumor immune suppression. In murine and human melanoma cells, SNAIL transduction induced EMP, and these E/M-induced clones demonstrated immunosuppressive features both in cancer-immune cell co-culture experiments *in vitro* and experiments performed *in vivo* (150). In these hybrid E/M clones, SNAIL mediates the immunosuppressive function of Treg cells, resistance to CTL lysis, and decreases the function of DCs through TGFβ and thrombospondin 1 (TSP1) signaling (150). In an *in vivo* model of ovarian cancer, SNAIL was also found to have important immunomodulatory effects whereby SNAIL knockdown reduced tumor growth, increased CTL tumor infiltration, and decreased tumor-infiltrating MDSCs (77). Dongre et al. (2017) developed a model of EMP in mouse mammary tumor virus (MMTV) breast cancers using a SNAIL-driven yellow fluorescent protein (YFP) to identify SNAIL-high and SNAIL-low cells, which were labelled “quasi-mesenchymal” (akin to hybrid E/M) and epithelial, respectively (85). While the epithelial cells in this study were immunogenic, quasi-mesenchymal cells promoted an immunosuppressive TME associated with CTL exclusion from the tumor core, M2-like pro-tumor macrophage polarization, and decreased response to anti-CTLA-4 therapy. Interestingly, only a small percentage of quasi-mesenchymal cells (10%) in mixed tumors was needed to cross-protect the surrounding epithelial malignant cells from immune attack, suggesting a potent immunosuppressive effect of the hybrid E/M state. A subsequent study from the same group identified genes that were enriched in quasi-mesenchymal cells yet did not affect hybrid E/M status to identify downstream mediators of immune suppression in the hybrid E/M state (66). Among these genes, CD73, CSF1, secreted phosphoprotein 1 (SPP1), galectin-3, MBL associated serine protease 1 (MASP1), and CXCL12 all negatively regulated the number and function of CTLs (Figure 2). However, knockdown of only CD73, CSF1, or SPP1 could mobilize CTLs into the tumor core. Of these, CD73 was the only gene that upon knockdown led to tumor regression and decreased metastasis in anti-CTLA-4 therapy. This is one of the first studies establishing a causal role of the hybrid E/M state in immune modulation and resistance to ICB therapy (66).

Like SNAIL, ZEB1 has also been shown to confer immune resistance. In a *K-Ras* model of lung adenocarcinoma, ZEB1 expression specifically in invading cancer cells directly upregulated the expression of immune checkpoint proteins PD-L1 and CD47, a surface protein that promotes TAM polarization to a tumor-promoting phenotype (78). The resultant PD-L1 induction and CD47-dependent polarization of TAMs created a protective niche for invading cancer cells that was deficient in CTLs. This study demonstrates a direct link between EMP and immune resistance (78). Regulation of PD-L1 by ZEB1 was also found in an experiment implementing the *K-ras* LA1/+p53R172HΔg/+ (KP) mouse model of NSCLC that demonstrated ZEB1 increases PD-L1 expression by repressing miR-200, a miRNA that binds PD-L1 mRNA (125) (Figure 3).

EMP and Macrophage Polarization

Macrophages play a key role in EMP, and in a mouse model of skin squamous cell carcinoma, macrophage depletion by anti-CSF1R and anti-CCL2 blocking antibodies decreased tumor formation and caused a shift of malignant cell states toward more

epithelial-like phenotypes (135). Positive feedback loops between malignant cells and TAMs promoted the induction of EMP and ultimately facilitated intravasation. In a breast cancer mouse model, macrophages induced EMP via CCL18 while malignant cells with an EMP phenotype could induce macrophage secretion of CCL18 through GM-CSF along with environmental factors of the TME including lactate (62) (Figure 2). The expression of this GM-CSF and CCL18 positive feedback loop was associated with metastasis in mouse models and worse outcomes in patients. Similar to the GM-CSF and CCL18 positive feedback loop, macrophage-secreted IL-6 has been shown to modulate EMP through activating signal transducer and activator of transcription 3 (STAT3) signaling in cancer cells, which increases metastasis and macrophage recruitment through CCL2 (151) (Figure 2). In a separate study focusing on NSCLC, TAMs were found to create a pro-tumor niche, including induction of EMP and recruitment of Tregs in early tumorigenesis and seeding of a NSCLC cell line injected through the tail vein (152). These studies illustrate that the hybrid E/M phenotype promotes macrophage polarization into a phenotype that both sustains hybrid E/M phenotypes and the tumor-promoting macrophage phenotype itself, ultimately contributing to tumor progression.

Opposing Roles of NK Cells in EMP

Evidence for the role of EMP in regulating NK cells is controversial due to pleotropic, even conflicting, observations across studies. In melanoma cells, induction of EMP was found to increase resistance to NK cell cytotoxicity, which the authors attributed to increased surface expression of MHC class I – a suppressive signal to NK cells – along with decreased expression of ligands that activate NK cells (153) (Figure 2). Interestingly, no significant difference was observed in the surface expression of the NK cell activating ligand NKG2D upon EMP induction (153). Increased NK cell resistance was also shown in mesenchymal subclones from lung adenocarcinoma (compared to epithelial subclones) via sustained hypoxic stress of IGR-Heu cells established from primary NSCLC tumor samples (74). Increased resistance to NK cell cytotoxicity in the mesenchymal clones was associated with reduced tyrosine phosphorylation in contact areas of NK cells with mesenchymal subclones indicative of decreased NK cell activation. Hybrid E/M states facilitated this resistance – inhibiting EMP in the hypoxia-driven mesenchymal subclones increased their susceptibility to NK cell-mediated killing.

