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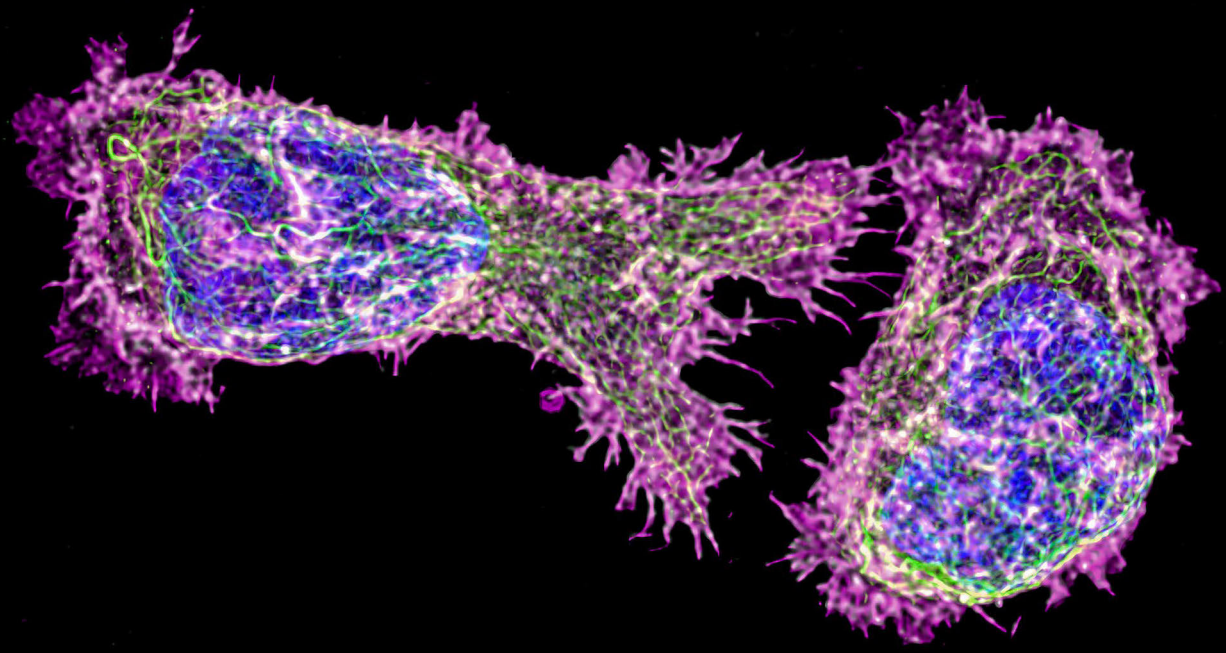

ADENOSINE METABOLISM IN CANCER: UNVEILING NEW THERAPEUTIC OPPORTUNITIES

Karolina Losenkova Mingeaud

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ABSTRACT

Tumor immune evasion, a hallmark of cancer, allows malignant cells to escape immune surveillance and elimination. While immune checkpoint inhibitors, such as anti-PD-1, anti-CTLA-4, and anti-PD-L1 antibodies, have revolutionized cancer therapy by enhancing T-cell responses, their efficacy is often limited by additional immunosuppressive mechanisms within the tumor microenvironment (TME). Elevated adenosine (Ado) levels, generated by the ectoenzymes CD39 and CD73, significantly contribute to immunosuppression by activating adenosine receptors (ARs) on immune cells. Although targeting the CD39-CD73-AR axis shows therapeutic promise, limited clinical success underscores the need for a deeper understanding of Ado metabolism complexity within the heterogeneous TME.

This thesis investigates cell-type-specific Ado metabolic pathways, focusing on human vascular endothelial cells, cancer cells, and T cells. I identify a complex network of purine-converting ectoenzymes, including CD39 and CD73, as well as ENPP1, NDPK, AK, and ADA, with distinct cell-specific distributions. While endothelial cells rely on the "classical" ATP-inactivating/Ado-producing pathway involving CD39 and CD73, breast and prostate cancer cells utilize an alternative pathway, incorporating ENPP1 and CD73 while lacking CD39. In T cells, CD73 is primarily expressed on naïve T cells, whereas CD39 is prevalent on activated T cells. Hypoxia further modulates Ado metabolic pathways in a cell-specific manner, increasing CD39 activity in endothelial cells and upregulating CD73 in cancer cells.

Beyond the "canonical" Ado signaling pathways, I uncover the underexplored role of intracellular Ado metabolism in cancer progression and immunomodulation. My research shows that extracellular Ado, taken up by cancer cells and T cells via the equilibrative nucleoside transporter ENT1, reduces cancer cell tumorigenic potential *in vivo* and exerts potent immunosuppressive effects on T cells by inhibiting their proliferation, cytokine production, and conventional ATP-generating pathways.

Taken together, this study emphasizes the importance of accounting for the complexity of the TME when developing effective therapies targeting Ado pathways. My findings also highlight intracellular Ado metabolism as a promising therapeutic target that merits further investigation.

KEYWORDS: adenosine metabolism, cancer, CD39, CD73, ENPP1, ENT1, ADK, tumor microenvironment, T cells

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TIIVISTELMÄ

Immuunipuolustuksen välttäminen on yksi syövän tunnusmerkeistä. Sen avulla pahanlaatuiset solut voivat paeta immuunivalvontaa ja edelleen eliminointia. Vaikka immuunijärjestelmän tarkistuspisteiden estäjät, kuten anti-PD-1-, anti-CTLA-4- ja anti-PD-L1-vasta-aineet, ovat mullistaneet syövän hoidon tehostamalla T-soluvasteita, kasvaimen mikroympäristössä esiintyvät immunosuppressiiviset mekanismit rajoittavat niiden tehoa. Ektoentsyymien CD39 ja CD73 tuottamat kohonneet adenosiinipitoisuudet vaikuttavat merkittävästi immunosuppressioon aktivoimalla immuunisolujen adenosiinireseptoreita. Vaikka hoidon kohdentaminen CD39-CD73-adenosiinireseptori-akseliin on ollut lupaavaa, rajallinen kliininen teho korostaa tarvettamme ymmärtää syvällisemmin adenosiiniaineenvaihdunnan monimutkaisuutta heterogeenisissä kasvaimissa.

Tutkin väitöskirjassani aineenvaihduntareittejä keskittyen ihmisen verisuonten endoteelisoluihin, syöpäsoluihin sekä T-soluihin. Tunnistin monimutkaisen verkoston puriinia konvertoivia solutyyppispesifisiä ektoentsyymejä, kuten CD39 ja CD73, sekä ENPP1, NDPK, AK ja ADA. Endoteelisolut käyttävät ”klassista”, ATP:tä inaktivoivaa ja adenosiinia tuottavaa reittiä, johon CD39 ja CD73 osallistuvat. Rinta- ja eturauhassyöpäsolut käyttävät vaihtoehtoista reittiä, johon kuuluvat ENPP1 ja CD73, mutta josta CD39 puuttuu. T-soluissa CD73 ilmentyy pääasiassa naiiveissa soluissa, kun taas CD39 esiintyy pääasiassa aktivoituneissa T-soluissa. Hypoksia moduloi adenosiinireittejä lisäämällä CD39:n aktiivisuutta endoteelisoluissa ja lisäämällä CD73:n määrää syöpäsoluissa.

Osoitan tässä kirjassa solunsisäisen adenosiinimetabolian roolin syövän etenemisessä ja immunomodulaatiossa. Tulokset osoittavat, että solun ulkoinen adenosiini, jota syöpäsolut ja T-solut ottavat vastaan nukleosidikuljettaja ENT1:n kautta, vähentää syöpäsolujen tuumorigeenisyyttä *in vivo,* ja vaikuttaa voimakkaan immunosuppressiivisesti T-soluihin estämällä niiden lisääntymistä, sytokiinituotantoa sekä klassisia ATP-synteesireittejä.

Tämä tutkimus korostaa, että on tärkeää ottaa huomioon kasvaimen mikroympäristön monimutkaisuus, kun kehitämme hoitoja, jotka kohdistuvat adenosiinireitteihin. Löytöni korostavat myös solunsisäistä adenosiinimetaboliaa lupaavana terapeuttisena kohteena, joka ansaitsee lisätutkimuksia.

AVAINSANAT: adenosiinimetabolia, syöpä, CD39, CD73, ENPP1, ENT1, ADK, kasvaimen mikroympäristö, T-solut

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АБСТРАКТ

Уклонение опухоли от иммунного ответа – характерная особенность рака, позволяющая злокачественным клеткам избегать уничтожения. Хотя ингибиторы контрольных точек, такие как антитела против PD-1, CTLA-4 и PD-L1, произвели революцию в терапии рака, усиливая Т-клеточный ответ, их эффективность часто ограничена дополнительными иммуноподавляющими механизмами опухолевого микроокружения (ОМО). Один из них – повышенный уровень аденозина (Адо), вырабатываемый эктоферментами CD39 и CD73 и вызывающий иммуносупрессию активацией аденозиновых рецепторов (AR) на иммунных клетках. Несмотря на то, что воздействие на ось CD39-CD73-AR многообещающе, ограниченный успех клинических испытаний подчеркивает необходимость глубже изучить метаболизм Адо в гетерогенном ОМО.

В данной диссертации исследованы метаболические пути Адо различных человеческих клеток: сосудистых эндотелиальных, раковых и Т-клеток. Выявлена сложная сеть пурин-превращающих эктоферментов, включая CD39, CD73, ENPP1, NDPK, AK, и ADA, с различным распределением в разных клетках. Эндотелиальные клетки используют «классический» путь генерации Адо с участием CD39 и CD73, а клетки рака молочной и предстательной желез – альтернативный путь с ENPP1 и CD73, без участия CD39. В Т-клетках CD73 находится преимущественно на наивных клетках, а CD39 – на активированных. Более того, гипоксия модулирует метаболические пути Адо, повышая CD39 в эндотелиальных клетках и CD73 в раковых клетках.

Помимо «канонических» сигнальных путей Адо, также изучалась роль внутриклеточного метаболизма Адо. Показано, что внеклеточный Адо, поглощаемый раковыми и Т-клетками через равновесный нуклеозидный транспортер ENT1, снижает онкогенный потенциал раковых клеток *in vivo* и оказывает мощное иммуносупрессивное действие на Т-клетки, подавляя их пролиферацию, выработку цитокинов и классические пути синтеза АТФ.

В данной диссертации подчеркивается важность учета сложности ОМО при разработке эффективных терапий, направленных на Адо-пути, а также значимость внутриклеточного метаболизма Адо как перспективной мишени.

КЛЮЧЕВЫЕ СЛОВА: метаболизм аденозина, рак, CD39, CD73, ENPP1, ENT1, ADK, микроокружение опухоли, Т-клетки

Table of Contents

Abbreviations

2DG	2-deoxy-D-glucose
AC	Adenylate cyclase
ADA	Adenosine deaminase
ADK	Adenosine kinase
Ado	Adenosine
ADP	Adenosine diphosphate
AK	Adenylate kinase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AP	Alkaline phosphatase
AR(s)	Adenosine receptor(s)
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
CAF(s)	Cancer-associated fibroblast(s)
CALHM1	Calcium homeostasis modulator 1
cAMP	Cyclic adenosine monophosphate
CD39	Nucleotide triphosphate diphosphohydrolase-1
CD73	Ecto-5′-nucleotidase
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
CNS	Central nervous system
CNT(s)	Concentrative nucleoside transporter(s)
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
Cx43	Connexin 43
DNMT(s)	DNA methyltransferase(s)
EC(s)	Endothelial cell(s)
ENPP	Ecto-nucleotide pyrophosphatase/phosphodiesterase
ENT(s)	Equilibrative nucleoside transporter(s)
FACS	Fluorescence-activated cell sorting
GACI	Generalized arterial calcification of infancy
GPCR(s)	G-protein-coupled receptor(s)
GPI	Glycosylphosphatidylinositol

GranzB	Granzyme B
HIF	Hypoxia-inducible factor
HUVEC(s)	Human umbilical vein endothelial cell(s)
Hyp	Hypoxanthine
IAP	Intestinal alkaline phosphatase
IBD	Inflammatory bowel disease
IF	Immunofluorescence
IFN	Interferon
Ino	Inosine
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MAC	Maxi-anion channel
NAD	Nicotinamide adenine dinucleotide
NBTI	S-(4-Nitrobenzyl)-6-thioinosine
NDPK	Nucleoside diphosphate kinase
NECA	5′-(N-Ethylcarboxamido)adenosine
NK	Natural killer
NTPDase	Nucleotide triphosphate diphosphohydrolase
OXPHOS	Oxidative phosphorylation
P2XR(s)	P2X receptor(s)
P2YR(s)	P2Y receptor(s)
Panx1	Pannexin 1
PD-1	Programmed death receptor 1
PD-L1	Programmed death receptor ligand 1
P_i	Inorganic phosphate
PNP	Purine nucleoside phosphorylase
PP_i	Inorganic pyrophosphate
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SCID	Severe combined immunodeficiency
TAM(s)	Tumor-associated macrophage(s)
TCR	T-cell receptor
TKI(s)	Tyrosine kinase inhibitor(s)
TLC	Thin-layer chromatography
TM	Transmembrane domain
TME	Tumor microenvironment
TNAP	Tissue non-specific alkaline phosphatase
TNF	Tissue necrosis factor

Treg(s)	Regulatory T cell(s)
UDP	Uridine diphosphate
UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor
VNUT	Vesicular nucleotide transporter
VRAC	Volume-regulated anion channel
WB	Western blotting
WT	Wild-type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Losenkova K.**[*], Zuccarini M.[*], Helenius M.[*], Jacquemet G., Gerasimovskaya E., Tallgren C., Jalkanen S., Yegutkin G.G. Endothelial cells cope with hypoxia-induced depletion of ATP via activation of cellular purine turnover and phosphotransfer networks. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 2018; 1864: 1804–1815.

II **Losenkova K.**, Zuccarini M., Karikoski M., Laurila J., Boison D., Jalkanen S., Yegutkin G.G. Compartmentalization of adenosine metabolism in cancer cells and its modulation during acute hypoxia. *Journal of Cell Science*, 2020; 133(10): jcs241463.

III **Losenkova K.**, Kreisig N., Svärd S., Weiler C., Jalkanen S., Takeda A., Yegutkin G.G. Adenosine suppresses T cell function through cellular uptake via equilibrative nucleoside transporter ENT1 and intracellular metabolism. *Manuscript.*

[*], equal contribution

1 Introduction

Adenosine triphosphate (ATP) and its derivative adenosine (Ado) are fundamental molecules present throughout cells. ATP serves as the primary energy source for most cellular processes, while Ado serves as a building block for DNA and RNA synthesis. Beyond their intracellular roles, both molecules are involved in extracellular signaling, known as purinergic signaling. This type of cell-to-cell communication regulates various physiological processes through specific P2 and P1 receptors, which respond to ATP and Ado, respectively.

One critical process regulated by purinergic signaling is the immune response. Generally, ATP initiates inflammatory responses, while Ado promotes immune suppression. Therefore, maintaining the proper balance between pro-inflammatory ATP and anti-inflammatory Ado is essential for immune homeostasis. However, this balance can be disrupted under certain pathophysiological conditions, such as chronic inflammation, autoimmune diseases, and cancer. In the context of cancer, this disruption is particularly significant as it directly contributes to disease progression.

Elevated Ado levels and the subsequent activation of P1 receptors in the tumor microenvironment (TME) are critical factors implicated in immune suppression, which favors tumor growth. CD39 and CD73, extracellular enzymes mediating the sequential conversion of ATP to Ado, are currently considered as key contributors to Ado production. Several clinical trials are investigating small-molecule inhibitors and antibodies targeting these enzymes in cancer. However, recent research has identified additional extracellular pathways involved in ATP and Ado metabolism. These redundant pathways, which include various ectoenzymes that degrade ATP and Ado, or regenerate ATP, work alongside CD39 and CD73 to modulate Ado levels within the TME. Additionally, certain features of the TME, such as hypoxia, nutrient deprivation, and other metabolic stresses, may influence the expression levels and activities of these ectoenzymes, adding further complexity to regulation of Ado levels within the TME.

In addition to its well-established signaling role through P1 receptors, recent findings indicate that Ado also exerts significant effects independent of receptor activation. Cells take up extracellular Ado and metabolize it intracellularly, leading

to alterations in cellular function. This intracellular Ado metabolism is potentially as important as receptor-mediated signaling, yet its role in cancer progression and tumor immunity remains underexplored.

Given these gaps, a deeper understanding of both extracellular and intracellular Ado metabolic pathways within the TME is crucial for developing novel anti-cancer strategies and improving the effectiveness of existing therapies. In this thesis, I investigated Ado metabolic pathways within various cell types, including human vascular endothelial cells, cancer cells, and T cells, with a special emphasis on the interplay between the extracellular and intracellular Ado metabolism. I also explored the effects of hypoxia, a key feature of the TME, on these metabolic pathways in endothelial and cancer cells.

The results of this PhD thesis reflect the complexity of Ado metabolic pathways, with various cell types exhibiting unique repertoires of purine-converting ectoenzymes and differing in their ability to adapt to hypoxia and other environmental challenges. This underappreciated complexity of Ado metabolism should be taken into account to enhance the therapeutic potential of targeting Ado pathways in cancer treatment. Additionally, this work provides novel insights into the role of intracellular Ado metabolism in cancer progression and immunomodulation, revealing its involvement in reducing the tumorigenic potential of cancer cells *in vivo* and suppressing T-cell proliferation, cytokine production, and their conventional ATP-generating pathways.

2 Review of the Literature

2.1 Purinergic signaling system

The intracellular role of adenosine triphosphate (ATP) as a key regulator of cellular homeostasis has been known for several decades (Khakh & Burnstock, 2009). ATP serves as the primary energy-carrying molecule in cells, capable of storing and releasing energy when needed. ATP is a purine nucleotide composed of three subunits: the purine nucleobase adenine, ribose (which together form the nucleoside adenosine), and three phosphate groups (α, β, and γ phosphates) (**Figure 1**). The phosphate groups are linked together by two phosphoanhydride bonds, often referred to as "high-energy" bonds. The energy within ATP is particularly stored in these bonds and is released during their hydrolysis. For instance, the hydrolysis of the terminal phosphoanhydride bond of ATP, which produce adenosine diphosphate (ADP) and inorganic phosphate (P_i), releases 11-13 kcal/mol of free energy, depending on intracellular conditions (Alberts et al., 2008). The released energy is utilized by cells for various intracellular processes, such as biosynthesis, active transport, cellular movements, and body temperature maintenance.

The importance of ATP extends beyond its intracellular functions. In the early 1970s, Geoff Burnstock identified ATP as a neurotransmitter in the nervous system and introduced the term "purinergic signaling" (Burnstock, 1971, 1972, 2014). At first, the concept of ATP as an extracellular signaling molecule was not widely accepted until the first purinergic receptors were discovered (Burnstock, 1990; Ralevic & Burnstock, 1998; Zimmermann, 2021). Presently, the signaling roles of extracellular ATP, along with its derivatives ADP and adenosine (Ado), mediated through their respective receptors, are well-established in a wide range of processes, including neurotransmission, immune regulation, blood flow distribution, angiogenesis, cell proliferation, and differentiation (Abbracchio et al., 2009; Burnstock, 2017; Junger, 2011; Yegutkin, 2021). In addition, various components that regulate purinergic signaling have been identified, collectively forming what is now recognized as the purinergic signaling system.

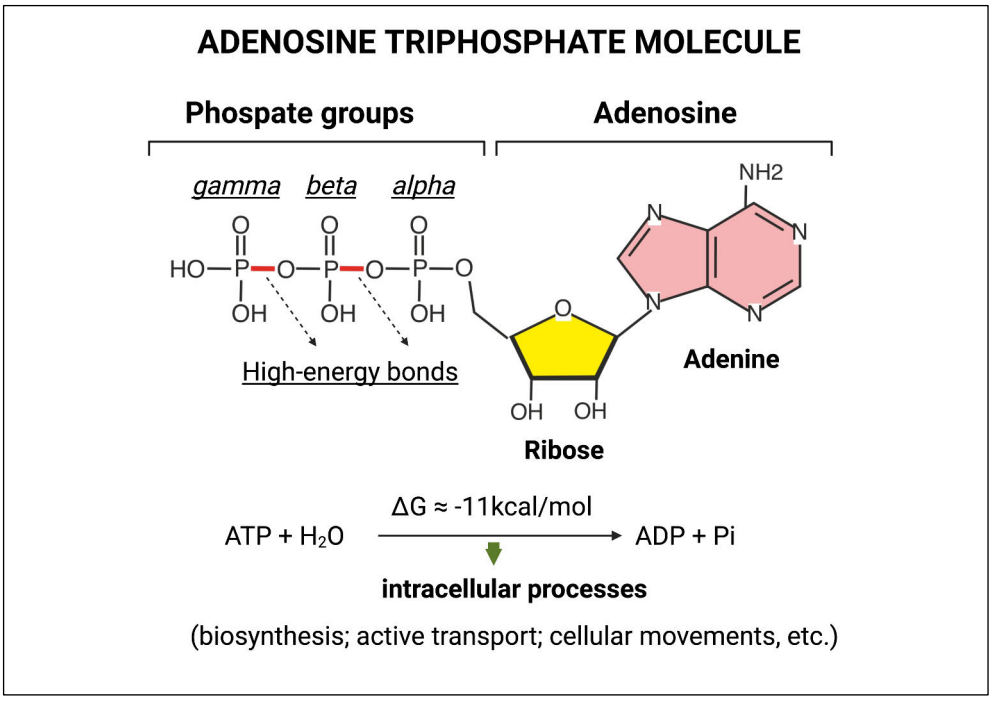


Figure 1. ATP structure and its key intracellular functions. ATP molecule consists of three subunits: the purine nucleobase adenine, ribose, and three phosphate groups (α, β, and γ phosphates). This structural composition classifies ATP as a purine nucleotide. Adenine and ribose together form adenosine (Ado), which is designated as a purine nucleoside. The hydrolysis of the high-energy bond between the γ and β phosphates releases free energy, which cells utilize for various crucial intracellular processes. Created with Biorender.com.

According to the current concept, the purinergic signaling system is a complex multistep cascade that can be divided into the following steps: (1) the release of endogenous ATP into the extracellular milieu by cells; (2) subsequent signaling events via purinergic receptors; (3) the inactivation of extracellular ATP by purine-converting ectoenzymes; and (4) the cellular uptake of nucleotide-derived Ado and its intracellular conversion to ATP through complex phosphotransfer reactions (**Figure 2**) (Yegutkin, 2021). The proper functioning of the entire system is crucial for physiological homeostasis, and any disruption within it can lead to various pathologies (Antonioli et al., 2019).

It is important to note that dividing the purinergic signaling system into steps is arbitrary and not entirely accurate, as all the components are interrelated. Nevertheless, for the sake of clarity, I will adhere to this concept and discuss each step of purinergic signaling system in the following chapters separately.

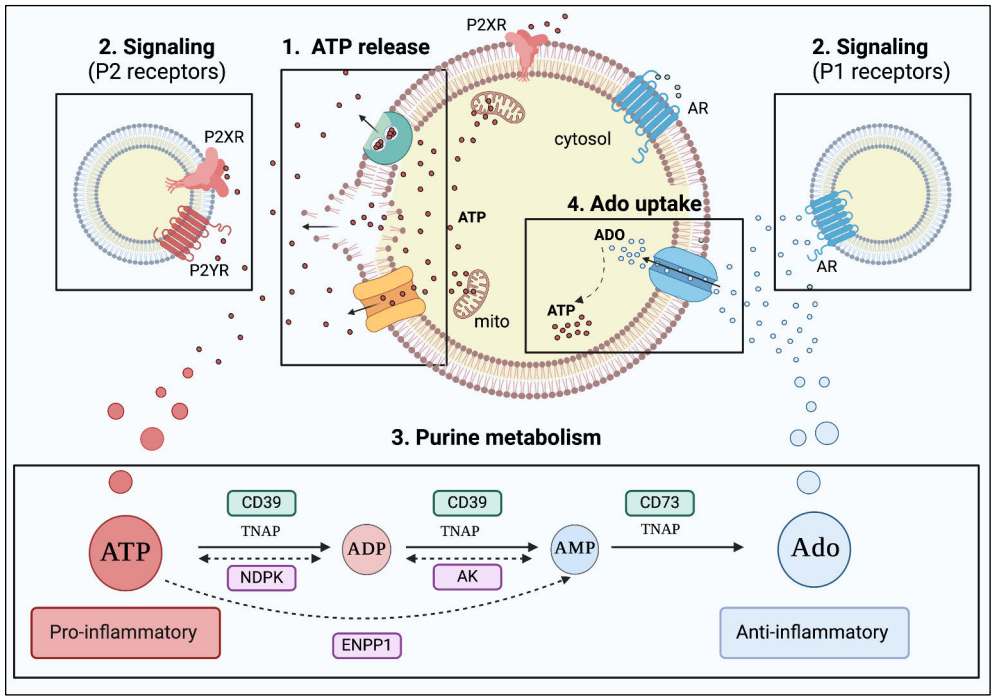


Figure 2. **Current concept of purinergic signaling system**. It includes four interrelated steps: 1. Release of endogenous ATP by cells through various mechanisms, such as cell death, membrane leakage, vesicular exocytosis, and channel-mediated pathways. Mitochondria (mito) are essential organelles, playing a crucial role in ATP production within the cells. 2. ATP and Ado signaling via purinergic P2 (P2X and P2Y) and P1 receptors, respectively, in both paracrine and autocrine manners. ATP generally has a pro-inflammatory effect, whereas Ado exhibits an anti-inflammatory effect, symbolized by their red and blue colors, respectively. 3. Extracellular purine metabolism comprises a complex network of purine-converting ectoenzymes, responsible for both nucleotide inactivation and Ado production, as well as ATP regeneration. Ectoenzymes marked in green squares are well-studied, while those in purple squares are less appreciated. Tissue non-specific alkaline phosphate (TNAP), with its broad substrate dephosphorylation capability, may also play a role in regulating extracellular levels of ATP and its derivatives. 4. Cellular uptake of nucleotide-derived Ado and its subsequent intracellular phosphorylation into ATP. Created with Biorender.com.

2.1.1 Release of endogenous ATP in the extracellular milieu

Cellular respiration is a fundamental process by which cells convert the chemical energy from nutrients into ATP. The two primary pathways for ATP production are mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis, both of which rely on glucose as a key energy source (Alberts et al., 2008). OXPHOS, which takes place in the mitochondria and requires oxygen, is the most efficient ATP-generating pathway, producing up to 38 ATP molecules per glucose under optimal conditions. In contrast, glycolysis, which occurs in the cytoplasm and does not require oxygen, produces only 2 ATP molecules per glucose through its partial oxidation. Despite its

lower efficiency, glycolysis plays a crucial role in ATP synthesis during oxygen-deprived states, such as in skeletal muscle cells during intensive physical activity (Alberts et al., 2008).

Under quiescent conditions, intracellular ATP concentration is in the millimolar range, whereas its extracellular concentration remains in the nanomolar range (Bours et al., 2006). However, extracellular ATP levels can be dramatically increased under certain conditions. For instance, damage or necrosis cause non-specific lytic release of ATP due to cell membrane disruption (**Figure 3A**) (Cook & McCleskey, 2002; Sikora et al., 2015). Additionally, cells can actively release endogenous ATP in response to various stimuli, including shear stress, hypoxia, oxidative stress, and inflammation (Idzko et al., 2014; Yegutkin, 2008). In this case, ATP release occurs through two distinct mechanisms: vesicular exocytosis and channel-mediated conductive pathways (Lazarowski, 2012). Vesicular exocytosis, a regulated process, relies on the vesicular nucleotide transporter (VNUT) (**Figure 3B**). VNUT actively transports cytosolic ATP into vesicles, which subsequently release their contents into the extracellular space by exocytosis (Moriyama et al., 2017). Neuronal cells, platelets, lymphocytes, and microglia utilize this mechanism to release ATP, either alone or in combination with other cellular mediators (Evans et al., 1992; Imura et al., 2013; Tokunaga et al., 2010). Alternatively, active ATP release can occur through channel-mediated conductive pathways, involving connexin hemichannels, pannexin channels, maxi-anion channels (MACs), volume-regulated anion channels (VRACs), and calcium homeostasis modulator 1 (CALHM1) channels (**Figure 3C**) (Taruno, 2018).

