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Review Article

Mesenchymal traits at the convergence of tumor-intrinsic and -extrinsic mechanisms of resistance to immune checkpoint blockers

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Targeting of immune checkpoint blockers (ICBs), such as cytotoxic T-lymphocyte antigen-4 and programmed-death 1/programmed-death ligand 1, has dramatically changed the landscape of cancer treatment. Seeing patients who were refractory to conventional therapy recover after immunotherapy, with high rates of objective durable responses and increased overall survival, has raised great enthusiasm in cancer care and research. However, to date, only a restricted portion of patients benefit from these therapies, due to natural and acquired resistance relying on the ever-evolving cross-talk between tumor and stromal cells. Here, we review the convergence of tumor-intrinsic and -extrinsic cues, both affecting tumor plasticity and tumor stroma leading to an immunosuppressive tumor microenvironment, which may account for the heterogeneous responses and resistance to ICB therapies. A deeper knowledge of the mechanisms and fingerprints involved in natural and acquired resistance is likely to bring clinical benefit to the majority of patients, offering important clues for overcoming drug resistance and boosting the effectiveness of treatment. We discuss the need to define tumor subtypes based on the tumor, immune and stromal gene signature and propose that the better we understand tumor mesenchymal traits, the more we will be able to identify predictive biomarkers of response to ICB treatments.

Introduction

The simplistic view of tumors as masses of neoplastic cells has governed cancer research for more than 30 years, although pioneer work demonstrated that the embryonic environment can suppress Src-driven transformation [1]. It is now clearly evident that tumors contain various non-cancer cells including fibroblasts, vascular endothelial cells, immune cells — with T-cells as potent mediators of antitumor immunity — and soluble factors [2–4] that, in dynamic reciprocity with cancer cells, make up the tumor microenvironment (TME). TME is organ-specific in terms of interstitial cells and extracellular matrix (ECM), which condition tissue oxygen levels and pH, adding further layers of complexity [5,6].

As the environment does in natural selection and evolution, TME imposes a strong selective pressure that advantages proliferation and survival of tumor cells [7] accompanied by stromal cell modification. Indeed, tumor and stromal cells progressively co-evolve [8] and their interaction determines phenotypic and functional plasticity, which may lead to tissue architecture deconstruction, alteration of matrix geometry and stiffness and, in turn, of immune cell functions, favoring or hindering cancer evolution and response to therapies [7,8].

Inflammation and cancer are closely connected [9,10], and inflammatory cells, chemokines and cytokines are key players in TME [11]. The signals connecting inflammation and cancer involve intrinsic and extrinsic pathways. The former include the activation of oncogenes or the inactivation of

tumor suppressor genes, leading transformed cells to produce inflammatory mediators [12]. The latter include key components of the inflammatory microenvironment, such as immune-infiltrating cells, cancer-associated fibroblasts (CAFs) and pro-inflammatory cytokines produced by these cells [11,13].

Conditioned by inflammation-associated signaling, tumor adopts plasticity to respond to the changes in the surrounding environment. Indeed, under the selective pressure of the immune system, tumor cells adopt a plethora of tricks for losing their immunogenicity [14,15]. Among these strategies, epithelial–mesenchymal transition (EMT) emerges as a program able to alter cell–cell adhesion, enable cell migration, confer mesenchymal traits, increasing cell heterogeneity and stemness and fueling resistance to cancer therapy [16]. However, in the clinical setting, validated markers of EMT are still missing, and the reciprocal impact between EMT and TME needs more insights.

Immune classification of cancer has provided innovative prognostic and predictive tools in different tumors, above all in colon cancer [17] and has recently been integrated by a stromal classification, indicating that the mesenchymal subtype is related to poor prognosis [18]. Of clinical relevance in the new era of immune check-point blockers (ICBs), tumor-enriched mesenchymal traits have emerged as a signature of resistance [19–22], whereas the highest objective response rates were observed in cancers with a high mutational load [i.e. melanoma and non-small cell lung cancer (NSCLC)] and are likely related to an enriched tumor-associated antigen (TAA) repertoire [23–27]. Targeting the major ICBs, such as cytotoxic T-lymphocyte antigen (CTLA)-4, programmed cell death (PD)-1 and programmed cell death ligand (PD-L)1, is revolutionizing cancer therapy [28,29]. Notwithstanding the unprecedented durable response rates observed, the benefit has been limited to a minority of patients and primary and acquired resistance emerge, based on mechanisms relying on the dynamic reciprocity modulated by TME, which interferes with antitumor immunity and in particular T-cell activity [30,31]. In resting T-cells, CTLA-4 resides intracellularly. Shortly after T-cell activation, CTLA-4 translocates to the plasma membrane where it acts as a competitive antagonist of the CD28–B7 interaction halting costimulation at the T-cell–antigen-presenting cell (APC) interface and abrogating activation of T-cell responses [32,33]. Moreover, CTLA-4 is highly expressed on regulatory T (T_{reg}) cells which further results in T-cell suppression [34]. This knowledge led to the hypothesis that CTLA-4 blocking could allow T-cell responses to persist; indeed, therapeutic blocking of CTLA-4 has reached routine clinical use [35–37] and pioneered the development of additional medications that modulate ICBs as the PD-1/PD-L1 axis. PD-1 is an immune cell-specific surface receptor [38,39] that, similarly to CTLA-4, is expressed on activated T-cells, where it serves as an inducer of T-cell anergy [36,40–43]. PD-1 has two ligands, PD-L1 and PD-L2. PD-L2 is predominantly expressed on APCs, whereas PD-L1 can be expressed on tumor cells, immune cells, epithelial cells and endothelial cells [37,43]. PD-L1 overexpression in tumor and immune cells reflects the adaptation to a dynamic TME and is one of the mechanisms of immune-evasion [44,45]. Indeed, Type I and Type II interferons (IFNs), mainly released by activated tumor-specific T-cells, induce PD-L1 up-regulation in neoplastic and stromal cells, impairing T-cell activity and favoring tumor immune-escape [44,46]. Given this key role, IFNs have been implicated in primary and acquired resistance to ICBs [47–49]. Accordingly, PD-L1, either constitutively expressed or induced by IFNs, is considered a predictive biomarker of response to ICBs [38,50]. However, the identification of additional reliable biomarkers to select patients who are more likely to respond to ICBs is urgently needed.

