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The metabolic milieu in melanoma: Role of immune suppression by CD73/adenosine

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Abstract

The mechanisms leading to immune escape of melanoma have been largely investigated in relation to its tumour immunogenicity and features of inflamed microenvironment that promote the immune suppression during the disease progression. These findings have recently led to advantages in terms of immunotherapy-based approaches as rationale for overcoming the immune escape. However, besides immune checkpoints, other mechanisms including the adenosine produced by ectonucleotidases CD39 and CD73 contribute to the melanoma progression due to the immunosuppression induced by the tumour milieu. On the other hand, CD73 has recently emerged as both promising therapeutic target and unfavourable prognostic biomarker. Here, we review the major mechanisms of immune escape activated by the CD39/CD73/adenosine pathway in melanoma and focus potential therapeutic strategies based on the control of CD39/CD73 downstream adenosine receptor signalling. These evidences provide the basis for translational strategies of immune combination, while CD73 would serve as potential prognostic biomarker in metastatic melanoma.

Keywords

Melanoma, CD73, adenosine, immunometabolism, immunotherapy

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Introduction

The incidence of cutaneous melanoma has increased worldwide in the last decade, although both progression free survival (PFS) and overall survival (OS) have been definitely improved by immunotherapy.[1–3] However, only some patients benefit from immune checkpoint inhibitors for the unavailability of biomarkers to select potential responders as well as to early identify resistant patients whose molecular mechanisms have been partly discovered.[4,5]

In this context, the majority of explorative studies failed to discover reliable biomarkers in the clinical setting, and thus, novel investigative approaches to strengthen anti-melanoma strategies have been addressed including the regulatory role of the immunometabolism.[6] Immunometabolism is a new chapter in immune oncology that describes the modifications occurring in intracellular metabolic pathways of immune cells during their activation or inhibition.[7–9] It has been previously demonstrated that the defective regulation of the cellular energy includes a metabolic reprogramming process activated in cancer cells[10,11] as in lymphocytes surrounding the melanoma cells within the tumour microenvironment.[12] The interplay of melanoma cells with components of the adaptive immune system drives both initiation and propagation of signals required to engage proliferative signals,[13] and the intracellular cascade activating T cells to counterattack the melanoma proliferation promotes a

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number of intracellular modifications mostly affecting the cellular immune metabolism.

It is noteworthy that naive T-lymphocytes mainly use the available nutrients including fatty acids and glucose to maximize energy production though oxidative phosphorylation, whereas effector T cells require an anabolic milieu. Furthermore, T cells recruited to peripheral sites become activated after both binding and stimulation of T-cell receptor (TCR) by co-stimulatory molecules that reprogram the metabolism of naive T cells towards an anabolic behaviour that sustains the proliferation resulting in the effector functions.

Moreover, this metabolic reprogramming is characterized by the engagement of the aerobic glycolysis, a process characterized by the conversion of glucose into lactate. In addition, one hallmark of this metabolic conversion is that, despite adequate oxygen levels to support complete oxidation of glucose, cellular adenosine triphosphate (ATP) production switches from oxidative phosphorylation to high-throughput glycolysis.[14] However, other T-cell subsets require alternative energetic and biosynthetic pathways to satisfy their demand and to balance the immune system activity,[15] and besides intrinsic modifications in immune cells that affect the energetic metabolism, the melanoma tumour microenvironment may negatively influence the efficiency of T-cell-mediated immune response, and particularly the intracellular metabolism. In this context, the adenosine signalling is a crucial pathway exploited by melanoma cells to evade the immune surveillance.[16] Moreover, it has been demonstrated that the extracellular adenosine protects melanoma cells from the cytotoxicity induced by T cells, thus actively participating in the generation of an immunosuppressive milieu.

Here, we review the molecular mechanisms that involve the CD39/CD73/adenosine pathway during the melanomagenesis and the potential role of CD73 expression for planning future therapeutic strategies aimed at targeting the metabolism of immune cells in metastatic melanoma.

