Regulation of purinergic signaling and T cell activation by mechanisms that define extracellular nucleotide concentrations

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Boston, August 29th, 2014

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Declaration

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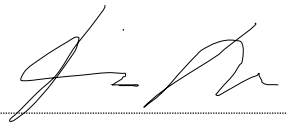

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Kurzfassung

T-Zellen sind essentiell für die Antigenerkennung und Regulierung der Immunabwehr. Funktionsstörungen dieser Zellen sind für die Entstehung zahlreicher Immunerkrankungen verantwortlich. Ein besseres Verständnis der Mechanismen, welche die T-Zellfunktionen regulieren, ist daher von großer Bedeutung. Die purinerge Signalübertragung spielt eine wichtige Rolle bei der Immunzellregulation. Eine angemessene T-Zellantwort erfordert die Freisetzung von ATP und die autokrine Stimulation von Purinrezeptoren. Allerdings ist nur wenig über die Mechanismen bekannt, welche die extrazelluläre ATP-Konzentration und den ATP-Abbau durch T-Zellen steuern. Das Ziel dieser Arbeit war es, diese Mechanismen genauer zu erforschen. Die Experimente wurden mit humanen, primären $CD4^+$ T-Zellen und der Jurkat-T-Zelllinie durchgeführt. Stimuliert wurden die Zellen mit Beads oder in Platten, die mit Antikörpern gegen CD3 im T-Zellrezeptor (TCR)-Komplex und gegen den Co-Rezeptor CD28 beschichtet waren. Die extrazellulären Konzentrationen von ATP, ADP, AMP und Adenosin wurden mit HPLC gemessen und die mRNA-Expression von Ektoenzymen mit quantitativer PCR. Eine optimale ATP-Freisetzung schien die gleichzeitige Stimulation von TCR und CD28 zu erfordern. Die Freisetzung von ATP ließ sich mit CBX oder Latrunculin B reduzieren. Dies deutet auf eine Beteiligung der Mitochondrien und Pannexin 1 bei der ATP-Freisetzung hin. Jurkat- und $CD4^+$ T-Zellen unterschieden sich in den Expressionsprofilen der Ektonukleotidasen. In beiden Zelltypen war Adenosindeaminase (ADA) am stärksten exprimiert. Die Expression war in Jurkat-Zellen etwa 10-fach höher als in $CD4^+$ T-Zellen. Neben ADA exprimierten Jurkat-Zellen auch ENTPD2, 5NTE und ENPP1. Die Expression dieser Enzyme war in stimulierten Zellen wesentlich erhöht. Zusätzlich zu ADA hatten $CD4^+$ T-Zellen auch hohe ENPTD1-, ENTPD2-, 5NTE-, ENPP2- und ENPP3-Level. Abgesehen von 5NTE war die Expression der Enzyme in $CD4^+$ T-Zellen nach Stimulation verringert. Weiters zeigten Jurkat- und $CD4^+$ T-Zellen Unterschiede in ihrem extrazellulären Nukleotidmetabolismus. $CD4^+$ T-Zellen entfernten ATP schneller, während Jurkat-Zellen Adenosin schneller abbauten. ADP war das primäre ATP-Abbauprodukt von Jurkat-Zellen und AMP das von $CD4^+$ T-Zellen. Die Zugabe von ATP oder ADP führte zu einem extrazellulären ATP-Anstieg. Nur der ATP-induzierte Anstieg konnte mit CCCP oder CBX reduziert werden, was darauf hindeutet, dass Mitochondrien und Pannexin 1/Connexin-Membrankanäle involviert sind. Die Hemmung der Adenylatkinase mit AP5A verringerte den ADP-induzierten ATP-Anstieg. Dies weist darauf hin, dass Adenylatkinasen an der Phosphorylierung von ADP zu ATP beteiligt sind. Zusammengefasst zeigt diese Arbeit, dass verschiedene Mechanismen die extrazelluläre Konzentration von purinergen Liganden in T-Zellen regulieren. Außerdem unterscheiden sich Jurkat- und $CD4^+$ T-Zellen in ihrem extrazellulären Nukleotidmetabolismus und der Enzymexpression. Diese Unterschiede könnten zu dem malignen Phänotyp der Leukämie-Zelllinie beitragen.

Schlagwörter: T-Zellen, Purinerge Signalübertragung, Ektonukleotidasen, Nukleotidmetabolismus

Abstract

T cells are essential for antigen recognition and regulation of host defense. T cell dysfunctions are involved in the pathogenesis of many immune disorders. A better understanding of the mechanisms that regulate T cell functions is therefore highly relevant. Purinergic signaling plays an important role in the regulation of immune cells. In T cells, ATP release and autocrine stimulation of purinergic receptors are required for a proper cell regulation. However, little is known about the mechanisms that govern extracellular ATP concentrations and the breakdown of ATP in T cells. The aim of this thesis was to study these mechanisms in more detail. Experiments were performed with human primary $CD4^+$ T cells and the Jurkat T cell line. The cells were stimulated with anti-CD3/CD28 antibody coated beads or with plate-bound antibodies to stimulate the T cell receptor (TCR) and the CD28 co-receptors. Extracellular concentrations of ATP, ADP, AMP, and adenosine were measured with HPLC. The mRNA expression of ectoenzymes was assessed via quantitative PCR. Optimal ATP release appeared to require simultaneous stimulation of T cells via TCR and CD28. ATP release was reduced by CBX or latrunculin B, indicating the involvement of mitochondria and pannexin 1 in the ATP release. Further, Jurkat cells and $CD4^+$ T cells differed in their expression profiles of ectonucleotidases. In both cell types, adenosine deaminase (ADA) was the most abundantly expressed enzyme. The expression in Jurkat cells was about 10-fold higher than in $CD4^+$ T cells. In addition to ADA, Jurkat cells also highly expressed ENTPD2, 5NTE, and ENPP1. The expression of these enzymes increased significantly after TCR/CD28 stimulation. Besides ADA, $CD4^+$ T cells also expressed high levels of ENPTD1, ENTPD2, 5NTE, ENPP2, and ENPP3. Except for 5NTE, the expression of all ectoenzymes decreased following TCR/CD28 stimulation of $CD4^+$ T cells. Jurkat cells and primary $CD4^+$ T cells showed differences in their metabolism of extracellular nucleotides. $CD4^+$ T cells were faster than Jurkat cells in removing ATP, while Jurkat cells degraded adenosine more rapidly than $CD4^+$ T cells. ADP was the primary ATP breakdown product formed by Jurkat cells and AMP that of $CD4^+$ T cells. Furthermore, the addition of ATP or ADP induced an increase in extracellular ATP. While this response to ATP was reduced by treatment with CCCP or CBX, neither CCCP nor CBX affected the response to ADP. This suggests that the ATP- but not the ADP-induced increase in extracellular ATP involves mitochondria and pannexin 1/connexin hemichannels. However, inhibition of adenylate kinase with AP5A reduced the ADP-induced ATP increase, which suggests that adenylate kinases are involved in the phosphorylation of ADP to ATP. In conclusion this work shows that various mechanisms regulate the extracellular concentrations of purinergic ligands in T cells. Jurkat and $CD4^+$ T cells differ from each other in their extracellular nucleotide metabolism and enzyme expression. These differences might contribute to the malignant phenotype of the leukemia cell line.

Keywords: T cells, purinergic signaling, ectonucleotidases, nucleotide metabolism

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1 Introduction

The immune system is arranged in a way to be effective while not being harmful [1]. This requires a tight regulation of the cells involved in this system. One of these cell types involved in immune regulation are the T lymphocytes. They are major regulators of the immune system and of inflammatory processes and are designed to recognize antigens. However, T cells need to be appropriately stimulated and their responses must be tightly controlled to allow their intended functions. How these cells become activated is still incompletely understood. Purinergic signaling is a novel mechanism of cell activation that has been shown to be involved in regulating T cells and other immune cell populations. This project focused on the mechanisms by which T cells regulate the extracellular concentrations of the ligands that stimulate purinergic receptors, which are involved in the control of T cell activation and function.

1.1 Human T lymphocytes possess several surface molecules that are important for their function

The entire lymphocyte population is comprised of two major subgroups, the B lymphocytes and the T lymphocytes also simply referred to as B and T cells [2]. It is a hallmark of these cell groups of the adaptive immunity that both subpopulations express antigen specific cell surface receptors [3]. B cells carry immunoglobulin molecules on the cell surface [2]. Once stimulated, B cells generate immunoglobulins that constitute an important aspect of humoral immunity [3]. Antibodies can reach pathogens in the blood and the extracellular environment, but they cannot reach bacteria, parasites and viruses that replicate inside cells. T lymphocytes are responsible for cell-mediated, adaptive immune responses and can also recognize and destroy intracellular pathogens. The receptors of T cells recognize peptide fragments of intracellular pathogens, which are transported to the cell surface by major histocompatibility complex (MHC) glycoproteins [2]. In addition to the peptide interaction the T cell also interacts with the MHC molecule to stabilize the interaction and effectively respond to the antigen [2].

The main two classes of T lymphocytes both develop in the thymus. One class carries the clusters of differentiation (CD) 8 cell surface protein and the other CD4. These markers are important for the function of the cells by specifying the interplay between T cells and other cells. When the naive T lymphocytes leave the thymus, the CD8 cells are determined to become cytotoxic T cells, whereas naive CD4 T cells can turn into a variety of effector T cells after they got activated by an antigen. In response to the diversity of pathogens, they exert a variety of effector activities [2]. Differentiated CD4 T lymphocytes release several lymphokines and thereby take part in the inflammatory response by activating macrophages [1], mobilizing neutrophils, aiding B cell antibody production or promoting allergic responses [2]. By secreting cytokines, T helper cells direct immune responses and contribute to humoral immune responses by activating innate killing mechanisms or

supporting the differentiation of B cells into plasma cells that are able to produce antibodies [3].

CD4 is a co-receptor necessary for the binding of the T cell to the MHC molecule of antigen presenting cells (APCs). T cells that activate other cells carry CD4 and recognize MHC class II, which is expressed by cells that take part in the immune response like dendritic cells, macrophages, B and T cells and thymic cortical epithelial cells. CD4 associates with the T cell receptor (TCR) on the cell surface during antigen recognition. Then it binds remote from the peptide-binding site to the non-polymorphic region of the MHC molecule. Thus, CD4 represents a co-receptor necessary to generate and enhances an effective T cell response [2]. The binding of both CD4 and T cell receptor to the same MHC class II peptide complex increases the sensitivity of the T cell to antigens considerably [2].
Variable antigen-binding chains and associated invariant chains constitute the antigen receptor and are responsible for signaling. The term T cell receptor is often used and will further be used in this thesis to refer to the whole T cell receptor complex together with the associated signaling subunits (Figure 1) [2].
The highly variable, antigen-binding TCR α:β heterodimer alone is not a complete cell surface antigen receptor. Other molecules, like the CD3 complex and the ζ chain, are needed for signaling and for the expression of the antigen binding chains on the cell surface. The CD3 complex consists of one CD3γ, one CD3δ, and two CD3ε chains. These CD3 proteins comprise extracellular immunoglobulin like domains, while the ζ chain, a disulfide-linked homodimer, has a short extracellular domain [2].
T cell receptor signaling is initiated by phosphorylation of tyrosine residues in the cytoplasma at the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3ε, γ, δ, and ζ chains, when the receptor binds to its ligand. CD3ε, γ, δ contain one ITAM each, while three ITAMs can be found on each ζ chain [2].

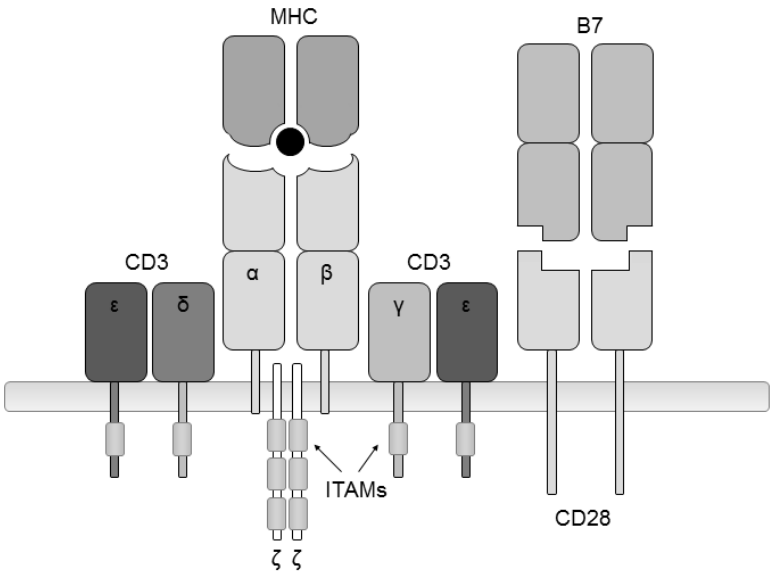


Figure 1: T cell receptor complex with co-stimulatory molecules.
The T cell receptor α:β heterodimer is highly variable and responsible for binding antigen (black) on MHC molecules of antigen presenting cells. It forms a complete cell surface antigen receptor with other molecules like the CD3 proteins and ζ chains. The CD3 complex consists of one CD3γ, one CD3δ, and two CD3ε chains. Tyrosine-based activation motifs (ITAMs) of the chains are important for signaling. CD3ε, γ, and δ contain each one ITAM, while three ITAMs can be found on each ζ chain. For activation of T cells co-stimulatory molecules like CD28 are necessary. This molecule binds to a B7 molecule on antigen presenting cells [2].

Signaling via the T cell receptor complex is not sufficient by itself to activate naive T cells. Antigen presenting cells that can activate these naive T cells express co-stimulatory molecules on their cell surface. These molecules interact with co-stimulatory receptors on the cell surface of the naive T cells to transmit a required signal (signal 2), along with the antigen stimulatory signal [2]. The cell surface protein CD28 is a co-stimulatory molecule. It exists on all naive T cells and binds the co-stimulatory ligands CD80 (B7.1) and CD86 (B7.2). Both ligands are only expressed on specialized APCs. Such co-stimulatory ligands are induced on APCs by infection, which ensures the activation of T cells only in case of infection. Both antigen and co-stimulatory ligand on the same APC are needed for the naive lymphocyte to become activated [2].
Binding of B7 to CD28 leads to the tyrosine phosphorylation on tyrosine residues in the cytoplasmic domain of CD28. This starts the intracellular signaling cascade and stimulates IL-2 gene expression. This cytokine is necessary for promoting proliferation and differentiation of T cell into effector cells [2].

1.2 Jurkat T cell line

The Jurkat cell line originates from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia (ALL). The cell line was established from cells obtained during the

first of four relapses. This cell line shows T cell characteristics. Cells grow without attachment to the culture vessel surface. The ultrastructure of the cells displays sparse cytoplasm with a smoothly contoured cell membrane and a dentate nucleus with marginal chromatin distribution and commonly a prominent nucleolus [4].

1.3 Purinergic signaling

The term “purinergic signaling” was first coined by Dr. Burnstock who described the finding that the principal active substance released from nerves is the purine nucleotide adenosine triphosphate (ATP) [5]. Since then nucleotide signaling was found to be involved in many different cell types and tissues and now purinergic signaling can be considered as an ubiquitous signaling mechanism of mammalian, and perhaps all eukaryotic cells [6]. However, the current knowledge of this multistep cascade of purinergic signaling is rather limited [7].

Figure 2 illustrates a basic idea of purinergic signaling in T lymphocytes. T cell receptor stimulation leads to Ca^{2+} influx, which is buffered by mitochondria resulting in ATP synthesis and release of ATP via pannexin 1 hemichannels. The released ATP acts as a co-stimulatory signal that activates purinergic P2 receptors in an autocrine fashion and might also activate purinergic receptors on APCs in a paracrine fashion [8]. Extracellular ATP is also degraded to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine via ectonucleotidases. Adenosine can be taken up by the cell or be further degraded by adenosine deaminase, but it can also activate P1 receptors [9].
The individual parts of this briefly described signaling pathway are further explained in the next subchapters.

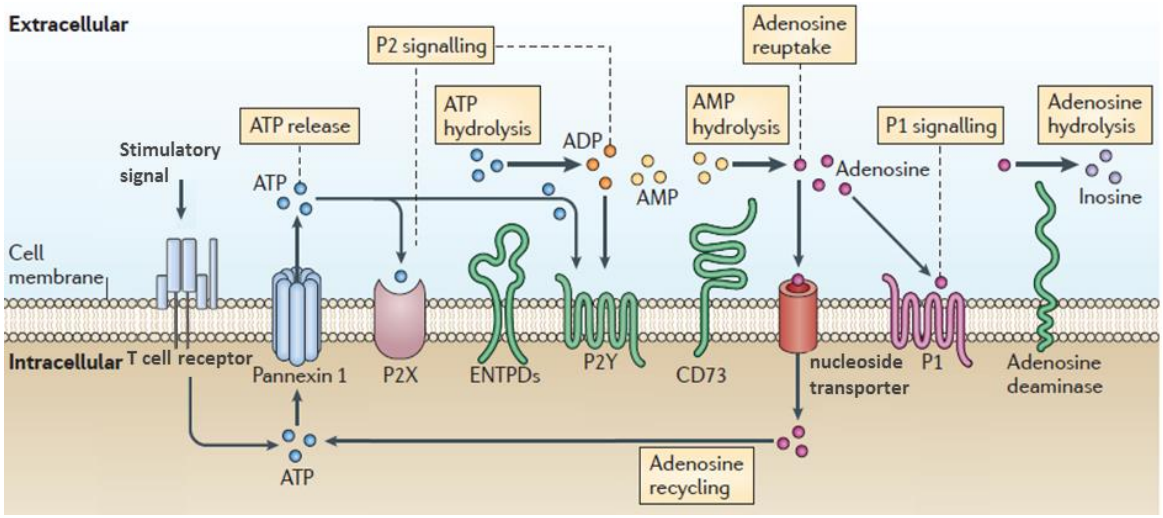


Figure 2: Proposed purinergic signaling cascade in human T lymphocytes (modified from [9]).
Key molecules involved in the purinergic signaling cascade are ATP and its metabolites, P1 and P2 receptors, transport molecules like pannexin 1 and adenosine transporters, and various enzymes. Upon receptor stimulation ATP is released from the cell through pannexin 1 hemichannels. In the extracellular space it activates P2 receptors in an autocrine way. This extracellular ATP is also degraded to ADP, AMP and adenosine via ectonucleotidases like ectonucleoside triphosphate diphosphohydrolases (ENTPDs) and ecto-5'-nucleotidase (CD73). The formed adenosine can be taken up by the cell via transporter molecules or gets further degraded by adenosine deaminase to inosine. Adenosine also activates P1 receptors. Intracellular adenosine can be recycled back to ATP.

The specificity of purinergic signaling is directed by the extracellular nucleotide source, specific receptor expression and ectonucleotidases [6]. Various enzymes participate in the control of extracellular nucleotide and nucleoside concentrations and are thereby key regulators of purinergic signaling. The duration and magnitude of purinergic signaling depends on the consumption, generation and interconversion of nucleotides, and the expression of nucleotide and nucleoside receptors on the cell surface [3].
The roles of endogenous signaling molecules, such as ATP and adenosine, are very complex during inflammatory and immune responses. Depending on the extracellular concentration, expression of purinergic receptors and ectoenzymes, their effects seem to switch from immune stimulatory to immune regulatory or vice versa. Thus, purinergic signaling is important for the fine-tuning of the immune response and inflammation to remove the threat, while only minimally damaging healthy tissue [3].

1.3.1 Nucleotides, nucleosides, purinergic receptors, and their involvement in purinergic signaling

Nucleotides and nucleosides are essential in all cells and organ systems for intracellular as well as extracellular mechanisms [7]. Intracellularly, they are essential for energy

metabolism, enzyme regulation and nucleic acid synthesis [10]. Extracellularly, nucleotides and adenosine can act as signaling molecules in various biological systems [3,10]. They have essential and diverse effects on most if not all biological processes including neurotransmission, smooth muscle contractility, platelet aggregation and immune response [3,6,7,11,12]. Various conditions, such as inflammation, hypoxia and ischemia can increase extracellular ATP and adenosine concentrations [3]. Nucleotides interact with surface purinergic receptors expressed on virtually all cells and can be metabolized by ectonucleotidases [13]. Extracellular effects of ATP and adenosine, its degradation product, are mediated by purinergic P1 and P2 receptors. Ectonucleotidases are effective regulators that might also have protective functions to keep extracellular ATP and adenosine levels in physiological ranges [12].

