



Immunosuppressive circuits in tumor microenvironment and their influence on cancer treatment efficacy

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Abstract

It has been for long conceived that hallmarks of cancer were intrinsic genetic features driving tumor development, proliferation, and progression, and that targeting such cell-autonomous pathways could be sufficient to achieve therapeutic cancer control. Clinical *ex vivo* data demonstrated that treatment efficacy often relied on the contribution of host immune responses, hence introducing the concept of tumor microenvironment (TME), namely the existence, along with tumor cells, of non-tumor components that could significantly influence tumor growth and survival. Among the complex network of TME-driving forces, immunity plays a key role and the balance between antitumor and protumor immune responses is a major driver in contrasting or promoting cancer spreading. TME is usually a very immunosuppressed milieu because of a vast array of local alterations contrasting antitumor adaptive immunity, where metabolic changes contribute to cancer dissemination by impairing T cell infiltration and favoring the accrual and activation of regulatory cells. Subcellular structures known as extracellular vesicles then help spreading immunosuppression at systemic levels by distributing genetic and protein tumor repertoire in distant tissues. A major improvement in the knowledge of TME is now pointing the attention back to tumor cells; indeed, recent findings are showing how oncogenic pathways and specific mutations in tumor cells can actually dictate the nature and the function of immune infiltrate. As our information on the reciprocal interactions regulating TME increases, finding a strategy to interfere with TME crosstalk becomes more complex and challenging. Nevertheless, TME interactions represent a promising field for the discovery of novel biomarkers and therapeutic targets for improving treatment efficacy in cancer.

Keywords Immunosuppression · Oncogenes · Metabolic reprogramming · Extracellular vesicles · Myeloid-derived suppressor cells · Immune checkpoint inhibitors

Introduction

Cancer cells need nutrients to proliferate, differentiate, increase the tumor mass, and invade the surrounding tissue. During tumor development, several genetic and epigenetic alterations occur in cancer cells leading to the activation of oncogenic signals that are involved in the reprogramming of tumor metabolism. The metabolic reprogramming mediated by the activation of oncogenes and signaling pathways ensures that tumor cells have all the elements necessary to grow

autonomously [1]. Dysregulated metabolism and oncogenic mutations of cells lead to the activation of inflammatory signals, which in turn sustain the growth and aggressiveness of cancer. Specifically, the dysfunctional metabolisms induce the release of cytokines, chemokines, growth factors, and acid metabolites including protons (H⁺) and lactate. These are fundamental factors for recruiting immunosuppressive cells, such as Tregs, tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) at tumor site, and fostering their immunosuppressive functions [2]. Moreover, the alteration of oncogenic pathways is correlated with lower or higher immune infiltration [3] and promotes immune evasion [4] and poor response to immunotherapy [5, 6]. In addition, tumor cells release immune-related factors and extracellular vesicles (EVs), among which exosomes, involved in intercellular crosstalk by the transfer of various molecular constituents. These tumor EVs target immune cells and

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induce a reprogramming toward an immunosuppressive phenotype [7]. Thus, by the release of immune-related factors and exosomes in the blood, cancer cells stimulate the bone marrow myelopoiesis toward the generation of dysfunctional myeloid cells such as MDSCs [8]. Once recruited at the tumor site from the circulation, together with TAMs, these cells support tumor growth and proliferation through the production of factors that stimulate the formation of new tumor vessels and metastatization and impair T and natural killer (NK) cell effector functions [9]. The increment of MDSCs in cancer patients is associated with tumor aggressiveness and poor prognosis [10] and thus MDSCs are deemed as target cells to improve the response to immune checkpoint inhibitors (ICIs) [11]. In this review, we summarize recent findings about the role of cancer cells in shaping the metabolic and cellular composition of the TME, focusing our attention on tumor immunosuppression.

Oncogenes and tumor mutations influence the immune microenvironment and response to immunotherapy

Activation of oncogenic pathways in tumor cells promotes immune evasion

The function of aberrant oncogenes and tumor suppressor genes has been investigated as a cell-intrinsic tumor characteristic in the setting of an immunosuppressive microenvironment. Most of the causal evidence comes from studies with genetically engineered mouse tumor models uncovering the molecular pathways by which tumor mutations impact the immune contexture of the TME [4]. Alterations involving *TP53*, *PTEN*, *RAS*, *MYC*, and *CTNNB1* genes have been shown to shape the immune infiltrate by increasing tumor cell production of cytokines and chemokines promoting the recruitment and activation of macrophages, monocytes, neutrophils, mast cells, or Tregs while concurrently reducing CD8+ T cell levels [4]. Such evidence has been obtained for several tumor types, including malignant melanoma, non-small cell lung cancer (NSCLC), colorectal cancer (CRC), breast cancer, hepatocellular carcinoma (HCC), prostate cancer, pancreatic cancer, and the production of factors such as TGF- β , IL-1 β , CCL-2, CCL-5, CCL-9, and CCL-17 recruiting myeloid cells to the tumor has been involved in these studies [4].

