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ATP and adenosine metabolism in cancer – exploitation for therapeutic gain

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Adenosine in cancer

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Abbreviations: ADA, adenosine deaminase; ADK, adenosine kinase; ADK-L, long isoform of adenosine kinase; ADK-S, short isoform of adenosine kinase; AK, adenylate kinase; Ap_nA, diadenosine polyphosphates; AR, adenosine receptor; bFGF, basic fibroblast growth factor; cADPR, cyclic ADP-ribose; CAF, cancer associated fibroblast; CAR-T cells, T cells with chimeric activating receptors; CD, cluster of differentiation; cGAMP, cyclic GMP-AMP; cGAS, cGAMP synthase; CNT, concentrative nucleoside transporter; CRISPR, clustered regularly interspaced short palindromic repeats; CTL, cytotoxic T-lymphocyte; CTLA-4, cytotoxic T lymphocyte antigen 4; EC, endothelial cell; ENPP, ecto-nucleotide pyrophosphatase phosphodiesterase; ENT, equilibrative nucleoside transporter; FDA, Food and Drug Administration; IL, interleukin; NDPK, nucleoside diphosphate kinase; NK cells, natural killer cells; NTPDase, nucleoside triphosphate diphosphohydrolase; P receptors; purinergic receptors; PAP, prostatic acid phosphatase; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1; PNP, purine nucleoside phosphorylase; PEG, polyethylene glycol; SAH, S-adenosyl-L-homocysteine; SAHH, SAH hydrolase; SAM, S-adenosyl-L-methionine; SCID, severe combined immunodeficiency disease; scRNA-Seq, single cell RNA sequencing; STING, stimulator of interferon genes; TAM, tumor associated macrophages; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor beta; TIL, tumor infiltrating lymphocytes; TME, tumor microenvironment; TNAP, tissue-nonspecific alkaline phosphatase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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***Abstract* – Adenosine is an evolutionary ancient metabolic regulator linking energy state to physiological processes including immunomodulation and cell proliferation. Tumors create an adenosine-rich immunosuppressive microenvironment through the increased release of ATP from dying and stressed cells and its ectoenzymatic conversion into adenosine. Therefore, the adenosine pathway becomes an important therapeutic target to improve the effectiveness of immune therapies. Prior research has focused largely on the two major ecto-nucleotidases, ecto-nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39) and ecto-5'-nucleotidase/CD73, which catalyze the breakdown of extracellular ATP into adenosine, and on the subsequent activation of different subtypes of adenosine receptors with mixed findings of anti-tumor and pro-tumor effects. New findings, needed for more effective therapeutic approaches, require consideration of redundant pathways controlling intratumoral adenosine levels, including the alternative NAD-inactivating pathway through the CD38-ENPP1-CD73 axis, the counteracting ATP-regenerating ectoenzymatic pathway, as well as cellular adenosine uptake and its phosphorylation by adenosine kinase. This review provides a holistic view of extracellular and intracellular adenosine metabolism as an integrated complex network, and summarizes recent data on the underlying mechanisms through which adenosine and its precursors ATP and ADP control cancer immunosurveillance, tumor angiogenesis, lymphangiogenesis, cancer-associated thrombosis, blood flow, and tumor perfusion. Special attention is given to differences and commonalities in the purinome of different cancers, heterogeneity of the tumor microenvironment, subcellular compartmentalization of the adenosine system, and novel roles of purine-converting enzymes as targets for cancer therapy.**

Key words: adenosine deaminase; adenylate kinase, NDPK/NME/Nm23; cancer purinome; immune checkpoint

Significance statement

The discovery of the role of adenosine as immune checkpoint regulator in cancer has led to the development of novel therapeutic strategies targeting extracellular adenosine metabolism and signaling in multiple clinical trials and preclinical models. Here we identify major gaps in knowledge that need to be filled to improve the therapeutic gain from agents targeting key components of the adenosine metabolic network and, on this basis, provide a holistic view of the cancer purinome as a complex and integrated network.

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I. Introduction

Adenosine is an evolutionary ancient regulator of energy homeostasis and a biochemical component of a large array of biomolecules including ATP, ADP, AMP, NAD, FAD, S-adenosyl-L-methionine (SAM), S-adenosyl-L-homocysteine (SAH), and RNA. Life started with RNA, not DNA, and messenger RNAs have poly-A tails. A prerequisite for the evolution of life was the development of a simple and efficient system that allows a cell to save energy under conditions of an energy crisis, such as a lack of nutrients or oxygen. The easiest way to achieve this is to couple the breakdown of ATP, as a function of energy consumption to the production of adenosine, and then utilize adenosine as a feedback inhibitor to broadly block pathways and reactions that consume energy. Because of the early evolutionary role of the ATP/adenosine regulatory system, the maintenance of well-balanced purine homeostasis is of crucial importance for health and disease in a variety of (patho)physiological conditions, including inflammation and cancer (Allard et al., 2020; Boison and Yegutkin, 2019; Eltzschig et al., 2012; Spychala, 2000; Vijayan et al., 2017). The main conclusion is that cancer tissue creates its own adenosine-rich immunosuppressive microenvironment through increased ATP release and adenosine production. Thereby, the adenosine pathway may significantly limit the effectiveness of immune therapies. Therefore, the adenosine system emerges as an important therapeutic target in cancer. However, there are major knowledge gaps that prevent the development of more effective adenosine-based therapeutics: (i) redundant extracellular and intracellular pathways, which control ATP and adenosine levels have received little consideration; and (ii) receptor-dependent and -independent effects of adenosine have not been distinguished in the past (Boison and Yegutkin, 2019). This review provides a holistic view of adenosine metabolism and signaling as a complex and

integrated network and further highlights the emerging roles of purine-converting enzymes as novel targets for cancer therapies.

II. Overview of cellular purine turnover

A. Pathways for ATP release

In line with the early evolutionary role of purines, any condition of stress initiates purinergic signaling by the release of endogenous ATP, which occurs by regulated vesicular exocytosis or via ion channels and transporters **(Figure 1)**. Cellular mechanisms of cargo-vesicle trafficking include nucleotide secretion from specialized exosomes or through ion transporters of the solute carrier (SLC) family, such as vesicular nucleotide transporter (VNUT/SLC17A9). ATP release through conduction includes facilitated diffusion through pore-forming channels and nucleotide transporters, such as connexin and pannexin hemichannels, volume-regulated anion channels, ATP-binding cassette (ABC), and P2X7 receptors (Junger, 2012; Linden et al., 2019; Yegutkin, 2008). Along with stress-induced ATP release, cells are able to maintain ATP and related metabolites (ADP, AMP, adenosine) at steady-state levels, thereby contributing to the sustained activation and/or desensitization of purinergic receptors (Corriden and Insel, 2010; Helenius et al., 2012; Yegutkin et al., 2006).

B. Signal transduction pathways

Extracellular ATP and related nucleotides are ligands of the two major receptor subfamilies, P2X and P2Y (**Figure 1**). P2X receptors are ligand-gated ion channels that comprise of seven subtypes (P2X1 through P2X7), whereas G-protein coupled P2Y receptors are divided into eight subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) based on agonist selectivity and

signal tranducstion pathways (Antonioli et al., 2019; Bours et al., 2006; Jacobson and Muller, 2016; Ralevic and Burnstock, 1998). Another mechanism of ATP action is conveyed via its ectoenzymatic breakdown into adenosine, which in turn binds to G-protein coupled adenosine receptors, which are selectively expressed on tumor cells, lymphocytes, stromal cells, and vascular endothelium and function by activating ($A_{2A}R$ and $A_{2B}R$) or inhibiting (A_1R and A_3R) adenylyl cyclase (Antonioli et al., 2019; Borea et al., 2018; Bours et al., 2006; IJzerman et al., 2022; Jacobson and Muller, 2016; Ralevic and Burnstock, 1998). Activation of P2Y and P2X receptors serves largely pro-inflammatory and pro-thrombotic functions, whereas adenosine counteracts the role of ATP and/or ADP by attenuating inflammation and tissue damage (Antonioli et al., 2019; Bours et al., 2006; Cekic and Linden, 2016; Eltzschig et al., 2012; IJzerman et al., 2022). In line with this protective role, adenosine mediates additional cardioprotective, neuroprotective, anti-nociceptive, vasoactive, and pro-angiogenic effects (Borea et al., 2018; Chen et al., 2013; IJzerman et al., 2022; Mustafa et al., 2009). The latter function is particularly pertinent in view of the critical role of angiogenesis for supplying cancer cells with oxygen and the nutrients needed to sustain their growth, as well as for promoting metastatic spread (Ghesquiere et al., 2014; Li et al., 2019b; Lugano et al., 2020).

C. Extracellular ATP metabolism

The equilibrium between extracellular ATP and adenosine depends on an intricate network of purine-converting ectoenzymes which are co-expressed to a variable extent among mammalian tissues and share similarities in substrate specificity (Yegutkin, 2008; Zimmermann et al., 2012). In light of opposing effects of ATP and adenosine in regards to tumor-promotion and -suppression, the tumorigenic role of key ectoenzymes mediating the sequential hydrolysis of ATP

into adenosine has received much attention: ecto-nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39) and ecto-5'-nucleotidase/CD73 (Allard et al., 2020; Demaria et al., 2019; Li et al., 2019c; Maj et al., 2017; Perrot et al., 2019; Vijayan et al., 2017). In contrast to traditional paradigms that focus mainly on the CD39-CD73 axis, it has now become clear that a more complex interplay between redundant ATP-consuming and counteracting ATP-regenerating pathways controls the balance between extracellular ATP and adenosine (Yegutkin, 2014) **(Table 1)**. In the following chapters we discuss in more detail the major purinergic ectoenzymes and their role in cancer biology.

D. Cellular adenosine uptake and metabolism

Extracellular adenosine appears in the interstitial milieu only transiently and acts as a short-term signaling molecule that needs to be removed in a coordinated fashion, either through extracellular adenosine deaminase (ADA) or by transport into the cell via nucleoside transporters followed by intracellular metabolism **(Figure 1)**. Four energy-independent equilibrative nucleoside transporters (ENT1 through ENT4) are included in the human SLC29 family, while three sodium-dependent concentrative nucleoside transporters (CNT1, CNT2, and CNT3) belong to the human SLC28 family of transporters (Pastor-Anglada and Perez-Torras, 2018; Young et al., 2013). The ability of cancer cells to take up adenosine through ENT1 and to sequentially phosphorylate it to ATP (Losenkova et al., 2020; Virtanen et al., 2014) provides evidence for the existence of a highly dynamic interplay between extracellular and intracellular purine-converting pathways, which converge into a tuned control of cellular ATP and adenosine levels. In addition to their role of regulating the uptake of adenosine needed for the salvage pathways of nucleotide synthesis, ENTs also mediate the uptake of cancer therapeutics from the class of nucleoside analogues

(Young et al., 2013), as well as the uptake of deoxyadenosine by human macrophages and other immune cells and its further conversion into cytotoxic deoxyATP (Winstel et al., 2018).

III. Major enzymes and enzyme families involved in cellular adenosine metabolism

A. Nucleotide-inactivating ectoenzymes

The first steps of extracellular ATP and ADP breakdown are primarily mediated by NTPDase1/CD39 and three additional members of this family (NTPDase 2, 3, and 8) **(Table 1)** (Robson et al., 2006; Yegutkin, 2008; Zimmermann et al., 2012). The ATP/ADP-derived AMP is then further hydrolyzed by ecto-5′-nucleotidase/CD73, which is linked to the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor (Alcedo et al., 2021; Yegutkin, 2014; Zimmermann et al., 2012). Both CD39 and CD73 are expressed to variable extents in vascular and lymphatic vessels, hematopoietic cells, gastrointestinal tract, brain, eye (Alcedo et al., 2021; Eltzschig et al., 2012; Hesse et al., 2021; Losenkova et al., 2022; Yegutkin, 2014), and in the tumor microenvironment (TME) (Allard et al., 2020; Boison and Yegutkin, 2019; Li et al., 2019c), where they regulate hemostasis, thrombosis, and inflammation through the coordinated control of balanced ATP, ADP, and adenosine levels. Consequently, these two ecto-nucleotidases have received much attention as immune checkpoint regulators and therapeutic targets for cancer therapy (see below).

Along with the canonical CD39-CD73 axis, additional ectoenzymes contribute to the metabolism of extracellular nucleotides. For instance, ATP can directly be converted into AMP and diphosphate (PP_i) through ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) activity. Two members of this multigene enzyme family, ENPP1 and ENPP3, are particularly relevant in the context of the purinergic signaling cascade due to their ability to hydrolyze ATP,

diadenosine polyphosphates (Ap_nA), cyclic ADP-ribose (cADPR), and 2′ 3′ cyclic GMP-AMP (cGAMP) (Carozza et al., 2020b; Gasparrini et al., 2021; Linden et al., 2019; Yegutkin, 2021). Adenosine can also be generated from ATP and inorganic polyphosphates via tissue-nonspecific alkaline phosphatase (TNAP) activity (Muller et al., 2019; Yegutkin, 2008; Zimmermann et al., 2012), by AMP hydrolysis through prostatic acid phosphatase (PAP) (Yegutkin, 2014), and from NAD through the CD38-ENPP1-CD73 axis (Boison and Yegutkin, 2019; Chini et al., 2018; Gasparrini et al., 2021; Hesse et al., 2021; Malavasi et al., 2008) (**Table 1** and **Figure 2**).

B. ATP synthesis via reverse phosphotransfer reactions

Whereas traditional paradigms focus on adenosine-producing pathways, additional enzymes of the 'classical' intracellular adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK/NME/Nm23) families also contribute to the metabolism of extracellular nucleotides. Tissues with a high-energy demand, like brain, heart and skeletal muscle express AK1 in their cytoplasm, where it catalyzes reversible phosphoryl transfers: ATP + AMP ↔ 2ADP (Dzeja et al., 2007; Yan and Tsai, 1999) **(Table 1)**. In addition, AK1 is expressed on the surface of vascular endothelial cells (EC), epithelial cells, lymphocytes, neoplastic cells (Donaldson et al., 2002; Muller et al., 2019; Yegutkin, 2021; Yegutkin et al., 2006), and it can also circulate as a soluble enzyme in the bloodstream (Yegutkin et al., 2012) and vitreous fluid (Zeiner et al., 2019). Another member of this family, AK2, is expressed in the mitochondrial intermembrane and intra-cristae space and facilitates high-energy phosphoryl exchanges between mitochondria and the cytosol (Dzeja et al., 2007; Klepinin et al., 2020). Mutations in the AK2 gene and loss of AK2 expression in humans compromise mitochondrial energy metabolism and result in reticular dysgenesis, a rare form of severe combined immunodeficiency (SCID). This condition is characterized by an arrest

of maturation in the lymphoid and myeloid lineages, recurrent and overwhelming infections, and bilateral sensorineural deafness (Six et al., 2015). Interestingly, in some cells, AK2 is also localized in the nucleus, where it forms a complex with dual-specificity phosphatase 26 (DUSP26) and controls cell growth via dephosphorylation of fas-associated protein with death domain (FADD) (Kim et al., 2014).

Two main members of the NDPK family, NDPK-A/NME1 and NDPK-B/NME2, are ubiquitously expressed as both intracellular (cytosolic and intranuclear) and extracellular (membrane-bound and soluble) enzymes in different tissues, with the highest expression in brain, kidney, liver, heart, and neoplastic cells **(Table 1)** (Boissan et al., 2018; Lodillinsky et al., 2021; Yegutkin, 2014; Yokdang et al., 2011). Along with a critical role in cellular bioenergetics via regulation of the exchange of a γ-phosphate between nucleoside tri- and diphosphates, these enzymes can contribute to multiple processes including cell proliferation and tumor metastasis through mechanisms not connected to their catalytic activity. These effects can be mediated by binding of NDPK/NME proteins to components of the cytoskeletal machinery and formation of a complex with a member of the phosphoesterase protein family h-Prune, by activation of protein histidine kinases (Boissan et al., 2018), by promotion of dynamin-2 (DNM2) oligomerization and activation of GTPase activity (Boissan et al., 2014; Khan et al., 2019), and also by inhibition of clathrin-mediated endocytosis and cancer cell migration via binding to long-chain fatty acyl coenzyme A (Zhang et al., 2022). Strikingly, recent data have shown that NDPK/NME is also capable of regulating glucose-stimulated insulin secretion, beta-cell function and calcium dynamics via formation of a functional complex with fatty-acid-binding protein 4 (FABP4) and ADK (named Fabkin) and subsequent activation of ATP- and ADP-specific $P2Y_1Rs$ (Prentice et al., 2021).

