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Canonical and non-canonical adenosinergic pathways

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ABSTRACT

Adenosine (ADO) is an immunosuppressive molecule with multiple functions in different human organs. ADO is released through the concerted action of surface molecules endowed with enzymatic functions, that belong to two different adenosinergic pathways. The canonical pathway is started by CD39, that converts ATP to AMP. On the other hand, the non-canonical pathway metabolizes NAD^+ to ADPR, through the action of CD38. The latter byproduct is then converted to AMP by CD203a/PC-1. Both pathways converge to CD73, that fully degrades AMP to the final product ADO.

In this Review we take into account the most relevant finding regarding the expression of ectoenzymes belonging to both adenosinergic pathways in different cell types, including regulatory cell subsets and neoplastic cells. Moreover, we summarize the role of these molecules in different physiological and pathological settings. Finally, we discuss potential therapeutic application of specific inhibitors of ectoenzymes and/or ADO receptors.

1. Adenosine: a pleiotropic molecule

Adenosine (ADO) is a purine nucleoside, which plays important roles in different human organ systems (i.e. nervous, reproductive, cardiac, renal, respiratory, hepatic and immune systems) [1]. ADO can be produced through the metabolism of adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD^+). Once released, ADO can signal through four different G-protein coupled receptors, namely A1, A2A, A2 B and A3 receptors, that are expressed on different cell populations [2]. A1 and A2A are high affinity receptors, while A2B and A3 are low affinity receptors. The effects of stimulation largely depend on the type of receptor engaged by ADO. Adenosine receptors work through inhibition or stimulation of adenylate-cyclase to decrease or increase intracellular cyclic adenosine monophospahate (cAMP) levels. In particular, A1 and A3 receptors are preferentially coupled to inhibitory regulative Gi/o proteins, inhibiting adenylate-cyclase and cyclic AMP production, whereas the receptors of A2 family are generally coupled to stimulative regulative Gs protein that trigger intracellular cAMP accumulation [3]. It has also been reported that adenosine receptors can exert their signaling through the activation of mitogen-activated protein kinase signaling pathway in some cell types [4] (Fig. 1).

The concentration of this molecule in biological fluids and extracellular spaces is in general very low ($< 1\,\mu M$). However, ADO levels dramatically increases upon stress conditions, like hypoxia, cancer, tissue injury, inflammatory responses or sepsis [5–8].

Two different adenosinergic pathways for ADO production, composed by different surface molecules with ectoenzymatic functions, have been characterized in the last years, namely canonical and non canonical pathway. Here we summarized the most relevant findings regarding these two pathways and their role in the control of the immune responses in different pathological settings.

2. Canonical adenosinergic pathway

The canonical pathway for ADO production is started by nucleoside triphosphate diphosphohydrolase/CD39, that converts ATP to AMP [9]. The latter molecule is then fully converted to ADO by ecto-5′-nucleotidase/CD73[10] (Fig. 1).

This adenosinergic pathway has been firstly characterized by Yegutkin et al. on human endothelial cells and normal and leukemic lymphocytes [11]. CD39 and CD73 are expressed in several cell types: in particular CD39 is expressed on monocytes, B cells and a subsets of T cells [12,13], whereas CD73 is expressed in subsets of T cells, myeloid

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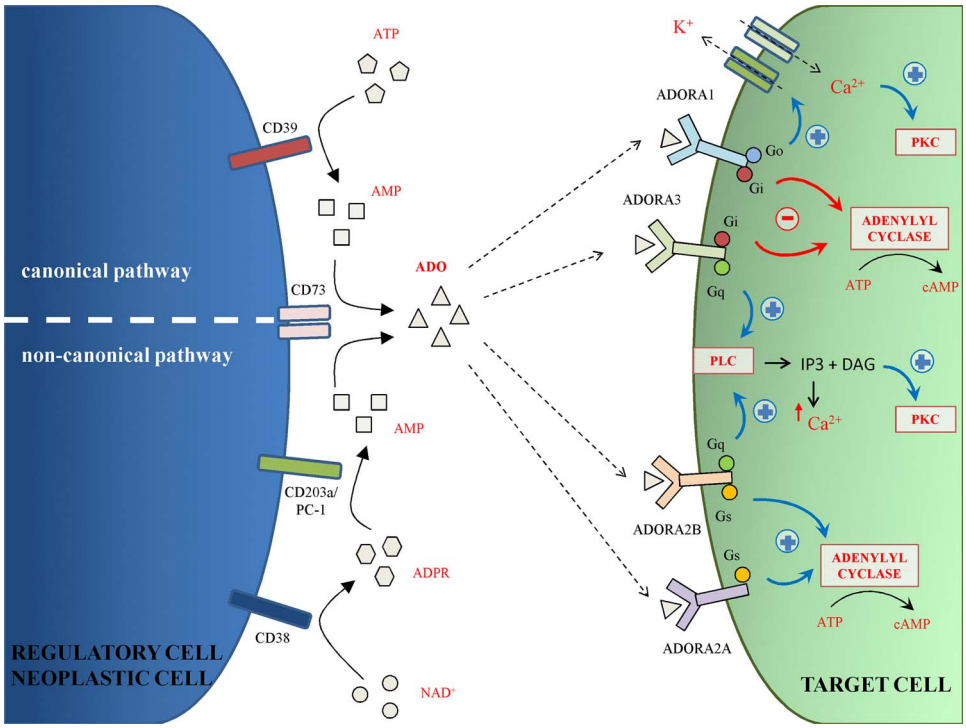