The reshaping of the secretome of tumor cells consequent to aberrant signaling of specific genes is a well-known effect in human cancer. A variety of oncogenic pathways has been associated with production of cytokines or chemokines able to regulate the recruitment of immunosuppressive cells. Classic examples are gene mutations hyperactivating the MAPK pathway occurring in *RAS*, *BRAF*, and *RET* oncogenes, which characterize several solid tumor types. Constitutive activation of MAPK pathway by *BRAF* mutation in melanoma was

shown to regulate the expression of a plethora of genes which by increasing immunosuppressive cytokines and reducing MHC/melanoma antigen complexes impair antitumor immunity by promoting an immunosuppressive environment with low numbers of activated T cells, dendritic cells (DCs), and NK cells [12]. Conversely, inhibition of MAPK pathway through *BRAF* and *MEK* inhibitors showed favorable effects on TME, including decreased immunosuppressive cytokines, increased antigen expression, and recruitment of a CD8+ T cell infiltrate [13–15]. In this context, we showed that the production of CCL-2 is increased in both patient tumor and serum and contributes to treatment resistance and that co-targeting of CCL-2 or downstream miRNAs may be beneficial [16].

Oncogenes expressed in immune cells of the tumor microenvironment

It has been recently proposed that oncogenic pathways in immune cells can be implicated in the spontaneous regression of tumors, and thus, proto-oncogenes might represent novel therapeutic targets for cancer treatment [17]. *MYC* oncogene is a main physiological regulator of the immune system and of immune cell functions in the context of cancer. *MYC* and *KIT* pathways are important for the cytotoxic activity of NK cells, and their decreased expression was implicated in the poor antitumor activity of NK cells in cancer patients [18]. *MYC* also regulates macrophage functions, and the activation of *MYC* controls the macrophage polarization into M2 type and promotes macrophage protumoral activity [19]. The polarization of macrophages into M2 type is also regulated by p53; the usage of genetically engineered mouse models revealed that a low expression of p53 is associated with the stabilization of *MYC* and consequently an increased transcription of M2 genes [20]. The tumor suppressor gene *TP53* is also involved in the regulation of T cell differentiation, and p53 and *MYC* may act synergistically in the regulation of T cell proliferation [21].

Tumor genetic profile shapes antitumor immune response

The association between tumor genetics and immune infiltrate has been studied in the context of the high-resolution mapping of tumor immune landscape by RNA-based information and genetic data from The Cancer Genome Atlas (TCGA) [22]. A recent study identified immune subtypes spanning cancer tissue types and impacting prognosis, where the relation to genome state (including aneuploidy, copy number variation, loss of heterozygosity) was determined and specific driver mutations correlated with lower (*CTNNB1*, *NRAS*, *IDH1*) or higher (*BRAF*, *TP53*, *CASP8*) immune infiltration [3]. Indeed, specific mutations in *EGFR*, *ALK*, and *JAK* genes have been

associated to poor response to immunotherapy and to a poor immune TME in NSCLC and MM respectively [5, 6] (reviewed in Table 1).

Tumor genomic instability can determine the gain or loss of large genomic regions, resulting in somatic copy number alterations. The aneuploid setting has been associated to immune escape and to poor response to immunotherapy with anti CTLA-4 and PD-1 in several tumor types, including metastatic melanoma [29, 30]. Genetic deletions in the tumor cells may deeply influence the characteristics of the leukocytes infiltrating the tumor. Two seminal examples are the deletion of *IL15* in CRC [33] and the deletion of *IFNG* locus in melanoma [31, 34] determining poor lymphocyte infiltration in the TME. In particular, the latter is of major importance given the role of IFN signaling in cytotoxic immune cells and in the regulation of the expression of immune checkpoints (ICs) [35], and in consideration of the dysregulation of IFN-stimulated genes in several cancer types and in resistance to ICIs [36].

Tumor mutational burden (TMB), defined as the number of mutations per coding area of a tumor genome, reflects the genomic instability of tumor cells and it is usually associated to neoantigen formation, thus determining increased tumor immunogenicity. For this reason, high TMB and neoantigen formation are considered crucial factors for the shaping of the immune infiltrate and predictors of response to immunotherapy in diverse cancers [23, 37]. Notably, a correlation between TMB, as the number of coding mutations per megabase (Mb), and the objective response rate to anti PD-1 and PD-L1 therapy has been reported when the data from 27 tumor types were analyzed [24]. However, it is also clear that there are a number of patients in which response or treatment resistance cannot be associated to TMB [27, 38]. Neoantigen formation can result also from small-scale insertion and deletion mutations (indel), which may generate highly immunogenic neoantigens by

creating novel open reading frames. Different types of renal cell carcinoma were reported to have the highest indel rate compared to other tumor types, and the indel number was reported to be associated with checkpoint inhibitor response in melanoma [25].

Melanoma is the tumor with the highest TMB among the cancer types analyzed by TCGA, with a mean mutation rate of 16.8 mutations/Mb; in particular, melanoma has a high fraction of C>T transitions at dipyrimidines consistent with UV radiation's signature [39]. Several studies have reported an association between TMB with high predicted neoantigen load and response to immunotherapy in melanoma [40–42].