In this review, we detail the pathways involved in the dialog that animates TME, shapes the anticancer immune response and leads to the acquisition/enrichment of mesenchymal traits, which have emerged as a common signature in tumors resistant to ICB therapies (Figure 1).

TME-related mechanisms leading to mesenchymal traits and resistance to ICB
A glimpse at EMT in cancer

A multitude of work have indicated that EMT, known by embryologists for long time [51,52], represents a biological process able to regulate cancer invasion, metastasis and immune-escape [53–57]. The program is mediated by the convergence of a plethora of signaling networks triggered by different cellular components of the TME, and soluble factors, including growth factors critical to cancer, as well as inflammatory cytokines [53,57–61]. In all tissue contexts during EMT, cells down-regulate the expression of epithelial proteins, with an alteration of the epithelial cell–cell junctions, and redirect their gene expression program to promote changes in cytoskeletal architecture reorganization and cell shape [54]. In parallel, the activation of genes, related to a
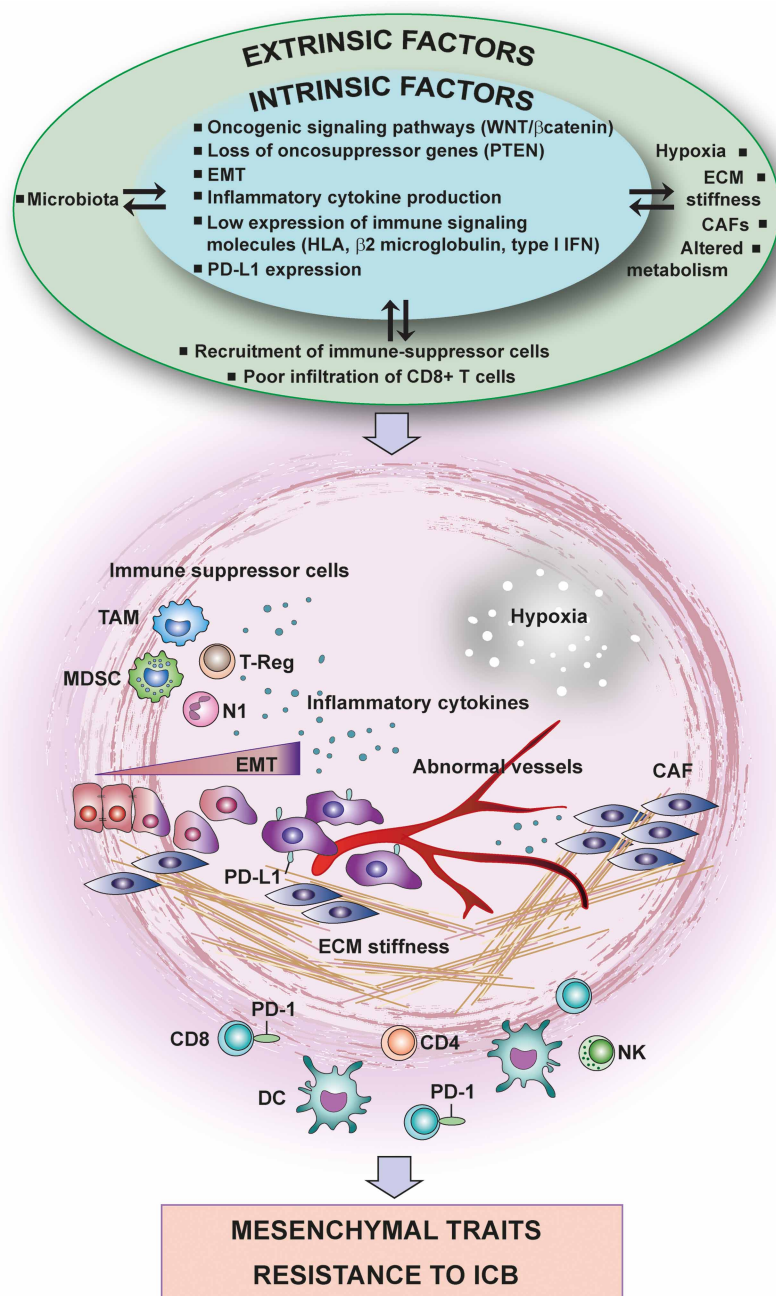
Figure 1. Mesenchymal traits resulting from tumor-intrinsic and -extrinsic factors influence T-cell trafficking and function determining resistance to ICB treatment.