The CD39/CD73/adenosine pathway: the physiological function

Adenosine is a nucleoside released in extracellular and intracellular space that shows pleiotropic effects in different organs and tissues in relation to its interplay with its receptors, namely A1, A2A, A2B, and A3.[17] Recent studies have demonstrated that adenosine regulates several functions in human pathology while drives intracellular signals critical for the regulation of cardiovascular system, thus suggesting its measurement for both therapeutic and diagnostic purposes.[18,19] Moreover, adenosine is an important central neuromodulator implicated in the control of the neuronal excitability and synaptic/non-synaptic transmission.[20] The intracellular adenosine is produced during the biosynthesis of purine or following the ATP degradation (Figure 1), and then crosses the cell membrane by dedicated nucleoside transporters. Moreover, it is also produced as result of the progressive dephosphorylation of the ATP mediated by the intracellular 5′-nucleotidase or the hydrolysis of SAH (S-adenosylhomocysteine),[21] while it is recruited for the regulation of the energetic metabolism.[22] By contrast, the extracellular form of the adenosine is mostly implicated in the control of intracellular signalling, thus acting as powerful inhibitor of the immune response within the tumour microenvironment.[23] In fact, extracellular adenosine is a negative immune checkpoint molecule that plays a role in establishing an immunosuppressive melanoma microenvironment through different mechanisms. On the other hand, the defective immune editing exerted on melanoma cells mostly depends on both over-production and release of negative modulators of the immune system as adenosine, tumour necrosis factor-β, vascular endothelial growth factor, and indoleamine 2,3-dioxygenase. Moreover, the loss of class I–II antigens of the major histocompatibility complex as well as the overexpression of inhibitory checkpoints on immune cells participate to immune evasion in melanoma.[13] In this context, further evidence suggest that, under hypoxic conditions, adenosine limits the maturation of natural killer (NK) cells while enhancing the immune suppression by recruiting both T regulatory (Treg) cells and myeloid-derived suppressor cells (MDSCs) within the melanoma microenvironment. This activity strongly restrains T-cell effector functions through paracrine signals that favour the melanoma growth.[24] However, these inhibitory effects are differently induced by the adenosine receptors. The A2A receptor (A2AR) is, indeed, the most common subtype activated in both innate and adaptive immunity [25] that induces inhibitory signals restraining the activity of T cells, NK cells, NK-T cells, neutrophils, macrophages and dendritic cells (DCs).[26] As represented in Figure 2, the cytotoxicity, proliferation, cytokine production and effectiveness of antigen processing and presentation activated by these cell populations are impaired by CD73 through A2AR activation.[27]

The amounts of extracellular adenosine are extremely low as effect of the enzymatic degradation induced by both CD39 (ectonucleoside triphosphate diphosphohydrolase-1, E-NTPDase1) and CD73 (ecto-5′-nucleotidase, Ecto5′NTase). In fact, the activation of CD39 induces the dephosphorylation of the ATP to adenosine diphosphate (ADP) and thus to adenosine monophosphate (AMP), while CD73 catalyzes the hydrolysis of AMP into adenosine and phosphate. Finally, both extracellular and intracellular adenosine levels are regulated by the activity of adenosine deaminase (ADA),[28] whose major function is dedicated to