The degree of similarity of the mutational landscape or more in general of the whole genomic alterations of primary and metastatic tumors remains an unsolved question, giving rise to a number of recently published studies [43–45]. Although the results are in some instances contradictory, it could be concluded that a different genetic profile could be found in 40% of matched primary versus metastatic cancers [45]. The majority of metastatic cancer tissues retain the genomic features of their corresponding primary tumor. However, the gene mutation rate is higher in the metastatic forms, as evidenced in six different metastatic cancers (CRC, NSCLC, soft tissue sarcoma, bladder, thyroid, and ovarian cancers) [43, 44], leading to the acquisition of new signatures associated to cancer progression. Among others, *TP53* is the most frequent altered gene in metastatic lesions [43]. In pancreatic and colon adenocarcinoma, the second most common altered gene in metastatic tissues is *KRAS* [43]. Moreover, mutational exclusiveness of *ALK-BRAF* and *ERBB4-BRAF* was found in metastatic melanoma and also *EGFR-JAK3* and *CTNMB1-TP53* only in metastatic NSCLC [44].

Collectively, these data support the view that integrated analysis of genetic and immune makeup of tumors may improve precision medicine. Taken together, the studies

Table 1 Intrinsic tumor genetic features associated to response to immunotherapy

Genetic alteration	Tumor type	^a Fq	^b ICIs	ICI outcome	TME	Ref
Mutational load	Melanoma and 27 tumor types	^f ND	CTLA-4, PD-1, PD-L1	Response	^g Immune signatures in R	[23, 24]
Indel number	Melanoma	ND	PD-1	Response	ND	[25]
^c DNA MMR	^d MMRD colorectal cancer	ND	PD-1	Response	High CD8 and PD-L1	[26]
<i>BRCA2</i>	Melanoma	28%	PD-1	Response	^g Immune signatures in R	[27]
<i>TP53</i>	Lung adenocarcinoma	33%	PD-1	Response	high CD8 and PD-L1	[28]
Aneuploidy	Melanoma	ND	CTLA-4	Poor response	^h Immune signature in NR	[29]
Copy number loss	Melanoma	ND	CTLA-4 and PD-1	Poor response	^h Immune signature in NR	[30]
<i>IFNG</i> -related gene loss	Melanoma	50%	CTLA-4	Poor response	ND	[31]
<i>EGFR</i>	^e NSCLC	ND	PD-1	Poor response	Low CD8 and PD-L1 staining	[5]
<i>ALK</i>	NSCLC	ND	PD-1	Poor response	Low CD8 and PD-L1 staining	[5]
<i>JAK1</i>	Melanoma	< 5%	PD-1	Poor response	Absence of CD8 PD-L1 PD-1 staining	[6]
<i>JAK2</i>	Colorectal cancer	< 10%	PD-1	Poor response	Absence of CD8 PD-L1 PD-1 staining	[6]
<i>STK11/LKB1</i>	Lung adenocarcinoma	16%	PD-1	Poor response	Absence of PD-L1 staining	[32]

^a Fq, frequency; ^b ICIs, immune checkpoint inhibitors; ^c DNA MMR, DNA mismatch repair; ^d MMRD, mismatch repair-deficient; ^e NSCLC, non-small cell lung cancer; ^f ND, not determined; ^g Increased expression of genes associated to adaptive immunity in responders (R); ^h Reduced expression of genes associated to adaptive immunity in non-responders (NR)

highlight tumor genetic profile as a key factor for selecting patients that will benefit from immunotherapy. However, tumor specimens are not always available for genetic characterization and whole exome sequencing (WES) for the determination of TMB is expensive and time consuming. For these reasons, alternative strategies are needed to measure TMB, such as targeted sequencing, or strategies able to surrogate it, such as assessment of circulating biomarkers. Initial data on TMB assessment through sequencing of circulating DNA have been recently discussed at the American Society of Clinical Oncology (ASCO) meeting [46]. Moreover, the optimization of a pipeline aimed at precisely defining the threshold of high and low TMB and evaluating the immunogenicity of neoantigens generated by TMB is also still required.

Tumor-driven biochemical remodeling of the tumor microenvironment and its role in immune suppression

Metabolic reprogramming and immune evasion are two important hallmarks of cancer. The metabolic reprogramming supports the high proliferation rate and growth of tumor cells by providing the required energy and the carbonic precursors necessary for the synthesis of new biomolecules. Conversely, by immune evasion, tumor cells grow escaping the immune surveillance operated by different immune cell subsets, belonging to innate or adaptive immunity subsets. These two hallmarks, cancer dysmetabolism and immune evasion, are interdependent and linked via the tumor-driven metabolic changes occurring in TME. Activated oncogenes and inactivated tumor suppressors regulate the metabolic reprogramming of tumor cells that in turn changes the availability of nutrients and leads to the accumulation of metabolites in the TME. This altered metabolic environment imposes the biochemical remodeling of tumor infiltrating stromal and immune cells and affects their phenotype and functional specification. Thus, besides the secretion of specific cytokines, chemokines, and growth factors, the competition for nutrients and the metabolic milieu enriched in tumor-derived metabolites favors the setting of an immunosuppressive and protumor environment.

Metabolic changes in cancer cells

In cancer cells, glucose is one of the primary sources of energy and it is metabolized via aerobic glycolysis, a process known as Warburg effect. This metabolic dysfunction is crucial for tumor maintenance and progression since, in addition to provide energy in the form of ATP (although with low efficacy as compared to oxidative phosphorylation (OXPHOS), compensated by enhanced glucose uptake), it also ensures the accumulation of precursors for macromolecule synthesis and

opens the possible commitment of mitochondria to anabolic metabolism.