The convergence of tumor-intrinsic and -extrinsic factors, as above schematically illustrated, generates mesenchymal traits with immune cell exclusion and/or dysfunction involved in resistance to ICB treatments.

mesenchymal phenotype, leads to increased cell protrusions and motility, which enables the ECM degradation [62]. Alternative splicing is an additional regulator of EMT plasticity and epithelial splicing regulatory proteins (ESRP)1/2 have been involved in EMT in different cancers [63]. The actin cytoskeleton regulator hMENA (ENAH gene) has been found among the ESRP1/2 targets [64,65]. We have shown that the tissue-specific splicing program of Mena, with the switch from hMENA[11a] to hMENAΔv6 isoform expression, may represent a crucial node in the convergence of EMT-related signaling pathways [66–68]. Transforming growth factor

(TGF)-β, a known inhibitor of host immunosurveillance [69] and a potent activator of EMT, down-regulates hMENA[11a] and up-regulates hMENAΔv6 [68].

hMENA[11a], spliced by ESRP1/2 [64,65], when silenced, reduces E-cadherin. Conversely, the overexpression of hMENAΔv6, in the absence of hMENA[11a], increases vimentin and fibronectin overexpression as well as matrix metalloproteinase (MMP)2 secretion [68] (and unpublished results), all mesenchymal markers belonging to an EMT signature across various human cancers, as explored in The Cancer Genome Atlas (TCGA) [70]. In agreement, we have suggested that the pattern of hMENA isoforms may represent a powerful prognostic factor in NSCLC and pancreatic ductal adenocarcinoma (PDAC), hMENA[11a] being a good and hMENAΔv6 a poor prognostic factor [67,68].

The process of EMT has been broadly studied and developed in cell line models that do not resemble the TME and its immune contexture, thus raising the question as to how to define the EMT signature in the clinical setting. Recently, the importance of integrating tumor classification with an 'EMT score' has highlighted the need to consider the contribution of the rich stromal contingent present within the TME to better define the mesenchymal status of a tumor [22]. This is a crucial node to interpret, in light of the different genomic and proteomic signatures that have evidenced tumor mesenchymal traits associated with an immunosuppressive TME and resistance to ICBs [19–21].

Here, dissecting both cancerous autonomous and non-cancerous signaling pathways and their convergence, we discuss and suggest that tumor mesenchymal traits may be markers to select patients for anti-ICB therapy and identify targets to overcome resistance.

Tumor cell-intrinsic pathways fostering ICB resistance

Tumor cell-intrinsic factors account for a portion of the whole mechanism responsible for resistance to ICBs preventing T-cell infiltration and function within the tumor [71].

Besides the loss of TAA expression or defects in the processing and presentation machinery, β-2-microglobulin (B2M) loss and human leukocyte antigen (HLA) down-regulation [72,73], different mechanisms of resistance have been reported to rely on multiple factors strictly connected to the EMT process and include activation or repression of signaling pathways that prevent T-cell response. The β-catenin pathway is the first mechanistically identified oncogene pathway able to mediate the exclusion of immune cells from TME. When activated, it correlates with the absence of a T-cell gene expression signature in metastatic melanoma patients, as demonstrated in a seminal work by Spranger et al. [74]. β-catenin-mediated T-cell exclusion occurs by inducting the transcriptional repressor cyclic AMP-dependent transcription factor (ATF)3, which inhibits the chemokine (C–C motif) ligand (CCL)4 gene transcription, leading to the lack of CD103^{+} dendritic cells (DCs), hence hampering cross-priming of antitumor T-cells. High expression of β-catenin-related genes and lack of T-cell infiltration result in resistance to anti-CTLA-4- and anti-PD-L1-based therapies in murine models [74]. It is noteworthy that β-catenin is an important contributor of EMT, since WNT signaling activation results in the inhibition of glycogen synthase kinase (GSK)3β, stabilizing β-catenin into the nucleus to regulate gene expression of several EMT-inducing transcription factors [75]. Furthermore, β-catenin co-operates with TGF-β, the major inducer of EMT [76].