Connexin hemichannels and pannexin channels, assembled from connexin and pannexin subunits, respectively, are membrane channels with central pores that are permeable to selective ions and signaling molecules (Alberts et al., 2008; Boassa et al., 2007). Examples of extensively studied members include Connexin 43 (Cx43) and Pannexin 1 (Panx1), both of which participate in active ATP release during various (patho)physiological processes. ATP released via Cx43, found in a variety of cells, such as astrocytes, neutrophils, macrophages, endothelial cells (ECs), and epithelial cells, plays a role in immune modulation, retinal development, and the progression of diseases such as sepsis, atherosclerosis, and glaucoma (Dosch et al., 2019; Eltzschig, et al., 2006a; Pearson et al., 2005; Pfenniger et al., 2013; Zhao et al., 2023). Panx1-mediated ATP release from apoptotic cells, erythrocytes under shear stress and hypoxia, platelets, and ECs during inflammation, has been shown to promote phagocytosis of apoptotic cells, regulate vascular tone, and modulate immune responses (Chekeni et al., 2010; Locovei et al., 2006; Lohman et al., 2015; Taylor et al., 2014).

Another group of ATP-permeable channels, including MACs, VRACs, and CALHM1, are anion channels that allow the passage of selective ions across the cell

membrane upon activation by specific stimuli. For instance, MACs, which are found in ECs, cardiac myocytes, glial cells, and lymphocytes, release ATP when activated by ischemia, hypoxia, and osmotic swelling (Sabirov et al., 2016; Sabirov & Okada, 2005). VRACs, present on astrocytes, microglia, and macrophages, also respond to osmotic swelling and regulate cell volume by transporting various organic anions, including ATP (Akita & Okada, 2014; Burow et al., 2015; Fujii et al., 2017; Pedersen et al., 2015). CALHM1, widely expressed in the brain, nasal epithelial cells, and taste buds, has been shown to mediate ATP release from type II bud cells, contributing to the perception of sweet, bitter, and umami tastes (Ma et al., 2016; Taruno et al., 2013; Workman et al., 2017).

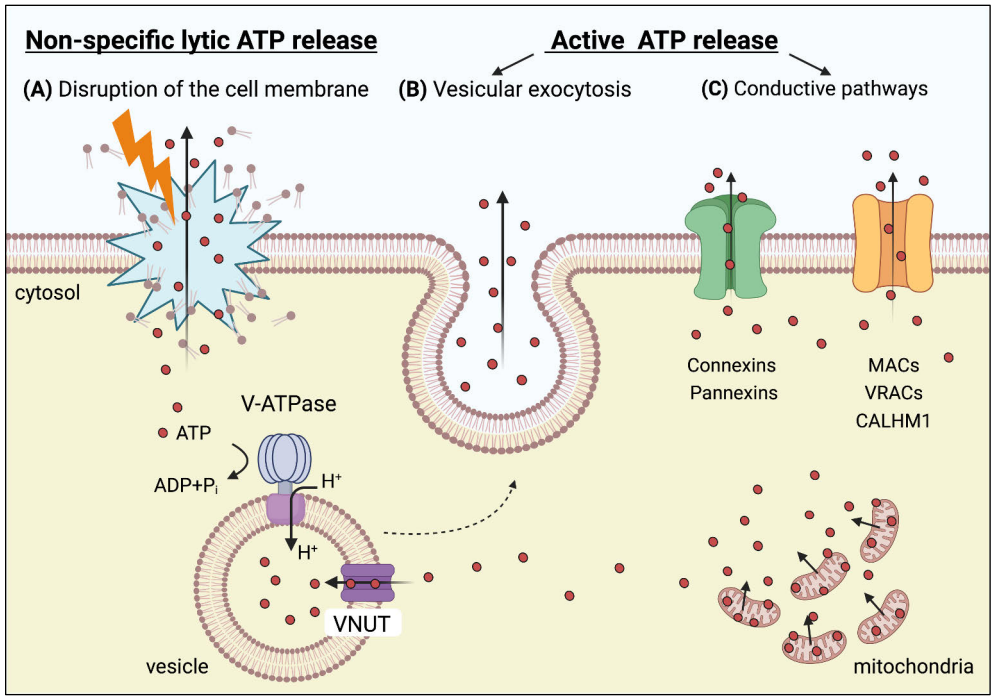


Figure 3. ATP-releasing pathways. Endogenous ATP, primarily produced through mitochondrial respiration, can be released into the extracellular milieu via several mechanisms, including non-specific lytic and active ATP release. **(A)** Non-specific lytic ATP release occurs when the cell membrane ruptures due to mechanical stimuli, apoptosis, or necrosis, leading to the release of intracellular contents, including ATP and other nucleotides and nucleosides. **(B)-(C)** Active ATP release is triggered by specific stimuli and involves two major mechanisms: vesicular exocytosis and conductive pathways. **(B)** During vesicular exocytosis, ATP is actively transported into vesicles by the vesicular nucleotide transporter (VNUT), which relies on the electrochemical gradient of protons mediated by vacuolar proton ATPase (V-ATPase). These vesicles then fuse with the cell membrane, releasing their contents into the extracellular space. **(C)** Conductive pathways facilitate ATP release from the cytosol through connexin hemichannels, pannexin channels, and ATP-permeable anion channels, such as MACs, VRACs, and CALHM1. Created with Biorender.com.

Cells can release ATP through several ATP-permeable channels simultaneously, complicating the determination of individual contributions. One common approach to study the channels is the use of pharmacological inhibitors, though their limited selectivity can complicate result interpretation. Several other ion channels and transporters, including voltage-dependent anion channel-1 (VDAC-1) and cystic fibrosis transmembrane conductance regulator (CFTR), have been proposed to act as ATP-releasing channels (Okada et al., 2004; Reisin et al., 1994). Nevertheless, there are contradictory results regarding their involvement in ATP release (Lazarowski, 2012; Lazarowski et al., 2011). In addition, evidence suggests that the ATP-activated purinergic receptor P2X7 may self-regulate extracellular ATP levels by forming an intrinsic ATP-permeable pore (Brandao-Burch et al., 2012; Suadicani et al., 2006).

2.1.2 Purinergic receptors

The initiation of signaling events by extracellular ATP and its derivative Ado is the next step in the purinergic signaling cascade. Purinergic receptors for extracellular ATP and Ado, known as P2 and P1 receptors, respectively, are present in almost every cell in the human body. Activation of these receptors mediates a broad range of (patho)physiological processes, including neurotransmission, pain, vascular homeostasis, smooth muscle contraction, and platelet aggregation (Burnstock, 2018). Furthermore, extracellular ATP and Ado play a crucial role in regulating immune responses in a receptor-dependent manner. In particular, extracellular ATP, acting as a danger-associated molecular pattern, promotes immune responses by binding to P2 receptors on immune cells (Idzko et al., 2014). In contrast, extracellular Ado suppress immune functions through P1 receptor signaling. Most immune cells co-express both P2 and P1 receptors, indicating the dual nature of cellular regulation through purinergic signaling (Junger, 2011). Thus, balanced and coordinated activation of P2 and P1 receptors is vital for maintaining immune homeostasis as well as other crucial physiological processes.

2.1.2.1 P2 receptors

P2 receptors are membrane-bound receptors that trigger biological responses when activated by ATP and several other nucleotides. These receptors are divided into two distinct families: ionotropic P2X receptors (P2XRs) and metabotropic P2Y receptors (P2YRs) (Ralevic & Burnstock, 1998).

The ionotropic P2XRs are a family of ATP-gated ion channels characterized by rapid activation (Burnstock & Kennedy, 2011). These receptors have a trimeric structure with a channel pore formed by three identical subunits (**Figure 4A**). When

ATP binds to a P2XR, it induces conformational changes that open the channel pore, allowing ions to pass across the cell membrane. This results in plasma membrane depolarization and subsequent initiation of various downstream cellular responses. To date, seven different subunits (P2X1-7) have been identified, resulting in the formation of seven unique receptor subtypes (P2X1-7Rs), each named after its corresponding subunit (North & Jarvis, 2013). In addition, certain subunits can form heterotrimeric complexes, such as P2X2/3R, P2X1/5R and several others, adding further diversity to the biophysical and functional properties of the P2XR family.

P2XRs are widely distributed across various cell types, including neurons, glial cells, smooth muscle cells, platelets, ECs, epithelial cells, and immune cells. Activation of these receptors triggers diverse effects, such as neuromodulation, smooth muscle contraction, platelet aggregation, and inflammation (Surprenant & North, 2009). Among the P2XRs, P2X7R is the most extensively studied due to its significant role in modulating immune system (Di Virgilio et al., 2017). Predominantly expressed on immune cells, including macrophages, dendritic cells, mast cells, and microglia, P2X7R activation promotes inflammation. Specifically, P2X7R is a potent activator of the NLRP3 inflammasome, leading to caspase-1 cleavage and the secretion of pro-inflammatory cytokines such as IL-1β and IL-18 (Gombault et al., 2013; Karmakar et al., 2016). While NLRP3 inflammasome is a crucial component of innate immunity, its extensive activation can cause diseases such as Alzheimer's disease, atherosclerosis, diabetes, and osteoarthritis (Wang & Hauenstein, 2020). Therefore, there is significant therapeutic potential for P2X7R antagonists to treat a range of diseases (Burnstock & Knight, 2018).

The metabotropic P2YRs are a family of G-protein-coupled receptors (GPCRs) activated by several purine and pyrimidine nucleotides. This receptor family is typically associated with slower activation compared to P2XRs, with eight currently characterized subtypes: $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, and $P2Y_{11}R$-$P2Y_{14}R$ (**Figure 4B**) (Burnstock, 2018). The missing numbers in the subtype names represent either non-mammalian receptors or receptors that share some sequence homology with the P2YR family but are not activated by nucleotides (Abbracchio et al., 2006). Each P2YR subtype exhibits unique characteristics related to their agonists and signaling transduction pathways. Unlike P2XRs, where ATP acts as an agonist for the entire family, only two members of the P2YR family, $P2Y_2R$ and $P2Y_{11}R$, are activated by ATP. The activation of other P2YR subtypes is mediated by ADP, which activates $P2Y_1R$, $P2Y_{12}R$, and $P2Y_{13}R$, and by several pyrimidine nucleotides, which activate $P2Y_4R$, $P2Y_6R$ and $P2Y_{14}R$ (Abbracchio et al., 2006). Upon binding with their respective agonists, P2YR subtypes initiate various downstream signaling pathways. Specifically, $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$ activate the phospholipase C pathway, resulting in the release of calcium ions (Ca^{2+}), while $P2Y_{12}R$, $P2Y_{13}R$, and $P2Y_{14}R$ inhibit the adenylate cyclase (AC) pathway, leading to reduced levels of

cyclic AMP (cAMP). $P2Y_{11}R$ possesses unique properties within the family, as it can stimulate both the phospholipase C and the AC pathways (Abbracchio et al., 2006). This diversity in agonist specificities and downstream signaling pathways enables a wide range of physiological responses mediated by the activation of P2YRs.

P2YRs are critical regulators of various physiological processes, including platelet aggregation, vascular homeostasis, neurotransmission in the central nervous system (CNS), and immune system regulation (Burnstock, 2008; Eltzschig et al., 2012). Clopidogrel, widely used in antithrombotic therapy, was the first clinically applied P2YR-targeting drug. Its mechanism of action involves inhibition of $P2Y_{12}R$ on platelets, which subsequently leads to inactivation of glycoprotein IIb/IIIa receptors and prevents platelet aggregation (Eltzschig et al., 2012). The next generation of anticoagulants targeting $P2Y_{12}R$ on platelets includes Prasugrel and Ticagrelor (Rollini et al., 2016). Another clinical agent modulating P2YRs is Diquafosol, approved for the treatment of dry eye symptoms in Japan. It acts as a $P2Y_2R$ agonist on conjunctival epithelial cells and goblet cells, enhancing water and mucin secretion (Keating, 2015). Established roles of several P2YR family members in immune responses suggest their potential for treating inflammatory and infectious diseases (Eltzschig et al., 2012; Idzko et al., 2014). For instance, ATP released by apoptotic cells acts as a danger-associated molecular pattern and promotes $P2Y_2R$-dependent recruitment of macrophages and neutrophils, facilitating the phagocytic clearance of apoptotic cells (Chen et al., 2006; Elliott et al., 2009). Activation of $P2Y_6$ receptors on macrophages leads to the release of chemokines, such as CCL2 and IL-8, attracting inflammatory myeloid cells to the site of infection or inflammation (Warny et al., 2001; Yebdri et al., 2009; Zhang et al., 2011). $P2Y_{12}Rs$ on microglia, the resident macrophages in the CNS, mediate cell chemotaxis to the site of brain injury, a crucial step in the immune response and tissue repair (Haynes et al., 2006). Additionally, $P2Y_{12}Rs$ are suggested to mediate the neuroprotective role of microglia under homeostatic conditions (Cserép et al., 2020). Other P2YR family members, due to their crucial role in neurotransmission, have been proposed as potential therapeutic targets in CNS disorders, with various agonists and antagonists modulating their functions (Burnstock, 2008). Nevertheless, further studies aimed at understanding the mechanisms of action of different P2YR subtypes in health and disease, as well as developing their selective and potent modulators, are essential. These investigations could pave the way for novel therapeutic approaches and treatment strategies for a wide range of diseases.

2.1.2.2 P1 receptors

P1 receptors, also known as Ado receptors (ARs), are a family of GPCRs that respond to Ado. This family comprises four distinct subtypes: A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR, encoded by the genes *ADORA1*, *ADORA2A*, *ADORA2B*, and *ADORA3*, respectively (**Figure 4C**) (Ijzerman et al., 2022). Upon activation, all family members initiate signal transduction through the AC pathway, affecting its activity in different ways: A_1AR and A_3AR inhibit AC, reducing cAMP levels, while A_{2A}AR and A_{2B}AR stimulate AC, increasing cAMP levels. Each subtype also exhibits varying affinities for Ado, with A_1AR presumably having the highest affinity and A_{2B}AR being the least sensitive and potentially inactive under homeostatic conditions (Cekic & Linden, 2016; Ijzerman et al., 2022). Additionally, all P1 receptor subtypes undergo desensitization during prolonged agonist exposure, with A_3AR desensitizing the fastest, often within minutes (Klaasse et al., 2008). These pharmacological properties of P1 receptors, along with their distribution in the body, define a wide range of physiological effects.

A_1AR and A_{2A}AR are extensively studied P1 receptor subtypes, abundantly distributed in both the CNS and peripheral organ systems. In the CNS, A_1AR is particularly prevalent at excitatory synapses, where its activation reduces the release of excitatory neurotransmitters, leading to pain alleviation and other modulatory effects (Borea et al., 2018). A_{2A}AR, widely expressed in the striatum, affects motor activity though neurotransmitter release. Both receptors also regulate critical CNS processes such as the sleep-wake cycle, memory, and learning, although their precise mechanisms are not fully understood (Chen, 2014; Rasch & Born, 2013). Widely distributed in the periphery, A_1AR exerts various physiological functions, including the suppression of cardiac activity through negative inotropic, chronotropic, and dromotropic effects; renal vasoconstriction, reduction of glomerular filtration rate and renin secretion; and inhibition of insulin secretion and lipolysis (Borea et al., 2018). Conversely, A_{2A}AR has a more restricted peripheral distribution, with high abundance in the vascular system, particularly in vascular smooth muscle and ECs (Ralevic & Burnstock, 1998). Activation of A_{2A}AR in this system generally induces vasodilation, including in coronary and renal vessels (Borea et al., 2018; Shryock et al., 1998). Additionally, A_{2A}AR is widely present on various hematopoietic cells, such as platelets, neutrophils, T cells, B cells, monocytes, macrophages, and dendritic cells. Activation of A_{2A}AR on platelets suppresses their aggregation, contributing to anti-coagulant effects (Boncler et al., 2019; Johnston-Cox & Ravid, 2011). In immune cells, A_{2A}AR-mediated signaling induces strong anti-inflammatory responses, regulating immune homeostasis (Cekic & Linden, 2016; Junger, 2011). However, this potent immunosuppression is also recognized as one of key mechanisms that impairs cancer immunosurveillance (Vijayan et al., 2017).

Less explored within the P1 receptor family, the functions of A_{2B}AR and A_3AR in health and disease remain to be fully elucidated. Emerging evidence suggests that both receptors are involved in immune system modulation. A_{2B}AR is highly expressed in macrophages, dendritic cells, mast cells, fibroblasts, and ECs, and in some cases, co-expressed with A_{2A}AR (Feoktistov & Biaggioni, 2011). Activation of A_{2B}AR on immune cells induces suppressive immune functions; however, due to its low affinity for Ado, A_{2B}AR is suggested to be activated only under pathological conditions (Cekic & Linden, 2016). For instance, in addition to A_{2A}ARs, A_{2B}AR signaling on immune cells is currently considered as another Ado-mediated immunosuppressive mechanism in cancer (Vijayan et al., 2017). A_3AR is also expressed on immune cells, and predominantly found in neutrophils, monocytes, macrophages, eosinophils, and mast cells, where its activation can lead to both pro-inflammatory and anti-inflammatory effects, depending on the context (Borea et al., 2015). For instance, A_3AR signaling mediates neutrophil chemotaxis and regulates cytokine production in macrophages. However, its activation has been shown to either enhance or suppress these functions depending on the research model used (Jacobson et al., 2018).

P1 receptors hold significant promise as targets for therapeutic interventions (Jacobson et al., 2019). Several modulators of P1 receptors, including both antagonists and agonists, are already in clinical use. For instance, caffeine, a widely consumed psychoactive drug, exerts its stimulating effects by non-selectively inhibiting P1 receptors in the CNS (Chen et al., 2013). Other examples include commercially available Ado, employed for the treatment of arrythmias; the non-selective ARs antagonist Theophylline, used for the treatment of asthma; and the recently approved selective A_{2A}AR antagonist Istradefylline, representing a first-in-class drug for alleviating symptoms of Parkinson's disease (Borea et al., 2018). Of particular interest is the role of A_{2A}AR and A_{2B}AR in immune suppression in cancer, which has prompted clinical investigations exploring therapeutic strategies to target these receptors (Di Virgilio et al., 2024; Offringa et al., 2022; Yegutkin & Boison, 2022). Beyond cancer, P1 receptors have therapeutic potential for treating various other conditions, including epilepsy, Alzheimer’s disease, cardiovascular diseases, and immune-related disorders (Borea et al., 2018). Numerous ongoing clinical trials are investigating selective A_1AR and A_{2A}AR agonists and antagonists, both alone and in combination with other pharmacological agents, for these conditions (Kutryb-Zając et al., 2023). Additionally, two A_3AR agonists, Piclidenoson and Namodenoson, are currently in clinical trials for the treatment of psoriasis and liver diseases, respectively (Kutryb-Zając et al., 2023). Given their diverse therapeutic potential, continued research and clinical trials on P1 receptors could significantly expand therapeutic strategies for various diseases.

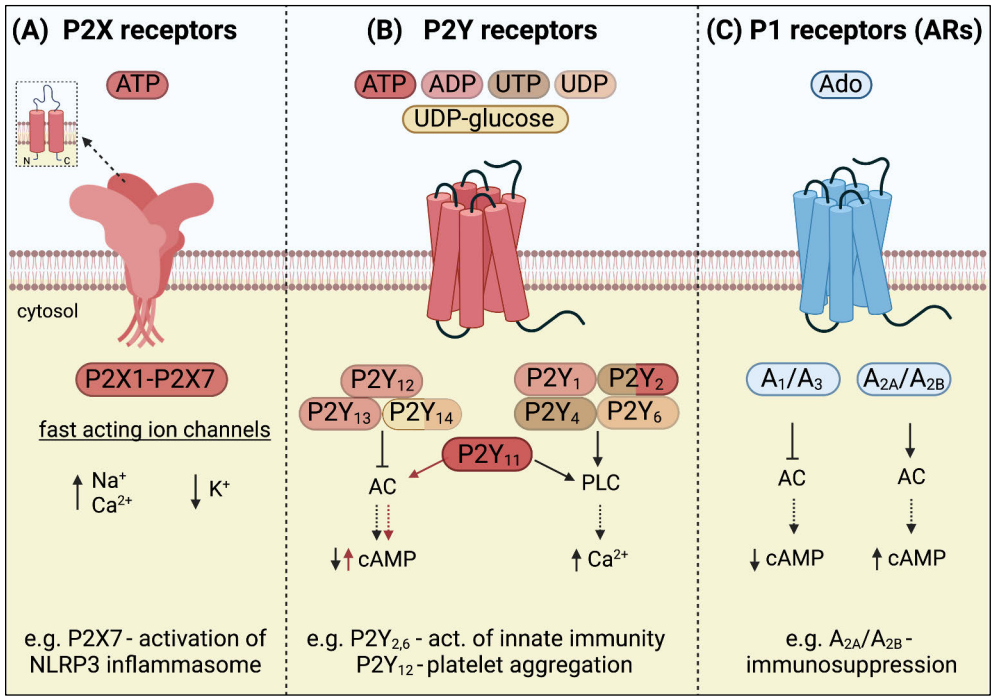


Figure 4. **Purinergic receptors.** ATP and Ado mediate their signaling functions via P2 receptors **(A, B)** and P1 receptors **(C)**, respectively. P2 receptors are further divided into two families: ionotropic P2X receptors and metabotropic P2Y receptors. **(A)** The P2X receptor family consists of ATP-gated channels that, upon activation, mediate the passage of selective ions across the cell membrane, leading to plasma membrane depolarization and the initiation of various downstream cellular responses. This receptor family includes 7 subtypes: P2X1-P2X7 receptors. Each receptor is formed by three identical subunits (P2X1-P2X7), with each subunit containing two transmembrane domains (at the N- and C-terminus) and an extracellular domain housing the ATP binding site. **(B)** The P2Y receptor family comprises G-protein-coupled receptors (GPCRs) activated by various purine and pyrimidine nucleotides, including ATP, ADP, uridine triphosphate (UTP), uridine diphosphate (UDP), and uridine diphosphate glucose (UDP-glucose). Currently, eight subtypes have been identified: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$- $P2Y_{14}$, each activated by a specific ligand. The frame color of each receptor subtype matches the frame color of its corresponding ligand. The downstream signaling pathways of the P2Y receptor family include inhibition of the adenylate cyclase (AC) pathway, leading to reduced cyclic AMP (cAMP) levels ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$), or activation of the phospholipase C (PLC) pathway, leading to the release of Ca^{2+} ($P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$). The $P2Y_{11}$ receptor possesses unique properties, as it can stimulate both AC and PLC pathways. **(C)** The P1 receptor family includes GPCRs activated by Ado, hence also referred to as Ado receptors (ARs). It includes four distinct subtypes: A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR, that mediate their downstream signaling pathways via the AC pathway. A_1AR and A_3AR inhibit AC, reducing cAMP levels, whereas A_{2A}AR and A_{2B}AR stimulate AC, increasing cAMP levels. The lower section of the figure reflects representative functions of selective receptors. Created with Biorender.com.

The outcome of purinergic signaling depends on multiple factors. These include the pharmacological properties of receptors, such as specificity, ligand affinity, desensitization, and downstream signaling pathways. The concentration of extracellular nucleotides and nucleosides determines the intensity and duration of purinergic receptor activation. A delicate balance between P1 and P2 receptor ligands, regulated by specific purine-converting ectoenzymes, is essential for the proper functioning of purinergic signaling (Yegutkin, 2014). Furthermore, the expression levels of purinergic receptors can vary significantly across different cell types and tissues and can be further modulated by various molecules, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and lipopolysaccharides (LPS), indicating the dynamic nature of purinergic signaling (Bours et al., 2006). This intricate interplay of receptor characteristics, ligand concentrations, and regulatory factors ensures the proper biological outcomes of purinergic signaling, thereby maintaining cellular and tissue homeostasis.

2.1.3 Purine-converting ectoenzymes

Purine-converting ectoenzymes are another critical component of the purinergic signaling system, responsible for regulating the extracellular levels of various nucleotides and nucleosides. ATP and ADP signaling, mediated through P2 receptors, is terminated by specific nucleotide-hydrolyzing ectoenzymes, including four main families of enzymes: nucleoside triphosphate diphosphohydrolases (NTPDases), 5′-nucleotidases, ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs), and alkaline phosphatases (APs). Key enzymes, including NTPDase 1 (CD39), ecto-5′-nucleotidase (CD73), ENPP1, and tissue non-specific AP (TNAP), catalyze the dephosphorylation of ATP or its derived nucleotides, ultimately forming Ado in the extracellular space (**Figure 5**). Once generated, Ado activates P1 receptor signaling, which is then terminated by Ado clearance from the extracellular space. This elimination occurs either through Ado uptake into cells via nucleoside transporters or its degradation by nucleoside-inactivating ectoenzymes, including the extracellular form of adenosine deaminase (ADA) (Boison & Yegutkin, 2019) (more detailed information is presented in section 2.1.4). Additionally, extracellular adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK) enable the reverse generation of extracellular ATP via phosphotransfer reactions, adding further complexity to the regulation of purinergic signaling (Boison & Yegutkin, 2019).

Purine-converting ectoenzymes are primarily membrane-bound enzymes, with some also present in soluble forms and on the surface of extracellular vesicles (Boison & Yegutkin, 2019; Clayton et al., 2011; Jiang et al., 2014; Schneider et al., 2021; Yegutkin et al., 2003). Although ADA, AK, and NDPK were originally considered to be exclusively intracellular, their extracellular forms have been

additionally identified (Donaldson et al., 2002; Eltzschig et al., 2006b; Yegutkin et al., 2001; Yegutkin, 2014). Different cell types express unique repertoire of purine-converting ectoenzymes. Some cells co-express several ectoenzymes simultaneously, while others express only certain types, relying on interaction with neighboring cells (Schneider et al., 2019). This diversity and the complexity of purine-converting pathways provide precise control of purinergic signaling, tailored to the specific characteristics of each tissue and its microenvironment. Next, I will summarize the current knowledge of purine-converting ectoenzymes, with a specific focus on their roles in purinergic signaling.

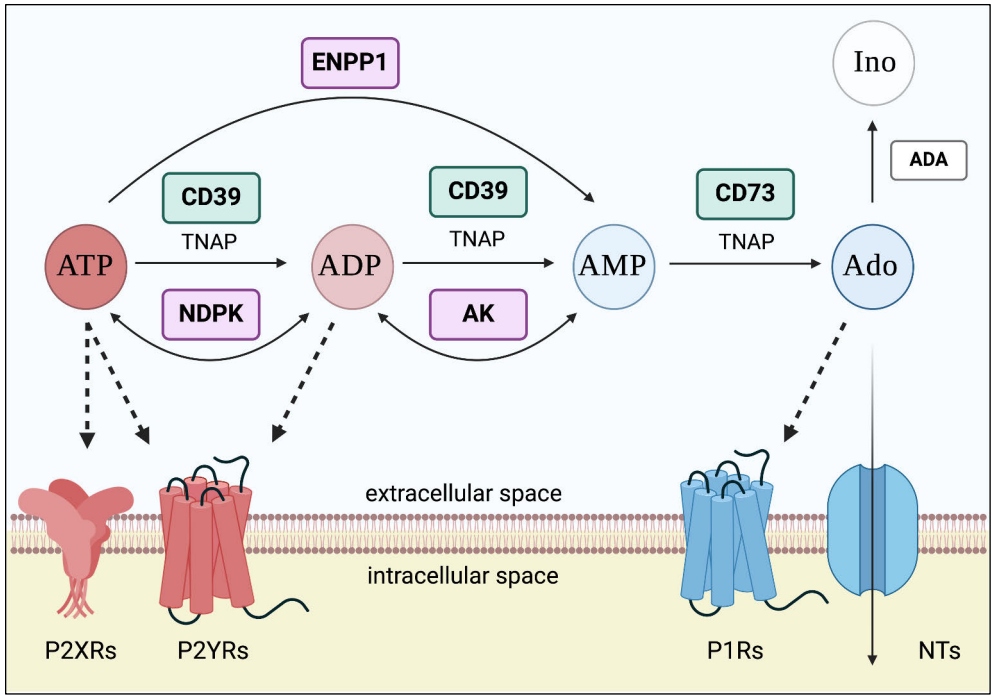


Figure 5. Schematic presentation of purinergic metabolic and signaling pathways. A broad network of ectoenzymes governs the extracellular metabolism of ATP and Ado, maintaining balanced concentrations of P1 and P2 receptor ligands. Several nucleotide-hydrolyzing ectoenzymes are involved in the termination of P2 receptor (P2XR or P2YR) signaling. Among these, CD39 (NTPDase1) and CD73 are considered the primary ectoenzymes responsible for ATP inactivation and Ado generation via the sequential dephosphorylation of ATP to ADP, AMP, and Ado. An alternative pathway for ATP hydrolysis is mediated by ENPP1, producing AMP and pyrophosphate (PPi). TNAP (tissue non-specific AP) may also contribute to the extracellular production of ADP, AMP, and Ado by dephosphorylating various nucleotides. Additionally, a reverse ATP-generating pathway exists, involving extracellular forms of AK and NDPK. Once extracellular Ado is produced, it mediates signal transduction through P1 receptors. Ado can then be either degraded into inosine (Ino) by extracellular form of ADA or taken up by cells through nucleoside transporters (NTs). Ectoenzymes in green squares are well-characterized, whereas those in purple squares are less studied, particularly in the context of cancer progression. Adapted from (Boison & Yegutkin, 2019). Created with Biorender.com.