Oncogenic mutations and the activation of several pathways important for cell proliferation regulate the aerobic glycolysis. For example, BRAF suppresses the OXPHOS in melanoma, RAS increases the transcription of glucose transporter 1 (*GLUT1*), and PI3K/AKT controls the uptake of glucose increasing the GLUT-1 on the cell surface. Oncogenic KRAS induces activation of MYC through Raf/Mek/Erk pathway enhancing glucose consumption. Furthermore, MYC also increases lactate production boosting the expression of lactate dehydrogenase A (LDHA) [1].

As a consequence of tumor development, the oxygen and nutrients supplied from normal vasculature are not sufficient to support the high metabolic rate of tumor cells, thus, low O₂ (hypoxia) and nutrient availability, high apoptosis, and necrosis characterize fast-growing tumors. Hypoxia restricts OXPHOS and it further pushes the tumor cell metabolism toward the conversion of glucose to lactate by O₂-independent glycolysis (Warburg effect). Thus, lactate, carbonic dioxide (CO₂), and protons (H⁺), produced by cancer cells and accumulated in the extracellular milieu, are the main causes of low pH and acidity in the TME [2]. Moreover, the necrotic tumor cells release their intracellular potassium (K⁺) producing a high concentration of K⁺ in the TME [47].

The growth of cancer cells is not only dependent on glucose, as for their active proliferation, cancer cells require lipids and amino acids, whose synthesis or uptake is also regulated by oncogenes or signaling pathways. For example, KRAS regulates the glutamine metabolism in pancreatic cancer [1] and BRAF inhibitors favor the overgrowth and proliferation of glutamine-dependent melanoma cells [48]. The oncogene MYC is involved in the promotion of glutamine uptake and in the conversion of glutamine into acetyl-CoA in the mitochondria through the upregulation of glutamine transporters like SLC1A5 (ASCT2) and glutaminase 1 (GLS1), respectively [49]. The glutamine converted into glutamate is an important source for the glutathione-based anti-oxidant system, and it provides nitrogen used for the synthesis of asparagines and precursors of nucleic acids. Moreover, the glutamine can be converted into α -ketoglutarate (α -KG) and, in condition of hypoxia, α -KG can be used as precursor of cholesterol synthesis [50]. To satisfy their lipid requirement, tumor cells enhance lipid and cholesterol synthesis through the activity of sterol regulatory element-binding protein (SREBP1) regulated by the PI3k/Akt/mTORC1 pathway [51].

Tumor-derived metabolites and competition for nutrients favor the establishment of a protumor environment

Immune cells are highly plastic, and in response to external stimuli, they polarize toward different functional states. The

competence for different effector functions is regulated by programmed changes in the cellular metabolism. Importantly, the metabolic environment in which the immune cells operate influences the metabolism of immune cells and, consequently, their functions. In this perspective, the tumor-driven metabolic milieu imposes a metabolic reprogramming to infiltrating immune cells, which leads to the acquisition of protumor and immune suppressive features.

Hypoxia, acidity, K^+ , and metabolites such as lactate accumulated in the TME as result of metabolic dysfunctions of cancer cells affect the survival, proliferation, and effector functions of immune cell populations (T cells, Tregs, macrophages, and MDSCs) creating an immunosuppressive and protumorigenic microenvironment.

Although evidence mainly produced by *in vitro* experiments indicated that hypoxia and the consequent expression of hypoxia-inducible factor 1 α (HIF-1 α) enhances the effector functions of T cells [52], this condition is not met in the hypoxic TME. Indeed, complex hypoxia-driven interactions occur in the TME which result in the setting of T cell immune suppression. Hypoxia stimulates the expression of PD-L1 in tumor cells, TAMs, MDSCs, and the PD-L1/PD-1 axis leads to anergy in T cells [53]. Moreover, the secretion of IL-2 and IFN γ from T cells is impaired by the high acidity and K^+ level, which characterize the highly hypoxic TME [47, 54]. Hypoxia and acidity also promote an environment that stimulates the immunosuppressive properties of Tregs and TAMs which actively block T cell functions. HIF-1 α induces the expression of Foxp3 in Tregs favoring their survival in an acidic TME [55, 56]. Hypoxia not only supports Tregs, but, by inducing the production of the CCL-28 and CCL-2 chemokines in cancer cells, it also facilitates the recruitment of CCR10-positive Tregs and CCR2-positive MDSCs [57, 58]. HIF-1 α promotes the release of the immune suppressive cytokine IL-10 in PD-L1-positive MDSCs and increases the expression of CD39 and CD73, ectoenzymes involved in the conversion of ATP to adenosine, which exerts immune suppressive function [53, 59]. Tumor zones with a low O_2 are highly infiltrated with M2-type TAMs [60] and hypoxia regulates the proangiogenic phenotype of M2 cells [61]. HIF-1 α and acidic TME increase the immunosuppressive capacity of TAMs by the production of vascular endothelial growth factor (VEGF), arginase 1 (Arg1), and nitric oxide synthase (iNos) [62]. We demonstrated that limiting the extrusion of H^+ by the usage of omeprazole, a proton pump inhibitor, the viability and aggressive features of tumor cells and concomitantly the protumor and immunosuppressive functions exerted by the TAMs were reduced in HCC, likely favoring an antitumor response mediated by T cells [63].

