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Impact of hypoxic tumor microenvironment and tumor cell plasticity on the expression of immune checkpoints

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ABSTRACT

Compared to traditional therapies, such as surgery, radio-chemotherapy, or targeted approaches, immunotherapies based on immune checkpoint blockers (ICBs) have revolutionized the treatment of cancer. Although ICBs have yielded long-lasting results and have improved patient survival, this success has been seriously challenged by clinical observations showing that only a small fraction of patients benefit from this revolutionary therapy and no benefit has been found in patients with highly aggressive tumors. Efforts are currently ongoing to identify factors that predict the response to ICB. Among the different predictive markers established so far, the expression levels of immune checkpoint genes have proven to be important biomarkers for informing treatment choices. Therefore, understanding the mechanisms involved in the regulation of immune checkpoints is a key element that will facilitate novel combination approaches and optimize patient outcome. In this review, we discuss the impact of hypoxia and tumor cell plasticity on immune checkpoint gene expression and provide insight into the therapeutic value of the EMT signature and the rationale for novel combination approaches to improve ICB therapy and maximize the benefits for patients with cancer.

1. Immune checkpoint blockade in cancer therapy

For over a century, scientists have attempted to eradicate cancer cells by activating and harness the patient's immune response. The majority of the cancer immunotherapies developed over the past two decades have been dedicated to the potentiation of anti-tumor adaptive immune response mediated mostly by T lymphocytes. Neither systemic administration of high-doses of interleukin (IL)-2 or autologous T lymphocyte transplantation has yielded very little to no therapeutic benefit and very high toxicity.

After decades of bench research and the resulting deeper understanding of the mechanisms underlying anti-tumor immunity, cancer immunotherapy has now finally moved into the clinic. The immune checkpoint blockade-based cancer immunotherapy revolution has just started and yet it has transformed the field of onco-immunology and the way we treat cancer. Immune checkpoint blockers (ICBs) such as anti-CTLA-4 and anti-PD-1/PD-L1 antibodies have reshaped the clinical approaches to multiple different types of cancers. ICBs have emerged as a revolutionary treatment for cancers including, but not limited to, breast, lung, kidney, bladder, prostate, lymphoma, and melanoma [1,2]. Ipilimumab (anti-CTLA-4) was approved in 2011 and pembrolizumab and nivolumab (anti-PD-1) were approved in 2014 by the U.S. Food and Drug Administration (FDA) for the treatment of advanced melanoma.

Despite the exciting and encouraging clinical responses, the majority of patients treated with ICB-based monotherapies (e.g., anti-CTLA-4, anti-PD-1, or PD-L1) only have partial responses and fail to achieve higher objective responses. Only 20–30% of patients with non-small-cell lung carcinoma (NSCLC), renal cell carcinoma (RCC), and melanoma benefited from cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death 1 (PD-1) blockade. ICB-unresponsive patients can be classified into two types: (1) primary resistance patients who did not respond at all and (2) acquired resistance patients who initially responded but later relapsed. It is now well established that enduring therapeutic benefit and prolonged survival can be achieved by combining several ICBs such as anti-CTLA-4 and anti-

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Abbreviations

ANGPTL4	Angiopoietin like 4
BRAFi	B-raf inhibitor
CAFs	cancer associated fibroblasts
ccRCC	Clear cell renal cell carcinoma
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cells
DNMT1	DNA-methyltransferase 1
DNMT3A	DNA methyltransferase 3A
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
EMT-TFs	EMT-Transcription factors
FDA	Food and drug administration
GITR	glucocorticoid-induced TNFR family related gene
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HLA-G	Human leukocyte antigen G
HRE	Hypoxia response element
ICBs	Immune checkpoint blockers
IDO	Indoleamine 2, 3-dioxygenase
IL-6	Interleukin 6
IL-10	Interleukin 10
LAG3	Lymphocyte-activation protein 3
MDSC	Myeloid derived suppressor cells
MET	Mesenchymal-to-epithelial transition
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
NK cells	Natural killer cells
NSCLC	Non-small-cell lung carcinoma
pADC	Pulmonary adenocarcinoma
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
RCC	Renal cell carcinoma
SIRPα	Signal regulatory protein alpha
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
TILS	Tumor-infiltrating lymphocytes
TKIs	Tyrosine kinase inhibitors
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells
TSP-1	Thrombospondin
VEGFA	Vascular endothelial growth factor A and
VHL	Von Hippel-Lindau

PD-1 [3,4].