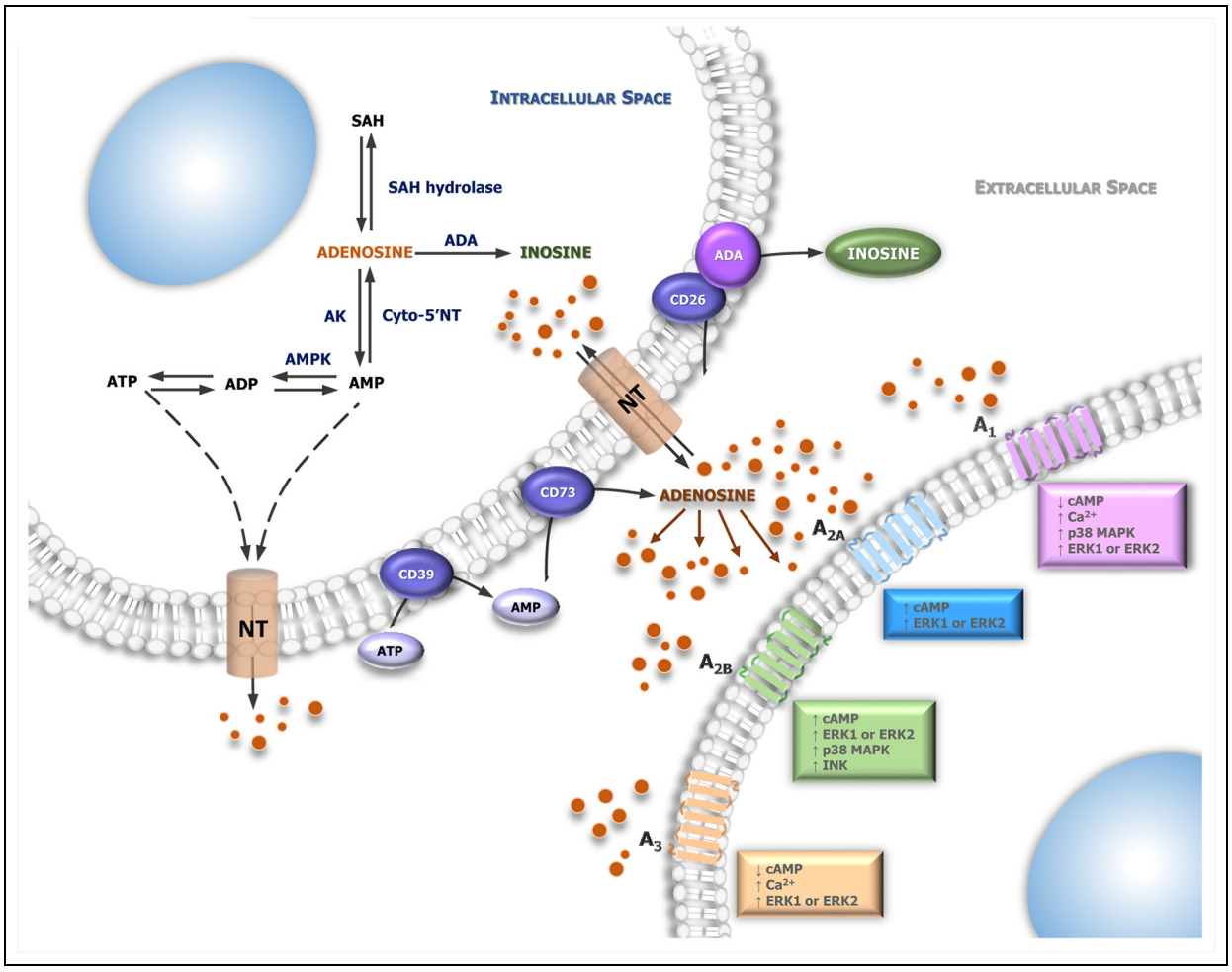

Figure 1. Adenosinergic signalling. ATP and adenosine are continuously recycled in relation to the energy request through a series of dephosphorylation and phosphorylation steps mediated by enzymes such as cyto-5′NT, AK, and AMPK. In physiological conditions the rate limiting enzyme is AK that phosphorylates adenosine in AMP. Alternatively, intracellular adenosine can also be produced from the hydrolysis of SAH. In response to high metabolic activity, both ATP and adenosine are extruded to the outside of the cell via secondary active transporters and the bioavailability of the extracellular adenosine is extremely low since derives from the enzymatic systems that include CD39 and CD73. In this context, the activation of CD39 induces the dephosphorylation of the ATP to adenosine diphosphate (ADP) and, subsequently, then to adenosine monophosphate (AMP), while CD73 catalyzes the hydrolysis of AMP into adenosine and phosphate. Finally, adenosine is irreversibly deamidated to inosine by the ADA enzyme while the extracellular adenosine binds adenosine receptor with high affinity (A1, A2A, A2B, A3).
ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; AMPK: adenosine monophosphate-activated protein kinase; AK: adenosine kinase; cyto-5′NT: Cytosolic 5′-nucleotidase; SAH: S-adenosylhomocysteine; ADA: adenosine deaminase; NT: nucleoside transporters; cAMP: cyclic AMP; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase.

