Adenosine Signaling in the Tumor Microenvironment

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Abstract

Adenosine, deriving from ATP released by dying cancer cells and then degradated in the tumor environment by CD39/CD73 enzyme axis, is linked to the generation of an immuno-suppressed niche favoring the onset of neoplasia. Signals delivered by extracellular adenosine are detected and transduced by G-protein-coupled cell surface receptors, classified into four subtypes: A_1, A_{2A}, A_{2B}, and A_3. A critical role of this nucleoside is emerging in the modulation of several immune and non-immune cells defining the tumor microenvironment, providing novel insights about the development of novel therapeutic strategies aimed at undermining the immune-privileged sites where cancer cells grow and proliferate.

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9.1 Introduction

Until a few years ago, the term cancer defined an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metastasize [1]. It has been for long conceived that hallmarks of cancer were intrinsic genetic features eliciting tumor development, proliferation, and progression and that targeting such cell

pathways could be sufficient to reach a therapeutic cancer control [1].

This simplistic view of the neoplastic disease has been radically changed since the introduction of the concept of tumor microenvironment (TME) [2]. Basically, the neoplastic microenvironment is a dynamic niche consisting of multiple cell types, including several immune cell populations (i.e., macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, NK cells, T and B lymphocytes), cytokines, blood vessels, cancer cells, and their surrounding stroma (which consists of fibroblasts, endothelial cells, pericytes, and mesenchymal cells) all collaborating to shape tumor progression [2]. The TME is an immuno-suppressed *milieu*, characterized by the presence of mediators able to defuse the immune surveillance, impairing T-cell infiltration and favoring the accrual and activation of regulatory cells and thus contributing to cancer dissemination [3].

At the end of the 1990s, Blay et al. provided the first evidence about a marked increase of adenosine levels, a metabolite resulting from the degradation of ATP, in the cancer microenvironment [4, 5]. This sparked the interest of the scientific community about the role of this nucleoside in tumor onset and progression. Indeed, the concentrations of adenosine, physiologically present at low levels in the interstitial fluids of unstressed tissues, increase rapidly in response to pathophysiological conditions, such as hypoxia, ischemia, inflammation, or trauma, in order to generate a variety of cellular responses aimed at restoring tissue homeostasis [6, 7]. The extracellular adenosine accumulation following an acute injury exerts protective effects, shielding cells and tissues from an excessive inflammatory response and immune-mediated damage and promoting the healing processes [6, 8–11]. However, the persistence of increased adenosine concentrations beyond the acute injury phase can become detrimental to tissues paving the way to the generation of an immunosuppressed niche, an ideal condition for the onset and development of neoplasia [12]. Interestingly, further investigations allowed to observe that elevated adenosine concentrations within neoplastic environment exerted an immunosuppressant activity and counteracted the effec-

tor T-cell functions, mainly via the engagement of the adenosine A_{2A} receptors [13, 14].

It needs to be emphasized that the release of adenosine is actively increased as a result of specific genetic alterations that occur during tumor progression [15]. Several tumors are characterized by an altered purine metabolism, which facilitates the production of adenosine or reduces its degradation resulting in a "protective adenosine halo," spurring the cancer development and progression [15]. In this regard, it has observed that the purine metabolism undergoes a marked reorganization within the cancer niche, with a marked increase in the expression and activity of the main adenosine-generating enzyme ecto-5′-nucleotidase, also designated CD73 [16]. Several types of neoplasia display a high expression of CD73, which quickly dephosphorylates the AMP to adenosine, leading to the generation of an immunosuppressive and pro-angiogenic environment, which facilitates the development of neoplasia [17]. Following these studies, the CD73-adenosine axis has emerged as one of the most promising pathways in immuno-oncology. Indeed, several preclinical studies highlighted the value of CD73 as a potential therapeutic target for cancer management [17]. The pharmacological blockade of CD73, via monoclonal antibodies (mAbs) or small molecules, was effective in counteracting cancer cell growth and proliferation and angiogenesis and in curbing neoplastic cell spreading [18–21]. In parallel, several authors reported a significant downregulation of the ecto-adenosine deaminase and its cofactor CD26 (also known as dipeptidyl peptidase 4) and/or nucleoside transporters in several tumor cells, which determines the accumulation of adenosine within the intratumoral milieu [15, 22–24].

9.2 Adenosine Signaling: Receptors, Enzymes, and Transporters

Adenosine signaling strictly depends on the levels of extracellular concentrations reached in the receptor biophase [25, 26]. The extracellular levels of this nucleoside are regulated by a complex ecto-enzyme machinery and uptake system, aimed at

shaping the purinergic signaling based on the variations of tissue health condition [16, 25, 27].

Physiologically, the main source of adenosine is the S-adenosylhomocysteine [28]. Following the synthesis, adenosine is released in the extracellular space via nucleoside transporters [28]. Based on their molecular and functional characteristics, these transporters are classified into: (*1)* equilibrative nucleoside transporters (ENTs), which carry nucleosides across cell membranes in either directions, based on their concentration gradient. They include four subtypes, named as ENT1, ENT2, ENT3, and ENT4 [7, 29]; (*2)* concentrative nucleoside transporters (CNTs), comprising CNT1, CNT2, and CNT3, which allow the intracellular influx of nucleosides against their concentration gradient, exploiting the sodium ion gradient across cellular membranes as source of energy [7].

Under adverse conditions, comprising inflammation, hypoxia, ischemia, trauma, or neoplastic microenvironment, the extracellular levels of adenosine increase significantly, reaching micromolar range [30, 31]. In these contexts, adenosine accumulation stems from increased extracellular dephosphorylation of ATP, mediated in a sequential manner by ecto-nucleoside triphosphate diphosphohydrolase-1 (also named CD39) and by ecto-5′-nucleotidase (CD73) (Fig. 9.1) [9]. A number of studies identified CD73 as a critical checkpoint in regulating the duration and the magnitude of the "adenosine halo" surrounding immune cells [32]. Of note, adenosine can be generated through an alternative catabolic pathway, which is initiated by the nicotinamide adenine dinucleotide (NAD^+) glycohydrolases/CD38 enzyme axis that converts extracellular NAD^+ into adenosine diphosphate ribose (ADPR) [33]. ADPR is then metabolized by CD203a into AMP and then converted by CD73 into adenosine [33].