Fig. 1. Schematic representation of adenosinergic pathways. The expression and function of ectoenzymes belonging to canonical non-canonical adenosinergic pathways is shown. In addition, the cartoon shows the most relevant effects of ADO on target cells, that are mediated by (i) the interaction of ADO with ADO receptors A1, A2A, A2B and A3 and (ii) different intracellular signaling pathways.

cells, bone marrow stromal cells, thymic epithelial cells and human B cells [14]. Moreover, induction of CD39 is promoted upon exposure to pro-inflammatory cytokines (e.g., IL-6 and IL-27), oxidative stress and hypoxia. B cells, Tregs, Th17 cells, NK cells, and myeloid-derived suppressor cells can co-express CD39 and CD73.

2.1. Regulatory T cells

The canonical adenosinergic pathway has an important role in the control of the immune responses. Indeed, it has been demonstrated that in mice CD73 is expressed by $CD25^+/FoxP3^+$ regulatory T cells (Tregs) and by $CD25^-$ uncommitted primed precursor Th cells. Those cell subsets are able to produce ADO from AMP at inflammatory site, thus suppressing T cell proliferation and cytokine secretion, dampening excessive immune reactions [15]. Similar results were obtained by Borsellino et al., who have established that in mice CD39 is expressed primarily by Tregs and it is present on virtually all $CD4^+/CD25^+$ T cells. Moreover, they showed that CD39 expression is driven by the Treg-specific transcription factor Foxp3 and its catalytic activity is increased upon T-cell receptor (TCR) ligation, thus suggesting that activated Treg cells are able to abrogate ATP-related effects. Finally, they have proved that, in humans, CD39 expression is restricted to a subset of $Foxp3^+$ regulatory effector/memory-like T cells, and this cell subset is reduced in peripheral blood of patients with remitting/relapsing multiple sclerosis (MS). This data suggested that CD39 identifies a Treg subset involved in the control of the inflammatory autoimmune disease in humans [16]. In this respect, it has been demonstrated that $CD39^+$, but not $CD39^-$ Tregs are able to suppress IL-17 production by pathogenic Th17 cells in MS patients. This function is related to ADO production, since similar effects were obtained by adding exogenous ADO to Th17 cells [17]. On the other hand, it has been observed that Tregs in the synovial joints of patients with rheumatoid arthritis are $CD39^{hi}CD73^{low}$ and display suppressive functions, whereas $CD39^-$ counterparts do not. However, $CD39^{hi}CD73^{low}$ Tregs suppressed IFN-γ, TNF-α and IL-17F, but not IL-17A secretion [18].

Co-expression of CD39 and CD73 is peculiar on Tregs as compared to other T cell subsets. Tregs are able to produce ADO through the canonical pathway, leading to the inhibition of effector T cell functions, mainly through the interaction of ADO with ADO A2A receptor on activated T effector cells [19,20]. Indeed, T regs from *cd39*$^{-/-}$ mice showed impaired immunosuppressive functions *in vitro* and *in vivo*, thus confirming that ADO production is pivotal for the regulatory function of Tregs [19].