While some studies show decreased susceptibility of malignant cells to NK cell killing associated with EMP (153), others show increased susceptibility of malignant cells to NK cell cytotoxicity upon induction of EMP. For instance, in human lung cancer cell lines, TGFβ-mediated EMP induction triggered the initiation of metastasis yet increased susceptibility to NK cell-mediated killing by both a human NK cell line and NK cells isolated from healthy donors, which acted as a rate limiting step in EMP-driven metastasis (154). In this study, spontaneous metastasis of lung cancer cells was observed upon NK cell depletion in $RAG1^{-/-}$ mice, which lack T and B cells but retain functional NK cells. In addition to the findings in lung cancer, this study showed a similar effect of increased NK cell susceptibility in EMP-induced estrogen receptor positive breast cancer cell lines (154). A potential mechanism for these findings is that EMP induces the expression of the NK cell activating ligand cell adhesion molecule 1 (CADM1), while downregulating

type 1 E-cadherin ligand that inhibits NK cells (154). Interestingly, while EMP induction in this study was associated with increased expression of NKG2D ligands, namely UL16 Binding Protein 1 (ULBP1) and ULBP4, blocking NKG2D on NK cells did not affect NK cell-mediated lysis. However, another study has shown that inhibition of the NKG2D receptor with blocking antibodies decreased NK cell cytotoxicity against EMP-induced malignant cells (155). The contrasting findings of these studies may be due to differences in disease models and/or heterogeneity of EMP across cancer types. Further investigation of EMP and NK cell interactions will be critical to resolve these seemingly contradictory results and might reveal context-specific cues for these observations.

Although prior studies have provided limited insight into how malignant cells may leverage EMP to manipulate the surrounding immune cells, a recent study explored the role of NK cell-mediated cytotoxicity towards CTCs in a single cell form compared to CTCs in clusters using an *in vivo* mammary tumor model (94). Interestingly, CTC clusters had higher expression of an epithelial-related gene set (specifically genes which are suppressed by ZEB1 during EMT) and CDH1-related genes (whose low expression indicates a downregulation of E-cadherin). These more epithelial-like CTC clusters showed relatively decreased susceptibility to NK cell-mediated cytotoxicity relative to CTCs travelling as a single cell, which provided a metastatic advantage to the CTC clusters. Perturbation of EMP by the ZEB1 inhibitory miRNA, miR-200c, led to a more epithelial-like phenotype in the mammary cancer cells accompanied by a switch from single-cell dissemination to a collective dissemination as CTC clusters. Increased susceptibility of single cell CTCs to NK cell cytotoxicity upon induction of EMP was dominantly mediated by the NKG2D signaling pathway (Figure 2). Thus, this study suggests that EMP may confer mesenchymal features at the primary site to promote invasion while subsequently inducing epithelial features in circulation within CTC clusters to protect from NK cell cytotoxicity.

EMP and the Antigen Presentation Pathway

Signaling events associated with EMP contribute to decreased MHC class I presentation and result in an immune evasion phenotype (Figure 3). Peptides presented on MHC class I are generated in the proteasome or the immunoproteasome, the latter of which is the primary producer of MHC class I binding peptides and is induced by interferon gamma (IFNγ) (156). The immunoproteasome is defined by inclusion of catalytic subunits PSMB8, PSMB9, and PSMB10 (157). Increased expression of PSMB8 and PSMB9 has been associated with improved patient survival and slower disease progression in several cancers, including NSCLC (64) and melanoma (156). In melanoma, PSMB8 and PSMB9 expression was the most predictive marker of a response to anti-CTLA-4 or anti-PD-1 therapy, more so than an IFNγ gene signature, mutational load, or CTL infiltration (156). A clinical association between EMP and the immunoproteasome has been described in patient-derived samples of NSCLC, in which an intermediate mesenchymal phenotype (akin to hybrid E/M) with dual expression of epithelial and mesenchymal markers showed decreased immunoproteasome expression (64) (Figure 3). Similarly, EMP induced by TGFβ in NSCLC cell lines was associated with decreased protein levels of PSMB8 and PSMB9 (64) (Figure 3). These mesenchymal-like, hybrid E/M NSCLC cell lines showed increased levels of phospho-STAT3 and decreased levels of phospho-STAT1 relative to epithelial-like

NSCLC cell lines. STAT1 is activated by IFNγ and is a main promoter of NLR family CARD domain containing 5 (NLRC5) protein, a key promoter of genes in the MHC class I antigen presentation pathway including the immunoproteasome subunits (158,159). Thus, as expected, treatment of mesenchymal NSCLC cell lines with IFNγ increased phospho-STAT1 levels, immunoproteasome subunit expression, and lysis by CTLs (64). However, a pathway connecting EMP explicitly with STAT1 and NLRC5 in their regulation of antigen presentation has yet to be well defined, representing an opportunity to uncover new pharmacologic targets.