In addition to low pH, tumor metabolic-conditioned TME includes the accumulation of lactate which mediates pleiotropic immune suppression activities. The selective uptake of lactate by macrophages induces their conversion from proinflammatory M1 into immune suppressive M2 subtype

[62]. Moreover, lactate limits the proliferation and the functions of conventional T cells [64]. Conversely, the immune suppressive Tregs will survive in a microenvironment rich in lactate and poor in glucose. In fact, Tregs reprogram their metabolism by Foxp3 transcription which suppresses MYC and glycolysis and potentiates the OXPHOS [56].

In this hostile environment, cancer and immune and stromal cells compete for glucose and glutamine nutrients and cancer cells limit the availability of glucose to tumor-infiltrating lymphocytes (TILs) [65, 66]. Thus, by promoting their own proliferation, cancer cells simultaneously counteract immune surveillance.

Cancer cells are particularly addicted to glutamine. The high request of glutamine by tumor cells can be supported by cancer-associated fibroblasts (CAFs) and TME-educated TAMs that are able to supply glutamine and support tumor growth [67, 68], thereby exerting a strong protumor function.

It was also recently reported that nutrient readiness affects epigenetic regulation of cancer cells and TME. Specifically, in melanoma, low amount of glutamine promotes epigenetic changes associated with histone hypermethylation, resulting in the induction of an aggressive, dedifferentiated, and BRAF inhibitor-resistant phenotype [69]. Moreover, epigenetic reprogramming associated with nutrient availability was also reported to be associated with immunosuppression. The conversion of glutamine into α -KG is linked to increased oxidation of fatty acids and the transcription of M2 genes in macrophages by the epigenetic regulation exerted by Jmid3 [70]. Furthermore, another epigenetic regulator, the methyltransferase EZH2, regulates the glycolysis and effector functions of T cells. Ovarian cancer cells restrict the expression of EZH2 on T cells, thus improving immune evasion [71].

In conclusion, the metabolic interplay between tumor and immune cells in the TME is crucial for the regulation of the antitumor immune response. The microenvironmental metabolism driven by metabolically dysfunctional cancer cells imposes the reprogramming of immune and stromal cells leading to the acquisition of protumor and immune suppressive functions. Thus, “metabolic checkpoints” regulating tumor immunity might define new targets for therapeutic interventions (Fig. 1).

The role of extracellular vesicles in tumor-induced immunosuppression targeting immune cells

EVs are membrane surrounded structures that can be released by most types of cells. EVs are important carriers of biological materials and can be found in body fluids, including plasma, serum, lymph, urine, saliva, tears, and milk. The family of EVs is composed by different types of vesicles called

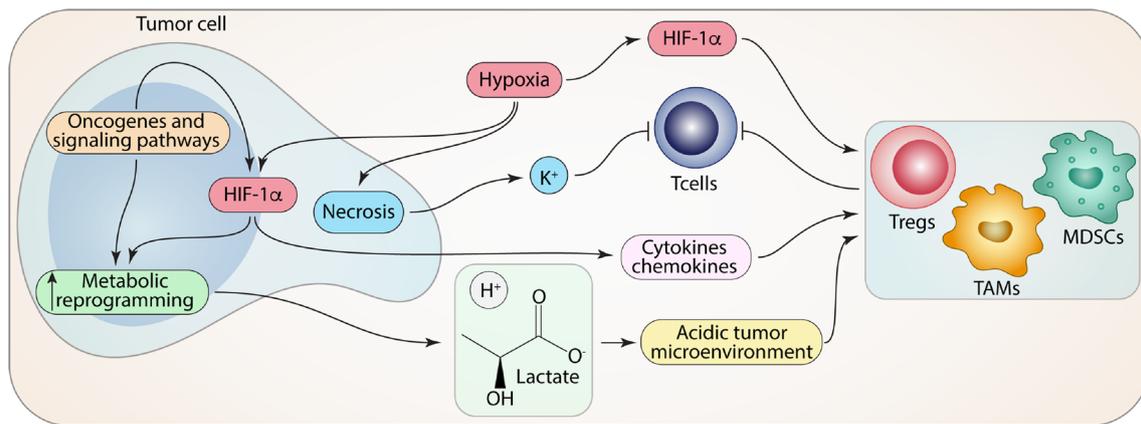


Fig. 1 Biochemical remodeling of the tumor microenvironment (TME). Oncogenes, signaling pathways, and hypoxia can reprogram tumor cell metabolism. The metabolic reprogramming contributes to the generation

of metabolites involved in the acidification of TME. Acidic TME, necrosis induction, and cytokine/chemokine production mediated by hypoxia generate an immunosuppressive milieu

exosomes, microvesicles, and apoptotic bodies that differ for their biogenesis, size, and cargo. Discovered in the early 1980s, EVs attracted attention only after a decade later, when Raposo and colleagues described that exosomes have immunomodulatory properties [72]. Originating from the endosomal compartment through the degranulation of multivesicular bodies, exosomes mediate intercellular crosstalk working as carriers for the active transfer of functional nucleic acids, such as miRNAs, proteins, and lipids, a process with several immunoregulatory effects [73].