Additional evidences support the pivotal role of canonical adenosinergic pathway in the immunosuppressive function of Tregs. Alam and coworkers confirmed that CD39 and CD73 are predominantly expressed by Tregs either from human peripheral blood or gastric mucosa, and CD73 expression was significantly increased upon activation. Inhibition of CD73 increased the production of IFN-γ. Moreover, gastritis in mice infected with Helicobacter felis was significantly worse in $CD73^{-/-}$ than in wild-type mice due to a loss of Treg inhibitory functions, that lead to an increased level of pro-inflammatory cytokines [21]. They have also demonstrated that $CD73^{-/-}$ mice have significantly higher levels of pro-inflammatory cytokines and reduced anti-inflammatory responses. $CD73^{-/-}$ mice were more resistant to Salmonella infection, showing a greater inflammatory responses and a significantly lower bacterial load than wild-type mice. These data suggested that CD73 expression attenuates inflammation and impairs immunity, leading to increased bacterial colonization and prolonged infection [22].

The function of canonical adenosinergic pathway is crucial for responsiveness of methotrexate (MTX) treatment in patients with rheumatoid arthritis. Indeed, Tregs from unresponsive patients showed a lower expression of CD39, a lower ADO production and a lower suppressive function than those from responsive patients or healthy controls after MTX treatment. Same results have been obtained in a prospective study on unresponsive and responsive patients before MTX treatment. Moreover, MTX treatment was not effective in mice treated with CD39-blocking agents, thus confirming the crucial role of ADO production triggered by CD39/CD73 [23].

An increased ADO production by Tregs may represent an immune escape mechanism in several human tumors. The expression of CD39 and CD73 in Treg was higher in patients with head and neck squamous cell carcinoma (HNSCC) than in normal controls. Consequently, Tregs from patients produced higher levels of ADO from ATP and showed a higher suppressive function on effector T cells than those from controls.

Thus, canonical adenosinergic pathway represents promising immunotherapeutic strategy for patients with HNSCC [24]. In this line, hepatic growth of melanoma metastatic tumors was strongly inhibited in *cd39*$^{-/-}$ mice and in wt mice with circulating *cd39*$^{-/-}$ bone marrow-derived cells. Indeed, functional CD39 expression on Tregs suppressed antitumor immunity mediated by natural killer (NK) cells *in vitro* and *in vivo*. Finally, inhibition of CD39 enzymatic activity significantly inhibited tumor growth, thus confirming that CD39 is crucial for Tregs function [25]. An interesting finding comes from the work of Maj et al. who have demonstrated that Tregs in tumor microenvironment undergo apoptosis due to their high vulnerability to oxygen species produced within the tumor. Apoptotic Tregs expressed very high levels of CD39 and CD73 and produced high amounts of ADO that strongly contributed to create an immunosuppressive microenvironment by inhibiting effector cell functions, mainly through ADO A2A receptor [26].

2.2. Th17 cells

Th17 cells is a subsets of T cells that produce cytokines including IL-17, GM-CSF and IFNγ and that is involved in immune and tissue inflammatory responses resulting beneficial in controlling extracellular bacteria and fungal pathogens. CD39 and CD73 has been detected on human Th17 cells generated *in vitro* with IL-6 and TGF-β. These cells produce ADO and suppress effector T cell functions, and adoptive transfer of these cells in mice promoted tumor growth in a CD39-dependent manner. STAT3 is activated through IL-6 stimulation *in vitro* and acts on the promoter of the CD39 and CD73 genes while downregulation of Gfl-1, caused by TGF-B, displays the opposite function. Indeed, Th17 cells generated with IL-1β, IL-6 and IL-23 without TGF-β did not express CD39 and CD73 and were not immunosuppressive [27]. Consequently, Th17 cells generated in microenvironments were TGF-β is released by resident cells acquired CD39 and CD73 expression and cooperate to immunosuppression. The expression of CD73 was detected on inflammatory cytokine-producing Th17 cells isolated from mice with experimental autoimmune encephalomyelitis (EAE). Such expression was increased in parallel with disease progression. However, EAE development was not altered in *cd73*$^{-/-}$ mice in terms of cytokine production or recruitment of inflammatory or regulatory cells in the central nervous system, suggesting that while CD73 expression is regulated during EAE, this enzyme is not essential to promote or limit Th17 cell expansion or EAE severity [28].