2.1.3.1 Nucleotide-hydrolyzing ectoenzymes

2.1.3.1.1 Nucleoside triphosphate diphosphohydrolase family

The family of NTPDases is a class of hydrolases that cleave the γ- and β-phosphate groups from nucleotide tri- and diphosphates. The NTPDase family consists of eight enzymes (NTPDase1-8), but only four of them (NTPDase1, 2, 3, and 8) are ectoenzymes involved in extracellular nucleotide hydrolysis (Robson et al., 2006). These ecto-NTPDases are proteins with a molecular weight of ~70–80 kDa, consisting of two transmembrane domains at the N- and C-terminus and an extracellular loop with catalytic activity (**Table 1**). They are enzymatically active in pH range of 7-8.5 and require Mg^{2+} and Ca^{2+} cations for their function. Ecto-NTPDases exhibit broad substrate specificity for nucleotide tri- and diphosphates, including ATP, ADP, UTP, and UDP. However, different family members display variations in their substrate preferences (Zimmermann et al., 2012). For instance, NTPDase1, also known as CD39, efficiently hydrolyzes both ATP and ADP, whereas NTPDase2 exhibits a preference for ATP over ADP, leading to the sustained accumulation of ADP. NTPDase3 and NTPDase8 function as intermediates between CD39 and NTPDase2, converting ATP to AMP with a transient accumulation of ADP. These substrate preferences partially explain the distinct functional roles of the ecto-NTPDases.

CD39, the most extensively studied family member, exhibits high expression levels on vascular ECs and smooth muscle cells, as well as on circulating immune cells, including monocytes, neutrophils, dendritic cells, and certain subsets of B and T cells (Eltzschig et al, 2012; Yegutkin, 2021; Zimmermann et al., 2012). It plays a crucial role in maintaining vascular and immune system homeostasis by effectively removing pro-inflammatory ATP and pro-thrombotic ADP from the bloodstream (Atkinson et al., 2006; Deaglio & Robson, 2011; Kaczmarek et al., 1996). Furthermore, CD39 contributes to immune suppression by producing extracellular AMP, which is a primary source of anti-inflammatory Ado (Deaglio et al., 2007; Kanthi et al., 2014). NTPDase2, primarily expressed in the adventitial layer of blood vessels, promotes blood coagulation through the sustained accumulation of pro-thrombotic ADP (Atkinson et al., 2006). This accumulation leads to the ADP-mediated activation of P2Y receptors on platelets (Sévigny et al., 2002). Knowledge about the remaining ectoenzymes, NTPDase3 and NTPDase8, is still limited. NTPDase3 is found in the gastrointestinal tract, salivary glands, and pancreas, where it is proposed as a specific marker for human pancreatic beta-cells (Lavoie et al., 2011; Saunders et al., 2019). Although the significance of NTPDase3 is not well understood, its global deletion in mice has been linked to increased basal energy metabolism, providing protection against diet-induced obesity (Sandhu et al., 2021).

NTPDase8 is predominantly expressed in the liver, with particular abundance in bile canaliculi, and has been additionally detected in human intestinal epithelial cells (Fausther et al., 2007; Salem et al., 2022). Recent studies using a mouse model of intestinal inflammation suggest a potential protective role for NTPDase8 by restricting $P2Y_6$ receptor activation on colonic epithelial cells (Salem et al., 2022). However, similar to NTPDase3, a thorough understanding of the physiological significance of NTPDase8 requires further investigation.

Due to the critical immunomodulatory role of CD39, its dysregulated activity is implicated in several immune-related disorders (Antonioli et al., 2019; Eltzschig et al., 2012; Takenaka et al., 2016). In the context of cancer, elevated CD39 levels within the tumor microenvironment (TME) contribute to tumor immune evasion, making it an attractive target for therapeutic intervention in advanced solid tumors (Allard et al., 2017; Moesta et al., 2020). Currently, clinical trials are evaluating several anti-CD39 antibodies and small-molecule inhibitors, both as standalone therapies and in combination with other immunotherapies (Di Virgilio et al., 2024; Xia et al., 2023). In contrast, reduced CD39 expression has been implicated in inflammatory bowel disease (IBD), with CD39 polymorphism linked to disease progression (Friedman et al., 2009; Longhi et al., 2017; Robles et al., 2020). In addition, CD39 dysregulation has been associated with other chronic inflammatory conditions, including multiple sclerosis and sepsis (Zeng et al., 2020). Beyond CD39, other ecto-NTPDases may also contribute to the pathogenesis of these diseases (Feldbrügge et al., 2017; Haas et al., 2021; Longhi et al., 2017; Salem et al., 2022). However, despite these findings, the therapeutic potential of ecto-NTPDases in immune dysregulation and chronic inflammation remains underexplored due to the limited understanding of their regulatory functions in both healthy and diseased states.

2.1.3.1.2 Ecto-5´-nucleotidase

The 5'-nucleotidase family is a class of hydrolases that catalyze the dephosphorylation of nucleotide monophosphates by releasing α-phosphate group. To date, seven human 5'-nucleotidases have been identified, six of which are located intracellularly and participate in *de novo* nucleotide synthesis (Hunsucker et al., 2005). Ecto-5'-nucleotidase (CD73) is the only member of this family that exhibits extracellular localization. It hydrolyzes extracellular AMP to Ado, thereby terminating the ATP-to-Ado conversion initiated by ecto-NTPDases (**Figure 5**). Encoded by the *NT5E* gene, CD73 has a molecular weight of ~60–80 kDa (Yegutkin, 2014; Zimmermann et al., 2012). Enzymatically active CD73 forms a dimeric structure with two identical subunits that are noncovalently bound and anchored to the outer plasma membrane by glycosylphosphatidylinositol (GPI) (**Table 1**). Each

subunit comprises a C-terminal domain containing a substrate binding site and an N-terminal domain responsible for binding Zn^{2+} ions and other divalent metal cofactors (Knapp et al., 2012). These domains are linked by an alpha helix, allowing CD73 to dynamically switch between "open" and "closed" conformations. In the "open" state, AMP binds to the enzyme, while in the "closed" state, the catalytic reaction occurs (Knapp et al., 2012). Notably, the enzymatic activity of CD73 is competitively inhibited by binding of ATP or ADP to the catalytic site (Zimmermann et al., 2012).

CD73 is a multifunctional enzyme with a crucial role in regulating both immune and vascular systems (Allard et al., 2017; Yegutkin, 2021). It is abundantly expressed on various immune cells, including subsets of T and B cells, myeloid cells, and natural killer (NK) cells, as well as on ECs (Allard et al., 2019; Colgan et al., 2006). Through its enzymatic activity, CD73 generates Ado, which exerts strong immunosuppressive effects by activating ARs on immune cells (Allard et al., 2019). On the endothelium, CD73-derived Ado helps maintain barrier integrity, a function particularly evident under hypoxic conditions, as CD73-deficient mice exhibit severe vascular leakage when exposed to hypoxia (Eltzschig et al., 2003; Thompson et al., 2004; Yegutkin et al., 2015). In addition to its enzymatic function, CD73 acts as an adhesion molecule, facilitating the interaction between lymphocytes and ECs and promoting lymphocyte adhesion and transmigration (Airas et al., 1995, 2000; Salmi & Jalkanen, 2005).

The role of CD73 in maintaining tissue integrity has been documented across various organs, including the brain, heart, kidneys, liver, and gastrointestinal tract, where it is expressed in multiple cell types, such as fibroblasts, epithelial cells, and ECs (Alcedo et al., 2021; Minor et al., 2019). While the precise mechanisms of CD73 action in these organs are not entirely understood, recent technological advancements, such as single-cell sequencing, are providing novel insights. For instance, in the gastrointestinal tract, CD73 is highly expressed on the luminal side of enterocytes at the villus tips within the intestinal epithelium (Moor et al., 2018), suggesting its potential role in mediating immune tolerance toward host commensal bacteria (Alcedo et al., 2021). Additionally, its presence at the villus tips, where oxygen levels are low, suggests a protective function against hypoxia. The upregulation of CD73 in the intestinal epithelium, mediated by hypoxia-inducible factor-1 (HIF-1), is crucial for maintaining epithelial barrier function and counteracting hypoxia-induced intestinal permeability (Synnestvedt et al., 2002).

The therapeutic potential of CD73 is currently under evaluation for cancer treatment. While Ado generated by CD73 helps maintain immune homeostasis and prevents excessive inflammation (Cekic & Linden, 2016), its immunosuppressive effects can also enable cancer cells to evade the immune system (Allard et al., 2019; Vijayan et al., 2017). Several clinical trials are investigating the efficacy of targeting CD73 with pharmacological agents, such as monoclonal antibodies and small-

molecule inhibitors (Di Virgilio et al., 2024; Xia et al., 2023; Yegutkin & Boison, 2022). Nevertheless, despite the promising rationale, many trials were unable to demonstrate therapeutic benefits of CD73-inhibiting drugs over existing immunotherapies (Di Virgilio et al., 2024). One major challenge impeding the development of effective Ado-based therapeutics is the complexity of the TME, which can vary significantly between different types of tumors. Additionally, redundancy and compensatory mechanisms in the purinergic signaling cascade may undermine the effectiveness of CD73 targeting (Yegutkin & Boison, 2022). Therefore, it is essential to comprehensively evaluate CD73's role in the TME and identify patient cohorts that could benefit from such therapy. Until this is achieved, developing effective therapies targeting CD73 remains a challenge in cancer treatment.

2.1.3.1.3 Ecto-nucleotide pyrophosphatase/ phosphodiesterase family

The ENPP family includes seven structurally and functionally diverse enzymes (ENPP1-7) that hydrolyze pyrophosphate and phosphodiester bonds in various substrates (Zimmermann et al., 2012). ENPP1, ENPP3, ENPP4, and ENPP5 primarily hydrolyze extracellular nucleotides, whereas ENPP2, ENPP6, and ENPP7 prefer phospholipids as substrates (Borza et al., 2022). With the exception of ENPP2 and ENPP6, which are secreted and GPI-anchored enzymes respectively, all other ENPP family members are anchored to the cell membrane via a single transmembrane domain (**Table 1**). The catalytic activity of this enzyme family is attributed to a conserved phosphodiesterase (PDE) domain containing two Zn^{2+} ions bound in the active site. In addition to the PDE domain, ENPP1-3 possess two somatomedin B-like domains and inactive nuclease domain, which provide their structural stability (Borza et al., 2022). This variability among the members of ENPP family determines their unique characteristics, including substrate specificities and corresponding biological functions.

ENPP1, also known as plasma cell membrane glycoprotein-1 (PC-1), is one of the best characterized and studied members of the ENPP family. It is a type II transmembrane protein consisting of disulfide-linked homodimers with a molecular weight of 110-125kDa (Goding et al., 2003; Yegutkin, 2014). ENPP1 is highly expressed in bone and cartilage, where it plays a crucial role in mineralization of skeletal and soft tissues by hydrolyzing ATP to AMP and pyrophosphate (PP_i) (Roberts et al., 2019). PP_i inhibits the deposition of hydroxyapatite, an inorganic mineral essential for the structure of bones and teeth. Dysregulated ENPP1 activity leads to impaired hydroxyapatite metabolism, contributing to pathophysiological conditions such as soft tissue calcification, osteoarthritis, and cardiovascular diseases (Goding et al., 2003). One notable condition linked to ENPP1 mutations is

generalized arterial calcification of infancy (GACI) (Albright et al., 2015). This rare but lethal disease is characterized by calcifications of the large arteries, severe hypertension, and cardiac failure in infants (Ferreira et al., 2024). Subcutaneous administration of an ENPP1-Fc fusion protein has been shown to prevent mortality in animal models of GACI, highlighting its therapeutic potential (Albright et al., 2015).

Beyond its role in mineralization, recent studies have identified cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) as another substate for ENPP1, indicating its role in innate immunity (Decout et al., 2021). cGAMP is a crucial second messenger in the cGAS-STING pathway, which is activated when the cGAS sensor protein detects cytosolic DNA. Typically, cytosolic DNA is present outside the nucleus only under pathophysiological conditions, such as viral infections, cellular damage, or tumorigenesis. Upon recognizing cytosolic DNA, cGAS generates cGAMP, which activates the STING protein in the endoplasmic reticulum membrane, triggering the production of type I interferons and pro-inflammatory cytokines to stimulate immune responses (Decout et al., 2021).

While the cGAS-STING pathway primarily functions within cells, cGAMP can also be transported across cell membranes, acting as an immunotransmitter. Several transporters facilitate this process, enabling the release and uptake of cGAMP to mediate intracellular communication (Dvorkin et al., 2024). ENPP1 plays a key role in regulating extracellular cGAMP levels by hydrolyzing it into AMP and GMP, thereby controlling systemic inflammation by attenuating the STING pathway (Carozza et al., 2022). Moreover, ENPP1 may also contribute to Ado-mediated immunosuppression by producing AMP, which CD73 then converts into Ado. This ENPP1's dual role in immunosuppression has been proposed to be particularly important in maintaining intestinal homeostasis (Chang et al., 2020). However, the immunosuppressive functions of ENPP1 are not solely beneficial, as it has also been shown to promote metastasis and tumor evasion by dampening immune responses against tumors (Carozza et al., 2020; Li et al., 2021; Ruiz-Fernández de Córdoba et al., 2023; Wang et al., 2023).

ENPP2, also known as Autotaxin, is another well-studied member of the ENPP family. It is a secreted enzyme found at high levels in plasma, primarily produced by ECs, fibroblasts, and adipocytes (Kanda et al., 2008; Perrakis & Moolenaar, 2014; Takeda et al., 2016). In contrast to ENPP1, which mediates purinergic signaling, ENPP2 plays a critical role in lipid signaling. Specifically, ENPP2 converts extracellular lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA) (Yung et al., 2014). LPA is a potent bioactive lipid mediator that binds to a family of GPCRs known as LPA receptors. These receptors are expressed in virtually every organ, including the heart, brain, liver, lymphoid organs, where they regulate various physiological processes, including embryonic development, tissue homeostasis,

angiogenesis, and cell trafficking (Geraldo et al., 2021; Knowlden & Georas, 2014). Elevated levels of ENPP2, and consequently increased LPA signaling, are implicated in a range of pathophysiological conditions, including cancer, pulmonary fibrosis, arthritis, and Alzheimer's disease, making ENPP2 a promising therapeutical target (Magkrioti et al., 2019; Matas-Rico et al., 2021).

Other members of the ENPP family involved in purinergic signaling include ENPP3, ENPP4, and ENPP5. However, their specific substrates and functions are not yet well understood. ENPP3 is found on basophils and mast cells, where it modulates allergic responses by hydrolyzing extracellular ATP (Tsai et al., 2015). ENPP4 is present on the surface of vascular endothelium and contributes to blood coagulation (Albright et al., 2012). Specifically, it activates ADP-sensitive $P2Y_1R$ and $P2Y_{12}R$ on platelets by producing ADP from the hydrolysis of diadenosine triphosphate (Ap3A). ENPP5 is proposed to play a role in neurotransmission by cleaving nicotinamide adenine dinucleotide (NAD) (Gorelik et al., 2017). Further research is needed to elucidate the precise roles of these enzymes in various biological processes.

2.1.3.1.4 Alkaline phosphatase family

The AP family is a group of membrane-bound enzymes that catalyze the hydrolysis of phosphate groups from various substrates under alkaline conditions (pH 8 to 11) (Millán, 2006; Yegutkin, 2014). In humans, the AP family includes four members: tissue-nonspecific AP (TNAP), intestinal AP (IAP), placental AP, and germ cell AP, encoded by the respective genes *ALPL*, *ALPI*, *ALPP*, and *ALPP2* (Zimmermann et al., 2012). These enzymes are typically homodimeric, consisting of GPI-anchored monomers with a molecular weight of approximately 80 kDa (**Table 1**). Each monomer's catalytic site contains three metal ions, including two Zn^{2+} and one Mg^{2+}, crucial for catalytic activity. The AP family exhibits broad substrate specificity, hydrolyzing various nucleotides, as well as PP_i, glucose-phosphate, pyridoxal phosphate, β-glycerophosphate, and other phosphate-containing molecules (Millán, 2006; Yegutkin, 2014). Given this diversity of substrates, the AP family is likely involved in a wide range of cellular processes, complicating a comprehensive understanding of its functions.

TNAP is the most extensively studied member of the AP family. This membrane-bound enzyme is ubiquitously expressed in bone, liver, and kidney, where it is sometimes referred to as bone AP, liver AP, and kidney AP, respectively. The functional diversity of these isoforms arises from differences in the post-translational glycosylation of TNAP in these tissues (Haarhaus et al., 2017). In bone, TNAP is present on mineralizing cells such as osteoblasts and chondrocytes, where it hydrolyzes PP_i, generated by ENPP1, into two molecules of inorganic phosphate

(P_i). Together, TNAP and ENPP1 ensure proper bone mineralization by controlling extracellular levels of PP_i, a potent inhibitor of hydroxyapatite deposition, and P_i, a crucial component for hydroxyapatite crystal growth (Hessle et al., 2002). In the liver, TNAP is primarily found in hepatocytes and the biliary epithelium, where it likely regulates bile formation and secretion (Goettsch et al., 2022). In the kidney, TNAP is present in renal tubules and may contribute to LPS detoxification (Goettsch et al., 2022). However, the exact functions of TNAP in these organs are still under investigation.

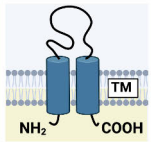
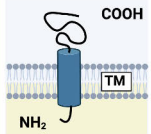
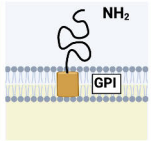
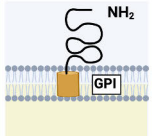
Several studies have additionally identified a substantial presence of TNAP in human neutrophils, suggesting its potential anti-inflammatory effects (Bessueille et al., 2020; Corriden et al., 2008). Notably, TNAP levels are higher in neonatal neutrophils compared to adults, where it serves as a major source of Ado formation through its AMPase activity (Pettengill et al., 2013). TNAP also exists in a soluble form within the bloodstream due to cleavage of membrane-bound TNAP by phospholipases (Anh et al., 2001; Magnusson et al., 2002; Poupon, 2015). Intriguingly, recent studies have suggested an additional intracellular localization of this enzyme. In murine thermogenic adipose cells, TNAP has been detected in mitochondria, where it is suggested to play a role in thermogenesis by hydrolyzing phosphocreatine (Sun et al., 2021).

Due to its key role in bone mineralization under homeostatic conditions, TNAP dysregulation can lead to ectopic mineralization in conditions such diabetes, atherosclerosis, chronic kidney disease, and certain genetic disorders (Goettsch et al., 2022). TNAP inhibition led to a significant reduction in vascular and soft tissue calcification in several preclinical models (Li et al., 2019a; Tani et al., 2020). Increased TNAP activity has been reported in patients with a rare genetic vascular disease due to CD73 loss, leading to extensive arterial calcification (Jin et al., 2016; St. Hilaire et al., 2011). This observation suggests that TNAP may compensate for insufficient Ado production while contributing to pathological mineralization. Elevated TNAP levels in blood serve as a diagnostic marker for liver and bone disorders, such as hepatitis, cirrhosis, and osteoporosis. Notably, our recent findings detected TNAP in the human optic nerve head and its soluble form in the vitreous fluid, with increased activity observed in patients with diabetic retinopathy (Zeiner et al., 2019). Further research is needed to understand TNAP regulation in these pathological conditions and explore its therapeutic potential.

IAP, predominantly expressed by intestinal epithelial cells, plays a crucial role in maintaining gut homeostasis (Lallès, 2019). IAP mediates nutrient absorption and detoxifies bacterial LPS (Beumer et al., 2003; Ghosh et al., 2021; Narisawa et al., 2003). Additionally, IAP preserves the gut microbiota and promotes its growth by dephosphorylating ATP and other luminal nucleotide triphosphates, which have been shown to directly inhibit bacterial growth (Malo et al., 2010, 2014). In several

rodent models of colitis, oral supplementation with exogenous IAP attenuated intestinal inflammation (Ramasamy et al., 2011; Tuin et al., 2009). Correspondingly, reduced levels of IAP in humans have been associated with IBD, such as Crohn's disease and ulcerative colitis (Parlato et al., 2018; Tuin et al., 2009). Given its important role in gut homeostasis, a deeper understanding of the mechanisms and therapeutic applications of IAP could significantly advance the treatment of IBD.

Table 1. Major nucleotide-hydrolyzing ectoenzymes. This table highlights key members of enzyme families involved in the degradation of extracellular nucleotides. The structures depicted represent the monomeric forms of selected family members: CD39, ENPP1, CD73, and TNAP, respectively. Notably, some enzymes exhibit catalytic activity only in their dimeric forms. The catalytic reactions listed focus on well-studied pathways for adenine nucleotides and their derivatives, although these ectoenzymes can act on other substrates not detailed in the table. Abbreviations: TM, transmembrane domain; GPI, glycosylphosphatidylinositol anchor. Adapted from (Boison & Yegutkin, 2019; Zimmermann, 2001). Created with Biorender.com.

	STRUCTURE	ISOZYME	GENE NAME	ALIASES	CATALYTIC REACTIONS
Ecto-NTPDases		NTPDase1 NTPDase2 NTPDase3 NTPDase8	*ENTPD1* *ENTPD2* *ENTPD3* *ENTPD8*	CD39 apyrase ecto-ATPase	ATP + H_2O → ADP + Pi ADP + H_2O → AMP + Pi
Ecto-NPPs		ENPP1 ENPP3 ENPP4 ENPP5	*ENPP1* *ENPP3* *ENPP4* *ENPP5*	PC-1 CD203a/c	ATP + H_2O → AMP + PPi cGAMP + H_2O → AMP + GMP Ap3A → AMP + ADP
Ecto-5´-nucleotidase		-	*NT5E*	CD73 AMPase	AMP + H_2O → Ado + Pi
Alkaline phosphatases		TNAP IAP	*ALPL* *ALPI*	Basic phosphatase	ATP + 3H_2O → Ado + 3Pi PPi → 2Pi

2.1.3.2 The reverse ATP-generating pathway

The reverse ATP-generating pathway involves two nucleotide kinase families: adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK). These enzymes, typically located intracellularly, also exist extracellularly in both membrane-bound and soluble forms (Boison & Yegutkin, 2019; Donaldson et al., 2002; Yegutkin et al., 2001). Ecto-nucleotide kinases, along with nucleotide-hydrolyzing ectoenzymes, regulate extracellular nucleotide levels, thereby modulating purinergic signaling

(**Figure 5**). Under homeostatic conditions, ecto-nucleotide kinases are thought not to compete with nucleotide-hydrolyzing ectoenzymes for the limited pool of released nucleotides (Yegutkin, 2008). However, during inflammation, characterized by massive ATP release and the feed-forward inhibition of CD73 by high levels of ATP and ADP, ecto-nucleotide kinases may become activated, leading to reverse ATP generation (Yegutkin, 2008). Nevertheless, the mechanisms governing their extracellular localization and functional roles remain poorly understood, making the significance of the reverse ATP-generating pathway in purinergic signaling regulation under both homeostatic and pathological conditions unclear.

2.1.3.2.1 Adenylate kinase family

The AK family is a group of transferases that catalyze the reversible transfer of a phosphoryl group from ATP to AMP, producing two ADP molecules (**Table 2**). Structural studies of bacterial AKs have identified three functional domains: the CORE domain, and two smaller domains, LID and NMP (Formoso et al., 2015). During the enzymatic reaction, the LID and NMP domains undergo significant conformational changes, switching between “open” and “closed” states to facilitate catalysis, while the CORE domain remains stable. Mg^{2+} cations are essential cofactors in AK activity, facilitating these conformational changes and accelerating the catalyzed reaction (Kerns et al., 2015).

The human AK family includes nine isozymes (AK1-9), primary located intracellularly (Panayiotou et al., 2014). AK1, the most studied isozyme, is a 20kDa protein predominantly found in the cytosol. AK1 is particularly abundant in high-energy-demand tissues, such as skeletal muscles, highlighting its role in cellular energy regulation (Yegutkin, 2008). By maintaining adenine nucleotide equilibrium, AK1 is proposed to regulate intracellular AMP levels, that reflect the cell’s energy status (Hardie et al., 2012). Under homeostatic conditions, ATP is the most abundant intracellular nucleotide, primarily generated through aerobic glycolysis and mitochondrial respiration. In this state, AK1 catalyzes the conversion of ATP and AMP into two ADP molecules, keeping AMP levels low (Hardie & Hawley, 2001). However, during metabolic stress, as ATP is rapidly consumed and ADP levels rise, AK1 reverses the reaction, converting two ADP molecules into ATP and AMP. This increases AMP levels, leading to the activation of AMP-activated protein kinase (AMPK), the key regulator of cellular energy balance (Hardie et al., 2012; Hardie & Hawley, 2001). Activated AMPK triggers pathways to restore energy balance by stimulating ATP production through processes such as glycolysis and fatty acid oxidation, while inhibiting ATP consumption in processes such as protein and lipid synthesis (Hardie et al., 2012; Herzig & Shaw, 2018). Furthermore, AK1 has been proposed to function similarly to creatine kinase, serving as an additional mechanism

for transferring high-energy phosphoryl groups from mitochondria to myofibrils (Dzeja et al., 1999). Notably, AK1-deficient mice exhibited impaired energy expenditure during contractile activity, especially under hypoxic conditions, highlighting the importance of AK1 in maintaining efficient cellular energy homeostasis (Janssen et al., 2000).

In addition to its well-known intracellular localization, AK has also been found extracellularly. A specific isoform, AK1β, which features 18 additional amino acids at the N-terminus, has been identified on the cell plasma membrane and may play a role in p53-dependent regulation of cell-cycle arrest (Collavin et al., 1999). Extracellular AK activity has been reported in various cell types, including vascular ECs, lymphocytes, hepatocytes, and airway epithelial cells, as well as in a soluble form in the bloodstream (Donaldson et al., 2002; Yegutkin, 2008; Yegutkin et al., 2012). Furthermore, ecto-AK activity has been detected in the vitreous fluid of patients with diabetic retinopathy, correlating with elevated levels of ATP and ADP, particularly in those with diabetic vitreous hemorrhage (Zeiner et al., 2019). This correlation could be explained by the involvement of extracellular AK in the ATP-regenerating pathway, sustaining high levels of ATP and ADP (Zeiner et al., 2019). However, despite these findings, the mechanisms underlying the extracellular presence of AK and its specific functions remain unclear.

2.1.3.2.2 Nucleoside diphosphate kinase family

The NDPK family, encoded by the *NME* gene, is a group of transferases that catalyze the reversible exchange of phosphoryl groups between nucleotide tri- and diphosphates (**Table 2**). NDPK functions via a “ping-pong” mechanism, involving the formation of a phosphohistidine enzyme intermediate (Lascu & Gonin, 2000). Although ten human *NME*-encoded proteins have been identified, not all exhibit enzymatic activity (Boissan et al., 2009). The most studied members, NDPK-A and NDPK-B, encoded by the *NME1* and *NME2* genes, respectively, are hexameric enzymes composed of 17 kDa subunits. Their catalytic activity requires the presence of Mg^{2+} and other divalent cations (Yegutkin, 2014). These enzymes are ubiquitously expressed in the cytosol and nucleus of various tissues, with the highest levels found in the brain, kidney, liver, and heart (Bilitou et al., 2009).