Axl, a member of the tyrosine-protein kinase receptor (TYRO)3, AXL and MER proto-oncogene, tyrosine kinase (MERTK) (TAM) family of receptor tyrosine kinases [77], is not a traditional oncogene driver, but rather a regulator of tumor plasticity [78,79]. Overexpression of Axl or its ligand growth arrest-specific (GAS)6 induces EMT-related transcription factors and promotes tumor invasion by different mechanisms, including regulation of cytoskeleton remodeling by GTPase proteins Rho and Rac, and regulation of the expression of MMP-9, enzyme with prominent matrix-degrading activity [78,80]. AXL activation has been associated with T-cell exclusion in melanoma and with an immunosuppressive TME, caused by DC inhibition and expression of myeloid supporting cytokines [81]. Moreover, Axl was found as a component of the innate PD-1 resistance (IPRES) signature in melanoma patients [82], further strengthening its critical role as a negative regulator of antitumor immune responses [81].

Recently, deletions and loss-of-function mutations in the phosphatase and tensin homolog deleted on the chromosome 10 (*PTEN*) gene, one of the most frequently disrupted tumor suppressors in cancer [83], have been found to be higher in tumors with low T-cell infiltration, suggesting that PTEN loss contributes to resistance to immunotherapy in melanoma by a mechanism not overlapping with β-catenin-mediated T-cell exclusion [84]. PTEN loss also contributes to an immunosuppressive TME by producing vascular endothelial growth factor (VEGF), which is involved in the recruitment of suppressive immune cells and inhibitory effects on

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T-cells [85,86]. Of note, PTEN loss has been associated with increased PD-L1 expression in gliomas and colorectal cancers [87,88].

PTEN loss may contribute to EMT by activating phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling essential to the EMT process, mediated by both TGF-β and receptor tyrosine kinases [89].

Inactivation of the tumor suppressor p53 through somatic mutations has been observed in 50% of human cancers [90] and a link between p53 and immune responses has been found. Cancer cells respond to genotoxic stress and DNA damage by up-regulating an immunoglobulin superfamily receptor death domain 1α and related checkpoint molecules, such as PD-1 and PD-L1 molecules on cancer cells, in a p53-dependent manner, enabling their interactions with T-cells to suppress immune response and escape from immune-surveillance [91]. Moreover, p53 regulates different microRNAs involved in antitumor immune response, including miR34, known to regulate PD-L1 expression [92,93].

The p53 inactivation induces EMT through several mechanisms mainly affecting EMT-related transcription factor expression [94,95]. Moreover, wild-type p53 directly activates transcription of the miR-200 and miR-34 gene loci, associated with zinc finger E-box-binding homeobox transcription factor 1 and snail family transcriptional repressor 1 expression by reciprocal regulation [96,97].

Table 1 summarizes tumor cell-intrinsic pathways fostering ICB resistance.

Tumor-extrinsic factors fostering ICB resistance
Immune cell infiltration and CAFs

The assumption that T-cell activity is the final effector mechanism leading to tumor elimination has been extensively demonstrated [98]. T-cell-mediated tumor control requires T-cells to infiltrate tumors, and the abundance, spatial distribution and functional activity of tissue-infiltrating T-cells have been considered as prognostic and predictive parameters [17,99], as well as a focal trait of tumor responsiveness to ICB therapy [100,101]. Recently, the segregation of immune response into three main phenotypes has been suggested as a predictor of response to the PD-1/PD-L1 blockade [36,102]. The inflamed phenotype includes the simultaneous presence in the tumor parenchyma of antitumor CD8 and CD4 T-cells along with immune-inhibitory cells (i.e. suppressor myeloid and B cells, T_{reg} cells, macrophages and CAFs), which affect T-cell functionality and up-regulate several inhibitory receptors, leading to T-cell dysfunction and exhaustion [101]. Since these cells are the best candidates to be re-invigorated by ICB therapy [100,103–106], this TME-inflamed phenotype may be related to ICB sensitivity, as indicated in multiple cancer types [103]. In renal cancer, higher ratios of baseline tumor-derived T-effector-to-T_{reg} signatures are correlated with responses to the anti-PD-L1 [107]. In melanoma, accumulation of pre-existing infiltrating PD-1^{+}CTLA-4^{+} [108] or PD-1^{+}PD-L1^{+} CD8^{+} T-cells, with a

Table 1 Tumor-intrinsic factors fostering ICB resistance

Tumor-intrinsic factors	Mechanisms	Effects	Refs
β-catenin pathway	Inhibition of CCL4 transcription by increasing the transcriptional repressor ATF3	Lack of CD103+ dendritic cells, T-cell exclusion	[74]
Axl pathway	Suppression of antigen presentation, production of immunosuppressive cytokines	Suppression of antitumor immune response, T-cell exclusion	[81]
PTEN loss	Increase of immunosuppressive cytokines (i.e. VEGF)	Recruitment of suppressive immune cells (immature dendritic cells, MDSCs and regulatory T-cells)	[84]
p53 activity	• Increase of DD1α, PD-1 and PD-L1 on cancer cells • PD-L1 regulation via miR-34	Suppression of T-cell-mediated immune response	[91,92]