ATP is an important extracellular signaling molecule

ATP is a purine nucleotide and is naturally occurring in every living cell of the body [12]. It is used intracellularly as energy source [13] for energy dependent processes, while extracellular ATP is an important signaling molecule in various tissues and cells [3,7]. Intracellular ATP is synthesized via complex mechanisms to maintain or quickly restore the intracellular level [13]. Thus, intracellular ATP concentrations in eukaryotic cells are high with 3–10 mM compared to very low physiological extracellular concentrations in plasma of 400–700 nM [3,10,14]. This low concentration is achieved by several mechanisms like minimal permeation of cytosolic ATP across lipid bilayers, and due to ubiquitous ectoenzymes that hydrolyze extracellular nucleotides. This ensures that putative signaling agents like ATP are only transiently present and only in response to specific physiological and/or pathological situations [10,14].

Cytosolic ATP can be the source of extracellular ATP when the cells break or get damaged [10]. Further, ATP can be released via exocytosis of secretory granules or vesicles [10,15] and via intrinsic plasma membrane channels or pores. Thus, ATP might accumulate locally in the extracellular space for example during infection and inflammation. Due to the widespread nucleotide receptor expression on various cell types that release ATP, it seems obvious that the release of ATP evokes positive feedback and feed-forward reactions [10]. In most cells and tissues ATP-induced responses correlate with changes in cellular Ca^{2+} homeostasis [10].

Low micromolar extracellular ATP concentrations can affect a variety of biological processes in various cells and tissues [10,13] like platelet aggregation, neurotransmission, cardiac function and muscle contraction [13]. In immune cells ATP activates the immune system and leads to pro-inflammatory responses [16].

Its effect is mediated by binding to purinergic P2X or P2Y receptors on the cell surface [7,10]. Extracellular ATP is not only a P2 receptor ligand, but also a substrate for ectonucleotidases and thus, an adenosine source [7]. A rapid inactivation of extracellular nucleotides to adenosine after signal transduction is needed [7]. Various ectoenzymes

rapidly catabolize extracellular ATP, and these enzymes are differently expressed on distinct tissue and cell surfaces [10].

Adenosine is an important anti-inflammatory and immunosuppressive agent

Adenosine is a purine nucleoside that is composed of adenine and ribose linked via a glycosidic bond [17]. Adenosine is generated by the hydrolysis of released ATP by ectonucleotidases [15]. In addition, intracellular hydrolysis of ATP or ADP might also result in the formation of cytosolic adenosine which can be released from intact cells under physiological as well as pathological situations [10]. Thus, adenosine is a metabolite of adenine nucleotides but also a precursor of them. All cells use energy from ATP catabolism and as adenosine can be formed from intracellular and extracellular adenine nucleotide catabolism, all cells are possible adenosine sources [17]. Most intracellularly formed adenosine is deaminated to inosine or diffuses out of the cell [17].
Although nearly all cells can produce adenosine, it seems to be produced on demand and is not stored in vesicles. With a half-life of a few seconds, extracellular adenosine can diffuse several millimeters from its source, having a wider area of action compared to a synapse but a more localized reach than a circulating hormone [15]. Adenosine deaminase degrades adenosine to inosine [17]. However, extracellular adenosine can also be taken up by the cell via nucleoside transporters [18,19] and is then used to restore intracellular purine nucleoside and nucleotide pools [10].

Various cells in the blood and vascular system can release ATP and ADP, which can be the source of adenosine in blood. Adenosine is transported from blood cells into plasma via passive carriers like nucleoside transporters and via diffusion from the interstitium between endothelial cells into the vascular space [17]. However, the concentration of adenosine in the blood and interstitial fluids is kept low. The half-life of adenosine in the blood is only a few seconds [17,20]. Adenosine plasma concentrations *in vivo* are hard to determine due to rapid formation and metabolism by blood cells [17,21] and individual differences [20].

Adenosine has anti-inflammatory and immunosuppressive properties and can act as an inhibitor of inflammation [22,23] with a tissue protective effect [24]. Adenosine has a dampening effect on inflammation via the A2A receptor that is found in nearly all immune cells [25]. In T cells, stimulation of the A2A receptor inhibits T cell activation and proliferation [25–27] as well as inflammatory cytokine production [24,25,27], whereas anti-inflammatory cytokine production is enhanced [25]. Therefore, adenosine contributes to immunosuppressive mechanisms [26].

1.3.2 Purinergic receptors and their ligands

Various biological effects of extracellular purines, such as adenosine, ADP and ATP, acting as important signaling molecules are mediated by interaction with cell surface receptors. These purine receptors are divided into two main families, the P1 and P2 receptors. Adenosine is recognized by and acts through P1 receptors [11,28]. Thus, these receptors are also called adenosine receptors and they are ubiquitously distributed [17]. They are subdivided into A1, A2A, A2B and A3, which all couple to G proteins [29] and possess seven transmembrane-spanning domains [28].

In contrast, P2 receptors recognize nucleotides like ATP and ADP [11]. These receptors are subdivided into P2X and P2Y receptors [30,31]. P2X are ligand-gated cation channels [11,14,32] permeable to Na^+, K^+ and Ca^{2+} [29,33] and mediate fast responses [31], while P2Y receptors are single membrane spanning [11] G-protein coupled receptors [14,29] mediating slower responses [31]. ATP acts via P2X receptors, while P2Y receptors respond not only to ATP but also to other substrates like ADP. However, the agonist specificity differs between subtypes [6]. Subclasses of these receptors are numbered consecutively [31]. To date, seven mammalian P2X (P2X1 to 7) [6,11,34] and eight P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14 [9]) receptor subtypes have been identified and characterized [6,34]. These receptors are widely distributed in mammalian tissues [11].

1.3.3 Ectoenzymes and their principal roles

Like all signaling pathways, purinergic signaling requires mechanisms that can inactivate the signaling cascade [35,36]. To remove or inactivate extracellular signaling molecules, they can be taken up by the cell or extracellularly hydrolyzed. Besides, receptors can be desensitized or downregulated. Nucleotides are hydrolyzed extracellularly, which results in the formation of the respective nucleoside and free phosphate. The cleaved off phosphate can be recycled and used for nucleotide resynthesis [35] and the nucleosides can be taken up by the cell via specific transporters and rephosphorylated inside the cell [37].

Ectonucleotidases belong to the ectoenzymes [6]. A main function of ectonucleotidases is the termination of nucleotide signaling [35]. Ectonucleotidases usually hydrolyze nucleoside triphosphates, nucleoside diphosphates, nucleoside monophosphates and dinucleoside polyphosphates, and thereby produce nucleoside diphosphates, nucleoside monophosphates, nucleosides, phosphate and inorganic pyrophosphate [37]. Divalent cations like calcium or magnesium and an alkaline pH are prerequisites for maximal catalytic activity of many enzymes. Hence, the maximal catalytic activity is adjusted to the extracellular environment. The catalytic site of membrane bound ectonucleotidases is directed towards the extracellular space [35].

Ectonucleotidases terminate the signal transduction induced by ATP and ADP via rapid breakdown that reduces the extracellular concentrations of these nucleotides in the

proximity of purinergic receptors. Thus, the expression of ectoenzymes on the cell surface controls the ligand availability for purinergic receptors and thereby influences the magnitude and duration of the signal [10,37]. In addition to removing the stimulatory P2 ligands ATP and ADP, these enzymes generate adenosine at the same time [10]. The differential expression of ectonucleotidases in particular cell types might regulate the local production of adenosine for signaling purposes [10]. In cells expressing both adenosine and P2 receptors extracellular ATP activates P2 receptors, while adenosine receptors will be activated upon ATP metabolization to adenosine. Depending on the cell type and the expression pattern of adenosine receptor subtypes, adenosine can either attenuate or potentiate the responses that are triggered by ATP receptors [10].
There is a competition between ectonucleotidases and P2 receptors for the limited amounts of released nucleotides. Thus, ectonucleotidases are involved in the modulation of P2 receptor function [6], the termination of receptor activation by hydrolysis of the ligand [6,37], in the prevention of receptor desensitization, or in the activation of counter-acting receptors by the generation of hydrolysis products. All this is important for purinergic signal transmission [37].

Moreover, various enzyme families are able to hydrolyze extracellular nucleotides. Different enzymes are able to hydrolyze the same nucleotide, depending on tissue and cellular expression. Further, different enzymes are co-localized on cell or tissue surfaces. Soluble ectonucleotidases are transported by body fluids and can diffuse within the interstitial medium. Cleaved and soluble extracellular isoforms exist that then may be termed “exonucleotidases” [35].
However, some of the enzymes have multiple functions. They do not only have catalytic activity, but also interact with extracellular matrix proteins and might have a role in cell adhesion or transmembrane receptor functions [35,37]. They might also be involved in intracellular signaling by interacting with intracellular proteins and modulation of signaling pathways [6,7].

1.3.4 The major groups of ectonucleotidases

Currently four families are known that share to some extent tissue distribution and substrate specificity [3]. These four main groups of ectonucleotidases include the ectonucleoside triphosphate diphosphohydrolase (NTPDase) family, ectonucleotide pyrophosphatase/phosphodiesterase (NPP) family, ecto-5′-nucleotidase (CD73), and alkaline phosphatases (APs) [3,7,34,35,37]. Different distributions can be observed among individual members of the families. However, many of these enzymes show overlapping tissue distribution, like co-expression of members of the NTPDase family in the same tissue or co-expression of these members with members of other families like the ENPP family or ALPs [35].

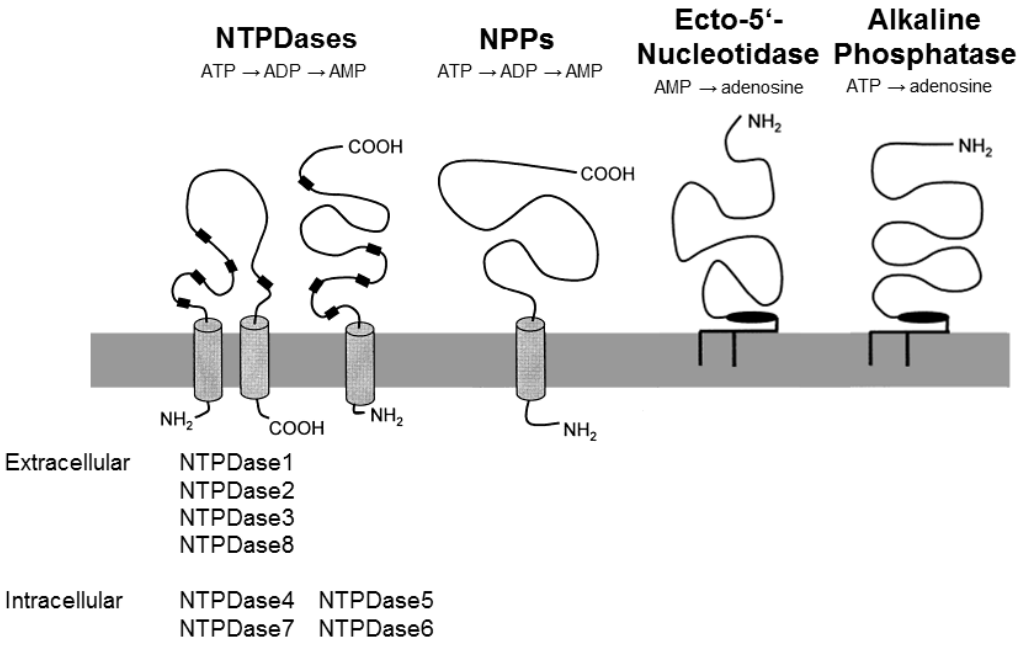


Figure 3: Major groups of ectonucleotidases (modified from [38]).

The ectonucleoside triphosphate diphosphohydrolase (NTPDase) family consists of eight members, located at the cell membrane or intracellular. NTPDase1, 2, 3 and 8 are bound to the membrane; NTPDase4 and 7 are located intracellularly, as well as NTPDase5 and NTPDase6, which might be released from the cell. NPP family members are also located at the cell membrane and/or form soluble forms. Alkaline phosphatases and ecto-5'-nucleotidase are glycosylphosphatidyl inositol (GPI) anchored to the membrane and might be released after cleavage [6,38].

Ectonucleoside triphosphate diphosphohydrolase family

The NTPDase protein family is encoded by eight ENTPD (ectonucleoside triphosphate diphosphohydrolase) genes. NTPDase1, 2, 3 and 8 are membrane-bound, cell-surface located enzymes; NTPDase4 and 7 are located exclusively intracellularly next to the lumen of cytoplasmic organelles, and NTPDase5 and 6 are located intracellularly and undergo secretion after heterologous expression [6].

NTPDases hydrolyze nucleoside triphosphates [6,37,39] and/or diphosphates [6,37] to nucleoside monophosphates, but they do not hydrolyze AMP [37]. They have the ability to dephosphorylate ATP stepwise via ADP to AMP accompanied by the cleavage of two phosphates [7]. Their preferred hydrolysis substrates vary among distinct members of this family [6,7,35,39]. Further, the NTPDase subtypes vary in their cellular location and functional properties [6,38]. Ca^{2+} or Mg^{2+} ions in millimolar concentrations are required by these enzymes for maximal activity [6,7,40–43]. In the absence of these cations the enzymes are inactive [6].

The surface-located NTPDase1, 2, 3 and 8 can be distinguished according to their substrate preference, usage of divalent cations and product formation [6,38].

These NTPDases control extracellular nucleotides and thus, the agonist availability at P2 receptors [6]. In doing so they can terminate agonist signaling of P2 receptors [39]. They are also involved in the recycling of nucleosides [6]. It is proposed that NTPDases have a dual function. First, they degrade ATP and thereby prevent receptor activation and second, they generate ADP from ATP and thus, contribute to activation of other receptors [44].

As the cell surface-located enzymes ENTPD1, 2, 3 and 8 are the only ones that can hydrolyze extracellular substrates [6], this study focuses on these enzymes, which are further discussed below.

NTPDase1 (CD39)

NTPDase1 and CD39 have been identified to be the same protein [43]. NTPDase1 is a membrane bound glycoprotein with its active site facing the extracellular milieu [43]. Nucleoside triphosphates and nucleoside diphosphates but not nucleoside monophosphates are hydrolyzed by NTPDase1. Accordingly, it hydrolyzes extracellular ATP and ADP to AMP [42,43,45–48]. However, it hydrolyzes ATP almost directly to AMP by producing only small amounts of ADP. This mainly bypasses the P2Y receptor activation for nucleoside diphosphates [6] and also terminates the action of ATP as P2 receptor ligand [45,49]. Thus, a major role of this enzyme is the inhibition of ATP or ADP induced signal transduction through P2 receptors [43]. Furthermore, it may also prevent receptor desensitization [50].

In lymphoid organs CD39 is expressed in the majority of paracortical B and T lymphocytes, low expressed in mantle zone cells, and absent from thymic lymphocytes and germinal center cells [51]. NTPDase1 is expressed on the cell surface of leukocytes [43,52]. However, less than 2 % of resting peripheral blood T, B, NK cells, monocytes, neutrophils and bone marrow cells express CD39 [51]. Human thymocytes, peripheral blood T lymphocytes, Jurkat T lymphoblasts, some other T cell lines and malignant lymphocytes have low or no extracellular ATP degradation abilities [53]. Further, normal peripheral blood T cells lack intracellular CD39 [51].

CD39 was primarily found on activated immune cells like activated T cells [45,51]. Due to the fact that it is expressed only on a subset of activated T cells, there might be functional differences between cells that express this enzyme and cells that do not [51].

In immune cells NTPDase1 might also be important for the protection of the cells from the potential lytic effect of extracellular ATP, which is able to induce cell death in these cells, but also in some tumor cell lines [45].

NTPDase1 might not only participate in cell adhesion but also in cell-cell interactions [43,51]. Thus, another role might be the interaction with other cells of the immune system [51].

NTPDase2

In contrast to NTPDase1, NTPDase2 has a higher preference for nucleoside triphosphates than for nucleoside diphosphates [7,35,42,49]. It is able to hydrolyze ATP and marginally also ADP [36]. As it preferentially hydrolyses ATP over ADP [41,48,49], it might provide a major extracellular ADP source [49]. This ADP released from the hydrolyzed ATP is then slowly degraded to AMP [6]. The conversion of ATP to ADP facilitates the activation of ADP specific P2 receptors [39].

Due to the substrate selectivity of NTPDase1 and 2 they may have different roles in regulation of P2Y receptor-mediated signaling [49].

NTPDase3 and NTPDase8

NTPDase3 and NTPDase8 hydrolyze nucleoside triphosphates and nucleoside diphosphates efficiently, but prefer ATP over ADP as hydrolysis substrate. This efficient ATP hydrolysis leads to the transient formation of the respective nucleoside diphosphate and an accumulation of ADP can be observed [42]. Thus, the transient accumulation of nucleoside diphosphates and a simultaneous presence of nucleoside triphosphates are the result of NTPDase3 and NTPDase8 action [6]. Therefore, these enzymes may attenuate and/or terminate ATP specific receptor activation, but favor the ADP specific receptor activation [42].

Ectonucleotide pyrophosphatase/phosphodiesterase family

Seven ectoenzymes, ENPP (Ectonucleotide pyrophosphatase/phosphodiesterase) 1–7, numbered according to their discovery, belong to the NPP family. They have a broad substrate specificity [37], but a clear distinction of NPP and NTPDase members is often difficult due to co-expression and similar substrate specificity [35]. Some NPPs can dephosphorylate ATP in a NTPDase-like fashion via ADP to AMP [7]. However, these families show no phylogenetic relationship [35].

NPP1, NPP2 and NPP3 are able to hydrolyze nucleotides and thus, only these three members of the ENPP family are relevant for purinergic signaling [7].

NPPs display a phosphodiesterase and nucleotide phyrophosphatase activity that allows them to hydrolyze phosphodiester and pyrophosphate bonds [35,54–56] in a variety of extracellular compounds like nucleoside triphosphates and diphosphates, but not AMP [37]. Thus, they are able to hydrolyze ATP and ADP to AMP [35,36,55]. For the catalytic activity NPPs need divalent cations like Ca^{2+} or Mg^{2+} [35,55]. In addition, NPPs might also have non-catalytic functions [56].

NPPs can be found at the cell surface [56], but they also exist as soluble forms, for example in body fluids such as serum that contains cleaved [55] or secreted [56] forms.

NPP1 is present in nearly every tissue [41] and expressed in various regions of the body [7]. However, it does not seem to be expressed on resting peripheral T cells [55]. In addition to the membrane spanning form, a soluble form exists, which is most likely cleaved of the membrane form [55,57]. In comparison to NPP1, NPP2 is not a transmembrane protein, but synthesized as a pre-pro-enzyme and secreted after removal of the signal peptide and further trimming [58]. NPP3 is membrane bound, but also soluble forms exist [59].

Ecto-5'-nucleotidase/CD73

Only one of the seven human 5'-nucleotidases that have been characterized until now, is attached to the plasma membrane. Five are found in the cytosol and one is located in the mitochondrial matrix [7].

The membrane-bound ecto-5'-nucleotidase is a glycosylphosphatidylinositol (GPI)-anchored surface protein that is also known as CD73 [35,60,61]. It is nucleotide specific and hydrolyses exclusively nucleoside 5'-monophosphates to the respective nucleoside and inorganic phosphate [35–37,60–62]. 5'-AMP is the preferred substrate [61].

The plasma membrane-bound form CD73 only hydrolyzes extracellular substrate and is not involved in intracellular nucleotide synthesis or degradation [53,63]. Thus, it mainly contributes to the purine metabolism by hydrolyzing extracellular nucleoside 5'-monophosphates [63] and is the principal enzyme that produces extracellular adenosine from AMP [37]. As CD73 is mainly involved in the hydrolysis of extracellular 5'-AMP to adenosine and no cellular release mechanism for AMP has been described, CD73 represents the final enzymatic step in the cascade leading from extracellular trinucleotides to nucleosides [61].

CD73 does not depend on divalent cations, but Mg^{2+} in millimolar concentrations can increase its activity [61]. Competitive inhibitors are for example ADP and ATP [61].

In its surface-located form, CD73 can be found in virtually all tissues, but not on every cell type [35,61]. Certain cell types like lymphocytes [53], especially circulating T cells and B cells, express this enzyme [64], while other blood cells, like neutrophils, erythrocytes and platelets express little or no CD73 [7]. The CD73 expression in various cell types is variable and seems to be regulated, for example developmentally [61]. During proliferation of the cells its activity is increased [62]. CD73 represents a T cell and B lymphocyte maturation marker, as its expression strongly correlates with the maturity of the cells and changes during T cell maturation [7,35,53,61]. Peripheral T cells show a higher enzyme activity than thymocytes [60]. While the CD73 activity is low in immature T cells, thymocytes and cord blood mononuclear cells, which are considered to be immature, it is significantly higher in mature blood T lymphocytes [53,65] and B cells [53].

CD73 has different effects on different cells and tissues, which might be important in regulating diverse functions [66]. Besides its role in the regulation of the purinergic

signaling cascade by generating adenosine that activates adenosine receptors, it plays also an important role in the synthesis of nucleosides for the purine salvage and might also have non-enzymatic functions [67]. These would be the signal transduction across the membrane [67], involvement in cell adhesion [66,67], like cell-cell and cell-matrix interactions [61,67] and co-signaling in T cell activation and proliferation [66].