As described under physiological conditions, adenosine massively released under pathological conditions can be re-up taken into the cells through the nucleoside transporter or converted into inosine via adenosine deaminase both inside and outside the cell [1, 34, 35] and then into the stable end product uric acid via xanthine oxidase activity [36].

Most of the biological actions of adenosine are mediated by G-protein-coupled cell surface receptors, classified into A_1, A_{2A}, A_{2B}, and A_3 [37]. A_1 and A_3 receptors are coupled with pertussis toxin-sensitive G proteins of the G_i, G_q, and G_o family [17]. Of note, the engagement of A_1 and A_3 receptors can also elicit the intracellular Ca^{2+} mobilization [17]. The signaling mechanism of A_{2A} and A_{2B} receptors relies on the activation of adenylyl cyclase via G_s or G_{olf}. Moreover, A_{2B} receptors can also stimulate phospholipase C via G_q [17].

In addition, all adenosine receptors couple to mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinase 1 (ERK1), ERK2, and p38 MAPK [38]. Adenosine also shows receptor-independent effects, since it can cross the cell membrane, acting on less well-defined intracellular mechanisms, comprising the S-adenosylhomocysteine hydrolase systems [39], AMP-activated protein kinase (AMPK) [40], and adenosine kinase [41].

9.3   The Adenosine System and Immune Cell Interactions in Neoplastic Microenvironment

9.3.1   Endothelial Cells

Endothelial cells constitute the inner layer, commonly termed as endothelium, of blood and lymphatic vessels. These cells play a crucial role in the control of hemostasis, blood fluidity, platelet aggregation, and vascular tone, as well as lymphocytes trafficking and vascular permeability [42].

Into the TME, many soluble factors, including vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and chemokines derived from cancer cells or inflammatory cells, stimulate the proliferation and migration of endothelial cells, which form new tumor blood vessels surrounded by perivascular stromal cells, also known as pericytes [43]. Aberrant tumor vascularization promotes tumor progression and metastasis because tumor endothelial cells (TECs) overexpress
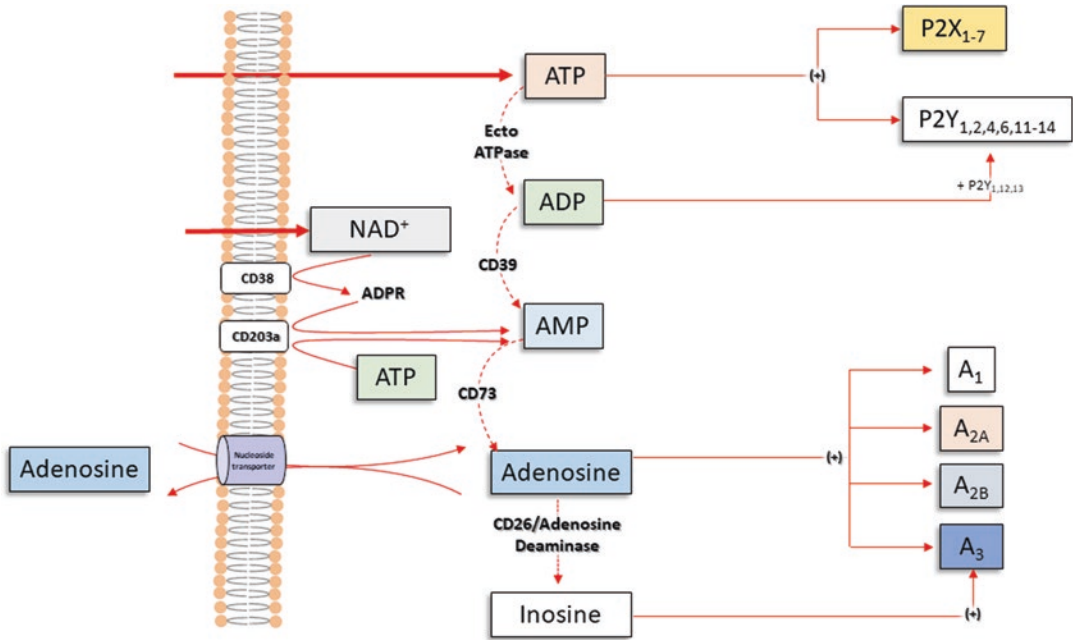
Fig. 9.1 The purinergic pathway. Once released into the extracellular environment, ATP exerts its effects via P2 receptor engagement (P2X and P2Y). ATP is catabolized via a set of nucleotidases [ecto-ATPase, ecto-apyrase (CD39), and ecto-5'nucleotidase (CD73)], leading to the sequential dephosphorylation of ATP to ADP and AMP and subsequent production of the active metabolite adenosine, which stimulates P1 (A_1, A_{2A}, A_{2B} and A_3) receptors. The CD38-CD203a enzyme axis on the cell surface, operating independently or in synergy with the conventional CD39/CD73 pathway, also contributes to the generation of adenosine. Several cell types express nucleoside transporters (NT) and adenosine deaminase, which mediate the uptake or deamination of extracellular adenosine, respectively, thus actively participating in the termination of adenosine signaling. *ADP* adenosine diphosphate, *ADPR* ADP-ribose, *AMP* adenosine monophosphate, *ATP* adenosine triphosphate, *NAD+* nicotinamide adenine dinucleotide

matrix metalloproteinases (MMPs) and secrete cytokines and growth factors that facilitate tumor cells migration, enhancing their invasive behavior [44].

Adenosine has emerged as modulator factor of endothelium functions, acting on endothelial cells through several mechanisms. The stimulation of adenosine receptors on endothelial cells modulates positively the expression of many proteins, such as CD73 [45], VEGF [46–48], IL-8 [48], and basic fibroblast growth factor [48, 49] while inhibits the production of the antiangiogenic factor thrombospondin-1 [50, 51]. Furthermore, adenosine causes the downregulation of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin on endothelial cells, impairing leukocyte extravasation [52–55].