C. Extracellular and intracellular adenosine metabolism

Extracellular adenosine signaling needs to be terminated. This is accomplished by a concerted effort between ecto-ADA activity, nucleoside transport, and the intracellular metabolism of adenosine. ADA has two isoforms, ADA1 (usually referred to as ADA) and ADA2, characterized by different affinities towards adenosine with K_m values in the range of 40 μM and ~2 mM, respectively (Blackburn and Kellems, 2005; Yegutkin, 2014) **(Table 1)**. Along with cytosolic expression, ADA is expressed as an ectoenzyme on surfaces of lymphocytes, dendritic cells and other lymphoid and non-lymphoid tissues (Cortes et al., 2015; Kaljas et al., 2017; Spychala, 2000). Approximately 10–15% of all SCID cases are caused by inborn ADA deficiency and presents as a rare metabolic condition characterized by impaired immune cell development and function, complete absence of humoral and cellular immunity, and excessive infections (Blackburn and Kellems, 2005; Cortes et al., 2015). The deficiency of ADA2 due to autosomal-recessive loss-of-function mutations in the ADA2 gene (CECR1) presents as a condition with small- and medium-size vessel vasculitis (Moens et al., 2019). The next enzyme in the purine catabolic chain, purine nucleoside phosphorylase (PNP), converts adenosine-derived inosine to hypoxanthine, which is then metabolized to uric acid via xanthine (Helenius et al., 2012; Yegutkin, 2014). Human PNP mutations result in severe T-cell immunodeficiency, as well as immune and neurological dysfunction (Grunebaum et al., 2013).

Further, intracellular adenosine kinase (ADK) provides the major metabolic route of adenosine clearance under physiological conditions (Boison, 2013; Boison et al., 2010; Spychala, 2000) **(Table 1)**. This high affinity, low capacity enzyme exists in two isoforms, ADK-S and ADK-L, derived from alternative splicing and promoter use and is expressed in the cytoplasm and

cell nucleus, respectively (Boison, 2013). ADK-S and cytosolic 5'-nucleotidase form a highly active substrate cycle between AMP and adenosine, which allows a cell to rapid adjustments to changes in energy homeostasis (Bontemps et al., 1983). Since the concentrations of intracellular ATP, ADP and AMP are much higher compared to adenosine, even minor changes in the ADK-mediated flow of the adenosine/AMP cycle would translate into major changes in intracellular adenosine levels without having a major impact on concentrations of the phosphorylated nucleotides (Boison et al., 2010). As outlined above, recent findings provide evidence for the ability of ADK to control extracellular ATP and ADP levels in the vicinity of islet cells via formation of catalytically active hormonal complex with FABP4 and NDPK/NME (Prentice et al., 2021). Increased levels of ADK have been found in human gliomas of astrocytic origin, where high expression levels have been associated with tumor-related epilepsy (de Groot et al., 2012). Interestingly, human macrophages can also take up extracellular deoxyadenosine through ENT1 and further convert it into cytotoxic deoxyATP through cytosolic ADK and other phosphotransfer reactions. This purine salvage pathway is used by Staphylococcus aureus capable of producing deoxyadenosine from host DNA through adenosine synthase A to kill phagocytes (Winstel et al., 2018).

D. Intranuclear adenosine metabolism

The nuclear isoform of ADK, ADK-L, plays a major role in controlling the flux of methyl groups through the SAM-dependent transmethylation pathway, which drives DNA and histone methylation (Williams-Karnesky et al., 2013) **(Figure 2)**. Through this epigenetic role, ADK-L is in a unique position to contribute to the regulation of gene expression. The epigenetics of cancer is an emerging research area, and includes changes in DNA and histone methylation, as well as their

interplay. Those epigenetic alterations are thought to play a major role in driving the high proliferative rate of cancer cells (Casciello et al., 2015; Du et al., 2015; Huang et al., 2015; Zahnow et al., 2016). A direct role for ADK-L in the regulation of cell growth and differentiation is supported by findings in the brain (Boison, 2013; Williams-Karnesky et al., 2013). Young dividing and plastic cells are characterized by high levels of ADK-L, whereas mature and terminally differentiated cells are characterized by high levels of ADK-S – and in the case of mature neurons, a complete lack of ADK-L (Gebril et al., 2021; Gebril et al., 2020). Those findings from the brain suggest the possibility that cancer cells regain or maintain 'youth', plasticity, and capacity to divide, by reprogramming the developmental clock to assume (in the case of mature cells) or maintain (in the case of stem cells) a high ADK-L / low ADK-S ratio. High ADK-L is expected to promote cell proliferation, whereas low ADK-S – by enhancing adenosine in the TME – is expected to suppress immune functions and to promote angiogenesis. ADK-L is considered an important therapeutic target to regulate the epigenome in cancer cells, as genetic mutations in cancer cells can theoretically be overridden by epigenetic therapeutic manipulations (Martinez-Outschoorn et al., 2017). Cytosolic and intranuclear levels of adenosine are also regulated by SAH hydrolase (SAHH), which cleaves SAH, the product of transmethylation, into L-homocysteine and adenosine (**Figure 2**). High levels of SAH block transmethylation reactions by product inhibition. This implies that effective removal of adenosine by metabolism through ADK is a necessity to maintain transmethylation (Bjursell et al., 2011; Boison et al., 2002; Moffatt et al., 2002; Williams-Karnesky et al., 2013). Therefore genetic deletion or inherited deficiency of ADK in mice (Boison et al., 2002), plants (Moffatt et al., 2002), and humans (Bjursell et al., 2011) cause major transmethylation defects and developmental abnormalities. Because SAHH has a central role in a major metabolic pathway it can contribute

both to the formation (under methylating conditions) or removal (under conditions of elevated adenosine) of adenosine.

IV. Hallmarks of cancer cell metabolism

Cancer biology in general is tightly linked to metabolism. Cancer cells support their growth primarily by the generating energy via the anaerobic catabolism of glucose in contrast to oxidative catabolism of pyruvate in healthy cells, a metabolic change known as the Warburg effect. Tumor hypoxia and resulting lactate accumulation have been associated with poor disease outcome. Therefore, hypoxia and anaerobic metabolism are considered the hallmarks of cancer (Hanahan and Weinberg, 2011). Recent studies confirmed the significance of metabolic derangements in cancer through the identification of altered mitochondrial function (Martinez-Outschoorn et al., 2017; Seyfried et al., 2014). Because mitochondria are the primary source of ATP production, there is a central role of ATP linking cancer cell metabolism and bioenergetics. Therefore, the selective targeting of metabolic pathways linked to cellular ATP constitutes a rational therapeutic approach to target deregulated energy homeostasis in cancer cells (Gentric et al., 2017; Hanahan and Weinberg, 2011; Martinez-Outschoorn et al., 2017).

It is important to consider that the TME is highly heterogeneous and comprised of a broad spectrum of neoplastic and non-neoplastic cell types. Heterogeneity of the TME determines the metabolism of cancer cells and their ability to metastasize and colonize. For instance, access to nutrients and oxygen is superior to cancer cells in proximity to the blood vasculature, which enables them to generate ATP through aerobic metabolism via oxidative phosphorylation and up-regulated anabolic pathways (Lugano et al., 2020; Martinez-Outschoorn et al., 2017). Along with the significant heterogeneity of malignant cells including dormant and metastatic cells as well as

hyper-proliferative cancer stem cells (Hanahan and Weinberg, 2011; Peinado et al., 2017), benign components of the TME also show heterogeneity and differ from their healthy counterparts in terms of morphology, metabolism and function (**Figure 3**). This applies in particular to abnormal tumor vessels extensively branched throughout tumor parenchyma (Ghesquiere et al., 2014; Li et al., 2019b; Lugano et al., 2020), the wide diversity of stromal cancer-associated fibroblast (CAF) subsets (Ghesquiere et al., 2014; Wu et al., 2020), formation of heterotypic platelet-tumor cell aggregates (Haemmerle et al., 2018), as well as the presence of a broad repertoire of tumor-infiltrating lymphocytes (TIL), tumor-associated macrophages (TAM), and other immune cells (Guerra et al., 2020; Peinado et al., 2017). The TME is dynamically shaped by the immune interactions of a tumor, in both a pro-tumorigenic and anti-tumorigenic manner. Effector T cells (T_{eff}), comprised of $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ T helper cells, natural killer (NK) cells, mature dendritic cells (DC), and type 1 macrophages (M1) can cause the elimination tumor cells, whereas regulatory T cells (T_{reg}), tissue-resident memory T cells, myeloid-derived suppressor cells (MDSC), tolerogenic DC, and M2 macrophages are associated with immune suppression, tissue remodeling and angiogenesis (Galon and Bruni, 2019; Guerra et al., 2020; Hanahan and Weinberg, 2011; Peinado et al., 2017; Tauriello et al., 2021).

Understanding the mechanisms by which tumor cells escape from immune surveillance is a major clinical challenge. Classification of cancers according to their status of immune infiltration led to an immune-based, rather than a cancer-based, classification system for tumors (Galon et al., 2006). The resulting Immunoscore is a standardized scoring system, which quantifies two lymphocyte populations (CD3 and CD8) in the center of the tumor and in its invasive margin (Galon et al., 2014). Subsequently, tumors have been categorized into hot, altered-excluded, altered-immunosuppressed, and cold, an important step in further classification, which guides the

choice of combination immunotherapies (Galon and Bruni, 2019). Immune checkpoints are attractive therapeutic targets. Thus, programmed cell death protein 1 (PD-1), programmed cell death-1 ligand-1 (PD-L1), and cytotoxic T lymphocyte antigen 4 (CTLA-4) have been targeted to achieve improved outcome in several cancers based on the rationale that the blocking of immuno-inhibitory signals enables patients to produce an effective antitumor response (Galon and Bruni, 2019; Gentric et al., 2017; Martinez-Outschoorn et al., 2017). Recent data provide evidence for an important tumorigenic role of cytokine transforming growth factor-β (TGF-β), which is released by both tumor and benign cells in the TME and enforces immune tolerance, suppresses inflammation, and regulates angiogenesis (Chen et al., 2019; Li et al., 2017; Tauriello et al., 2021). These findings have sparked the development of a wide range of therapeutic modalities aiming to inhibit the TGF-β pathway in clinical immuno-oncology (Tauriello et al., 2021). It has become evident that adenosine metabolism can be co-opted by tumors to promote their growth and impair immunity (Allard et al., 2020; Boison and Yegutkin, 2019; Hatfield and Sitkovsky, 2020; Vijayan et al., 2017) (**Figure 3**). Therefore, targeting this pathway has the potential to further improve the response rates in cancer patients. In the following we will discuss the complexity of cancer metabolism in more detail with a specific emphasis of dysregulated adenosine metabolism and the prospects for the development of personalized purinergic therapies.

V. Dysregulation of adenosine metabolism in cancer

A. The counteracting roles of ATP and adenosine in immunomodulation and tumor immune evasion

In cancer cells, the hypoxic environment is a trigger for the release of ATP. The in vivo measurement of extracellular ATP concentrations using cell surface-targeted luciferase revealed

the ability of solid tumors to maintain peritumoral ATP within the range of 10^{-5}-10^{-4} mol/L, which is much higher than the concentration of this nucleotide detected in the interstitium of healthy tissues (De Marchi et al., 2019; Di Virgilio et al., 2018). Activation of excitatory ATP-specific receptors by ATP derived from stressed or dying cells induces a fast-acting inflammatory response by the amplification of T cell receptor signaling in lymphocytes and inflammasome activation (Cekic and Linden, 2016; Di Virgilio et al., 2018; Eltzschig et al., 2012; Ma et al., 2013; Michaud et al., 2011), and in addition acts as a 'find-me signal' to promote phagocytic clearance by macrophages (Elliott et al., 2009) (**Figure 3**). However, because tumor cells can efficiently scavenge nucleotides, they uniquely contribute to rapid termination of pro-inflammatory effects of ATP within the metabolically abnormal TME. The metabolic clearance of ATP leads to the enhanced production of adenosine and the resulting A_{2A}R-dependent inhibition of effector immune cells, including NK cells, cytotoxic T-lymphocytes, macrophages, and DCs (Ma et al., 2013; Maj et al., 2017; Vijayan et al., 2017; Young et al., 2014). Because both innate and adaptive cellular components of the immune system contribute to immune surveillance and tumor suppression (Galon and Bruni, 2019; Guerra et al., 2020; Hanahan and Weinberg, 2011), and because adenosine effectively suppresses inflammation and immune function (Hatfield and Sitkovsky, 2020; Sidders et al., 2020; Sitkovsky et al., 2014), a tumor maintaining adenosine in its microenvironment will suppress the host's immune defense mechanisms. For example, the analysis of biochemical and functional properties of T_{reg} cells in the TME in human ovarian cancer has shown that adenosine itself, rather than classical suppressive factors such as PD-L1, CTLA-4, TGF-β, interleukin-35 (IL-35) or IL-10, triggered immunosuppression via activation of A_{2A}Rs (Maj et al., 2017). Therefore, targeting the immunosuppressive adenosinergic pathway represents a highly attractive target to improve the clinical outcome in cancer patients.

The pharmacological inhibition of $A_{2A}R$-mediated signaling in T cells, or the pretreatment of tumor-reactive T cells with $A_{2A}R$ siRNA before adoptive transfer, initiated robust antitumor effects of the transferred T cells and rejection of lung metastases. At the same time, those treatments reduced neovascularization of tumors and markedly inhibited tumor growth (Ohta et al., 2006; Sitkovsky et al., 2014). Furthermore, blockade of the adenosinergic pathway could potentially be combined with the targeting of co-stimulatory receptors to potentiate antitumor immune responses via the TGF-β pathway (Chen et al., 2019; Sidders et al., 2020), adoptively transferred tumor-reactive $CD8^+$ T cell or NK cells, cancer vaccine–induced tumor-reactive T cells, T cells with chimeric activating receptors (CAR-T cells) (Leone and Emens, 2018; Sek et al., 2020; Sitkovsky et al., 2014; Vijayan et al., 2017), as well as anti-hypoxic oxygenation agents (Hatfield and Sitkovsky, 2020; Sek et al., 2020).

B. The CD39-CD73 pathway in immuno-oncology

The recent identification of CD39 and CD73 as one of the major immune checkpoint regulators provided a basis for a more thorough investigation of this ATP-degrading and adenosine-producing pathway in a wide range of cancer types. The up-regulation of CD39 and CD73 on the surface of epithelial, vascular endothelial, lymphoid, and tumor cells as a result of tissue hypoxia and inflammation supports the current emphasis that adenosine metabolism constitutes an important anti-cancer target (Allard et al., 2017; Boison and Yegutkin, 2019; Chiu et al., 2017; Eltzschig et al., 2012; Losenkova et al., 2020; Sitkovsky et al., 2014). These findings are based on early studies, which identified a hypoxia-responsive element in the promoter of the CD73 (NT5E) gene, and demonstrated that CD73 is a transcriptional factor of the hypoxia-inducible factor 1α (HIF-1α) (Synnestvedt et al., 2002). A wealth of evidence also indicates the

presence of high levels of TGF-β in the TME due to enhanced production and release of this cytokine by tumor cells, myeloid cells and CAFs (Tauriello et al., 2021), which in turn may create an adenosine-rich immunosuppressive microenvironment via the up-regulation of CD39 and/or CD73 on T cells and other TIL (Chen et al., 2019; Kurnit et al., 2021; Li et al., 2017; O'Connor et al., 2021). Additional factors potentially contributing to increased phosphohydrolysis in the TME may include secretion of IFN-γ and TNF-α by activated T cells with subsequent up-regulation of CD73 on CAF in patients with non-small cell lung cancer (O'Connor et al., 2021), as well as an increased expression of CD39 on tumor-associated macrophages (TAM) and T cells driven by activation of the aryl hydrocarbon receptor on glioblastoma cells (Takenaka et al., 2019).