The EMT inducer AXL is implicated in downregulating MHC class I presentation genes in several cancer types, including NSCLC and melanoma (160) (Figure 3). AXL signaling mechanistically links EMP to an immune evasion phenotype. In the MMTV breast cancer model, a radiation therapy-resistant cell line was found to exhibit an EMP state along with increased AXL expression and decreased MHC class I expression relative to a radiation-sensitive cell line (161). AXL knockdown promoted a more epithelial state, restored MHC class I expression, and promoted tumor elimination *in vivo* (161). A role of EMP in decreased MHC class I expression has also been noted in melanoma tumor samples, in which SNAIL was the highest expressed gene in HLA-low tumors (162). In this study, 7 out of the 16 melanoma tumor samples with anti-PD-1 resistance had downregulation of MHC class I, which was associated with an increase in EMP and its associated gene expression and pathway activation, including AXL, SNAIL, and TGFβ signaling.

Using the MMTV breast cancer model and a SNAIL-YFP reporter in malignant cells, Dongre et al. also identified a link between the hybrid E/M phenotype, SNAIL, and the antigen presentation machinery (66). In this study, cells in the quasi-mesenchymal state (akin to hybrid E/M) had lower expression of PSMB9 and STAT1 (Figure 3). Additionally, relative to epithelial-like cells, the quasi-mesenchymal cells showed downregulation of proteins in the MHC class I antigen presentation pathway, including transporter associated with antigen processing 1 (TAP1), TAP binding protein (TABP), and immunoproteasome subunit PSMB9. A chromatin immunoprecipitation sequencing (CHIP-seq) assay identified that SNAIL binds DNA in proximity to these MHC class I presentation genes, suggesting SNAIL may inhibit transcription of these genes (66). Collectively, these studies suggest that a more mesenchymal phenotype is associated with decreased MHC class I antigen presentation, which mechanistically may be explained by aberrant AXL signaling, STAT1/STAT3 signaling, and SNAIL activity.

The Immunologic Synapse and EMP

The immunological synapse is an organized clustering of membrane-bound signaling and adhesive proteins between a T cell and any cell presenting an antigen, including malignant cells. Immunologic synapse disruptions have been observed *in vitro* between CTLs and mesenchymal malignant cells, namely decreased TCR signaling and reduced CTL killing of malignant cells with mesenchymal features (73–75). Adhesive proteins in the interaction of a CTL with an APC, including malignant cells, play an important role in effecting the tumor lysis capacity of CTLs. The interaction of CD103 (ITGAE) on CTLs with E-cadherin on malignant cells is one such adhesive interaction (Figure 4). The CD103-E-

cadherin interaction mediates polarization of cytolytic granules and subsequent exocytosis to the surrounding cancer cells that mediates cancer cell lysis (163). CD103 expression is upregulated in CTLs by TGFβ signaling, and CD103+ CTLs have increased anti-cancer activity compared to CD103− CTLs as well as increased secretion of TGFβ in the TME, which self-sustains their CD103 expression (164). Interestingly, the CD103+ population of CTLs has been shown to include the vast majority of tumor-specific CTL clones in several malignancies (164,165). Moreover, CD103+ CTLs exhibit localization to cancer cells expressing E-cadherin in lung cancer samples, and *in vitro* CTL migration toward cancer cell lines expressing E-cadherin was inhibited by an anti-CD103 blocking antibody (164). It stands to reason that hybrid E/M malignant cells are uniquely suited to induce CD103 expression on CTLs due to their increased TGFβ expression and their localization to the tumor-stroma border, while their mesenchymal features prevent CTL-mediated lysis. Furthermore, sustained E-cadherin expression in a hybrid E/M state may promote recruitment of CD103+ CTLs to hybrid E/M cells at the tumor-stroma border that in turn contributes to the induction of a hybrid E/M state via CD103+ CTL secretion of TGFβ, a major inducer of EMP (7) (Figure 4). As immune cells are often excluded from the tumor core and positioned at the tumor-stroma interface adjacent to hybrid E/M malignant cells, investigation of adhesive interactions and associated signaling between hybrid E/M malignant cells and CTLs may reveal determinants of immune exclusion from the tumor core.

EMP and its Modulation of CTL Motility

Structural proteins within the ECM may be specifically secreted or regulated by hybrid E/M malignant cells, representing an additional layer of interaction with the immune system. For example, laminin-332 is a structural protein enriched in the hybrid E/M state and serves as an archetypal example, contributing to decreased CTL migration and effector functions (166). This heterotrimeric protein consists of laminin α3, laminin β3, and laminin γ2 and can be secreted to the apical surface to promote migration via interactions with α3β1 and α6β1 integrin receptors located at the apical surface of cancer cells (167,168). Laminin-332 subunit enrichment at the leading edge has been observed in several cancer types, such as NSCLC, HCC, lung adenocarcinoma, and esophageal squamous cell carcinoma (27,166,169,170). Additionally, in three studies characterizing hybrid E/M states in primary samples or cancer cell lines, laminin-332 subunits were specifically enriched in the hybrid E/M state (27,35,171). Laminin γ2 expression can be induced by TGFβ through JNK/AP1 signaling (166). Importantly, the laminin γ2 subunit of laminin-332 has been shown to decrease T cell chemotaxis, downregulate TCR expression, and be a predictor of poor response to anti-PD-1 therapy in NSCLC and esophageal squamous cell carcinoma patients and cancer models (166). Although the precise effect of laminins expressed by cancer cells on immune cell functions is not well understood, given that immune cells express a variety of integrins capable of binding laminins (172), the laminin-immune interactions appear to be an important source of immune modulation by hybrid E/M malignant cells.