Tumor endothelial cells express both CD39 and CD73, and several studies have demonstrated the critical role of these ecto-nucleotidases in promoting tumor angiogenesis. At this regard, it has been observed that deficiency of CD39 or pharmacological inhibition of CD39 activity reduced significantly tumor growth and metastasis, and these effects are associated with reduced tumor angiogenesis [56–58]. The expression of CD73 on endothelial cells promotes both growth and migration of endothelial cells and can also stimulate the formation of tube-like structures [59]. Indeed, in mice lacking CD73 tumor size and angiogenesis were reduced when compared to *wild-type* animals, and CD73^{+/+} pulmonary microvascular endothelial cells (PMECs) showed an increased capability to form capillary-like structures than CD73^{−/−} PMECs, especially when cultured in cancer-conditioned medium. Interestingly,

it emerged that this effect was related both to enzymatic and nonenzymatic activity of CD73 [59]. Later Allard and collaborators clearly demonstrated the role of CD73 and the effects of anti-CD73 therapy on angiogenesis in a mouse model of breast cancer [18]. In particular, they showed that CD73 on cancer cells induces the release of VEGF, via adenosine release, whereas the expression of CD73 on endothelial cells reduced the tube formation and the migration of endothelial cells independently on its enzymatic activity [18]. Therefore, the inhibition of enzymatic and nonenzymatic effects of CD73 reduces the levels of VEGF and angiogenesis in the TME in mice [18]. Another study by Wang et al. showed that CD73 on tumor endothelial cells can also inhibit the adhesion and transmigration of T cells into tumor lesions, through the reduction of ICAM-1 expression [60].

The adenosine-induced effects on tumor angiogenesis are mainly dependent on A_{2A} and A_{2B} receptors expressed on endothelial cells, cancer cells, and immune cells [61–63]. Indeed, mice lacking A_{2B} receptors [62] or treated with a selective A_{2B} receptors antagonist [63, 64] displayed a reduced release of VEGF in the tumor lesions as well as an inhibition of tumor growth. Furthermore, Sitkovsky's group demonstrated that the inhibition of hypoxia in the TME, by using supplemental oxygen therapy, can induce tumor regression in mice [65]. Tumor levels of VEGF and vascularization were inhibited as consequence of reduced intratumoral hypoxia and hypoxia inducible factor (HIF)- 1α. In other words, oxygenation is able to downregulate the expression of adenosine-generating enzymes CD39 and CD73 and thus the accumulation of extracellular adenosine in the TME, which are driven by HIF-1α [65]. Altogether these studies indicate a clear role of adenosine in promoting vascularization and release of angiogenic factors such as VEGF into the TME.

9.3.2   Pericytes

Pericytes are an heterogeneous cell population distinct in different subtypes located within the basement membrane of capillaries [66]. Each subtype, besides to exert common functions, can play different roles within the microcirculation [67]. In particular, the common functions of pericytes include vessel formation and stabilization as well as the regulation of blood flow, via relaxation/contraction mechanisms [68–70]. Pioneering studies reported that such cells display macrophage-like characteristics, suggesting a possible participation of these cells also in immune responses [71–73]. Increasing evidences described the pericytes in the TME as important modifiers of disease progression, contributing directly or indirectly to tumor angiogenesis, growth, and metastasis, despite, the mechanisms still remain to be clarified.

During the angiogenic process, the endothelial cells release several signals able to induce pericyte recruitment in order to provide physical and chemical support for new blood vessels. Of note, in TME, the vascular architecture does not accomplish complete maturation, resulting in several structural and functional abnormalities [74–77]. Indeed, it has been reported that an excessive pericyte coverage along tumor microvessels improves the vasculature stability and perfusion, paving the way to the tumor growth. By contrast, low pericyte coverage compromises vessel structure integrity, which becomes leaky, facilitating tumor cell invasion/extravasation [78–81]. In this regard, several authors demonstrated that the blockade of pericyte recruitment, via PDGFβ pathway inhibition, induced an epithelial cell loss with subsequent regression of tumor vessels [82, 83]. Interestingly, a recent study, performed by Birbrair and colleagues, allowed to demonstrate that only type 2 pericytes, a subtype located within both large blood vessels and small capillaries, participate in new blood vessel formation during tumor angiogenesis, thus representing an intriguing target for antiangiogenic cancer therapy [84].

At present, the evidence about the role of adenosine in the modulation of pericyte functions are scanty. In this regard, it has been reported that adenosine is able to promote pericyte relaxation, triggering an increment of capillary diameter [85]. Indeed, adenosine is able to activate ATP-

sensitive potassium (K_{ATP}) currents in pericytes through the activation of both A_1 and A_{2A} adenosine receptors [86]. By contrast, extracellular ATP triggers an increase in intracellular calcium levels, through the activation of $P2X_7$ receptors, thus determining the pericyte contraction [87, 88].

The pericytes are endowed with CD39 and CD73, which undergo to a rapid increase in their expression and activity under hypoxic conditions, thus leading to a reduction of extracellular concentration of ATP and to a marked presence of adenosine levels [89]. The high extracellular adenosine concentrations activate A_{2A} adenosine receptors on pericytes, determining an increase in K_{ATP} channel activity and promoting the dilatation of microvessels with a marked influx of nutrient and oxygen delivery [86]. In parallel, other authors reported a critical role of CD39 in regulating pericyte activity in new vessel formation [56]. The CD39-null mice displayed a reduced angiogenesis characterized by a poor recruitment of surrounding pericytes, thus supporting the evidence about an involvement of this enzyme in the process of neovascularization [56]. A recent study performed by Zhu et al. focused their attention on the role played by the adenosine deaminase protein cat eye syndrome critical region protein 1 (CECR1) in tumor angiogenesis [90]. Such protein, highly expressed in the macrophage lineage, beyond to regulate the differentiation of monocytes into macrophages and to stimulate T helper and macrophage proliferation, is involved in tumor cell proliferation and migration [91]. In this context, Zhu and colleagues reported that CECR1, produced by tumor-associated macrophage, promotes pericyte recruitment and migration, thus supporting the tumor angiogenesis [90].