High expression levels of CD39 were detected in patients with colorectal and lung cancers (Kunzli et al., 2011; Simoni et al., 2018), pancreatic cancer (Kunzli et al., 2007), melanoma (Sade-Feldman et al., 2018), multiple myeloma (Yang et al., 2020), acute myeloid leukemia (Aroua et al., 2020), high-grade serous ovarian cancer (Bareche et al., 2021; Gaudreau et al., 2016), and other solid tumors (Bastid et al., 2012; Moesta et al., 2020), where it has been selectively associated with both neoplastic cells and adjacent host tissues, including CAF, $CD8^+$ T-cells and other TIL. Overexpression of another member of this family, ENTPD2, was also found as a poor prognostic indicator for the patients with hepatocellular carcinoma (Chiu et al., 2017). Likewise, the increased expression or activity of CD73 is associated with poor outcome in a variety of cancers, including prostate cancer, glioblastoma, triple negative breast cancer, high-grade serous ovarian cancer and melanoma (Allard et al., 2020; Bareche et al., 2021; Goswami et al., 2019; Loi et al., 2013; Yu et al., 2020). However, conflicting studies reported positive prognostic links to increased CD73 expression in other cancer types such us endometrial carcinomas (Bowser et al., 2016; Kurnit et al., 2021), colorectal cancer (Cushman et al., 2015),

and neuroblastoma (Jain et al., 2021). Thus, with certain caveats, the general assumption is that increased extracellular adenosine, linked to increased expression or activity of CD39 and/or CD73 dampens spontaneous tumor-associated antigen-specific T-cell immunity and equates to a poor prognosis.

C. The role of adenosine metabolism in tumor angiogenesis and vasculogenesis.

Numerous clinical observations have established a clear relationship between cancer and angiogenesis, which can participate in multiple stages of chronic inflammation, tumorigenesis and metastasis (Lugano et al., 2020; Yamauchi et al., 2018). Intratumoral hypoxia induces production of a plethora of pro-angiogenic factors within the tumor parenchyma, which in turn leads to chaotic blood vessel formation, uneven blood flow and enhanced permeability and interstitial fluid pressure (Lugano et al., 2020). Recent data also provide evidence that tumors cells, through angiogenesis, can enforce an embryonic-like gene expression program in vascular EC to ultimately suppress leukocyte infiltration and compromise antitumor immunity (Huijbers et al., 2022). Robust data sets suggest that adenosine is an important regulator of neovascularization, wound healing, and tissue repair in different organs and tissues, including cancer. Adenosine and its phosphorylated precursor nucleotides (ATP and ADP) modulate key pathways of angiogenesis and vasculogenesis, which include the enhancement of EC proliferation and migration (Allard et al., 2014; Borea et al., 2018; Feoktistov et al., 2009; IJzerman et al., 2022; Yegutkin et al., 2011a), as well as maintenance of endothelial (Strassheim et al., 2020; Yegutkin et al., 2015; Yegutkin et al., 2011a) and epithelial (Kurnit et al., 2021; Synnestvedt et al., 2002) barrier integrities. While ATP primarily controls vascular remodeling and permeability through activation of $P2Y_1$ and $P2Y_{13}$ receptors (Strassheim et al., 2020), the pro-angiogenic role of adenosine is based activating

endothelial A_{2A}Rs and A_{2B}Rs and, consequently, regulate the production and release of VEGF, basic fibroblast growth factor (bFGF), and insulin-like factor-1 (Borea et al., 2018; Feoktistov et al., 2009). Given the prominent role of the A_{2B}R in the regulation of tumor-induced angiogenesis and marked up-regulation of both A_{2A} and A_{2B} receptors in human cancers (Ahmad et al., 2009; Allard et al., 2014; Feoktistov et al., 2009; IJzerman et al., 2022; Ryzhov et al., 2014), the development of A_{2B}R antagonists is considered a promising strategy for the development of novel anti-cancer treatments.

A critical role for the CD39-CD73 axis in governing adenosinergic signaling and supporting tumor angiogenesis and vasculogenesis was established both in vitro and in vivo. CD73 derived from cancer cells contributes to VEGF production by tumors, whereas CD73 derived from the host is thought to promote optimal angiogenic responses to VEGF/bFGF (Allard et al., 2014). In addition, TGF-β contributes to the regulation of tumor growth and angiogenesis in the TME by driving the expression of both CD39 and CD73 on myeloid and innate lymphoid cells (Chen et al., 2019; Li et al., 2017; Vijayan et al., 2017). Disruption of TGF-β signaling prevented differentiation of MDSC into pro-tumorigenic $CD39^+/CD73^+$ terminally differentiated myeloid mononuclear cells, in conjunction with increased infiltration of T lymphocytes, reduced blood vessel densities, and attenuated progression of Lewis lung and spontaneous mammary carcinomas (Ryzhov et al., 2014). In turn, loss of CD73 in advanced stage endometrial cancer cells shifted TGF-β from tumor suppressor to promoter, and it was associated with poor tumor differentiation, increased fibrosis and vascular space invasion (Kurnit et al., 2021). Studies performed in CD39 knockout mice have shown that CD39 plays a significant role in the activation of integrin-associated signaling pathways and attenuation of tumor angiogenesis and metastasis (Jackson et al., 2007). In line with an anti-inflammatory role of adenosine, it was recently established that

ADK activity plays a key role in driving vascular inflammation through an epigenetic mechanism, highlighting the potential therapeutic value of treatments targeting the nuclear isoform of the enzyme (Xu et al., 2017). Overall, the role of adenosine metabolism in angiogenesis is likely crucial for the promotion of new vasculature in tumors, tumor growth, and metastasis.

D. The role of adenosine metabolism in tumor lymphangiogenesis

The "seed and soil" hypothesis posits that tumor cells that have entered the blood circulation need to extravasate through a multilayer vessel wall in order to colonize and metastasize at predetermined locations (Peinado et al., 2017). The lymphatic vasculature is an important route for the metastatic spread of human cancer. Indeed, the homeostatic functions of the lymphatic vasculature in trafficking immune cells between peripheral tissues, lymph nodes, and circulation can be co-opted by tumor cells (Li et al., 2019b; Peinado et al., 2017). The lymphatic vasculature is characterized by remarkable plasticity and can be considered as an active tissue-specific component of various physiological and pathological processes (Petrova and Koh, 2020). CD73 in particular has emerged as lymphatic EC-specific marker for the identification of lymphatic capillaries and collecting vessels (Kato et al., 2006). A selective distribution of CD73 in germinal centers, high endothelial venules, afferent lymphatic vessels, and lymph has been confirmed in histochemical analyses of human lymph nodes (LN) (Algars et al., 2011; Junker et al., 2019; Yegutkin, 2014; Yegutkin et al., 2015).

In addition, studies with CD73 knockout mice have shown that adenosine generated via CD73 may prevent maturation of DCs in the draining LN (Eichin et al., 2021) and restrict the migration of lymphocytes across high endothelial venules into the LN after an inflammatory stimulus (Takedachi et al., 2008). Interestingly, human dermal microvascular lymphatic EC displayed

higher CD73 activity than blood ECs isolated from the same donor (Algars et al., 2011; Yegutkin et al., 2015). Knocking out CD73 on lymphatic ECs also leads to the up-regulation of inflammation-associated genes and a more pro-inflammatory phenotype of interacting DCs (Eichin et al., 2021). However, in contrast to ECs from the blood, lymphatic ECs were unresponsive to adenosinergic signaling during the regulation of endothelial sprouting and permeability control (Yegutkin et al., 2015). Adenosine also inhibited the proliferation and migration of lymphatic ECs in vitro but stimulated lymphangiogenesis in vivo in Matrigel plug assays with the stable analogue of adenosine, 2-chloro-adenosine (Lenoir et al., 2014). A recent study with Adora2a –deficient mice confirmed the important role of $A_{2a}R$ signaling in promoting lymphangiogenesis and LN metastasis, and further demonstrated the existence of positive correlations between genes encoding lymphatic markers and $A_{2A}R$ (ADORA2A), CD73 (NT5E), and CD39 (ENTPD1) in multiple human tumors (Allard et al., 2019). Additional studies are needed to further understand the contributions of the CD73-adenosine axis in the regulation of tumor lymphangiogenesis and lymphogenous metastasis.

E. The role of adenosine metabolism in cancer-associated thrombosis

Cancer patients frequently display an increased risk of thrombotic occlusion of vessels as a result of tumor-induced activation of platelets and the coagulation system. This phenomenon is mainly due to elevated expression of prothrombotic factors, either directly by the tumor itself or by platelets and other adjacent cells present in the TME (Haemmerle et al., 2018; Palacios-Acedo et al., 2019). Interestingly, tumor cells can exhibit so called “platelet mimicry” via formation of platelet-tumor cell aggregates shielding them from NK cell- and TNF-α-induced cell death (Haemmerle et al., 2018), as well as “vascular mimicry”, whereby aggressively growing tumor

cells can form vessel-like structures to source sufficient blood supply and nutrients (Lugano et al., 2020). The formation of thromboxane-A2 (TXA2) by cyclooxygenase-1 (COX1) or the secretion of ADP from dense granules with the subsequent activation of $P2Y_1/P2Y_{12}$ receptors on platelets initiates the process of platelet activation and recruitment with subsequent hemostatic plug formation. Platelet reactivity is controlled by the vascular endothelium controls, which can prevent thrombus formation nitric oxide and prostaglandin-I_2 synthesis or ADP scavenging by CD39 (Eltzschig et al., 2012; McFadyen et al., 2018; Yegutkin, 2021). It is pertinent to note that intravascular concentrations of proinflammatory ATP and prothrombotic ADP may be markedly elevated during acute vascular injury, sepsis, and ischemia-reperfusion (Eltzschig et al., 2012). Approaches to offset the reduced effectiveness of endothelial nucleotidases at sites of vascular injury and inflammation include up-regulation of endogenous CD39 in injured vessels (Baek et al., 2017), the employment of soluble NTPDase (apyrase), targeting of soluble CD39 (targ-CD39) recombinantly fused to an activated platelet adhesion receptor GPIIb/IIIa-specific single-chain antibody (Hohmann et al., 2013), fusion of CD39 to the essential platelet collagen receptor glycoprotein VI (CD39-GPVI-Fc) (Degen et al., 2017), as well as the generation of a bi-functional enzyme made up from the ecto-domains of CD39 and CD73 and acting as a potent antiplatelet drug (Zhong et al., 2021). Taking into account data on the maintenance of high concentrations of ATP in the TME (De Marchi et al., 2019; Di Virgilio et al., 2018; Michaud et al., 2011), and the ability of soluble apyrase to modulate tumor growth by local scavenging of intratumoral ATP (Feng et al., 2011; Yegutkin et al., 2011b), the directional manipulation of soluble and/or membrane-bound nucleotidase activities might be a promising strategy for the prevention of thrombosis in cancer. On the other hand, clinical trials with different $P2Y_{12}R$ antagonists (clopidrogel, ticagrelor or plasugrel), reported an increased risk of worsening tumor growth and

bleeding complications in cancer patients (Haemmerle et al., 2018), thus highlighting the need for developing novel therapeutic approaches selectively targeting interactions of platelets with tumor cells without interfering with their normal thromboregulatory functions.

F. Purinergic mechanisms of blood flow and tumor perfusion

Adenosine and ATP can also affect the functionality of blood vessels through the modulation of coronary and microvascular blood flow. Hypoxia and shear stress induce the release of intravascular ATP from the EC, which in turn stimulates vasodilatation through the direct binding to endothelial $P2Y_1$, $P2Y_2$ and/or P2X4 receptors, and further conversion to adenosine and activation of A_{2A}Rs and, to some extent, A_{2B}Rs on vascular endothelial and smooth muscle cells (Borea et al., 2018; Mustafa et al., 2009). In addition, the activation of endothelial nucleotide- and nucleoside-selective receptors induces the release of endothelium-derived nitric oxide, hyperpolarizing factor, and prostacyclin, which act to inhibit platelet aggregation and thereby improve circulation (Mustafa et al., 2009). ATP can also be produced by erythrocytes in response to shear stress, strenuous exercise, acidosis, and hypoxia (Pernow et al., 2019). The released ATP and, consequently, adenosine following the breakdown of ATP, act at endothelial nucleotide- and nucleoside-specific receptors to induce vasodilatation and an increase in local blood flow. This promotes oxygen delivery to the tissue and thereby leads to the rebalancing of oxygen supply with metabolic demand. CD39 and CD73 are selectively expressed in different arterial, venous, microvascular, cerebral and retinal blood vessels (Algars et al., 2011; Losenkova et al., 2022; Yegutkin, 2021; Zeiner et al., 2019) and also circulate as soluble enzymes in the bloodstream (Yegutkin et al., 2012), thus preventing vessel occlusion and keeping the hemostatic process tightly regulated. On the other hand, since the half-life of adenosine in circulation is very short

(<10 seconds) (Chen et al., 2013; Moser et al., 1989), the directional manipulation of blood flow and vascular tone via intravenous administration of adenosine is not practical for long-term applications. Interestingly, the widely used oral anti-thrombotic drug ticagrelor is capable to trigger pleiotropic effects, by inhibiting platelet aggregation through reversible binding to platelet $P2Y_{12}$ receptors and at the same time, blocking adenosine reuptake through the inhibition of ENT1 on erythrocytes and platelets (Cattaneo et al., 2014; D'Amario et al., 2020). By potentiating adenosine's bioavailability, ticagrelor might have beneficial effects through an increase of coronary blood flow and the promotion of additional adenosine-induced physiological responses in the vascular system. A novel poly(ethylene glycol) (PEG)-based hydrogel delivery system for CD73 has also been designed recently, which allows the improvement of blood flow via continuous generation of adenosine locally in the target tissue (Sayegh et al., 2021). Despite a high vascular density, tumor vessels are usually functionally and structurally perturbed by the creation of a hostile milieu, that is deprived of oxygen and nutrients and from which cancer cells attempt to escape and metastasize to more favorable sites in the body (Huijbers et al., 2022; Li et al., 2019b; Lugano et al., 2020; Peinado et al., 2017). Therefore, it is tempting to speculate that directional shifts in ATP and adenosine levels in the TME might affect local vascular supply and tumor perfusion, and in this way, improve the delivery of chemo- and immuno-therapeutics, a hypothesis requiring further validation.