A major limitation in existing knowledge related to EMP and immune interactions is the use of approaches that merely capture a “snapshot” of *in vitro* CTL-hybrid E/M malignant cell interactions. *In vivo* studies of CTL-tumor interactions have found that CTLs remain motile

while on a target cell, have heterogenous killing rates ranging from zero to twelve target cells per day, and that malignant cells are more likely to die with multiple CTL interactions (173–175). Thus, assessing CTL killing *in vitro* as a snapshot of functional synapses as has been done in EMP and CTL interaction studies to date does not capture the complex cellular dynamics essential to tumor elimination. Indeed, CTL migration and scanning for antigens across the tumor-stroma border is important for CTL-mediated lysis: A study of CD44-knockout CTLs showed that these CTLs were less efficient in tumor killing due to decreased ability to migrate across the tumor despite retaining antigen-TCR interactions (176). Given that CTL motility across a tumor is crucial for tumor elimination and hybrid E/M malignant cells are localized at the tumor edge, investigation into the relation of hybrid E/M states and CTL interactions may reveal insights into mechanisms of tumor immune evasion that are associated with EMP.

EMP Confers Resistance to CTL Cytotoxicity

Cancer cells that retain MHC class I expression and antigen presentation for recognition by CTLs have mechanisms related to EMP that may prevent CTL-mediated lysis, including resistance to caspase- or mitochondrial-dependent apoptosis and the induction of autophagy. The EMP-associated TFs brachyury and MUC1 mediate CTL resistance (67,68,177) (Figure 3). Death ligands expressed on CTLs, such as Fas ligand (FASL) and TNF-related apoptosis inducing ligand (TRAIL), bind death receptors on target cells to induce intracellular signaling pathways in the target cell that lead to cell death (178). CTL cytotoxicity is also mediated by perforins that perforate the membrane and serine proteases called granzymes that enter the target cell (178). Brachyury has been shown to prevent caspase-mediated nuclear laminin degradation through CDK1 in the death receptor/death ligand pathway of CTL cytotoxicity in pancreatic, breast, lung, and colon cancer cell lines (177) (Figure 3). In CRC cell lines, brachyury has been shown to stabilize MUC1 mRNA, which in turn conferred increased resistance to mitochondrial-induced apoptosis and perforin- and granzyme-mediated lysis (67) (Figure 3). While EMP can support an anti-apoptosis phenotype in malignant cells, the anti-apoptotic proteins can also, in turn, induce EMP. For instance, the anti-apoptotic Bcl-2 family of proteins are involved in the induction of E/M states in cancer while also functioning to prevent granzyme-mediated apoptosis in malignant cells (179–181). Additionally, though classically described as anti-tumor interactions, the FAS/FASL interaction and the TRAIL-R1 and -R2 receptors that are implemented in CTL tumor killing also play important roles in modulating EMP in malignant cells (182,183).

Autophagy is a mechanism of CTL resistance that acts by neutralizing granzymes as seen in breast cancer cell lines exposed to hypoxia, a known inducer of hybrid E/M states (184). The breast cancer cell line MCF-7 transfected with the EMP TF SNAIL displayed greater resistance to CTL lysis than the parental, epithelial-like MCF-7 cells due to autophagy induction (73). A role for autophagy induction in CTL resistance was also identified in a CRISPR screen using several mouse and cancer cell lines involving 19,069 protein-coding genes (185). In both the CRISPR screen and SNAIL transfection studies, pharmacologic inhibition of autophagy increased CTL-mediated lysis of cancer cells. While EMP and autophagy are associated with CTL resistance, the mechanistic links between these two processes and signaling pathways are still undetermined. For instance, while the induction of

autophagy resists CTL-mediated lysis in hybrid E/M-induced MCF-7 cells, the induction of autophagy can also inhibit EMP through destabilization of EMT TFs (69). As the autophagy pathways involve stabilization or degradation of EMT TF proteins, we suspect there may be relevant post-translational interactions between the autophagy pathway and EMT TFs contributing to broader EMP regulation and CTL resistance.