9.3.3 Myeloid Cells (Monocytes/ Macrophages, DCs, and MDSCs)

Mononuclear phagocytes family comprises cells originated in the bone marrow from myeloid precursor cells, which firstly differentiate in blood monocytes. These latter circulate in bloodstream and turn into macrophages or dendritic cells (DCs) when they enter tissue. Mononuclear phagocytes play a critical role during inflammation, wound healing, and immune response by producing several molecules, including cytokines, prostanoids, angiogenic factors, and antimicrobial peptides.

Macrophages can infiltrate the TME, where they exhibit an anti-inflammatory phenotype (M2-like) and in this context are referred to as tumor-associated macrophages (TAMs) [92]. Macrophage infiltration into the tumor stroma is driven by chemokines (CCL2, CCL5, CCL5, and CX3CL1), VEGF, and macrophage colony-stimulating factor (M-CSF) [93, 94], and accumulation of these cells in the TME can strongly favor tumor progression. Several studies demonstrated that TAMs potently suppress the antitumor immune response, stimulate angiogenesis, and promote metastasis dissemination, through a facilitation of the extracellular matrix (ECM) remodeling [95–99].

Adenosine has an important role in regulating the differentiation and maturation of mononuclear cells, as well as their recruitment into TME [100]. Interestingly, adenosine promotes M2 differentiation of macrophages, through activation of A_{2B} receptors and A_{2A} receptors [9, 100, 101]. In particular, the engagement of A_{2A} receptors reduces the production of interleukin (IL)-12, tumor necrosis factor (TNF) [102], nitric oxide [103], and superoxide [104], while it increases the production of VEGF [105].

In support of a role exerted by adenosine in controlling macrophage functions into the tumor, Yegutkin and collaborators demonstrated that CD73-deficient mice showed a reduced tumor infiltration of M2-like macrophages [106]. Later, an elegant work by Cekic and collaborators, by using myeloid-specific A_{2A} receptors deficient mice, clearly reported the role of A_{2A} receptors in myeloid cells in tumor-bearing mice. Indeed, this study showed that signaling of A_{2A} receptor in myeloid cells favors the polarization to M2-like macrophages that in turn suppress CD8$^+$T cells and NK cell-mediated antitumor response [107]. In human ovarian ascites, Montalban Del Barrio et al observed that TAMs

highly express both CD39 and CD73, and adenosine generated by ovarian cancer cells promotes the differentiation of M2 macrophages, able in turn to produce adenosine that impaired T-cell responses [108].

A large number of evidence indicate that adenosine signaling can also markedly affect the features of dendritic cells (DCs), which are specialized antigen-presenting cells (APCs) fundamental in orchestrating adaptive immune responses. Under physiological conditions, mature DCs expressing high levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules, such as CD80/86, modulate the activation and the differentiation of T cells into Th1-like cells [109]. Conversely, in pathological conditions, including cancer, the maturation and differentiation of DCs can be altered becoming tolerogenic and thus able to induce T-cell anergy, T- cell death, or regulatory T-cell (Treg) expansion [110].

Within TME, adenosine can critically impair the maturation of DCs favoring the differentiation of a tolerogenic DC subset that produce transforming growth factor (TGF)-β, arginase 2, VEGF, IL-8, IL-10, and IL-6, while the secretion of IL-12, TNF, and chemokines was downregulated [111–116]. A study from Novitskiy et al. demonstrated that in the absence of adenosine deaminase, the adenosine levels into microenvironments were increased, resulting in an enhanced tolerogenic function of DCs [113]. These results were confirmed by Naval-Macabuhay et al. [117]. In this study, the authors observed that the addition of adenosine deaminase to DC:T cell cultures reduced the adenosine levels and promoted the priming of effector T cells, while the induction of Tregs was suppressed [117].

Adenosine affects the maturation of DCs through the activation of A_{2B} receptors [114, 118]. In tumor-bearing hosts, the blockade of A_{2B} receptors inhibited the tumor growth by promoting DC activation and consequent T-cell activation [119]. When maturing as tolerogenic, DCs lose their ability to induce T-cell proliferation thus contributing to tumor growth, as observed in several cancer models [119, 120].

Interestingly, in a colon cancer mouse model, the inhibition of CD39 enhances the recruitment of DCs into the tumor lesion together with T cells, restoring the efficacy of chemotherapeutic agents against autophagy-deficient cancer cells [121]. These effects depend on increased concentrations of extracellular ATP as consequence of CD39 inhibition that otherwise would be degraded to adenosine. In support, other studies have demonstrated that inhibition of adenosine generation from ATP increases infiltration of CD103+DCs and enhanced CD8+T cell/Treg ratio, improving radiation therapy-induced tumor control [122, 123].

In tumor-bearing hosts, immature myeloid cells (IMCs) can generate an immature subset of myeloid cells with regulatory functions called myeloid-derived suppressor cells (MDSCs) [124–127]. MDSCs can differentiate into TAMs and DCs, or they cannot differentiate at all [128, 129]. This cell population, together with TAMs and tolerogenic DCs, contribute to strongly induce immunosuppression and angiogenesis in the TME.

MDSCs limit antitumor immune response through multiple mechanisms, including arginase 1 (Arg 1), nitric oxide synthase 2 (NOS2), NADPH oxidase 2 (NOX2), indoleamine 2,3-dioxygenase, suppressive cytokines, and pro-angiogenic factors. Moreover, MDSCs that express high levels of CD39 and CD73 produce adenosine that contributes to MDSC-mediated immunosuppression in the TME [120]. Adenosine can stimulate MDSCs expansion by binding the A_{2B} receptors, expressed by myeloid precursors [130]. In this study, Ryzhov and collaborators observed that tumor infiltration of MDSCs that express high levels of CD73 is reduced in mice deficient of A_{2B} receptor [130]. Accordingly, in a mouse melanoma model, the blockade of A_{2B} receptors with a selective antagonist leads to a significant decrease of the number of tumor-infiltrating MDSCs [63, 131, 132]. These studies clearly indicate a role for A_{2B} receptor in promoting expansion and accumulation of MDSCs in the tumor lesions, where these cells contribute to promote tumor progression by suppressing T-cell-mediated response and favoring tumor