2. Hypoxia in the tumor microenvironment

Hypoxia is a hallmark of all solid tumors' microenvironment and it is strongly associated with malignant progression, therapeutic resistance, and poor clinical outcome [5,6]. All solid tumors contain areas of variable oxygen concentration [7]. Within solid tumors, there are areas that are well oxygenated, poorly oxygenated, and, finally, necrotic in which tumor cells have died due to inadequate oxygenation [5].

Tumor cells adapt to the hypoxic microenvironment through the hypoxia-inducible factor (HIF) family of transcription factors. HIFs are heterodimeric proteins composed of an oxygen-sensitive alpha subunit (HIF-1α, HIF-2α, HIF-3α) and a beta subunit (HIF-β/ARNT). Both HIF-1α and HIF-2α are regulated by oxygen-dependent von Hippel-Lindau (VHL)-mediated degradation [6]. HIF-1α and HIF-2α share overlapping target genes and each one also regulates a set of unique targets. These hypoxia-dependent HIF-1α- and HIF-2α-induced genes play important roles in regulating different aspects of tumor biology such as angiogenesis, cell survival, chemo- and radio-resistance, proliferation, tumor cell plasticity, invasion and metastasis, pH regulation and metabolism, resistance to the immune system, and maintenance of cancer stem cells [6,8].

It is well established that cancer progression is not only regulated by the cancer cells but also influenced by the surrounding stroma. The tumor microenvironment (TME), in addition to cancer cells, includes a diversity of cells and factors such as cancer-associated fibroblasts (CAFs), endothelial cells, immune cells, growth factors, cytokines, and extracellular matrix proteins [9]. Loss of HIF-1α and its target gene VEGF-A but not HIF-2α enhanced tumor growth in MMTV-PyMT transgenic mice by reducing vascular density with less leaky vessels and decreased tumor-associated macrophage infiltration [10].

Hypoxia-mediated HIF activation has opposing effects in tumor and stromal cells. HIF1 activation in different cell types within the TME can either promote or repress tumorigenesis; HIF1 activation was shown to be the former in CAF by promoting autophagy and aerobic glycolysis, which in turn provides nutrients to the surrounding cancer cells and, thereby, promotes their growth. In contrast, HIF1 activation exhibited tumor suppressor activity in breast cancer cells [11].

3. Tumor cell plasticity and the epithelial mesenchymal transition

"Epithelial-to-mesenchymal transition" (EMT) was first described as a mechanism driving critical morphogenetic steps in the development of most metazoans and in wound-healing and carcinoma progression [12].

"Epithelial-to-mesenchymal transition" (EMT) is a reversible, dynamic cellular program during which either healthy or neoplastic epithelial cells transform into a more motile, invasive, and aggressive mesenchymal cell type. The resulting mesenchymal-like cells can revert back to the epithelial state, which is known as the mesenchymal-to-epithelial transition (MET). Upon EMT initiation, the expression of epithelial markers (e.g., E−cadherin) is repressed, which leads to the loss of the polygonal, round, cobblestone morphology of epithelial cells. The cells then acquire a spindle-shaped, elongated mesenchymal morphology and express mesenchymal markers (e.g., neural cadherin, vimentin, and fibronectin). EMT is orchestrated by a series of master EMT-inducing transcription factors (EMT-TFs) including ZEB, SNAIL, and TWIST, which inhibit or represses the expression of genes associated with the epithelial state [13]. To quantitatively measure the interplay between EMT and cancer progression, universal computed EMT scoring has been defined from cancer-specific transcriptomic EMT signatures of ovarian, breast, bladder, lung, colorectal and gastric cancers. The EMT score was defined based on the expression of multiple EMT markers including the major EMT-drivers, SNAI1 and ZEB1. Thus, Samples with a positive (high) EMT score were more mesenchymal, whereas those with a negative (low) score were more epithelial [12].

Several cell-intrinsic signaling pathways (TGF-β, WNT, STAT, and NOTCH) also induce EMT-TFs leading to a phenotypic transition to a mesenchymal or partially mesenchymal cell state [14]. Importantly, several stromal cells (e.g., CAFs) and immune cells (e.g., $CD4^+$ helper and $CD8^+$ cytotoxic T cells, Treg, MDSCs, TAMs) in the TME are known to secrete various cytokines (e.g., TGF-β, IL6, TNF-α), chemokines (e.g., CCL18), and growth factors (e.g., VEGF, HGF, and EGF). These secreted factors act in a paracrine fashion to induce EMT in the surrounding

cancer cells either by activating EMT-TFs or by inducing effector molecules that inhibit the epithelial state [13].