VI. Adenosine metabolism in cancer: exploitation for therapeutic gain

A. CD39 and CD73 as targets in cancer immunotherapies

As outlined above, upregulation of CD39 and/or CD73 as a result of tissue hypoxia, inflammation, oxidative stress, and epithelial-to-mesenchymal transition is a pathological hallmark of the

metabolically abnormal TME in many cancers. This metabolic abnormality emerges as a potential mechanism for the development of resistance to checkpoint-blockade. Preclinical studies have demonstrated tumor regression after blocking of adenosine receptors, inhibition of adenosine-producing ectoenzymes, or reversal of tissue hypoxia (Allard et al., 2020; Vigano et al., 2019; Vijayan et al., 2017; Yang et al., 2020). In autophagy-deficient tumors the recruitment of immune cells was reestablished through the inhibition of extracellular ATP-degrading enzymes, which in turn increased pericellular ATP levels and restored responses to chemotherapeutic agents in immunocompetent hosts (Michaud et al., 2011). CD39 has been targeted with a variety of approaches including the small-molecule inhibitors POM-1 and ARL-67156, or a CD39-blocking antibody (OREG-103/BY40), as a strategy to significantly reduce the $CD39^+$ tumor cell–mediated suppression of $CD4^+$ and $CD8^+$ T-cell responses and thereby to induce enhanced cytotoxic activity of CTL and NK cells, and thereby to promote the ablation of tumor cells (Bastid et al., 2015; Sun et al., 2010). A recent innovative approach targeted a single nucleotide polymorphism in the Entpd3 gene associated with risk of renal cell carcinoma to upregulate lncRNA-ENTPD3-AS1. The authors of this study demonstrated the suppression of renal cell carcinoma through a mechanism involving miR-155/HIF-1alpha signaling (Wang et al., 2021a). The inhibition of CD39 ecto-ATPase activity with genetic and pharmacological means blocked mitochondrial reprogramming due to cytarabine treatment and significantly enhanced its cytotoxicity in acute myeloid leukemia cells (Aroua et al., 2020). In line with those findings, treating B16-10 melanoma-bearing mice with a combination of the CD39 inhibitor POM-1, and anti-PD-1 and CTLA-4 antibodies led to a significant reduction in tumor growth and increased survival (Sade-Feldman et al., 2018; Sun et al., 2010). Noteworthy, along with acting as a potent CD39 inhibitor,

POM-1 and related polyoxometalates can inhibit the activities of additional members of the NTPDase family and ENPP1 (Lee et al., 2015).

Recent therapeutic approaches have focused on the directional manipulation of adenosinergic signaling in the TME by blocking ATPase/ADPase activity with anti-CD39 antibodies (Moesta et al., 2020; Perrot et al., 2019; Spatola et al., 2020). Based on this premise, several humanized and fully human anti-CD39 antibodies are now in Phase I clinical trials involving patients with lymphoma and advanced solid tumors, either alone or in combination with immunomodulatory and chemotherapeutic agents (**Table 2**). Furthermore, the identification of CD73 as a 'master switch' which determines the shift towards adenosine formation or reverse ATP regeneration (**Figure 2**) has triggered numerous attempts to develop novel fully human or humanized anti-CD73 antibodies selectively targeting the immunosuppressive adenosinergic pathway (Alcedo et al., 2021; Allard et al., 2020; Boison and Yegutkin, 2019; Hay et al., 2016). The combination of an anti-CD73 therapy with immunogenic cytotoxic drugs would be a further strategy to target CD73 and its downstream effector $A_{2A}R$ in order to enhance anthracycline chemotherapy and improve clinical outcomes in patients with triple-negative breast cancer (Allard et al., 2014; Loi et al., 2013). Multiple clinical trials focused on combined therapies with CD73-inhibiting antibodies, $A_{2A}R$ antagonists and additional drugs targeting immune checkpoints, as well as chemotherapeutic agents have been initiated recently to evaluate the therapeutic potential of the CD73-$A_{2A}R$ pathway in immuno-oncology (**Table 2**).

A potential limitation of these approaches is that anti-CD39 and anti-CD73 antibodies may not penetrate well into solid tumors. Alternative strategies include the development of small-molecule inhibitors of CD39 (al-Rashida and Iqbal, 2014; Jeffrey et al., 2020; Murtaza et al., 2021) and CD73 (Alcedo et al., 2021; Bowman et al., 2019; Jeffrey et al., 2020). A broad spectrum of novel

nucleotide analogues with high metabolic stability and nanomolar inhibitory potency towards human CD73 have been developed recently and tested as potential anti-cancer drugs. In particular, these compounds include a broad spectrum of derivatives of the classical CD73 inhibitor α,β-methylene-ADP (AMPCP), focused on structural modifications of the N^6-benzyl residue, PSB-12489, PSB-19416, PSB-18332 (Bhattarai et al., 2019; Schmies et al., 2020), and AB680 (Bowman et al., 2019; Jeffrey et al., 2020) or the linker between the external phosphonic acid and the sugar ring (OP-5244) (Du et al., 2020), the replacement of the bisphosphonic acid moiety with a methylenephosphonic acid (Sharif et al., 2021), as well as synthesis of N^4-benzyloxyimino and 5'-O-[(phosphonomethyl)phosphonic acid] derivatives of purine and pyrimidine nucleosides (Junker et al., 2019; Scortichini et al., 2022). Several orally bioavailable CD73-inhibiting compounds such as AB680/Quemliclustat (Arcus Biosciences) and LY3475070 (Elly Lilly and Company) have progressed to clinical trials (**Table 2**), with additional small-molecule CD73 inhibitors such as ORIC-533 (ORIC Pharmaceuticals) and CB-708 (Calithera Biosciences) being tested in preclinical models.

B. Non-canonical nucleotide-inactivating/adenosine-generating pathways as therapeutic targets

Along with the canonical route of ATP breakdown via the CD39-CD73 axis, extracellular adenosine can also be generated from NAD through the CD38-ENPP1-CD73 pathway (**Figure 2**). Several evolving concepts on the metabolism, transport, and roles of these NAD pathway metabolites in disease states such as cancer, neurodegeneration, and aging have emerged in the last few years (Chini et al., 2018; Covarrubias et al., 2021). In this regard, special attention was given to the modulation of NAD metabolism and boosting cellular NAD levels via inhibition of

the key NAD-degrading enzyme CD38 by using monoclonal antibodies and small molecule inhibitors (Chini et al., 2018). Consequently, CD38 has been identified as an important immune checkpoint and also as a cell-surface marker in hematological cancers (Chen et al., 2018; Chini et al., 2018). Two CD38-targeting antibodies daratumumab (Janssen Biotech) and isatuximab (Sanofi) have been approved by the US Food and Drug Administration (FDA) for the treatment of multiple myeloma and hematological malignancies, with three other anti-CD38 antibodies MOR202 (Morphosys), TAK079 (Takeda), and SAR442085 (Sanofi) being progressed into clinical trials either alone or in combination with proteasome inhibitors or immunomodulatory agents (Boison and Yegutkin, 2019; Chini et al., 2018; Kassem et al., 2022; van de Donk et al., 2021).

Ectoenzymes of the ENPP family may also contribute to cancer cell proliferation and immunomodulation, either in conjunction with CD38 as an important constituent of the NAD-inactivating machinery or via direct conversion of extracellular ATP into AMP and PP_i (Boison and Yegutkin, 2019; Gasparrini et al., 2021; Zimmermann et al., 2012). The identification of the ENPP1-CD73 pathway as an alternative CD39-independent adenosinergic loop on cancer cells (Helenius et al., 2012; Losenkova et al., 2020) and lymphocytes (Hesse et al., 2021; Linden et al., 2019) might enable cancer cells to bypass CD39-based therapeutics. Furthermore, the metabolism of extracellular cGAMP by ENPP1 outlines an additional ATP-independent pathway, which enables chromosomally unstable cancer cells to evade immune surveillance and promote metastasis (Carozza et al., 2020a; Li et al., 2021). In response to double-stranded DNA the second messenger cGAMP is synthesized in the cytosol by the enzyme cGAMP synthase (cGAS). cGAMP subsequently binds to its endoplasmic reticulum surface receptor stimulator of interferon genes (STING) as a molecular pathway to stimulate the production of type I interferons. In

addition to its cell-intrinsic effects, cGAMP is exported to the extracellular space where it acts as an anticancer immunotransmitter by activating the immune innate STING pathway in host cells (Carozza et al., 2020a). One mechanism by which cancer cells have evolved to cope with cGAS–STING activation is through rapid ENPP1-mediated degradation of the immune-stimulatory metabolite cGAMP into immunosuppressive adenosine (Carozza et al., 2020a; Li et al., 2021).

Given the highly selective expression of another isozyme ENPP3 in renal cell carcinoma, a conjugate comprised of an anti-ENPP3 antibody linked with maleimidocaproyl monomethyl auristatin-F has been tested in binding, internalization and cytotoxicity assays, as well as in in vivo studies using xenograft tumor models (Donate et al., 2016). This approach allowed the identification of ENPP3 as a potential human cancer-specific antigen. The anti-ENPP3 antibody (AGS-16M8F)-drug conjugate has been tested in a Phase I trial in patients with advanced refractory renal cell carcinomas (ClinicalTrials.gov ID: NCT01114230) (Thompson et al., 2018). A wide range of highly selective inhibitors of ENPP1 (al-Rashida and Iqbal, 2014; Carozza et al., 2020b), ENPP3 (Lee et al., 2021), and other members of the NTPDase family (NTPDase2, 3 and 8) (al-Rashida and Iqbal, 2014; Murtaza et al., 2021) have recently been tested for their therapeutic potential.

The important role of TNAP and other alkaline phosphatases in cancer and various (patho)physiological processes (Millan, 2006; Rao et al., 2017; Vijayan et al., 2017; Zimmermann et al., 2012) makes them an attractive target for tissue-specific therapeutic interventions. A number of small-molecule inhibitors of TNAP have been synthesized recently and tested as potential drug candidates for the treatment or prevention of hepatic and breast cancers and associated malignancies (Ashraf et al., 2018; Channar et al., 2018; Rao et al., 2017; Zaher et al., 2020). A recent study also identified alkaline phosphatase as a novel regulator of migration and

epithelial-to-mesenchymal transition in prostate cancer, where high enzyme expression correlated with a reduction in disease-free survival in patients (Rao et al., 2017). The authors further highlighted the promise of small-molecule inhibitors as anti-metastatic drugs inhibiting tumor-derived TNAP activity in patients with advanced prostate cancer. Though, taking into account the unique capabilities of the enzymes of the alkaline phosphatase families to hydrolyze a broad variety of phosphoric acid ester bonds, more thorough functional and competitive studies would be required to elucidate functional implications of these multifunctional ectoenzymes on the purinergic signaling cascade.

In addition, the adenosine-generating (ecto)enzyme PAP is the target antigen of the FDA–approved antitumor vaccine Sipuleucel-T, thereby validating PAP as a relevant prostate tumor antigen. Sipuleucel-T is a therapeutic cancer vaccine comprised of autologous peripheral-blood mononuclear cells, which have been activated ex vivo with a recombinant fusion protein PA2024, which in turn is a fusion of PAP to granulocyte–macrophage colony-stimulating factor (GM-CSF), an immune-cell activator (Kantoff et al., 2010). A recent Phase II clinical study investigated the effect of an investigational DNA vaccine encoding PAP, pTVG-HP [MVI-816], on 2-year metastasis-free survival (MFS) in patients with recurrent, non-metastatic prostate cancer (NCT01341652) (McNeel et al., 2019). While the results did not show an overall difference in MFS between the patients receiving pTVG-HP with GM-CSF adjuvant or GM-CSF alone, a subset of patients with rapidly progressing disease, as determined by a rapid doubling time of PSA, and which were treated with pTVG-HP had a longer MFS (McNeel et al., 2019).

C. Suppression of tumor metastasis via modulation of phosphotransfer reactions.

Coordinated phosphotransfer reactions are needed to mediate communication between ATP-producing and ATP-consuming cellular compartments in order to maintain normal growth, development and bioenergetics of the cell (Dzeja et al., 2007; Johnson et al., 2019). Recent data provide evidence that key nucleotide-phosphorylating enzymes of the AK (Klepinin et al., 2020; Maslah et al., 2021) and NDPK/NME/NM23 (Boissan et al., 2018; Romani et al., 2018; Zhang et al., 2022) families have been implicated in cancer cell proliferation and metastasis. There are some contradictory studies showing substantial down-regulation of AK1 in lung cancer and hepatoma (Klepinin et al., 2020), and the existence of a positive correlation between high AK1 levels and poor survival of cytarabine-treated acute myeloid leukemia patients (Frejno et al., 2020). The expression levels of another isozyme, AK2, were shown to be down-regulated in breast, liver, and lung cancers (Kim et al., 2014), whereas other studies have shown that high expression levels of AK2 correlate with a worse prognosis for patients with neuroblastoma, embryonic carcinoma, breast and lung adenocarcinomas (Klepinin et al., 2020), and T-cell acute lymphoblastic leukemia (Maslah et al., 2021). Interestingly, the diterpene lactone neoandrographolide extracted from the traditional medicinal herb Andrographis paniculata has potent anticancer properties most likely based on inhibition of AK2 activity (Klepinin et al., 2020; Maslah et al., 2021).

Compelling data suggest that the first two members of the NDPK/NME/NM23 family, NME1 and NME2, can also modulate various tumor-associated biological events (Boissan et al., 2018; Buxton et al., 2010; Zhang et al., 2022). Importantly, an inverse association between NME1 expression and the metastatic potential for human tumors of epithelial origin was reported in clinical studies (Boissan et al., 2018; Lodillinsky et al., 2021; Marino et al., 2012). Likewise, studies with syngeneic and xenograft animal models and with cancer cell lines confirmed the

ability of NME1 to suppress multiple steps of the metastatic cascade, including tumor cell invasion, survival, and colonization (Khan et al., 2019; Lodillinsky et al., 2021; Marino et al., 2012). On the other hand, NME2 enhanced tumorigenesis in solid tumors and hematological malignancies, including hepatocarcinoma, endometrial, cervical, leukemia, gastric, pleural mesothelioma, colorectal, lung, sarcoma, giant cell tumors (Li et al., 2015), and invasive breast cancer and ductal carcinoma in situ (Lodillinsky et al., 2021).

Several compounds have been tested for their ability to augment the suppression of tumor metastasis via up-regulation of NDPK activity, including thujone, a monoterpene natural product (Marino et al., 2012), medroxyprogesterone acetate, an agonist of the glucocorticoid receptor (Miller et al., 2014), as well as a natural small-molecule Nm23 activator-1 (NMac1) isolated from Zingiber cassumunar Roxb (Zungiberaceae) and its synthetic derivatives (Lee et al., 2018). Other therapeutic strategies for increasing the expression of this enzyme in solid tumors include gene therapy-based Nm23 delivery using an adeno-associated virus-mediated transduction or non-viral nanoparticle-mediated transduction, as well as the fused protein-based approach for delivering cell-permeable Nm23 protein (CP-Nm23-H1) (Marino et al., 2012). Strikingly, the role of ecto-NDPK seems to be different from that of the intracellular expression of this enzyme, with tumor-promoting functions of extracellular NME proteins being demonstrated in several tumor cohorts, including breast cancer (Romani et al., 2018; Yokdang et al., 2015). These data are not contradictory to the role of intracellular NM23 as a metastasis suppressor, but emphasize the challenges in successful targeting this multifunctional enzyme due to its ability to mediate different, often opposite, effects via extracellular and intracellular mechanisms.

D. Soluble and secreted enzymes as biomarkers and anti-metastatic targets

The presence of soluble nucleotide hydrolases and kinases in the bloodstream (Syn et al., 2016; Yegutkin et al., 2012) and other biological fluids (Donaldson et al., 2002; Yegutkin et al., 2015; Yokdang et al., 2011; Zeiner et al., 2019) could represent an important auxiliary effector system for spatial propagation or termination of purinergic signaling responses far distant from the sites of inflammation. Furthermore, the constitutive presence of soluble ADA1 and ADA2 isozymes in the blood circulation (Blackburn and Kellems, 2005; Yegutkin, 2014; Yegutkin et al., 2012), and their ability to selectively bind to different immune cell subsets (Kaljas et al., 2017) offers an opportunity for the therapeutic delivery of adenosine-removing enzymes in the TME with the goal to adjust local adenosine levels selectively in areas of tumor growth and at sites of inflammation. In addition, ecto-nucleotidases can also be incorporated into exosomes, a subclass of membrane-derived vesicles secreted into the extracellular milieu by most cell types (Pegtel and Gould, 2019). The increased expression of CD39 and/or CD73 in extracellular vesicles from glioblastoma (Wang et al., 2021c), prostate cancer (Salimu et al., 2017), other neoplastic cells (Syn et al., 2016), as well as from tumor-infiltrating B-cells (Zhang et al., 2019), effector $CD8^+$ T cells (Schneider et al., 2021), and bone marrow plasma cells (Morandi et al., 2019) could further promote an immunosuppressive environment via local scavenging of intratumoral ATP and subsequent blunting of the responses of immune effector T cells and suppression of DC function. Because extracellular vesicles released by bone marrow-derived mesenchymal stromal cells have increased expression levels of CD39 and CD73, they can inhibit extracellular matrix remodeling and tumor associated angiogenesis via the activation of adenosinergic signaling pathways (Angioni et al., 2020). The underlying mechanisms through which the extensive network of membrane-bound and secreted purinergic activities regulates intratumoral ATP and adenosine

concentrations, promises to be an area of intense future research, which may lead to the development of either soluble or microvesicle-wrapped enzymes as novel anti-metastatic drugs.