Beyond regulating intracellular purine and pyrimidine nucleotide homeostasis, NDPK-A and NDPK-B are involved in several cellular processes (Boissan et al., 2018). These enzymes are crucial for membrane remodeling through their interaction with the dynamin superfamily, a group of GTPases involved in endocytosis (Prunier et al., 2023). By maintaining high local GTP concentrations via their nucleoside diphosphate kinase activity, NDPK-A and NDPK-B ensure optimal dynamin function (Boissan et al., 2014). Additionally, these enzymes may function as protein

histidine kinases, potentially further supporting dynamin-mediated endocytosis (Khan et al., 2019). NDPK-B's histidine protein kinase activity has also been shown to activate Ca^{2+}-activated K^+ channels on $CD4^+$ T cells, leading to T-cell activation and cytokine production (Panda et al., 2016; Srivastava et al., 2006). However, the exact mechanisms by which the NDPK active site interacts with large protein substrates remain unclear (Boissan et al., 2018). Furthermore, both enzymes are proposed to have additional intracellular functions, such as transcriptional regulation and DNA cleavage, though these roles require further critical evaluation (Steeg et al., 2011).

Another significant aspect of NDPK-A and NDPK-B is their involvement in tumor metastasis. The *Nme* gene was first identified in cancer research, where its high expression in murine cancer cells was linked to a lower tumor metastasis potential, leading to its designation as the non-metastatic (*Nme/NME*) gene (Steeg et al., 1988). Accordingly, overexpression of *Nme1* in murine melanoma cells significantly suppressed tumor metastasis without affecting the size of the primary tumor (Leone et al., 1991). Additionally, *Nme1* knockout mice showed an increased rate of lung metastases in a model of spontaneously developing hepatocellular carcinoma (Boissan et al., 2005). In human cancers, a negative correlation between *NME1* expression and tumor metastasis has been observed in melanoma, ovarian, and colon cancers (Flørenes et al., 1992; Mandai et al., 1994; Xerri et al., 1994; Yamaguchi et al., 1993). This anti-metastatic role may be partly explained by NDPK/*NME* interaction with the dynamin superfamily, promoting endocytosis and suppressing cell motility (Khan et al., 2019; Prunier et al., 2023). However, this role appears to be context-dependent, as *NME1* expression is associated with a higher metastatic risk and poorer clinical prognosis in neuroblastoma and hematological malignancies (Almgren et al., 2004; Niitsu et al., 2011; Tan & Chang, 2018). Further research is essential to elucidate the precise mechanisms by which NDPK/*NME* regulates metastasis across different cancer types, potentially offering new avenues for therapeutic interventions.

In addition to their intracellular roles, NDPK-A and NDPK-B may also exhibit extracellular functions (Romani et al., 2018). Extracellular activity of these enzymes has been observed in various cell types, including human astrocytoma cells, vascular ECs, airway epithelial cells, and lymphocytes (Donaldson et al., 2002; Yegutkin, 2014). Furthermore, several solid tumors and hematological malignancies have been shown to secrete soluble forms of NDPK-A/B, with elevated extracellular levels potentially serving as a prognostic marker (Niitsu et al., 2011; Okabe-Kado et al., 2005; Yokdang et al., 2015). Notably, NDPK-B secreted by breast cancer cells has been linked to the promotion of vascular angiogenesis, potentially through the synthesis of extracellular ATP, leading to the activation of P2Y receptors on ECs (Buxton et al., 2010; Yokdang et al., 2011). However, despite these findings, the

biological significance of extracellular NDPK in both physiological conditions and cancer remains unclear and requires further investigation.

Table 2. ATP-regenerating and Ado-degrading pathways. Extracellular AK and NDPK comprise the extracellular ATP-regenerating pathway. Their intracellular forms, along with cytosolic adenosine kinase (ADK-S), contribute to the Ado-salvage pathway (for more details, see **Figure 6**). ADA and purine nucleoside phosphorylase (PNP) are involved in the Ado-degrading pathway, functioning both intra- and extracellularly. Abbreviations: Ino, Inosine; Hyp, Hypoxanthine; NDP, Nucleoside diphosphate; NTP, Nucleoside triphosphate. Adapted from (Boison & Yegutkin, 2019). Created with Biorender.com.

	ISOZYME	GENE NAME	ALIASES	CATALYTIC REACTIONS	LOCALIZATION
Adenylate kinase	AK1	*AK1*	myokinase	ATP + AMP ⟷ 2ADP	extracellular cytosol
Nucleoside diphosphate kinase	NDPK-A NDPK-B	*NME1* *NME2*	Nm23-H1, NME1 Nm23-H2, NME2	ATP + NDP ⟷ ADP + NTP	extracellular cytosol
Adenosine deaminase	ADA1 ADA2	*ADA1* *ADA2*	-	Ado + H_2O ⟶ Ino	extracellular cytosol
Purine nucleoside phosphorylase	PNP	*PNP*	inosine phosphorylase	Ino + P_i ⟷ Hyp + α-D-ribose 1-phosphate	extracellular cytosol
Adenosine kinase	ADK-S ADK-L	*ADK*	-	Ado + ATP ⟶ AMP + ADP	cytosol nucleus

2.1.4 Cellular Ado uptake and metabolism

After completing their signaling functions, ATP and its derivates must be cleared from the extracellular space. As outlined in **Table 1**, specific nucleotide-hydrolyzing ectoenzymes play a crucial role in clearing extracellular ATP, ADP, and AMP. In turn, extracellular Ado can be degraded by the extracellular forms of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP), which sequentially convert Ado via inosine (Ino) into hypoxanthine (Hyp) (**Table 2**). Alternatively, extracellular Ado can be efficiently removed through cellular uptake via nucleoside transporters (**Figure 6**) (Boison & Yegutkin, 2019).

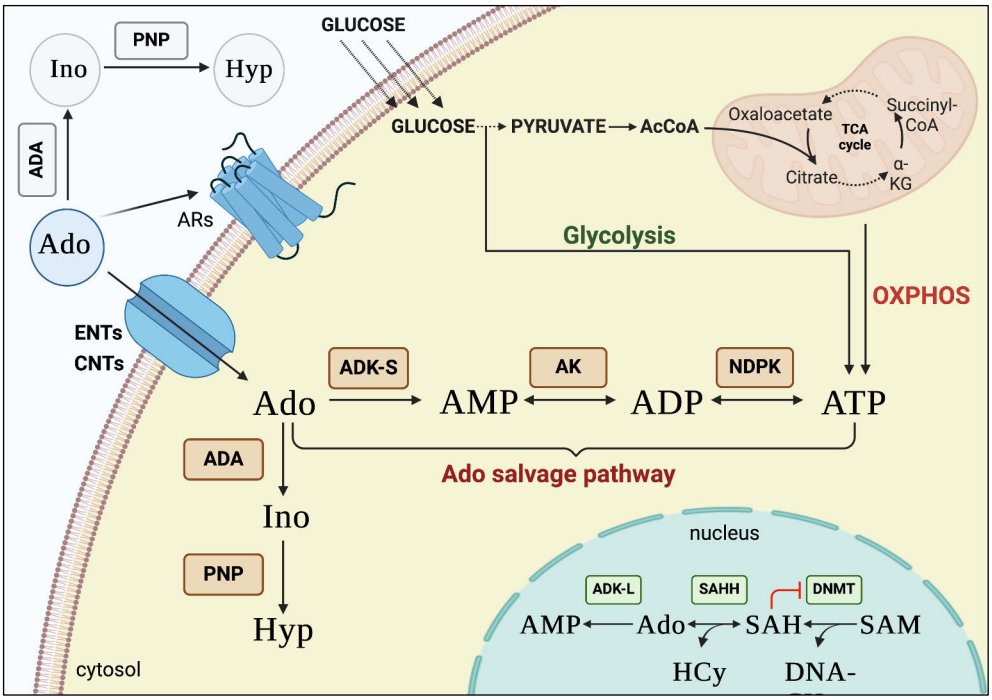


Figure 6. Cellular Ado turnover. Termination of AR signaling occurs through the removal of extracellular Ado. Extracellular Ado is either sequentially degraded by ADA and PNP into Ino and Hyp, or taken up by cells via nucleoside transporters (NTs), including equilibrative NTs (ENTs) and concentrative NTs (CNTs). ENTs facilitate the passive bidirectional transport of various nucleosides and their derivatives, including Ado, along the concentration gradient, while CNTs actively transport nucleosides and nucleobases solely into cells using the sodium gradient. Once inside the cells, Ado can either be degraded by intracellular ADA and PNP or phosphorylated. Cytosolic adenosine kinase (ADK-S) converts Ado into AMP, which is further phosphorylated to ADP and ATP via intracellular AK and NDPK. Mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis, both dependent on glucose uptake via glucose transporters, are the primary pathways for ATP production. However, ATP synthesis from internalized Ado, referred to here as the Ado salvage pathway, may also contribute to cellular ATP levels. While this pathway likely has minimal impact under physiological conditions, it may serve as an alternative ATP source in pathological states. In the nucleus, Ado metabolism is regulated by intranuclear adenosine kinase (ADK-L), which plays a role in epigenetic regulation. DNA methyltransferases (DNMTs) transfer methyl groups (-CH3) from S-adenosylmethionine (SAM) to DNA, generating S-adenosylhomocysteine (SAH) as a byproduct. Since SAH is a potent DNMT inhibitor, its removal is essential to maintain DNA methylation. ADK-L facilitates this process by reducing intranuclear Ado levels, allowing S-adenosylhomocysteine hydrolase (SAHH) to convert SAH into Ado and homocysteine (HCy). If Ado accumulates, it can drive the reverse SAHH-mediated reaction, leading to SAH accumulation and impaired DNA methylation. Adapted from (Boison & Yegutkin, 2019). Created with Biorender.com.

Once inside the cell, Ado can undergo a series of complex phosphotransfer reactions, starting with its conversion into AMP by cytosolic adenosine kinase (ADK-S) (Boison, 2013; Boison & Yegutkin, 2019). AMP can then be further

phosphorylated into ADP and ATP by intracellular forms of AK and NDPK. While nucleotide salvage pathways are typically associated with recycling free purine bases from degraded DNA and RNA to generate nucleotides (Pareek et al., 2021), this intracellular pathway for Ado may offer a route for nucleoside recycling, referred to here as the Ado salvage pathway (**Figure 6**) (Boison & Yegutkin, 2019). Alternatively, intracellular Ado can be degraded by intracellular forms of ADA and PNP, ultimately producing Hyp, which can either enter the nucleotide salvage pathway or be further metabolized into uric acid (Mandal & Mount, 2015).

Besides its well-established signaling roles via P1 receptors, recent research indicates additional regulatory functions of Ado through its intracellular metabolism (Boison & Yegutkin, 2019). For instance, erythrocytes rely on extracellular Ado uptake to maintain their intracellular nucleotide pool, which is crucial for proper erythropoiesis (Mikdar et al., 2021). In lung ECs, the metabolism of internalized Ado has been linked to mitochondrial oxidative stress, leading to impaired barrier function (Lu et al., 2012). Additionally, Ado treatment has been shown to reduce cancer cell invasiveness independently of AR activation (Virtanen et al., 2014). Another important regulatory role of Ado occurs within the nucleus (**Figure 6**). Elevated intranuclear Ado levels lead to the accumulation of S-adenosylhomocysteine (SAH), which inhibits DNA methyltransferases (DNMTs) and disrupts DNA methylation (Boison, 2013; Boison & Yegutkin, 2019). The nuclear isoform of ADK (ADK-L) reduces intranuclear Ado levels, thereby facilitating DNA methylation. This ADK-mediated epigenetic regulation has been shown to prevent epileptogenesis, as well as mediate vascular inflammation and angiogenesis (Williams-Karnesky et al., 2013; Xu et al.; 2017a; Xu et al., 2017b). However, the precise mechanisms linking Ado uptake, its cytosolic and nuclear metabolism, and their effects on various cellular outcomes remain unclear, highlighting the need for further investigation.

2.1.4.1.1 Nucleoside transporters

Nucleoside transporters are membrane proteins that facilitate the passage of nucleosides and their derivatives across cell membranes. They are divided into two distinct families: equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs) (Young, 2016). The human ENT family, encoded by the solute-carrier gene family 29 (*SLC29*), comprises four isoforms: ENT1-4. ENTs function as passive, bidirectional transporters, enabling nucleosides to move along their concentration gradient. Except for ENT4, whose role in nucleoside transport is not well-defined, all ENT isoforms demonstrate broad selectivity for transporting both purine and pyrimidine nucleosides, along with their derivatives (Pastor-Anglada & Pérez-Torras, 2018). In contrast, CNTs are active, Na^+-dependent

concentrative transporters that use the sodium gradient to move nucleosides and their derivatives exclusively into the cells (Young, 2016). The human CNT family consists of three isoforms, CNT1-3, encoded by the solute-carrier gene family 28 (*SLC28*). Each isoform exhibits specific substrate preferences: CNT1 transports pyrimidine nucleosides, CNT2 transports purine nucleosides, while CNT3 transports both pyrimidine and purine nucleosides. Compared to ENTs, CNTs generally have higher substrate affinities but lower turnover rates (Young et al., 2013). Additionally, CNTs show more specialized distribution, with some isoforms localized in the kidney, liver, and intestine, whereas ENTs are ubiquitously distributed throughout the body (Young et al., 2013).

The ENT family has been more extensively studied than the CNT family, partly due to the greater availability of selective pharmacological tools targeting ENTs (Pastor-Anglada & Pérez-Torras, 2018). Mammalian ENTs were initially categorized based on their sensitivity to the inhibitor S-(4-Nitrobenzyl)-6-thioinosine (NBTI) (Baldwin et al., 1999). Of the four identified ENT isoforms, ENT1 is the most sensitive to inhibition by NBTI at nanomolar concentrations, while other family members are either unaffected or inhibited only at micromolar concentrations (Young et al., 2013). Other ENT inhibitors, particularly those targeting ENT1 and ENT2, include clinically approved drugs such as Dilazep, Dipyridamole, and Ticagrelor. While Dilazep selectively inhibits ENTs, both Dipyridamole and Ticagrelor exhibit additional mechanisms by inhibiting phosphodiesterase 3 (PDE3) and $P2Y_{12}$ receptors, respectively. Nevertheless, the vasodilatory and antithrombotic effects of these drugs are at least partially related to their ability to increase extracellular Ado levels by blocking its cellular uptake, which leads to enhanced AR signaling (Cattaneo et al., 2014; Kaur et al., 2022; Molina-Arcas et al., 2009).

ENT1 and ENT2, in addition to terminating AR signaling via extracellular Ado uptake, plays crucial roles in cellular homeostasis by facilitating the uptake of various extracellular nucleobases and nucleosides essential for nucleotide salvage pathways (Boswell-Casteel & Hays, 2017; Yegutkin & Boison, 2022; Young et al., 2013). Recent studies have particularly highlighted the significance of ENT1-mediated extracellular Ado uptake in erythrocytes, where it maintains intracellular nucleotide homeostasis and sustains erythropoiesis (Mikdar et al., 2021). Notably, the link between erythrocytes and ENT1 gained new insight with the discovery that the previously known Augustin antigen on red blood cells originates from the *SLC29A1* gene (ENT1) (Daniels et al., 2015). This finding ultimately led to the recognition of the Augustine blood group system as the 36th human blood group (Storry et al., 2016). Several identified individuals with the Augustine-null blood type, who exhibit ENT1 deficiency, do not develop anemia, suggesting the presence of compensatory mechanisms that sustain erythropoiesis *in vivo* (van den Akker,

2021). However, the occurrence of ectopic mineralization in these individuals suggests an additional role for ENT1 in bone homeostasis (Daniels et al., 2015).

While ENT1 and ENT2 are primarily located on the plasma membrane, facilitating nucleoside transport between the extracellular and intracellular compartments, ENT3 is predominantly found within lysosomal and mitochondrial membranes, suggesting its involvement in organelle-specific nucleoside transport (Baldwin et al., 2005; Govindarajan et al., 2009). Although its precise role under normal homeostatic conditions remains unclear, ENT3 has been implicated in several pathological processes. For instance, it has been shown to promote viral infections by enabling the release of viral genomes from macrophage lysosomes, thereby facilitating viral replication within host cells (Hsieh et al., 2023). The histiocytosis observed in some patients with disorders linked to mutations in the *SLC29A3* gene (ENT3) (Kang et al., 2010), is likely due to impaired lysosomal function in macrophages, as indicated by studies in ENT3-deficient mice (Hsu et al., 2012). These mice exhibited defective clearance of apoptotic cells, leading to the accumulation of lysosomal nucleosides in macrophages and their pathological proliferation (Hsu et al., 2012). Further research is required to fully elucidate the role of ENT3 in both homeostatic and pathological conditions.

CNTs, due to their higher substrate specificity and specific distribution compared to ENTs, are thought to play a crucial role in maintaining nucleotide homeostasis by acting within specific tissues. In the small intestine, CNTs located on the apical membrane of epithelial cells are proposed to facilitate the absorption of dietary nucleosides (Young et al., 2013). Their high expression in hepatocytes and biliary epithelia suggests that CNTs contribute to the liver's production of non-dietary nucleotides by transporting nucleosides and their derivates required for nucleotide synthesis (Young et al., 2013). Studies on CNT1-deficient mice highlight the importance of CNT1 in kidney function, particularly its role in renal nucleoside reabsorption (Persaud et al., 2023). Despite these insights, the full range of CNTs' physiological functions remains largely unexplored.

The therapeutic relevance of both ENTs and CNTs is currently linked to their role in transporting nucleoside analog drugs used in cancer therapy (Hruba et al., 2023; Young et al., 2013). Both transporter families mediate the uptake of these drugs into cancer cells, where they are incorporated into nucleic acids, disrupting DNA and RNA synthesis and ultimately causing cancer cell death. Gemcitabine is one such example, with high levels of ENT1 in pancreatic cancer patients being associated with improved drug efficacy (Farrell et al., 2009; Greenhalf et al., 2014). CNT1 has also been shown to influence Gemcitabine efficacy, as studies in CNT1 knockout mice with pancreatic tumors demonstrated increased renal excretion of the drug, leading to reduced treatment efficacy and poorer outcomes (Persaud et al., 2023). Several strategies are being explored to enhance the delivery of nucleoside

analog drugs via nucleoside transporters (Kaur et al., 2022). However, the limited understanding of nucleoside transporter functions, their structures (with ENT1's structure only resolved in 2019) (Wright & Lee, 2019), and their involvement in nucleoside analog pharmacokinetics continues to hinder the potential for targeting nucleoside transporters in cancer therapies.

2.1.4.1.2 Adenosine kinase

ADK is a class of transferases that catalyzes the transfer of a phosphoryl group from ATP to Ado, forming AMP and ADP (Boison & Yegutkin, 2019). The active form of ADK is a monomeric protein with a molecular weight of approximately 40 kDa, comprising two domains: a large αβα domain and a small lid domain. The phosphorylation of Ado by ADK is proposed to follow an ordered Bi-Bi mechanism (Boison, 2013). Structural data for bacterial ADK reveal that Ado first binds to the catalytic site on the large domain, causing its rotation. This conformational change increases the enzyme's affinity for ATP. The subsequent binding of ATP to the Ado-enzyme complex induces additional conformational changes that facilitate the catalytic reaction. Once Ado phosphorylation is complete, the substrates are released, with AMP being the final product to dissociate (Park & Gupta, 2008).

Human ADK is encoded by the *ADK* gene, which undergoes alternative splicing to produce two isoforms: short (ADK-S) and long (ADK-L) isoforms. In ADK-S, the first four N-terminal amino acids are replaced by 21 N-terminal amino acids in ADK-L (McNally et al., 1997). Both isoforms function as high-affinity, low-capacity enzymes involved in the intracellular metabolism of Ado but differ in their subcellular localization: ADK-S is located in the cytoplasm, whereas ADK-L is found in the nucleus (Boison, 2013). ADK-S and ADK-L are prominently expressed in the kidney, liver, and lung, with additional tissue-specific distributions (Murugan et al., 2021). However, their precise tissue-specific localization and respective functions remain largely unknown.

The distinct subcellular localization of ADK-S and ADK-L suggests their unique functional implications. ADK-S, located in the cytosol, regulates AR signaling by clearing intracellular Ado, thus promoting the uptake of extracellular Ado through ENTs that transport nucleosides along the concentration gradient (Yegutkin & Boison, 2022). Beyond its role in cytosolic Ado clearance, ADK-S is thought to have broader implications for cellular metabolism, though these are not yet fully understood (Boison, 2013). It contributes to the Ado-salvage pathway by generating AMP, which can then be phosphorylated into ADP and ATP (Boison & Yegutkin, 2019). Additionally, AMP produced by ADK-S may activate AMPK, indicating a potential signaling role for ADK-S (Han et al., 2022). In a mouse model of type 2 diabetes, elevated extracellular Ado levels has been shown to impair muscle stem

cell regeneration via the ENT-ADK-AMPK signaling pathway (Han et al., 2022). ADK-L, present in the nucleus, functions as a regulator of DNA transmethylation, a fundamental process that mediates gene expression, maintains genomic stability, and determines epigenetic modifications within cells (Boison, 2013). This process involves the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to DNA, catalyzed by DNA methyltransferases. As a by-product of this reaction, S-adenosylhomocysteine (SAH) is generated, with its accumulation inhibiting DNA methyltransferases. Therefore, regulation of SAH levels is essential for maintaining DNA methylation. S-adenosylhomocysteine hydrolase (SAHH) controls SAH levels by catalyzing its reversible hydrolysis into Ado and L-homocysteine. The direction of this reaction depends on the thermodynamic state of the system, with continuous removal of SAH-derived Ado by ADK-L favoring SAH hydrolysis. If ADK-L is impaired, the reaction shifts towards SAH generation, leading to a decrease in DNA methylation and subsequent gene alterations (Boison, 2013). This epigenetic regulatory function of ADK-L has been shown to prevent epileptogenesis, regulate vascular inflammation, and modulate angiogenesis (Williams-Karnesky et al., 2013; Xu et al., 2017a; Xu et al., 2017b). Disruptions in the transmethylation pathway have been identified in patients with ADK deficiency, clinically manifesting as severe encephalopathy and liver pathology (Bjursell et al., 2011). The overexpression of ADK-L and its associated DNA hypermethylation has been documented in several other medical conditions, including brain injury, epilepsy, and atherosclerosis (Murugan et al., 2021).

The therapeutic potential of ADK has been previously explored, particularly due to strong evidence of its role in epileptogenesis. However, the high toxicity and severe side effects associated with several ADK-targeting compounds have reduced interest in further therapeutic development (Boison, 2013). Despite these challenges, ADK remains a promising target, with its relevance also suggested in cancer (Yegutkin & Boison, 2022). Given ADK's role in mediating Ado effects via both AR-dependent and AR-independent mechanisms, selective targeting of its isoforms may offer a novel approach to combat cancer by simultaneously interfering with multiple pathways (Köse et al., 2016; Toti et al., 2016; Yegutkin & Boison, 2022). Nevertheless, limited knowledge of ADK's physiological functions, its involvement in various pathologies, and the lack of selective and safe inhibitors continue to hinder its therapeutic application.

2.1.4.1.3 Adenosine deaminase

ADA is a class of hydrolases that catalyze the irreversible deamination of Ado and deoxyadenosine (dAdo) into Ino and deoxyinosine (dIno), respectively (Yegutkin, 2014). This enzyme family includes two isoenzymes, ADA1 and ADA2, each with

distinct catalytic, biochemical, and physiological roles. ADA1, which accounts for the majority of ADA activity in the body, is predominantly found intracellularly in its monomeric form (Meyts & Aksentijevich, 2018). It is characterized as a low-affinity, high-capacity enzyme with a molecular weight of approximately 41 kDa. Its physiological function includes regulating intracellular Ado levels, particularly when the limited capacity of ADK is exceeded (Boison, 2013; Spychala, 2000). Another crucial role of ADA1 is its involvement in dAdo metabolism, with ADA1 deficiency linked to severe combined immunodeficiency (SCID) due to the accumulation of dAdo and its toxic derivatives (Blackburn & Kellems, 2005). The absence of ADA1 is particularly detrimental to lymphocytes and NK cells, which are absent in patients with ADA1 deficiency (Flinn & Gennery, 2018). In addition to its intracellular localization, the extracellular presence of ADA1 has been documented on several cell types, as well as in its soluble form in blood circulation (Franco et al., 2007; Pettengill et al., 2013; Yegutkin, 2014). On immune cells, extracellular ADA1 is associated with glycoprotein CD26 or ARs (Kameoka et al., 1993; Moreno et al., 2018), where it is proposed to mediate immune responses both enzymatically, by removing local extracellular Ado (Dong et al., 1996), and non-enzymatically, by facilitating the formation of the immunological synapse (Pacheco et al., 2005). Recent findings further suggest the potential significance of extracellular ADA1 through its production of Ino. Extracellular Ino has been shown to support T-cell growth and function in the absence of glucose (Wang et al., 2020) and to induce a stem-cell-like phenotype in chimeric antigen receptor (CAR) T cells, enhancing their therapeutic potency (Klysz et al., 2024). Additionally, Ino produced by intestinal bacteria has been reported to significantly improve the efficacy of immune checkpoint inhibitors for cancer treatment (Mager et al., 2020). Although these effects may not be directly linked to ADA1, they suggest a potential role for ADA1 in Ino-mediated immune regulation.

ADA2 is a less-studied isoenzyme of the family, with a currently limited understanding of its physiological significance. It is a homodimeric enzyme with a molecular weight of 59 kDa, primarily secreted by myeloid cells into the extracellular space (Meyts & Aksentijevich, 2018). Compared to ADA1, ADA2 exhibits a significantly lower affinity for Ado, suggesting its negligible role in Ado metabolism under normal physiological conditions (Yegutkin, 2014). However, in pathological contexts where extracellular Ado levels are significantly elevated, ADA2 may become activated (Zavialov & Engström, 2005). A deficiency in ADA2 (DADA2) leads to several clinical manifestations, particularly affecting the immune and vascular systems (Meyts & Aksentijevich, 2018). Although the exact mechanisms underlying these symptoms remain unclear, disruptions in Ado metabolism are proposed to contribute to the vasculitis observed in DADA2 patients. In the absence of ADA2, enhanced AR signaling on neutrophils promotes the

formation of neutrophil extracellular traps (NETs), which in turn induce macrophage activation (Carmona-Rivera et al., 2019). This leads to the secretion of several pro-inflammatory cytokines by macrophages, including TNF-α and IL-6, further amplifying neutrophil activity and creating a viscus cycle of inflammation (Carmona-Rivera et al., 2019). Notably, TNF-α inhibitors are the primary treatment currently used in clinics for DADA2 patients. Beyond its enzymatic function, ADA2 also exhibits growth factor activity (Zavialov et al., 2010), though its physiological importance remains to be fully elucidated.

2.2 Current anti-cancer therapies and their limitations

According to the World Health Organization, cancer ranks among the top causes of global mortality, accounting for approximately 10 million deaths in 2020. This complex and heterogeneous group of diseases can be characterized by several key features, often referred to as the "hallmarks" of cancer. These include genome instability, immune system evasion, dysregulated cellular energetics, and tumor-associated inflammation (Hanahan & Weinberg, 2011). Among these characteristics, genome instability, leading to numerous mutations and disruption of the cell growth and division cycle, is often recognized as the initial trigger that drives normal cells into a cancerous state (Hahn & Weinberg, 2002). Nevertheless, the tumor microenvironment (TME), with its complex interactions among cancer cells, stromal cells, ECs, immune cells, and the extracellular matrix, plays a critical role in determining tumor cell fate (de Visser & Joyce, 2023). The TME can either suppress and eliminate tumor cells or promote cancer hallmarks, facilitating tumor growth, invasion, and metastasis. This understanding of TME's importance has led to a paradigm shift in cancer research, expanding the focus beyond the sole examination of cancer cells to a deeper exploration of TME's integral role in tumorigenesis. Although the composition of the TME varies depending on the origin and localization of cancer, extensive studies of specific cell types within the TME, such as ECs, cancer-associated fibroblasts (CAFs), and certain immune cells, including T cells, and tumor-associated macrophages (TAMs), have been conducted to understand their involvement in cancer progression (Hanahan & Coussens, 2012). These insights have driven significant advancements in cancer therapy, with modern strategies now aiming to disrupt the pro-tumor activity within the TME (Guelfi et al., 2024; Rannikko & Hollmén, 2024; Sharma et al., 2023). As a result, therapies such as anti-angiogenic therapy and immune checkpoint inhibitor therapy are now widely used in clinical settings, strategically targeting critical aspects of the TME (Ferrara & Adamis, 2016; Sharma et al., 2023).