Once activated in cancer cells, EMT promotes resistance to cell death inducers including chemotherapy. EMT was shown to contribute to cyclophosphamide resistance in a spontaneous breast-to-lung metastasis model [15]. Similarly, EMT suppression through the deletion of Snail or Twist in a murine model of pancreatic cancer led to increased sensitivity to gemcitabine treatment [16].

EMT supports stemness, immune evasion, immune suppression, and resistance to immunotherapy [13,17–19]. Both human and mouse melanoma cells transfected with snail1 underwent EMT and became more metastatic than their parental cells. Snail1-expressing melanoma cells secreted TGFβ and thrombospondin, which favored the emergence of highly immunosuppressive regulatory T cells. Snail small interfering RNA (siRNA) injected *in vivo* reduced the immunosuppressive and metastatic potential of these melanoma cells [20]. Likewise, the CTL-mediated lysis of MCF-7 snail cells decreased considerably due to the induction of autophagy [21]. Interestingly, tumors that best respond to CTLA-4, PD-L1, and PD1 (e.g., melanomas, renal, bladder and lung cancers [2]) have higher EMT scores [12].

4. Hypoxia-dependent regulation of immune checkpoints in cancer

Hypoxia contributes to the immunosuppressive tumor microenvironment in many cancers by activating intrinsic mechanisms that allow tumor cells to escape from the innate and adaptive immune defenses [8]. Moreover, several lines of evidence suggest that the establishment of this immunosuppressive microenvironment could be attributed to the ability of hypoxia to regulate immune checkpoints. We have previously shown that the mRNA and protein expression of programmed cell death ligand 1 (PD-L1), but not programmed cell death ligand 2 (PD-L2), in myeloid-derived suppressor cells (MDSCs) infiltrating several tumor types, is higher than splenic MDSCs indicating that factor(s) in the tumor microenvironment is (are) involved in the upregulation of PD-L1. We have further identified hypoxia as a major factor involved in the upregulated expression of PD-L1 in tumor-infiltrating MDSCs since the exposure of MDSCs isolated from the spleen to hypoxic conditions dramatically increased the expression of PD-L1 but not PD-L2. Further investigation revealed that, upon its stabilization in hypoxic cells, HIF-1α binds the hypoxia-response element (HRE) located in the proximal PD-L1 promoter. The functional consequences of the overexpression of PD-L1 on the surface of MDSCs include the increased production of IL-6 and -10 under hypoxic conditions and significantly decreased proliferation of $CD8^+$ T cells; taken together, these phenotypic changes are indicative of enhanced immunosuppressive function when MDSCs are exposed to hypoxia. Treatment with an anti-PD-L1 antibody significantly decreased both the expression of IL-6 and -10 and the $CD8^+$ T cells anti-proliferative property of MDSC [22]. Moreover, a combination therapy targeting tumor hypoxia with an antibody against PD-L1 may be beneficial for stimulating the anti-tumor immune response in patients with cancer. Our data were supported by Barsoum et al., who showed that hypoxia upregulates the expression of PD-L1 on the surface of human breast and prostate cancer cells, as well as in mouse melanoma and mammary carcinoma cells. Increased expression of PD-L1 leads to tumor cell resistance to CTL-mediated lysis [23] (Fig. 1A).

In addition to PD-L1, HIF-1α also regulates the expression of macrophage immune checkpoint marker CD47 under hypoxic conditions (Fig. 1A). CD47 is a “don't eat me” signal found on the surface of several cancer cell types. Following its interaction with signal regulatory protein alpha (SIRPα) and thrombospondin-1 (TSP-1), which are expressed by tumor phagocytic cells, the phagocytosis of tumor cells is inhibited by the coordinated blockade of the “eat me” signal and the activation of the “don't eat me” signal [24]. As anti-CD47 blockade-based therapy is now moving into the clinic [25], understanding the molecular mechanism involved in the regulation of CD47 is a highly prioritized area of research toward enhancing cancer immunotherapeutic approaches using CD47 blockade. Thus, it has been shown that HIF-1α regulates the transcription of the CD47 gene by directly binding to its promoter in breast cancer cells. Targeting CD47 increases the phagocytic ability of macrophages against breast cancer cells. According to The Cancer Genome Atlas (TCGA), in an analysis of thousands of patients with breast cancer, CD47 expression is correlated with HIF target gene expression such as angiopoietin-like 4 (ANGPTL4) and vascular endothelial growth factor A (VEGFA). Increased CD47 mRNA levels are associated with decreased patient survival [26]. Furthermore, the induction of HIF-1α by chemotherapy directly activates PD-L1, CD47, and CD73 gene transcription in triple-negative breast cancer (TNBC) making their TME more immunosuppressive by impairing the adaptive anti-tumor immune response (Fig. 1A) [27].