angiogenesis [34, 63, 120, 131, 132]. TGF-β signaling could have a critical role in promoting the recruitment of CD39 and CD73 expressing MDSCs in the tumor lesions [120]. In line to this, Li et al. demonstrated that tumor-derived TGF-β activates HIF-1α that stimulates the expression of CD39 and CD73 on MDSCs isolated from tumor tissues and peripheral blood of patients with non-small cell lung cancer [133]. In the same study, it has been observed that the accumulation of CD39+CD73+MDSCs is closely correlated with tumor progression in cancer patients and with their response to chemotherapy [133]. An additional study from Li et al. reported that metformin can reduce the expression and the activity of CD39 and CD73 in MDSCs of diabetic patients with ovarian cancer, through activation of the protein kinase α (AMPKα) which suppresses HIF-1α [134]. These patients treated with metformin show lower circulating CD39+CD73+ MDSCs and longer overall survival than patients not receiving metformin [134]. Elevated levels of CD39/CD73-expressing MDSCs have also been reported in colorectal cancer patients [135]. To note, other studies have also demonstrated that CD73-derived adenosine can promote resistance to targeted therapy including anti ErbB2 mAb in cancer patients [136]. At this regard, it has been shown that inhibition of CD73 can potentiate the therapeutic efficacy of an anti-ErbB2 mAb in a mouse model of breast cancer, by decreasing the number of tumor-infiltrating MDSCs, and this effect is associated with an increased number of CD8+T cells [136].

9.3.4 Lymphocytes

Lymphocytes are white blood cells originated from the bone marrow-derived progenitors and include three main families: T cells, B cells, and natural killer (NK) cells, all involved in immune response against pathogens and tumor cells.

Cancer patients with high frequency of infiltrating lymphocytes into the TME generally show enhanced survival rates [137]. Analysis of several solid tumors has pointed out that two different phenotypes exist: T-cell infiltrated tumors and non-T-cell infiltrated tumors [138]. In the first case, chemokines promote the influx of CD8+ effector T cells [138], but these get functionally inhibited by many inhibitory stimuli, including regulatory cells and/or inhibitory mediators, such as IDO, anti-inflammatory cytokines, and adenosine. In the second case, a lack of chemokines leads to reduced infiltration of T cells [138]. Interestingly, it has been observed that the type, density, and location of immune cells within the tumor lesions could predict survival of patients affected by colorectal cancer [139]. Of note, it has been observed that an elevated frequency of Tregs in TME can weaken an efficient immune response against cancer [138, 140].

Adenosine plays a critical role in suppressing lymphocytes activity within TME. The enzymes involved in the adenosine production, CD39 and CD73, as well as the adenosine receptors are widely expressed on lymphocyte populations [141–144]. Most of the adenosine-induced suppressive effects on T cells are dependent on the predominantly expressed A_{2A} receptors. The stimulation of A_{2A} receptors on lymphocytes negatively regulates the production of cytokines and proliferation. Specifically, A_{2A} receptor activation on cytotoxic CD8+T cells results in reduced secretion of IL-2, TNF-α, and interferon (IFN)-γ [145]; likewise its stimulation on NK cells inhibits the production of IFN-γ [146] and TNF-α [147, 148]. On T cells, A_{2A} receptor stimulation also reduces the expression of CD25 and CD40 ligand (CD40L) while increases the expression of programmed cell death protein (PD)-1 and cytotoxic T-lymphocyte antigen 4 (CTLA)-4 [149]. Additionally, this receptor is involved in promoting peripheral tolerance by inducing T-cell anergy [143]. A_{2A} receptor has been observed to be involved in driving naive CD4+ T cell differentiation toward regulatory CD4+FOXP3+ T cells, which also express CD73 and CD39 [150]. Furthermore, Tregs cultured with A_{2A} receptor agonists showed a stronger immunosuppressive activity [150]. All these suppressive effects on T cells are dependent on A_{2A} receptor-induced accumulation of intracellular cAMP, which causes the activation of the protein kinase A (PKA) and the exchange protein directly

activated by cAMP (EPAC) [151]. PKA phosphorylates the transcription factors cAMP response element-binding protein (CREB) and the nuclear factor of activated T cells (NF-AT) [152–154]. Additionally, EPAC exerts its action by sequestering Raf-1, causing thus the inhibition of T- cell receptor (TCR)-induced MEK-ERK activation [155, 156]. Furthermore, activation of PKA in T cells inhibits the TCR/CD28 transduction signaling during activation by reducing the phosphorylation of ZAP-70 [157]. This effect strongly impairs the production of IL-2, proliferation, and release of effector cytokines such as IFN-γ and granzyme B [157, 158].

The first evidence for a role of A_{2A} receptors in controlling antitumor T-cell responses was provided by Ohta and collaborators in 2006 [61] . In this work, the authors demonstrated that mice lacking A_{2A} receptor rejected immunogenic tumors in a CD8+T-cell-dependent manner. Accordingly, blockade of this receptor with a selective antagonist delayed tumor growth [61]. Later many studies have been focused on the therapeutic potential of targeting A_{2A} receptors with selective antagonists in many types of tumors in mice. Selective blockade of this receptor increases the tumor infiltration of CD8+T cells, NK cells, and the tumor levels of cytokines while reducing the expression of T- cell co-inhibitory receptors (reviewed in [159]). Interestingly, other studies have also demonstrated that pharmacological inhibition of A_{2A} receptor can enhance the efficacy of immunotherapeutic agents, including inhibitors of immune checkpoint PD-1 [160, 161], PD-L1 [162], and CTLA-4 [162, 163], by improving T-cell-mediated antitumor response. Because blockade of A_{2A} receptors decreases the expression of multiple checkpoint pathways, including PD-1 and LAG-3, on both CD8+ effector T cells (Teff) and Tregs in the TME, A_{2A} receptor antagonists synergistically enhance also the antitumor immune response of adoptively transferred T cells [164–166].