Alkaline phosphatase (ALP)

ALP shows a broad substrate specificity towards various phosphated compounds [7]. For instance, ALPs hydrolyze nucleoside triphosphates, diphosphates, and monophosphates [3,37]. Thus, one enzyme could be able to catalyze the whole hydrolysis cascade from nucleoside triphosphate to the corresponding nucleoside [35]. Still it is less efficient in producing adenosine from AMP than ecto-5'-nucleotidase [7].

Compared to NTPDases and ecto-5'-nucleotidase, little is known about the role of NPPs and ALPs in controlling purinergic signaling [37].

1.3.5 Adenosine deaminase (ADA)

ADA exists in the cytosol or as ecto-ADA, however there are no differences in the catalytic activities of these molecules [28]. Only one gene exists, therefore intracellular and ecto-ADA seem to be identical at the protein level, lacking signaling peptide and putative transmembrane domain [28]. ADA can form a complex with dipeptidyl peptidase IV (DPPIV), also called CD26, which is a surface located receptor on T cells [68,69]. Larger complexes are often formed between extracellular ADA and CD26/dipeptidyl peptidase IV on lymphoid cells. ADA can also be anchored by interaction with A1 [70] or A2B adenosine receptors [71].
ADA is an ubiquitous enzyme [28] present in many tissues and cells [72]. It can be found in lymphoid and non-lymphoid tissues, such as intestine, thymus and spleen [7,72] and in the cytosol and on the cell surface [3,7]. Immune cells, like lymphocytes and dendritic cells, express this ectoenzyme on their cell surface [7,72,73]. It can also be found on monocytes, neutrophils and erythrocytes or in the serum [72].

ADA as an ectoenzyme is necessary in the purine metabolism [73] and is strongly involved in the purine inactivating chain [7] by converting adenosine to inosine via deamination [3,6,28,68,74]. Due to its participation in the purine metabolism it controls the extracellular adenosine concentration [28].
Adenosine represents a powerful immunosuppressant [68]. Thus, its anti-inflammatory role and the abundant ADA expression in lymphoid tissues cause a sustained activation of T cells and dendritic cells during inflammation [7]. However, the cellular ADA content in T cells decreases as maturation proceeds [75]. The catalytic activity to reduce adenosine,

represents the main role of ADA in the adaptive immune system and in T cell proliferation [68].
However, ecto-ADA also has a non-catalytic function as a co-stimulatory molecule, as the interaction between ADA and CD26 is essential for the activation of peripheral T cells [28]. In the total population of peripheral blood lymphocytes and the $CD4^+$ subpopulation, CD26 and ecto-ADA expression increases notably upon activation. Thus, the expression level correlates with the degree of activation. Resting T cells show low ecto-ADA expression and anergy is the result if ecto-ADA is absent. This suggests that the expression of ADA and signaling via the interaction with CD26 are necessary for maintaining and expanding activation [28].

1.4 Aims

T cells are central effector cells of the immune system. They need to be tightly regulated, but the underlying mechanisms are still not completely understood. Autocrine purinergic signaling has emerged as one important mechanism that controls immune cell functions [9]. The release of ATP and autocrine activation of purinergic P2 receptors is being increasingly recognized as a key event in T cell activation [8,9,76,77]. However, comparatively little is known about the mechanisms by which extracellular concentrations of purinergic ligands regulate T cells.

In my thesis, I focused on the mechanisms that regulate the extracellular concentrations of nucleotides in T cells. Specifically, the following key questions were addressed in this study:

- Does the release of ATP from stimulated T cells differ depending on the stimulation via the T cell receptor with or without the co-stimulation via CD28?
- Which mechanisms are involved in the release of ATP?
- How are ATP, ADP, AMP and adenosine metabolized by T cells, which ectonucleotidases are responsible, and does the expression of ectonucleotidases in T cells change in response to cell stimulation?
- Do unstimulated and stimulated T cells differ in the way how they metabolize extracellular nucleotides?
- Are there differences between Jurkat cells and primary $CD4^+$ T cells in the expression profile of ectonucleotidases and the metabolism of extracellular nucleotides?

2 Material and Methods

2.1 Materials, Reagents and Buffers

A list of all used reagents can be found in the appendix.

2.1.1 Coating of Dynabeads with anti-CD3/CD28 antibodies

Dynabeads Pan Mouse IgG (Invitrogen, Life Technologies, Grand Island, NY, USA) were coated with antibodies against human CD3 (BD Biosciences, San Jose, CA, USA) and against human CD28 (BD Biosciences). These stimulate simultaneously the T cell receptor CD3 complex and the co-receptor CD28, which is required for T cell activation [78,79]. The beads (20 µl) were washed with a magnet and 500 µl buffer containing phosphate buffered saline (PBS; HyClone, Thermo Scientific, Logan, UT, USA) with 0.05 % BSA (Sigma-Aldrich, St. Louis, MO, USA) for 1 min. Then they were resuspended in 500 µl buffer and 3 µl anti-CD3 and 3 µl anti-CD28 antibodies (~2 µg/10^7 beads) were added and incubated at room temperature for 60 min on a shaker. After that the beads were washed three times with the buffer, resuspended in the same buffer and adjusted to 25-30 x 10^4 beads/µl. The beads were kept sterile and refrigerated, so that they are stable for several weeks. The beads were washed twice and resuspended in RPMI (Roswell Park Memorial Institute) 1640 medium (ATCC, Manassas, VA, USA) before the experiment. The concentration of beads for the experiments was adjusted to one to two beads per cell.

2.1.2 Coating 96-well plates with antibodies and preparing soluble anti-CD3

96-well plate wells were coated with 30 µl 5 µg/ml human CD3 and human CD28 antibodies in PBS, incubated at 37°C for 1 h and at 4°C over night. On the next day the wells were washed twice with 200 µl PBS. All wells used for the experiment were incubated for at least 5 min with RPMI 1640 medium supplemented with 10 % heat inactivated fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA). Then the wells were washed once with 200 µl RPMI and 100 µl RPMI was added to all wells needed for the experiment. For the soluble anti-CD3 wells the RPMI was discarded shortly before the experiment and 100 µl soluble anti-CD3 in RPMI (1 µg/ml) was added instead.

2.1.3 Preparation of luciferase reagents

All reagents were from the ATP Determination Kit (Molecular Probes, Life Technologies, Grand Island, NY, USA). One milliliter of a 10 mM D-luciferin stock solution was prepared by adding 50 µl of the 20 x reaction buffer (500 mM Tricine buffer, pH 7.8, 100 mM $MgSO_4$, 2 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM sodium azide) and 950 µl of cell

culture grade water (HyClone, Fisher Scientific, Pittsburgh, PA, USA) to one vial containing 3 mg lyophilized D-luciferin. This stock was aliquoted in 1.5 ml tubes with 50 µl per tube and stored at -20°C, protected from light.
A 100 mM dithiothreitol (DTT) stock solution was prepared by adding 1.62 ml of cell culture grade water to the bottle containing 25 mg of DTT. This was aliquoted into 40 µl volumes and stored frozen at -20°C.
ATP standards were prepared by diluting the 5 mM ATP solution (in Tris-EDTA buffer) with RPMI. These diluted solutions were stored at -20°C for maximal a month. Usually 5 µM, 2.5 µM, 1 µM, 0.5 µM, 0.25 µM and 0.1 µM concentrations were used as standards.

The prepared or aliquoted components were combined as follows to make 1 ml of a standard reaction solution: 889.75 µL dH2O, 50 µL 20 x Reaction Buffer, 10 µL 0.1 M DTT, 50 µL of 10 mM D-luciferin and 0.25 µl of the 5 mg/ml firefly luciferase stock solution (in 25 mM Tris-acetate, pH 7.8, 0.2 M ammonium sulfate, 15 % v/v glycerol and 30 % v/v ethylene glycol) were added and stored on ice and in the dark until use.

2.1.4 Preparation of buffer A and buffer B for high performance liquid chromatography (HPLC)

All needed glass vessels were rinsed with distilled water, ddH_2O (Milli-Q, EMD Millipore, Billerica, MA, USA) and then with HPLC water (Macron Fine Chemicals, VWR, Radnor, PA, USA) before using.
For buffer A roughly 900 ml HPLC water was inserted into a 1000 ml glass beaker and placed on a stir plate. Then 0.1 M (13.61 g) potassium phosphate monobasic (KH_2PO_4; Fluka, Sigma-Aldrich, St. Louis, MO, USA) and 4 mM (1.358 g) tetrabutyl ammonium hydrogen sulfate (Fluka, Sigma-Aldrich), as the ion-pairing reagent, was added to the HPLC water. The pH was adjusted to 6.0 with potassium hydroxide (KOH; Fluka, Sigma-Aldrich). The solution was transferred to a glass cylinder and adjusted to one liter using HPLC water. The buffer A solution was filtered through a 0.2 µm sterile filter (EMD Millipore, Billerica, MA, USA) and stored in glass bottles at 4°C until use.
To make buffer B 70 % of buffer A was mixed with 30 % methanol (Fluka, Sigma-Aldrich) on a stir plate. Buffer B was filtered through the same filter as used for buffer A and stored in glass bottles at 4°C until use.

2.1.5 Chemicals and inhibitors

Adenosine 5'-[γ-thio]-Triphosphate (ATPγS)

ATPγS (Sigma-Aldrich) is an ATP analog that is resistant to hydrolysis [50,80,81].

Bafilomycin A

Bafilomycin A (Sigma-Aldrich) is an inhibitor of vesicular H^+-ATPase [82,83], it neutralizes acidic compartments [84] and prevents the refilling of vesicles. Cellular release via exocytosis can be reduced or abolished with bafilomycin [82].

Brefeldin A

Brefeldin A (Sigma-Aldrich) impairs endosomal traffic pathways, inhibits membrane transport and transport of newly synthesized proteins into the Golgi apparatus [85].

Carbenoxolone (CBX)

CBX (Sigma-Aldrich) is a water soluble derivative of the liquorice root derived glycyrrhetinic acid [86] and is a relatively nonspecific gap junction channel blocker [76,87–90] that is able to block pannexin 1 [76,90], but also connexins [91].

Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP)

CCCP (Sigma-Aldrich) is a proton translocator that interferes with the mitochondrial function by making the inner mitochondrial membrane permeable for protons and causing dissipation of the proton gradient across the inner mitochondrial membrane. Due to the loss of electrochemical gradient, CCCP causes the uncoupling of mitochondria. This means that the electron transfer through the electron transport chain is no longer coupled to ATP production and thus, mitochondrial ATP production is inhibited [92].

Diadenosine pentaphosphate (AP5A)

AP5A (Sigma-Aldrich) is an inhibitor of the adenylate kinase, which catalyzes 2 ADP ↔ AMP + ATP [93]. With its adenosine groups it interacts with the binding sites for ATP and AMP on the adenylate kinase [94].

Dipyridamole

Dipyridamole (Sigma-Aldrich) inhibits the nucleoside transporter and thus, the carrier mediated adenosine transport [17]. This leads to the inhibition of adenosine uptake into cells [17,95,96], but also reduces adenosine release by cells [17].

Erythrohydroxynonyladenine (EHNA)

EHNA (Sigma-Aldrich) inhibits the adenosine deaminase and thereby, prevents the adenosine metabolism to inosine, leading to an increase in interstitial adenosine [15,17].

Latrunculin B

Mitochondria move and accumulate at the immune synapse. This movement can be prevented by latrunculin B (Sigma-Aldrich), which inhibits actin filament polymerization [84].

2.2 Cells

2.2.1 Cell culture

Jurkat cells (E6-1 clone, American Type Culture Collection (ATCC), Manassas, VA, USA) and primary human $CD4^+$ T cells were cultured in RPMI 1640 medium supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) at 5 % CO_2 and 37°C in a humidified atmosphere.

2.2.2 Counting cells and adjusting cell concentrations

A 10 µl sample was diluted with 10 µl trypan blue (dilution factor = 2) and 8 µl of this mixture was pipetted into a hemocytometer (Sigma-Aldrich). The living, unstained cells in the four corner squares were counted under the microscope. The cell concentration was calculated as follows:
cell concentration [cells/ml] =
count of four chambers : 4 x dilution factor (e.g. 2) [cell/µl] x 10^3 µl/ml

2.2.3 Isolation of $CD4^+$ T cells

Primary human $CD4^+$ T cells were purified from heparinized peripheral venous blood of healthy volunteers. Blood draw was approved by the Institutional Review Board of Beth Israel Deaconess Medical Center. Typically, 60 ml blood was drawn and diluted 1 + 1 with prewarmed RPMI medium. Then 20 ml of the diluted blood was carefully layered onto 10 ml Ficoll-Paque Plus (GE Healthcare Life Sciences, Pittsburgh, PA, USA) in a 50 ml tube. The tubes were centrifuged for 30 min at room temperature at 400 x g. During centrifugation, cells separated according to their density: erythrocytes aggregated at the bottom of the tube while peripheral blood mononuclear cells (PBMC) remained at the plasma-Ficoll interphase. Due to their higher density, granulocytes separated from PBMCs and moved towards the bottom. The upper plasma layer was discarded and the PBMC layer was collected and transferred into a new 50 ml tube. The tubes were filled up with RPMI to reach 50 ml and centrifuged at 300 x g for 12 min. Washing was repeated one more time. Cells were counted and after that washed again.
The $CD4^+$ cell isolation was done according to the MACS separation (Miltenyi Biotec, San Diego, CA, USA) manufacturer's instructions with slight modifications. The RPMI was completely aspirated and the cells were resuspended in cold buffer (PBS + 0.5 % BSA + 2 mM EDTA); 80 µl of the buffer was used for 1 x 10^7 cells. This suspension was

transferred into a 15 ml tube and 20 µl CD4 microbeads (Miltenyi Biotec) were added per 1 x 10^7 cells. The suspension was mixed well by slightly flicking the tube, incubated for 15 min at 4°C and washed with 15 ml buffer (300 x g, 10 min). The supernatant was aspirated and the pellet was resuspended in 500 µl buffer. The MS column (Miltenyi Biotec) was stuck into the magnet and prepared by rinsing it with 500 µl buffer. 500 µl cell suspension was pipetted onto the column. The column was washed three times with 500 µl buffer. The column was removed from the magnet, placed onto a new 15 ml tube and 1 ml buffer was flushed through the column with a plunger to get the magnetically labeled cells. The $CD4^+$ cells were washed once with RPMI and rested overnight in RPMI supplemented with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at a density of ~5 x 10^5 cells/ml.

2.2.4 Evaluation of $CD4^+$ cell purity by FACS

Flow cytometry was used to determine the purity of isolated $CD4^+$ cells. For that 2 x 10^5 PBMCs or $CD4^+$ cells were stained with anti-CD3 phycoerythrin (PE; BD Biosciences) or anti-CD4 allophycocyanin (APC; BioLegend, San Jose, CA, USA) or both or the respective isotype controls (BD Biosciences) at 1:100 dilution in staining buffer consisting of PBS/5 % FBS for 20 min at 4°C in the dark. After that cells were washed twice with 200 µl staining buffer and resuspended in 200 µl fixing solution (BD sheath fluid/0.5 % v/v formaldehyde (Sigma-Aldrich)). The samples were stored at 4°C until measurement with a FACSCalibur flow cytometer (BD Biosciences).

2.3 Measurement of ATP, ADP, AMP and adenosine

All experiments were performed at 37°C in a water bath placed on a vibration isolation table to minimize mechanical stimulation.

2.3.1 Stimulation-induced ATP release

Jurkat cells were resuspended in RPMI medium at a density of 5 x 10^6 cells/ml. Aliquots of 150 µl (5 x 10^5 cells) were transferred to 1.5 ml tubes and cells rested for 30 min. After that 50 µl RPMI, beads without antibodies or beads coated with anti-CD3/CD28, anti-CD3 or anti-CD28 (1–2 beads/cell) were added to the cells and incubated for 0.5 min or 1 min. Cells were placed on ice for 10 min. Then the samples were spun down at 400 x g for 5 min at 0°C. The supernatant was harvested, centrifuged again (400 x g, 5 min, 0°C) and 110 µl of the supernatant was transferred into new prechilled tubes and analyzed immediately using the luciferase assay.

Jurkat cells were resuspended in RPMI medium at 5 x 10^6 cells/ml and 130 µl aliquots were transferred to wells of a 96-well plate and rested for 30 min. After that, 100 µl of the cell suspension (5 x 10^5 cells) was pipetted with a multichannel pipette into wells containing 100 µl RPMI, soluble anti-CD3 (1 µg/ml) or to wells that had been coated with anti-CD3

and anti-CD28 antibodies (see 2.1.2). Cells were stimulated for 5 min, 1 min or 0.5 min. Stimulation was stopped by placing the plate on ice for 10 min. Then the samples were carefully transferred to 1.5 ml tubes and spun down at 400 x g for 5 min at 0°C. The supernatant was harvested, centrifuged again (400 x g, 5 min, 0°C) and 110 µl of the supernatant was transferred into new prechilled tubes and analyzed immediately using the luciferase assay.

2.3.2 Stimulation with ATP, ATPγS or ADP

Jurkat cells or $CD4^+$ T cells were resuspended at 3.3×10^6 cells/ml in RPMI medium, and 150 µl aliquots (5×10^5 cells) were transferred to 1.5 ml tubes. After 30 min incubation at 37°C, CCCP, CBX, AP5A, anti-CD3/CD28 coated beads or RPMI (control) was added in a volume of 25 µl. Cells were incubated for another 10 min. Then RPMI or ATP (Sigma-Aldrich), ATPγS or ADP (Sigma-Aldrich) at a final concentration of 2 µM, 5 µM or 10 µM was added with a multichannel pipette. Cells were immediately placed on ice or incubated at 37°C for 0.5 min, 1 min or 10 min. The samples were treated and centrifuged as described above (2.3.1) and immediately measured with luciferase.

2.3.3 Extracellular metabolism of ATP, ADP, AMP and adenosine

Jurkat cells (5×10^5 cells/tube or 7.5×10^5 cells/tube) or primary $CD4^+$ T cells (5×10^5 cells/tube) in 200 µl RPMI were treated with ATP, ADP, AMP (Sigma-Aldrich) or adenosine (Sigma-Aldrich) at the indicated concentratios. For stimulation anti-CD3/CD28 beads were used at 1–2 beads/cell. The experiment was stopped after 3 s (as fast as possible), 0.5 min, 1 min, 10 min or 60 min by placing samples on ice for 10 min. The samples were treated and centrifuged as described above (2.3.1) and 120 µl of the supernatant was transferred into a new tube. After that, 6 µl of 400 mM perchloric acid (PCA; Sigma-Aldrich) was added to the supernatant. The tubes were vortexed and stored at -80°C until processing for HPLC analysis (2.5).
To assess if adenosine is broken down by ADA or taken up by the cell 10 µM EHNA and/or 10 µM dipyridamole were added to the cells 10 min prior to adenosine addition (5 µM to Jurkat cells and 13 µM to $CD4^+$ T cells). Cells were centrifuged and further processed as described above.

2.4 Bioluminescence assay

The ATP determination kit was used to measure the extracellular ATP concentrations. The luciferin/luciferase assay is a sensitive method to measure ATP allowing the detection of ATP ranging from low nanomolar to millimolar concentrations [78]. According to the instructions, the quantitative determination of ATP is done with recombinant firefly luciferase and D-luciferin, its substrate. Luciferase needs ATP to produce light, which is the basis of this assay.

The assay was performed according to the manufacturer's instructions with slight modifications.
Before measurement the Thermo Scientific Luminoskan Ascent Microplate Luminometer was primed with 5 ml 70 % ethanol (Decon Laboratories, King of Prussia, PA, USA), 5 ml ddH_2O and two times with 1 ml reaction solution.
Thereafter, 25 µl of the appropriate samples were pipetted into wells of a 96-well plate, placed into the luminometer and 75 µl of the reaction solution was added by the machine. A blank sample containing cell culture grade water and the standards were measured first. Then the experimental samples were measured. A standard curve was generated from the prepared ATP standards. The ATP concentrations in the samples were calculated using this standard curve.

2.5 HPLC analysis

The nucleoside adenosine and the nucleotides AMP, ADP and ATP were separated by ion-pair reversed-phase chromatography. With this HPLC it was possible to assess the extracellular ATP, ADP, AMP or adenosine removal or breakdown capacity of the cells with a simultaneous observation of the degraded or arisen products.