Abbreviations: CCL4, C–C motif chemokine ligand 4; ATF3, cyclic AMP-dependent transcription factor 3; PTEN, phosphatase and tensin homolog; VEGF, vascular endothelial growth factor; MDSC, myeloid-derived suppressor cell; DD1α, death domain 1α; PD-1, programmed-death 1; PD-L1, programmed-death ligand 1.

restricted T-cell receptor (TCR) repertoire, correlates with anti-PD-1 responsiveness, and CD8$^+$ T-cell activation, proliferation and clonal TCR expansion [109]. On the other hand, a broad TCR diversity has been associated with tumor mutational load, a predictive marker of ICB sensitivity [110]. The analysis of longitudinal samples demonstrated that tumors responding to ICBs showed an intra-tumoral oligoclonal T-cell expansion [111,112]. This selective pressure induced by a long-lasting T-cell response may lead to the emergence of acquired resistance determined by Janus kinase (*JAK*)1 or *JAK*2 inactivating mutations as found in melanoma patients after anti-PD-1 or anti-CTLA4 therapies [49].

The immune-excluded phenotype is characterized by abundant immune cells held in the stroma surrounding tumor nests, as dictated by physical barriers (i.e. stiffened tissue with high matrix fiber mass and dense collagen network) [113] or the low expression of specific chemokines involved in T-cell recruitment [13,31,114]. Often, the hindrance of lymphocyte infiltration and trafficking is accompanied by the recruitment of suppressive cells and may also be ascribed to abnormal neovasculature derived from VEGF tumor overexpression [21,115,116] and down-modulation by tumor cells of adhesion and chemotactic signals on tumor endothelium, all features found in signatures related to ICB resistance [21,30,117]. Furthermore, CCL2, a chemokine derived from stromal or tumor cells, chemoattracts macrophages and myeloid derived suppressor cells (MDSCs) [118,119], and is highly expressed in ICB-resistant melanoma [21]. Also, crucial in T-cell exclusion is the C–X–C motif chemokine 12 (CXCL12) produced by fibroblast activation protein (FAP)$^+$ CAFs, which, when inhibited, promotes T-cell infiltration and synergizes with the anti-PD1 therapy in a mouse model of PDAC, suggesting the need for a combination of ICBs and stroma-derived targets in this subgroup of tumors [120].

The third immune-desert phenotype, characterized by few or no CD8$^+$ T-cells and the presence of MDSCs and macrophages, both in the tumor parenchyma and in the stroma, reflects the lack of a spontaneous pre-existing antitumor response ascribed to the absence of productive T-cell priming, immunological ignorance or induction of tolerance and, as expected, is resistant to ICB therapy [121,122]. The lack of T-cell priming may be ascribed to a low tumor immunogenicity, which may be driven by a low tumor antigen quality or by the selection of tumor variants immunoedited and not recognized by T-cells. On the other hand, numerous tumor-derived soluble factors including VEGF and indoleamine 2,3-dioxygenase (IDO) [123] may impair DC maturation and antigen-presenting function. In parallel, the presence of suppressive immune cells such as T_{reg} cells and MDSCs activates a circuit inhibiting DC maturation and, in turn, T-cell expansion and activation. In this scenario, CTLA-4 expression on T_{reg} cells reinforces this circuit by triggering forkhead box O3 (FOXO3)-dependent signaling and up-regulating IDO in DCs [124]. In these patients, the combination of two different checkpoints may be the most effective approach, since anti-CTLA-4 therapy may inhibit this circuit, rendering this nonpermissive phenotype responsive to anti-PD-1 therapy.

As cited above, MDSCs represent one of the major populations of regulatory cells promoting tumor invasiveness by impairing antitumor innate and adaptive responses and also supporting EMT [55,125,126]. MDSCs show a plastic phenotype [126] and monocytic MDSCs induce, while granulocytic MDSCs suppress EMT [127]. MDSC presence in TME has been linked to ECM modification and in turn to EMT, as reported for high secreted protein acidic- and cysteine-rich (SPARC) expression in a murine model of breast cancer [128,129]. High levels of MDSCs are clearly involved in resistance to ICB therapy in patients [130–133]. Hence, inhibition of the colony-stimulating factor (CSF)-1/CSF-1R signaling, responsible for MDSC tumor infiltration, has been proposed in combination with CTLA-4 blockade therapy, as it induces antitumor T-cell responses and tumor regression [134].

Tumor-associated macrophages (TAMs), most of the immune cell population within the tumor stroma, can be polarized to an M2 anti-inflammatory and pro-tumoral phenotype under the influence of the TME [135]. TAMs have been shown to dramatically affect T-cell responses through PD-L1 in hepatocellular carcinoma [136] and B7-H4 in ovarian carcinoma [137]. Notably, PD-1/PD-L1 responses correlate, at least partially, to stromal PD-L1 expression [103,109], consistent with a role for TAMs among other stromal cells in inducing T-cell anergy. In addition, TAMs are able to recruit T_{reg} cells by CCL22 [138], thus favoring an immunosuppressive environment. Notably, pro-inflammatory M1 and pro-tumorigenic M2 TAMs, mainly located at the invasive front of tumors, have been associated with EMT induction via TGF-β [139,140]. Noteworthy, in a positive feedback loop, cancer cells themselves recruit TAMs by secreting CSF-1 and TAMs, in turn, edit cancer cells to a mesenchymal metastatic phenotype by releasing the epidermal growth factor [141]. Despite the lack of direct proof, we speculate that the ability of TAMs to induce EMT on tumor cells may indirectly lead to immune resistance, which may converge with the direct induction of T-cell exhaustion.