Hypoxia increases the expression of CD137 (4-1BB) in tumor-infiltrating T lymphocytes (TILs) in colon carcinomas, melanoma, and spontaneous breast adenocarcinomas (Fig. 1A). CD137 is expressed on both innate (NK cells) and adaptive immune cells (activated T cells). The CD137 ligand (CD137L) is expressed exclusively on the surface of activated antigen-presenting cells (macrophages, dendritic cells [DCs] and B cells). CD137L binding to CD137 induces a strong antitumor immune response mediated by activated T and NK cells. In HIF-1α knockout T cells, hypoxia fails to induce CD137 expression on the surface of TILs and they remain CD137 negative even when becoming TILs. Combining anti-CD137 agonists results in a synergistic effect on the PD-L1 blockade [28]. The same group also showed that a soluble form of CD137 (sCD137) was induced under hypoxia in multiple murine and human cancer cell lines. This secreted sCD137 blocks CD137L mediated co-stimulation of activated T cells [29].

Human leukocyte antigen G (HLA-G) is another immune checkpoint marker that contributes to tumor immune evasion. A correlation between the expression of HLA-G and poor clinical patient outcome has

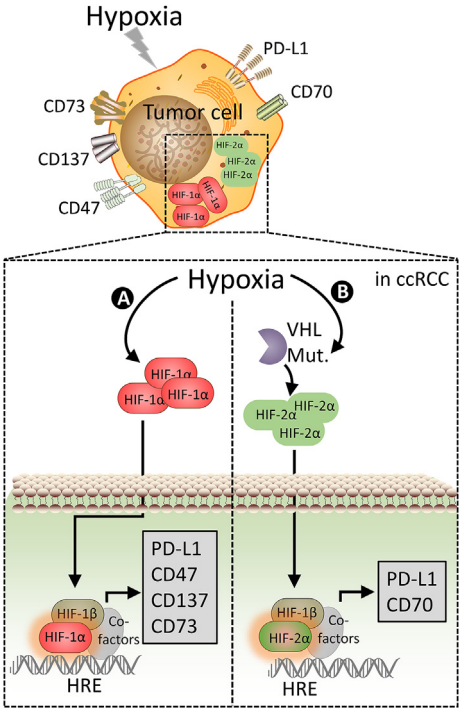

Fig. 1. The impact of hypoxia on the regulation of immune checkpoints in tumor cells. Under hypoxic conditions, stabilized HIF-1α **(A)** or mutated VHL-dependent stabilized HIF-2α in ccRCC cells **(B)** translocates to the nucleus and forms a complex with HIF-1β and its co-factors. This complex binds to the hypoxia-response element (HRE) motif and induces the expression of several immune checkpoint genes such as PD-L1, CD47, CD137, CD73, and CD70.

been described [30]. In melanoma, the expression of HLA-G positively correlated with the hypoxic status of these tumors [31]. In glioma cells, HLA-G gene expression is mediated by HIF-1α through binding to the HRE motif located in exon 2 [32].