Clinical Trials Coupling Immune Checkpoint Blockade with Agents Targeting EMP

As studies elucidate the critical signals mediating EMP and immune escape, there has been a corresponding increase in clinical trials addressing these pathways. Targeting EMP immune escape via inhibition of TGFβ, AXL, or IL-8 in combination with ICB is being evaluated in 88 clinical trials ranging from phase I to III in several solid cancer types. Most notably due the prominent role of TGFβ in EMP, 70 clinical trials – including phase I to phase III – feature TGFβ inhibition combined with ICB. These anti-TGFβ agents include small molecule inhibitors of TGFβ signaling including vactosertib (NCT03724851, NCT03732274, and NCT04064190) and LY3200882 (NCT02937272 and NCT04158700), the anti-PD-L1/TGFβ decoy receptor fusion proteins bintrafusp alfa (e.g., NCT04220775) and SHR-1701 (e.g., NCT04856774), and anti-TGFβ antibodies including BCA101 (NCT04429542), NIS793 (NCT04390763 and NCT02947165), and SAR439459 (NCT04524871). AXL inhibition combined with ICB is in 13 clinical trials ranging from phase I to phase III. AXL inhibitors include multi-tyrosine kinase inhibitors that inhibit AXL including cabozantinib (e.g., NCT04471428), INCB081776 (NCT03522142) and sitravatinib (NCT03941873 and NCT03666143), the AXL-specific inhibitor bemcentinib (NCT03654833 and NCT03184571), and the decoy receptor batiraxcept (NCT04004442 and NCT04019288) that binds the AXL ligand Gas6. Inhibitors of IL-8 being used in combination with ICB include the anti-IL-8 antibody BMS-986253 (NCT03689699 and NCT04572451) and SX-682 (NCT04599140, NCT04477343, and NCT03161431), which inhibits the IL-8 receptors CXCR1 and CXCR2.

The results of these clinical trials and mechanistic study of these combination therapies have yet to be completed. As such, the efficacy and mechanisms related to targeting the hybrid E/M cell-immune crosstalk from these combination therapies remain uncertain but will provide critical insight into the role of targeting EMP to improve ICB efficacy and patient outcomes. Given that even a small percentage (10%) of hybrid E/M cells can provide immune evasion to a tumor, identifying these mechanisms of immune evasion may reveal novel therapeutic targets to enhance ICB (85). Indeed, Dongre et al. (2021) found that CD73 expressed specifically in hybrid E/M cells supported tumor immune evasion, and upon knockdown, increased efficacy of ICB in a pre-clinical model of breast cancer (66). Future research that focuses on identifying hybrid E/M-specific mechanisms of tumor immune evasion may similarly define potential novel targets to increase ICB efficacy.

Conclusion

The study of EMP and immune interactions is an exciting field due to expanding evidence of a spectrum of hybrid E/M states with unique effects on the anti-tumor immune response

and therapeutic interventions. The spatial localization of malignant cells with a hybrid E/M phenotype at the tumor-stroma interface and in CTCs physically positions hybrid E/M cells in proximity to immune cells. EMP confers resistance to cytotoxicity from the anti-tumor immune response, while capitalizing on immune cell plasticity to polarize immune cells to an immunosuppressive phenotype that further promotes EMP. Indeed, this arrangement supports both soluble and contact signaling in the crosstalk between EMP and immune cells, which in general leads to immune suppression. Moreover, the spatial proximity to immune cells and the immunomodulatory effects of hybrid E/M malignant cells indicates that targeting the hybrid E/M state may overcome tumor immune evasion, especially in combination with ICB. Many studies to date have focused on classical EMT pathways in immune evasion and have identified associations between EMT and immune resistance. The distinction between a complete EMT and a hybrid E/M state and their presence in patient tumors are not always clear in prior studies. We believe that future investigations focused on establishing the mechanistic, causal roles of more subtle and distinct hybrid E/M states and their comparison to full mesenchymal states on immune evasion will be critical for advancing our understanding of tumor immunology and improving cancer treatment.