2.2.1.1.1 Anti-angiogenic therapy

Angiogenesis, the formation of new blood vessels, plays a crucial role in the development of solid tumors. The uncontrolled proliferation of cancer cells often leads to oxygen deprivation and persistent hypoxia within the TME. In response to these low-oxygen conditions, various cell types activate HIF, a key regulator of cellular adaptation and survival under hypoxic environments, by modulating the expression of specific genes (Semenza, 2003). One important gene activated by HIF is *VEGF*, which encodes the cytokine vascular endothelial growth factor (VEGF), crucial for angiogenesis. Under normal conditions, VEGF-induced angiogenesis is a critical mechanism for tissue growth and repair. By binding to the specific tyrosine kinase receptor VEGFR2 on ECs, VEGF triggers the transition of these cells from a quiescent state to an activated state (Potente et al., 2011). This process involves the disruption of tight junctions between ECs, degradation of the vessel basement membrane, and pericyte detachment, enabling ECs to migrate and form new vascular sprouts. However, in the context of cancer, VEGF-induced angiogenesis becomes detrimental. Elevated VEGF levels within the TME, produced by cancer cells, CAFs, and TAMs, lead to the formation of poorly organized, chaotically branched and leaky vessels (Goel & Mercurio, 2013). This tumor-induced vascular remodeling not only impedes tissue perfusion, further contributing to the progression of the primary tumor, but also facilitates the process of metastasis (Mazzone et al., 2009; Weis et al., 2004).

Anti-angiogenic therapy is a pivotal strategy for targeting angiogenesis in solid tumors and other diseases characterized by pathological blood vessel formation (Cao et al., 2023). Among these, anti-VEGF therapy is a widely used approach with applications in both cancer and ocular diseases. This category includes FDA-approved drugs, such as Bevacizumab, a monoclonal anti-VEGF antibody used to treat multiple cancers; Ranibizumab and Faricimab, approved for ocular conditions like neovascular age-related macular degeneration and diabetic macular edema; and Aflibercept, a VEGF trap with applications in both ocular diseases and metastatic colorectal cancer (Cao et al., 2023). Other anti-angiogenic strategies target the VEGF receptor or its downstream tyrosine kinase signaling pathways and are primarily used in cancer therapy. These include Ramucirumab, an anti-VEGFR2 antibody, and tyrosine kinase inhibitors (TKIs) such as Sorafenib, Sunitinib, Axitinib, and Pazopanib, which block downstream signaling pathways (Cao et al., 2023). Currently, anti-angiogenic therapy serves as the first-line treatment for several cancers. For instance, Bevacizumab is used as a first-line treatment in combination with chemotherapy for metastatic colorectal cancer, while TKIs serve as a first-line monotherapy for metastatic renal cell carcinoma (Cao et al., 2023; Jayson et al., 2016). Nevertheless, despite its therapeutic benefits, anti-angiogenic therapy has notable limitations. Some cancer patients exhibit primary resistance to this therapy,

while others may develop resistance over time (Bergers & Hanahan, 2008). Compensatory pro-angiogenic growth factors, such as angiopoietin, fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor, are implicated in this acquired resistance (Lugano et al., 2020). Additionally, other signaling molecules, including cytokines and chemokines, along with secreted extracellular matrix-remodeling enzymes by TAMs and neutrophils further support tumor angiogenesis (De Palma et al., 2017). The heterogeneity of ECs of blood vessels within the TME adds another layer of complexity, as some vessels respond to anti-VEGF treatment while others remain resistant (Helfrich et al., 2010; Sitohy et al., 2012; Zeng et al., 2023). Notably, studies using mouse tumor models suggest that discontinuation of anti-VEGF therapy leads to the promotion of liver metastasis (Yang et al., 2016). Although this phenomenon has not yet been confirmed in humans, it highlights the need for further research to evaluate its clinical relevance and identify optimal treatment strategies. In light of these challenges, continued research is essential to refine anti-angiogenic therapy, particularly by exploring its potential in combination with immune checkpoint inhibitors (Apte et al., 2019; Kuo et al., 2024; Schmittnaegel et al., 2017), and to develop novel strategies to overcome resistance, ultimately improving cancer patient outcomes.

2.2.1.1.2 Immune checkpoint inhibitors

Immune system evasion is another critical factor in tumorigenesis. The human immune system serves as a vital defense mechanism, protecting the body against external invaders such as bacteria, viruses, and parasites, as well as its own mutated cells. Cancer cells, characterized by genome instability, numerous mutations, dysregulated RNA splicing, and disordered post-translational modifications, generate neoantigens absent in normal cells (Xie et al., 2023). These antigens from highly mutated tumors are recognized as foreign by the immune system, leading to their elimination. Conceptually, the development of immunity to cancer is termed as the “cancer-immunity” cycle (Chen & Mellman, 2013). This cycle commences with dendritic cells, professional antigen-presenting cells, capturing tumor-associated antigens within the TME and presenting them to naïve T cells in the draining lymph node. Upon encountering these antigens, naïve T cells undergo activation and subsequent differentiation into three distinct types of T cells: cytotoxic, helper, or regulatory T cells (Tregs). Cytotoxic T cells, also known as $CD8^+$ T cells, play a pivotal role in eradicating cancer cells. Following differentiation, cytotoxic T cells migrate to the site of inflammation where they recognize cancer cells through specific antigens presented on major histocompatibility complex-I (MHC-I) molecules, leading to the elimination of these malignant cells. This process of cancer cell elimination triggers the release of additional tumor-associated antigens,

subsequently initiating the next cycle of the "cancer-immunity" response (Chen & Mellman, 2013).

Once inflammation has resolved, it is crucial to terminate immune responses to prevent autoimmunity and damage to normal tissues and organs. Immune checkpoint molecules, such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), expressed on effector T cells, play vital roles in negatively regulating immune responses. Upon binding to their respective ligands, PD-L1 and CD80 or CD86, expressed on various immune cells, including dendritic cells and macrophages, these immune checkpoint molecules transmit inhibitory signals to T cells and suppress their functions (Pardoll, 2012). While this regulatory mechanism is essential for maintaining immune homeostasis, tumors can exploit these pathways to their advantage. Specifically, within the TME, tumors induce the expression of ligands for immune checkpoint molecules, resulting in the inhibition of effector T-cell function and evasion of immune surveillance (Pardoll, 2012). This discovery led to the development of immune checkpoint therapies, which includes several FDA-approved antibodies targeting PD-1 (Pembrolizumab, Nivolumab, and Cemiplimab), PD-L1 (Atezolizumab, Avelumab, and Durvalumab), as well as CTLA-4 (Ipilimumab and Tremelimumab) (Patel et al., 2024; Sharma et al., 2021). These drugs are widely used in cancer treatment, either as monotherapies, in combination with other immune checkpoint inhibitors, or alongside additional therapies, including chemotherapy and radiation therapy (Sharma et al., 2023). However, despite the potency of these treatments, a significant portion of cancer patients do not respond to immune checkpoint therapies. Moreover, some patients who initially exhibit sensitivity to these therapies may eventually develop adaptive resistance over time (Sharma et al., 2017). Additionally, the use of immune checkpoint therapies can lead to the development of serious immune-related adverse events, which in some cases can be fatal (Boutros et al., 2016; Johnson et al., 2022).

Ongoing research continues to uncover suppressive factors and mechanisms of cancer immune surveillance that have the potential to enhance existing immunotherapies. In addition to well-known targets such as PD-1, PD-L1, and CTLA-4, other immune checkpoint molecules, contributing to immune suppression, have emerged, including TIGIT, TIM3, LAG3, and VISTA (Kraehenbuehl et al., 2022). Clinical trials are actively evaluating antibodies against these targets. Notably, Relatlimab, the anti-LAG3 antibody, has recently obtained approval for treating advanced melanoma in combination with Nivolumab, the anti-PD-1 antibody (Amaria et al., 2022; Tawbi et al., 2022). Furthermore, intrinsic properties of tumor cells and specific characteristics of the TME contribute to immune suppression and resistance to current immune checkpoint therapies (Sharma et al., 2017). Cancer cells utilize various mechanisms to evade recognition by T cells, including the absence of antigenic mutations, loss of MHC-I expression, and

alterations in antigen processing machinery (Sharma et al., 2017). Another crucial factor contributing to the failure of immune checkpoint therapies is IFN-γ, with its complex and context-dependent role in cancer (Gocher et al., 2022). Prolonged exposure to IFN-γ has been demonstrated to induce epigenetic and transcriptomic modifications in cancer cells, leading to immune checkpoint resistance (Benci et al., 2016). Furthermore, chronic IFN-γ signaling within the TME has been suggested to inhibit the maintenance, clonal diversity, and proliferation of intra-tumoral stem-like T cells, which are crucial for mediating the response to immunotherapies (Mazet et al., 2023; Miller et al., 2019). However, in other studies, impaired IFN-γ signaling in cancer cells have been associated with resistance in melanoma patients to anti-CTLA-4 and anti-PD-1 therapies, underscoring the importance of a deeper understanding of the multifaceted role of IFN-γ in tumorigenesis (Gao et al., 2016; Zaretsky et al., 2016).

Similarly, TGF-β, another abundant cytokine within the TME, plays a dual role in cancer progression (David & Massagué, 2018). It regulates various cellular processes, such as growth, differentiation, and apoptosis, and dysregulation of its signaling can promote tumorigenesis (David et al., 2016; Siegel & Massagué, 2003). Alterations in TGF-β receptor genes have been observed in pancreatic and ovarian cancer patients (Goggins et al., 1998; Wang et al., 2000). In mouse models of colorectal and pancreatic cancer, TGF-β has been shown to suppress tumor progression (Becker et al., 2004; David et al., 2016). However, as tumors advance, TGF-β can exhibit a tumor-promoting role, along with hindering the efficacy of existing immunotherapies. Elevated TGF-β levels in the TME have been shown to exclude T cells from tumors, thus impairing the effectiveness of anti-PD-1 blockade (Mariathasan et al., 2018; Tauriello et al., 2018). Furthermore, TGF-β signaling activates genes that facilitate the metastatic invasion of cancer cells, promoting their infiltration and colonization of specific organs (Kang et al., 2005; Padua et al., 2008). Additionally, TGF-β-induced epithelial-to-mesenchymal transition, crucial for tissue (re)generation during development and wound healing, holds the potential to further promote tumor invasion and metastatic dissemination when it occurs in cancer cells (Dongre & Weinberg, 2019). Nonetheless, despite the complexity of TGF-β signaling in cancer, ongoing preclinical and clinical trials are evaluating various approaches to inhibit TGF-β signaling in cancer patients, either alone or in combination with other targeted therapies (Batlle & Massagué, 2019). However, to effectively harness the therapeutic potential of TGF-β targeting, a comprehensive understanding of its multifaceted roles is essential. This knowledge will facilitate precise patient selection for successful clinical trials, thus enabling the development of effective TGF-β-based therapeutic strategies.

In addition to the suppressive factors mentioned above, the purinergic signaling system represents another significant obstacle to the effectiveness of current immune

checkpoint therapies. Its potential as a therapeutic target in cancer will be discussed in the following chapter.

2.3 Purinergic signaling system in cancer

2.3.1 Therapeutic potential of the CD39-CD73-AR axis in cancer

The purinergic signaling system has recently gained significant attention in cancer research (Di Virgilio et al., 2024; Junger, 2011; Yegutkin & Boison, 2022). The TME is characterized by high levels of extracellular Ado, primarily due to the metabolism of ATP, which is extensively released by various cells in response to stimuli such as inflammation, hypoxia, apoptosis, and necrosis. The high expression of purine-converting ectoenzymes, with CD39 and CD73 currently considered the primary contributors to Ado generation within the TME, leads to potent immunosuppression through AR activation (Allard et al., 2017; Vijayan et al., 2017). Among these receptors, T-cell A_{2A}AR and A_{2B}AR are recognized as key mediators of immunosuppressive signaling (Allard et al., 2020; Ohta et al., 2006; Vijayan et al., 2017). In particular, A_{2A}AR activation on cytotoxic T cells triggers a cAMP-dependent signaling pathway that inhibits T-cell proliferation and effector function (Allard et al., 2020; Cekic & Linden, 2016). This pathway activates protein kinase A (PKA), which phosphorylates C-terminal Src kinase (Csk), a negative regulator of lymphocyte-specific protein tyrosine kinase (Lck). Since Lck is a critical component of the signaling cascade following TCR activation, its inhibition ultimately suppresses the transcription factors such as NF-kB, NFAT, and AP-1, required for T-cell proliferation and cytotoxicity, thereby weakening the immune response against tumors (Allard et al., 2020; Cekic & Linden, 2016).

Beyond suppressing effector T cells, A_{2A}AR signaling plays a broader role in dampening anti-tumor immunity within the TME through the impairment of NK maturation and promotion of Tregs expansion (Ohta et al., 2012; Vijayan et al., 2017; Young et al., 2018; Zarek et al, 2008). Supporting its role in immune evasion, A_{2A}AR-deficient mice exhibit significant tumor growth suppression, leading to the rejection of about 60% of immunogenic tumors compared to wild-type (WT) mice (Ohta et al., 2006). Furthermore, CRISPR/Cas9-mediated deletion of A_{2A}AR on CAR-T cells significantly enhances their tumor-killing efficacy in a murine cancer model (Giuffrida et al., 2021). Similar to A_{2A}AR, activation of A_{2B}AR on various cell types, with its signaling typically linked to pathological conditions, also contributes to tumorigenesis (Allard et al., 2020). For instance, stimulation of A_{2B}AR on dendritic cells disrupts their differentiation and maturation (Novitskiy et al., 2008; Wilson et al., 2009). A_{2B}AR signaling on cancer cells and other cell types within the

TME promotes tumor angiogenesis by inducing the production of IL-8 and VEGF (Merighi et al., 2007, 2009; Ryzhov et al., 2008; Vijayan et al., 2017). Given these detrimental roles of both A_{2A}AR and A_{2B}AR in cancer progression, several A_{2A}AR and A_{2B}AR antagonists are currently under active investigation in preclinical and early-phase clinical trials for cancer treatment (Di Virgilio et al., 2024; Kutryb-Zając et al., 2023; Offringa et al., 2022).

Targeting the upstream mechanisms of Ado production in cancer represents another promising strategy. Extensive research has focused on identifying the key enzymes responsible for its generation within the TME, with CD39 and CD73 currently regarded as the major players (Allard et al., 2017). In several preclinical cancer models, genetic knockout or pharmacological inhibition of these enzymes has resulted in significant resistance to tumor growth and metastasis, underscoring their critical role in tumorigenesis (Jackson et al., 2007; Stagg et al., 2010, 2011; Sun et al., 2010; Allard et al., 2017; Perrot et al., 2019; Moesta et al., 2020; Liu et al., 2022). Multiple cell types within the TME, including immune, endothelial, stromal, and cancer cells, express CD39 and CD73, each contributing to pathological immunosuppression and angiogenesis (Allard et al., 2019; Moesta et al., 2020).

Among the immune cells generating immunosuppressive Ado, Tregs play a particularly significant role (Allard et al., 2017; Borsellino et al., 2007; Deaglio et al., 2007). In addition to inhibiting antigen-presenting cell function through CTLA-4-mediated signaling and secreting immunosuppressive cytokines like IL-10, IL-35, and TGF-β, Tregs also suppress immune responses via Ado production (Tay et al., 2023; Togashi et al., 2019). By expressing both CD39 and CD73, murine Tregs have been shown to inhibit the cytotoxic functions of T cells and NK cells through AR-mediated signaling (Borsellino et al., 2007; Deaglio et al., 2007). While this Ado-mediated suppression is essential for maintaining immune homeostasis, in cancer it contributes to immune evasion, promoting tumor growth and metastasis (Antonioli et al., 2013; Sun et al., 2010). Intriguingly, apoptotic murine Tregs exhibit even greater immunosuppressive capacity compared to their live counterparts, driven by higher Ado production, which further promotes cancer progression (Maj et al., 2017). In particular, this enhanced immunosuppression has been shown to undermine the effectiveness of PD-1/PD-L1 checkpoint inhibitors in murine cancer models (Maj et al., 2017; Togashi & Nishikawa, 2017). Notably, while human Tregs express CD39, their expression of CD73 remains controversial, highlighting differences in purinergic profiles between species (Mandapathil et al., 2009; Schneider et al., 2021; Schuler et al., 2014). Nevertheless, Ado-mediated immunosuppression by human Tregs likely depends on CD73 from other sources, such as T cells, cancer cells, or $CD73^+$ extracellular vesicles, to sustain Ado production and contribute to the immunosuppressive environment (Clayton et al., 2011; Gourdin et al., 2018; Schneider et al., 2021; Schuler et al., 2014).

In addition to Tregs, CD39 expression has been also observed on other T-cell subtypes within the TME, including exhausted T cells (Canale et al., 2018; Duhen et al., 2018; Sade-Feldman et al., 2018; Simoni et al., 2018). These tumor-infiltrating $CD8^+$ T cells recognize tumor-specific antigens but, due to chronic stimulation within the TME, exhibit reduced cytotoxic capacity (Simoni et al., 2018). Although the exact role of CD39 on this subset of T cells is not fully understood, CD39 deletion in terminally exhausted T cells significantly enhanced the efficacy of anti-PD-1 and anti-CTLA-4 therapies in a murine melanoma model, indicating its immunosuppressive function (Vignali et al., 2023).

TAMs represent another immune cell subset that drives Ado-mediated immune suppression within the TME, with studies demonstrating their expression of both CD39 and CD73 (d'Almeida et al., 2016; Goswami et al., 2020; Montalbán del Barrio et al., 2016; Takenaka et al., 2019; Zhang et al., 2022). CD39 activity on TAMs has been shown to not only induce AR signaling on T cells, suppressing their proliferation (Montalbán del Barrio et al., 2016), but also to shape the TAMs' own immunosuppressive profile in an autocrine manner, as indicated by reduced IL-10 secretion following CD39 blockade in LPS-stimulated TAMs (d'Almeida et al., 2016). Notably, beyond its functional role, CD39 has been also proposed as a cellular marker for targeting TAMs and other cell types with abnormal CD39 expression within the TME, including tumor ECs (Zhang et al., 2022). The use of engineered anti-CD39 antibody, which induce antibody-dependent cellular cytotoxicity against both of these cell subsets, has been shown to significantly reduce tumor neovascularization and growth *in vivo* (Zhang et al., 2022).

The important regulatory role of CD39 and CD73 on ECs in maintaining vascular homeostasis has been demonstrated by numerous studies. By producing Ado, these enzymes mediate hemostasis, blood flow and vascular tone, as well as control endothelial barrier function under hypoxia (Synnestvedt et al., 2002; Robson et al., 2006; Yegutkin, 2021). In cancer, the functional implication of CD39 on ECs has been shown by using CD39 knockout mice (Jackson et al., 2007). Tumor-bearing CD39 knockout mice exhibited reduced tumor neovascularization due to disrupted activation of integrin-associated signaling pathways in ECs (Jackson et al., 2007). The high levels of CD39 observed in tumor ECs (Zhang et al., 2022) suggest that, in addition to its impact on angiogenesis, EC-derived CD39 may further contribute to generate Ado-enriched TME. However, further studies are required to define the roles of endothelial CD39 and CD73 in tumorigenesis.

CAFs are another contributor to Ado-mediated immunosuppression within the TME, primarily through their CD73 expression. High CD73 levels have been documented in CAFs from breast, ovarian, and colorectal cancers (Costa et al., 2018; Montalbán del Barrio et al., 2016; Yu et al., 2020). In triple-negative breast cancer, one of the four identified CAF subsets is characterized by high CD73 expression,

along with other markers such as B7H3 and DPP4 (Costa et al., 2018). This subset has been shown to promote Treg differentiation and enhance their immunosuppressive function toward effector T cells. Silencing CD73 in this CAF subset led to reduced Treg survival, underscoring the functional role of CD73 in mediating immunosuppression by CAFs (Costa et al., 2018). The observed effects are likely mediated by CD73-generated Ado, which promotes Treg expansion and enhances their immunosuppressive capacity through AR signaling (Ohta et al., 2012; Zarek et al., 2008).

Expression of both CD39 and CD73 has been additionally documented on cancer cells and cancer-derived vesicles originating from various tissues, with their enzymatic activities contributing to Ado-mediated immunosuppression (Allard et al., 2019; Bastid et al., 2015; Clayton et al., 2011; Jin et al., 2010; Sade-Feldman et al., 2018). The detrimental role of CD73 derived from breast cancer cells has been particularly highlighted in several *in vivo* studies, where it was linked to tumor growth, metastasis, and angiogenesis (Stagg et al., 2010; Beavis et al., 2013; Allard et al., 2014). Moreover, CD73 on cancer cells has been shown to promote resistance to anthracycline-based chemotherapy, highlighting its role in reducing the effectiveness of this cancer treatment (Loi et al., 2013).

Based on these premises, numerous clinical trials aim to evaluate small-molecule inhibitors and antibodies targeting CD39 and CD73 in cancer patients, either as monotherapies or in combination with other treatments (Di Virgilio et al., 2024; Yegutkin & Boison, 2022). However, despite the promising potential of targeting Ado metabolism in cancer, a significant number of the trials have been terminated or have failed to demonstrate outcomes superior to standard-of-care treatments (Di Virgilio et al., 2024). With only two agents advancing to Phase III, including the anti-CD73 antibody Oleclumab (PACIFIC-9/NCT05221840) and the small-molecule CD73 inhibitor Quemliclustat (PRISM-1/NCT06608927), the majority of ongoing trials remain in Phase I or I/II, including AR targeting ones. This difficulty in translating preclinical success into clinical outcomes underscores the need for a deeper understanding of the mechanisms driving Ado metabolism in the TME, as well as the discovery of additional pathways and targets that could enhance the therapeutic efficacy of Ado-based treatments.

2.3.2 Challenges in leveraging Ado-based cancer therapies

The complexity of the TME presents significant challenges in translating Ado-based therapies into clinical applications. The dynamic regulation of CD39 and CD73 expression by various stimuli, including hypoxia, immune-modulating molecules such as IFN-β/γ and TGF-β, as well as chemotherapy agents, highlights the intricate nature of Ado metabolism within the TME (Chen et al., 2019; Kiss et al., 2007;

Kurnit et al., 2021; Li et al., 2017; Loi et al., 2013; Vijayan et al., 2017). Moreover, focusing solely on CD39 and CD73 as mediators of Ado metabolism may provide an overly simplistic view, as other extracellular purine-converting ectoenzymes, such as ENPP1, NDPK, AK, and ADA, may not only regulate extracellular nucleotide and nucleoside levels but also contribute to tumor progression and immune modulation (Yegutkin & Boison, 2022). For instance, recent research has highlighted the detrimental role of ENPP1 in tumorigenesis (Carozza et al., 2020; Ruiz-Fernández de Córdoba et al., 2023; Wang et al., 2023). By hydrolyzing cGAMP, ENPP1 suppresses the pro-inflammatory cGAS-STING pathway, thereby facilitating tumor growth. Moreover, through its ability to hydrolyze both ATP and cGAMP into AMP, ENPP1, along with CD73, may contribute to immunosuppression within the TME via AR signaling. Additionally, the involvement of ENPP1, CD73, and the ectoenzyme CD38 (NADase) in metabolizing NAD into Ado further complicates our understanding of the redundant Ado-generating pathways in the TME (Yegutkin & Boison, 2022).

The composition and activity of purine-converting ectoenzymes differ across various cell types, underscoring the need for a more comprehensive understanding of purine metabolic networks within the TME. This is further emphasized by the variable prognostic significance of CD73 across different cancers: while CD73 is associated with poor outcomes in breast, ovarian, and colorectal cancers (Loi et al., 2013; Turcotte et al., 2015; Wu et al., 2012), its high expression correlates with better prognosis in endometrial and bladder cancers (Bowser et al., 2016; Koivisto et al., 2019; Wettstein et al., 2015). Furthermore, even within the same cancer type, patients with immune “cold” and “hot” tumors exhibit distinct purinergic signatures (Yegutkin & Boison, 2022), highlighting the need to additionally account for the immune landscape of the TME when designing Ado-based therapies.

In addition to extracellular Ado metabolism, the non-receptor mediated effects of Ado remain largely underexplored, emphasizing the necessity for further investigation (Yegutkin & Boison, 2022). While several studies have demonstrated the functional role of intracellular Ado metabolism in ECs, erythrocytes, and several other cell types, its broader implications in both physiological and disease contexts remain under-studied (Virtanen et al., 2014; Xu et al., 2017a,b; Mikdar et al., 2021; Yegutkin & Boison, 2022). This gap is particularly relevant within the Ado-rich and nutrient-deprived TME, enabling cells to internalize Ado and potentially utilize the Ado-salvage pathway as an alternative energy source. The interplay between extracellular and intracellular Ado metabolism has been largely overlooked, yet it may significantly influence cellular behavior, with potential implications for cancer progression and immune evasion.

Finally, while much of the data on immunosuppressive Ado pathways in cancer has been derived from mouse models, significant species differences between mice

and humans must be acknowledged (Shay et al., 2013; Yegutkin & Boison, 2022). For instance, CD73-deficient mice and humans exhibit markedly different clinical outcomes, with CD73 deficiency in humans leading to severe vascular calcification, a condition not observed in mice (Joolharzadeh & St Hilaire, 2019). Additionally, variations in purinergic signaling among immune cells, including Tregs and B cells (Deaglio et al., 2007; Schneider et al., 2021; Shay et al., 2013), further emphasize the importance of studying Ado metabolism in human-derived materials for clinical translation.

Taken together, addressing these gaps is essential for advancing Ado-based therapies in cancer. A more detailed exploration of both extracellular and intracellular Ado pathways in humans, considering the diverse cellular composition of the TME and cell-specific purinergic signatures, will not only deepen our understanding of the intricate role of Ado metabolism in tumor progression and immune evasion but also facilitate the development of more targeted and effective therapeutic strategies.

3 Aims

The purinergic signaling system has recently emerged as a promising target for addressing immune checkpoint therapy-resistant cancer. Prior research has primarily focused on immunosuppression mediated by the ATP-inactivating/Ado-generating pathway, involving ectoenzymes CD39 and CD73, and $A_{2A/2B}$AR signaling. This focus has led to the development of specific antibodies and small molecule inhibitors targeting this axis, some of which are currently in preclinical and clinical trials. However, several significant knowledge gaps limit the therapeutic potential of leveraging Ado metabolic and signaling pathways in cancer treatment. Extracellular Ado levels and subsequent AR activation are regulated not only by CD39 and CD73 but also by other ATP-inactivating, ATP-regenerating, and Ado-inactivating ectoenzymes. Additionally, the expression levels and activities of these ectoenzymes vary across different cell subsets within the TME and can be modulated by factors such as hypoxia and various signaling molecules present in the TME. Moreover, while the receptor-mediated functions of Ado are well established, the role of its intracellular metabolism in regulating cellular processes remains poorly understood, particularly in the context of cancer progression and immune evasion. Therefore, the goal of this study was to thoroughly investigate both the extracellular and intracellular Ado metabolic pathways across different cell subtypes and evaluate their modulation under hypoxic and Ado-enriched conditions, which reflect key characteristics of the TME.

The specific aims of the study were:

I. To investigate adaptive changes in extracellular and intracellular Ado metabolic pathways in human vascular ECs under hypoxia

II. To investigate adaptive changes in extracellular and intracellular Ado metabolic pathways in human cancer cells under hypoxia

III. To evaluate the impact of Ado-enriched conditions on human T-cell activation and function through AR-dependent and AR-independent pathways

4 Materials and Methods

This chapter provides an overview of the materials and methods employed in the original publications **I-III**. The summary of these methods is listed in **Table 3**. For more detailed description, refer to the original publications and their supplements.

Table 3. Summary of methods used in the original publications.

Method	Publication
Cell isolation, culture, and experimental conditions	I, II, III
Immunofluorescence microscopy	I, II, III
Flow cytometry	I, II, III
Thin-layer chromatography	I, II, III
Quantification of ATP levels	I, II, III
Phospho-kinase array	I
Western blotting	I, II
T-cell metabolic assay	III
Single-cell sequencing	III
Enzyme histochemistry	II
In vivo experiments	II
Statistical analysis	I, II, III

4.1 Antibodies

The list of primary antibodies utilized in the following methods: immunofluorescence microscopy (IF), flow cytometry (fluorescence-activated cell sorting, FACS), and Western blotting (WB), is presented in **Table 4**.