Tumor cells release a considerable amount of EVs which are responsible for the setting of a protumorigenic and immunosuppressive TME. In fact, these vesicles can act on different immune cell subsets of both innate and adaptive immunity and can induce their functional commitment to immune suppression through several mechanisms [74]. Their modulatory potential may derive from their heterogeneity in size, thereby generating a vast variety of subpopulations. These differences in dimensions might in turn be linked to particular molecular compositions that induce specific biological effects [75]. Indeed, it has been reported that the presence of tetraspanins and integrins, the cargo of nucleic acids, or the protein content can direct EV adhesion or uptake by specific recipient immune cells and guide their acquisition of immunosuppressive features [76, 77]. In hematological malignancies, it was found that large vesicles, as well as the small exosomes, are important actors in the crosstalk between the tumor and the microenvironment [78]. In this regard, our group recently showed that plasma samples from melanoma patients contain a higher concentration and larger EVs compared to healthy donors. In vitro studies demonstrated that these EVs have immunomodulatory properties and are able to confer a MDSC phenotype to CD14₊ cells [79].

In summary, tumor-derived circulating EVs can promote immunosuppressive circuits that contribute to the generation of suppressive myeloid cells, which represent key targets for

therapeutic intervention to restore antitumor immunity [80]. Table 2 summarizes the immune suppressive effects of tumor EVs on immune cells.

Effect of tumor-derived extracellular vesicles on functional activities of myeloid cells

Our group has focused on the study of the role of tumor EVs in tumor immune escape mechanisms and inhibition of immune response. We first described that tumor EVs have immunomodulatory properties by impairing monocyte differentiation into DCs and promoting the generation of MDSCs [80, 100]. Specifically, we showed that CD14₊ monocytes isolated from healthy donors exposed to melanoma EVs, even in the presence of IL-4 and GM-CSF (cytokine known to induce DC differentiation), maintained the CD14 expression and change into cells with HLA-DR(-/low) and low expression of the costimulatory molecules CD80 and CD86. This phenotype is associated to the release of immunosuppressive cytokines (IL-6 and TGF- β), and to a strong suppressive activity on T cell-proliferation and cytolytic functions [7]. Moreover, tumor EVs interact with DCs via miR-203 and contribute to the dysfunction of DCs by controlling TLR4 expression and production of cytokines, such as TNF and IL-12 [81]. In addition, tumor exosomes promote IL-6 secretion from DCs via TLR2 and TLR4 signaling through a membrane-associated mechanism that relies on HSP72 expressed by the exosomes [82].

Additionally, tumor exosomes promote also MDSC differentiation. Breast, lung, and ovarian cancer exosomes that express membrane HSP70 can interact and activate MDSCs via TLR2. Importantly, the frequency of HSP70-expressing exosomes is higher in cancer patients compared with healthy donors [83]. Furthermore, in renal cell carcinoma, exosomal HSP70 can determine the suppressive activity of MDSCs via phosphorylation of STAT3 in a TLR2-MyD88-dependent manner, justifying exosomal HSP70 as a significant target in this

Table 2 Tumor extracellular vesicles target immune cells and suppress the immune system

Target cell	Tumor	Effects/mechanisms	Ref
Dendritic cell	Melanoma	^c ↑Immunosuppressive activity via IL-6 and TGF-β	[7]
	Pancreatic cancer	^d ↓TLR4 expression and cytokines via miR-203	[81]
	Melanoma	↑IL6 via HSP72/ HSP105 in a TLR2/4 manner	[82]
^a MDSC	Breast/lung/ovarian	↑Activation via HSP70/TLR association	[83]
	Renal cell carcinoma	↑Immunosuppressive activity via p-STAT3	[84]
	Multiple myeloma	↑Proliferation activation via STAT3	[85]
Macrophage	Breast	↑M2 phenotype via STAT3 transferring gp130	[86]
	Breast	↑Pro-inflammatory activity via TLR2/NF-kB	[87]
	Ovarian	↑M2 phenotype via miR-222	[88]
	Pancreatic cancer	↑M2 phenotype via miR-301a	[89]
	Gastric cancer	↑PD-1 and M2 phenotype	[90]
Monocyte	Glioblastoma	↑M2 phenotype and PD-L1	[91]
	Neuroblastoma	↑Chemotherapy resistance by miR-21, miR-155	[92]
	Melanoma	↑MDSC phenotype	[79]
Treg	Nasopharyngeal	↑Cell recruitment via CCL20	[93]
	Head and neck	↑TGF-β, IL-10, COX-2, CD39, CD73	[94]
	Colorectal cancer	↑Treg induction via TGF-β	[95]
T CD8+	Head and neck	↑Suppressor phenotype by loss of CD27/CD28	[96]
	Breast	↓Proliferation	[97]
^b NK	Acute myeloid leukemia	↓NKG2D expression and cytolytic activity	[98]
	Breast	↓Cytotoxic activity	[97]
	Lung carcinoma	↓Activity via TGF-β, miR23a	[99]

^a MDSC, myeloid-derived suppressor cell; ^b NK, natural killer cells; ^c Increase; ^d Decrease

context [84]. Finally, tumor exosomes can mediate an increased proliferation of MDSCs in the bone marrow of multiple myeloma-naïve mice via STAT3 [85] and a decrease of normal hematopoiesis in acute myeloid leukemia (AML) [101].