A less studied but important population of infiltrating immune cells are tumor-associated neutrophils [142], able to promote tumor cell plasticity [143] and cancer migration through the secretion of MMPs and interleukin (IL)-1β which activates endothelial cells and inhibits natural killer cells [144]. High levels of neutrophils and neutrophil-to-lymphocyte ratio in peripheral blood of melanoma patients have been associated with resistance to anti-CTLA-4 [145].

CAFs are the most abundant non-neoplastic cells in TME, key players in cancer progression through the biomechanical and biochemical remodeling of the ECM and the secretion of a plethora of soluble factors [146] and contributors to therapeutic outcome. In the context of tumor–stroma coevolution, CAFs have been shown to feed cancer progression endowing tumor cells with mesenchymal traits [147]. In NSCLC, the dynamic crosstalk between tumor cells and CAFs, by secreting IL-6, forces tumor cells toward a mesenchymal phenotype correlated to chemoresistance [148]. Incipient data are showing the role of the AXL in the paracrine feedback loop between cancer and fibroblastic cells with significant effects on resistance to conventional therapies, and likely, immunotherapy [149], paving the way for the design of new therapeutic strategies combining AXL targeting with ICBs. Growing evidence shows that FAP, selectively expressed on CAF subtypes and associated with ECM remodeling, induces tumor-promoting inflammation [150–152]. Furthermore, as mentioned before, FAP targeting has been shown to synergize with ICB-based immunotherapy [120,150,152].

ECM and hypoxia

The ECM furnishes an organized network whose primary function is to support tissues. Mechanical properties of ECM integrate biochemical cues from TME to regulate tumor progression and metastasis [153]. With respect to normal tissues, tumor tissues are characterized by increased ECM deposition leading to higher stiffness that activates mechano-signaling with strong impact on survival, migration and invasion [154]. Recently, a novel role for the non-receptor tyrosine focal adhesion kinase (FAK1) in driving immune-escape has been revealed in PDAC, enforcing the concept that physical properties of ECM contribute to the generation of an immunosuppressive TME. Hyperactivated FAK1 associates with extensive collagen deposition and matrix stiffness and drives cytokine production which results in increased myeloid cell recruitment and pro-tumor polarization of macrophages, leading to immunosuppressive TME. Of clinical relevance, FAK1 inhibition dampens immunosuppressive inflammatory cell infiltration, sensitizing previously unresponsive PDACs to immunotherapy [155].

That ECM composition and remodeling play a key role in mediating response to ICBs is also confirmed by the signatures of resistance to ICBs that evidence the up-regulation of ECM components or factors that contribute to ECM remodeling [19–21].

It is noteworthy that hypoxia, together with ECM components and enzymes, participates in the IPRES signature that defines tumors not-responding to ICBs [21]. Indeed, hypoxia is strictly linked to ECM deposition and remodeling, as hypoxia-inducible factor (HIF)1 and 2 regulate the expression of enzymes involved in biosynthesis of collagen fibers and in collagen degradation [154]. Moreover, under hypoxic conditions, tumor cells secrete paracrine signaling that contribute to fibrosis, promoting the conversion of precursor cell types into CAFs [154]. The cross-talk between hypoxia and immune-escape mechanisms is a novel and recently emerging aspect in tumor progression and drug resistance. Metabolic shift from oxidative phosphorylation to glycolysis is one of the adaptation strategies of cancer cells to survive in hypoxic conditions [156]. The accumulation of lactate as waste product, together with the strong metabolic competition between cancer and immune cells, leads to local immunosuppression [157]. Increased hypoxia contributes to the generation of an immunosuppressive TME also by the release of different immunosuppressive molecules that recruit and activate multiple myeloid and lymphoid immune suppressor cells, such as M2 TAMs, MDSCs and T_{reg} cells [158]. Moreover, PD-L1 expression is up-regulated under hypoxic conditions in a panel of mouse and human tumor cell lines through a HIF-1α-dependent mechanism [159]. Future studies will clarify the role of hypoxia in regulating immune checkpoint signaling and resistance to ICBs.