To proceed with preparation for HPLC analysis, samples were thawed in ice water and vortexed. Then they were centrifuged at 15,700 g for 10 min at 0°C to remove protein precipitates. To neutralize the pH 80 µl of the supernatant was taken out, placed into a new 1.5 ml tube and 8 µl of 4 M K_2HPO_4 (Sigma-Aldrich) was added. After that the samples were vortexed for 10 s, chilled on ice for 10 min and spun at 15,700 g for 10 min at 4°C. After that 50 µl of the supernatant was transferred to a new 1.5 ml tube in an ice bath and 200 µl of HPLC water was added.
For the removal of lipids 50 µl tri-n-octylamine (Thermo Fisher Scientific, New Jersey, USA) and 50 µl 1,1,2-trichlorotrifluoroethane (SynQuest Laboratories, Alachua, FL, USA) were added, vortexed for 20 s, chilled on ice for 10 min and spun at 15,700 g for 5 min at 4°C.
The next step, ethenoderivatization, was performed to obtain fluorescent adenine compounds according to the method described by Lazarowski et al. [97]. For this, 150 µl of the upper aqueous phase was transferred to a new 1.5 ml tube; 20 µl 250 mM Na_2HPO_4 (Sigma-Aldrich) and 32 µl 1M chloroacetaldehyde (Sigma-Aldrich) were added and vortexed for 10 s. In parallel to samples, 150 µl negative control (HPLC water) and 150 µl standards containing 50 nM of each, ATP, ADP, AMP and adenosine, were prepared by adding 20 µl 250 mM Na_2HPO_4 and 32 µl 1 M chloroacetaldehyde and also vortexed for 10 s. All samples, controls, and standard were incubated at 72°C in a water bath for 30 min. After the incubation the samples were chilled on ice. To alkalize the samples 50 µl 0.5 M NH_4HCO_3 (Sigma-Aldrich) were added into each tube. The samples were filtered with one milliliter syringes through 0.2 µm syringe filters into new tubes.

The identification and quantification of the ethenylated samples were accomplished with an automated Waters HPLC apparatus with a fluorescence detector [97]. A sample volume of 60 µl was transferred into glass inserts (Thermo Scientific) and placed into the glass vessels of the Waters 717plus Autosampler (Waters Corporation, Milford, MA, USA) carrousel, which was placed afterwards into the machine and kept at 4°C. Via the Waters 510 HPLC Pump Millipore a 40 µl sample was injected into the 3 µm Supelcosil LC-18-T HPLC column (Sigma-Aldrich). The elution times for the standard were typically about 11 min for adenosine, about 12 min for AMP, about 15 min for ADP, and about 17 min for ATP (Figure 4). The fluorescent purine derivatives were detected at an excitation wavelength of 254 nm and an emission wavelength of 410 nm via a scanning fluorescence detector (Waters 474). Data were analyzed by using Empower2 software (Waters), and results of test samples were compared against the standard samples with known concentrations of ATP, ADP, AMP and adenosine.

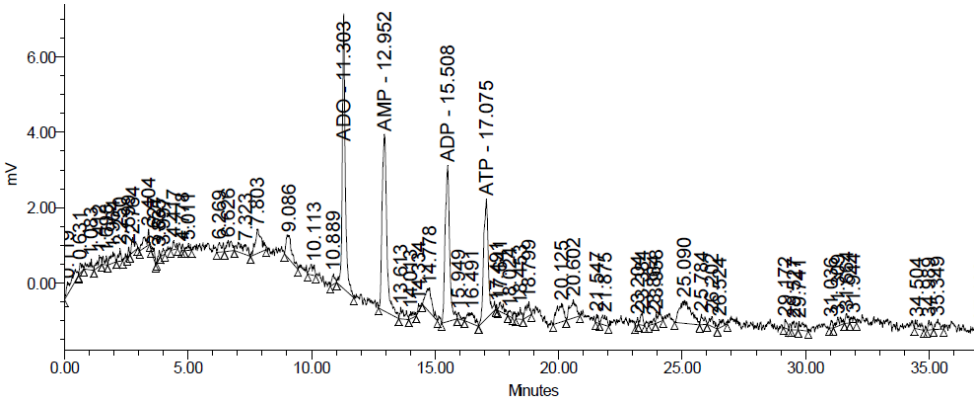


Figure 4: Chromatogram of an ethenoderivatized standard.

The elution times with an ion-pair reversed-phase high performance liquid chromatography of a standard containing adenosine, AMP, ADP and ATP were typically about 11 min, 12 min, 15 min and 17 min, respectively. The ethenoderivatized fluorescent purine derivatives were detected at an excitation wavelength of 254 nm and an emission wavelength of 410 nm via a scanning fluorescence detector.

2.6 mRNA expression

2.6.1 Cell stimulation

The expression of ectonucleotidases in stimulated and unstimulated Jurkat cells and $CD4^+$ T cells was examined via quantitative polymerase chain reaction (qPCR). Cells (1×10^6/ml) were incubated in RPMI medium supplemented with 10 % FBS in the absence or presence of anti-CD3/CD28 coated beads (1-2 beads/cell) for 4 h. The 4-h stimulation period was chosen because of the observation that IL-2 mRNA levels rise 2 to 3 h after stimulation and reach the maximum at about 5 h, after which they decrease again in Jurkat [98] and $CD4^+$ T cells [99]. Then cells (4×10^5 cells/sample) were pelleted by centrifugation (300 x g,

5 min, 23°C), resuspended in 350 µl RLT lysis buffer (RNeasy mini kit, Qiagen, Valencia, CA, USA) and homogenized by vortexing for 1 min. If RNA was not isolated immediately, samples were stored at -80°C.

2.6.2 RNA isolation

RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Samples were thawed and 350 µl 70 % ethanol (Sigma-Aldrich) was added. The sample was transferred to a spin column and centrifuged at 16,100 x g for 30 s at room temperature. The flow-through was discarded, 700 µl of the Buffer RW1 was added and again centrifuged with the same speed and time. After that the column was washed with 500 µl Buffer RPE and centrifuged with the same speed and time as previously. It was again washed with 500 µl Buffer RPE, but centrifuged for 2 min with the same speed. The column was placed in a new 2 ml collection tube and centrifuged for 1 min at high speed and placed on a new 1.5 ml collection tube. Then 30 µl of RNase free water was added to the column and centrifuged for 1 min again with the same speed. The eluate was pipetted into the column to elute it a second time and centrifuged for 1 min. The eluate was stored at -80°C.

2.6.3 Reverse transcription

RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions.
For the genomic DNA elimination reaction 4 µl of the 7 x gDNA Wipeout Buffer was added to 24 µl template RNA. The mixture was incubated for 2 min at 42°C and then placed on ice.
The reverse transcription master mix was prepared and kept on ice. A master mix consisting of 8 µl 5 x Quantiscript RT Buffer including Mg^{2+} and dNTPs, 2 µl RT Primer Mix, and 2 µl Quantiscript Reverse Transcriptase containing RNase inhibitors was prepared per sample. This 12 µl reverse transcription master mix was added to 28 µl of the genomic DNA elimination, mixed and stored on ice. The solution was incubated for 25 min at 42°C and then incubated for 3 min at 95°C to inactivate the Quantiscript Reverse Transcriptase.
The tubes containing cDNA were placed on ice and either proceeded directly with real-time PCR or stored at -20°C.

2.6.4 Quantitative real-time PCR

Real-time qPCR primers were purchased from Qiagen. They are designed to be highly specific and have a high efficiency of ~100 %.

The cDNA was diluted 1:5 in PCR grade water.

For the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) all components were thawed at room temperature. Then they were mixed vigorously, and centrifuged to collect contents to the bottom of the tube before using. The SYBR Green was kept in the dark as much as possible. For the master mix 3 µl PCR grade water, 2 µl primer mix (final concentration 250 nM) and 10 µl iQ SYBR Green Supermix were added per sample. This 15 µl master mix was pipetted into each well of a 96-well plate and 5 µl diluted cDNA was added. One master mix was used as negative control with water.
The cycling program consisted of 10 min 95°C, 20 s 95°C, 45 times of 30 s 60°C, 30 s 72°C and the following melting curve was composed of 95°C for 15 s, 60°C for 15 s, gradually increasing temperatures (from 60 to 95°C) over 20 min, 95°C for 15 s and 4°C as a hold step.

The comparative threshold cycle (C_t) method was used for relative quantification of gene expression. Relative expression ratios were calculated by normalizing the target gene expression against the expression of the internal reference gene TATA-box-binding protein (TBP), which is stably expressed in T cells [100] ($2^{(Ct\ reference\ gen\ -\ Ct\ target\ gen)} = 2^{\Delta Ct}$). The fold expression change after stimulation of cells was then determined by dividing the ΔCt of stimulated cells through the ΔCt of unstimulated cells.

2.7 Statistics

If not stated otherwise data are expressed as mean values ± standard deviation (SD) of $n \geq 3$ experiments. For comparison between groups unpaired Student's t-test or Mann-Whitney U test were applied when data were normally or not normally distributed, respectively. Differences between groups were considered statistically significant at $p < 0.05$. If data represent $n \geq 3$ experiments and are not marked by an asterisk (*), differences were not statistically significant.

3 Results

3.1 Evaluation of cell isolation and stimulation

3.1.1 Purity of $CD4^+$ Cells

To determine the purity of isolated $CD4^+$ T cells, cell preparations were stained with anti-CD3 and anti-CD4 antibodies and analyzed using flow cytometry. The results were compared to PBMCs stained before isolation of $CD4^+$ cells. Double staining with the respective isotype control antibodies was used to identify cells staining positive for CD3 and CD4. As shown in figure 5 the isolation procedure proved high efficiency. While $CD3^+CD4^+$ T cells accounted for about 25 % of PBMCs (Figure 5 A right), this number increased to > 99 % after magnetic cell sorting (Figure 5 B right).

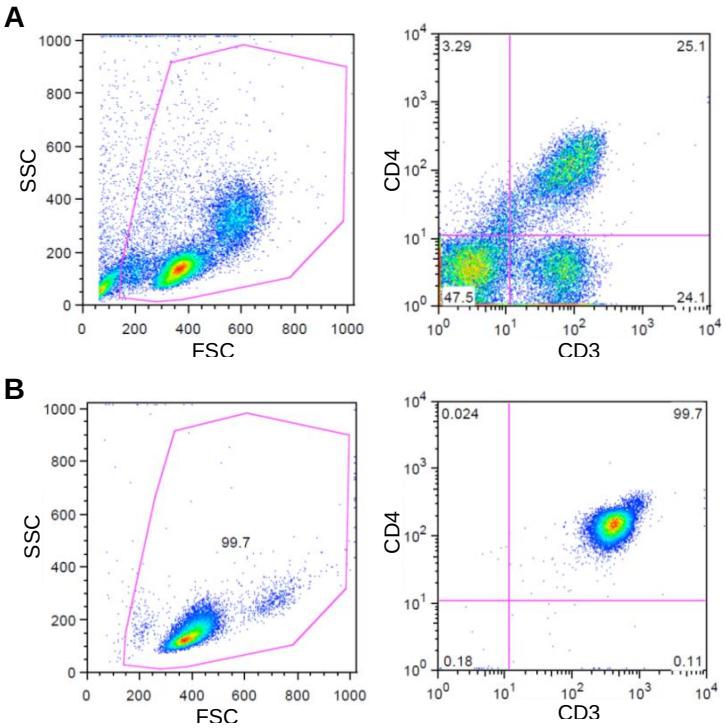


Figure 5: High purity of $CD4^+$ cells isolated from human blood.

PBMCs were isolated from human blood using Ficoll-Paque Plus gradient centrifugation and $CD4^+$ cells were isolated from PBMCs using MACS separation. The cells were stained with anti-CD3 phycoerythrin (PE) and anti-CD4 allophycocyanin (APC) antibodies. Then they were fixed in 0.5 % v/v formaldehyde and analyzed using flow cytometry. (A; left) Forward (FSC) and side scatter (SSC) and (right) CD3 and CD4 expression properties of PBMCs isolated from human blood. (B; left) FSC and SSC and (right) CD3 and CD4 expression properties of cell preparations isolated using MACS separation with anti-CD4 magnetic beads. Numbers indicate the percentages of cells in the respective gates.

3.1.2 T Cell stimulation with anti-CD3/CD28 antibody coated beads

To confirm that the activation of Jurkat and primary $CD4^+$ T cells with anti-CD3/CD28 coated beads was effective under our experimental conditions, interleukin-2 (IL-2) levels were examined. Both cell types revealed high IL-2 mRNA levels after stimulation for 4 h indicating a good activation with these coated beads (Figure 6). The increase in IL-2 mRNA levels after stimulation was stronger in $CD4^+$ T cells (876-fold) than in Jurkat cells (76-fold).

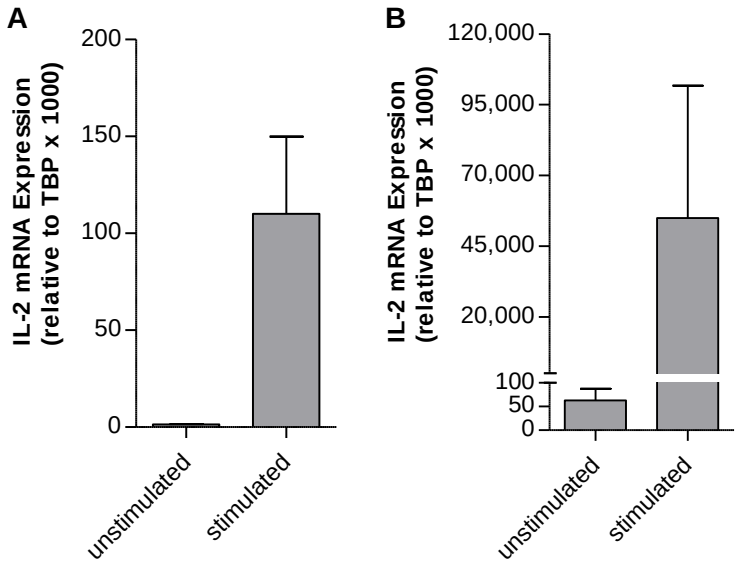


Figure 6: IL-2 mRNA expression is up-regulated in stimulated T cells.
(A) Jurkat cells or (B) primary human $CD4^+$ T cells were suspended in RPMI supplemented with 10 % FBS (10^6 cells/ml) and incubated in the presence or absence of anti-CD3/CD28 coated beads (1–2 beads/cell) for 4 h. IL-2 mRNA expression was determined by qPCR and normalized against the expression of TATA-box-binding protein (TBP). Data shown are means + SD of (A) n = 2 or (B) n = 3 independent experiments measured in duplicates.

In addition, stimulated cells were examined under the microscope (Figure 7). Both Jurkat cells and primary $CD4^+$ T cells formed characteristic aggregates when they were stimulated with beads.

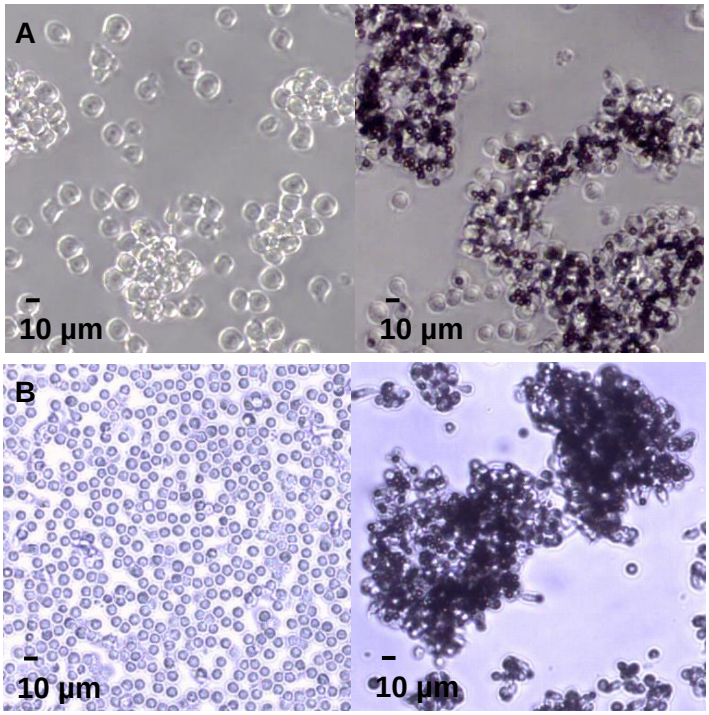


Figure 7: Cell cluster formation after treatment with anti-CD3/28 coated beads.
(A) Jurkat cells or (B) $CD4^+$ T cells isolated from human blood were stimulated with anti-CD3/CD28 coated beads (1–2 beads/cell) for 4h (right). Unstimulated control cells are shown on the left. Cells were observed under the microscope (100 x magnification).

3.2 T cell stimulation via CD3/CD28 triggers ATP release

3.2.1 Basal ATP release by unstimulated T cells

To be able to assess increasing or decreasing ATP levels in the supernatant of T cells, the basal ATP levels were examined. It was found that even resting T cells release a certain amount of ATP. Basal ATP levels reached concentrations of 298.26 ± 153.10 nM (n = 12, measured in duplicates, ± SD) in the cell culture supernatant of Jurkat cells (5 x 10^5 cells in 200 µl RPMI). In $CD4^+$ T cells, the basal ATP level was 8-fold lower (38.37 ± 6.57 nM in the supernatant of 5 x 10^5 cells in 200 µl RPMI; n = 3, measured in duplicates, ± SD).

3.2.2 T cell stimulation via CD3/CD28 may be more effective in inducing ATP release than via anti-CD3 alone

Complete activation of T cells and the induction of appropriate effector functions require stimulation of both TCR and co-stimulatory receptor CD28. It is well known that T cell stimulation increases the ATP concentration in the supernatant [77]. However, it is not clear to what extent this ATP release depends on stimulation of CD3, CD28, or both. To investigate this question, Jurkat cells were stimulated with uncoated beads or with beads coated with anti-CD3, anti-CD28 or both (Figure 8). Cells treated with RPMI or antibody-free beads served as non-stimulation controls and revealed the mechanically induced ATP

increase, for example due to pipetting. Mechanical stimulation by adding RPMI led to an increase in the extracellular ATP concentration compared to untreated cells. However, the supernatant of cells incubated with antibody-free beads showed even higher ATP levels. Stimulation with beads coated with anti-CD28 alone induced less ATP release than stimulation with antibody-free beads. Extracellular ATP concentrations were highest in Jurkat cells that had been stimulated with anti-CD3/CD28 coated beads, however the intra-experimental variation was high. Beads coated with anti-CD3 induced an ATP concentration nearly as high as beads coated with both antibodies. After one minute the ATP levels were lower than after 30 s stimulation. Stimulation with anti-CD3 coated beads caused the highest release of ATP at this time point, while there was no difference in extracellular ATP levels when cells were stimulated with anti-CD28, anti-CD3/CD28 or antibody-free coated beads.

To corroborate these findings, the experiment was repeated, however the results could not be replicated (data not shown). The ATP increase was lower in all settings compared to the previously done experiment and no difference was found between the supernatant of unstimulated cells and cells treated with the different stimuli described above.

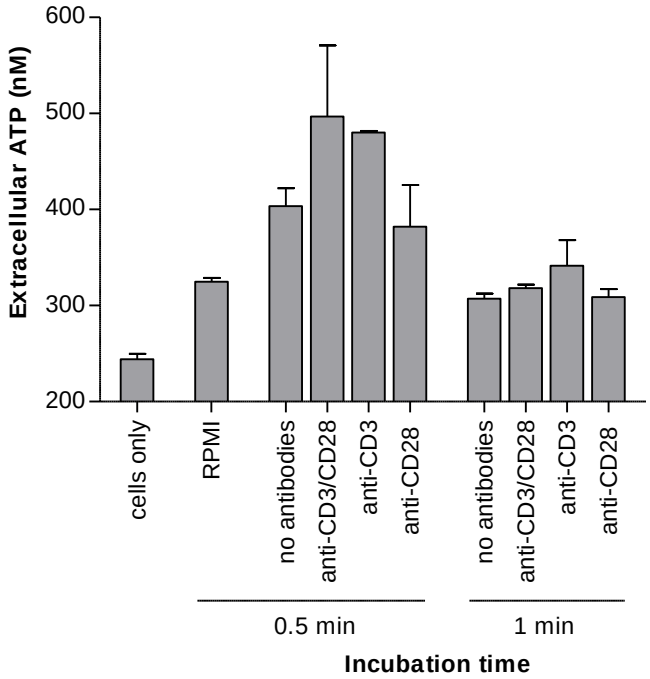


Figure 8: Combined stimulation via CD3 and CD28 may increase ATP release from Jurkat cells.
Jurkat cells suspended in RPMI (5×10^6 cells/ml) were stimulated by adding beads carrying the indicated combinations of antibodies against CD3 and CD28 (1–2 beads/cell). Cells treated with RPMI or antibody-free beads (no antibodies) served as non-stimulation controls. After stimulation cells were placed on ice, and ATP levels in the supernatant were assessed using a luciferase assay. Data shown are means + SD of a single experiment measured in duplicates.