2.3. B cells

It is well known that human peripheral blood B cells are able to utilize the ADO pathway. Co-expression of CD39 and CD73 in resting circulating B cells suggests they can hydrolyze ATP, yielding AMP and ADO. It has been demonstrated that the *in vitro* activation of human B cells with CD40L and IL-4 leads to significant upregulation of surface CD39 expression levels and of its enzymatic activity [29]. ADO is predominantly consumed by B cells themselves to self-regulate their function, through ADO A3 receptor. Resting B cells promoted activated T cell proliferation through cytokine production, whereas ADO is degraded by adenosine deaminase (ADA) expressed on T cells. In contrast, activated B cells downregulated CD73 and upregulated CD39 expression and act mainly metabolizing ATP to 5′-AMP, that inhibit T cell responses through the interaction with ADO A1 receptor [29]. Figueirò and coworkers identified a subset of B cells expressing high levels of CD39 with regulatory properties. This subset of CD3^{9hi} B cells, expanded upon *in vitro* activation, shows increased expression of CD73 and the ability to secrete higher levels of IL-10 and to produce higher levels of ADO compared to CD39^{low} and CD39^{-} counterparts. These characteristics are crucial for their observed inhibitory activity on T cell functions [30].

2.4. Mesenchymal stromal cells

ADO production by canonical adenosinergic pathway is also important for the function of mesenchymal stromal cells (MSC), bone marrow-derived cells of nonhematopoietic origin with immunoregulatory properties. CD39 and CD73 are co-expressed on murine MSCs, that are able to produce ADO, which can directly inhibit T cell functions through ADO A2A receptor. Indeed, blocking of the adenosinergic pathway using (i) A2A receptor antagonist or (ii) CD39 inhibitors completely blocked MSC-mediated suppression of T-cell proliferation [31]. On human MSCs CD39 is expressed at low levels and such expression was increased upon co-cultures of the latter cells with activated T lymphocytes. Accordingly, ADO levels were higher than those detected in MSCs cultured alone [32]. In other studies, it has been confirmed that the highest levels of ADO production, and consequently the highest suppression of T cell proliferation, are achieved when MSCs are co-cultured with activated T cells *in vitro*. However, the authors suggested that this effects occur through the co-operation between CD73 on MSCs and CD39 highly expressed on T cells [33,34].

MSC also inhibited Th17 cell function, in terms of proliferation and secretion of IFN-γ and IL-17. Here, as well, ADO production driven by CD39/CD73 pathway is crucial for such inhibition. Moreover, the expression of CD39 and CD73 was increased on human Th17 cells upon co-culture with MSC, suggesting that ADO triggered an immunosuppressive loop by increasing its own production [35]. Another evidence that support the crucial role of adenosinergic pathway for the immunosuppressive function of MSCs comes from the study of Chen et al., who have shown evidence that the therapeutic effects of MSCs on experimental autoimmune uveitis is lost by pre-treatment with a specific inhibitor of CD73 [33]. MSCs isolated from tumor microenvironment (i.e. cervical cancer) expressed higher levels of CD39 and CD73 than those isolated from normal tissue, produced large amounts of ADO and strongly inhibited cytotoxic T cell functions *in vitro* [36].

2.5. Myeloid cells

Neutrophils are professional phagocytes that play a critical role in host defense against infection and in the pathogenesis of many inflammatory diseases. Neutrophils co-express CD39, CD73, and all four adenosine receptors. Therefore, these cells have the capacity either to generate or to bind adenosine, which can activate or inhibit various neutrophil functions, depending on its concentration. Activated neutrophils, the major source of tissue ADO in inflammatory microenvironment, are able to release also ATP, which is in turn processed by CD39 and CD73 to ADO. In addition, both ATP and ADO have a pivotal role for neutrophil chemotaxis to inflammatory sites [37–39].

CD39/CD73 expression has been detected also on myeloid-derived suppressor cells (MDSC) isolated from non-small cell lung cancer (NSCLC) patients. MDSC are an heterogeneous population of cells, involved in the inhibition of T cells function in different diseases. In NSCLC, TGF-β secreted by tumor cells induced the phosphorylation of mTOR, that in turn activated hypoxia-inducible factor-1α (HIF-1α). The latter molecule increased the expression of CD39 and CD73 on MDSC, thus increasing their suppressive function on T and NK cells, protecting tumor cells from cell-mediated cytotoxicity [40]. Similarly, Ryzhov and coworkers have demonstrated the presence of terminally differentiated myeloid mononuclear cells (TDMMCs) in the tumor microenvironment. These cells originated from MDSC upon stimulation with TGF-β, and are characterized by a high expression of CD39 and CD73 and high production of ADO, thus contributing to immunosuppression and tumor progression. When TGF-β signaling is disrupted in mice, TDMMCs are decreased in tumor microenvironment, and an increased T lymphocytes infiltration is observed, leading to reduced tumor progression [41].