Tumor mechanics and metabolism

The alteration of tumor architecture and the dynamic ECM remodeling are strictly interconnected to the metabolic reprogramming adopted by cancer cells during tumor progression, and mechano-signalings promote tumor progression and metabolic rewiring [6]. Matrix stiffness, via integrin-mediated activation of FAK and downstream pathways, can result in PI3K signaling activation that regulates glucose metabolism, sustaining tumor cell glucose addiction, and glucose uptake and utilization [160]. Moreover, via integrin activation of

β-catenin and MYC, matrix stiffness also induces miR-18a expression that inhibits PTEN expression, again activating PI3K [161]. The tumor progression results in an intense competition between cells in the TME and tumor-imposed metabolic effects can limit the responsiveness of TILs by competing for nutrients [162]. PD-L1 expression has been found to be associated with the Akt/mammalian target of rapamycin activation and glycolysis in tumor cells, resulting in a metabolic impairment of T-cells, which dampens their effector function and allows tumor progression. ICB therapy restores glucose in the TME, permitting T-cell glycolysis and IFN-γ production [162]. Of clinical relevance, the up-regulation of genes corresponding to proteins associated with metabolic and solute transport functions has been found in tumors of renal cell carcinoma patients, non-responding to anti-PD-1 therapy [163], confirming the involvement of an altered metabolism in response to ICB-based immunotherapy.

Microbiota

Accumulating evidence in the last 3 years has revealed that the microbiota, and particularly the gut microbiota, engages in mutualistic interactions with the host, thus modulating systemic metabolic functions and immune-surveillance, which have an impact on cancer initiation, progression and response to chemo- and immunotherapies [164,165]. Notably, Vetizou et al. [166] and Sivan et al. [167] in a back-to-back study showed that intestinal microbiome may and do influence response and resistance to ICBs. Indeed, *Bacteroides* spp. and *Bifidobacterium* have been found to be instrumental for anti-CTLA4 and anti-PD-L1 therapy, respectively [166,167], since their dysbiosis correlated with ICB resistance over-ridden only by fecal microbial transplantation. The incipient evidence that the heterogeneity in immunotherapeutic efficacy and toxicity is related to the inter-individual differences of the gut microbiota suggests how relevant is to manipulate the microbiota to restore homeostatic gut immunity to improve the therapeutic index of ICBs.

Cancer EMT signatures and resistance to ICBs

The rationale to investigate mesenchymal traits as potential predictive markers of response to ICBs stems from a growing number of studies focusing at unraveling the link between EMT and immunosuppression [70]. In this Review, the enrichment of mesenchymal traits in different signatures of resistance to ICBs has been evidenced (Table 2), highlighting the point that the mesenchymal status of the tumor mass *in toto* encompasses and goes beyond the single value of PD-L1 as a predictor of responsiveness to ICBs.

Recently, in the transgenic mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) model of breast carcinoma, Dongre et al. [168] showed that epithelial tumor-bearing mice were completely responsive to anti-CTLA4 immunotherapy, while mesenchymal tumor-bearing mice were refractory, indicating that epithelial and mesenchymal carcinomas differ in their susceptibility to immune attack.

Critical evidence of the role of mesenchymal traits in determining tumor resistance to ICBs comes from the comparison of transcriptomes of responding versus non-responding tumors from melanoma patients treated with anti-PD-1 therapy [21]. Among the genes differentially expressed in pretreatment tumors of non-responding patients, mesenchymal transition genes such as *AXL*, *ROR2*, *WNT5A*, *LOXL2*, *TWIST2*, *transgelin* (*TAGLN*) and *FAP* were enriched. Of note, among highly expressed genes in resistant tumors, several codes for proteins involved in ECM composition and remodeling such as collagens, fibulin and inhibin βA and MMPs (MMP13 or MMP1).

Gene ontology analysis showed that genes up-expressed among non-responding tumors were enriched for cell adhesion and ECM organization [21]. Of note, E-cadherin (*CDH1*), typically down-regulated in mesenchymal cancer cells, was expressed at low levels in non-responding tumors. On the other hand, genes with putative roles in modulating ICB sensitivity were not differentially expressed. Importantly, from the co-enrichment of a group of 26 transcriptomic signatures, the authors gained the IPRES signature, which defines a distinct transcriptomic program across various cancer histology. This analysis indicated that mesenchymal transition, together with angiogenesis, hypoxia and wound healing, correlates with resistance to ICBs. Collectively, these data support the notion that the reciprocal tumor cell and TME plasticity are critical barriers to anti-PD-1 responses.

The proteomic analysis of formalin-fixed, paraffin-embedded metastatic melanoma tissues collected prior ICBs evidenced 106 significantly different proteins between responding and non-responding groups [19]. The analysis showed that non-responding tumors were characterized by elevated histone H3 lysine [27] trimethylation (H3K27me3) and decreased E-cadherin, features related to a more mesenchymal phenotype. Of note, calponin (CNN)1, serpin family F member (SERPINF)2, collagen type III α 1 chain (COL3A1) and matrix remodeling associated (MXRA7), all involved in ECM remodeling, were up-regulated in non-responding