However, in some tumors like glioblastoma, tumor exosomes have a predilection for targeting monocytes and inducing a M2 phenotype and PD-L1 upregulation via STAT3 pathway [91]. In addition, M2 polarization is also induced by breast cancer EVs via gp130/STAT3 activation causing a decrease of *IFNG* gene expression and increase the mRNA level of *IL1B*, *IL6*, *IL10*, *CCL2*, and *CXCR4* genes [86]. Moreover, breast cancer EVs modulate macrophage activity via TLR2 and NF-kB pathway [87]. Additionally, gastric cancer EVs are involved in the induction of a protumorigenic M2 phenotype by PD-1 expression in macrophages that are actually accumulated in advanced-stage gastric cancer [90].

A crucial role in immunomodulation is also played by the exosomal miRNA cargo. Specifically, miR-222 and miR-301a in ovarian and pancreatic cancers, respectively, induce an M2 phenotype in macrophages by SOCS3/STAT3 pathway or in a HIF-1α- or HIF-2α-dependent manner [88, 89]. Moreover, tumor exosomes are involved in the resistance to chemotherapy via miRNA by targeting human monocytes in neuroblastoma through miR-21/TLR8-NF-κB and miR-155/TERF1 pathways [92].

Role of extracellular vesicles in the T and NK cell modulation

The detrimental effects of cancer EVs involve also the T and NK cells. Indeed, tumor exosomes may directly affect T cell function by controlling the recruitment of Tregs via CCL-20 [93] and by increasing their immunosuppressive activity through the induction of Ca²⁺ influx [102]. Regulatory T cell-related genes such as TGF-β are upregulated by colorectal cancer EVs [94, 95]. In addition, exosomes derived from murine breast cancer cells can inhibit T cell proliferation, while EVs derived from human head and neck cancer induce a suppressor phenotype in human CD8+ T cells by loss of CD27/CD28 [96, 97].

Concerning NK cells, exosomes isolated from plasma of AML patients, enriched in TGF-β1/LAP, CD39/CD73, PD-1/PD-L1, or Fas/FasL proteins, all molecules exerting immunosuppressive activities, when incubated with NK-92 cells reduce the expression of NK cell cytotoxicity-related molecule NKG2D [98]. Moreover, in vitro murine breast cancer EVs reduced the lysis of tumor cells mediated by NK [97] and exosomes derived from lung carcinoma cultured in hypoxic conditions displayed strong inhibitory effects on NK cell cytolytic activity. The inhibitory activity of these hypoxic exosomes correlated to their enhanced levels of miR-23a and TGF-β [99].

In conclusion, since EVs reflect the dysregulations of the originating cells and since they are endowed with a

remarkable stability in the body fluids, they represent ideal non-invasive biomarkers to measure the systemic immune suppression status in cancer patients [103, 104]. Thus, exosomes are considered as a valuable target to identify novel biomarkers of cancer-induced immunosuppression that could potentially be used in the clinics to predict patient outcomes or treatment responses.

Myeloid cells in cancer, accrual of protumor effectors

A perfect example of how cancer can engage host responses to promote its own survival is the systemic crosstalk that tumor establishes with myeloid cells. The secretion into the blood stream of cytokines (GM-CSF, IL-6, CCL2), genetic material, membrane vesicles, and other factors by cancer triggers subtle but persistent stimulation of signaling pathways such as STAT3, PI3K, IRF8, NOTCH, adenosine receptor, and Rb1 in myeloid cells [8]. Bone marrow myelopoiesis is hence modified with the release in the peripheral circulation of immature and dysfunctional cells; concomitantly, differentiated monocytes and neutrophils in other immune organs (spleen, lymph nodes, blood) are reprogrammed to acquire a regulatory function. The whole process, leading to the generation of potentially immunosuppressive effectors, is part of a homeostatic response aimed at protecting tissues from excessive immunity and inflammation [105].

Dysfunctional myeloid cells are globally defined as MDSCs. They include a heterogeneous population of monocytic- and granulocytic-like elements morphologically and phenotypically resembling their normal counterparts (defined in human peripheral blood mononuclear cells as CD14+HLA-DRneg and CD15+HLA-DRneg for monocytic and granulocytic MDSCs, respectively) but differing for the broad protumor activity. Recent studies elucidating MDSC generation are showing that the process is made possible by the high plasticity of the myeloid subset that can be finely tuned and redirected to multiple differentiation programs by transcriptional, epigenetic, and metabolic regulation [106, 107].