In patients with clear cell renal cell carcinoma (ccRCC) and VHL bi-allelic inactivation, the expression of PD-L1 was increased compared to the ccRCC tumors with wild-type VHL. Using 786-O cells expressing different VHL mutants with stabilized HIF-2α, we demonstrated that HIF-2α and PD-L1 expression are positively correlated. Indeed, targeting HIF-2α in ccRCC cells significantly decreased PD-L1 mRNA and protein expression levels. Using chromatin immunoprecipitation and luciferase assays, we found that, similar to HIF-1α, HIF-2α regulates the expression of PD-L1 by binding directly to the HRE motif in the PD-L1 proximal promoter (Fig. 1B). In VHL-mutated RCC4 renal cells that express both HIF-1α and HIF-2α, the knock-down of HIF-1α or HIF-2α or both decreased the expression of PD-L1 suggesting that, in 786-O cells, PD-L1 is a direct target of HIF-2α; however, in RCC4 cells, PD-L1 expression is regulated by both HIF-1α and HIF-2α. These data highlight the rationale behind treating patients with RCC using anti-PD-L1/PD-1 immunotherapies and suggest that the VHL mutation status could potentially be used as a biomarker predictive of RCC response to anti-PD-L1/PD-1 immunotherapy [33]. Another study supported these data by showing a positive correlation between PD-L1 and HIF-2α target genes in ccRCC [34]. CD70 is an immune checkpoint factor from the tumor necrosis factor (TNF) family. The CD70 ligand activates T cells by binding to the CD27 receptor expressed on the surface of these lymphocytes [35]. The overexpression of CD70 has been well documented in RCC and seems to be driven by HIF in ccRCC tumors with defects in pVHL (Fig. 1B). In ccRCC tumors, the overexpression of CD70 seems to correlate with the release of soluble CD27 from the tumor-infiltrating lymphocytes, which thereby prevent T cell activation [36].

Cytotoxic T lymphocytes (CTLs) are the main weapons of destruction against various pathogens and tumor cells. The CTL-mediated anti-tumor immune response is regulated by hypoxia and controlled in part by HIFs and VHL. The deletion of VHL alters the differentiation of effector and memory $CD8^+$ T cells. VHL-deletion also resulted in elevated HIF expression, which sustained CTL effector function. Moreover, hypoxia modulated the expression of critical transcription factors, effector molecules, co-stimulatory receptors (4-1BB, GITR, and OX40) and activation-induced inhibitory receptors (LAG-3 and CTLA-4) in a HIF-1α- and HIF-2α-dependent manner (Fig. 2C) [37]. The hypoxia-mediated regulation of different immune checkpoints in various cancer types is summarized in Table 1.

5. Involvement of EMT in the regulation of immune checkpoints

In addition to its ability to regulate ICBs, several lines of evidence suggest that hypoxia could act as an EMT inducer and that the induction of EMT subsequently regulates the expression of immune checkpoint-associated factors. Indeed, the hypoxia-dependent induction of EMT causes morphological changes and loss of E-cadherin expression in hepatocellular carcinoma cell lines. Moreover, HIF-1α induces the expression of CCL20 in these cancer cells allowing for metabolism changes in macrophages characterized by the expression of indoleamine 2, 3-dioxygenase (IDO) and increased numbers of $Foxp3^+$ regulatory T cells (Treg cells) and subsequent decreased T-cell proliferation [38] (Fig. 3A). It seems that a complex interplay between hypoxia, EMT, and immune checkpoint gene expression exist. Nevertheless, little is known about how tumor cell plasticity regulates the expression of immune checkpoints. Our group has discovered that EMT-TF plays a crucial role in the regulation of PD-L1 [39] and CD47 [40] in human breast cancer cells undergoing EMT.

In keeping with this, we reported that driving EMT by overexpressing EMT-TF in epithelial MCF7 breast cancer cells induced the upregulation of PD-L1. PD-L1 upregulation in mesenchymal breast cancer cells resulted in tumor cell escape from CTL-mediated killing. Surprisingly, treatment of MCF7 with EMT-inducing factor TGF-β, or inhibiting TGF-β signaling in mesenchymal-like MDA-MB-231 cancer cells, had no impact on either mRNA or protein levels of PD-L1. Further investigation of EMT-activated breast cancer cells revealed that the EMT-dependent overexpression of PD-L1 requires the ZEB1/miR-200 axis and SNAI1 but not SLUG (Fig. 3B). Importantly, targeting PD-L1 by siRNA or treating mesenchymal cells with an anti-PD-L1 blocking antibody increased the susceptibility of tumor cells to CTL-mediated killing [39]. These findings were supported by Chen and colleagues who showed that ZEB1 removed the repression of miR-200 on PD-L1 protein levels. Therefore, ZEB1 expression during EMT leads to PD-L1 expression in tumor cells and the subsequent suppression of $CD8^+$ T-cell activity and metastasis. This observation was further experimentally supported in tumors generated from 393P cells overexpressing ZEB1. These tumors exhibited an increase of overall tumor burden, metastasis lung nodules, a significantly reduction in total $CD8^+$ TILs and an increased in exhausted $CD8^+$ T cells (PD-1 + TIM-3 +). Pharmacological treatment of tumors generated from 393P cells overexpressing ZEB1 with ICB (anti-PD-L1) resulted in a reduction in tumor size, metastasis and exhausted $CD8^+$ T cells [41]. In addition to $CD8^+$ T cells, Dongre and colleagues have observed that EMT impacted other immune cell populations. Thus, an increased percentage of suppressive Tregs, pro-tumor M2 markers arginase1 and reduced level of the anti-tumor M1 marker iNOS was observed in mice transplanted with mesenchymal cell line compared to epithelial cell line derived from MMTV-PyMT mouse model. It has been reported that the efficiency of anti-CTLA-4 was only observed in epithelial tumors but not in mesenchymal tumors [42].