E. Management of neoplastic diseases associated with abnormalities in intracellular adenosine metabolism

The concentration of extracellular adenosine also depends on intracellular metabolism, which is largely under the control of the multifunctional enzyme ADK (Boison, 2013). Therapeutic approaches to increase the metabolic clearance of intracellular adenosine via up-regulation of cytoplasmic ADK-S activity would turn an adenosine secreting cancer cell into a sink for adenosine. One method to increase the expression of ADK-S within a cancer would be through gene therapy. In this case, a complete penetrance of gene therapy virus expression within the target tissue would not be necessary as overexpression of ADK-S in a subset of cells would determine the therapeutic net effect on adenosine concentrations in the TME and lead to a sharp decline in the tissue tone of adenosine. Furthermore, the increased expression of the nuclear isoform ADK-L is associated with global DNA and histone hypermethylation (Boison, 2013; Williams-Karnesky et al., 2013). Through this epigenetic mechanism, ADK-L is poised to contribute to the regulation of gene expression. Importantly, epigenetic alterations in cancer cells, includes maladaptive alterations in DNA and histone methylation signatures, are thought to play major roles in promoting the proliferative potential of cancer cells (Du et al., 2015; Huang et al., 2015; Mentch and Locasale, 2016; Zahnow et al., 2016). Given the functional link of ADK-L expression with DNA and histone methylation status, and given the known disruption of normal DNA and histone methylation patterns, in particular global hypermethylation, ADK-L is a rational target for the therapeutic manipulation of epigenetic signatures in cancer cells. We recently

demonstrated that blocking or reducing ADK activity is a viable strategy to reduce vascular inflammation through epigenetic alterations (Xu et al., 2017). Novel small molecule inhibitors are currently in development, which could provide a more selective approach in targeting ADK isoforms (Toti et al., 2016). Ideally, therapeutic strategies that combine a reduction of ADK-L activity with an increase of ADK-S activity (e.g. by allosteric interaction), in conjunction with additional therapeutic approaches to manipulate the cancer purinome, will open new opportunities for therapeutic intervention.

In addition, the low affinity but high capacity enzyme ADA contributes to adenosine removal, especially under conditions of inflammation and stress during which adenosine accumulates to excessive levels (Blackburn and Kellems, 2005; Spychala, 2000). The involvement of ADA in cancer, has led to the development of ADA inhibitors as potential therapeutic agents. Those agents fall into four categories: (i) ground-state inhibitors with structures similar to the endogenous substrate adenosine; (ii) transition-state inhibitors mimicking the tetrahedral intermediate formed during the enzymatic deamination process, and (iii) second generation non-nucleoside-type inhibitors, such as FR221647, which were developed from structure-based drug design approaches using X-ray crystal structures of binary inhibitor–ADA complexes (Cortes et al., 2015), as well as (iv) a metal-binding pharmacophore strategy targeting the catalytic Zn^{2+} co-factor of this metalloenzyme (Adamek et al., 2020). Currently, the purine analog pentostatin (or 2'-deoxycoformycin, US trade name Nipent™), a powerful inhibitor of ADA, has been approved as a chemotherapeutic agent by the FDA for the treatment of hairy cell leukemia. Because ADA interacts with several intra- and extracellular metabolic pathways, additional studies are needed to determine whether the antitumor effects of pentostatin are mediated by inhibition of membrane-bound or cytosolic forms of this enzyme with concomitant modulation of adenosinergic signaling

pathways and/or intracellular purine homeostasis. In addition, pentostatin may act as immunotherapeutic drug by decreasing the SAM to SAH ratio (known as the methylation index) and lowering the methylation of cellular RNA, which in turn triggers toll-like receptor 3/TIR-domain-containing adapter-inducing interferon-β (TLR3-TRIF) signaling in tumors, local production of type I interferon and T-cell infiltration (Tusup et al., 2021). A recent study also identified ADA2 as a prognostic factor associated with increased survival in patients with breast, lung, pancreas, melanoma, and other solid tumors, and further supports the therapeutic concept to use enzymatic depletion of tumor-associated adenosine with engineered PEGylated ADA2 ($PEGADA2^{HCA}$) for cancer immunotherapy (Wang et al., 2021b).

Along with ADK and ADA, additional intracellular enzymes, such as cytosolic 5'-nucleotidase-I (cN-I, specific for AMP), cytosolic 5'-nucleotidase II (cN-II, specific for IMP-GMP), PNP, hypoxanthine guanine phosphoribosyl transferase, and xanthine oxidoreductase appear to play important roles in the regulation of purine salvage and catabolism in various pathological states, including cancer (Garcia-Gil et al., 2018; Johnson et al., 2019; Spychala, 2000). Therefore, the strategy to use directional manipulation of purine-converting activities for cancer cell ablation or to further enhance the cytotoxic activity of anticancer compounds might represent a viable solution for the development of new anticancer therapies. Interestingly, the tumor-directed transduction of Escherichia coli PNP has been applied to produce the adenine analogue 2-fluoroadenine (F-Ade) acting as a robust anti-cancer agent via ablation of RNA and protein synthesis (Behbahani et al., 2019). This gene-directed enzyme based drug therapy has shown promise in locally advanced, regional head and neck squamous cell carcinomas in human subjects (Behbahani et al., 2019). Moreover, Forodesine, a transition-state analogue that inhibits PNP activity, has been approved for the treatment of relapsed/refractory peripheral T-cell

lymphoma PTCL in Japan (Makita et al., 2018). Recent data also revealed the ability of $CD8^+$ T_{eff} cells to take up and utilize inosine as an alternative substrate thereby supporting their growth and function in the absence of glucose (Wang et al., 2020). Based on these findings, the authors suggested that supplementation with inosine and selective up-regulation of PNP in TIL (but not on tumor cells per se) has the potential to fuel key metabolic pathways in T_{eff} cells and restore their tumor-killing activity during nutrient restriction.

VII. Current advances and challenges in targeting ATP and adenosine metabolism in cancer

A. Genomic analysis of purinergic signatures of different cancers

The post-genomic era has led to a revolution in cancer therapies and implies the need for more personalized therapies. The molecular heterogeneity of cancer tissue can be evaluated by a variety of methods including RNAi and CRISPR screens, pharmacogenomics studies, single cell RNA sequencing (scRNA-Seq), whole-genome sequencing and whole-exome sequencing genome-wide approaches (Doherty et al., 2019; Nguyen and Caldas, 2021). This is particularly relevant for the multifaceted network of purine metabolism. High intratumoral levels of adenosine and up-regulation of the ADORA2A ($A_{2A}R$) and ADORA2B ($A_{2B}R$) genes are generally considered as poor prognostic factors associated with unfavorable clinical outcomes in tumors of all types from the combined The Cancer Genome Atlas (TCGA), TARGET, and Genotype-Tissue Expression dataset (Allard et al., 2020; Bareche et al., 2021; Sidders et al., 2020; Vijayan et al., 2017). Likewise, in a complementary analysis of the gene-expression landscape of other components of the adenosine pathway the high expression levels of the NT5E (CD73) and ENTPD1 (CD39) genes were associated with significantly worse overall survival in different cancers, including

lung and colorectal adenocarcinomas, ovarian cancer, melanoma, and glioblastoma (Allard et al., 2020; Allard et al., 2017; Bareche et al., 2021).

It is important to consider that the TME contains a broad spectrum of cell types, including immune cells, stromal fibroblasts, vascular and lymphatic EC, and extracellular matrix components (Lugano et al., 2020; Palacios-Acedo et al., 2019; Peinado et al., 2017). The co-expression of CD73 with additional purine-converting ectoenzymes both on malignant cells and adjacent non-neoplastic cells may lead to the gross underestimation of local adenosine levels and inaccurate prognostic estimates. For example, a recent scRNA-seq analysis demonstrated stromal cell diversity associated with immune evasion in patients with triple-negative breast carcinomas (Wu et al., 2020). Additional analysis of this public dataset identified significant differences in purine-converting and signaling pathways among the patients studied (**Figure 4**). In contrast to the high expression levels of NME2/NDPK-B on malignant and neoplastic cells and the significant accumulation of $CD39^{low}$ T-cells in immune hot tumors (**Figure 4A**), the immune cold TME was characterized by predominant expression of NME1/NDPK-A and high levels of CD39 on major T-cell subsets (**Figure 4B**). Interestingly, a comparative analysis of signaling pathways revealed highly selective expression of AR subtypes on T-cells ($A_{2A}R$), $KRT17^{+}$ tumor cells ($A_{2B}R$), and $TREM^{+}$ TAMs (A_3R) in the immune hot but not immune cold TME (**Figure 4**). The information gained from these scRNA-seq analyses may allow the construction of whole atlases of cell types and cellular and sub-cellular purinergic networks in the TME.

B. Innovative metabolomic and multiplexed imaging approaches to study the complexity of adenosine metabolism in the heterogeneous TME

The high heterogeneity of the tumor architecture requires the development of advanced tools to study the cross-talk between tumor cells and adjacent host cells. In synergy with dissociation-based single cell genomic and transcriptomic approaches, an unbiased view of the cellular heterogeneity and spatial organization of cancer tissue is necessary both at the protein and mRNA levels. ScRNA-seq genomic data can be further validated using single cell multi-omics platforms, such as the Hyperion technology (Fluidigm), mass cytometry (cytometry by time-of-flight; CyTOF), genomic cytometry (Citeseq or REAPseq) (Jackson et al., 2020; Zielinski et al., 2021), as well as recently reported single-cell metabolic regulome profiling (scMEP) approaches, which allow quantifying over 100 proteins implicated in different metabolic pathways by using high-dimensional antibody-based technologies in combination with cellular identity on the single-cell level (Hartmann et al., 2020). The quantitative analysis of immune infiltrates in primary melanomas demonstrated that a close distance between cytotoxic T-cells and macrophages was associated with poor prognosis and shortened overall survival, thus highlighting the importance of spatial information when measuring immune targets (Gartrell et al., 2018). More recent analyses of the temporal and spatial distribution of tumor-resident macrophages in human non-small cell lung carcinoma lesions revealed that macrophages localized in close proximity to tumor cells induce the epithelial-mesenchymal program and promote tumor cell invasiveness by protecting them from killing by $CD8^+$ T cells (Casanova-Acebes et al., 2021).

Clearly, understanding the complexity of the adenosine metabolic network in space and time by making use of these innovative approaches will become an area of increased interest in the future. For instance, most of the current imaging approaches lack information on high-resolution

three-dimensional (3D) mapping of the exact position of cells, the identity of cellular phenotypes, and their functional state within the context of tissue structure on a macroscale (Li et al., 2019a; Losenkova et al., 2022). Conventional histopathological analyses of formalin-fixed paraffin-embedded or cryo-embedded tissue sections can offer high-resolution images of protein expression or catalytic activity of a certain ecto-nucleotidase within the tissue, but the limited thickness of slices hampers the acquisition of more information on the z-axis. **Figure 5** displays representative images of a metastatic lymph node from a breast cancer patient, which enables a comprehensive analysis of the tissue-specific distribution of CD73/AMPase activity (**panel A**) and CD73 immunoreactivity (**panel B**), together with the visualization of the stereoscopic morphology of pan-cytokeratin^{+} cancer cells and their heterotypic interactions with additional components of the TME (**C, D**). Strikingly, while ADK is highly expressed in neoplastic cells, other key purinergic enzymes are mainly associated with cancer-associated stromal cells (CD73), TIL and tumor vessels (CD39), but not with tumor cells themselves. The information gained from these multiplexed 3D analyses may permit high-resolution volumetric analyses of different metabolic pathways, which ultimately extends analytic capacities beyond traditional histopathological and dissociation-based methods.

In summary, the most obvious challenge in targeting adenosine metabolism in cancer is our limited understanding of intratumoral adenosine turnover as a highly dynamic and spatially arranged network that coordinately controls multiple mitogenic, thromboregulatory, angiogenic, and immunomodulatory functions in the heterogeneous TME **(Figure 3)**. Despite advances made in the discovery of novel adenosine-targeting drugs and our understanding of their mechanisms of action as immune checkpoints, delivery of these compounds to their targets is continuously challenged by multiple factors, including their metabolic stability, therapeutic efficacy and tissue-

penetrating capability, as well as a multitude of biological effects of adenosine and its precursor nucleotides ATP and ADP both in neoplastic and healthy host tissues. Furthermore, adenosine appears only transiently in the extracellular milieu as an intermediate metabolite and its concentrations can rapidly fluctuate (Chen et al., 2013; Moser et al., 1989). Therefore, the cell-specific targeting of the CD39-CD73 axis to prevent adenosine formation in the immediate vicinity of certain populations of tumor, immune or stromal cells without interfering with bulk extracellular nucleotide and nucleoside levels in the TME and systemic circulation, could provide an exciting advance in the treatment of cancer patients and minimize potential side effects of adenosinergic drugs.

C. Translational value of preclinical tumor models

One of the key issues in interpreting preclinical findings from the cancer purinome is the question whether data obtained in established human cancer cell lines and genetically engineered mouse models translate to the clinic. Hundreds of human cancer cell lines have been established and are widely used to study cancer biology and to enable high-throughput functional genomic and proteomic screening (Frejno et al., 2020; Iorio et al., 2016; Nguyen and Caldas, 2021). Circulating cancer cells or samples derived from biopsies can be propagated into organoids as a model system designed to recapitulate the genetic, histological, and molecular features of the tumor of origin. Those organoids are amenable to drug screening, and predict clinical drug responses (Bleijs et al., 2019; Nguyen and Caldas, 2021). Although cancer cell lines and patient-derived tumor organoids and xenografts are powerful tools to define signaling, metabolic, and transcriptomic pathways implicated in tumor behavior, these model systems do not necessarily capture all molecular phenotypes of a particular tumor type and, in addition, do not preserve the tumor architecture and

microenvironment. For example, contrary to the high CD73 expression profile on MDA-MB-231 human breast adenocarcinoma cells (Losenkova et al., 2020; Virtanen et al., 2014), no tumor cell-associated AMPase activity and CD73 immunoreactivity can be detected in surgically removed breast cancer samples (**Figure 5**). Therefore, the comparative analysis of commonalities and differences in the purinergic signature of cancer cell lines versus primary human tumor samples may necessitate to revisit the current view of adenosine metabolism in cancer cells specifically because certain gene functions may be audited or represent in vitro artifacts.