While there was no reproducible difference between cells treated with the differently coated beads, even stimulation with antibody-free beads induced an increase in extracellular ATP that was higher than in cells treated with RPMI. To avoid unspecific mechanical stimulation caused by the beads plate bound anti-CD3/CD28 and soluble anti-CD3 were used next to assess the stimulation-induced ATP increase in Jurkat cells (Figure 9). Stimulation with plate bound anti-CD3/CD28 led to an increase in extracellular ATP levels. Extracellular ATP concentrations were highest after 5 min stimulation, but the difference between anti-CD3/CD28 stimulated and unstimulated cells was most pronounced after one minute. In contrast, stimulation with soluble anti-CD3 had no significant effect on extracellular ATP concentrations.

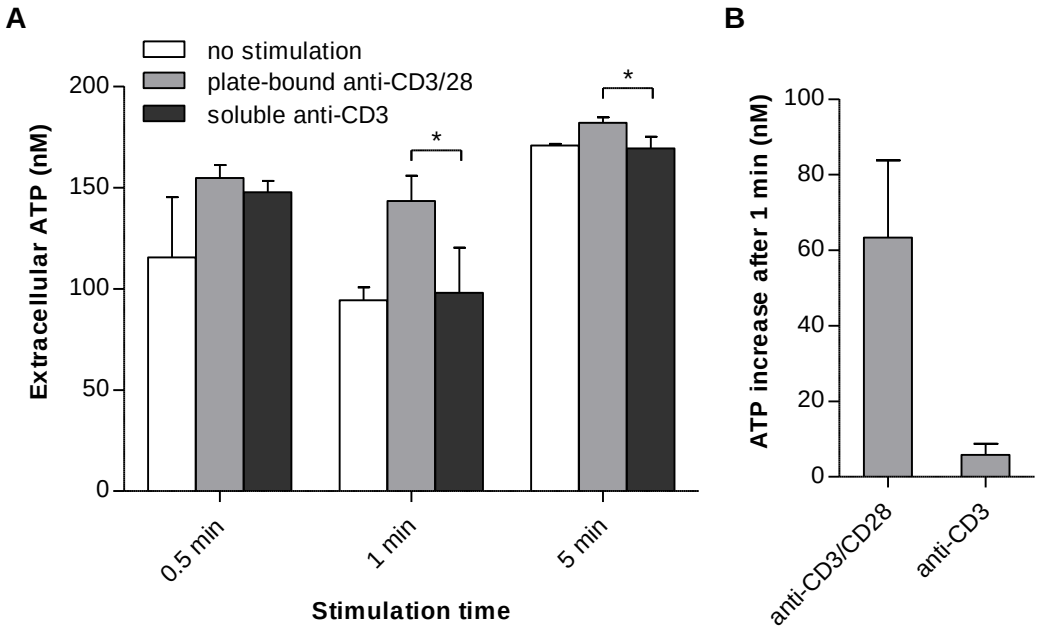


Figure 9: T cell stimulation via CD3/CD28 is more effective in increasing ATP than anti-CD3 alone. (A) Jurkat cells suspended in RPMI (5 x 10^6 cells/ml) were stimulated by placing cells in a 96-well plate coated with antibodies against CD3 and CD28 or by addition of soluble anti-CD3 (0.5 µg/ml). Treatment with RPMI was used as a negative control (no stimulation). Cells were incubated for the indicated times. The reaction was stopped by cooling the cells on ice, and ATP concentrations in the culture media were measured with a luciferase assay. Data are expressed as means + SD of a single experiment measured in duplicates (no stimulation) and triplicates; * $p < 0.05$, Student's t-test. (B) Jurkat cells were stimulated as described in (A) for 1 min with plate-bound anti-CD3/CD28 or with soluble anti-CD3 (0.5 µg/ml). Data show the increase in extracellular ATP as compared to unstimulated control cells. Data shown are means + SD of n = 2 independent experiments measured in triplicates.

Taken together, these findings show that anti-CD3/CD28 stimulation appears to be more effective in triggering ATP release than stimulation with soluble anti-CD3 alone. This indicates that T cells need a co-stimulatory signal in addition to the T cell receptor to

increase extracellular ATP levels. Therefore, further experiments were performed using antibodies against both CD3 and CD28 to stimulate cells.

3.2.3 CBX and latrunculin block the release of ATP

Next we studied in more detail which mechanisms are involved in the release of ATP from stimulated T cells. Treatment with latrunculin B was used to prevent actin polymerization and thus the translocation of mitochondria and other cellular organelles within the cell. CBX was used to block ATP release via pannexin 1 and connexin hemichannels. Both treatments reduced the release of ATP from cells stimulated with anti-CD3/CD28 antibody coated beads. Bafilomycin A is an inhibitor of exocytosis and only slightly decreased the release of ATP. Brefeldin A impairs endosomal transport and had no effect on ATP release (Figure 10).

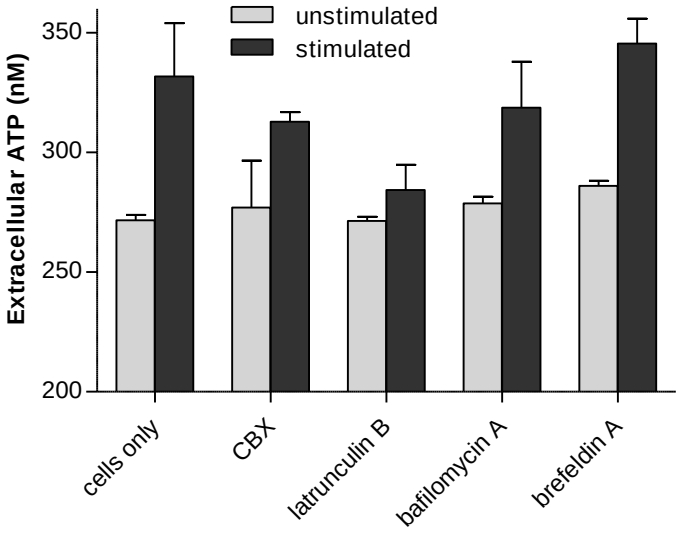


Figure 10: CBX and latrunculin B inhibit the ATP release from stimulated Jurkat cells.
Jurkat cells (3.3 x 10^6/ml) were not pretreated (cells only) or pretreated for 10 min with CBX (30 µM) or for 20 min with latrunculin (10 µg/ml), bafilomycin A (50 nM) or brefeldin A (10 µg/ml). Then the cells were either treated with media (unstimulated) or stimulated with anti-CD3/CD28 coated beads (1–2 beads/cell) for 1 min and placed on ice. The ATP concentration in the supernatant was measured using a luciferase assay. Data shown are mean values + SD of n = 1 experiment measured in duplicates.

Taken together with our published work [101], these results indicate that ATP release in response to CD3/CD28 stimulation requires mitochondrial translocation and ATP release via pannexin 1 hemichannels, whereas the contribution of vesicular ATP release seems to be negligible.

3.3 Expression of ectonucleotidases in unstimulated and stimulated T cells

Various ectonucleotidases located on the cell surface catalyze the degradation of extracellular nucleotides. These groups of enzymes differ in their substrate preferences and the products they form [37]. The expression pattern of ectonucleotidases has therefore a strong impact on extracellular nucleotide concentrations. QPCR was used to assess the expression of ENTPD1, 2, 3, 8, 5NTE, ENPP1, 2, 3, ALP and ADA in Jurkat cells und primary $CD4^+$ T cells. To test if stimulation modulates the expression of these enzymes, resting cells and cells that had been stimulated with anti-CD3/CD28 beads for 4 h were compared side by side.

3.3.1 Expression of ectoenzymes in Jurkat cells

ADA was the enzyme most abundantly expressed in unstimulated Jurkat cells. Besides ADA, Jurkat cells expressed also high amounts of 5NTE and low to moderate amounts of ENTPD2, ENPP1 and ENPP3 (Figure 11 A). In stimulated cells the expression of ENTPDase2, 5NTE and ENPP1 increased significantly compared to unstimulated cells, while the expression of ADA was significantly decreased after stimulation (Figure 11 B). All other tested enzymes were not or only marginally expressed.

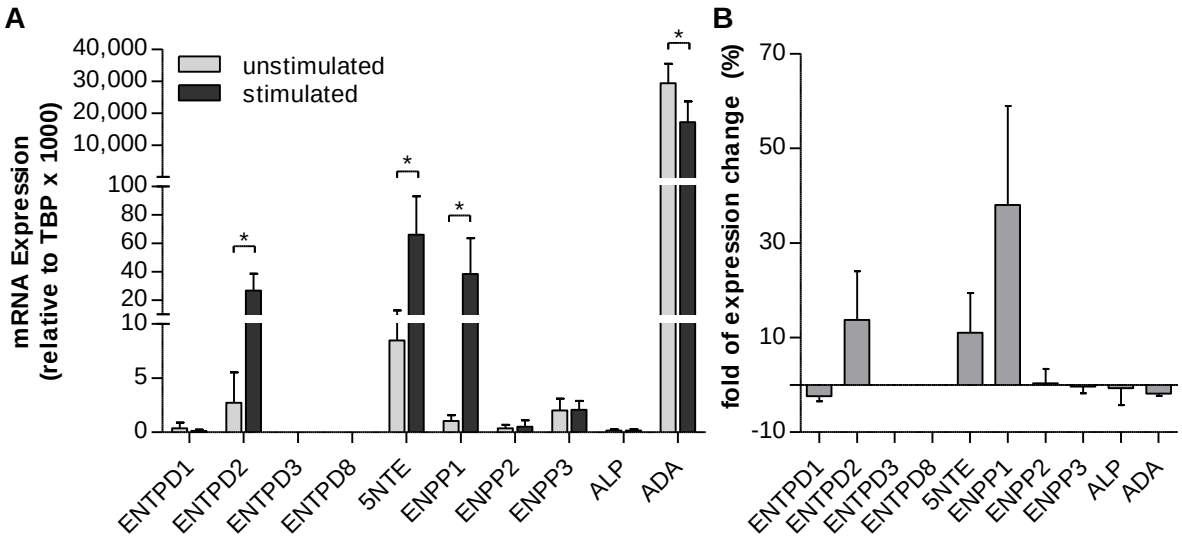


Figure 11: mRNA expression of ectoenzymes in unstimulated and stimulated Jurkat cells.
(A) Jurkat cells (1.11 x 10^6/ml) were treated with RPMI (unstimulated) or stimulated with anti-CD3/CD28 antibody coated beads (1–2 cells/bead) for 4 h. The mRNA of unstimulated and stimulated Jurkat cells was isolated, converted to cDNA and measured via qPCR. Data are shown as relative expression ratios normalized against the internal reference gene TATA-box-binding protein (TBP). (B) The ΔΔCt method was used to calculate the change in mRNA expression after stimulation. Data shown are mean values + SD of n = 4 independent experiments measured in duplicates; * $p < 0.05$, Student's t-test.

3.3.2 Expression of ectoenzymes in CD4$^+$ T cells

As in Jurkat cells, ADA was the enzyme with the highest expression in CD4$^+$ T cells. In addition, unstimulated CD4$^+$ expressed also ENTPDase1 and 2, 5NTE, ENPP2 and 3 (Figure 12 A). With the exception of 5NTE, which was significantly upregulated in stimulated cells, mRNA levels decreased after stimulation. Unlike Jurkat cells, primary CD4$^+$ T cells expressed very low levels of ENPP1 that did not change after stimulation (Figure 12 B).

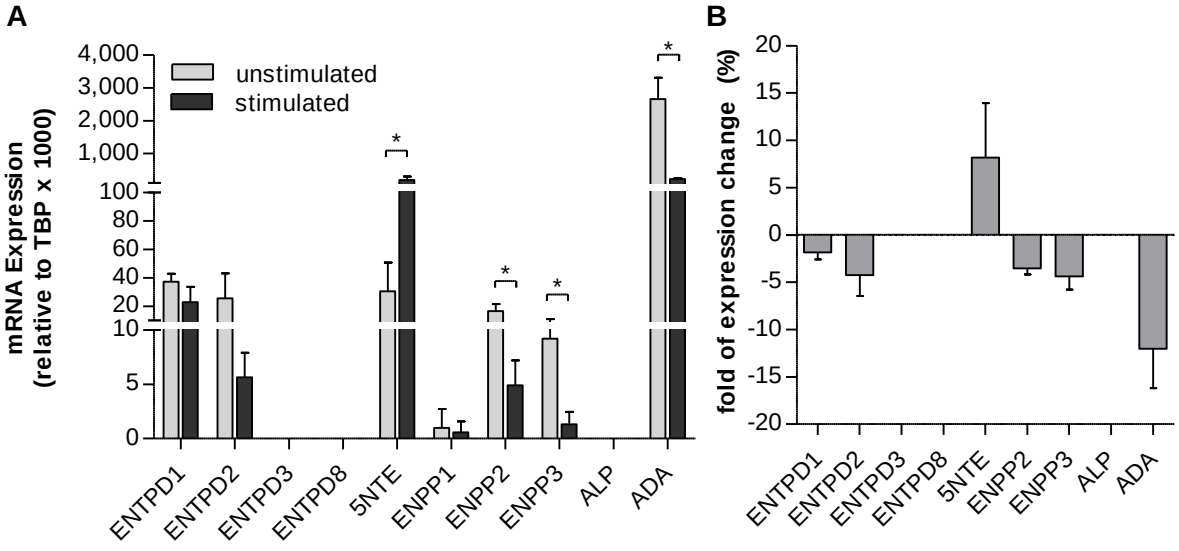


Figure 12: mRNA expression of ectoenzymes in unstimulated and stimulated CD4$^+$ T cells.
(A) CD4$^+$ T cells (1.11 x 10^6/ml) were treated with RPMI (unstimulated) or stimulated anti-CD3/CD28 coated beads (1−2 cells/bead) for 4 h. The mRNA of unstimulated and stimulated CD4$^+$ cells was isolated, converted to cDNA and measured via qPCR. Data are shown as relative expression ratios normalized against the internal reference gene TATA-box-binding protein (TBP). (B) The ΔΔCt method was used to calculate the change in mRNA expression after stimulation. Data shown are mean values + SD of n = 3 independent experiments measured in duplicates; * $p < 0.05$, Student's t-test.

Unstimulated CD4$^+$ T cells expressed ENTPD1, 2, 5NTE, ENPP2 and 3, which were lower expressed in unstimulated Jurkat cells (Figure 13). In contrast, ADA expression was about 10-times higher in unstimulated Jurkat cells than in unstimulated CD4$^+$ T cells. When comparing stimulated cells, ADA expression was still higher in Jurkat cells than in stimulated CD4$^+$ T cells. Furthermore, after stimulation Jurkat cells additionally showed higher ENTPD2 and ENPP1 levels than CD4$^+$ T cells.

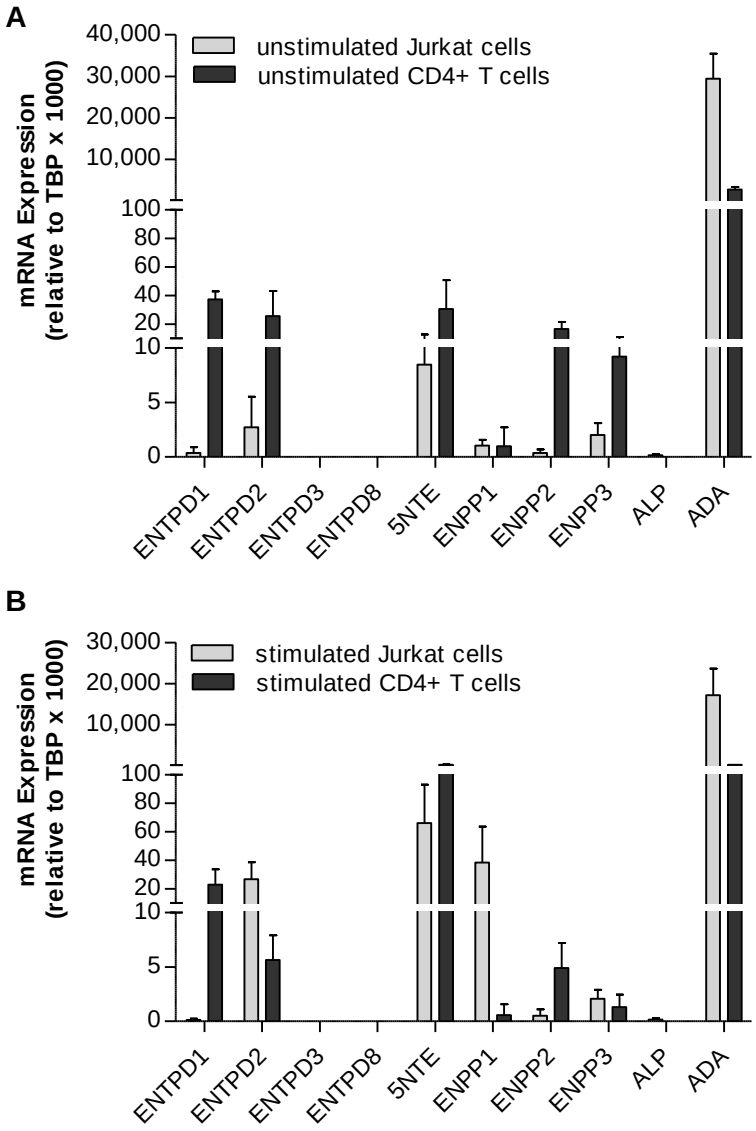


Figure 13: Side-by-side comparison of ectoenzyme expression in unstimulated and stimulated Jurkat and CD4$^+$ T cells.

(A) Unstimulated Jurkat and CD4$^+$ T cells (1.11 x 106/ml) were treated with RPMI (unstimulated) or (B) stimulated with anti-CD3/CD28 coated beads (1−2 cells/bead) for 4 h. The mRNA of unstimulated and stimulated CD4$^+$ cells was isolated, converted to cDNA and measured via qPCR. Data are shown as relative expression ratios normalized against the internal reference gene TATA-box-binding protein (TBP). Data shown are mean values + SD of n = 4 (Jurkat cells) or n = 3 (CD4$^+$ T cells) independent experiments measured in duplicates.

As shown in the figures 11, 12 and 13 T cells expressed a variety of ectonucleotidases that are essential for the breakdown of nucleotides like ATP, ADP and AMP. ADA is especially important for the adenosine breakdown. Consequently, ATP, ADP, AMP and adenosine metabolism was further examined in Jurkat and CD4$^+$ T cells.

3.4 Extracellular metabolism of adenines

3.4.1 Jurkat and CD4$^+$ T cells differ in their ATP degradation rates

To study the ATP breakdown characteristics of Jurkat and primary human CD4$^+$ T cells, ATP was added to cells that had been stimulated or not with anti-CD3/CD28 antibody coated beads and the concentrations of ATP, ADP, AMP, and adenosine in the supernatant was measured with HPLC at different time points following the addition of ATP. Jurkat cells and CD4$^+$ T cells showed differences in their degradation kinetics of ATP (Figure 14). Within 1 h unstimulated Jurkat cells degraded 2.2 µM (12 %) of 17 µM added ATP, while unstimulated CD4$^+$ T cells had degraded 5.4 µM (78 %) of 7 µM added ATP. In Jurkat cells cultures, extracellular ADP increased over time. In CD4$^+$ T cell cultures, extracellular AMP and to a lesser extent extracellular ADP increased.

It has to be noticed that 10 µM ATP was the intended amount to be added. However, when measuring the actual concentration of the ATP solution that had been added to the cells, some considerable variations were found. While Jurkat cells were actually treated with 17 µM ATP (Figure 14 A), only 7 µM had been added in the CD4$^+$ T cell experiment (Figure 14 B). This might be due to different stock solutions used, which might have differed in their ATP concentration. Thus, it needs to be considered, that these different concentrations could have affected the degradation behavior of the cells.