More recently, we reported that CD47 is upregulated in EMT-activated breast cancer cells compared with epithelial-like phenotypes. Indeed, CD47 levels were found to correlate with the expression of EMT marker genes in TCGA and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) databases. The overexpression of both SNAI1 and ZEB1 in epithelial MCF7 cells or their inhibition in mesenchymal MDA-MB-231 regulates CD47 expression at both the mRNA and protein levels. Therefore, we concluded that SNAI1 and ZEB1 can directly bind to two E-box motifs in the human CD47 proximal promoter region (Fig. 3B). Finally, we reported that targeting SNAI1, ZEB1, or CD47 rescues macrophage-mediated phagocytosis in mesenchymal MDA-MB-231. Our *in vitro* data highlight CD47 as a direct target of SNAI1 and ZEB1 and its blockade induces the phagocytosis of breast cancer cells undergoing EMT [40]. Overall, based on our data showing that EMT can regulate both CD47 and PD-L1, it is tempting to speculate that combining anti-CD47 and anti-PD-L1 agents will simultaneously reactivate both innate (macrophage checkpoint CD47) and adaptive immunity (T-lymphocyte checkpoint PD-1) and ultimately

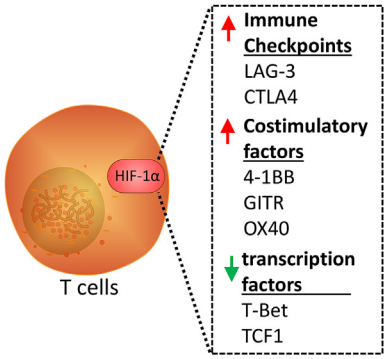

Fig. 2. The impact of hypoxia on the regulation of immune checkpoints in T cells. Through HIF-1a stabilization, hypoxia upregulates the expression of inhibitory immune checkpoints (LAG-3 and CTLA4) and the co-stimulatory factors (4-1BB, GITR, and OX40) but downregulates the transcription factors T-Bet and TCF1. Together, these events promote the effector function of $CD8^+$ T cells under hypoxic conditions.

Table 1
Summary of immune checkpoints that are regulated by either HIF-1α or HIF-2α in different cancer types.

Immune checkpoint	HIF-1 or HIF2	Cancer type	References
PD-L1	HIF-1α	Human breast	[23,27]
		Human prostate	[23]
		Mouse melanoma	[22,23]
		Human kidney	[23]
CD47	HIF-1α	Human breast	[26]
CD73	HIF-1α	Human breast	[27]
CD137 (4-1BB)	HIF-1α	Mouse colon	[28]
		Mouse melanoma	
		Mouse breast	
HLA-G	HIF-1α	Human melanoma	[31]
		Human glioblastoma	
CD70	HIF-1α	Human kidney	[35]
PD-L1	HIF-2α	Human kidney	[33]
CD70	HIF-2α	Human kidney	[36]