Human tumors can be grown and propagated in rodents. This is an important preclinical tool widely used to identify novel compounds with diagnostic and therapeutic anticancer properties predictive of clinical outcome (Mak et al., 2014). However, because there are important differences between murine and human cancer pathologies and in biochemical pathways, the rates of successful translation from promising murine models to approved clinical therapies may remain low. For example, while ADK is appears to be an exception, because of its high conservation in evolution (Park and Gupta, 2008) and because the genetic disruption of ADK expression in humans, mice, and even plants causes highly comparable phenotypes marked by transmethylation defects (Bjursell et al., 2011; Boison et al., 2002; Moffatt et al., 2000), this is not necessarily true for other purinergic pathways which have marked species-specific differences in their metabolic rates (Johnson et al., 2019). The comparative analysis of two compendia of transcriptional profiles collected from human (D-MAP) and mouse (ImmGen) cell lineages during immune system differentiation revealed that, while the expression patterns of most orthologous genes remain conserved between the species, several hundred genes show clearly divergent expression profiles across the examined cell lineages. Among them, the CD73/NT5E gene did so even under very strict criteria, with high NT5E mRNA expression profiles being detected on human, but not

mouse B cells (Shay et al., 2013). These transcriptomic data are consistent with flow-cytometric and enzymatic assays, showing high expression levels and activity of ecto-5'-nucleotidase/CD73 on $CD19^+$ B-lymphocytes isolated from human peripheral blood, efferent lymph and spleen (Hesse et al., 2021; Shay et al., 2013; Yegutkin et al., 2015), and human tonsillar (Junker et al., 2019; Yegutkin, 2014) and LN (**Figure 5A**) germinal centers, but not on $B220^+$ mouse B-cells and peripheral LN (Yegutkin et al., 2015; Yegutkin et al., 2010). CD73 deficiency in humans also results in the development of medial calcification and matrix remodeling of the lower-extremity arteries and joint calcifications, whereas CD73-deficiency in mice did not recapitulate the human vascular phenotype (Joolharzadeh and St Hilaire, 2019). In addition, experimental results can be influenced by the laboratory environment such as stress, which is a well-recognized confound in caged mice (Mak et al., 2014). An additional caveat in the extrapolation from preclinical rodent studies, specifically those with an immunological focus, is the artificially sterile and standardized environment in which rodents are normally housed. This is in stark contrast to the human immune system, which is constantly challenged by pathogens.

VIII. Conclusions and outlook

To date investigations on the role of adenosine in cancer have focused largely on the role of the CD39-CD73-A_{2A}R axis in immuno-oncology. The utility of A_{2A}R antagonists as checkpoint inhibitors designed to block adenosinergic signaling, as well as small-molecule inhibitors and antibodies against CD39 and CD73 designed to block the intratumoral adenosine production from ATP are well established clinically. It is also important to note that targeting of the CD39-CD73-A_{2A}R axis might not be without harm in certain circumstances as CD39 and A_{2A}R knockout mice develop liver cancer (Sun et al., 2013). Only the past decade has brought advances in our

understanding of the complexity of mechanisms determining the duration and magnitude of purinergic signaling. It is important to understand the roles of CD39 and CD73 as targets for cancer therapy within the broader network of several interacting pathways, which cooperate in the control of extracellular ATP and adenosine levels within the TME. Those targets include CD38, ENPP1, additional nucleotide-inactivating/ adenosine-generating ectoenzymes, as well as counteracting ATP-regenerating enzymes of the NDPK/NME/Nm23 and AK families. Along with canonical adenosine-receptor dependent pathways, adenosine can also exert additional biological effects via intrinsic receptor-independent mechanisms. Those can be linked to the intracellular uptake and phosphorylation of adenosine, as well as a specific epigenetic role of the nuclear isoform of adenosine kinase, ADK-L. Therefore, cellular adenosine uptake and its intracellular metabolism are expected to have an equally important, yet understudied role. In addition to acting as immune checkpoint, targeting the adenosine pathway in the TME may offer new therapeutic opportunities to inhibit or promote vessel growth, lymphangiogenesis, blood flow, and thrombosis. Overall, these data justify the reevaluation of existing models of adenosine metabolism and their role in tumor growth, metastasis and immunosurveillance.

Key gaps of knowledge are: Would an increased production of extracellular adenosine trigger a compensatory increase in intracellular adenosine clearance, or vice versa? Would a combination of extracellular adenosinergic therapies with drugs targeting intracellular purine metabolism support bioenergetic activity and effector function of T cells and in this way improve immunotherapy approaches and enable tumor regression? Does the purinergic signature differ between patient samples and preclinical tumor models? Are there commonalities and differences in the purinergic signature between different cancer types? Broadly speaking, the cellular purinome can be viewed as a rich complex of receptors, enzymes, channels, transporters, various

ligands and cofactors that are involved in fundamental aspects of the purinergic signaling cascade and cellular purine homeostasis in a highly dynamic and coordinated manner. Therefore, a better understanding of the mechanisms linking intratumoral adenosine metabolism to other components of the cancer purinome can aid in the development of novel adenosine-related biomarkers associated with treatment response and survival, and on this basis, identify rational enzyme-based strategies for therapeutic intervention, personalized for the specific needs of each individual cancer. There is also an increased need for the development of novel blood-based biomarker platforms that reliably detect cancer (Doherty et al., 2019; Haemmerle et al., 2018; Nguyen and Caldas, 2021). The measurement of soluble and/or exosomal purinergic activities in the blood of cancer patients might represent a compelling addition to this 'liquid biopsy' arsenal. To translate adenosine modulating approaches into clinical practice, it is important to improve our understanding of the complexity of the entire purinome in different types of cancers.

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Footnotes

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Table 1 – Major enzymes for metabolism of adenine nucleotides, adenosine, and related compounds.

Enzyme or enzyme family	Isozyme	Alternative names and aliases	Gene name	EC number	Catalytic reactions			Cellular distribution
Ecto-nucleoside triphosphate diphosphohydrolase (NTPDase)	NTPDase1 NTPDase2 NTPDase3 NTPDase8	CD39, ecto-apyrase, ecto-ATPase, ATP-diphosphohydrolase	*ENTPD1* *ENTPD2* *ENTPD3* *ENTPD8*	3.6.1.5	ATP ATPase:ADPase ATPase:ADPase ATPase:ADPase ATPase:ADPase	→ 	AMP + $2P_i$ ~1-1.5:1 10-40:1 3-4:1 2:1	PM, Soluble, MV
Ecto-nucleotide pyrophosphatase/ phosphodiesterase (ENPP)	ENPP1 ENPP3	PC-1, CD203a PDNP1, M6S1 PDE-1β, CD203c, B10, PDNP3, $gp130^{RB13-6}$	*ENPP1* *ENPP3*	3.6.1.9 3.1.4.1	ATP ADPR Ap_4A cGAMP	→ → → →	AMP + PP_i AMP + PP_i ATP + AMP AMP + GMP	PM, Soluble
5'-nucleotidase	Ecto-5'-nucleotidase	CD73, AMPase	*NT5E*	3.1.3.5	AMP IMP	→ →	ADO + P_i Inosine + P_i	PM, Soluble, MV
Alkaline phosphatase (ALP)	Tissue-nonspecific alkaline phosphatase (TNAP)	Basic phosphatase	*ALPL*	3.1.3.1	ATP PP_i $PolyP_n$ PLP	→ → → →	ADO + $3P_i$ $2P_i$ $PolyP_{n-1}$ + P_i Pyridoxal	PM, Soluble, MV
Prostatic acid phosphatase (PAP)		Acid phosphatase, TMPase	*ACP3*	3.1.3.2	AMP TMP	→ →	ADO + P_i Thiamine + P_i	PM, Soluble
CD38		NADase, cADPR hydrolase	*CD38*	3.2.2.5 3.2.2.6	NAD^+ cADPR	→ →	ADPR + NA ADPR	PM, Cytosolic
Adenylate kinase (AK)	AK1	Myokinase, ATP-AMP phosphotransferase	*AK1*	2.7.4.10	ATP + AMP	↔	2ADP	PM, Soluble, Cytosolic
Nucleoside diphosphate kinase (NDPK)	NDPK-A NDPK-B	Nm23-H1, NME1 Nm23-H2, NME2	*NME1* *NME2*	2.7.4.6	ATP + NDP	↔	ADP + NTP	PM, Soluble, Cytosolic
Adenosine deaminase (ADA)	ADA1 ADA2	Adenosine aminohydrolase CERC1	*ADA1* *ADA2*	3.1.3.1	ADO dADO	→ →	INO dINO	PM, Soluble, Cytosolic
Purine nucleoside phosphorylase (PNP)		Inosine phosphorylase	*PNP*	2.4.2.2	INO Guanosine	→	Hypoxanthine Guanine	PM, Soluble, Cytosolic
Adenosine kinase (ADK)	ADK-S ADK-L		*ADK*	2.7.1.20	ADO + ATP dADO + ATP	→ →	AMP + ADP dAMP + ADP	Cytosolic, Intranuclear
S-adenosyl-L-homocysteine hydrolase (SAHH)		S-Adenosyl-L-homocysteinase	*AHCY*	3.3.1.1	SAH	→	ADO + HCy	Cytosolic, Intranuclear

Abbreviations: ADO, adenosine; Ap4A, diadenosine tetraphosphate; ATPase:ADPase, ATP-to-ADP hydrolysis ratio; cADPR, cyclic ADP-ribose; CD, cluster of differentiation; CERC1, Cat eye syndrome critical region protein 1; cGAMP, 2'3' cyclic GMP-AMP; dADO, 2'-deoxy-adenosine; dAMP, 2'-deoxy-AMP; dINO, 2'-deoxy-inosine; EC number, Enzyme Commission number; INO, inosine; HCy, L-homocysteine; MV, microvesicles; NA, nicotinamide; NAD+, nicotinamide adenine dinucleotide; Nm23, non-metastasis protein 23; PC-1, plasma cell membrane glycoprotein-1; PDE-1β, phosphodiesterase 1β; PLP, Pyridoxal-5'-phosphate; PM, plasma membrane; PolyP, inorganic polyphosphate; SAH, S-adenosyl-L-homocysteine; SAHH, S-adenosyl-L-homocysteine hydrolase; TMPase, thiamine monophosphatase. For other abbreviations, see content of Table 1.

Table 2 – Ongoing clinical trials targeting key enzymes of adenosine metabolism CD39 and CD73 in patients with malignancies

Target	Lead Company	Drug	Combination therapy	Cancer type	*ClinicalTrials.gov identifier*	Study phase
CD39	Trishula Therapeutics	Human anti-CD39 Ab (TTX-030)	+/- immunotherapy (anti-PD-1 mAb pembrolizumab), and chemotherapy (docetaxel, gemcitabine, nab-paclitaxel)	Solid tumors, lymphoma	NCT03884556	I
CD39	Trishula Therapeutics/ AbbVie	Human anti-CD39 Ab (TTX-030)	+/- immunotherapy (anti-PD-1 mAbs budigalimab, pembrolizumab), and chemotherapy (mFOLFOX6, nab-paclitaxel, gemcitabine)	Advanced solid tumors	NCT04306900	I
CD39	Elpiscience Biopharma	Humanized anti-CD39 Ab (ES002023)	monotherapy	Locally advanced and metastatic solid tumors	NCT05075564	I
CD39	Surface Oncology	Human anti-CD39 Ab (SRF617)	+/- immunotherapy (anti-PD-1 mAb pembrolizumab), and chemotherapy (albumin-bound paclitaxel, gemcitabine)	Advanced solid tumors	NCT04336098	I
CD39	Surface Oncology	Human anti-CD39 Ab (SRF617)	+/- immunotherapy (anti-PD-1 mAb zimberelimab), and $A_{2A}R/A_{2B}R$ antagonist (etrumadenant)	Prostate cancer	NCT05177770	II
CD39	Purinomia Biotech Inc	Human anti-CD39 mAb (PUR001)	monotherapy	Advanced solid tumors	NCT05234853	I
CD73	MedImmune LLC	Human anti-CD73 mAb (MEDI9447/oleclumab)	+/- EGFR tyrosine kinase inhibitor (osimertinib), and A_2R antagonist (AZD4635)	NSCL carcinoma	NCT03381274	I/II
CD73	MedImmune LLC	Human anti-CD73 mAb (MEDI9447/oleclumab)	+/- immunotherapy (anti-PD-L1 mAb MEDI4736/durvalumab)	Solid tumors	NCT02503774	I
CD73	M.D. Anderson Cancer Center	Human anti-CD73 mAb (MEDI9447/oleclumab)	+/- immunotherapy (anti-PD-L1 mAb durvalumab), and chemotherapy (gemcitabine, nab-paclitaxel)	Pancreatic adenocarcinoma	NCT04940286	II
CD73	Jules Bordet Institute/ AstraZeneca	Human anti-CD73 mAb (MEDI9447/oleclumab)	+/- immunotherapy (anti-PD-L1 mAb durvalumab), and chemotherapy (paclitaxel, carboplatin)	TNBC	NCT03616886 (SYNERGY)	I/II
CD73	Jules Bordet Institute/ AstraZeneca	Human anti-CD73 mAb (MEDI9447/oleclumab)	+/- immunotherapy (anti-PD-L1 mAb durvalumab), and radiation therapy	Luminal B Breast Cancer	NCT03875573	II
CD73	M.D. Anderson Cancer Center/NCI	Human anti-CD73 mAb (MEDI9447/oleclumab)	+/- immunotherapy (anti-PD-L1 mAb durvalumab)	Recurrent, Refractory, or Metastatic Sarcoma	NCT04668300	II
CD73	Nordic Society for Gynaecologic Oncology	Human anti-CD73 mAb (MEDI9447/oleclumab)	+/- immunotherapy (durvalumab, tremelilumab), and anti-OX40/CD134 mAb (MEDI0562/ tavolimab)	Ovarian cancer	NCT03267589	II
CD73	Akeso	Humanized anti-CD73 mAb (AK119)	+/- immunotherapy (anti-PD-1/CTLA-4 bispecific Ab AK104)	Advanced and metastatic solid tumors	NCT04572152	I
CD73	Symphogen A/G	Human anti-CD73 mAb (Sym024)	+/- immunotherapy (anti-PD-1 Ab Sym021)	Advanced and metastatic solid tumors	NCT04672434	I
CD73	Incyte Corporation	Anti-CD73 mAb (INCA00186)	+/- immunotherapy (anti-PD-L1 mAb retifanlimab), and $A_{2A}R/A_{2B}R$ antagonist (INCB106385)	Advanced solid tumors	NCT04989387	I
CD73	I-Mab Biopharma Co Limited	Humanized anti-CD73 mAb (TJ004309/TJD5)	+/- immunotherapy (anti-PD-1 mAb)	Advanced solid tumors	NCT04322006	I/II

CD73	I-Mab Biopharma US Limited	Humanized anti-CD73 mAb (TJ004309/TJD5)	+/- immunotherapy (anti-PD-L1 mAb atezolizumab)	Ovarian cancer and selected solid tumors	NCT05001347	II
CD73	Novartis Pharmaceuticals	Human anti-CD73 mAb (NZV930/SRF373)	+/- immunotherapy (anti-PD-1 mAb PDR001), and A_2R antagonist (NIR178)	Advanced solid tumors	NCT03549000	I
CD73	Bristol-Myers Squibb	Anti-CD73 mAb (BMS-986179)	+/- immunotherapy (anti-PD-1 mAb nivolumab), and rHuPH20	Advanced solid tumors	NCT02754141	I/II
CD73	Corvus Pharmaceuticals	Humanized anti-CD73 mAb (CPI-006)	+/- immunotherapy (anti-PD-1 mAb pembrolizumab), and A_2R antagonist (CPI-444)	Advanced solid tumors, Non-Hodgkin lymphoma	NCT03454451	I
CD73	Institut Paoli-Calmettes	Anti-CD73 mAb (IPH5301)	+/- chemotherapy (paclitaxel), and anti-HER2 mAb trastuzumab	Advanced solid tumors	NCT05143970	I
CD73	Shanghai Henlius Biotech	Humanized anti-CD73 mAb (HLX23)	monotherapy	Advanced solid tumors	NCT04797468	I
CD73	Arcus Biosciences	Small-molecule CD73 inhibitor (AB680/ Quemliclustat)	+/- immunotherapy (anti-PD-1 mAb zimberelimab), A_2R antagonist (etrumadenant), and chemotherapy (mFOLFOX-6, bevacizumab)	Metastatic colorectal cancer	NCT04660812	I/II
CD73	Arcus Biosciences	Small-molecule CD73 inhibitor (AB680/ Quemliclustat)	+/- immunotherapy (anti-PD-1 mAb zimberelimab), and chemotherapy (nab-paclitaxel, gemcitabine)	Advanced pancreatic cancer	NCT04104672	I
CD73	Eli Lilly and Company/ Merck	Small-molecule CD73 inhibitor (LY3475070)	+/- immunotherapy (anti-PD-1 mAb pembrolizumab)	Advanced solid tumors	NCT04148937	I

Abbreviations: EGFR, epidermal growth factor receptor; FOLFOX is a chemotherapy regimen, made up of the drugs folinic acid (leucovorin, FOL), fluorouracil (5-FU, F), and oxaliplatin (Eloxatin, OX); NSCL cancer, non-small cell lung cancer; rHuPH20, novel drug delivery enzyme, fully human recombinant DNA-derived hyaluronidase; TNBC, triple negative breast cancer.