Table 4. Summary of the antibodies used in the original publications.

Target	Clone / catalogue number	Host	Manufacturer	Application / Publication
ADK	Polyclonal	Rabbit	Provided by Prof. Boison	IF, WB / II
CD25-PerCP-Cy5.5	BC96/ 302626	Mouse	BioLegend	FACS / III
CD3-Alexa Fluor 488	HIT3a/ 300320	Mouse	BioLegend	FACS / III
CD31-Alexa Fluor 488	390/ 102414	Rat	BioLegend	IF / II
CD39	hN1-1_C	Guinea pig	Provided by Prof. Sevigny	IF, FACS / I, II, III
CD39-BV711	A1/ 328228	Mouse	BioLegend	FACS / III
CD4-PE	RPA-T4/ 555347	Mouse	BD Biosciences	FACS / III
CD73	h5NT-1_L	Rabbit	Provided by Prof. Sevigny	IF, FACS / I, II, III
CD73	4G4	Mouse	Produced in house	IF, FACS / I, II
CD73	D7F9A/ 87661	Rabbit	Cell Signaling	WB / II
CD73-PE-CF594	AD2/ 562817	Mouse	BD Biosciences	FACS / III
CD8a-BV785	RPA-T8/ 301045	Mouse	BioLegend	FACS / III
ENPP1 (CD203a)	3E8	Mouse	Provided by Prof. Malavasi	IF, FACS / II
ENT1-Alexa Fluor 647	SP120/ Ab311105	Mouse	Abcam	IF, FACS / III
GranzB- PE-Cy7	QA16A02/ 372213	Mouse	BioLegend	FACS / III
HIF-1α	H1alpha67/ MA1-16504	Mouse	ThermoFisher	IF, WB / II
IFN-γ-BV785	4S.B3/ 502541	Mouse	BioLegend	FACS / III
p-MLC2	Polyclonal / 3674	Rabbit	Cell Signaling	IF / I
PD-1- BV650	EH12.2H7/ 329950	Mouse	BioLegend	FACS / III
Phosphotyrosine	4G10/ 05-321	Mouse	MERCK	IF, WB / I
Vimentin	Polyclonal/ 919101	Chicken	BioLegend	IF / I, II, II
β-tubulin	TUB 2.1/ T4026	Mouse	Sigma-Aldrich	IF, WB / I, II

4.2 Cell isolation, culture, and experimental conditions

4.2.1 Cell line culture and hypoxia treatment

4.2.1.1 Human cell line culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords of healthy pregnancies with the approval of the Ethical Committee of Turku University Hospital. The human triple-negative breast cancer cell line MDA-MB-231 and the prostate androgen-independent prostate carcinoma cell line PC3 were obtained from ATCC (Manassas, USA). Cell cultures were maintained in 75 cm^2 tissue culture flasks at 37°C in a humidified incubator with 21% O_2 and 5% CO_2. HUVEC were cultured in endothelial cell growth basal medium-2 (EGM-2, Lonza, CC-3156), supplemented with the EGM-2 endothelial SingleQuots Kit (Lonza, CC-4176), 100 U/ml penicillin, and 100 µg/ml streptomycin. The MDA-MB-231 and PC3 cell lines were cultured in Dulbecco's modified Eagle-medium-high glucose (DMEM-high glucose, Sigma-Aldrich, D6429), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. A summary of the utilized cell lines and their respective culture media is provided in **Table 5**.

4.2.1.2 Hypoxia treatment

HUVEC, MDA-MB-231, or PC3 were harvested using trypsin/EDTA and seeded into appropriate tissue culture plates. Cells designated as "normoxic" were cultured in a humidified incubator with 21% O_2 and 5% CO_2 for 4-24 hours, while "hypoxic" cells were exposed to 1% O_2 in a humidified hypoxic chamber (Biotrace Ruskinn InvivO2 Hypoxia Workstation C400C Plus) for the same duration. Subsequently, the cells were either processed immediately for experimental assays or subjected to an additional hour of incubation in a humidified atmosphere with 21% O_2 to generate "hypoxia-reoxygenation" conditions. All assays for hypoxic cells were conducted and terminated within the hypoxic chamber, using tightly adjusted sleeves to prevent uncontrolled reoxygenation.

Table 5. Summary of the used cell lines and their respective media.

Cell line	Cell type	Culture medium	Manuscript
HUVEC	Human umbilical vein endothelial cells	EGM-2 BulletKit	I
MDA-MB-231	Human triple negative breast cancer	DMEM-high glucose	II
MDA-MB-231-LUC-D3H2LN	Bioluminescent human triple negative breast cancer	DMEM-high glucose	II
PC3	Human androgen-independent prostate carcinoma	DMEM-high glucose	II

4.2.2 Primary human T cells and Ado treatment

4.2.2.1 T-cell isolation and activation

Primary T cells were isolated from healthy donors using the EasyStep™ T-cell Isolation Kit (Stem Cell Technology, 17951) according to the manufacturer's instructions. The cells were either maintained in a non-activated state in the presence of 10 ng/ml IL-7 (Miltenyi Biotec, 130-095-362) or activated for 3 days with Human T-Activator CD3/CD28 Dynabeads™ (ThermoFisher Scientific, 1131D) in the presence of 30 U/ml IL-2 (Miltenyi Biotec, 130-097-744). The cells were cultured in OpTmizer™ CTS™ T-Cell Expansion Medium (ThermoFisher Scientific, A1048501), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, in 24 or 96-well plates at 37°C in a humidified incubator with 21% O_2 and 5% CO_2.

4.2.2.2 T-cell treatment with Ado and other purinergic drugs

The effects of Ado on T-cell metabolism and function were evaluated under two different experimental conditions. In the first settings (set 1), Ado was added at the stage of T-cell activation. Specifically, 10 µM or 100 µM Ado (Sigma-Aldrich, 01890) was added to freshly isolated T cells one hour after the addition of CD3/CD28 Dynabeads. Ado either remained throughout the entire 3-day period of T-cell activation or was removed after 2 or 24 hours of incubation. In the second settings (set 2), isolated T cells were first activated with CD3/CD28 Dynabeads for 3 days, followed by treatment with 10 µM or 100 µM Ado for 2, 4, 8, 18 and 24 hours, respectively.

Additional treatments included the $A_{2A/2B}$AR antagonist AB-928 (Cayman Chemical, 31444) and the ENT inhibitors Dipyridamole (Sigma-Aldrich, D9766)

and S-(4-Nitrobenzyl)-6-thioinosine (NBTI, Sigma-Aldrich, N2255). In set 1, 1 µM of the respective inhibitors were added simultaneously with CD3/CD28 Dynabeads. In set 2, 1 µM of the inhibitors were added to activated T cells one hour before Ado addition. Alternatively, a subset of activated T cells was pre-treated with the inhibitor of glycolysis 2-deoxy-D-glucose (2DG, 80mM, Sigma-Aldrich, D8375) for 2 hours, followed by washing and subsequent Ado treatment. IL-2 was continuously present in the culture medium for all treatments in both sets.

4.3 Immunofluorescence microscopy

Adherent cells (HUVEC, MDA-MB-231, PC3) and T cells were seeded on 13-mm coverslips in 24-well plates. To facilitate T-cell attachment, the coverslips were pre-coated with 200 µL of Poly-L-lysine (Sigma-Aldrich, P4707) for 5 minutes, followed by two washes with H_2O and air-drying. After subjecting the cells to the respective treatments as specified above, they were washed once with PBS and fixed in PBS containing 4% paraformaldehyde. Next, the cells were processed for staining of purine-converting ectoenzymes. They were incubated with a blocking buffer (PBS supplemented with 2% bovine serum albumin and 0.05% saponin) for one hour, after which the primary antibodies (~10-20 µg/ml diluted in blocking buffer) were added. For staining intracellular molecules, including cytoskeleton proteins, tyrosine-phosphorylated proteins, and ADK, the cells were additionally permeabilized with PBS containing 0.2% Triton X-100 before incubation with the primary antibodies. In cases where unconjugated primary antibodies were used, the cells were subsequently incubated with appropriate dye-labeled secondary antibodies (~1:800 diluted in blocking buffer). After the staining procedure, the coverslips were mounted with Prolong™ Gold Antifade reagent (ThermoFisher Scientific, P36930 or P36931). Image acquisition was performed using a 3i CSU-W1 spinning disk confocal microscope equipped with a Photometrics Evolve EM-CCD camera. Image processing and analysis were conducted using ImageJ 1.52 and Imaris 8.4 (Bitplane) software.

4.4 Flow cytometry

HUVEC, MDA-MB-231, or PC3 were seeded in 24-well plates and subjected to hypoxia treatment when reaching confluence. After the hypoxic treatment, culture media were immediately replaced with FACS buffer (PBS containing 2% fetal bovine serum and 0.01% NaN_3) to terminate endocytosis and prevent uncontrolled reoxygenation. The cells were then detached using trypsin/EDTA and incubated with primary antibodies (~10-20 µg/ml diluted in FACS buffer). In cases where unconjugated primary antibodies were used, the cells were further incubated with

the appropriate dye-labeled secondary antibodies (~1:400 diluted in FACS buffer). Isotype-matched negative control antibodies were used as negative controls.

For T-cell staining, T cells were collected post-Ado treatment, washed with staining buffer (PBS containing 2% fetal bovine serum), and incubated with Human TruStain FcX™ (BioLegend, 422301) to block non-specific Fc-receptor-mediated binding. Next, the cells were stained with primary conjugated antibodies (2-5 µg/ml diluted in staining buffer). For intracellular staining, the cells were fixed and permeabilized using the Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD, 554714), followed by incubation with the respective antibodies (2-5 µg/ml diluted in permeabilization buffer). Cytokine staining additionally involved a pre-incubation step of cultured T cells with Brefeldin A (BioLegend, 420601) and Cell Activation Cocktail (BioLegend, 423301) 4 hours prior to FACS staining.

T-cell proliferation was evaluated by labeling freshly isolated T cells with CellTrace ™ Violet (CTV, ThermoFisher Scientific, C34557). In brief, isolated T cells were incubated with 5 µM of CTV for 20 minutes at 37°C. Staining of apoptotic and dead cells was performed using the APC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, 640930). Flow cytometry data were acquired using an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (FlowJo LLC).

4.5 Thin-layer chromatography

4.5.1 Evaluation of extracellular Ado metabolism

The enzymatic activities of purine-converting ectoenzymes were determined by thin-layer chromatography (TLC) using radioactively labelled nucleotides and nucleosides as tracer substrates. [^{3}H]ATP, [^{3}H]ADP, [^{3}H]AMP, and [^{3}H]Ado were purchased from American Radiolabeled Chemicals Inc. (Campro Scientfific, The Netherdlands), and [γ-^{32}P]ATP was obtained from Perkin Elmer (Boston, MA). For detailed methodology, refer to (Yegutkin, 2014).

Adherent cells (HUVEC, MDA-MB-231, PC3) were seeded overnight into 96-well flat bottom plates at a density of ~8000-10000 cells per well and subjected to hypoxia treatment as specified above. T cells were seeded into 96-well flat bottom plates at a density of 120000 cells per well on the day of the experiment. The catalytic activity of ectoenzymes was determined by incubating the cells with the respective radioactively labelled substrates at 37°C. The substrates used were: [^{3}H]ATP or [^{3}H]ADP for CD39 activity; [γ-^{32}P]ATP for ENPP1 activity; [^{3}H]AMP for CD73 activity; [^{3}H]AMP or [^{3}H]ADP in the presence of γ-phosphate-donating ATP for AK and NDPK activities, respectively; and [^{3}H]Ado for ADA activity. For specific incubation times, substrate concentrations, and the medium used for each cell type,

refer to the original publications (I), (II) and (III). When inhibitors were used, the cells were pre-treated for 30 minutes before adding the radioactive substrates with: 100 µM of the AK1 inhibitor P^1,P^5-di(adenosine-5')-pentaphosphate (Ap_5A; Sigma-Aldrich, D4022); 100 µM of the CD73 inhibitor adenosine 5′-(α,β-methylene)diphosphate (APCP, Sigma-Aldrich, M3763); or 10 µM of the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, Sigma Aldrich, 324630).

After incubation with the radioactive substrates, aliquots of the assay medium (9 µl) were applied onto TLC aluminum sheets ALUGRAMSIL G UV254 (Macherey-Nagel, 818133) for ^{3}H-labelled substrates or polyester sheets POLYGRAM GEL 300 PEI UV254 (Macherey-Nagel, 801063) for ^{32}P-labelled ATP. The samples were separated using appropriate solvent systems and then either exposed for four weeks at -70°C Kodak BioMax MS films (Carestream, Rochester, NY) for autoradiographic imaging or subjected to extraction of radioactively labeled substrates and their metabolites, which were then quantified using a Wallac-1409 β-spectrometer (Perkin Elmer, Boston, MA).

4.5.2 Evaluation of intracellular Ado metabolism

Adherent cells (HUVEC, MDA-MB-231, PC3) were seeded overnight onto 24-well flat bottom plates at a density of ~60000 cells per well and subjected to hypoxia treatment as specified above. T cells were seeded into 96-well flat bottom plates at a density of 120000 cells per well on the day of the experiment. The cells' ability to internalize extracellular Ado was assessed by incubating them with [^{3}H]Ado or [^{3}H]AMP for 45-60 minutes. For specific substrate concentrations and the medium used for each cell type, refer to the original publications (I), (II) and (III). When inhibitors were used, the cells were initially pre-treated for 45-60 minutes with: 1 µM of ENT inhibitors dipyridamole and NBTI; 100 µM of CD73 inhibitor APCP; 10 µM of ADA inhibitor EHNA; or 0.5 µM of ADK inhibitor ABT-702 (Sigma-Aldrich, A2721). After incubation with the radioactive substrates, the cells were washed with PBS and lysed using the Mammalian Lysis Solution (Perkin Elmer/Revvity, 6016943). Aliquots of the lysates were then applied to TLC aluminum sheets ALUGRAMSIL G UV254 (Macherey-Nagel, 818133), separated using appropriate solvent systems, and further quantified for cell-incorporated ^{3}H-labelled metabolites as described above.

4.6 Quantification of ATP levels

ATP levels were assessed using the ATPlite™ Luminescence Assay System (Perkin Elmer/Revvity, 6016943) according to the manufacture's protocol. Cells were washed with BSS and subsequently lysed with the Mammalian Lysis Solution. The

cell lysates were then transferred into white 96 well-plates, and the substrate solution was added. Luminescence signals were measured using a Tecan Infinite M200 microplate reader (Salzburg, Austria). To standardize the data, protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, 232257).

4.7 Phospho-kinase array

HUVECs were cultured in 6-well plates under either normoxic or hypoxic conditions. The phosphorylation of major kinases and their downstream signaling molecules was profiled using the Human Phospho-Kinase Antibody Array Kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. In brief, cell lysates were incubated overnight with the array membranes, which were then washed and incubated with a cocktail of biotinylated detection antibodies. After a two-hour incubation at room temperature, the membranes were washed again and incubated with HRP-conjugated streptavidin. Subsequently, the membranes were incubated with chemiluminescence reagents, and the array signals were captured by exposure to X-ray films. The X-ray films were scanned, and the obtained signals were inverted into 8-bit halftone images. Quantitative analysis of the pixel density was performed using ImageJ 1.51 software.

4.8 Western blotting

HUVEC, MDA-MB-231, or PC3 were seeded in 6-well plates and subjected to hypoxia treatment upon reaching confluence. After the designated treatment period, the cells were washed and lysed with a buffer, containing 10 mM Tris, 1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 5 mM dithiothreitol, 10 µM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin and 1 mM sodium orthovanadate. The resulting lysates were centrifuged, and the supernatants were diluted in Laemmli buffer supplemented with 2% β-mercaptoethanol. The lysates were then boiled at 95°C for 5 minutes and loaded onto a 10% SDS-polyacrylamide gel (~25 µg protein/ lane) for subsequent electrophoresis. Upon completion of electrophoresis, the separated cellular proteins were transferred to nitrocellulose blotting membranes (Amersham). The membranes were blocked with Tris-buffered saline (TBS buffer) containing 0.1% Tween-20 and 2.5% bovine serum albumin, then incubated overnight at 4°C with primary antibodies against: CD73 (D7F9A), phosphotyrosine (4G10), ADK, and β-tubulin as a loading control (~2-5 µg each). Subsequently, the membranes were washed with TBS buffer containing 0.1% Tween-20 and incubated with either appropriate HRP-conjugated or IRDye 680RD-conjugated secondary antibodies, depending on the detection method. For

chemiluminescence detection, the membranes were incubated with chemiluminescence reagents, and the signals were captured by exposure to X-ray films. Alternatively, for infrared imaging, signals were detected using an Odyssey CLX-1005 infrared imaging system (LI-COR Biosciences GmbH).

4.9 T-cell metabolic assay

T-cell metabolism was evaluated using the Agilent Seahorse XF T-cell Metabolic Profiling Kit (Agilent, 103772-100) according to the manufacturer's guidelines. One day prior to the experiment, the XFe96/XF Pro Sensor Cartridge was hydrated at 37°C in a non-CO_2 incubator. The Seahorse XF PDL cell culture plate was coated with 50 µl Poly-L-lysine solution for 5 minutes. The plate was then washed twice with H_2O, allowed to air-dry, and kept at 37°C in a non-CO_2 incubator. On the day of the experiment, T cells, treated as specified above, were harvested and washed in XF RPMI Medium (Agilent, 103576-100), which was supplemented with 10 mM glucose (Agilent, 103577-100), 1 mM pyruvate (Agilent, 103578-100), and 2 mM glutamine (Agilent, 103578-100). The cells were then resuspended in the same buffer at a concentration of 2 million cells/ml for activated T cells or 4 million cells/ml for non-activated T cells. Next, 50 µl of the cell suspension was added to the pre-coated XF cell culture microplate, and metabolic profiling was conducted using the Seahorse XFe96 Analyzer according to the Agilent user guide. The results were analyzed using Agilent's web-based software Seahorse Analytics.

4.10 Single-cell RNA sequencing

T cells, subjected to Ado treatment as specified above, were prepared for single-cell RNA-sequencing using the Evercode™ Fixation v2 kit and Evercode™ WT v2 kit from Parse Biosciences following the manufacturer's instructions. The resulting eight sub-libraries were pooled and sequenced on an Illumina NovaSeq sequencer (S2 flow cell). Detailed data processing, clustering, and analysis, as well as information on the single-cell sequencing of T cells activated by dendritic cells, are provided in the original publication (III).

4.11 *In vivo* experiments

In vivo experiments were conducted with 10-week-old female SCID mice from the Charles River Laboratories, Germany. The luciferase-expressing adenocarcinoma cell line MDA-MB-231-luc-D3H2LN, obtained from Caliper Life Sciences, was injected into the fourth mammary fat pads of the mice using two experimental setups. In the first setup, the cells were pre-treated overnight with 100 µM Ado or 10 µM

5′-(N-Ethylcarboxamido)adenosine (NECA, Sigma-Aldrich, E2387), detached the next day, incubated for an additional 15 minutes with the same treatment, and then injected into the mice. Tumor growth was monitored using the bioluminescence IVIS-Spectrum Imaging System (Caliper Life Sciences) after injection of the substrate D-luciferin. In the second setup, breast cancer xenografts were grown for 3 weeks. Then, 50 or 100 µM Ado, 10 µM NECA, or equal volumes of 0.02% DMSO or PBS were administered intratumorally on days 22, 25 and 29 post-inoculation. Mice were sacrificed on day 32, and tumors were extracted and measured for weight and volume using precision scales (Mettler Toledo) and an electronic caliper (Mitutoyo), respectively. All animal experiments were done in accordance with European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the Statutes 1076/85 and 1360/90 of The Animal Protection Law in Finland and EU Directive 86/609. The experimental procedures were approved by the Project Authorization Board under license ID ESAVI/5587/04.10.07/2014.

4.12 *In situ* enzyme histochemistry

Breast cancer xenografts were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek), sectioned into 10 µm slices onto Superfrost™ PLUS glass slides (Thermo Scientific), and processed for enzymatic histochemical staining, as described by Losenkova et al., 2020. In brief, to assess the activity of purine-converting ectoenzymes, the tissue slides were incubated at 37°C with 400 µM ATP for 30 min or 400 µM AMP for 15 minutes in the presence of $Pb(PO_4)_2$. After incubation, the slides were washed and immersed in a developing solution containing 0.5% $(NH_4)_2S$ for 30 seconds. The resulting brown precipitates, indicative of purine-converting ectoenzymatic activities, were subsequently assessed. For the evaluation of TNAP activity, the slides were incubated for 30 minutes with artificial chromogenic enzyme substrates, including 400 µM of 5'-bromo-4-chloro-3-indolyl phosphate (BCIP, Thermo Scientific, 34040) and 400 µM of nitro blue tetrazolium chloride (Invitrogen, N6495), followed by assessment of the resulting blue color reaction. Microscopic detection of the signals was performed using the Pannormanic-250 Flash slide scanner (3DHistech Ltd., Budapest, Hungary). Breast cancer xenograft samples were additionally analyzed for the expression levels of several key purine-converting enzymes (CD39, CD73, ADK) using immunofluorescence staining. For further methodological details, refer to the original publication (II).

4.13 Statistical analysis

The *in vitro* data were analysed for statistical significance using a two-tailed Student´s t-test and the Mann-Whitney U-test. For *in vivo* studies, statistical significance between the control and treated groups was determined using multiple t-test grouped analysis with the Holm–Sidak method. Levels of statistical significance were designated as **P<0.01 and *P<0.05. All analyses were performed using Prism GraphPad 7 software (GraphPad, San Diego, CA).

5 Results

5.1 Hypoxia-induced adaptations in ECs: Alterations in Ado metabolism (I)

ECs, which line the inner walls of blood vessels, play vital roles in normal physiological conditions. They regulate vascular permeability, allowing the passage of essential molecules such as oxygen, water, and nutrients to surrounding tissues. Additionally, ECs maintain vascular tone, promote angiogenesis, and orchestrate immune cell trafficking. However, in the context of cancer, these cells become significant drivers of disease progression, particularly in solid tumors. Hypoxia, a hallmark of cancer, induces EC-mediated neovascularization by modulating VEGF pathways in the TME. This process results in the formation of dysfunctional vascular networks, which further exacerbate hypoxia and promote tumor growth. Beyond VEGF pathways, hypoxia can additionally exert its modulatory effects on Ado pathways, which are currently recognized as one of the key immunosuppressive mechanisms utilized by cancer. Hypoxia can alter both intracellular and extracellular ATP levels by inhibiting OXPHOS and triggering ATP release. Additionally, it induces changes in the expression and activity of purine-converting ectoenzymes, potentially further promoting the generation of immunosuppressive Ado within the TME. However, the heterogeneity and complexity of the TME present significant challenges to fully understand the hypoxia-induced modulation of Ado pathways. Different cell types within the TME express diverse patterns of purine-converting ectoenzymes and selectively adapt their Ado metabolic pathways under hypoxic conditions. In this study, we focused on how ECs adapt to hypoxia, with a particular emphasis on the intricate interplay between extracellular and intracellular Ado metabolism. By unraveling the hypoxia-induced changes in Ado metabolic pathways and the subsequent cellular responses, we aimed to uncover additional mechanisms through which ECs might promote cancer progression in the hypoxic TME.

5.1.1 Hypoxia does not induce significant cytoskeletal remodeling in ECs

ECs play a key role in regulating vascular permeability, with their cytoskeleton being crucial for modulating cell shape. Given the well-known ability of hypoxia to increase vascular permeability, we initially characterized cytoskeletal reorganization in ECs under hypoxic conditions. For this purpose, major cytoskeletal components were stained in a well-established EC model, human umbilical vein endothelial cells (HUVECs). These cells exhibited strong positivity for vimentin, β-tubulin, F-actin, and myosin (p-MLC2), indicating the presence of intermediate filaments, microtubules, microfilaments, and motor proteins, respectively (I, Fig. 1, 2). Following 24 hours of hypoxia, modest formation of actin stress fibers along the cell periphery was observed (I, Fig. 1, 2). However, these hypoxic conditions did not induce significant remodeling of the HUVEC cytoskeleton.

5.1.2 Hypoxia induces depletion of intracellular ATP stores and upregulates selective signaling pathways in ECs

Hypoxia, a known inhibitor of mitochondrial respiration, led to a 30% reduction in global cellular ATP levels in HUVECs after 24 hours of exposure to 1% O_2 (I, Fig. 3F). This decrease was reversible, with ATP levels recovering after one hour of reoxygenation of hypoxic cells in normal atmospheric conditions (I, Fig. 3F). We then assessed the impact of hypoxia on various protein phosphorylation signaling pathways. Following 24 hours of hypoxia, no changes were detected in the total tyrosine phosphorylation levels. Staining for tyrosine phosphorylation sites using the 4G10 anti-phosphotyrosine antibody showed no differences in signal distribution and intensity between normoxic and hypoxic cells (I, Fig. 3B). These findings were further confirmed by WB analysis of cell lysates (I, Fig. S1A). However, an increase in the activity of several phospho-serine/threonine kinases was observed using the phospho-kinase array. Specifically, the phosphorylation levels of p53 (S15) and p70S6 (T389) were significantly upregulated in hypoxic HUVECs compared to normoxic cells (I, Fig. 4).

5.1.3 Effects of hypoxia on extracellular Ado metabolism in ECs

Next, we investigated the effect of hypoxia on extracellular Ado metabolism in human ECs. To achieve this, we characterized the expression pattern of purine-converting ectoenzymes on HUVECs and their hypoxia-induced modulation using IF staining, FACS, and TLC analysis. IF staining with anti-CD39 (hN1-1c) and anti-CD73 (4G4 and h5NT) antibodies confirmed the presence of the two “classical”

ATP-inactivating/Ado-producing ectoenzymes, CD39 and CD73 (I, Fig. 7A-B). FACS analysis showed no significant shifts in the cell surface expression levels of CD39 and CD73 between normoxic and hypoxic HUVECs (I, Fig. 7C-D). The enzymatic activities of CD39 and CD73 were further measured using TLC, with [^{3}H]ATP and [^{3}H]ADP serving as substrates for CD39, and [^{3}H]AMP for CD73 (I, Fig. 6A-C). After 24 hours of hypoxia, we observed a statistically significant upregulation of CD39 activity, resulting in up to a 50% increase in the rate of ATP and ADP dephosphorylation, with no changes in AMPase/CD73 activity (I, Fig. 6A-C). Additionally, TLC analysis revealed the presence of ecto-AK, which mediated the transfer of a phosphoryl group between [^{3}H]AMP and ATP. The production of both [^{3}H]ADP and [^{3}H]ATP, quantified as AK activity, indicates the coordinated action of AK and another ecto-kinase, NDPK. Although this study did not provide data on NDPK activity, previous research from our group confirmed high NDPK activity in HUVECs, capable of transphosphorylating [^{3}H]ADP and ATP into [^{3}H]ATP and ADP (Yegutkin et al., 2001). Exposure of HUVECs to 1% O_2 for 24 hours did not affect the activity of either ecto-AK (I, Fig. 6D) or ecto-NDPK (data not shown). However, the activity of ecto-AK became significantly upregulated after an additional hour of reoxygenation in normal atmospheric conditions (I, Fig. 6D).

5.1.4 Enhanced Ado uptake and its phosphorylation as a compensatory mechanism for hypoxia-induced ATP depletion

After evaluating the extracellular Ado metabolism in HUVECs, we assessed the effects of hypoxia and reoxygenation on its intracellular metabolism. Using TLC, we analyzed the cellular uptake and metabolism of [^{3}H]Ado. Cultured HUVECs incubated with [^{3}H]Ado showed an uptake of approximately 4–6% of the total radioactivity added to the wells. The prominent presence of [^{3}H]ATP among the major intracellular radioactive metabolites indicated efficient uptake of extracellular Ado and its phosphorylation within the cells (I, Fig. 5A-B). Pre-treatment of HUVECs with the ENT inhibitors Dipyridamole and NBTI significantly reduced intracellular radioactive metabolites by up to 80%, suggesting that ENTs are primary contributors to extracellular Ado uptake (I, Fig. 5C-D). HUVECs exposed to hypoxia for 24 hours showed an increase in intracellular radioactivity, indicating enhanced uptake of extracellular Ado and its subsequent phosphorylation into ADP and ATP (I, Fig. 5A, B). This may serve as a compensatory mechanism for hypoxia-induced ATP depletion; however, statistical significance was not achieved, likely due to variations between ECs from different donors. Following 24 hours of hypoxia, one hour of reoxygenation reversed the hypoxia-induced changes, restoring intracellular radioactive metabolites to normoxic levels (I, Fig. 5A-B).