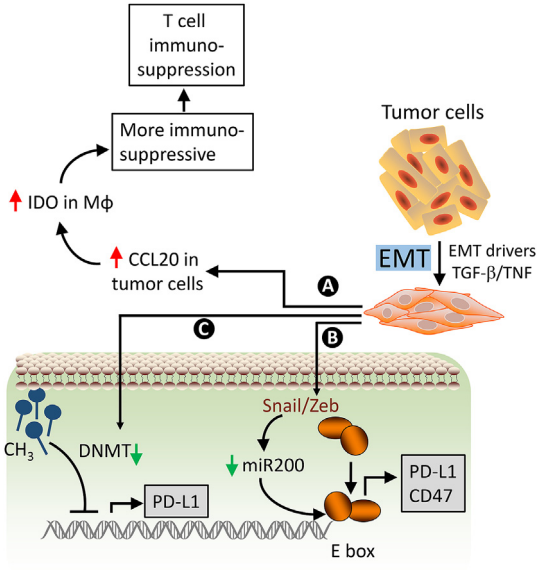

Fig. 3. The impact of EMT on the regulation of immune checkpoints. **A.** Driving EMT leads to an increase of CCL20 in tumor cells and a subsequent increase in indoleamine 2, 3-dioxygenase (IDO). IDO leads to metabolism changes in macrophages and an increase in Foxp3+ regulatory T cells (Treg cells) and a subsequent decrease in T-cell proliferation. **B.** EMT induces associated transcription factors Snail/Zeb. These transcription factors decrease miR-200, thus enhancing the expression of PD-L1. Snail/Zeb can also directly bind to the E box motif in the promoters of PD-L1 or CD47 and induce their expression. **C.** The overexpression of PD-L1 in cancer cells undergoing EMT by TGF-β/TNF-α treatment relies on the downregulation of DNA methyltransferase (DNMT), which prevents the methylation of the PD-L1 promoter.

provide more frequent, durable, and longer lasting responses in patients with breast cancer marked by highly aggressive, mesenchymal, and metastatic tumors. Consistent with our findings, it has been demonstrated that metastatic tumors have high CD47 expression levels compared to primary tumors from patients with melanoma and the *in vitro* blockade of CD47 in metastatic melanoma cells re-activated macrophage-mediated phagocytosis [43].

While the data described above clearly support the involvement of EMT in the transcriptional regulation of PD-L1, new evidence indicates that PD-L1 could also be regulated by epigenetic modification during the EMT process. This regulation is due to DNA demethylation and the NF-kβ pathway through a mechanism involving TGF-β and TNF-α respectively in NSCLC. Indeed, it has been shown that the overexpression of PD-L1 during EMT required both TGF-β and TNF-α treatment. TGF-β treatment decreased levels of DNA-methyltransferase 1 (DNMT1), resulting in PD-L1 promoter demethylation (Fig. 3C). However, TNF-α treatment induced the NF-kB pathway involved in promoting the expression of demethylated PD-L1 promoter in NSCLC [44]. Similarly, in prostate cancer cells, reduced expression of DNMT1 plays a role in the induction of EMT and a cancer stem cell phenotype [45]. In gastric cancer, the overexpression of DNA methyltransferase 3A (DNMT3A) represses E-cadherin expression and promotes cell migration and invasion. Indeed, DNMT3A expression levels are positively correlated with lymph node metastasis and poor prognosis in gastric cancer. Thus, multiple lines of evidence have illustrated the role of the DNMT family in promoting the regulation of immune checkpoints and EMT in a cancer-dependent manner [46].

Recently, Ricciardi and colleagues co-cultured T, B, and NK cells with multiple cancer cell lines undergoing EMT and found decreased lymphocyte proliferation and increased NK and T-cell apoptosis through a mechanism involving IDO but not the Fas ligand pathway [47].

More recently, it was reported that a subtype of breast cancer cells with low expression of claudin showed bidirectional crosstalk between PD-L1 expression and EMT. Indeed, PD-L1 downregulation in claudin-low breast cancer cells leads to CD44 and vimentin downregulation and CD24 upregulation [48].

According to a patient-derived pan-cancer analysis, EMT signatures strongly correlate with drug sensitivity and global molecular alterations at the DNA, RNA, and protein levels. In addition, a high mesenchymal EMT score was associated with high expression of immune checkpoints such as PD1, PD-L1, CTLA4, OX40L, and PD-L2 in 11 cancer types [49]. Additionally, a highly positive correlation exists between EMT and immune checkpoint-related genes including PD-L1, PD-L2, PD-1, TIM-3, B7–H3, BTLA, and CTLA-4 in colorectal cancer [50]. Furthermore, PD-L1 was upregulated in metastatic colorectal cancer compared to primary tumors [51] and this upregulation was closely related to EMT markers in patients with pulmonary adenocarcinoma (pADC) [52]. Indeed, in the high-risk group of patients with hepatocellular carcinoma (HCC), the expression of PD-L1 was correlated with an EMT phenotype and poor survival [53]. In addition, PD-L1 overexpression seems to be associated with developing resistance to a B-RAF inhibitor (BRAFi) in patients with metastatic melanoma [54]. In prostate cancer, N-cadherin upregulation was correlated to an immune regulatory signature comprised of IDO overexpression and increasing numbers of Treg cells [55]. However, therapy-induced E-cadherin downregulation leads to PD-L1 downregulation in lung cancer cell lines suggesting a common pathway for E-cadherin and PD-L1 [56]. Furthermore, the expression of newly emerging immune checkpoints CD276, OX40, and TGFB1 is correlated with the expression of EMT genes in kidney cancer [57]. The EMT-mediated regulation of PD-L1 and CD47 in various cancer types is summarized in Table 2.