While the effects of A_{2A} stimulation have been clearly investigated, the role of A_{2B} receptor on lymphocytes is still not completely understood. Antagonists of A_{2B} receptors, as well as A_{2A},

reduced the expression of FOXP3 in Tregs and the production of IL-10 [167]. In addition, A_{2B} antagonists have showed inhibitory effects on Tregs differentiation [167]. In some mouse tumor models, antagonism of A_{2B} receptors is able to inhibit tumor growth by enhancing the number of tumor-infiltrating CD8+ T cells [63, 119, 131], most likely as consequence of reduced expansion/recruitment of MDSCs.

As well as adenosine receptors, also CD73 plays an important role in controlling functions and behavior of T cells. It was observed that this nucleotidase is increased in anergic T cells and impairs the responsivity of these cells in vitro [168]. Interestingly, tumor CD73 has been shown to impair T-cell activity, while its inhibition improves the therapeutic antitumor effects of transferred tumor-specific T cells [169]. Based on the crucial role of CD73 in producing adenosine in the TME, targeting CD73 may inhibit tumor growth and improve antitumor CD8^{+}T-cell response [20, 170, 171], especially in combination with chemotherapeutic agents [172], radiotherapy [122, 123], and inhibitors of immune checkpoints [18, 163]. A recent paper by Young and collaborators (2016) showed that the simultaneous inhibition of CD73 and A_{2A} receptor in a mouse metastasis models decreased the metastatic burden enhancing the number and efficacy of infiltrating effector T cells [173]. These data demonstrated definitively that these two targets in the adenosine signaling pathway are nonredundant, but it is possible to reach an optimal therapeutic response by inhibiting both the A_{2A} receptor-mediated adenosine effects and enzymatic and nonenzymatic activity of CD73 [173].

Underlying the crucial tumor-promoting role of CD39^{+} Treg cells, it has been observed that adoptive transfer of Tregs from CD39-deficient mice into immunodeficient mice enhanced NK cell functions, conversely of what happened in *wild-type* animals [57]. In line with these findings, Stagg and co-workers demonstrated that also CD73 expression on Tregs was critical for Treg-mediated tumor growth [170]. Specifically, Treg depletion in *wild-type* mice abrogated significantly tumor growth, while Treg depletion in CD73-deficient mice showed no effect [170].

Very recently, a critical role of B cells in the adenosine-mediated pro-tumor effects also emerged [174]. In particular, this study demonstrated that B cells are able to produce CD19+ extracellular vesicles (EVs), which express both CD73 and CD39 and can hydrolyze ATP into adenosine [174]. Treatment with these EVs can reduce the chemotherapy efficacy in tumor-bearing mice, while pretreatment of EVs with a CD39 inhibitor, a CD73 inhibitor, or an anti-CD73 mAb prevented this effect [174]. Furthermore, Zhang et al. extended their investigation in gastric and colon cancer patients, whose progression-free survival resulted to be negatively correlated with EV levels in serum [174]. These interesting results give evidence of the contribution of B cells in immunosuppression and will be certainly the starting point for further studies in the characterization of adenosine-producing EVs.

9.4 Adenosine System and Neoplastic Cells

9.4.1 Apoptosis

Cell apoptosis is defined as a death program pivotally involved in the maintenance of tissue homeostasis [175]. Two major triggers can activate this process: the extrinsic pathway, which is mediated by extracellular signals, and the intrinsic pathway, promoted by the recruitment of signals of intracellular origin [176, 177]. Of note, cancer cells can be able to evade programmed cell death, representing another key mechanism, along with deranged proliferation, promoting their uncontrolled growth [175]. Malignant cells can become resistant to apoptosis through several mechanisms, including disruption of proapoptotic/antiapoptotic protein balance, reduced caspase activity, and impaired signaling of receptors involved in death program [178]. In this regard, the adenosine system is one of the pathways involved in the modulation of such mechanisms.

Adenosine can promote the apoptotic process in cancer cells through the modulation of extrinsic signaling pathways [33, 179–183]. In particu-

lar, this nucleoside displayed to increase the expression of TNF receptor 1 (TNFR1) and the TNF-related apoptosis-inducing ligand receptor-2 (TRAILR2, also known as DR5 and TNFRSF10B) in the human hepatoma cell line HepG2. Moreover, adenosine was also able to modulate the expression of downstream intracellular signaling proteins, including TNFR associated with a death domain (TRADD), receptor-interacting protein kinase 1 (RIPK1), and Fas-associated death domain (FADD). Globally, these events trigger the activation of caspase-8 and caspase-9 followed by the activation of the effector caspase-3 [33].

The majority of the extrinsic proapoptotic actions of adenosine are likely to be mediated by A_3 receptors [33, 179–183]. In in vitro experiments, A_3 receptor activation upregulated the extrinsic pathway by enhancing the expression of CD95 (Fas) [181], as well as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in leukemia cells [182]. In addition, such receptors increased the expression of CD95, caspase-8, and caspase-3 in a human hepatoma cell line [183].

In a thyroid cancer cell line, the activation of A_3 receptors elicited an increase in apoptosis through the activation of the proapoptotic cytokine TRAIL via stimulation of NF-κB and by potentiating the TRAILR2 expression [34]. Likewise, in thyroid cancer cells, A_3 receptor stimulation was associated with a reduced expression of the antiapoptotic proteins BCL-2 and cellular FLICE protein (FLIP), as well as phosphorylated AKT [34].

The activation of A_{2A} receptors resulted in the activation of intrinsic apoptotic process in human Caco-2 cancer cell line as well as in HepG2 cells [32, 184], through the reduction of mitochondrial membrane potential and the increase in cytochrome c efflux from mitochondria into cytosol. This effect results in the activation of caspase-9 and caspase-3 [32, 184]. These receptors can also reduce the expression of the antiapoptotic protein BCL-X_L and upregulate the proapoptotic factor BID in hepatocellular carcinoma cells, thereby disrupting mitochondrial membrane potential [32].