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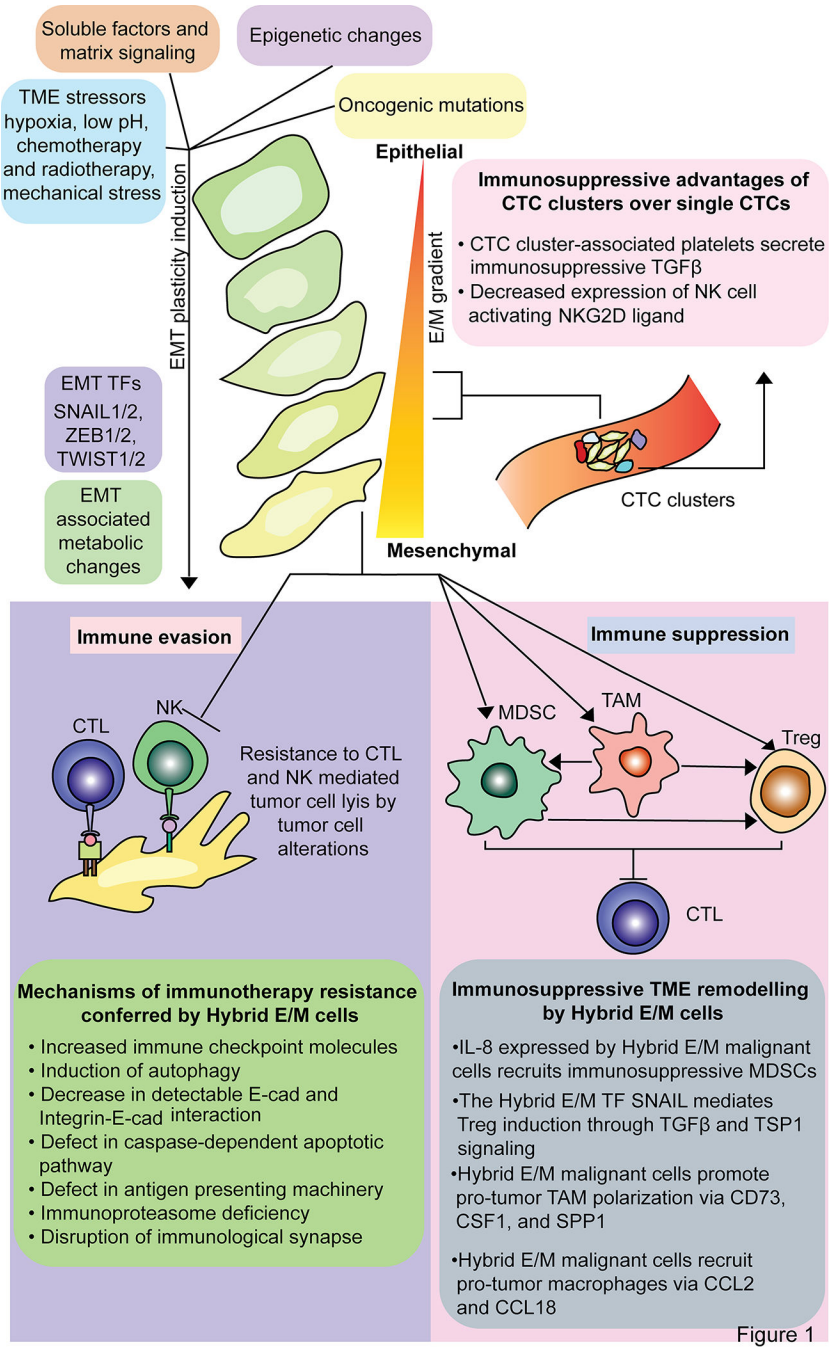

Figure 1. Induction of EMP promotes tumor immune escape.

EMP is induced by environmental and genomic factors that canonically activate SNAIL, ZEB, and TWIST transcription factor families. Hybrid E/M states mediate tumor immune escape through evading immune cell cytotoxicity and polarizing immune cells to an immunosuppressive phenotype.