the degradation of adenosine in inosine.[29,30] However, compared with the traditional catabolic pathway mediated by the CD39,[31] an alternative enzymatic cascade that increases the extracellular adenosine levels has been recently described and includes the nucleotide-metabolizing ectoenzymes as CD38 (NAD^+ nucleosidase), CD203α (ecto-nucleotide pyrophosphatase/phosphodiesterase 1) and CD73.

Therefore, either CD39/CD73 or CD38/CD203α/CD73 pathways are critically involved in the regulation of both NAD^+ nucleosidase and adenosine in cancer microenvironment [32] as well as in the composition of the so-called 'purinergic milieu' that surrounds the cells of immune system.[33,34]

The immunosuppressive role of the CD39/CD73/adenosine pathway in melanoma

Both reprogramming of energy metabolism and evading immune destruction have been recently described as emerging 'hallmark' of tumorigenesis.[8,35] In the context of the metabolic reprogramming, however, adenosine

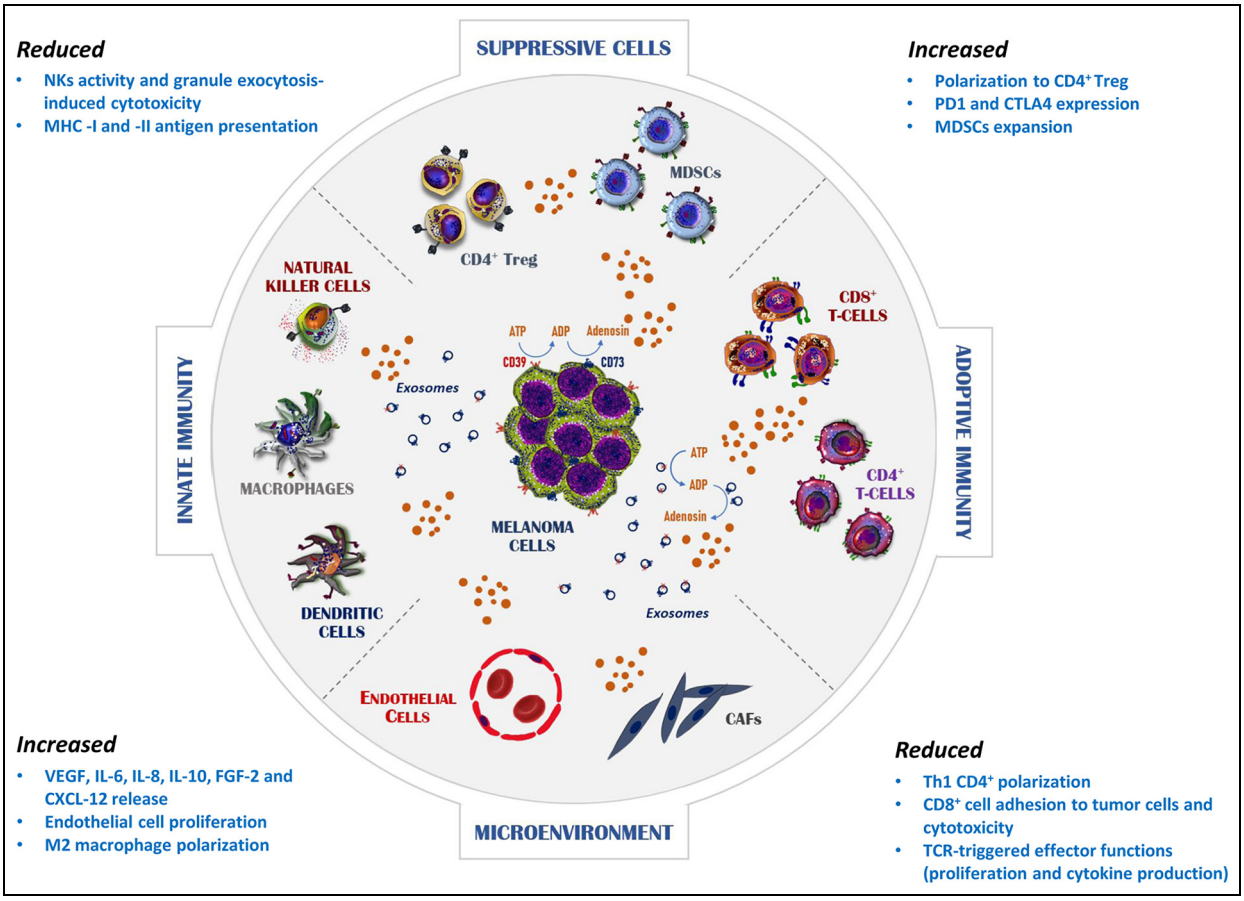

Figure 2. The CD39/CD73/adenosine pathway in melanoma microenvironment. Melanoma microenvironment is hypoxic since it is deprived of extracellular ATP quickly converted by CD39/CD73 ectonucleotidases complex in adenosine. The CD39 and CD73 ectonucleotidases are consistently expressed by melanoma cells and the high extracellular adenosine levels compromise the function of both adaptive and innate immunity while activating other pro-tumour and pro-angiogenic populations as CAFs and endothelial cells in tumour microenvironment. By binding the A2A receptor (A2AR) as the most common receptor subtype expressed by both innate and adaptive immunity, the extracellular adenosine induces inhibitory signals that restrain the activity of immune cells, thus favouring the growth and survival of melanoma cell. Tumour-derived exosomes also contribute to extracellular adenosine production, and hence indirectly modulate the immune effector cells.