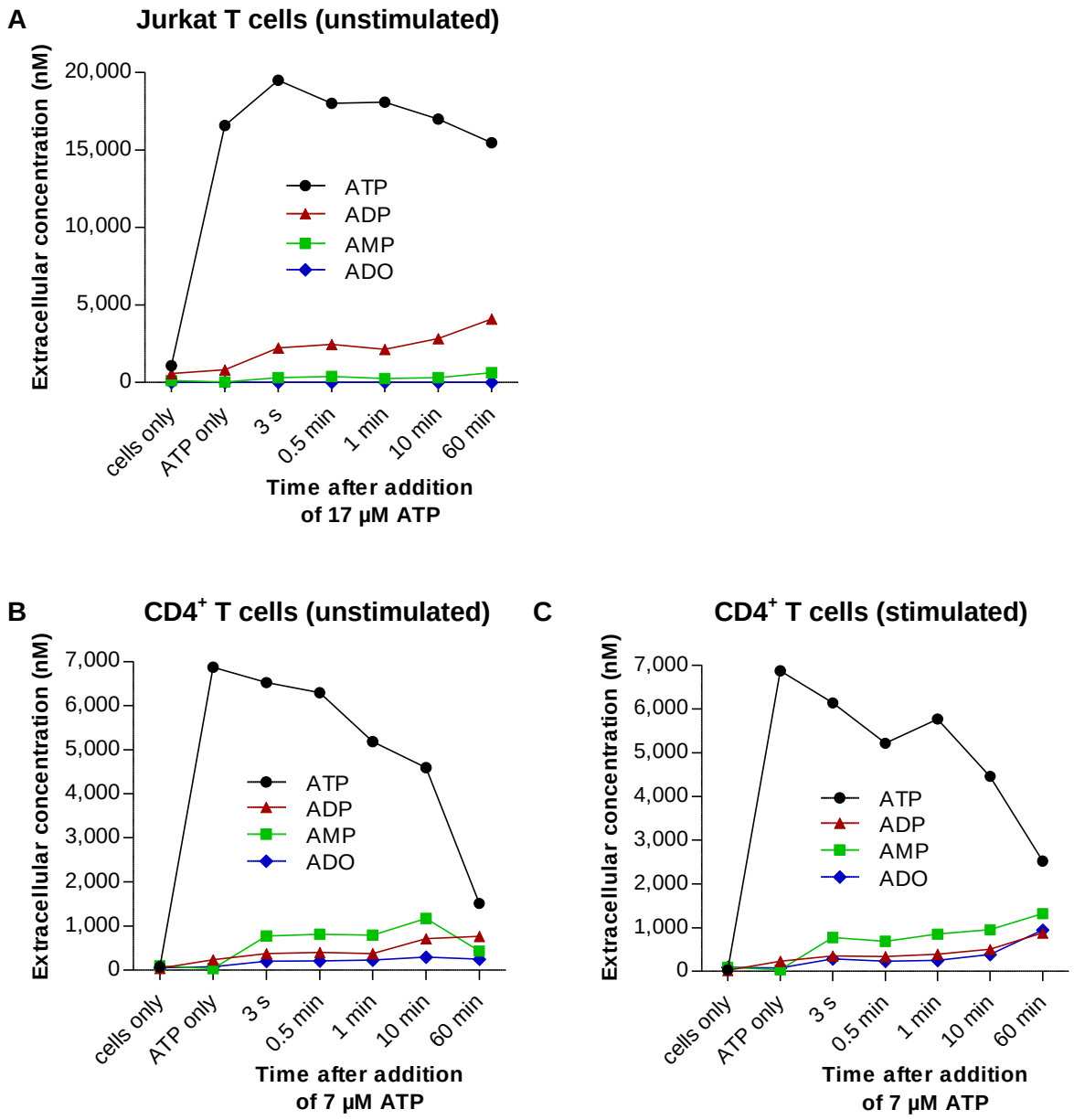


Figure 14: CD4$^+$ T cells degrade exogenously added ATP faster than Jurkat cells.
(A) Jurkat cells (3.75 x 10^6 cells/ml) were incubated with exogenous ATP (17 µM) for the indicated times. Cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) levels in the supernatant were measured using HPLC. A representative experiment of two separate experiments with similar results is shown in A. (B) Unstimulated CD4$^+$ T cells (3.3 x 10^6 cells/ml) or (C) CD4$^+$ T cells stimulated with anti-CD3/CD28 coated beads for 10 min were incubated with exogenous ATP (7 µM) for up to 1 h. The cells were placed on ice, and ATP, ADP, AMP and adenosine concentrations in the supernatant were measured using HPLC. Data of a single experiment are shown.

To get statistically relevant results, we selected the 10-min time point and repeated the above described experiment 3 times. After 10 min incubation with exogenously added

ATP, Jurkat cells showed a significant decrease in ATP and an increase in ADP (Figure 15). No significant difference was found between stimulated and unstimulated Jurkat cells (Figure 15 A). In comparison to Jurkat cells $CD4^+$ T cells degraded more ATP within 10 min (Figure 15 B). AMP was the predominant degradation product in stimulated and unstimulated $CD4^+$ T cells. In addition, ADP levels were slightly elevated. Adenosine did not change in either cell type.

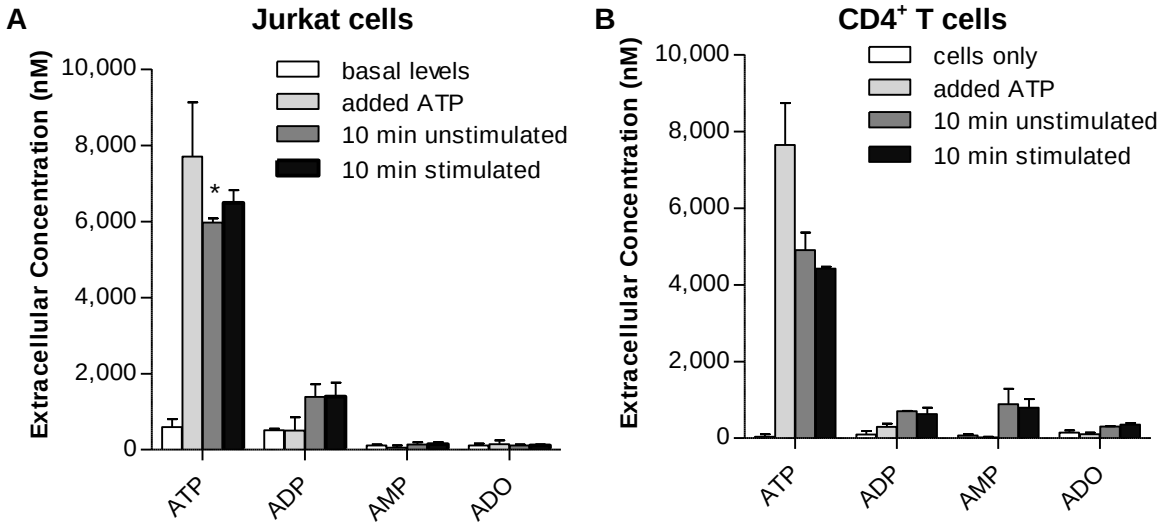


Figure 15: ATP is degraded to ADP in Jurkat cells and mostly to AMP in $CD4^+$ T cells.
(A) Jurkat cells and (B) human primary $CD4^+$ T cells (3.3×10^6 cells/ml) were suspended in RPMI and stimulated for 10 min with anti-CD3/28 coated beads (1–2 beads/cell). Unstimulated control samples were treated with RPMI instead of beads. Then ATP (8 µM) was added and the cells were incubated for another 10 min. After these 10 min cells were placed on ice and ATP, ADP, AMP and adenosine levels in the supernatant were measured using HPLC. Unstimulated control samples represent the basal ATP, ADP, AMP and adenosine (ADO) levels. Basal levels represent the ATP, ADP, AMP and adenosine concentration in the supernatant of untreated cells. Data shown are mean values + SD of (A) n = 3 or (B) n = 2 independent experiments; * $p < 0.05$ compared to 0 min (calculated as the sum of basal levels and added ATP), Student's t test.

Next it was tested whether increasing the stimulation time with anti-CD3/CD28 coated beads to 4 h would influence the degradation of ATP by $CD4^+$ T cells. As shown in figure 16, there was no major difference between stimulated and unstimulated cells. Stimulated cells degraded ATP slightly slower than unstimulated cells and the increase in AMP was less pronounced (Figure 16). This experiment was done once and would need to be repeated to confirm these observations.

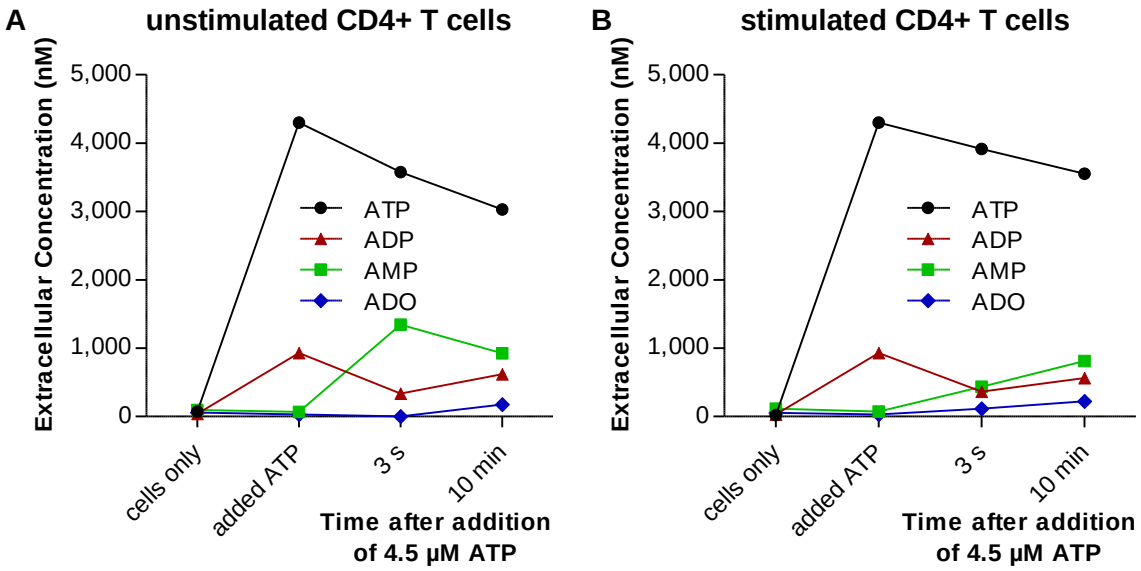


Figure 16: T cell stimulation has little effect on the breakdown of ATP.
$CD4^+$ T cells were isolated from human blood, suspended in RPMI supplemented with 10 % FBS (3.3×10^6 cells/ml) and incubated for 4 h in the (A) absence or (B) presence of anti-CD3/CD28 coated beads (1–2 beads/cell). Then ATP (4.5 µM) was added and incubated for the times indicated. After that cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) concentrations in the supernatant were measured using HPLC. Data of a single experiment are shown.

In conclusion, these data show that $CD4^+$ T cells degrade ATP more efficiently than Jurkat cells. AMP and ADP are the predominant degradation products in primary $CD4^+$ T cells and Jurkat cells, respectively.

3.4.2 ATP induces an increase in extracellular ATP

It has been reported previously for different cell types that ATP itself can induce the release of ATP [7,102]. Therefore, it was tested whether ATP could induce the release of ATP in T cells and whether the above described results were a combination of ATP degradation and release. The inhibitors CCCP and CBX were used to block mitochondrial ATP production and ATP release via pannexin 1 or connexin hemichannels, respectively.
Jurkat cells (Figure 17 A) and $CD4^+$ T cells (Figure 17 B) were pretreated for 10 min with CCCP or CBX. Then ATP was added and the ATP concentration in the supernatant was measured after 0.5, 1 and 10 min. As shown above (3.4.1) ATP added to Jurkat cells or $CD4^+$ T cells was gradually degraded. Interestingly, pretreatment with CCCP and CBX further reduced the extracellular ATP levels in Jurkat and $CD4^+$ T cells.

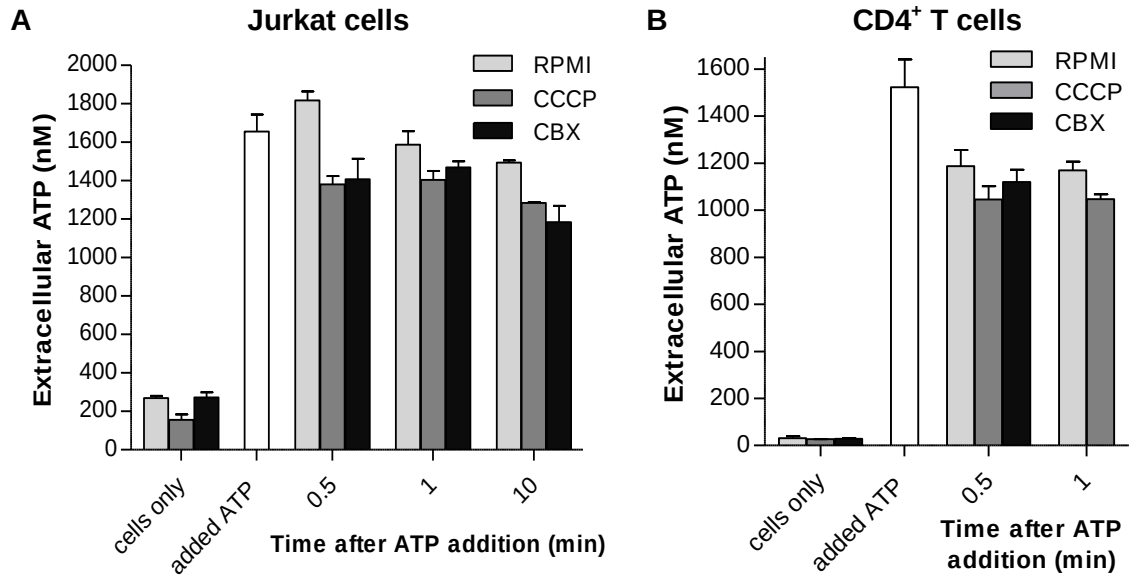


Figure 17: ATP is faster degraded when ATP production and release are blocked.
(A) Unstimulated Jurkat cells or (B) $CD4^+$ T cells (3.3 x 10^6 cells/ml) were suspended in RPMI and treated for 10 min with the mitochondrial blocker CCCP (10 µM), the pannexin 1 channel blocker CBX (20 µM) or RPMI (control). Then ATP (1.6 µM) was added and the cells were incubated for the times indicated. After that cells were placed on ice, and ATP concentrations in the supernatant were measured using a luciferase assay. Data shown are mean values + SD of a single experiment measured in duplicates.

To determine the ideal concentrations of these inhibitors dose response curves were performed (Figure 18). Incubating cells for 10 min with various concentrations of CCCP, ranging from 10 nM to 50 µM reduced the extracellular ATP concentration compared to cells treated without CCCP (Figure 18 A, B). All concentrations reduced the ATP levels to a similar extent. Thus, 10 µM was further used as final CCCP concentration in all experiments. Treatment with CBX at concentrations ranging from 500 nM to 100 µM also reduced the ATP concentrations in the supernatant (Figure 18 C, D). For further experiments CBX was used at a concentration of 20 µM.

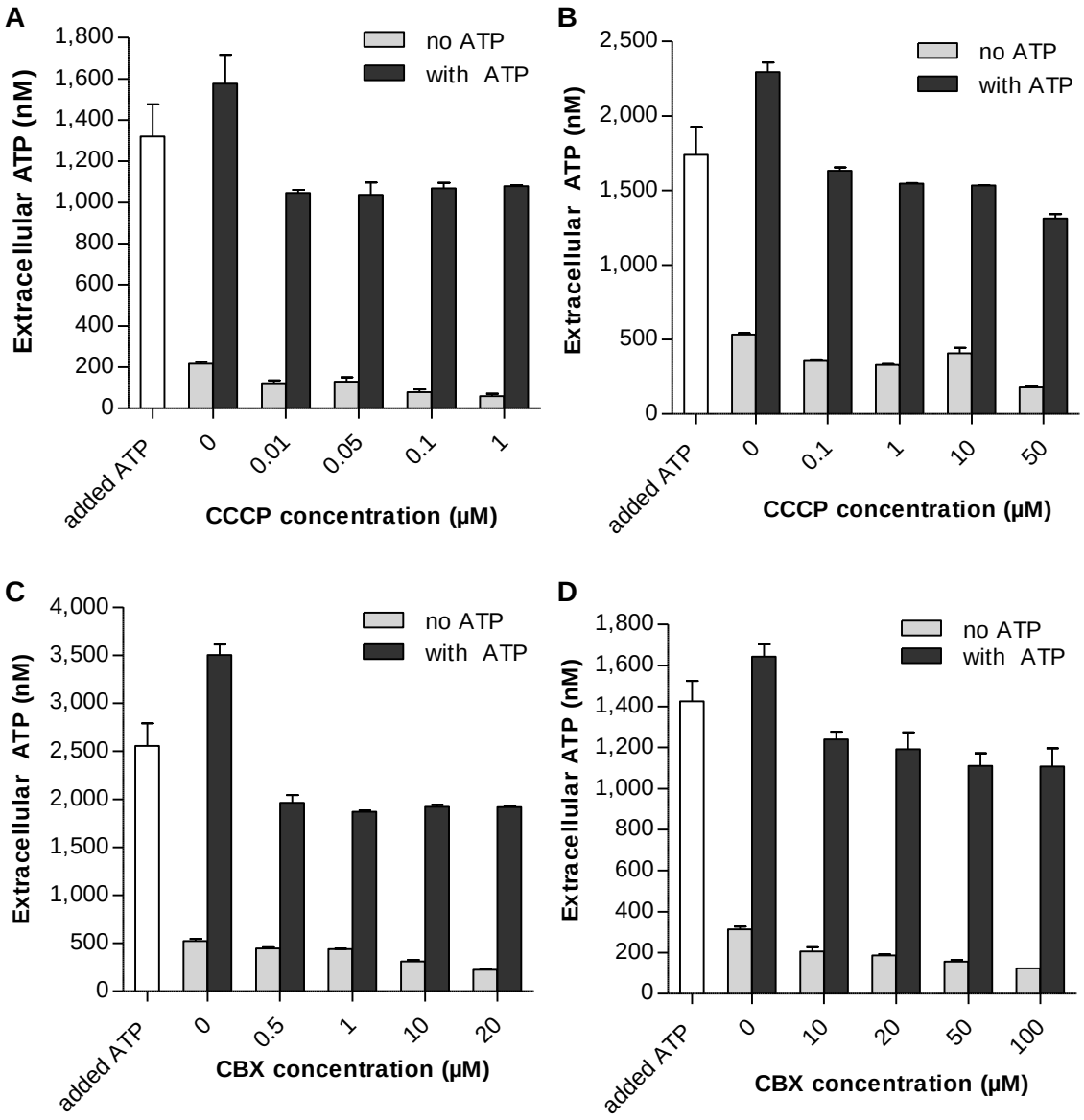


Figure 18: Dose response curves of CCCP and CBX in Jurkat cells.
Unstimulated Jurkat cells (3.3×10^6 cells/ml) were incubated for 10 min with the indicated concentrations of (A, B) CCCP or (C, D) CBX. After that 2 µM ATP was added to the cells and incubation continued for another 10 min. The cells were placed on ice and ATP concentrations in the supernatant were measured using a luciferase assay. Data shown are mean values + SD of duplicate measurements of a single experiment.

Exogenously added ATP decreased over time. CCCP and CBX further reduced the extracellular ATP levels, which indicate an involvement of mitochondria-produced ATP and the release via pannexin 1. Therefore, it was examined if the observed ATP decrease is reproducible and if CCCP and CBX also have an effect on stimulated cells.
Using the same experimental approach as above, the effect of CCCP and CBX on ATP degradation was studied in unstimulated Jurkat cells or in cells stimulated with anti-CD3/CD28 coated beads for 10 min. ATP levels were lower after CCCP and CBX

treatment (Figure 19). The reduction was stronger in unstimulated cells. However, stimulated Jurkat cells showed lower ATP levels in general.
Two repetitions of each experiment with CCCP or CBX (data not shown) revealed a reproducible reduction with CCCP and CBX; however, the extent of this effect varied considerably.

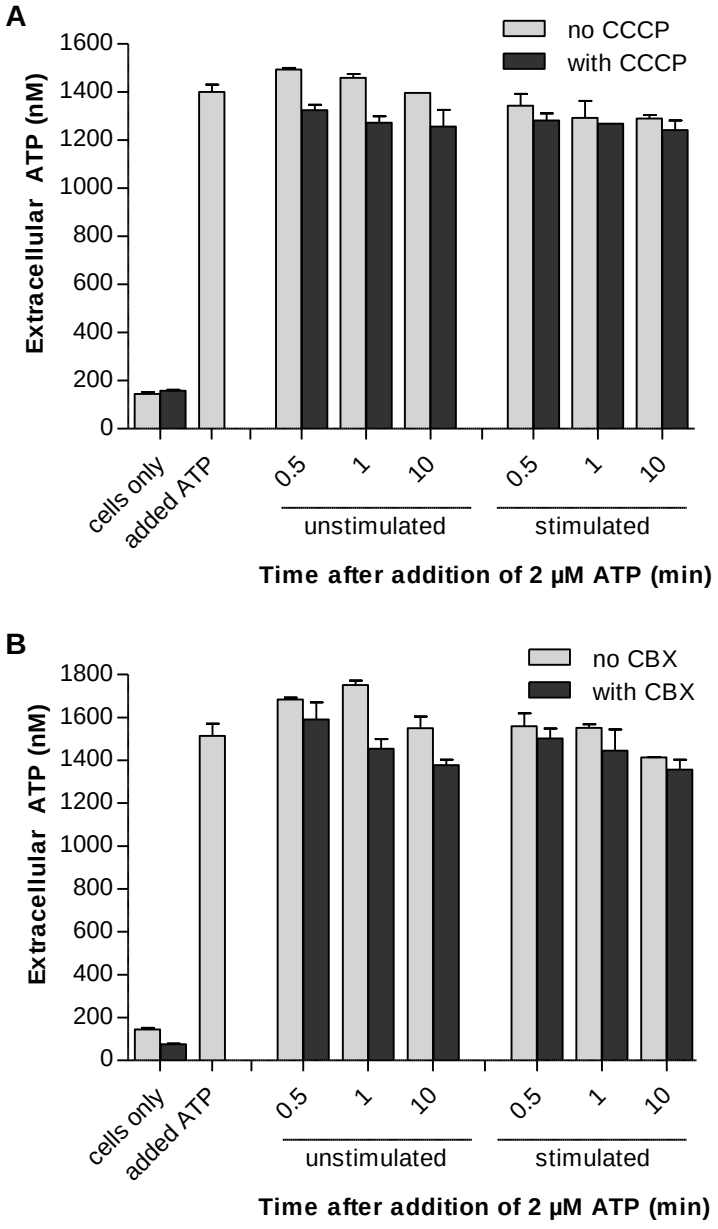


Figure 19: ATP levels are reduced in the presence of CCCP and CBX in Jurkat cells.
Jurkat cells (3.3×10^6 cells/ml) were preincubated for 10 min with (A) CCCP (10 µM) or (B) CBX (20 µM). Cells were incubated with RPMI (unstimulated) or stimulated with anti-CD3/CD28 coated beads (1–2 beads/cell) for 10 min. Thereafter, the cells were incubated with 2 µM ATP at the time points indicated. The cells were placed on ice and ATP concentrations in the supernatant were measured with a luciferase assay. Data shown represent mean values + SD of a single experiment measured in duplicates.