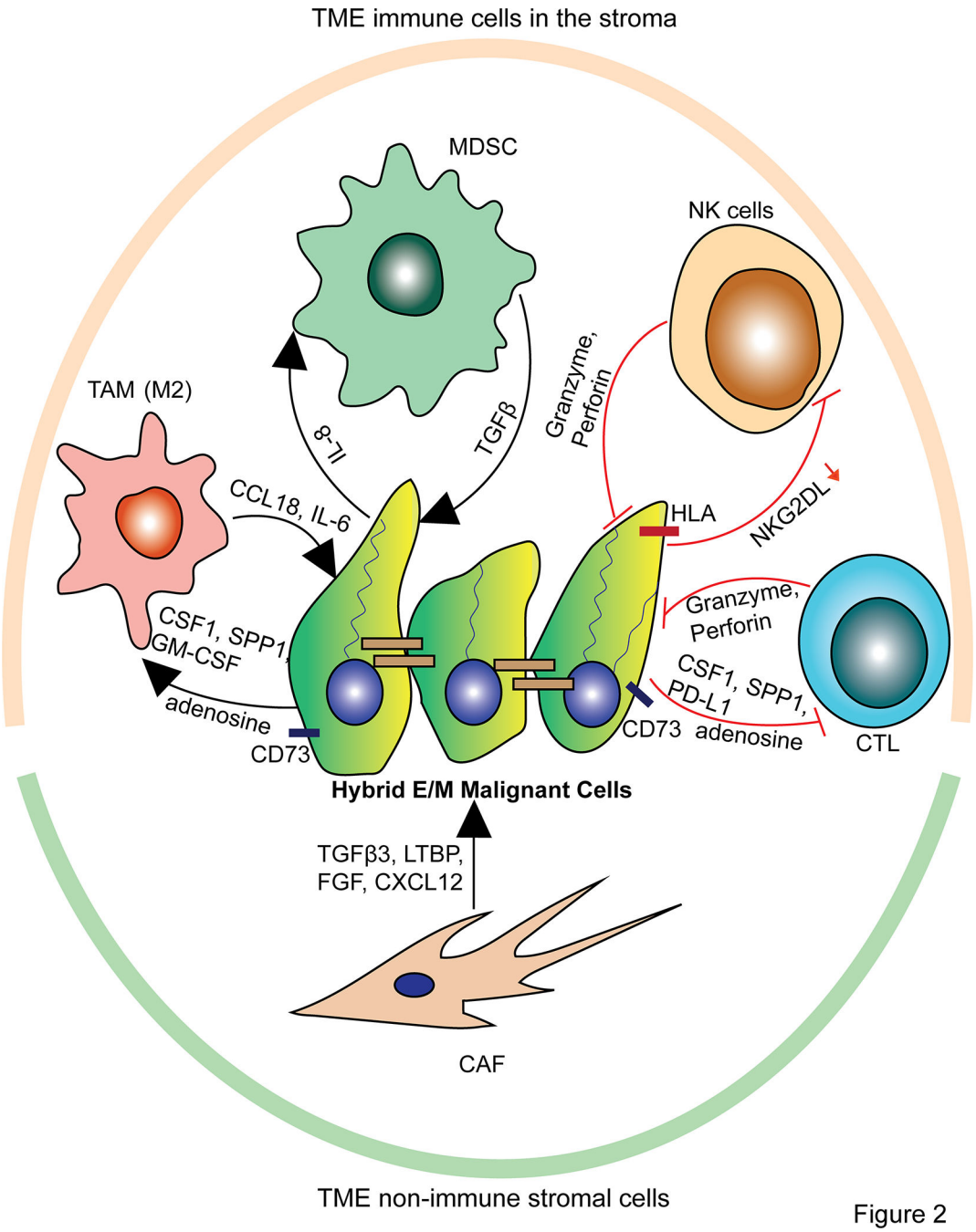

Figure 2

Figure 2. Crosstalk between hybrid E/M malignant cells and stromal cell populations.

MDSCs and TAMs are recruited and polarized to a pro-tumor phenotype by secreted factors from hybrid E/M malignant cells, and in turn, the polarized MDSCs and TAMs secrete factors that promote EMP. Additionally, CAFs promote EMP through their secreted factors. At the same time, hybrid E/M malignant cells resist NK cell- and CTL-mediated cytotoxicity via membrane bound proteins and secreted factors.

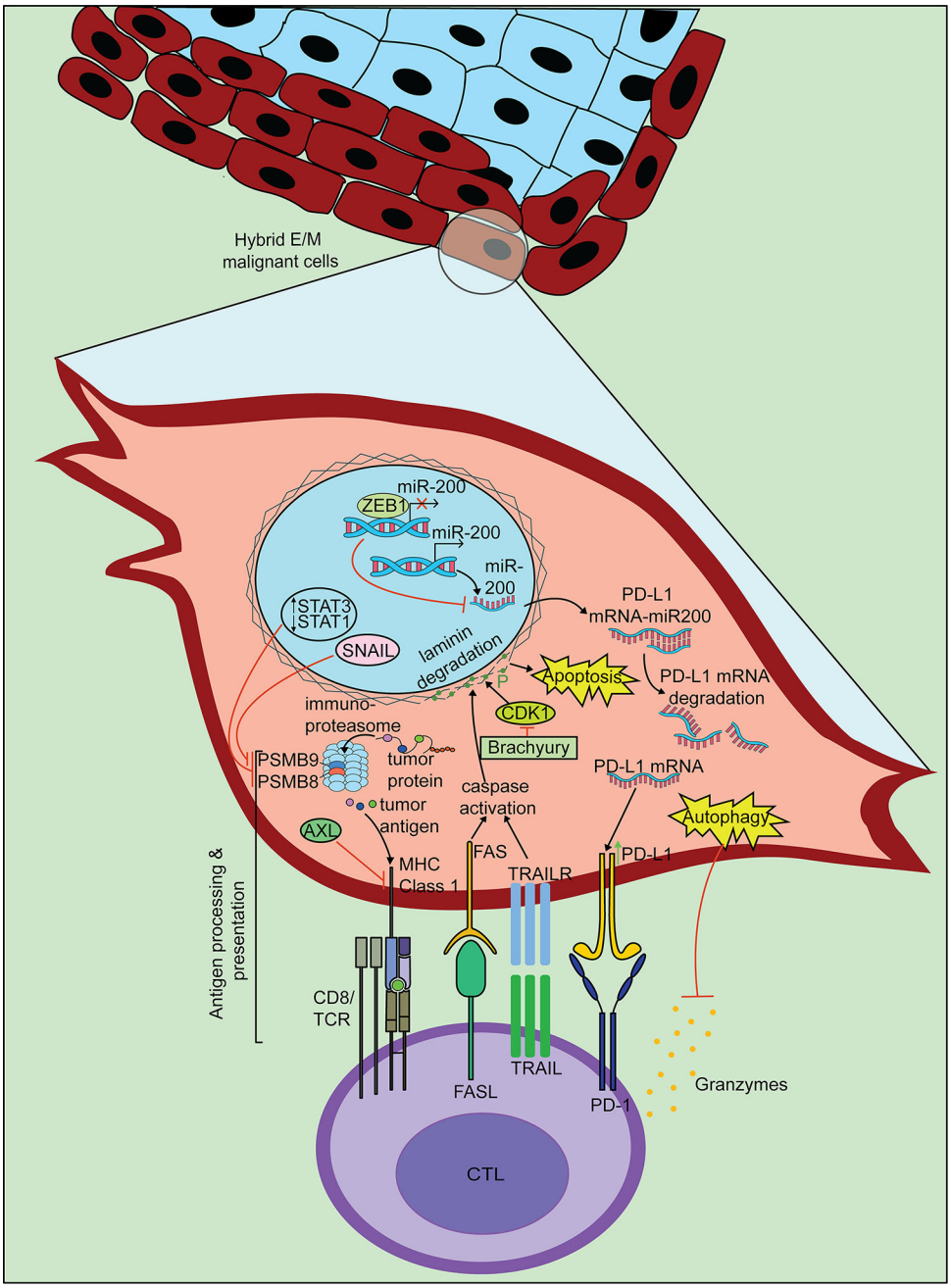

Figure 3

Figure 3. The hybrid E/M state prevents immune recognition and anti-tumor cytotoxicity of cancer cells by CTLs.