NK: natural killer; MHC: major histocompatibility complex; PD1: programmed cell death protein; CTLA4: cytotoxic T lymphocyte antigen-4; MDSCs: myeloid-derived suppressor cells; TCR: T-cell receptor; VEGF: vascular endothelial growth factor; IL: interleukin; FGF: fibroblast growth factor; CXCL: chemokine (C-X-C motif) ligand; M2: macrophages 2; ATP: adenosine triphosphate; ADP: adenosine diphosphate; CAFs: cancer-associated fibroblasts.

interferes with a number of intracellular events activated in cancer models of melanoma[36] including initiation, growth, angiogenesis, metastasis and regulation of immune response.

Other than ischemia, hypoxia, inflammation and trauma, the CD39/CD73/adenosine axis is apparently relevant for melanomagenesis[24,37,38] by inducing the dysfunction of the immune response.[39] Indeed, the immunosuppression mediated by adenosine on immune effector cells in melanoma microenvironment can be differently achieved. In first instance, adenosine can interfere signals mediated by interleukin-2 (IL-2) receptor by dephosphorylating SHP-2 or STAT-5 and inhibit the cytokine production. Furthermore, it has been demonstrated that melanoma cells exert a direct anti-proliferative effect on naive $CD4^+$ T cells through an adenosine-dependent mechanism and, to a lesser extent, on $CD8^+$ cells that was rapidly reverted by using specific CD73/CD38 inhibitors. Therefore, adenosine has been suggested as key player for the melanoma cell escape from the adaptive immune control.[40] In addition, the adenosine receptors are expressed by both immune and melanoma cells[41] and the specific activation of A1R regulates their motility and chemotaxis.[42] By contrast, A2R and A3R modulate angiogenesis,[43] metastasis and melanoma cell proliferation.[44,45] A recent finding concerning the role of CD73 in melanomagenesis demonstrated that the activation of mitogen-activated protein kinase (MAPK) pathway

promotes the CD73 over-expression by melanoma cells and, conversely, its reduction by blocking the BRAF/MEK signalling.[46]