To better dissect ATP degradation and ATP release, cells were in a next step stimulated with ATPγS, a non-hydrolysable ATP analogue, and extracellular ATP concentrations were determined. In the presence of ATPγS extracellular ATP levels increased in unstimulated Jurkat cells (Figure 20 A, C). The increase was strongest after 3 s. A small part of the added ATPγS was recognized by the luciferase (Figure 20 B). Figure 20 C shows the ATPγS-induced ATP increase after correction for this interference with the luciferase assay.

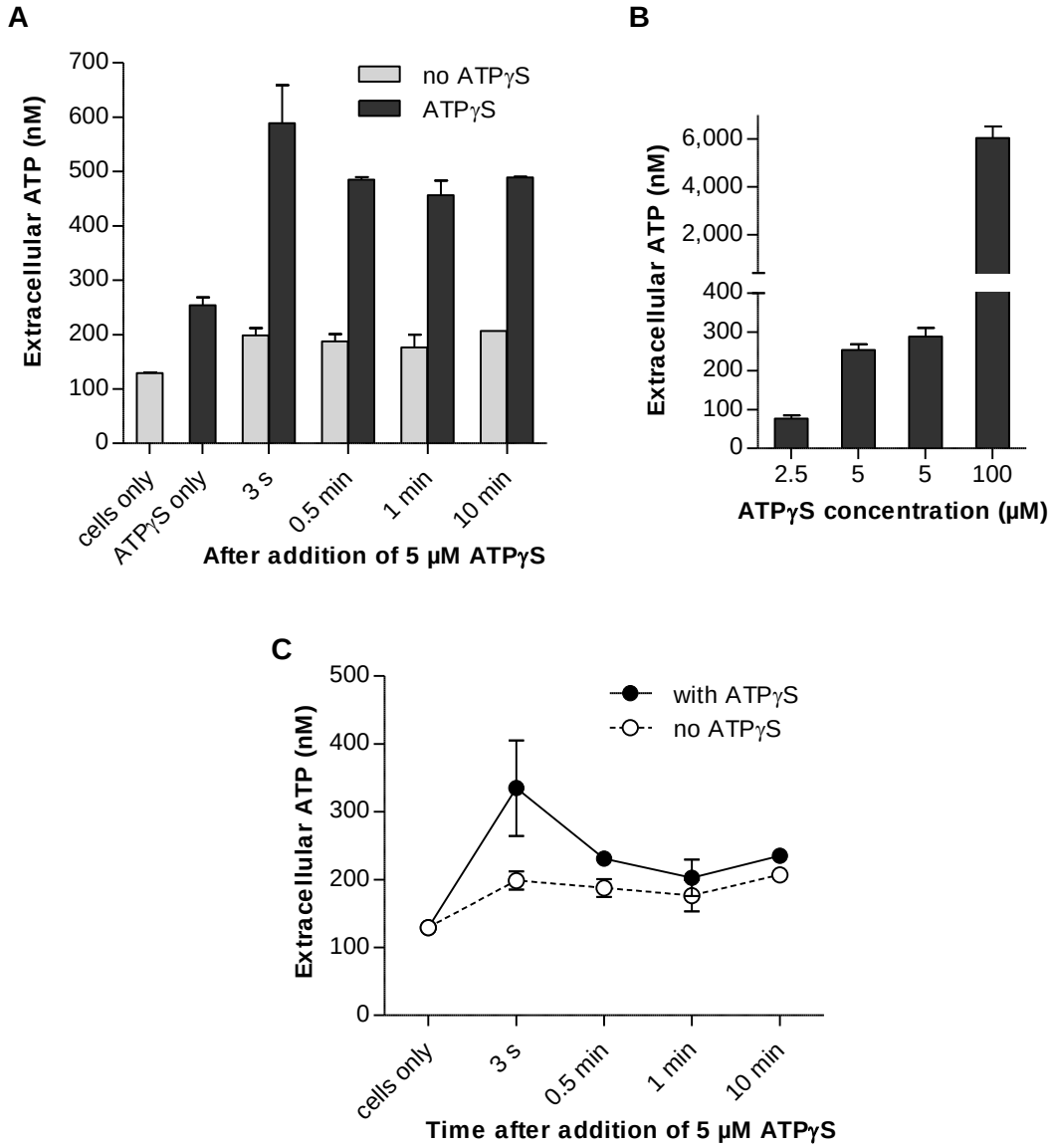


Figure 20: ATPγS induces the release of ATP from Jurkat cells.

(A) Unstimulated Jurkat cells (3.3×10^6 cells/ml) were incubated without or with ATPγS (5 µM) for the indicated time points. The cells were placed on ice and extracellular ATP concentrations were measured with a luciferase assay. (B) Different ATPγS concentrations without cells were measured with the luciferase assay. (C) The effect of ATPγS on the luciferase was subtracted and corrected extracellular ATP levels are shown in the line diagram. Data shown are mean values + SD of a single experiment measured in duplicates.

Taken together, these findings show that ATP induces the increase in extracellular ATP. This increased ATP might be produced by mitochondria and released via pannexin 1 hemichannels.

3.4.3 ADP degradation and ADP-induced extracellular ATP increase

Next, the extracellular metabolism of ADP was investigated. Jurkat and $CD4^+$ T cells degraded ADP in a similar way (Figure 21). In both cell types AMP increased over the observation period of 60 min. In Jurkat cells, an increase in ATP levels was detectable 10 min after addition of ADP and was even more pronounced after 60 min (Figure 21 A). $CD4^+$ T cells showed an increase of ATP and adenosine after 1 h. There was no apparent difference between unstimulated (Figure 21 B) and stimulated (Figure 21 C) $CD4^+$ T cells.

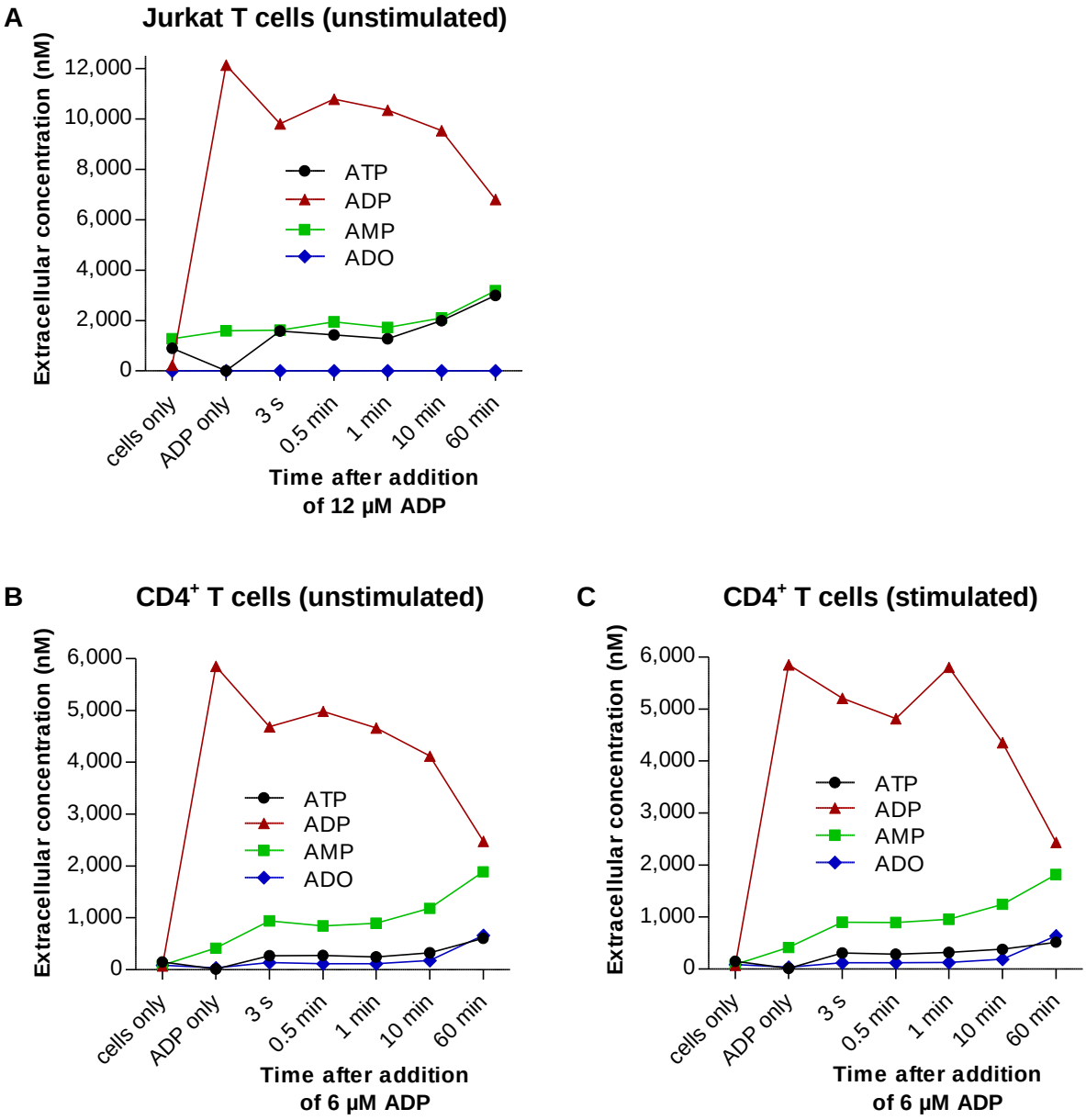


Figure 21: Extracellular metabolism of ADP in Jurkat and primary CD4$^+$ T cells.
(A) Jurkat cells (3.75 x 10^6 cells/ml) were incubated with exogenous ADP (12 µM) for the indicated times. Cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) levels in the supernatant were measured using HPLC. (B) Unstimulated CD4$^+$ T cells (3.34 x 10^6 cells/ml) or (C) CD4$^+$ T cells stimulated for 10 min with anti-CD3/CD28 coated beads were incubated with exogenous ADP (6 µM) for the indicated times. Cells were placed on ice and ATP, ADP, AMP and adenosine levels in the supernatant were measured using HPLC. Data of a single experiment are shown.

Exogenously added ADP decreased significantly after 10 min incubation with unstimulated or stimulated Jurkat cells (Figure 22 A). In parallel, extracellular ATP increased significantly. A marginal increase was observed for AMP and no increase of adenosine

was found. In contrast, $CD4^+$ T cells showed decreased ADP levels after 10 min incubation with exogenous ADP and increased AMP levels. ATP levels increased marginally and adenosine levels showed no increase. There was no difference between unstimulated and stimulated cells in either cell type.

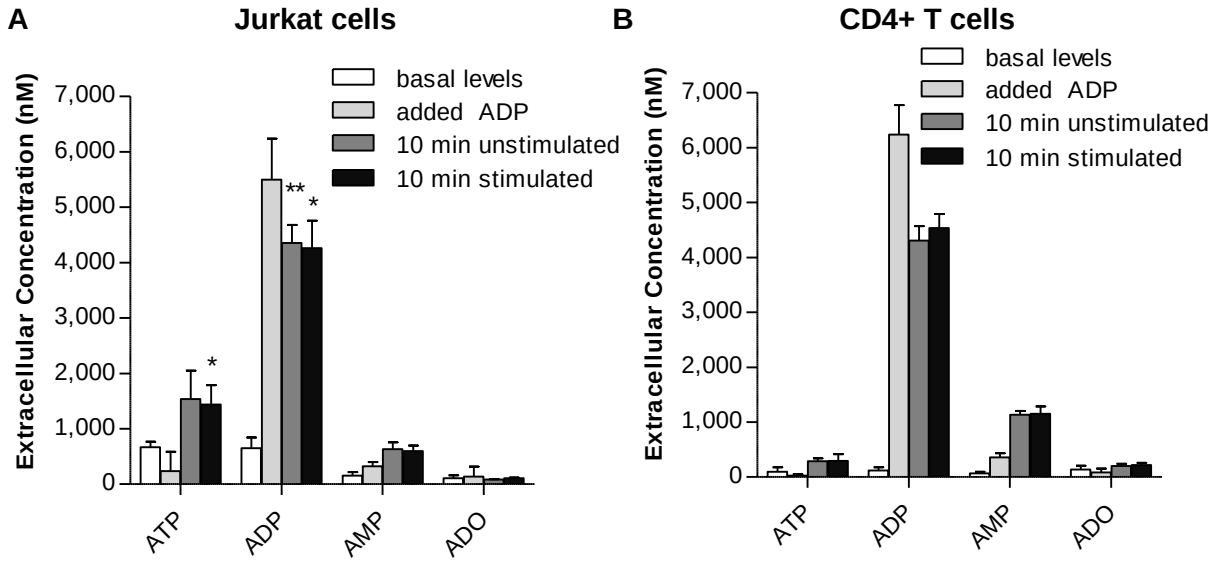


Figure 22: Exogenously added ADP is converted to ATP.
(A) Jurkat cells and (B) primary $CD4^+$ T cells (3.3×10^6 cells/ml) were suspended in RPMI and stimulated for 10 min with anti-CD3/28 coated beads (1–2 beads/cell). Unstimulated control samples were treated with RPMI instead of beads. Then ADP (6 µM) was added and cells were incubated for another 10 min. After these 10 min cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) levels in the supernatant were measured using HPLC. Basal levels represent the ATP, ADP, AMP and adenosine concentration in the supernatant of untreated cells. Data shown are mean values + SD of (A) n = 3, (B) n = 2 independent experiments; * $p < 0.05$, ** $p < 0.01$ compared to 0 min (calculated as the sum of basal levels and added ADP), Student's t-test.

To test the effect of a longer stimulation time, $CD4^+$ T cells were stimulated with anti-CD3/CD28 coated beads for 4 h and treated with exogenous ADP (Figure 23). Again there was no clear difference between unstimulated and stimulated cells. In stimulated as well as unstimulated cells, ADP levels decreased after 3 s and AMP increased. After 10 min, stimulated T cells had degraded 458 nM more ADP than unstimulated cells. ATP and adenosine levels did not change in unstimulated nor in stimulated cells. However, as this experiment was performed only once the results have to be considered preliminary and need to be confirmed in future experiments.

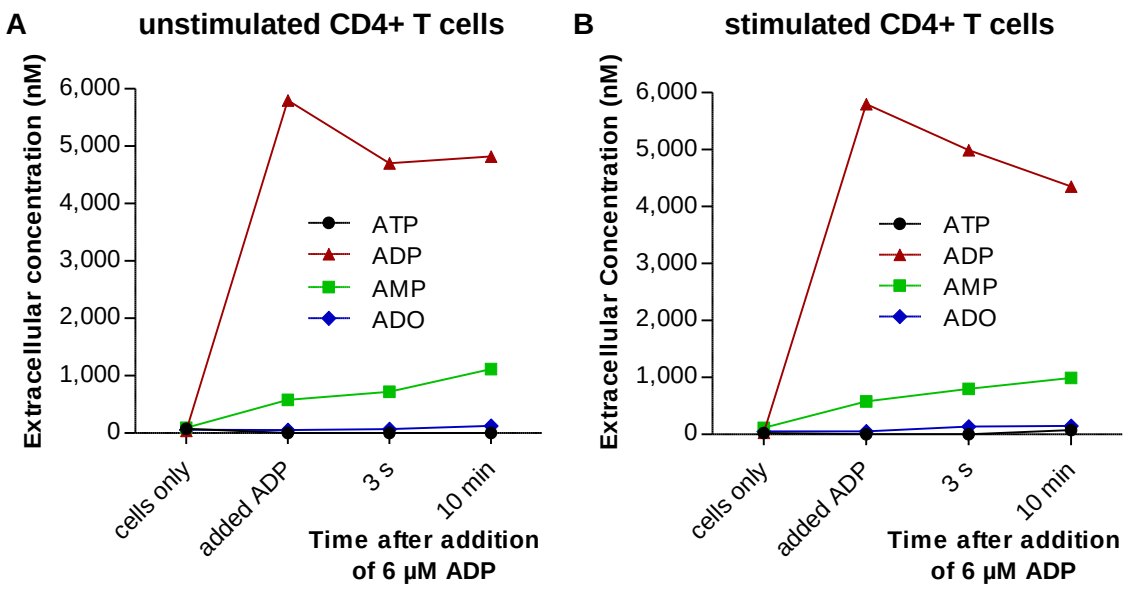
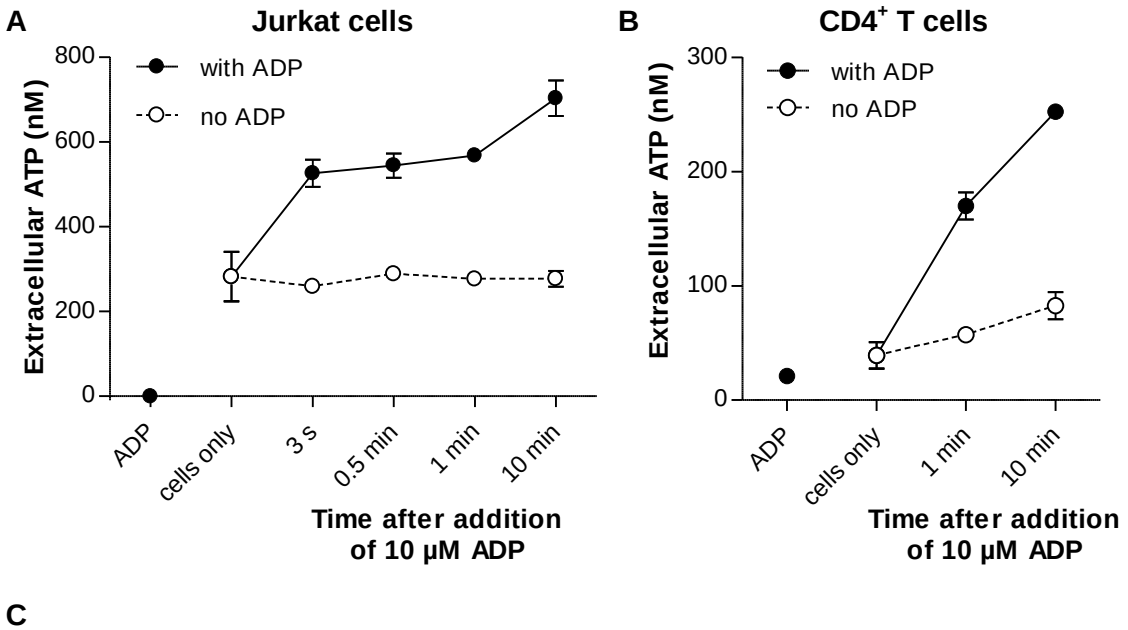


Figure 23: ADP metabolism by unstimulated and stimulated CD4$^+$ T cells.
CD4$^+$ T cells were isolated from human blood, suspended in RPMI supplemented with 10 % FBS (3.3 x 10^6 cells/ml) and incubated for 4 h in the (A) absence or (B) presence of anti-CD3/CD28 coated beads (1–2 beads/cell). Then ADP (6 µM) was added, incubated for the indicated times and placed on ice. ATP, ADP, AMP and adenosine (ADO) concentrations in the supernatant were measured using HPLC. Data of a single experiment are shown.