The hybrid E/M state prevents CTL recognition due to downregulation of the antigen presentation pathway. The tumor killing mechanisms of CTLs, including granzymes, FAS/FASL, and TRAIL/TRAILR, are blocked by hybrid E/M-associated signaling pathways including increased PD-L1 expression, autophagy induction, and inhibition of caspase-dependent cell death.

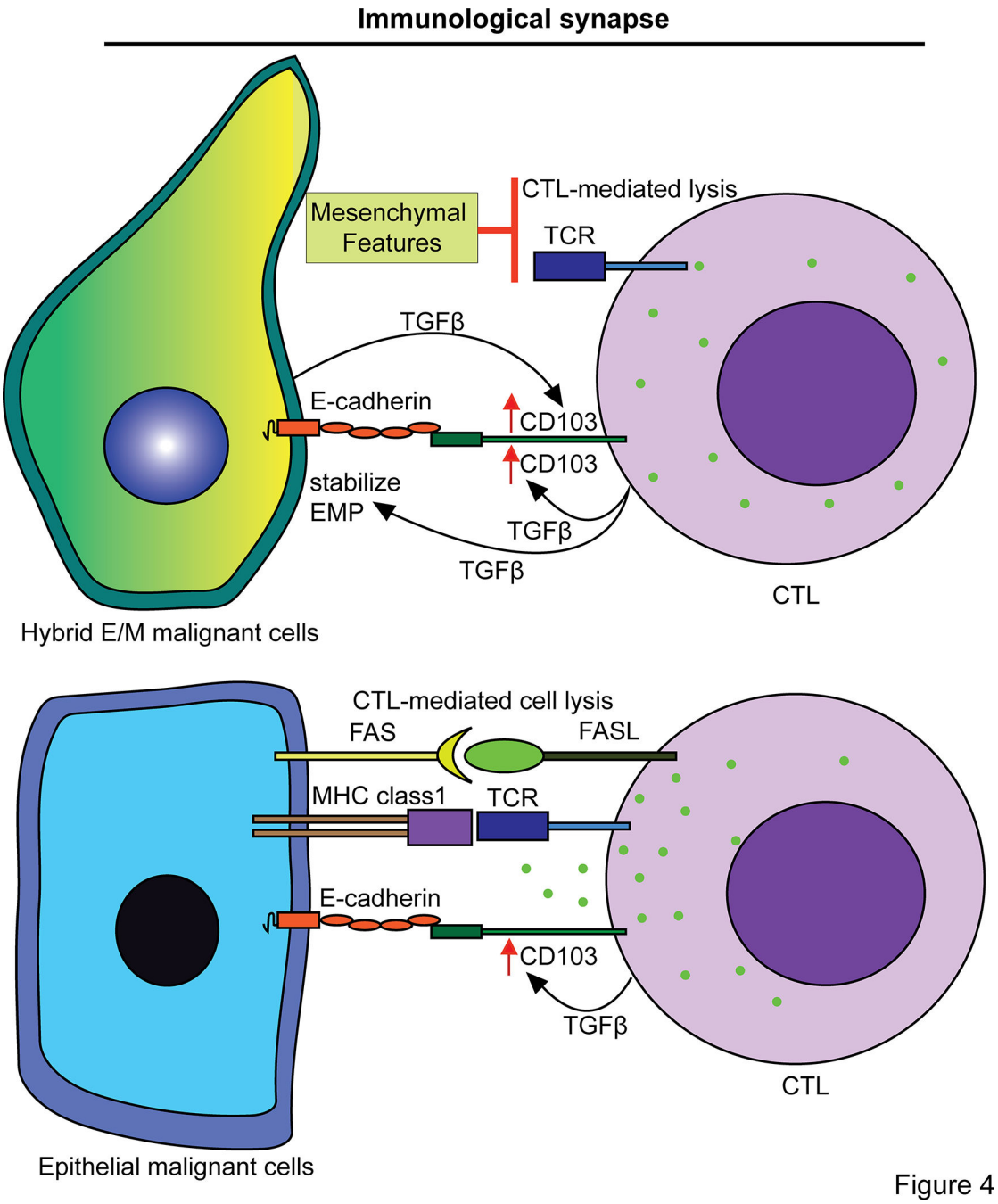

Figure 4. Potential regulation of the immunological synapse by hybrid E/M cells.

Hybrid E/M cells resist CTL-mediated cytotoxicity yet maintain adhesion proteins, such as E-cadherin, that are implemented in the immunological synapse with CD103+ CTLs, which secrete TGFβ and thereby may promote the hybrid E/M state.