Furthermore, CD73 concurs to the plasticity of melanoma cells and contributes to the switch from the proliferative to invasive phenotype.[47] In particular, the invasive profile is characterized by the low expression of melanocyte lineage transcription factor (MITF), the up-regulation of CD73 in parallel with the activation of putative genes regulating the epithelial-to-mesenchymal transition (EMT).[48,49] The molecular machinery activated for this purpose includes the high transcription of c-Jun/AP-1 complex through the TNF-α stimulation via MAPK signalling. A parallel chronic overproduction of pro-inflammatory cytokines has been also described in melanoma microenvironment, and this mechanism is enrolled for the acquisition of an adaptive non-genomic resistance to immunotherapy.[50,51] This finding provides the rationale for future therapeutic strategies mostly aimed at the inhibition of the CD73 signalling, the blocking of BRAF/MEK cascade and the antagonization of the A2AR.[52] Moreover, recent studies have also demonstrated that immunosuppressive populations including Treg and MDSCs[53,54] up-regulate the CD73/CD39 expression in both primary melanoma and lymph nodes, therefore enhancing their intrinsic immunosuppressive effect.[55]

An additional chapter in melanomagenesis includes small nanovesicles secreted by cancer cells, namely exosomes (Exo) that play an important role as mediators of immunosuppression and regulators of many cellular functions implicated in melanoma progression.[56] To this regard, Exo from different cell lines as bladder, colorectal, prostate and breast cancers express both CD39 and CD73[57] and contribute to the extracellular release of adenosine, thus negatively regulating the T-cell activity.[58]

Apart from these observations that support the protumorigenic role of the adenosine activation and overproduction, other studies are consistent with an opposite anti-tumour potential as reported in CD73 knockdown murine models that were characterized by an increased migratory capacity of melanoma cells for their metastatic potential.[59]

In conclusion, the combined activation of both CD39 and CD73 ectonucleotidases in melanoma microenvironment may modulate the immune response, thus reverting the ATP-mediated pro-inflammatory immune activity to an anti-inflammatory condition mostly driven by the extracellular adenosine.

Prognostic relevance of CD73 in metastatic melanoma

In the era of immunotherapy, prognostic and predictive biomarkers are urgently needed[60,61] since a considerable proportion of metastatic melanoma patients treated with checkpoint inhibitors[62] does not achieve clinical benefit or develop resistance.[63,64]

Based on the potential therapeutic role of adenosine, CD73 expression by cancer cells has been investigated for prognostic purposes in several histotypes producing conflicting results. Preliminary data demonstrated that CD73 is epigenetically regulated in malignant melanoma cells and drives the selective tropism towards metastatic sites. Indeed, clinical trials completed in a large cohort of patients revealed the high frequency of the CD73 CpG (—C—phosphate—G—) island methylation in both primary and metastatic melanoma as well as in normal melanocytes.[65] In addition, both visceral and brain metastatic sites are more frequently involved when CD73 is not methylated in primary melanoma, while in presence of CD73 methylation a limited metastatic potential or selective tropism towards the lymph nodes has been demonstrated, thus suggesting that CD73 is a potential epigenetic biomarker mirroring the disease severity. These findings further support the potential role of CD73 during tumorigenesis.[65]

To define the prognostic value of CD73 in melanoma, a peculiar association between levels of soluble CD73 and outcome has been found in patients with metastatic melanoma treated with anti-PD1 checkpoint inhibitors, thus suggesting the measurement of soluble CD73 for prognostic purposes. In particular, CD73 is proposed as the strongest prognostic factor for OS and PFS since high basal levels of soluble CD73 have been associated with the lowest response rate to immunotherapy.[66]