2.6. Tumor cells

Ectoenzymes belonging to canonical adenosinergic pathway can be expressed not only by immunosuppressive cell subsets, but also by cancer cells themselves. In this view, CD39 and CD73 expression was detected on ovarian cancer (OvCA) samples. In contrast, benign ovaries tested were negative for CD39, and CD73 was expressed at lower levels than those detected on tumor samples. OvCA cell lines and ascites-derived primary tumor cells expressed functional CD39 and CD73 and are able to produce ADO from ATP, leading to the inhibition of T and NK cell-mediated anti-tumor response *in vitro*. These results suggested that CD39 and CD73 enzymatic activity represents an intrinsic immune escape mechanism of tumor cells [42]. Indeed, blocking antibodies against CD39 or CD73 are able to dampen ADO production by OvCA cell lines and (i) restored cytotoxicity of NK cells or alloreactive cells against OvCA cell lines and (ii) de-inhibit proliferation of $CD4^+$ T cells in co-cultures with OvCA cell lines [43]. In another study, it has been showed that high levels of CD73 expression correlated with a worse prognosis of OvCA patients. Morover, CD73 and extracellular ADO increased tumor growth and expression of anti-apoptotic Bcl-2 family proteins in tumor cells *in vitro*. Finally, co-injection of OvCA cells and fibroblasts in mice showed that CD73 expression on the latter cells increased tumor immune escape and tumor growth [44]. CD39 and CD73 expression was increased also in endometrial tumors, as compared with normal tissues. Moreover, their expression correlated with higher tumor grade [45].

It has been demonstrated that exosomes derived from different human tumors (i.e. breast, prostate or colorectal cancer) expressed CD39 and CD73 and are able to metabolize ATP and 5′-AMP to ADO. Addition of exosomes and ATP to ADOR $A2A^+$ cells triggered a cAMP response, thus confirming that cancer-derived exosomes exerted immunosuppressive functions through ADO production [46].

The canonical pathway may function in a discontinuous fashion, when CD39 and CD73 are expressed on different cell subsets in a discrete microenvironment. Indeed, glioma cells are $CD39^+/CD73^+$, whereas infiltrating $CD4^+$ cells are $CD39^{hi}CD73^{low}$. Inhibition of effector T cells was observed only when the latter cells are cultured in the presence of both glioma cells and $CD4^+CD39^{hi}$ infiltrating cells. Such effect was reverted in the presence of CD39 or CD73 specific inhibitors. Moreover, downregulation of CD73 on glioma cells represented a good prognostic factor for patients with malignant glioblastoma, thus suggesting that CD39 and CD73 may represent a promising therapeutic tool [47].

3. Non-canonical adenosinergic pathway

Less information is still available regarding the production of ADO by the non-canonical adenosinergic pathway. This pathway is started by nicotinamide adenine dinucleotide (NAD^+)-glycohydrolase/CD38, that converts NAD^+ to ADPR. ADPR is then converted by CD203a(PC-1) to AMP, that is subsequently metabolized to ADO by CD73. The latter molecule represents the common link between the two adenosinergic pathways. By this set of events ADO production bypasses the canonical pathway mediated by CD39 (Fig. 1). The non-canonical pathway was firstly identified on leukemic Jurkat T cell line [45] and few data are reported about this pathway on normal and neoplastic cells [48].

3.1. Lymphoid cells

In Jurkat cell line CD38, CD203a(PC-1) and CD73 expression was increased by agents that augment intracellular cAMP [i.e. permeant cAMP analogue dibutyryl-cAMP (db-cAMP) and PMA]. The same effect was achieved on human peripheral blood T cells stimulated with PHA. Accordingly, db-cAMP-treated, but not untreated Jurkat cells are able to metabolize extracellular NAD^+ [48].