A_{2B} receptors also elicited the proapoptotic effects in osteosarcoma cells through the intrinsic pathway, by downregulating BCL-2 and BCL-X_L [28]. Interestingly, the study also showed that cisplatin incubation induced an increment of extracellular adenosine levels and p53-dependent expression of A_{2B} receptors, thus suggesting the occurrence of a p53-induced priming mechanism for cisplatin that uses A_{2B} receptor signaling as an additional death-inducing pathway [28].

A_3 receptors also mediate the activation of the intrinsic apoptotic pathway in cancer. Indeed, the stimulation of such receptors in human lung cancer cells elicited the upregulation of the proapoptotic gene *NOXA* through the activation of p53 [185]. Furthermore, A_3 receptor stimulation induced the loss of mitochondrial membrane integrity and caspase-3 activation, along with a decrease in the expression level of BCL-2 and an increase in BAX expression [30, 186]. Several lines of evidence suggest that adenosine can also induce the apoptotic process independently to adenosine receptor activation. In particular, it has been observed that apoptosis of astrocytoma cells can be activated by the internalization of adenosine into cancer cells, followed by its phosphorylation to AMP via adenosine kinase [31, 35, 187–189]. The subsequent raise in intracellular AMP activates the AMPK complex, resulting in apoptosis mediated by caspase-9 and caspase-3 [189]. However, the underlying mechanisms by which AMPK activation leads to caspase-9 activation remain unclear. The adenosine-AMP-AMPK pathway is also able to induce apoptosis in a human hepatocarcinoma cell line by the dissipation of mitochondrial membrane potential occurring through BCL-X_L phosphorylation and then its inactivation. This was followed by mitochondrial damage and release of several mitochondrial proteins, such as SMAC (also known as DIABLO) into the cytosol, eventually leading to the activation of caspase-3 [188]. In the same cancer cell line, the adenosine-mediated increment of AMP concentration triggered apoptosis by reducing the expression of FLIP, a specific inhibitor of caspase-8 [31]. In another study, the increase of intracellular AMP levels elicited an enhancement of p53 transcription, which in turn

activated caspase-independent apoptosis in pleural mesothelioma cells [35].

The apoptotic process can also occur in a programmed fashion but independently from caspase activation [190]. Indeed, the apoptotic-inducing factor (AIF), which is physiologically located into the mitochondrial intermembrane space, can be released in the cytosol in response to certain mitochondrial damaging insults. Then, the AIF, translocating into the nucleus and binds to nuclear DNA, induced a caspase-independent chromatin condensation [191]. In this context, the A_3 receptor activation in a human bladder cell line elicited caspase-independent apoptosis through increased nuclear accumulation of AIF [192]. Likewise, adenosine was found to induce the translocation into the nucleus of mitochondrion-associated inducer of death (AMID) [36, 193, 194], a homologous of AIF molecule [195]. This event was followed by the occurrence of caspase-independent apoptosis in several types of human cancer cell lines [36, 193, 194, 196], which was, in some cases, dependent from the recruitment of A_3 receptors [36, 193, 194].

Overall, it is likely that adenosine can induce cancer cell apoptosis mostly by the activation of A_3 receptors. Moreover, A_{2A} and A_{2B} receptors, as well as intracellular targeting of adenosine are also involved in promoting the apoptotic process.

9.4.2 Cell Proliferation

Cancer cells are characterized by an altered cell cycle, caused by genetic and epigenetic deregulation of genes, which, in turn, can lead to the progression of quiescent cells into S phase [197–199]. In this context, adenosine pathway plays dual and opposite actions on cell growth, both promoting and counteracting tumor cell proliferation. Such opposite effects were ascribed to the engagement of different adenosine receptors in particular tumors [200]. For instance, A_1 adenosine receptor subtype has been found to regulate cell growth in breast cancer. Indeed, mRNA and protein expression of this adenosine receptor

subtype significantly increased in various breast cancer cell lines and in primary breast tumor sections, and its activation promoted cancer cell proliferation through the downregulation of p27, a cyclin-dependent kinase (CDK) inhibitor, and the upregulation of CDK4 [201]. In addition, it has been reported that breast cancer cell proliferation induced by estradiol could involve A_1 adenosine receptor activation, since estradiol has been found to induce transcriptional upregulation of A_1 receptor [202]. In support of this view, both gene deletion and pharmacological blockade of A_1 receptors decreased MCF-7 breast cancer cell proliferation through the inhibition of estrogen receptor α (ERα)-dependent transcriptional activity [202]. These findings suggest that estradiol, ERα, and the A_1 adenosine receptor create a loop involved in the modulation of breast cancer cell proliferation [202].

Besides A_1 adenosine receptors, A_{2A} receptors expressed in MCF-7 cells have been found to promote ERα transcription and, in turn, stimulate cancer cell proliferation [203], thus suggesting that A_{2A} receptor subtype also regulates cell proliferation through the activation of estrogen signaling.

Of interest, an involvement of A_{2B} receptors in tumor cell proliferation has been also described. In particular, Ma et al. (2010) observed an increased expression of A_{2B} receptors in neoplastic colorectal tissues as well as in several human colorectal adenocarcinoma-derived cell lines [204]. The authors also demonstrated that the pharmacological blockade of A_{2B} receptors with the selective antagonist MRS1754 counteracted the neoplastic growth in colonic carcinoma cells, thus indicating that A_{2B} receptors have cancer-promoting properties [204]. Wei et al. (2013) showed that A_{2B} receptor activation increased cell proliferation in both androgen-dependent and independent-prostate cancer cell lines [205]. However, the mechanisms underlying these effects need to be clarified.

Increasing evidence also suggest that adenosine contributes to tumor cell proliferation through A_3 receptors [206–215]. In particular, it has been observed that the A_3 receptor activation inhibited the lymphoma cell proliferation, through the blockade of telomerase activity as well as cell cycle in G_0/G_1 phase [211]. In support of this view, the stimulation of A_3 receptors elicited the arrest of G_0/G_1 cell cycle in prostate cancer cell lines, through the downregulation of CDK4 and cyclin D1 and upregulation of p53 [186]. In addition, A_3 receptor stimulation counteracted cell proliferation in human melanoma cells by inhibiting G_1 phase of cell cycle [207]. Moreover, the proliferation of melanoma and colonic cancer cell lines was found to be inhibited by A_3 receptor activation [208, 210, 216]. The mechanisms underlying this antiproliferative effect were ascribed to the ability of A_3 receptor of downregulating the expression of protein kinase A catalytic subunit (PKAc), which, in turn, decreased the expression of protein kinase B (PKB). Such an effect was associated with a decrease in the phosphorylation of glycogen synthase kinase (GSK)-3β while increasing β- catenin phosphorylation and ubiquitination, with consequent inhibition of MYC and cyclin D1 transcription [208, 210, 216].