5.2 Ado metabolism in hypoxic cancer cells: The interplay between extracellular and intracellular pathways (II)

The CD39-CD73-$A_{2A/2B}$AR axis has emerged as a promising target for treating immune-checkpoint resistant cancers. Various pharmacological agents, including antibodies and small-molecule inhibitors that block these molecules, are currently in preclinical and clinical trials. However, no drugs targeting Ado metabolic and signaling pathways have been approved for clinical use in cancer treatment. A major challenge in leveraging Ado pathways for cancer therapy is the limited understanding of their regulation within the TME. Our previous study (I) demonstrated that hypoxia directly modulates Ado metabolism in vascular ECs, altering both extracellular and intracellular Ado metabolic pathways. This suggests that hypoxia, a hallmark of cancer, can shape Ado metabolism within the TME, potentially favoring cancer progression. To further explore this premise, our subsequent study investigated the effects of hypoxia on Ado metabolism in epithelial cancer cells. By maintaining consistent experimental settings, our goal was to elucidate how breast and prostate carcinoma cells adapt their intracellular and extracellular Ado metabolic pathways to hypoxia and compare these alterations with those observed in ECs. Exploring the link between hypoxia and Ado metabolism across different cell types will enhance our understanding of its regulation in cancer and may offer new perspectives for advancing cancer treatment strategies.

5.2.1 Characterization of purine-converting ectoenzymes in breast and prostate carcinoma cells

To characterize the pattern of extracellular Ado metabolism in human cancer cells, we assessed the enzymatic activities and expression levels of purine-converting ectoenzymes in two highly metastatic cancer cell lines, MDA-MB-231 (breast adenocarcinoma cell line) and PC3 (androgen-independent prostate carcinoma cell line). Using TLC with ^{3}H- and ^{32}P-labelled nucleotides as tracer substrates, we identified the presence of several purine-inactivating and purine-phosphorylating ectoenzymes. Both cell lines exhibited CD73 activity, as demonstrated by their ability to convert [^{3}H]AMP into [^{3}H]Ado (II, Fig. 1A). In accordance, the CD73 inhibitor APCP effectively blocked [^{3}H]Ado accumulation in the medium (II, Fig. 1A). Both cell lines additionally possessed ENPP1 activity, as evidenced by the direct conversion of [γ-^{32}P]ATP into AMP and $^{32}PP_i$ (II, Fig. 1C). Notably, ENPP1 activity was higher in MDA-MB-231 compared to PC3 cells (II, Fig. 1C). The presence of CD73 and ENPP1 on both cancer cell lines was validated independently by FACS and confocal microscopy using anti-CD73 (4G4 and h5NT) anti-ENPP1 (CD203a) antibodies, respectively (II, Fig. 1E, F). Importantly, no CD39-specific

immunoreactivity was observed when cells were stained with the anti-CD39 (hN1-1) antibody (II, Fig. 1E).

Further TLC analysis revealed extracellular presence of reverse ATP-generating and Ado-degrading pathways (II, Fig. 1B, D). Both cell lines exhibited high ecto-AK activity, converting [^{3}H]AMP into [^{3}H]ADP in the presence of γ-phosphate-donating ATP (II, Fig. 1A). Additionally, after incubation of the cells with [^{3}H]ADP, extracellular [^{3}H]AMP and [^{3}H]ATP were detected, further supporting the presence of ecto-AK (II, Fig. 1B). The AK inhibitor Ap_5A suppressed the [^{3}H]AMP production but not [^{3}H]ATP, suggesting the activity of ecto-NDPK (II, Fig. 1B). Furthermore, both cell lines efficiently degraded [^{3}H]Ado into [^{3}H]Ino and [^{3}H]Hyp, indicating the presence of ecto-ADA and ecto-PNP, respectively (II, Fig. 1D). Pre-treatment of the cells with the ADA inhibitor EHNA prior to addition of [^{3}H]Ado prevented its conversion into deaminated products [^{3}H]Ino and [^{3}H]Hyp, confirming the presence of high ecto-ADA activity in the studied cancer cells (II, Fig. 1D).

5.2.2 Ado uptake by cancer cells and its intracellular phosphorylation

After characterizing the purine-converting ectoenzymes on studied cancer cells, we aimed to explore the interplay between extracellular and intracellular Ado metabolic pathways in these cells. To achieve this, we evaluated the cellular uptake of [^{3}H]Ado and its intracellular metabolism using TLC. Similar to ECs, cancer cells efficiently took up [^{3}H]Ado and subsequently phosphorylated it, with [^{3}H]ATP accounting for approximately 90% of the total cell-incorporated radioactivity in both cell lines (II, Fig. 2A). The intracellular levels of [^{3}H]Ado and its deaminated products, [^{3}H]Ino and [^{3}H]Hyp, together accounted for only about 3% of the total intracellular radioactivity, suggesting that the primary pathway for incorporated Ado involves its phosphorylation to ATP (II, Fig. 2A). Inhibition of ADK by ABT-702 significantly increased [^{3}H]Ado accumulation relative to other intracellular radioactive nucleotides, indicating the crucial role of ADK in the phosphorylation of internalized Ado (II, Fig. 2E). Additionally, pre-treatment of cancer cells with the ENT inhibitors Dipyridamole and NBTI reduced intracellular radioactivity by 90%, indicating that ENTs are the primary transporters mediating cellular Ado uptake (II, Fig. 2C). Notably, ABT-702, an inhibitor of ADK, also inhibited ENT activity, preventing the cellular uptake of [^{3}H]Ado and reducing the total incorporated radioactivity by 70% (II, Fig. 2C).

5.2.3 Hypoxia-induced changes in extracellular and intracellular Ado metabolism in cancer cells

Given the persistent oxygen deprivation within the TME, our next objective was to assess the impact of hypoxia on both extracellular and intracellular Ado metabolic pathways in cancer cells. To evaluate changes in extracellular Ado metabolism, we compared the catalytic activities and expression levels of purine-converting ectoenzymes in normoxic and hypoxic cancer cells. Our results revealed an approximate 2-fold increase in AMPase/CD73 activity in both cell lines after their exposure to 1% O_2 for 24 hours (II, Fig. 4A). In addition, IF staining and WB analysis detected significantly elevated CD73 expression levels in hypoxic cancer cells (II, Fig. 4B, C). Notably, after one hour of reoxygenation, CD73 activity partially decreased, indicating its dynamic regulation in response to environmental changes (II, Fig. 4A). HIF-1α, which was upregulated in both cell lines under hypoxic conditions, may contribute to the remodeling of this metabolic pathway (II, Fig. 6A, Fig. S2A).

We then examined the effects of hypoxia on intracellular Ado metabolism by measuring cellular uptake of [^{3}H]Ado and its subsequent phosphorylation in both normoxic and hypoxic breast and prostate carcinoma cells (II, Fig. 5A). Although there was no significant difference in the total amount of cell-incorporated radioactivity between normoxic and hypoxic cells, hypoxia caused notable changes in the ratio of intracellular radioactive metabolites (II, Fig. 5B, C, D). In normoxic cells, [^{3}H]ATP accounted for approximately 90% of the total intracellular radioactivity, while in hypoxic cells, this proportion decreased to around 80%, accompanied by a statistically significant increase in [^{3}H]ADP (II, Fig. 5C, D). Despite these metabolic changes, no other alterations were observed, including ADK expression levels and total cellular ATP levels, highlighting the high plasticity of cancer cells (II, Fig. 6B, C).

5.2.4 Pre-treatment of breast cancer cells with Ado inhibits tumor growth *in vivo*

To assess the role of Ado metabolism in tumor initiation and growth, we injected luciferase-expressing MDA-MB-231-luc-D3H2LN cells, either untreated or pre-treated with exogenous Ado, into the mammary fat pads of SCID female mice. Tumor development was monitored over a 16-day period. Remarkably, Ado-treated cancer cells exhibited delayed tumor formation compared to untreated cells, with significant lag-phase in tumor growth observed on days 7 and 11 (II, Fig. 7A, C). Pre-treatment of cancer cells with the non-selective AR agonist NECA had no impact on tumor growth (II, Fig. S6). Additionally, intermittent injections of Ado or NECA into established tumors did not affect their volume or mass (II, Fig. 7B, D; Fig. S7).

To further explore Ado pathways within the TME, breast cancer xenografts were extracted and analyzed using *in situ* enzyme histochemistry and IF staining. High ATP-specific brown staining, primarily reflecting CD39-mediated ATPase activity, was predominantly detected in host connective tissues and blood vessels (II, Fig. 8A). Notably, TNAP was also abundantly present in large blood vessels (II, Fig. 8A). AMPase staining, indicative of CD73 activity, was primarily observed on cancer cells, with lower activity also detected in host blood vessels (II, Fig. 8A). IF staining confirmed the presence of ectoenzymes CD39 and CD73 on host blood vessels, with predominant CD73 expression in cancer cells (II, Fig. 8B, C). Additionally, a high abundance of intracellular ADK was observed in both cancer cells and host-derived vessels (II, Fig. 8D). Taken together, these findings underscore the complex interplay between various purine-converting enzymes within the TME and demonstrate that Ado can inhibit the tumor-initiating potential of breast cancer cells in a non-receptor-mediated manner.

5.3 ENT1-mediated Ado uptake: a potent mechanism for T-cell suppression (III)

Elevated Ado levels and the subsequent activation of ARs within the TME are critical factors implicated in immune suppression in cancer. In particular, A_{2A}AR-mediated suppression of T-cell proliferation and cytotoxic function is recognized as one of the key mechanisms of tumor evasion. However, the impact of intracellular Ado metabolism on T-cell function in an Ado-enriched TME remains largely unexplored. In the framework of this study, we aimed to investigate the immunosuppressive effects of high Ado levels on T cells, focusing on non-receptor mediated mechanisms related to its intracellular metabolic pathways. By examining T-cell bioenergetics and cytotoxic function, we intended to underscore the significance of intracellular Ado metabolism in T cells. A deeper understanding of the mechanisms underlying Ado-induced suppression could provide new insights and propose novel therapeutic strategies to enhance T-cell function in cancer patients.

5.3.1 Purinergic signaling system in T cells

To comprehensively profile the purinergic signaling system in T cells, we performed single-cell RNA sequencing on both naïve and activated T cells. Activated T cells were generated by stimulating peripheral blood naïve T cells with $CD1c^+$ allogenic dendritic cells isolated from human lymph nodes for 6 days. Unbiased clustering of the obtained data revealed 13 distinct clusters, primarily distinguished by CD4 and CD8 expression, markers of naïve (*TCF7*, *LEF1*) and activated (*IL2RA*, *IFNG*,

GZMB) T-cell states, and different cell cycle phases (III, Fig. 1B, S1). Clusters 11 and 12, representing $CD4^+$ and $CD8^+$ activated T cells, showed high expression of several purine-converting enzymes, including *ENTPD1* (CD39), *NME1*, *NME2*, *ADA*, *ADK*, and *PNP* (III, Fig. 1B). These clusters also displayed significant expression of *SLC29A1*, but not *SLC29A2* or *SLC29A3*, indicating that ENT1 is the predominant nucleoside transporter in activated T cells (III, Fig. 1B). Cluster 4 and 5, representing naïve $CD8^+$ T cells, demonstrated the highest expression of *ADORA2A* ($A_{2A}AR$) and *NT5E* (CD73) (III, Fig. 1B). Intriguingly, cluster 13 exhibited the signature of exhausted T cells, characterized by high expression of the transcription factor *TOX2*, along with *PDCD1* (PD-1), *ENTPD1* (CD39) and P2RX5 ($P2X_5$ receptor) (III, Fig. 1B).

To further validate our findings, we analyzed a publicly available dataset from (Szabo et al., 2019) where T cells were activated using CD3/CD28 Dynabeads. This analysis confirmed the consistent gene signature in both $CD4^+$ and $CD8^+$ activated T cells, characterized by high expression of *NME1*, *NME2*, *ADA*, *ADK*, and *PNP*, justifying the use of CD3/CD28 Dynabeads for T-cell activation in our subsequent experiments (III, Fig. S2). Notably, *ENTPD1* (CD39) was detected in Tregs but not in the activated T cells within this dataset, likely due to the relatively short 16-hour activation period (III, Fig. S2). In our following experiments, we extended the T-cell activation time with CD3/CD28 Dynabeads to 72 hours. Overall, our data indicate that the purinergic signaling system is broadly distributed across different T-cell subsets, each exhibiting distinct expression patterns.

5.3.2 Changes in purine-converting ectoenzymes upon T-cell activation

To evaluate the presence of purine-converting ectoenzymes at the protein level, we assessed their activities and expression levels in T cells under both non-activated and activated conditions. Peripheral blood naïve T cells were either maintained in a non-activated state (resting T cells) or activated with CD3/CD28 Dynabeads for 3 days (activated T cells), followed by TLC analysis. Activated T cells exhibited high CD39 activity, efficiently degrading [^{3}H]ADP into [^{3}H]AMP, whereas resting T cells showed its negligible activity (III, Fig. 2A, B). In contrast, CD73 activity, measured by the production of [^{3}H]Ado from [^{3}H]AMP, was significantly higher in resting T cells, particularly in $CD8^+$ T cells, consistent with our single-cell data (III, Fig. 1B, 2A, 2B). FACS analysis using anti-CD39-BV711 and anti-CD73-PE-CF594 antibodies, combined with IF microscopy using anti-CD39 (hN1-1) and anti-CD73 (h5NT) antibodies, revealed that CD39 expression levels increased following T-cell activation, while CD73 levels decreased (III, Fig. 2D, E). These changes in expression levels correspond with the observed shifts in enzymatic activities.

In addition to CD39 and CD73, TLC analysis identified the presence of other purine-converting ectoenzymes. Activated T cells exhibited ecto-AK and ecto-NDPK activities, converting [^{3}H]AMP into [^{3}H]ADP and [^{3}H]ADP into [^{3}H]ATP, respectively, in the presence of γ-phosphate-donating ATP (III, Fig. 2A, B). Furthermore, ecto-ADA activity was detected in activated T cells, as evidenced by the efficient degradation of [^{3}H]Ado into its deaminated products, [^{3}H]Ino and [^{3}H]Hyp (III, Fig. 2C). Notably, resting T cells exhibited only minor activity of these redundant ectoenzymes (III, Fig. 2A, B).

5.3.3 Elevated Ado uptake and its phosphorylation in activated T cells

Following the characterization of extracellular Ado metabolic pathways in both resting and activated T cells, we expanded our investigation to examine their intracellular Ado metabolism. Our single-cell data analysis identified ENT1 as the predominant nucleoside transporter, with particularly high abundance in activated T cells (III, Fig. 1B). To further explore intracellular Ado metabolic pathways, we measured the uptake of [^{3}H]Ado by T cells using TLC. Activated T cells exhibited significantly higher levels of intracellular radioactivity compared to resting T cells, indicating enhanced Ado uptake (III, Fig. 3A). Further analysis revealed that [^{3}H]ATP was the major intracellular radioactive metabolite, reflecting the rapid phosphorylation of the internalized [^{3}H]Ado (III, Fig. 3B). The addition of the ENT inhibitors Dipyridamole and NBTI reduced total intracellular radioactivity by 70%, indicating impaired Ado uptake (III, Fig. 3C, D). Taken together, these data suggest that activated T cells have an enhanced ability to uptake extracellular Ado, presumably mediated by ENT1, and subsequently phosphorylate it. FACS and IF microcopy analyses confirmed that activated T cells exhibited higher ENT1 expression levels compared to resting T cells, correlating with the observed increase in total intracellular radioactivity (III, Fig. 3E, F). Notably, the ADA inhibitor EHNA did not alter intracellular radioactivity levels, suggesting that ADA-mediated Ado degradation is not a major pathway for intracellular Ado metabolism in activated T cells (III, Fig. 3C, D).

5.3.4 Non-receptor mediated effects of Ado on T-cell suppression

Given the well-recognized immunosuppressive effects of Ado, we assessed its impact of T-cell proliferation and activation. Freshly isolated naïve T cells were treated once with either 10 μM or 100 μM Ado, which remained present throughout a 3-day activation period using CD3/CD28 Dynabeads (set 1) (III, Fig. 4A). FACS

analysis confirmed that Ado inhibited T-cell proliferation and suppressed the production of the inflammatory cytokine interferon gamma (IFN-γ) and the cytotoxic molecule Granzyme B (GranzB) in a concentration-dependent manner (III, Fig. 4C, D, E). Additionally, Ado treatment reduced the levels of activation markers such as PD-1 and CD25, as well as ENT1 (III, Fig. 4D, E). Notably, even a 12-hour exposure to 100 µM Ado significantly inhibited T-cell proliferation (III, Fig. 4E).

To determine whether the immunosuppressive effects of Ado are mediated through AR-dependent or AR-independent pathways, naïve T cells were pre-treated with the ENT inhibitors Dipyridamole and NBTI, or the potent $A_{2A/2B}$AR antagonist AB-928, one hour prior to Ado treatment (III, Fig. 4A). Remarkably, the ENT inhibitors effectively reversed the suppressive effects of 100 µM Ado, restoring T-cell proliferation, cytokine production, and activation marker levels (III, Fig. 4C, D, E). In contrast, the $A_{2A/2B}$AR antagonist AB-928 did not counteract Ado's effects, suggesting that the observed immunosuppression is driven by Ado cellular uptake rather than AR-mediated signaling (III, Fig. 4C, D, E). This conclusion is further supported by the finding that NECA, a non-selective AR agonist, had no impact on T-cell proliferation, cytokine production, or activation markers (III, Fig. 4C, D, E).

The suppressive effects of internalized Ado were further evaluated by exposing fully activated T cells to 10 µM or 100 µM Ado, either in the presence or absence of ENT inhibitors, for 24 hours (set 2) (III, Fig. 5A). FACS analysis revealed a significant downregulation of IFN-γ levels in activated T cells in the presence of 100 µM Ado, with its partial restoration upon inhibition of Ado uptake (III, Fig. 5B). GranzB levels remained unchanged, indicating that its regulation is not influenced by high Ado levels after T-cell activation (III, Fig. 5B). Furthermore, treatment with 100 µM Ado led to a statistically significant 1.3 to 1.5-fold increase in the percentage of Annexin V^+/7AAD$^+$ dead T cells, suggesting enhanced T-cell death (III, Fig. 5C, D). This increase may be partly attributed to intracellular Ado metabolism, as the percentage of double-positive cells was reduced in the presence of Dipyridamole and NBTI (III, Fig. 5C, D). Notably, incubation with 100 µM Ino had no effect on the viability of activated T cells (III, Fig. 5D).

5.3.5 Ado uptake in T cells suppresses conventional ATP-generating pathways while sustaining ATP levels via the Ado-salvage pathway

Next, we explored the impact of Ado uptake on T-cell bioenergetics by measuring global cellular ATP levels. When normalized to protein content, ATP concentrations were consistent between resting and activated T cells, indicating that T cells maintain stable intracellular ATP levels regardless of their activation status (III, Fig. 6B). The apparent increase in ATP levels per cell in activated T cells is likely due to their

larger size and higher intracellular content, correlating with elevated protein levels (III, Fig. 6A, B). When activated T cells were treated with 10 μM or 100 μM Ado, intracellular ATP levels increased during the first 2-4 hours, with a 1.6-fold rise observed at 100 μM Ado (III, Fig. 6C). This increase was blocked by the ENT inhibitors Dipyridamole and NBTI, indicating that Ado uptake and its subsequent phosphorylation are necessary for ATP elevation (III, Fig. 6F). Notably, treatment with 100 μM Ino did not affect ATP levels (III, Fig. 6D). After 24 hours of Ado exposure, intracellular ATP levels returned to baseline, suggesting that activated T cells undergo alterations in their ATP-generating pathways in response to prolonged exposure to high levels of Ado (III, Fig. 6C).

Given the crucial role of glycolysis and mitochondrial respiration in ATP generation, we examined their potential interplay with intracellular Ado metabolism (III, Fig. 6E). Inhibition of glycolysis with 2-deoxy-D-glucose (2DG) or OXPHOS with Oligomycin in activated T cells reduced total ATP levels by approximately 50%, underscoring the importance of both pathways in T-cell bioenergetics (III, Fig. 6F). Remarkably, ATP levels in glycolysis-inhibited T cells were fully restored after a 2-hour incubation with 40 μM Ado (III, Fig. 6F). However, this restoration of ATP levels was diminished when Ado uptake was inhibited (III, Fig. 6F). In contrast, ATP levels in activated T cells with impaired OXPHOS were not restored by Ado uptake, suggesting that the Ado-salvage pathway is closely linked to mitochondrial respiration in these cells (III, Fig. 6F).

Based on the observation that high Ado levels only temporarily increase global cellular ATP levels, we then investigated how prolonged Ado exposure affects T-cell bioenergetics using the Seahorse T-cell metabolic assay (III, Fig. 7A). After 24 hours of incubation with 100 μM Ado, activated T cells exhibited a significant inhibition of both glycolysis and mitochondrial respiration, with ATP production rates reduced by 50% and 60%, respectively (III, Fig. 7B). No inhibition was detected within the first two hours of Ado incubation, with partial inhibition emerged after 8 hours (III, Fig. 7B). Inhibition of Ado uptake with Dipyridamole and NBTI significantly restored ATP production rates by these pathways, underscoring the role of internalized Ado in suppressing both OXHPOS and glycolysis (III, Fig. 7B).

Taken together, these data suggest that the cellular uptake of Ado and its subsequent phosphorylation leads to an increase in global cellular ATP levels, initially working alongside glycolysis and OXPHOS to generate ATP. However, prolonged exposure to Ado ultimately inhibits these conventional ATP-generating pathways in activated T cells, resulting in their reliance on the Ado-salvage pathway to maintain stable ATP levels.

5.3.6 Ado-induced transcriptional changes in activated T cells

Given the suppressive effects of high Ado levels on T-cell function, we aimed to evaluate the underlying transcriptional changes using single-cell RNA sequencing. Activated T cells from two donors were either left untreated or exposed to 100 μM Ado for 24 hours before further processing (set 2) (III, Fig 5A). Unbiased clustering identified 12 distinct T-cell clusters, with no significant changes in cluster composition following Ado treatment (III, Fig. 8A). Most clusters were characterized by CD4 expression, while CD8 expression was restricted to clusters 1 and 9 (III, Fig. 8B). Cluster 1was identified as naïve $CD8^+$ T cells, marked by high *SELL* (L-selectin) expression, whereas cluster 9 was classified as $CD8^+$ effector T cells, characterized by high expression of *GZMA* (Granzyme A), *GZMB* (Granzyme B), and *IFNG* (IFN-γ) (III, Fig. S5A). Among the $CD4^+$ T-cell clusters, cluster 4 lacked the naïve T cell markers *TCF7* and *SELL* but displayed high expression of the transcription factor *MAF* (c-MAF), a marker for effector $CD4^+$ T cells (III, Fig. S5A). Notably, cluster 10 uniquely expressed *FOXP3*, identifying these cells as Tregs (III, Fig. S5A). The remaining clusters showed intermediate levels of *TCF7* and *SELL*, suggesting that they represent $CD4^+$ T-cell subsets in non-activated or intermediate activation states (III, Fig. S5A).

To compare differentially expressed genes between untreated (Ctrl) and Ado-treated T cells, we first performed a pseudo-bulk analysis of the entire dataset, irrespective of cluster identifications. This analysis revealed the upregulation of several histone genes, including *H2AC4*, *H2AC6*, *H2AC7* and *H3C12*, in Ado-treated T cells (III, Fig. S5B). Conversely, the most downregulated genes following Ado treatment included *MX1* (myxovirus resistance protein 1), *IFI44L* (interferon induced protein 44), and *CD70*, all of which encode inflammatory molecules (III, Fig. S5B). Further analysis of specific clusters, including cluster 4 (effector $CD4^+$ T cells), cluster 9 (effector $CD8^+$ T cells), and cluster 10 (Tregs), revealed that Ado treatment led to the upregulation of genes such as *ATF4* (activating transcription factor 4), *HNRNPA2B1* (heterogeneous nuclear ribonucleoprotein A2/B1), *ACLY* (ATP citrate lyase), *H2AC7* (H2A clustered histone 7), and *SFPQ* (splicing factor proline and glutamine rich) (III, Fig. 8B). In contrast, Ado downregulated the expression of *IL2RG* (IL2 receptor subunit-γ), *IL2RA* (IL2 receptor subunit-α), *TXNIP* (thioredoxin interacting protein), *CD70*, and *CCL5* in $CD4^+$ and $CD8^+$ effector T cells (cluster 4 and 9), but not in Tregs (III, Fig. 8B).

To further explore the functional implications of the gene expression changes, we performed gene enrichment analysis on the upregulated and downregulated genes in Ado-treated $CD8^+$ effector T cells (cluster 9). The analysis revealed that upregulated genes were significantly enriched in Gene Ontology terms related to "DNA packaging complex" and "Protein DNA complex", due to the increased

expression of histone genes (III, Fig. 8C). Conversely, downregulated genes were enriched in the “Cytokine-mediated signaling pathway”, attributed to the reduced expression of *IL2RG*, *CCL5*, and *CD70* (III, Fig. 8C). Taken together, these findings align with the immunosuppressive effects of Ado, leading to reduced expression of T-cell activation markers, particularly in $CD4^+$ and $CD8^+$ effector T cells. Notably, the significant upregulation of *ATF4*, a gene associated with stress responses in T cells, along with multiple histone genes in Ado-treated T cells, suggests a complex regulatory mechanism under prolonged Ado exposure, potentially modulating T-cell proliferation and function.

6 Discussion

6.1.1 Cell-specific nature of ATP-Ado metabolic pathways

The CD39-CD73-$A_{2A/2B}$AR axis has emerged as a promising target for overcoming resistance to immune-checkpoint inhibitors in cancer treatment (Allard et al., 2020; Vijayan et al., 2017). Current preclinical and clinical trials are actively exploring small-molecules inhibitors and antibodies targeting this axis (Di Virgilio et al., 2024; Xia et al., 2023). However, leveraging Ado-based therapies in clinical settings remains challenging due to the complex nature of the TME. Another significant obstacle is that much of the research on immunosuppressive Ado pathways predominantly relies on preclinical mouse models, which differ considerably from humans (Joolharzadeh & St Hilaire, 2019; Yegutkin & Boison, 2022). To address these gaps and gain a deeper understanding of the complexity of Ado regulation in humans, we investigated the cell-specific nature of ATP-Ado metabolic pathways across several human cell types, including vascular ECs, cancer cells, and T cells.

Vascular ECs expressed the "classical" ATP-inactivating/Ado-producing pathway involving both CD39 and CD73 (I), which is consistent with their role in maintaining vascular homeostasis. This pathway facilitates the removal of pro-inflammatory ATP and pro-thrombotic ADP while generating Ado, which is crucial for regulating vascular permeability (Deaglio & Robson, 2011; Kaczmarek et al., 1996; Yegutkin, 2021; Yegutkin et al., 2015). In contrast, cancer cells, represented by triple-negative breast cancer and prostate cancer cell lines, lacked CD39 but instead exhibited high levels of CD73 and another ATP-inactivating ectoenzyme ENPP1 (II). The absence of CD39 suggests that these epithelial-derived cancer cells may evade CD39-targeted therapies by relying on the alternative ENPP1-CD73 pathway to generate Ado. Additionally, ENPP1 can hydrolyze cGAMP, a potent activator of the pro-inflammatory STING pathway, thereby further promoting immunosuppression (Li et al., 2021; Wang et al., 2023).