Table 2 Mesenchymal traits identified in signatures related to ICB resistance

Cancer type	ICB type	Analysis	Patient (n)	Clinical response	Differentially expressed (n)	Genes/proteins		Refs
Melanoma	Anti-PD-1	RNA-Seq	28	15 Resp. 13 non-Resp.	693 genes	*AXL*, *ROR2*, *WNT5*, *LOXL2*, *TWIST2*, *TAGLN*, *FAP*, *MMP1*, *MMP13*, *COL8A1*, *COL12A1*, *INHBA*, *FBLN1*, *ADAMTS7*	↑	[21]
Melanoma	Anti-CTLA-4 or anti-PD-1	MS/MS	8	4 Resp. 4 non-Resp.	106 proteins	CNN1, SERPINF2, COL3A1, MXRA7, H3K27me3 CDH1	↑ ↓	[19]
Melanoma	Anti-CTLA-4 → anti-PD-1	RNA-Seq	1 (10 lesions)	6 Resp. 4 non-Resp.	138 genes	*LAMA3*, *CST2*	↑	[20]

tumors. It is noteworthy that *CNN1* and *COL3A1* genes have also been found to be associated with ICB-resistant tumors by Hugo et al. [21].

The analysis of multiple melanoma cutaneous metastases from a single patient revealed a heterogeneous response to anti-PD-1 therapy, among the lesions [20]. The differences were not related to PD-L1 expression in tumor or infiltrating immune cells, neither to T-cell abundance, location or major genomic events. The authors identified a 13-gene profile that differentiated progressing versus regressing lesions after anti-PD-1 therapy. Among these genes, the most highly differentially expressed gene, overexpressed in progressing metastases, is *laminin subunit alpha* (*LAMA*)*3*, coding for a subunit of Laminin-5, a secreted molecule that plays an important role in tissue architecture and adhesion of normal epithelial cells to the basement membrane, and is associated with EMT [169]. Another identified gene is *cystatin SA* (*CST2*), a member of cysteine proteinase inhibitors, with a role in ECM remodeling [20]. In a phase 2 clinical trial where metastatic urothelial carcinoma patients received the PD-L1 antibody Atezolizumab, the response was significantly lower in basal than in luminal subtype patients [105], the former being a phenotype associated with the expression of mesenchymal biomarkers [170].

Conclusion

The thread we have followed in this review supports the assumption that the tumor-intrinsic and -extrinsic pathways, related to mechanisms of resistance to ICBs, converge in the TME, are linked to tumor plasticity and exert profound effects on tumor stroma with an induction or enrichment of mesenchymal traits.

As the integration of technological and computational innovations in this exciting era of cancer immunotherapy improve, we can expect to identify markers resembling the complexity of TME signaling pathways, enabling more effective treatments.

The signatures gathered by clinical trials indicate mesenchymal traits as a common feature of ICB resistance. We suggest that the definition of tumor, immune and stromal gene signature should be the focus of future studies, to identify more precise, multiparametric and standardized markers of ICB response.

Summary

- The dynamic interaction of cancer cells and tumor stroma leads to an alteration of matrix geometry and stiffness, and in turn, immune cell functions, which may favor or hinder cancer evolution and response to therapy.

- Epithelial–mesenchymal transition, a program in which epithelial cells acquire motile phenotype and the characteristics of mesenchymal cells, has been involved in immune-escape and resistance to therapy.

- Immune checkpoint blockade represents a successful strategy of cancer immunotherapy. However, the majority of patients show primary and acquired therapy resistance.

- Immunotherapy resistance mechanisms rely on the dynamic reciprocity between tumor and its TME, which interferes with antitumor immunity and, in particular, T-cell activity.

- Tumor and stroma enrichment of mesenchymal traits emerges as a common feature of different signatures of resistance to ICBs.

Abbreviations

APC, antigen-presenting cell; CAF, cancer-associated fibroblast; CCL, C–C motif chemokine ligand; CDH, cadherin; CNN, calponin; COL3A1, collagen type III α 1 chain; CSF, colony-stimulating factor; CST, cystatin SA; CTLA-4, cytotoxic T-lymphocyte antigen-4; DC, dendritic cell; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; ESRP, epithelial splicing regulatory proteins; FAK, focal adhesion kinase; FAP, fibroblast activation protein; H3K27me3, histone H3 lysine (27) trimethylation; HIF-α, hypoxia-inducible factor-α; ICB, immune checkpoint blockers; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; IPRES, innate PD-1 resistance; JAK, Janus kinase; LAMA, laminin subunit α; MDSC, myeloid-derived suppressor cell; MERTK, MER proto-oncogene, tyrosine kinase; MMP, matrix metalloproteinase; MXRA, matrix remodeling associated; NSCLC, non-small cell lung cancer; PD-1, programmed-death 1; PDAC, pancreatic ductal adenocarcinoma; PD-L1, programmed-death ligand 1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatase and tensin homolog; SERPINF, serpin family F member; TAA, tumor-associated antigen; TAGLN, transgelin; TAM, tumor-associated macrophage; TCGA, The Cancer Genome Atlas; TCR, T-cell receptor; TGF-β, transforming growth factor β; Th, T helper; TME, tumor microenvironment; T_{reg}, regulatory T; VEGF, vascular endothelial growth factor.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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