Figure Legends

Figure 1. Cellular ATP turnover. The extracellular turnover of purines is comprised of several steps: (i) the release of endogenous ATP after lytic cell injury, or via non-lytic processes of vesicular exocytosis (VE), P2X7 receptors, ATP-binding cassette (ABT), connexin (Cx) and pannexin (Panx) hemichannels; (ii) the initiation of signaling events via nucleotide (P2XR and P2YR) and adenosine (AR) receptors; (iii) the further metabolism of nucleotides and nucleosides; and (iv) the intracellular uptake of nucleotide-derived adenosine via equilibrative (ENT) and concentrative (CNT) nucleoside transporters. Purine-inactivating pathways include nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39), ecto-nucleotide pyrophosphatase/ phosphodiesterase-1 (ENPP1), ecto-5'-nucleotidase/CD73, and adenosine deaminase (ADA) (lower right). Acute changes in the ratio between nucleoside tri- and diphosphates together with feed-forward inhibition of CD73 through ATP and ADP, can influence the directional shift from adenosine-producing pathways towards the re-synthesis of ATP via adenylate kinase-1 (AK1) and nucleotide diphosphate kinase (NDPK) (lower left).

Figure 2. Compartmentalization of adenosine biochemistry. Along with the canonical route of sequential ATP and ADP breakdown through CD39, additional ectoenzymatic pathways can contribute to the generation of AMP and its subsequent CD73-mediated hydrolysis to adenosine. Those include the direct conversion of ATP into AMP and PP_i by ENPP1, AK1-mediated transphosphorylation of ADP into ATP and AMP, as well as alternative adenosine-producing pathways from extracellular NAD and cGAMP, mediated via the CD38-ENPP1-CD73 and ENPP1-CD73 axes, respectively. The extracellularly generated adenosine can further be converted into inosine and hypoxanthine via sequential ADA and PNP reactions or taken up by

the cells via nucleoside-selective transporters. The intracellular adenosine metabolism depends on the concerted action of cytoplasmic forms of purine-inactivating (ADA and PNP) and phosphorylating (ADK-S, AK, and NDPK) enzymes. Mitochondria produce ATP through oxidative phosphorylation (OxPhos). Therefore, there is a tight link between mitochondrial bioenergetics and adenosine homeostasis. In the cell nucleus adenosine is part of the transmethylation pathway, which adds methyl groups from S-adenosyl-L-methionine (SAM) to DNA (DNA-CH_3) via DNA methyltransferase (DNMT). S-adenosyl-L-homocysteine (SAH) can also be hydrolyzed to adenosine and L-homocysteine (HCy) by SAH hydrolase (SAHH). Importantly, SAHH catalyzes a bidirectional reaction with the thermodynamic equilibrium favoring the production of SAH from adenosine and HCy. Through this mechanism nuclear ADK-L drives the flux of methyl groups through the biochemical pathway leading to increased DNA and histone methylation. For other abbreviations, see Table 1.

Figure 3. Diversity of biological effects of adenosine and other purinergic agonists in the tumor microenvironment. Adenosine and its precursor metabolites (ATP, ADP, NAD, and cGAMP) trigger diverse pro-tumorigenic and anti-tumor effects in virtually all components of the TME, mediated via both extracellular and intracellular mechanisms. ATP generally functions as a 'danger sensor' and 'find me' signal, promoting tumor cell cytotoxicity and phagocytosis by pro-inflammatory NK cells, cytotoxic T cells, M1-type macrophages and additional tumor-associated effector immune cells. Furthermore, ATP has a variety of vasoactive, mitogenic, and pro-angiogenic effects in the TME, whereas its metabolite ADP acts as a potent pro-thrombotic molecule implicated in cancer-associated thrombosis. ATP-derived adenosine, in turn, attenuates the inflammation and promotes immune evasion by tumor cells, and in addition, maintains

intratumoral blood flow, angiogenesis, lymphangiogenesis, and vascular endothelial and epithelial barrier functions. Abbreviations: BEC, blood endothelial cells; CAF, cancer associated fibroblasts; CAP, cancer-associated platelets; LEC, lymphatic endothelial cells; MDSC, myeloid derived suppressor cells; MTC, metastatic tumor cells; NF, normal fibroblasts; NLRP3 inflammasome, NLR family pyrin domain containing 3 inflammasome; RBC, red blood cells; TAM, tumor-associated macrophages; TC, tumor cells; TIL, tumor-infiltrating lymphocytes.

Figure 4. Variations in the purinergic signatures between immune hot and immune cold breast tumors. To identify purinergic mechanisms of the tumor escape in the context of the T-cell-inflamed and non-T-cell-inflamed TME, the gene expression profiles for major purinergic enzymes and ARs were analyzed at single-cell resolution via publicly available scRNA-Seq datasets of immune hot (A) and immune cold triple negative breast carcinoma (TNBC) samples obtained from two patients (Wu et al., 2020). Raw data were analyzed by Seurat (ver 4.0) for graph-based clustering and the analysis of gene expression. Sctransform was used for data normalization and variance stabilization of molecular count data (Hafemeister C, Genome Biology). Principle component analysis (PCA) and a graph-based clustering approach were used by running the functions FindNeighbors and FindClusters. The clustering was visualized with Uniform Manifold Approximation and Projection (UMAP). Cells were typed based on the expression of known marker genes, as indicated. Dot plots show the expression of key purinergic enzymes and ARs. The average expression levels of the indicated genes are shown on a pseudocolor scale, with the size of the dot representing the percentage of cells in a subset where the gene is detected relative to other subsets.

Figure 5. Cell- and tissue-specific distribution of purinergic enzymes in the breast tumor microenvironment. Tumor-draining lymph nodes (LN) were obtained from a treatment-naïve breast cancer patient undergoing mastectomy surgery with axillary lymph node dissection. The tumor in this example is a hormone-receptor-negative/Her2-positive infiltrative ductal carcinoma (GIII, Ki-67 25%, LN status 4/13). (A) CD73 activity was assayed by incubating tissue cryosections (6 μm thickness) with AMP in the presence of $Pb(NO_3)_2$, followed by detection of AMP-derived P_i as a brown precipitate. LNs were also stained with hematoxylin and eosin (H&E), as well as with antibodies against CD73, together with the stromal marker α-smooth muscle actin (SMA-α) and a pan-cytokeratin AE1+AE3 antibody cocktail, which differentiates epithelial tumors from non-epithelial tumors. The bright-field (A) and fluorescence (B) images were captured using Pannoramic Midi and Pannoramic-1000 slide scanners, respectively (3DHistech Ltd., Budapest, Hungary). (C, D) Spatial distribution of purinergic enzymes was also analyzed by high-resolution 3D microscopy. LN tissues were embedded in low melting point agarose, sectioned at 150 μm thickness using a vibrating microtome, and incubated in free-floating staining assays with antibodies against CD73, CD39, and adenosine kinase (ADK), together with anti-SMA-α and anti-cytokeratin antibodies, as indicated. Z-stacks were captured using a spinning disk confocal microscope, and presented as reconstructed 3D images. The right-hand panels display merged images with nuclei counterstained with DAPI. Abbreviations: BV, blood vessels; F, fibroblasts; GC, germinal center; LV, lymphatic vessels; TC, tumor cells. Scale bars, 2 mm (A), 600 μm (A, insets; B), and 60 μm (C,D). For experimental details, refer (Losenkova et al., 2022; Losenkova et al., 2020).

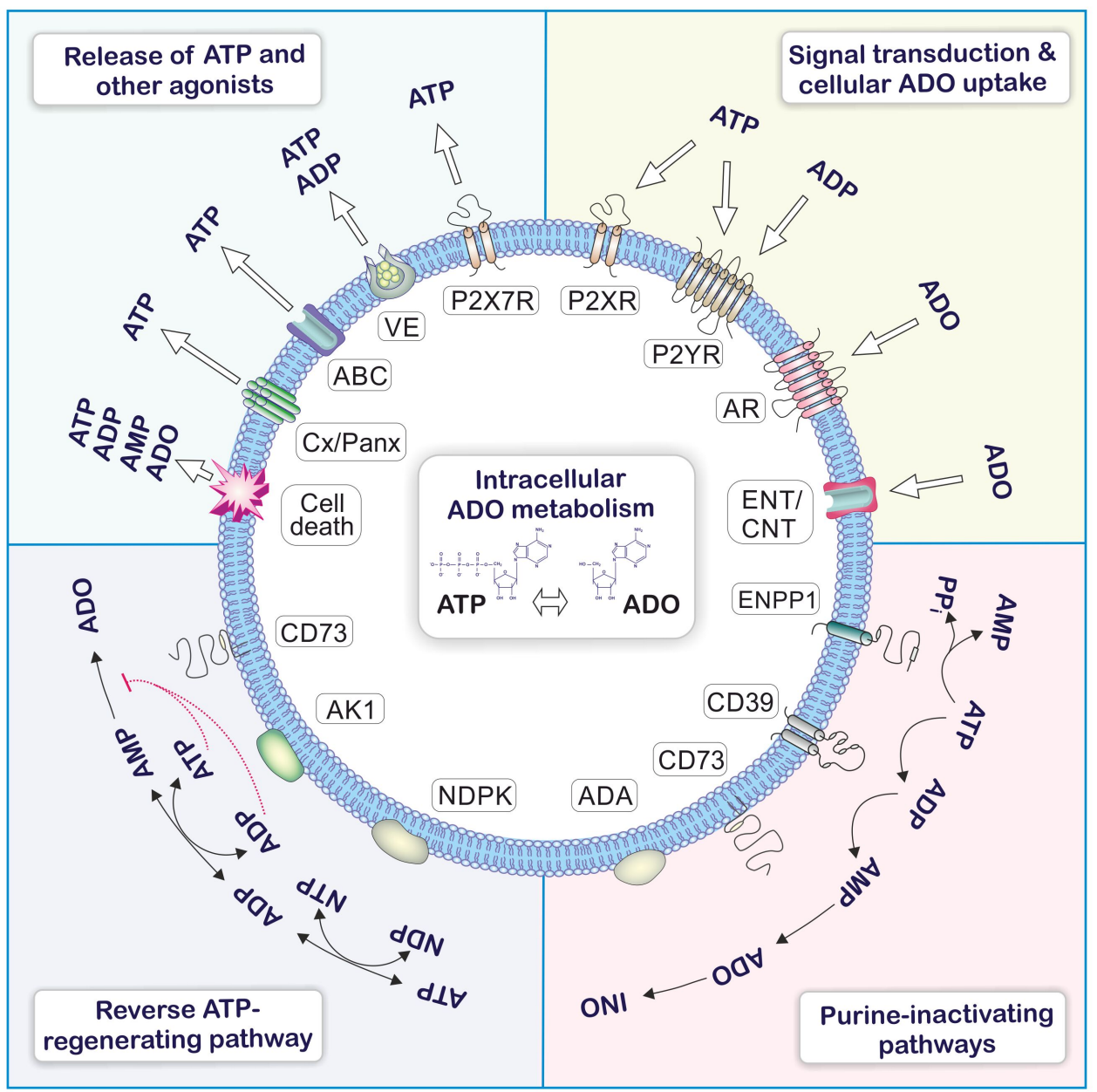

Release of ATP and other agonists
Signal transduction & cellular ADO uptake
Reverse ATP-regenerating pathway
Purine-inactivating pathways
Intracellular ADO metabolism
ATP
ADO
ATP
ADP
ATP
ATP
ADP
ATP
ADP
AMP
ADO
P2X7R
P2XR
P2YR
VE
ABC
Cx/Panx
Cell death
AR
ENT/ CNT
ENPP1
CD39
CD73
ADA
NDPK
AK1
CD73
ADO
AMP
ATP
ADP
ADP
NTP
NDP
ATP
AMP
ADO
INO
PPi
AMP
ATP
ADP

Fig.1

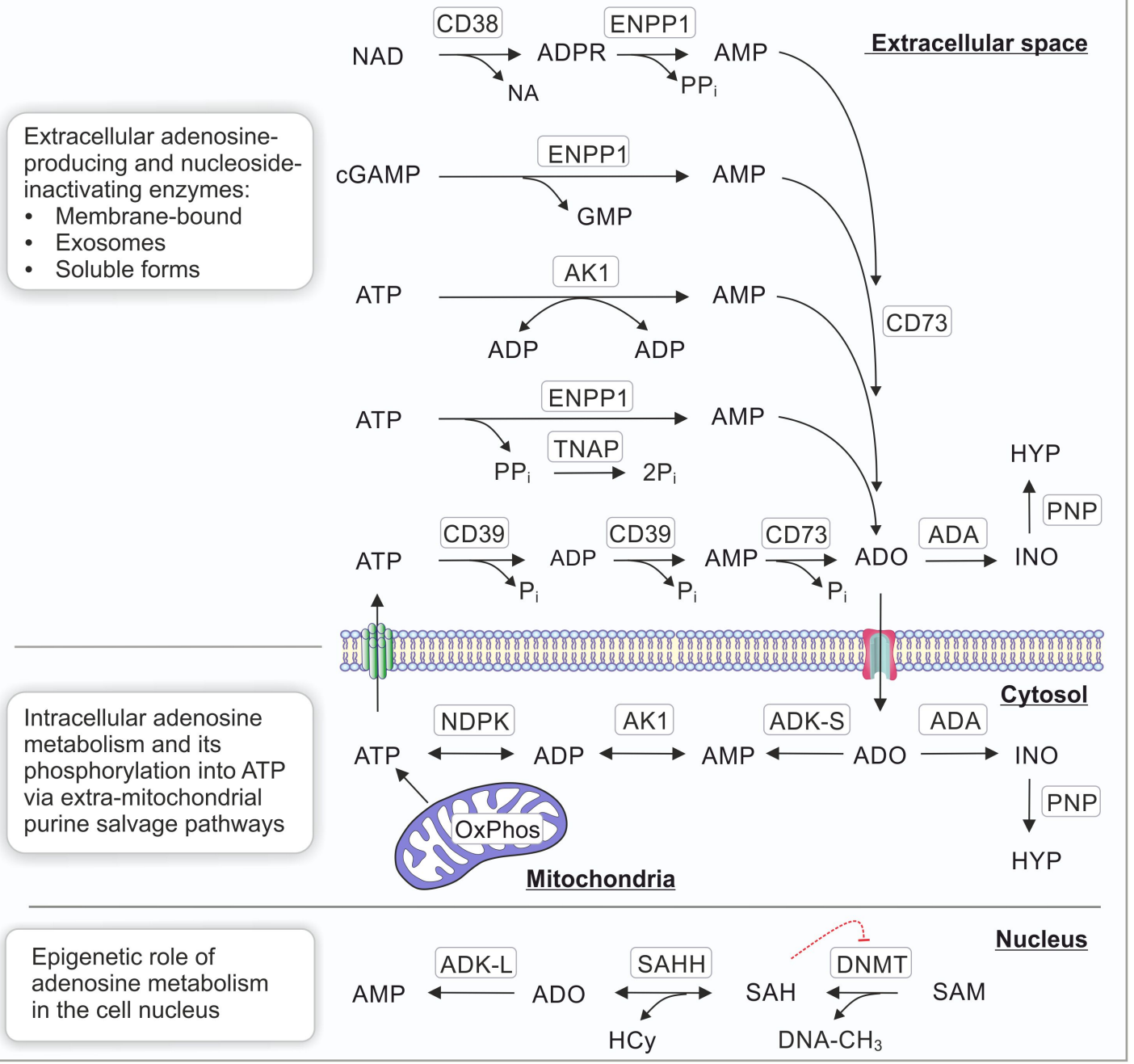

Extracellular adenosine-producing and nucleoside-inactivating enzymes:
• Membrane-bound
• Exosomes
• Soluble forms
Extracellular space
NAD
CD38
NA
ADPR
ENPP1
PPi
AMP
cGAMP
ENPP1
GMP
AMP
ATP
AK1
ADP
ADP
AMP
CD73
ATP
ENPP1
PPi
TNAP
2Pi
AMP
HYP
PNP
ATP
CD39
Pi
ADP
CD39
Pi
AMP
CD73
Pi
ADO
ADA
INO
Cytosol
Intracellular adenosine metabolism and its phosphorylation into ATP via extra-mitochondrial purine salvage pathways
ATP
NDPK
ADP
AK1
AMP
ADK-S
ADO
ADA
INO
PNP
HYP
OxPhos
Mitochondria
Epigenetic role of adenosine metabolism in the cell nucleus
Nucleus
AMP
ADK-L
ADO
SAHH
HCy
SAH
DNMT
SAM
DNA-CH3