T cells exhibited a dynamic expression pattern of CD39 and CD73 throughout activation (III). Naïve T cells displayed high levels of CD73 and low levels of CD39, with CD73-derived Ado potentially maintaining cellular quiescence through A_{2A} receptor signaling (Cekic et al., 2013). Upon activation, this pattern shifts, with CD39 being upregulated and CD73 reduced (III). This observed increase in CD39

levels on activated T cells is likely driven by TCR signaling. Notably, the relatively short 16-hour activation time used in the study by Szabo et al. was insufficient for CD39 upregulation, suggesting that prolonged TCR stimulation is required (Schneider et al., 2021). While *in vitro* activation does not fully recapitulate *in vivo* conditions, growing evidence indicates that CD39 serves as a marker of $CD8^+$ exhausted T cells in the TME and chronic viral infections, highlighting the role of persistent TCR stimulation in regulating CD39 expression (Canale et al., 2018; Duhen et al., 2018; Gupta et al., 2015). This dependence on TCR signaling is further supported by the observation that bystander $CD8^+$ tumor-infiltrating T cells, which do not recognize cancer antigens and thus not chronically stimulated through the TCR, lack CD39 (Simoni et al., 2018).

Despite these insights, evidence for the functional implications of CD39 on effector T cells remains limited. In a mouse model of melanoma, CD39 on $CD8^+$ exhausted T cells enhanced tumor growth and worsened responsiveness to immunotherapy in an Ado-mediated manner (Vignali et al., 2023). Additionally, CD39 enzymatic activity on $CD4^+$ effector T cells has been suggested to contribute to immune resolution by promoting metabolic stress and increasing the susceptibly of these cells to apoptosis (Fang et al., 2016). However, whether this mechanism is relevant *in vivo* or holds similar significance for $CD8^+$ effector T cells remains to be elucidated.

Tregs represent another T-cell subset characterized by high CD39 expression. While murine Tregs express both CD39 and CD73, enabling them to generate Ado that suppresses effector T-cell function, human Tregs express only CD39 and show little to no expression of CD73 (Borsellino et al., 2007; Deaglio et al., 2007; Gourdin et al., 2018; Schneider et al., 2021; Schuler et al., 2014). Our analysis of the publicly available dataset from Szabo et al. confirms that human Tregs express CD39 but lack CD73 (III). It has been suggested that CD39 on human Tregs induces immune suppression by cooperating with $CD73^+$ extracellular vesicles derived from activated $CD8^+$ T cells (Schneider et al., 2021). This release of $CD73^+$ extracellular vesicles could explain the reduced CD73 levels on the surface of activated T cells observed in our study (III). Nevertheless, our single-cell RNA sequencing data suggests that CD73 is transcriptionally downregulated during T-cell activation, though this mechanism remains to be elucidated (III).

In addition to ATP-inactivating/Ado-generating pathways, all studied cell types exhibited an extracellular ATP-generating pathway involving extracellular forms of AK and NDPK (I-III). Although less widely recognized, this pathway may offer an additional mechanism for regulating extracellular nucleotide levels (Boison & Yegutkin, 2019). The presence of extracellular ADA activity across all cell types adds another layer of complexity to our understanding of extracellular Ado metabolism (I-III). While exploring the functional roles of these pathways was

beyond the scope of our studies, our findings highlight the redundancy of ATP-Ado metabolic networks. Focusing exclusively on CD39 and CD73 in current therapeutic strategies risks overlooking the broader purinergic landscape within the TME, where other purine-converting ectoenzymes, such as ENPP1, AK, NDPK, and ADA, also regulate extracellular ATP and Ado levels. Moreover, the cell-specific enzymatic profiles observed in our studies suggest that various cell types within the heterogenous TME possess distinct patterns of purine-converting ectoenzymes. Without considering this complexity, ongoing clinical trials targeting Ado metabolism may not achieve optimal therapeutical outcomes. Therefore, to maximize the efficacy of these therapies, it is essential to develop tailored and combinational therapeutic strategies that account for the unique enzymatic profiles across different malignant and benign cell types.

6.1.2 Cell-specific modulation of purine-converting ectoenzymes under hypoxia

Hypoxia is a pathological condition characterized by reduced oxygen levels. Different cell types adapt to it in a cell-specific manner, rewiring their metabolic pathways to cope with this stress (Chi et al., 2006). In the context of cancer, hypoxia not only drives genetic instability in cancer cells but also promotes tumorigenesis by altering the metabolism of various cell subsets within the TME, leading to abnormal blood vessel formation and immune suppression (Chen et al., 2023; Harris, 2002). Given the well-established role of Ado-mediated immune evasion in the TME, it is crucial to understand how ATP-Ado metabolic pathways are regulated across different cell types under acute or chronic oxygen deprivation. To investigate this, we exposed vascular ECs and cancer cells to hypoxic conditions, aiming to uncover adaptations in their purine-converting ectoenzyme profiles and potentially provide novel insights into their hypoxia-induced metabolic reprogramming.

We observed that vascular ECs enhanced their CD39 activity (I), whereas cancer cells upregulated CD73 in response to acute hypoxia (II). Other purine-converting ectoenzymes remained unaffected in both cell types (I, II). The upregulation of CD39 in ECs likely plays a protective role against hypoxia-induced vascular permeability, as CD39 knockout mice exhibit increased endothelial barrier dysfunction during hypoxia compared to WT mice (Eltzschig et al., 2003). This protective CD39 upregulation in ECs is mediated by a transcription factor Sp1 (Eltzschig et al., 2009). Importantly, CD73 knockout mice also display increased vascular leakage under hypoxia, highlighting the crucial role of both CD39 and CD73 in maintaining endothelial barrier integrity through Ado production (Eltzschig et al., 2003; Thompson et al., 2004). Notably, while we observed a selective increase in CD39 activity in HUVECs after 4-24 hours of hypoxia (I), a separate study reported

upregulation of both CD39 and CD73 in human microvascular ECs (HMECs) under short-term hypoxia (Eltzschig et al., 2003). These differences may result from both experimental variations and the inherent heterogeneity of ECs (Aird, 2012; Feoktistov et al., 2002; Trimm & Red-Horse, 2023), as HUVECs originate from veins, while HMECs represent capillaries. Given the distinct functions of different vascular beds, the roles of CD39 and CD73 in ECs, as well as their modulation under hypoxia, likely vary based on their vascular origin. For instance, CD73 is critical for regulating endothelial permeability and leukocyte trafficking in large veins and capillaries (Airas et al., 1995, 2000; Yegutkin et al., 2015), whereas in arterial ECs, it appears to be essential for regulating mineralization, as evidenced by the severe arterial calcification observed in patients lacking CD73 (St. Hilaire et al., 2011). Nevertheless, the specificity of Ado metabolic pathways across different human endothelial subtypes, as well as their modulation under hypoxia, remains largely unexplored.

The link between oxygen levels and Ado production within the TME has been extensively investigated. In a mouse model of respiratory hyperoxia, tumor-bearing mice exposed to 60% oxygen for 3 hours showed reduced hypoxia and Ado levels within the TME, leading to inhibited tumor growth (Hatfield et al., 2014). This tumor regression is attributed to lower hypoxia-driven Ado production, which attenuates A_{2A}AR-mediated suppression of effector T cells and NK cells (Hatfield et al., 2015; Hatfield & Sitkovsky, 2020). The current recognition of CD39 and CD73 as major contributors to Ado production within the TME suggests that hypoxia-induced upregulation of CD39 on ECs (I) and CD73 on cancer cells (II) may further facilitate tumor progression.

In murine cancer models, CD39 is significantly elevated in tumor ECs compared to noncancerous tissues, suggesting its TME-driven upregulation (Zhang et al., 2022). Similarly, in our *in vivo* cancer model, we also observed high CD39 levels in the tumor vasculature (II). Administration of a glycoengineered anti-CD39 antibody to tumor-bearing mice, designed to induce antibody-dependent cytotoxicity against CD39-expressing cells, including ECs within the TME, effectively inhibited both tumor growth and neovascularization (Zhang et al., 2022). Notably, these effects were primarily driven by the elimination of CD39-expressing cells rather than blocking CD39 enzymatic activity. However, the role of CD39 in promoting tumor angiogenesis has been demonstrated in CD39 knockout mice, where impaired tumor neovascularization was observed (Jackson et al., 2007). This reduced angiogenesis has been linked to disrupted P2Y receptor signaling and defective integrin function in CD39-null ECs, although the precise mechanisms remain unclear (Jackson et al., 2007). Moreover, given that CD39, in conjunction with CD73, contribute to Ado generation, its expression on ECs may further promote tumor angiogenesis through AR-mediated signaling. *In vitro* studies with HUVECs, which express both CD39

and CD73, suggest that hypoxic conditions stimulate VEGF secretion by these cells in an A_{2B}AR-dependent manner (Feoktistov et al., 2002, 2004). In addition to ECs, other cell types, including macrophages and cancer cells, can also release VEGF in response to A_{2B}AR activation, potentially further enhancing tumor angiogenesis (Vijayan et al., 2017). For instance, in a murine model of breast cancer, CD73 expressed by cancer cells promotes VEGF secretion via Ado generation, and its inhibition reduces VEGF levels, impairing tumor blood vessel formation (Allard et al., 2014).

Beyond promoting angiogenesis, CD73 on cancer cells has been shown to induce potent AR-mediated immunosuppression within the TME, and the observed hypoxia-driven upregulation of CD73 on cancer cells (II) may exacerbate these effects. Supporting this, CD73 overexpression in mouse breast cancer cells reduces the efficacy of Doxorubicin, an anthracycline drug commonly used in cancer treatment, by suppressing immune responses though A_{2A}AR activation *in vivo* (Loi et al., 2013). In humans, high CD73 expression in triple-negative breast cancer patients was similarly associated with increased resistance to Doxorubicin and served as a poor prognostic marker (Loi et al., 2013). Notably, consistent with our findings, CD73 upregulation in response to hypoxia has also been reported in three different human triple-negative breast cancer cell lines, including the MDA-MB-231 cell line used in our study (Samanta et al., 2018). This increase was shown to be mediated by HIF, aligning with our observation of elevated HIF-1α levels after hypoxia exposure (Samanta et al., 2018).

Taken together, we highlighted the cell-specific modulation of extracellular Ado metabolism under hypoxia. The observed upregulation in CD39 and CD73 in ECs and cancer cells, respectively, can promote enhanced Ado production within the TME, contributing to tumor progression. However, as previously highlighted, the complexity of the TME, characterized by cellular heterogeneity, the cell-specific distribution of purine-converting ectoenzymes, and their modulation under hypoxia and various signaling molecules, complicates our understanding of Ado metabolism and signaling within the TME. Consistent with this, while CD73 is a poor prognostic marker in several cancers, including renal cell carcinoma, ovarian cancer, and breast cancer (Loi et al., 2013; Tripathi et al., 2020; Turcotte et al., 2015; Vijayan et al., 2017), its high expression is associated with better prognosis in endometrial and bladder cancers (Bowser et al., 2016; Koivisto et al., 2019; Wettstein et al., 2015). Further research is crucial to deepen our understanding of the cell-specific regulation of Ado metabolism and its broader implications across different tumor types, advancing therapeutic strategies that effectively target Ado metabolism in cancer.

6.1.3 Intracellular Ado metabolism as an alternative ATP source and its cell-specific functional role

Current research in the field has primarily focused on extracellular Ado metabolic and signaling pathways and its role in cancer immune evasion. Given the elevated Ado levels within the TME and the capacity of cells to uptake and metabolize Ado, we aimed to elucidate the functional significance of intracellular Ado metabolism across different cell types.

Vascular ECs, breast and prostate cancer cells, and activated T cells efficiently took up extracellular Ado and phosphorylated it into ATP, while non-activated T cells showed only minimal capacity for such intracellular Ado metabolism (I-III). This negligible ability of non-activated T cells to uptake Ado is attributed to the low expression levels of ENTs, whereas activated T cells exhibited high ENT1 expression at both the mRNA and protein levels (III). As T cells become activated, their energy demands increase, along with the need to synthesize various essential molecules, including purines and pyrimidines, to support their proliferation and effector functions. The upregulation of ENT1 in activated T cells likely reflects its crucial role in maintaining the purine salvage pathway by facilitating the transport of extracellular nucleosides and nucleobases utilized for DNA and RNA synthesis.

Although we did not directly confirm the presence of various ENT isoforms in ECs and cancer cells, it is likely that ENT1 mediates Ado uptake in these cell types as well. The significant inhibition of Ado uptake by 50 nanomolar Dipyridamole (an ENT1/ENT2 inhibitor) and NBTI (an ENT1 inhibitor) in vascular ECs supports the involvement of ENT1, as this low concentration of Dipyridamole effectively inhibits ENT1 without affecting ENT2 (Wang et al., 2013). In cancer cells, treatment with NBTI alone also led to a significant reduction in cellular ATP levels derived from extracellular Ado, indicating the presence of ENT1 and the Ado salvage pathway in both breast and prostate cancer cells (II).

ATP synthesized through the Ado-salvage pathway likely makes a negligible contribution to overall cellular ATP levels under normal conditions. However, this may change under metabolic stress or pathological conditions, such as cancer, which are characterized by elevated extracellular Ado levels. Generally, cells rely on two major pathways for ATP production: OXPHOS and glycolysis. Different cells favor one pathway over another based on their energy demands, functional roles, and environmental conditions. ECs predominantly rely on glycolysis, a metabolic preference that is particularly critical for supporting vessel sprouting (Li et al., 2019b). However, mitochondrial respiration may also contribute to ATP production in vascular ECs. In our study, exposure of HUVECs to hypoxia, which impaired OXPHOS due to limited oxygen availability, led to a partial reduction in global cellular ATP levels (I). Under these hypoxic conditions, we observed an increased uptake of extracellular Ado and its subsequent phosphorylation to ATP, suggesting

that such intracellular Ado metabolism may serve as a compensatory mechanism for ATP synthesis to cope with hypoxia-induced ATP depletion (I). Future experiments are required to clarify the role of intracellular Ado metabolism not only in sustaining ATP levels of ECs during hypoxia, but also in determining its broader functional significance.

Previous research has also reported the non-receptor-mediated effects of Ado in ECs. For instance, sustained Ado exposure has been shown to impair lung endothelial barrier function through ENT-mediated cellular Ado uptake, which leads to increased reactive oxygen species (ROS) levels and the activation of several stress-related signaling pathways, including p38, c-Jun N-terminal kinase (JNK), and the RhoA signaling cascades (Lu et al., 2012). Additionally, elevated intracellular Ado levels, resulting from the inhibition of ADK, led to reduced activity of transmethylation pathways, contributing to anti-inflammatory effects in ECs (Xu et al., 2017a). However, the precise mechanisms underlying these observations, involving intracellular Ado pathways and the resulting cellular metabolic changes, as well as alterations in epigenetic pathways influenced by nuclear Ado levels, remain unknown.

The Warburg effect, a metabolic shift characterized by a reliance on glycolysis even in the presence of sufficient oxygen, is a hallmark of cancer cells (Warburg, 1956; Pavlova et al., 2022). This adaptation enables cancer cells to sustain rapid proliferation and adapt to the hypoxic conditions of the TME. Consistent with this, global cellular ATP levels remained stable in cancer cells under both normoxic and hypoxic conditions (II). Furthermore, the lack of significant changes in Ado uptake or ADK expression levels under hypoxia (II) further suggests that cancer cells maintain stable ATP levels without requiring the Ado-salvage pathway as an alternative source of ATP production.

Our group has previously shown that the treatment of both MDA-MB-231 and PC3 cancer cells with Ado inhibited cellular invasion and migration *in vitro* in an AR-independent manner (Virtanen et al., 2014). In line with these findings, the present study observed a reduction in the tumorigenic potential of MDA-MB-231 cells pre-treated with Ado, but not with the AR agonist NECA, in SCID mice, further emphasizing the non-receptor mediated effects of Ado in modulating tumor progression (II). Additionally, other studies have reported non-receptor mediated effects of Ado in inducing cancer cell apoptosis (Antonioli et al., 2013). For instance, in human cervical and breast cancer cells, the cellular uptake of Ado and its subsequent conversion to AMP triggered AMPK-induced apoptosis, independent of caspase-mediated pathways (Mello et al., 2014; Saitoh et al., 2004). However, the precise molecular pathways responsible for these non-receptor-mediated effects of Ado on cancer cells remain largely unclear and require additional exploration.

T cells exhibit a dynamic metabolic nature depending on their activation state. Naïve T cells primarily rely on mitochondrial respiration for ATP production, whereas upon stimulation, they undergo metabolic reprogramming to favor glycolysis (Chapman et al., 2020; Reina-Campos et al., 2021). Inhibition of glycolysis with 2DG or blockade of OXPHOS with Oligomycin each resulted in approximately a 50% reduction in ATP levels in activated T cells, indicating that they rely on both metabolic pathways to sustain ATP production (III). Cellular Ado uptake and its intracellular metabolism selectively restored ATP levels in activated T cells treated with 2DG, but not in those treated with Oligomycin, underscoring the interplay between the Ado salvage pathway and mitochondrial respiration in these cells. Another notable observation is that treatment of unchallenged activated T cells with Ado led to a significant increase in global cellular ATP levels within the first few hours, but under prolonged exposure, these levels returned to baseline (III). This effect could be attributed to findings that prolonged incubation of activated T cells with Ado inhibits both glycolysis and mitochondrial respiration (III). It is likely that cells are reprogramming their metabolic pathways to restore steady-state ATP levels, with the Ado salvage pathway becoming a significant contributor to ATP synthesis. Nevertheless, the functional role and importance of this metabolic shift remain to be elucidated.

In addition to changes in T-cell bioenergetic pathways, Ado triggered immunosuppressive responses in T cells in a non-receptor mediated manner. Cellular uptake of Ado by activated T cells led to impaired IFN-γ production, as well as increased levels of apoptotic and necrotic T cells (III). Similar to our observations, ENT1-mediated cellular uptake of Ado has been documented to induce apoptosis in activated T cells and decrease their proliferation (Festag et al., 2020). Given the crucial role of glycolysis, not only as an ATP-producing pathway but also as a mediator of cytokine production, including IFN-γ (Chang et al., 2013), the observed inhibition of glycolysis by Ado uptake may explain the reduced levels of this cytokine (III). Additionally, single cell-data from Ado-treated activated T cells revealed a significant upregulation of the *ATF4* gene (III), a stress response gene in T cells (Ameri & Harris, 2008; Sundrud et al., 2009). This observation may indicate that these T cells are under metabolic stress and that *ATF4* potentially mediates the modulatory effects of Ado on T-cell proliferation and function.

The presence of Ado during T-cell activation further revealed its significant immunosuppressive effects, mediated through non-receptor mechanisms. ENT1-facilitated Ado uptake in these cells led to a marked reduction in T-cell proliferation, along with decreased expression of the activation markers PD-1 and CD25, as well as lower levels of the inflammatory cytokine IFN-γ and cytotoxic molecule GranzB (III). This strong immunosuppressive pathway was completely abolished following pharmacological blockade of ENT-1 (III), underscoring the potential therapeutic

relevance of targeting ENT1. While our study demonstrated that targeting ARs with the dual $A_{2A/2B}$AR antagonist AB-928 or the non-selective AR agonist NECA did not induce any changes in T cells (III), other studies have documented Ado-induced immunosuppressive and cytotoxic effects in ADA-deficient murine thymocytes via both A_{2A}AR-dependent and -independent mechanisms (Apasov et al., 2000; Apasov & Sitkovsky, 1999).

Given these findings, further investigation into Ado-mediated immunosuppression is required, with a clear distinction between receptor-mediated and non-receptor-mediated effects. While AR-mediated suppression of T-cell effector function within the TME is considered the primary immunosuppressive mechanism (Allard et al., 2020), the contribution of intracellular Ado metabolism may also be significant. Elucidating the precise molecular mechanisms behind the metabolic shifts in human T cells and their associated immunosuppressive effects, potentially driven by cellular uptake of Ado and its intracellular phosphorylation, could provide novel strategies to overcome immune suppression in the TME.

6.1.4 Limitations and further perspective

Despite the valuable findings of our studies, several limitations should be considered. First, the cells used were either commercially available cancer cell lines or derived from peripheral blood or umbilical cords of healthy donors, which do not fully capture the unique characteristics of cells present within the TME. Additionally, the hypoxic conditions in studies (I) and (II) were acute, lasting 4 to 24 hours, whereas the TME often experiences chronic hypoxia over years, characterized by alternating oxygen dynamics. Another limitation is that our research primarily focused on Ado-generating pathways arising from ATP, overlooking NAD metabolism as an alternative source of Ado. Specifically, cyclic ADP ribose hydrolase (CD38), along with ENPP1 and CD73, mediates the conversion of the oxidized form of NAD into Ado, potentially influencing extracellular Ado levels (Di Virgilio et al., 2024; Yegutkin & Boison, 2022).

The use of two-dimensional (2D) cultures limits the relevance of our findings to *in vivo* settings, as these models do not replicate the intricate tissue architecture and dynamic cellular interactions present in real tissues. Future studies should incorporate more advanced models, such as three-dimensional (3D) cancer organoids, which would allow for more accurate investigation of ATP-Ado metabolic pathways in a more physiologically relevant context (Nguyen & Caldas, 2021). Additionally, patient-derived organoids present a promising tool to explore cancer heterogeneity and evaluate the responses of diverse tumor types to Ado-based therapies (Iorio et al., 2016).

Given the limited success of clinical trials targeting the CD39-CD73-$A_{2A/2B}$AR axis with small-molecule inhibitors and antibodies, current efforts are focused on refining therapeutic strategies to improve patient outcomes. Besides improving pharmacological agents, including their specificity and potency (Jacobson et al., 2023), other approaches have been suggested, such as the development of predictive gene signatures to identify responder patients (Augustin et al., 2022; Nguyen & Caldas, 2021; Yegutkin & Boison, 2022), therapeutic oxygenation to alleviate Ado-mediated immunosuppression driven by hypoxia (Hatfield et al., 2019), and the use of engineered antibodies to selectively eliminate cells expressing high levels of Ado-generating enzymes within the TME (Zhang et al., 2022). However, a deeper understanding of the complex regulation of ATP-Ado metabolism within the TME remains essential for refining and improving future therapeutic interventions.

One of the obstacles hindering progress in this field is the lack of tools for accurately measuring Ado concentrations within the TME (Di Virgilio et al., 2024). Although elevated Ado levels in the TME have been reported (Graziano et al., 2023; Ohta et al., 2006), the transient nature of extracellular Ado as an intermediate metabolite, with its rapid inactivation and cellular uptake, complicates precise quantification (Chen et al., 2013; Moser et al., 1989). Additionally, the observed heterogeneity and cell-specific modulation of ATP-Ado metabolic pathways across different cell types pose further challenges, as various cells regulate extracellular ATP and Ado levels differently. Therefore, novel approaches are needed not only to quantify Ado levels within the TME but also to assess the contribution of specific cellular subsets to Ado generation.

Given the significant effects of intracellular Ado metabolism observed in our studies, further exploration into the underlying mechanisms is essential. While pharmacological agents such as Dipyridamole and NBTI have provided valuable insights, genetic modifications targeting ENT1, ADK, AK, or NDPK, may offer a deeper understanding of the Ado-salvage pathway or uncover additional mechanisms. Although use of relatively high micromolar concentrations of Ado in our studies complicate direct translation to physiological conditions, intracellular Ado metabolism remains highly relevant in the Ado-rich TME. Of particular interest are the immunosuppressive effects of ENT1-mediated Ado uptake by T cells, which dampen their proliferation, cytokine production, and conventional ATP-generating pathways, underscoring the need for further investigation. Exploring the intracellular Ado metabolic pathways in T cells in greater detail may not only enhance our understanding of Ado-mediated immunosuppression within the TME but also reveal novel therapeutic strategies for cancer treatment.

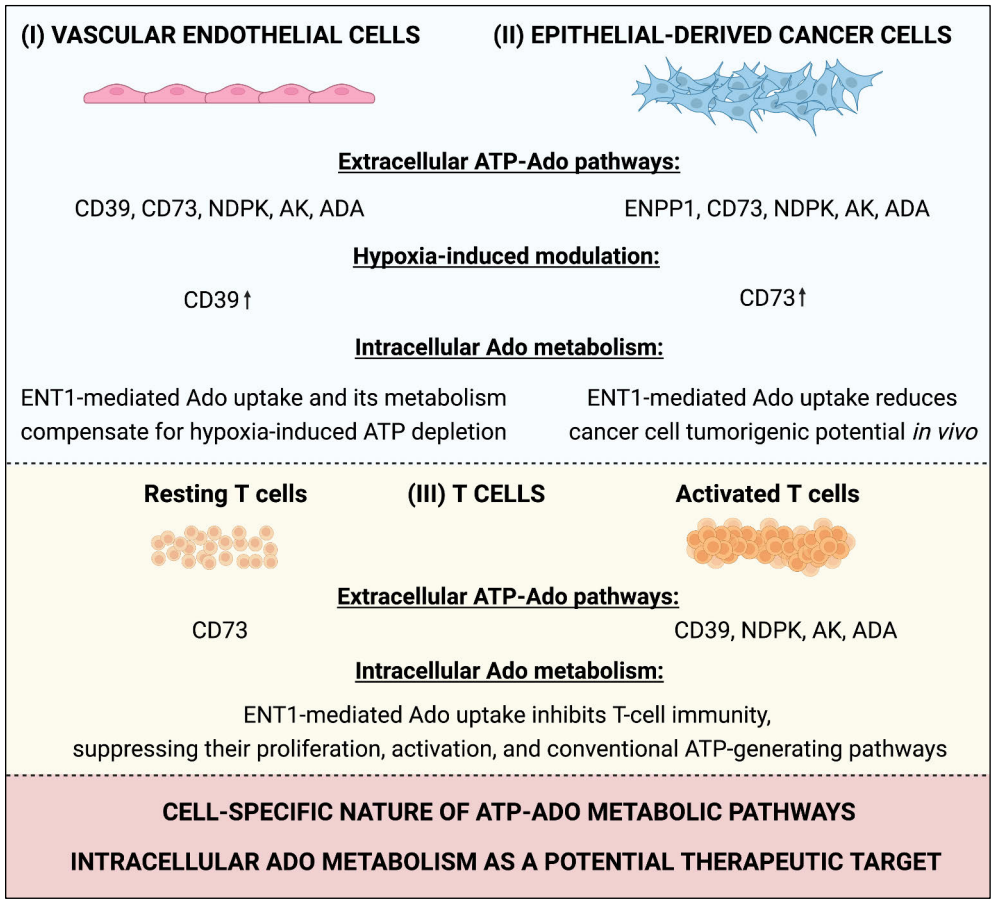


Figure 7. Graphical summary of key findings from studies (I-III). The cell-specific nature of extracellular ATP-Ado metabolic pathways is demonstrated across vascular ECs, breast and prostate carcinoma cells, and peripheral blood T cells, along with hypoxia-driven modulation of these pathways in ECs and cancer cells (not studied in T cells). Intracellular Ado metabolism is further highlighted for each cell type, with its potential therapeutic relevance, particularly in T cells.

7 Summary/Conclusions

This study highlights the complexity of ATP-Ado metabolic pathways across human vascular ECs, breast and prostate carcinoma cells, and T cells. We have demonstrated that each cell type exhibits a unique profile of purinergic enzymes and transporters, and further showed selective modulation of this purinergic signature under acute hypoxia in ECs and cancer cells, or TCR activation in T cells. While CD39 and CD73 are widely recognized as the primary producers of extracellular Ado, particularly within the TME, our findings uncover the presence of additional redundant ectoenzymes capable of regulating extracellular ATP and Ado levels. Furthermore, our data emphasize the underappreciated role of intracellular Ado metabolism, which has modulatory effects on both tumorigenesis and immune regulation. A more comprehensive approach to targeting Ado metabolism in cancer that accounts for the complexity of extracellular ATP-Ado pathways, as well as intrinsic non-receptor mediated effects of Ado, could help maximize the therapeutic potential of Ado-based strategies.

In summary, the key findings of this study include the following:

1. Human vascular ECs, cancer cells, and T cells express a cell-specific repertoire of purine-converting enzymes that, along with ENT1, regulate the dynamic cycling between extracellular and intracellular ATP and Ado pools.
2. Vascular ECs express the “classical” nucleotide-inactivating ectoenzymes CD39 and CD73, whereas breast and prostate cancer cells express ENPP1 and CD73 but lack CD39. Naïve T cells exhibit high levels of CD73, whereas activated T cells predominantly express CD39.
3. Under hypoxic conditions, vascular ECs upregulate CD39 activity, while cancer cells increase CD73. The extracellular ATP-regenerating (AK and NDPK) and Ado-degrading (ADA) pathways remain unchanged during acute hypoxia.
4. ENT1-mediated Ado uptake reduces the tumorigenic potential of cancer cells and suppresses T-cell immunity by inhibiting their proliferation, cytokine production, and conventional ATP-generating pathways.

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