Of note, treatment with selective A_3 receptor agonists inhibited cell proliferation in breast cancer cell lines, through the downregulation of ERBB2 expression and reduction of the activity of its downstream effector, ERK, thus corroborating the antiproliferative effect of A_3 receptor subtype [217, 218]. Likewise, A_3 receptor activation inhibited the proliferation in human papillary carcinoma cells via the reduction of ERK phosphorylation and the inhibition of the G_1 phase of cell cycle [219].

Overall, these findings suggest that adenosine pathways through the modulation of its receptors play a dual role in cancer cell proliferation. In particular, A_1, A_{2A}, and A_{2B} receptors have proliferative effects, while A_3 receptors decrease cancer cell proliferation.

9.4.3 Metastatic Process

Metastasis is a cancer expansion process where tumor cells can spread throughout the body, increase, and flourish in other tissues [220]. 90% of all cancer-related death is associated with the

development of tumor metastases [221]. In this context, increasing evidence highlight a pivotal involvement of adenosine pathway in the dissemination of several cancer cells [56, 57, 222, 223]. In a previous paper, Jackson et al. (2007) showed that CD39 knockout animals, inoculated intravascularly with melanoma cells, displayed fewer and smaller pulmonary metastases than *wild-type* mice [56]. In addition, Sun et al. (2010) observed that the expression of CD39 in Tregs inhibited NK cell activity and promoted hepatic metastatic tumor growth in mice, thus suggesting the involvement of CD39 enzymatic activity in metastatic processes [57].

Of interest, besides the relevance of CD39 in metastasis, a pivotal involvement of CD73 has been also described [224]. Indeed, several studies have shown a correlation between the expression and activity of this enzyme in cancer cells and their ability to spread, invade, and adhere to extracellular matrix (ECM) in different tissues [106, 170, 225–227]. Zhi et al. (2007) observed that silencing CD73 with small interfering RNA prevented human breast cancer cell adhesion to ECM and inhibited their migration [228]. In addition, the forced increase in CD73 expression enhanced invasiveness and adhesiveness to the ECM in breast cancer cell lines. Such an increase was counteracted by pharmacological blockade of CD73 activity and reproduced upon incubation of exogenous adenosine [225, 226]. These findings indicate a role of CD73-derived adenosine in the metastatic potential of cancer cells [225].

Of note, the employment of CD73 knockout mice allowed to better characterize the role of this enzyme in metastatic processes. Indeed, the CD73 deletion in mice protected against the development of melanoma metastases as compared with wild-type mice [106, 170]. The mechanisms underlying the pro-metastatic effect of CD73 were ascribed to its ability of supporting transendothelial migration of cancer cells [170]. However, the role of extracellular adenosine in mediating the effect of CD73 in in vivo models needs further investigations.

A pioneering study by Stagg et al. (2010) reported that adenosine increased tumor cell migration in vitro and metastasis in vivo, through the activation of A_{2B} receptors, recently regarded as a target of the metastasis-inducing transcription factor FOS-related antigen-1 (FRA-1) in breast cancer cells [20, 229]. Indeed, gene deletion as well as the pharmacological blockade of A_{2B} receptors inhibited the survival and metastatic potential of breast cancer cells in vitro [229]. In addition, treatment with theophylline and a chemotherapeutic agent suppressed breast cancer colonization in mouse lungs via the blockade of A_{2B} receptors [229], thus confirming the role of adenosine A_{2B} receptors in the promotion of metastasis. In support of this view, Ntantie et al. (2013) showed that the activation of A_{2B} receptors elicited an increase in cell scattering through a reduction of cell-cell adhesion and impairments of adherens cell junctions via the suppression of the small GTPase RAP1 prenylation [230].

Besides A_{2B} receptors, an involvement in cancer metastasis for A_{2A} receptor subtype has been identified [231, 232]. Ohta et al. (2006) reported that adoptively transferred CD8+ T cells in mice treated with A_{2A} receptor antagonist decreased metastasis in a melanoma model [61]. Shi et al. (2019) observed that an increased expression of A_{2A} receptors in gastric cancer tissues correlated positively with disease stage and promoted metastatic processes [233]. In particular, adenosine through A_{2A} receptors promoted gastric cancer cell migration and invasion via activation of PI3K-AKT-mTOR signaling [233].

In addition, in a pioneering study, Young et al. (2016) observed that the combination of A_{2A} receptor antagonist and antibody anti-CD73 counteracted tumor development and metastasis, thus suggesting a role of adenosine via A_{2A} receptor in the promotion of metastasis, and, that the concomitant inhibition of CD73 and A_{2A} could represent an innovative pharmacological strategy in cancer therapy [173].

Of interest, over the last years, a crucial role in metastatic processes has been ascribed to exosomes, vesicles with a diameter of 30–100 nm secreted by neoplastic cells. They promote the survival and outgrowth of disseminated tumor cells through the formation of pre-metastatic

niches [234]. One of the mechanisms underlying the metastatic effects of the cancer exosomes was ascribed to their ability of increasing the adenosine levels within the tumor microenvironment, through the CD39 and CD73 enzymes. These results indicate that exosome participate to cancer immune evasion via the CD39/CD73/adenosine signaling [235]

However, the cellular and molecular mechanisms underlying the role of the adenosine system in regulating metastasis remain to be clarified. For instance, the involvement of adenosine in regulating neoplastic cell motility cycle as well as epithelial-to-mesenchymal transition needs to be investigated. In addition, data on the role of adenosine on lysis oxidases and proteins involved in promoting metastasis are still lacking.

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