Moreover, several trials evaluating the immmunohistochemistry (IHC) expression of CD73 in human cancers[67] have reinforced its role in tumorigenesis,[68–72] and with regard to melanoma, a recent report showed high CD73 expression in the majority of melanoma cells. However, although similar values have been also demonstrated in endothelial, stromal, tumoral and immune cells, CD73 over-expression by tumour cells correlates with lower OS, while its levels in tumour-infiltrating mononuclear cells were associated with improved survival[73] and, furthermore, intensity of CD73 expression by melanoma cells paralleled the disease worsening. These findings were also detected in patients with non-small-cell lung cancer (NSCLC) whose CD73 levels by cancer cells were an independent indicator of poor prognosis as OS and recurrence-free survival. In addition, in these tumours CD73 harboured genetic defects such as *EGFR* mutations and *ALK* translocations, and this may explain, at least in part, the modest responsiveness to immunotherapy.[74,75] Finally, it has also been reported that the high levels of CD73 in EGFR-mutant NSCLC cell lines are inversely related to the low IFN-γ signature as compared with wild-type cell lines.[76] Therefore, anti-EGFR targeted

therapy by tyrosine kinase inhibitors appears a functional strategy to restrain the CD73-driven cascade.

Thus, high CD73 levels by malignant cells may reflect a more aggressive disease in metastatic melanoma patients, albeit at present this is not considered a reliable prognostic factor.

Targeting immunosuppressive adenosine in melanoma

The emerging role of CD73 in melanoma growth and progression has emerged for its immunosuppressive function in tumour microenvironment, thus representing a potential opportunity for developing targeted treatments.[77] Two different modalities have been recently proposed in mouse melanoma models, including molecular inhibitors of adenosine receptors or monoclonal antibodies targeting CD73, and showing similar efficiency in limiting melanoma cell growth.

In this context, recent studies showed that the single A2A receptor (A2AR) blockade or the combination with either PD-L1 or CTLA4 inhibitors[78] induces a defined T-cell activation, restrains the tumour growth, leads to Th1 gene expression signature consistent with immune activation, and reinforces the memory immune cells, although a parallel and compensatory increase of CD73 expression has been found in the majority of studies.[79,80]

Moreover, targeting the adenosine in combination with CTLA4 inhibitors improves the PFS in murine melanoma model, enhances the infiltration of $CD8^+$, $CD4^+$ T cells, and reduces the number of Tregs within tumour microenvironment, through both inhibition of adenosine production or blocking of A2AR. In addition, it has been demonstrated a different sensitivity of adenosine receptors to dedicated antagonists and this reinforces the hypothesis that restraining the adenosine pathway by blocking the A2AR signalling, or reducing its production, may increase the activity of anti-CTLA4 inhibitors.[81] Therefore, the antagonism of adenosine receptors could strengthen the anti-melanoma immune response by limiting the tumour growth and angiogenesis.

Regarding the knowledge of mechanisms implicated in the adaptive immune resistance, Chen et al. highlighted that the unresponsiveness to PD1/PDL1 blocking in lung and melanoma murine models is probably mediated by the tumour up-regulation of CD38 induced by levels of IFN-β as well as those of all-trans retinoic acid in tumour microenvironment. In particular, CD38 is an alternative ectoenzymatic pathway[40] that inhibits the cytotoxic activity of $CD8^+$ T cells through the activation of adenosine receptor signalling. Thus, the inhibition of CD38 cascade or the blockade of the adenosine receptors is potentially useful for overcoming the acquired resistance.[82] Nowadays, the anti-CD38 monoclonal antibody (mAb) is a current strategy for patients with refractory or relapsed multiple myeloma,[83] although many patients develop resistance. Therefore, an interesting proposal would be the evaluation in clinical trials strategies of sequencing or combination also targeting the adenosine alternative enzymatic pathway.

Finally, the disruption of the hypoxia-adenosine cascade may probably reduce the onset of resistance to MAPK inhibition, thus reverting the switch to an invasive melanoma phenotype as recently demonstrated in BRAF-mutated melanoma model.[46] In addition, CD73 expression in human melanoma could represent a marker of cell plasticity mirroring a non-genomic mechanism of resistance. Hence, MAPK-dependent regulation of CD73 exerts relevant translational implications for planning future therapies combining novel targeted agents in metastatic melanoma.