Similar results have been obtained by our group, using Jurkat cell line [49]. Indeed, the parental Jurkat cell line constitutively expressed CD38, and upon activation expressed CD203a(PC-1). In contrast, CD39 and CD73 were not express either in resting or activated cells. CD73 transfected cells (Jurkat/B-NT5.1 cells) expressed constitutively CD38 and CD73, but not CD203a(PC-1) and CD39. We have demonstrated that resting Jurkat cells were able to convert NAD^+ to ADPR *via* the enzymatic activity of CD38. Upon activation, the metabolization of NAD^+ by Jurkat cells produced AMP and Nicotinamide (Nic) in addition to ADPR, *via* the enzymatic activity of CD203a(PC-1). This conclusion was reinforced by the finding that activated Jurkat cells were able to produce AMP and Nic when ADPR was directly applied. Moreover, these cells were able to directly convert ATP to AMP, thus suggesting that CD203a(PC-1) was functional. Indeed, the latter molecule directly converted ATP to AMP, whereas CD39 produced ADP and AMP from ATP. Finally, we have demonstrated ADO can be produced when supernatants from Jurkat cell lines were transferred to Jurkat/B-NT5.1 ($CD73^+$) cell line, thus suggesting that ectoenzymes can operate in a discontinuous fashion when expressed on different cell subsets [49].

Moreover, we have recently demonstrated that $CD16^-CD56^{bright}$ NK cells expressed higher levels of CD39 and CD73 than $CD16^+CD56^{dim}$ counterparts. In addition, the expression of CD203a (PC-1) was restricted to $CD16^-CD56^{bright}$ NK cells. The latter cells were able to produce ADO from different substrates and to inhibt autologous $CD4^+$ T cell proliferation, mainly through ADO produced by non-canonical adenosinergic pathway, as witnessed by experiments performed using specific inhibitors. Finally, we have demonstrated that this regulatory function of $CD16^-CD56^{bright}$ NK cells may be lost during autoimmune/inflammatory diseases and enhanced during acute inflammatory responses [50]. Fedele et al. described the induction of unconventional suppressor T cells by dendritic cells exposed to pertussis vaccine (BPZE1-DC). These suppressor T cells expressed both canonical and non-canonical adenosinergic pathways and were able to metabolize exogenously added ATP and NAD^+, and to suppress effector cells through ADO production [51].

3.2. The myeloma niche

CD203a(PC-1) expression has been detected in the major lymphoid organs, predominantly on the surface of stromal cells within germinal centers. In the myeloma niche, CD203a(PC-1) expression was detected on MSC, that are also equipped with CD38, CD39 and CD73. Thus, MSC are able to produce ADO through canonical and non-canonical adenosinergic pathways. CD203a(PC-1) was found also on surface osteoblasts, where it regulates tissue mineralization by increasing extracellular levels of PPi derived from ATP metabolization. In conclusion, both pathways for ADO production are present and functional in the myeloma niche, although ectoenzymes are expressed on different cell populations [52]. Accordingly, we have demonstrated that ADO is present in bone marrow (BM) plasma samples from multiple myeloma (MM) patients, and ADO levels were higher than those detected in patients with asymptomatic MGUS/SMM and other hematological malignancies. Moreover, ADO levels in BM plasma samples correlated with disease stage, thus suggesting that ADO production increased with disease severity. Experiments performed on primary MM cells revealed that these cells express functional CD38 and CD203a(PC-1), and are able to produce ADPR and AMP starting from NAD^+. Co-culture experiments between MM and other cell populations revealed that AMP can be fully converted to ADO through the action of CD73, that is mainly expressed by osteoblasts, osteoclasts and MSC in the MM niche [53].

3.3. Human melanoma

The presence of non-canonical adenosinergic pathway for ADO production was also demonstrated by our group in the context of

human melanoma. Indeed, we have demonstrated that primary melanoma cell lines generated from patients expressed CD38, CD39, CD203a (PC-1) and CD73, and produced ADO from different substrates. ADO production through non-canonical pathway was involved in the inhibition of proliferation $CD4^+$ and $CD8^+$ T cells, since such inhibition was reverted by adding specific inhibitors of CD38 and CD73 [54].

4. Conclusions

In this review, we have described canonical and non-canonical adenosinergic pathways and we have summarized the most relevant studies describing their role in the modulation of the immune response in different pathological settings. Collectively, these findings demonstrated that ADO and adenosine-producing ectoenzymes [CD39, CD38, CD203a(PC-1) and CD73] represent promising therapeutic tools for patients autoimmune/inflammatory diseases and cancer. In this line, there are preclinical studies that underline as the inhibition of ADO signaling can synergize with other co-inhibitory molecules (i.e. CTLA4 and PDL1) in tumor control [55–58], and there are evidence that inhibition of CD73, A2A, or A2B has been shown to enhance the activity of chemotherapy [59,60]. Likewise, CD73 blockade has been shown to augment the efficacy of radiotherapy [61].

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