6. Conclusions

While the role of EMT in promoting an aggressive and metastatic phenotype in tumor cells is well established and extensively documented, new evidence is accumulating that points to the critical role of

Table 2
Summary of immune checkpoints that are regulated by EMT transcription factors in different cancer types.

Immune checkpoint	EMT	Cancer types	References
PD-L1	SNAI1/ZEB1/miR-200	Human breast	[39]
	ZEB1/miR-200	Human lung	[41]
	SNAI1	Mouse breast	[42]
	TGF-β reduces DNMT1	Mouse lung	[44]
	TNF-α induces NF-κB	Mouse lung	[44]
CD47	ZEB1/SNAI1	Human breast	[40]

this process in the regulation of the expression of immune checkpoint genes. The classification of primary tumors according to their EMT score revealed that mesenchymal-like tumors were highly enriched in immune checkpoint markers compared to epithelial-like tumors. Similarly, the tumor EMT score could be a valuable biomarker for stratifying patients who will benefit from ICB immunotherapies. Moreover, and consistent with the fact that the EMT process could regulate many immune checkpoints, we strongly believe that considering the EMT score of tumors could help tailor immunotherapy-based treatments and, thereby, optimize patient outcome.

In addition to serving as a biomarker for immunotherapy, EMT is a remarkable therapeutic target for cancer treatment [58]. It has been proposed that therapies able to revert the mesenchymal state of a tumor back into an epithelial one can prevent metastasis [59]. However, the complex interplay between hypoxia and EMT should be considered when using inhibitors targeting either mechanism. It will be interesting to know whether combining drugs that target hypoxia or HIF with EMT inhibitors helps to overcome therapy resistance or/and to block tumor cell dissemination and metastasis. We strongly believe that such a combination will have an additive or possibly even synergistic effect in combating those elements, which are critical for cancer's virulence.

Many protocols for tumor immunotherapy in the clinic have not taken into consideration the role of hypoxia and EMT. So far, to enhance the effectiveness of immune checkpoint blockade-based cancer immunotherapies, the combination of various existing immuno-therapeutic strategies have been used. These strategies include the administration of multiple immunotherapies that together confer a more efficacious and longer-lasting response. However, given the wide spectrum of changes that occur during EMT and hypoxia, new combination therapies, including EMT and hypoxia inhibitors, are also being investigated. Indeed, accumulating evidence indicates that improving the therapeutic response to PD-1/PD-L1 blockade in urothelial cancer could be achieved by simultaneously targeting the EMT-inducing TGF-β pathway [60]. The therapeutic value of this combination has also been demonstrated in esophageal squamous cell carcinoma [61]. Additional studies are warranted to determine if other cancer types share these features. Moreover, it has been proposed that targeting the EMT driver AXL would have the dual benefit of being an anticancer therapeutic as well as synergistically activating the antitumor immune response [62,63]. In addition to EMT, recent data showed the ability of reduced hypoxia, achieved using a hypoxia-activated prodrug, to sensitize multiple preclinical models of prostate cancer to the T cell checkpoint blockade. In keeping with these data, a clinical trial is currently ongoing to evaluate the therapeutic benefit of combining hypoxia prodrug TH-302 and anti-CTLA4 against several cancers including prostate, melanoma, and pancreatic tumors (NCT03098160).

As the response to cancer immunotherapy varies considerably from one patient to another, it remains critical to identify biomarkers predicting how patients with cancer will respond to different treatments, notably to the combination therapy given its increased toxicity. While several biomarkers have been identified, including microsatellite instability, the expression of PD-L1 in the tumor as valuable biomarker predicting the response to PD-1/PD-L1-based cancer immunotherapy is still currently debating [64]. Indeed, in certain tumor types, a benefit with immunotherapy was observed irrespective of PD-L1 expression level [65,66].

The number of immunotherapy combination trials initiated each year has been increasing dramatically. However, three major questions arise when designing a successful combination approach to cancer immunotherapy: i) what to combine? ii) how to combine? and iii) when to combine? Nevertheless, all of these clinical trials will soon provide an avalanche of data that should provide insight into how best to tailor cancer immunotherapy treatments for each patient with cancer.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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