Based on the central role of CD73/adenosine axis in the defective regulation of anti-tumour response as proved in preliminary preclinical tumour models, different strategies targeting CD73 ectonucleotidase are under intensive clinical investigation. The CD73 blockade approaches are hitherto based on the use of either small molecule inhibitors or humanized mAbs. In this context, small molecules overcame mAbs in terms of both bioavailability and feasibility of administration. By contrast, anti-CD73 mAbs are a valid alternative for their high specificity, longer half-life and binding affinity that, however, result mostly dependent on their different steric hindrance. Finally, both direct and indirect effects of mAbs on target cells as well as on immune system cells have been clearly demonstrated. The anti-CD73-based strategies including the relative ongoing clinical trials in monotherapy or combination with conventional chemotherapy, targeted therapies or immune checkpoint inhibitors (i.e. anti-CTLA4 or anti-PD1 mAbs) are summarized in Table 1. Nowadays, preliminary data of anti-CD73 mAb and PD1 blockade appear as a promising approach with an acceptable safety profile, thus encouraging future therapeutic application in metastatic melanoma.

Conclusions

Malignant melanoma remains the most aggressive and life-threatening skin cancer, albeit remarkable therapeutic advances have achieved. In this context, immunotherapy combinations are relevant strategies aimed at restoring the anti-cancer immunity as well as restraining primary and acquired resistance to immune checkpoint inhibitors. To this regard, a better understanding of mechanisms involved in melanoma progression and immune evasion is required. Therefore, as

Table 1. Current development status of anti-CD73 strategies.

Drug	Pharmaceutical company	Mechanism of action	Phase of development	Clinical trial	Condition or disease	Drugs combination
MEDI9447 (Oleclumab)	MedImmune	Fully mAb	I	NCT02503774	Advanced solid rumours	MEDI9447; MEDI4736
			I	NCT03736473	Advanced solid tumours	MEDI9447
			I/II	NCT03611556	Metastatic pancreatic carcinoma	MEDI9447; MEDI4736; Chemotherapy
			I/II	NCT03381274	EGFRm NSCLC	MEDI9447; Osimertinib; AZD4635
			I/II	NCT03616886	Unrecesctable/Metastatic TNBC	MEDI9447; MEDI4736; Chemotherapy
BMS-986179	BMS	Fully mAb	I/II	NCT02754141	Advanced solid tumours	BMS-986179; Nivolumab
CPI-006	Corvus Pharmaceuticals	Fully mAb	I	NCT03454451	Advanced solid tumours	CPI-006; CPI-444; Pembrolizumab
SRF373/NZV930	Surface Oncology/Novartis	Fully mAb	Pre-clinical	–	–	–
IPH5301	Innate Pharma	Fully mAb	Pre-clinical	–	–	–
AB680	Arcus	CD73 inhibitor	Pre-clinical	–	–	–

Source: ClinicalTrials.gov.
mAb: monoclonal antibody; EGFR: epidermal growth factor receptor; NSCLC: non-small-cell lung cancer; TNBC: triple negative breast cancer; MEDI4736: Durvalumab; AZD4635: adenosine A2A receptor antagonist; CPI-444: adenosine A2A receptor antagonist.

further mechanisms of immune evasion, the modulation of adenosine signalling and interconnected pathways appears a novel approach for improving the anti-tumour immune response resulting in ameliorated clinical outcome, as well as overcoming the resistance to conventional immunotherapy by both CTLA4 and PD1 blockers. In fact, the CD73 blockade may limit both production and activation of immunosuppressive adenosine within the tumour microenvironment, thus overcoming the development of immune resistance. Indeed, targeting the adenosinergic pathway prevents the melanoma immune escape and the tumour-induced immunosuppression as recently described in preclinical models of melanoma.

In conclusion, based on these findings, clinical studies aiming at translating anti-CD73 strategies in melanoma treatment are required to evaluate the targeted blockade of adenosine signalling as an alternative and effective future approach.

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