Together with tissue resident macrophages, myeloid cells exert in TME a tight and pleiotropic support to tumor growth involving, among others, (1) the release of proangiogenic factors and the transdifferentiation into endothelial cells, to sustain tumor neoangiogenesis [108]; (2) the remodeling of the stroma component, through the action of TGF- β and metalloproteinases, thus favoring tumor cells spreading and metastatization; (3) the conditioning of local fibroblasts and their increment by the transdifferentiation of MDSCs into fibrocytes exerting indoleamine-pyrrole 2,3-dioxygenase (IDO)-mediated immunosuppressive activity [109]; (4) impairment of T and NK cell function via metabolic block (mediated by Arg1, NO, and IDO), T cell receptor signaling impairment by reactive oxygen species, inhibition of T cell function and Treg recruitment

(through TGF- β and IL-10), and induction of T cell anergy by means of PD-L1 [110]; and (5) direct support of tumor cell proliferation via novel pathways involving caspase 1 and tumor-intrinsic MyD88-dependent carcinogenesis [111].

In addition to MDSCs, other myeloid cell populations exerting protumor activities found at the tumor site include TAMs, tumor-associated neutrophils (TANs), whose relationship with monocytic (M) and polymorphonuclear (PMN) MDSCs is still a debated issue, tolerogenic DCs, and mast cells (MCs). All these exert normal physiological functions in tissue homeostasis, but in cancer, they are exploited by the tumor cells to support their own growth and progression [112]. These cells share suppressive functions with MDSCs and they are active in attracting and stimulating the protumor activity of Tregs. Moreover, they produce factors which support the recruitment of immature myeloid cells, thus ensuring the constant replenishment of the TME with immune suppressive dysfunctional myeloid cells [112, 113]. Of note, a synergistic interaction can also occur between MCs and MDSCs at the tumor site. In fact, it has been shown that MCs are spatially associated with MDSCs in the human TME of colorectal carcinoma, and in an experimental model, MCs were found to increase the immunosuppressive capacity of MDSCs [113].

MDSCs as biomarker of systemic immunosuppression and new therapeutic target

The chronic cancer-myeloid interaction occurring with disease progression causes the incremental accumulation of myeloid cells in blood and TME of cancer patients, which is constantly a sign of aggressive disease and bad prognosis in virtually all cancers analyzed [10]. Recently, data are also emerging about the potential role of MDSCs in reducing response to ICIs [11]. Albeit no evidence about the pure predictive role of systemic myeloid accumulation in resistance to ICIs has been reported, it is conceivable that an increased level of systemic immunosuppression may not only worsen the disease course but also reduce the immunostimulating activity of immunotherapy.

Indeed, a local and systemic enrichment in myeloid cells is usually associated with poor tumor T cell infiltrate, an epithelial-to-mesenchymal transition profile, and poor response to ICIs [27, 114]. Similarly, the physical or functional removal of MDSCs represents a strategy to recover ICI activity in pre-clinical tumor models [115].

Despite the abovementioned evidence and the recent description of novel biomarkers specifically defining MDSCs [116], the quantification of these cells in blood or TME still needs to be acknowledged as a valid approach in clinical practice to assess cancer-associated systemic immunosuppression and predict resistance to therapy. Nevertheless, clinicians have recently reported that an increase in neutrophil and monocyte blood counts, or their relative ratios to lymphocytes, is associated with progression during ICI administration [117].

These parameters, known for long time to be a negative prognostic factor in several cancers [118], are likely reflecting the expansion of granulocytic or monocytic MDSCs, as indirectly demonstrated by recent preclinical in vitro studies [119].

As MDSC expansion represents a central mechanism for cancer to mediate immunosuppression at systemic level, drugs interfering with the generation or function of these cells could significantly improve the benefit of immunological therapies. The heterogeneous nature of these cells and their immunosuppressive pathways make a selective targeting rather problematic, but several molecules interfering with key MDSC mediators (for instance Arg1, CSF1, IDO, STATs, TRAILR) are under study or in clinical development [120, 121]. However, it is here worth to mention that standard cancer therapies such as certain chemotherapeutics or antiangiogenics can profoundly interfere with MDSC accrual through their well-known myelotoxicity [122, 123]. In line with this observation is the very recent evidence that ICI efficacy is remarkably increased by combination with these therapeutic strategies [124, 125].

Conclusions

The extraordinary success achieved by immunotherapy demonstrates how modifying the balance of immune components in TME can lead to a remarkable tumor control. In spite of that, the efficacy is still obtainable in a subset of patients, while the vast majority is receiving marginal or no benefit from treatment. Predictive biomarkers identify and direct resistant patients to alternative or combinatorial therapies, and the discovery of the mechanisms underlying resistance is a priority of current cancer research. In this review, we illustrated how some pillar pathways should be taken into account for developing new potential strategies limiting immunosuppression and favoring the efficacy of immunotherapy: (1) oncogenic pathways involved in the promotion of tumor evasion by altering the recruitment, cellular composition, and functions of immune infiltration, and as such influencing the response to immunotherapy; (2) oncogene-driven cancer dysmetabolism and “metabolic checkpoints” of cancer and immune cells, which contribute to the establishment of an immunosuppressive and protumorigenic environment; (3) the contribution of tumor EVs in the generation of immunosuppressive cells; (4) the crosstalk between myeloid and cancer cells, reminiscent of a physiological mechanism of wound healing turning into a strong protumor force. Finally, we can conclude that after the discovery of ICs, cancer-related immunosuppression in TME and at systemic level seems to be the ultimate barrier to maximize the therapeutic potential of cancer immunotherapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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