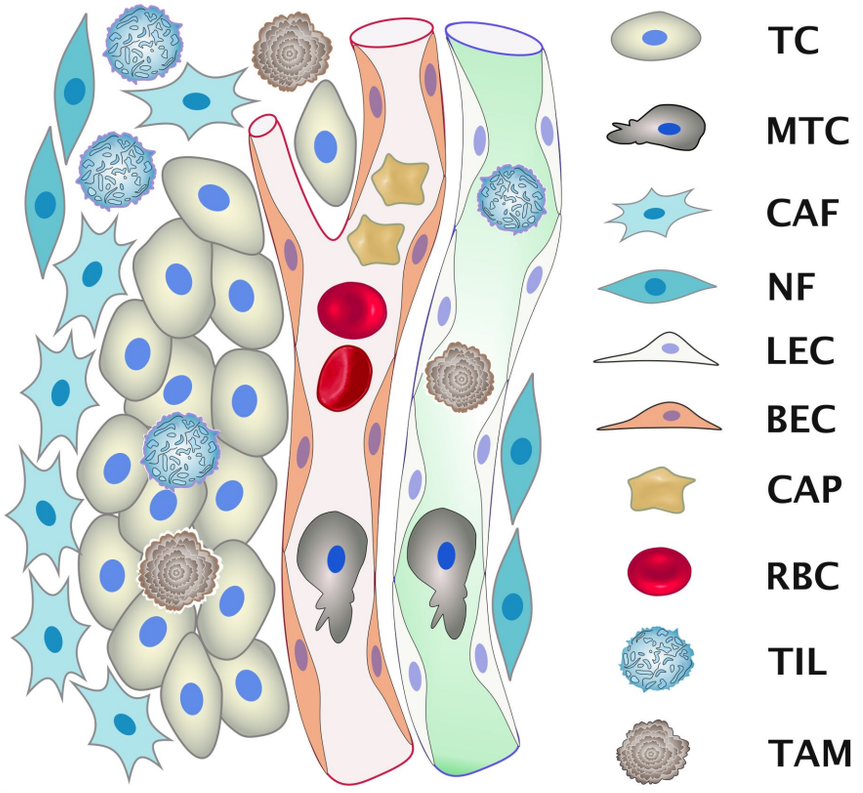
Fig.2

Tumor cells

- Inhibition or activation of tumor cell invasion, metastasis and proliferation (ATP, adenosine, cGAMP)
- Promotion of tumor cell bioenergetics, motility and cytoprotection (ATP, NAD)
- Epigenetic effects via inhibition of DNA and histone methylation (adenosine)

Tumor stroma

- Modulation of the epithelial to mesenchymal transition (adenosine)
- Maintenance of epithelial integrity and paracellular permeability (adenosine)
- Secretion of TGF-β and other immuno-suppressive cytokines, chemokines and exosomes (ATP, adenosine)



Effector immune cells

- "Danger sensor" and activation of NLRP3 inflammasome (ATP, NAD)
- Activation of NK cell- and T cell-mediated tumor cell cytotoxicity via production of inflammatory cytokines (TNF-α, IFN-γ) (ATP)
- "Find me" signal, promotion of tumor cell phagocytosis by M1 macrophages (ATP)

Suppressive immune cells

- Induction of pro-tumorigenic effects and promotion of immune evasion by tumor cells via expansion of immunosuppressive cells (MDSCs, T_{reg} cells, exhausted T cells, M2 macrophages), and concurrent inhibition of immunostimulatory effector immune cells (adenosine)

Tumor vasculature

- Promotion of tumor angiogenesis and vasculogenesis (ATP, adenosine)
- Maintenance of vascular endothelial barrier function (adenosine)
- Sensing and controlling of blood flow and tumor perfusion (ATP, adenosine)
- Cancer-associated thrombosis (ADP)

Tumor lymphatics

- Regulation of immune cell trafficking between peripheral tissues and lymph nodes (ATP, adenosine)
- Promotion of tumor lymphangiogenesis and lymph node metastasis (adenosine)
- Control of lymphatic endothelial cell permeability and sprouting (adenosine?)

Fig. 3

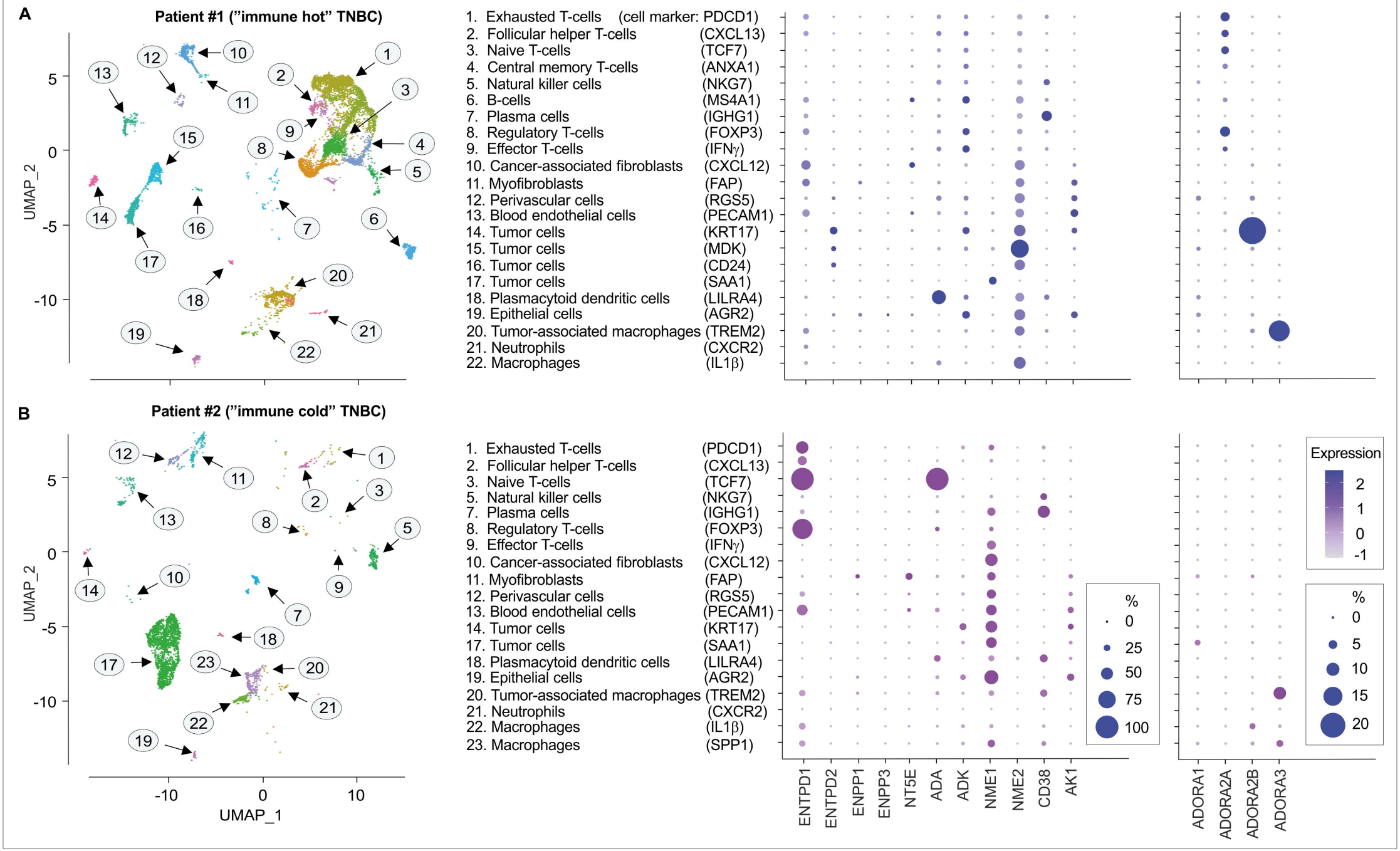
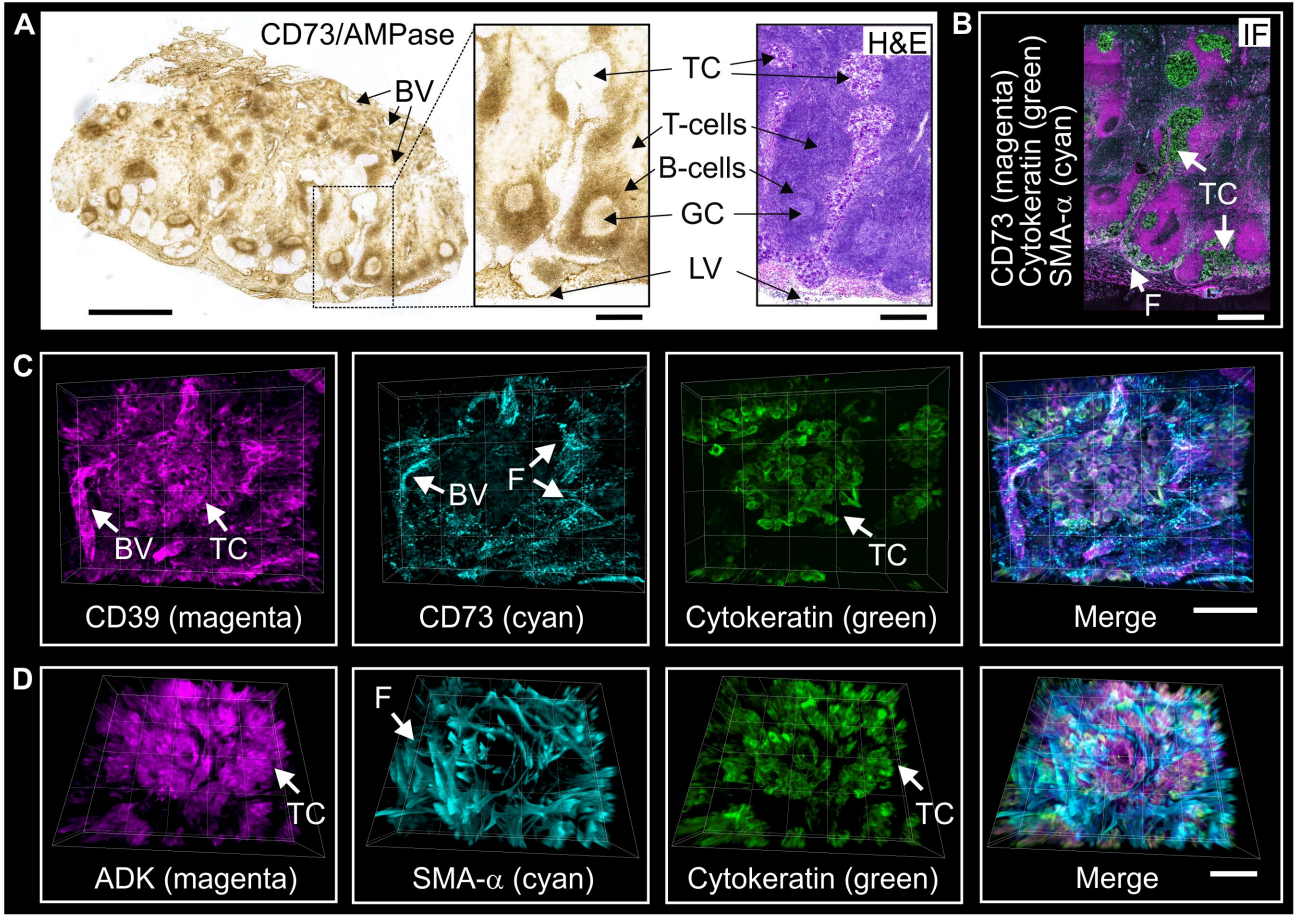

A
Patient #1 ("immune hot" TNBC)
UMAP_2
1. Exhausted T-cells (cell marker: PDCD1)
2. Follicular helper T-cells (CXCL13)
3. Naive T-cells (TCF7)
4. Central memory T-cells (ANXA1)
5. Natural killer cells (NKG7)
6. B-cells (MS4A1)
7. Plasma cells (IGHG1)
8. Regulatory T-cells (FOXP3)
9. Effector T-cells (IFNγ)
10. Cancer-associated fibroblasts (CXCL12)
11. Myofibroblasts (FAP)
12. Perivascular cells (RGS5)
13. Blood endothelial cells (PECAM1)
14. Tumor cells (KRT17)
15. Tumor cells (MDK)
16. Tumor cells (CD24)
17. Tumor cells (SAA1)
18. Plasmacytoid dendritic cells (LILRA4)
19. Epithelial cells (AGR2)
20. Tumor-associated macrophages (TREM2)
21. Neutrophils (CXCR2)
22. Macrophages (IL1β)
B
Patient #2 ("immune cold" TNBC)
UMAP_2
UMAP_1
1. Exhausted T-cells (PDCD1)
2. Follicular helper T-cells (CXCL13)
3. Naive T-cells (TCF7)
5. Natural killer cells (NKG7)
7. Plasma cells (IGHG1)
8. Regulatory T-cells (FOXP3)
9. Effector T-cells (IFNγ)
10. Cancer-associated fibroblasts (CXCL12)
11. Myofibroblasts (FAP)
12. Perivascular cells (RGS5)
13. Blood endothelial cells (PECAM1)
14. Tumor cells (KRT17)
17. Tumor cells (SAA1)
18. Plasmacytoid dendritic cells (LILRA4)
19. Epithelial cells (AGR2)
20. Tumor-associated macrophages (TREM2)
21. Neutrophils (CXCR2)
22. Macrophages (IL1β)
23. Macrophages (SPP1)
%
0
25
50
75
100
Expression
2
1
0
-1
%
0
5
10
15
20
ENTPD1
ENTPD2
ENPP1
ENPP3
NT5E
ADA
ADK
NME1
NME2
CD38
AK1
ADORA1
ADORA2A
ADORA2B
ADORA3

Fig. 4


A
CD73/AMPase
BV
H&E
TC
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B-cells
GC
LV
B
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Cytokeratin (green)
SMA-α (cyan)
TC
F
C
BV
TC
CD39 (magenta)
BV
F
CD73 (cyan)
TC
Cytokeratin (green)
Merge
D
TC
ADK (magenta)
F
SMA-α (cyan)
TC
Cytokeratin (green)
Merge

Fig. 5