Using HPLC a significant ATP increase after incubation with ADP had been observed in the supernatant of Jurkat cells, but only a marginal one in CD4$^+$ T cells (Figure 22). Therefore, this phenomenon was further examined with a luciferase assay, which is more sensitive in determining lower ATP concentrations. Using the luciferase assay an increase in extracellular ATP was observed in Jurkat and CD4$^+$ T cells after the incubation with ADP (Figure 24 A, B). ATP levels increased over time; the highest ATP level in both cell types was observed after 10 min incubation. At this time point Jurkat cells showed a 2.54-fold increase and CD4$^+$ cells a 3.05-fold increase in the ATP levels compared to cells without ADP. The ATP increase in Jurkat cells was significant after 10 min incubation with ADP (Figure 24 C).



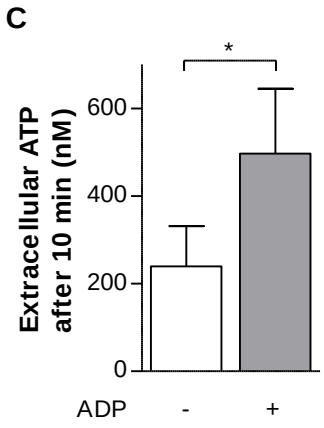


Figure 24: Treatment of Jurkat and CD4$^+$ T cells with ADP increases the ATP concentration in the cell culture supernatant.
(A) Jurkat and (B) CD4$^+$ T cells (3.3 x 10^6 cells/ml) were incubated with 10 µM ADP for the time indicated. The cells were placed on ice and extracellular ATP concentrations were measured with a luciferase assay. Data shown are mean values ± SD of a single experiment. (C) Jurkat cells (3.3 x 10^6 cells/ml) were left untreated or incubated with 5 µM ADP for 10 min. The cells were placed on ice and extracellular ATP concentrations were measured with a luciferase assay. Data shown are mean values + SD of n = 5 independent experiments; * $p < 0.05$, Student's t-test.

To further assess if this increase of ATP was due to the cellular release of ATP the experiment was repeated in the presence of CCCP, which inhibits the mitochondrial ATP production, and CBX, which inhibits the ATP release via pannexin 1 and connexin hemichannels. CCCP and CBX lowered the basal ATP levels of Jurkat cells (Figure 25). However, the ADP-induced ATP increase was not blocked by these inhibitors.

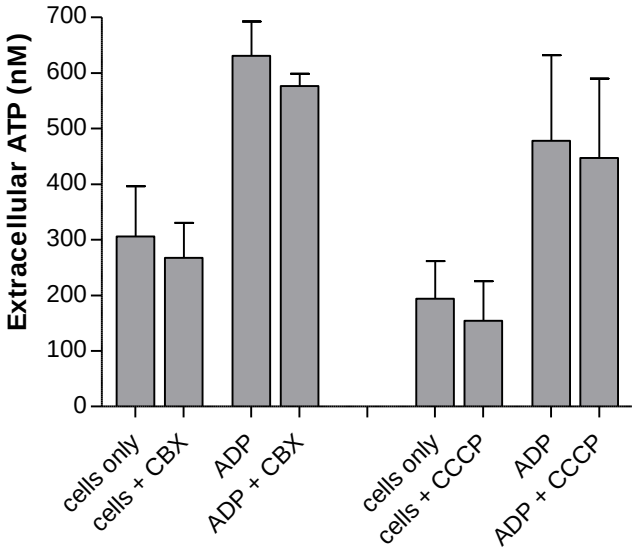


Figure 25: CCCP and CBX do not influence the ADP-induced ATP increase.
Jurkat cells (3.3 x 10^6 cells/ml) were preincubated with RPMI (cells only, ADP), CCCP (10 µM) or CBX (20 µM) for 10 min. Then cells were incubated for 10 min with 5 µM ADP or RPMI (cells only, cells + CBX, cells + CCCP). The cells were placed on ice and extracellular ATP concentrations were measured using a luciferase assay. Data shown are mean values + SD of n = 2 independent experiments measured in duplicates.

This suggests that mitochondrial ATP production and ATP release via pannexin 1 are not involved in the ADP-induced ATP increase. To test whether an extracellular kinase was responsible for this increase, Jurkat cells were treated with diadenosinepentaphosphate (AP5A), an inhibitor of adenylate kinase, prior to addition of ADP. As shown in figure 26 A and B, the ADP-induced ATP increase was less pronounced in the presence of AP5A. This effect was concentration dependent. In three experiments, maximum inhibition of 40% was reached at a concentration of 10 µM AP5A.

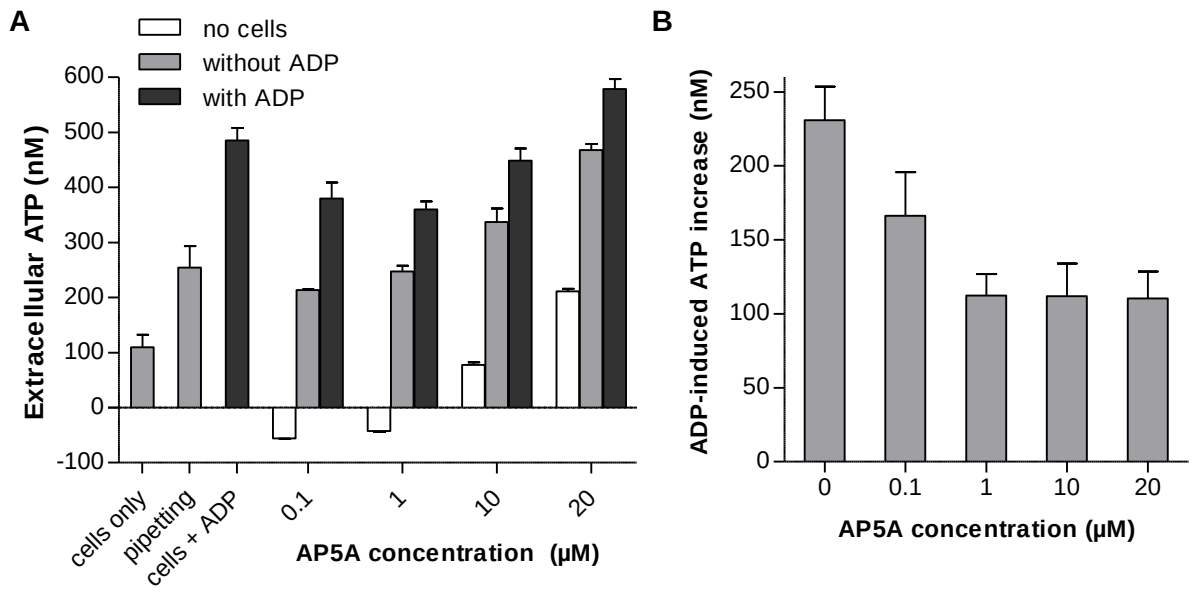


Figure 26: Inhibition of adenylate kinase reduces the ADP-induced ATP increase.
Jurkat cells (3.3 x 10^6 cells/ml) were pretreated with the indicated concentrations of the adenylate kinase inhibitor AP5A for 10 min and afterwards incubated with ADP (5 µM) for further 10 min. The cells were placed on ice and ATP concentrations in the supernatant were measured using a luciferase assay. (A) Raw data and (B) analyzed data of the experiment are shown as mean values + SD of one representative of n = 3 experiments measured in duplicates.

To assess if stimulation affects the observed ATP-inducing effect of ADP, cells were stimulated with anti-CD3/CD28 coated beads for 10 min and then treated with ADP. In both stimulated and unstimulated cells ADP treatment increased the extracellular ATP levels. There was no difference between stimulated and unstimulated cells (Figure 27).

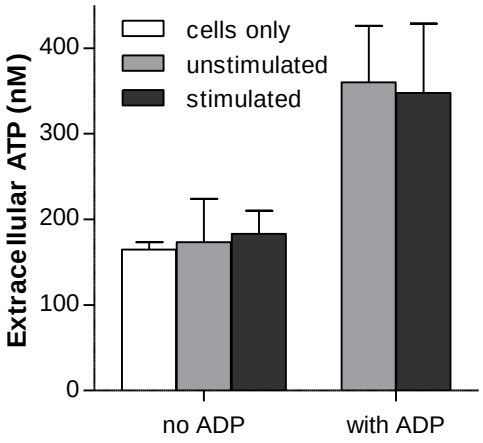


Figure 27: The ATP increase after ADP incubation is not influenced by stimulation.
Jurkat cells (3.3 x 10^6 cells/ml) were left untreated (cells only), incubated with RPMI (unstimulated) or stimulated with anti-CD3/CD28 coated beads (1–2 beads/cell) for 10 min and incubated with 5 µM ADP for 10 min. Cells were placed on ice and ATP concentrations in the supernatant were measured using a luciferase assay. Data shown are mean values + SD of a single experiment measured in duplicates.

Taken together these findings show that T cells degrade ADP to AMP. In addition, some of the ADP is converted back to ATP via adenylate kinase. This ADP-induced ATP increase is not influenced by cell stimulation.

3.4.4 Exogenous AMP does not alter ADP or ATP levels

The AMP metabolism of T cells was examined next. Within 30 s the levels of exogenously added AMP decreased by about 1 µM in the supernatant of Jurkat cells. After that AMP concentrations remained stable and did not further decrease for at least 60 min. ATP, ADP and adenosine levels remained nearly unchanged at all time points (Figure 28 A). In contrast, $CD4^+$ T cells showed decreased AMP and increased adenosine levels after 1 h incubation with exogenously added AMP (Figure 28 B). There was no difference between unstimulated and stimulated $CD4^+$ T cells (Figure 28 C).

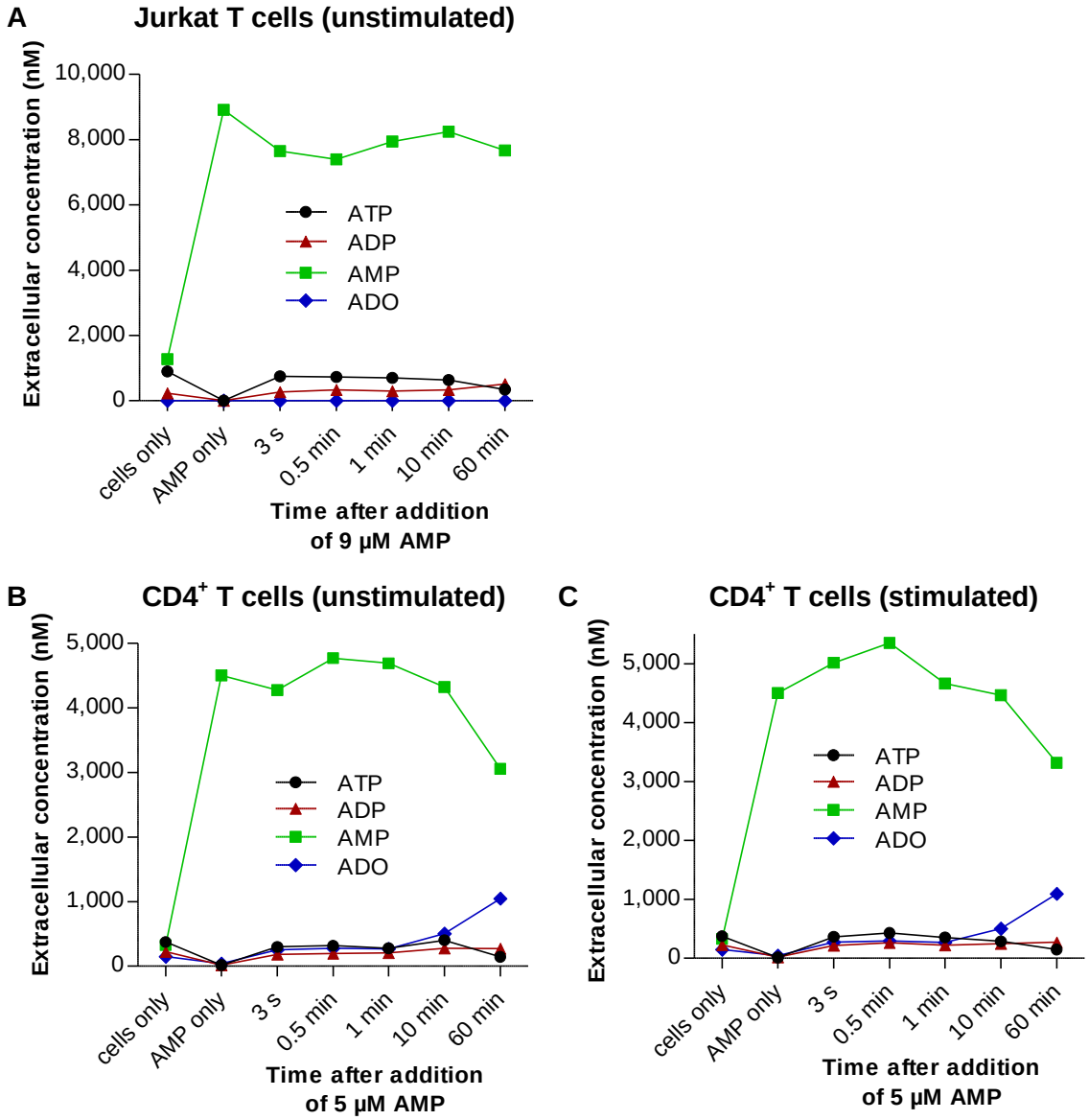


Figure 28: Jurkat cells degrade AMP initially faster than $CD4^+$ T cells.

(A) Jurkat cells (3.75×10^6 cells/ml) were incubated with exogenous AMP (9 µM) for the indicated times. (B) Unstimulated $CD4^+$ T cells (3.34×10^6 cells/ml) or (C) $CD4^+$ T cells stimulated for 10 min with anti-CD3/CD28 coated beads were incubated with exogenous AMP (5 µM) for the times indicated. The cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) concentrations in supernatant were measured using HPLC. Data of a single experiment are shown.

These results were confirmed by analyzing the combined results of three experiments. AMP concentrations tended to be lower in Jurkat cells 10 min after addition of AMP, while ATP, ADP and adenosine levels did not change (Figure 29 A). In $CD4^+$ T cells, AMP levels were decreased and adenosine levels increased. No change was found in the ADP or ATP levels. Furthermore, no significant difference was found between unstimulated and stimulated Jurkat and $CD4^+$ T cells.

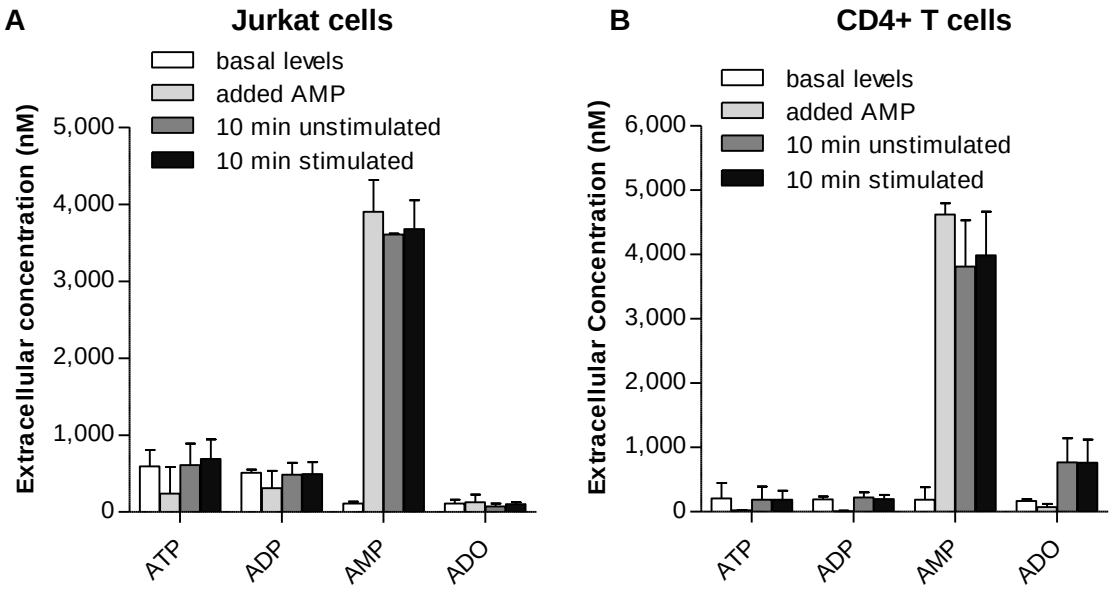


Figure 29: Jurkat and $CD4^+$ T cells degrade AMP, but adenosine increases only in $CD4^+$ T cells.
(A) Jurkat cells and (B) human primary $CD4^+$ T cells (3.3×10^6 cells/ml) were suspended in RPMI and stimulated for 10 min with anti-CD3/28 coated beads (1–2 beads/cell). Unstimulated control samples were treated with RPMI instead of beads. Then AMP (4 µM A or 5 µM B) was added and the cells were incubated for another 10 min. After these 10 min cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) levels in the supernatant were measured using HPLC. Basal levels represent the ATP, ADP, AMP and adenosine concentrations in the supernatant of untreated cells. Data shown are mean values + SD of (A) n = 3, (B) n = 2 independent experiments.

To assess if a longer stimulation with anti-CD3/CD28 coated beads had an effect on the AMP metabolism $CD4^+$ T cells were stimulated for 4 h prior to addition of AMP (Figure 30). Unstimulated and stimulated cells revealed similar decreasing AMP levels. The adenosine levels increased as AMP decreased especially after 10 min incubation.

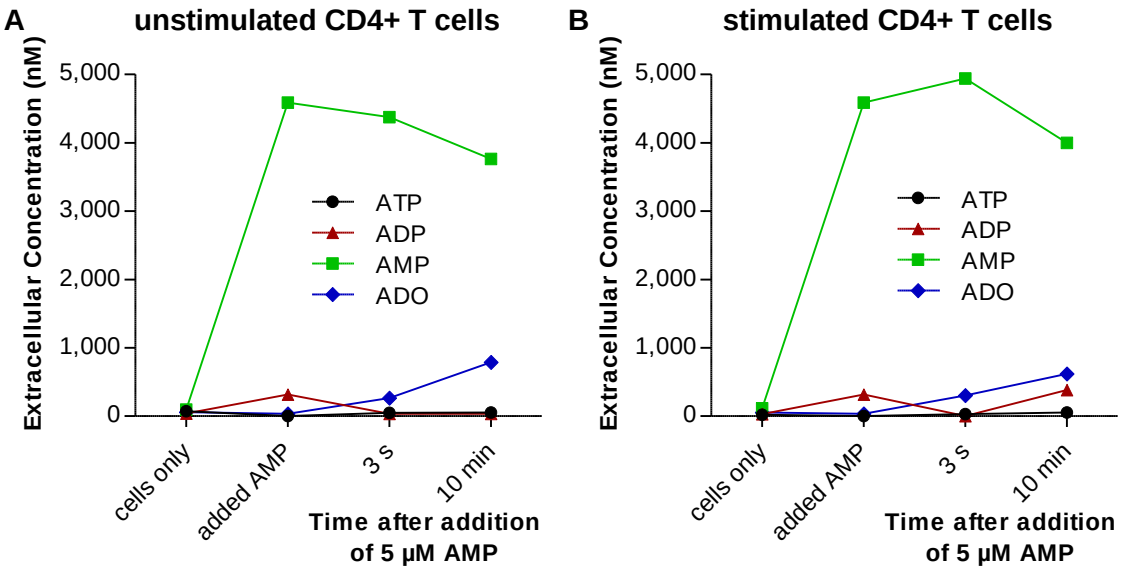


Figure 30: Unstimulated and stimulated CD4$^+$ T cells show a similar AMP metabolism.
CD4$^+$ T cells were isolated from human blood, suspended in RPMI supplemented with 10 % FBS (3.3 x 10^6 cells/ml) and incubated for 4 h in the (A) absence or (B) presence of anti-CD3/CD28 coated beads (1–2 beads/cell). Then AMP (5 µM) was added and incubated for the times indicated. After that the cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) concentrations in the supernatant were measured using HPLC. Data of a single experiment are shown.

These data indicate that AMP is degraded to adenosine in CD4$^+$ T cells. How adenosine is metabolized by T cells was investigated next.

3.4.5 Exogenous adenosine is rapidly degraded by ADA

The last metabolism to be observed is the adenosine metabolism. Exogenously added adenosine (10 µM) was completely removed from the supernatant of Jurkat cells within a few seconds after its addition. This phenomenon was observed in unstimulated and as well as in stimulated Jurkat cells (Figure 31 A, B). Also in unstimulated and stimulated CD4$^+$ T cells a rapid adenosine removal (about 2 µM) was observed within 3 s. However, adenosine decreased slower within the next 1 h (about 2 µM), but still one third of the initial concentration (about 2 µM) remained in the supernatant (Figure 31 C, D). AMP, ADP and ATP levels stayed unchanged in both cell types. The chromatograms of these experiments with exogenous adenosine removal from the supernatant of unstimulated Jurkat and CD4$^+$ T cells are shown in figure 32.

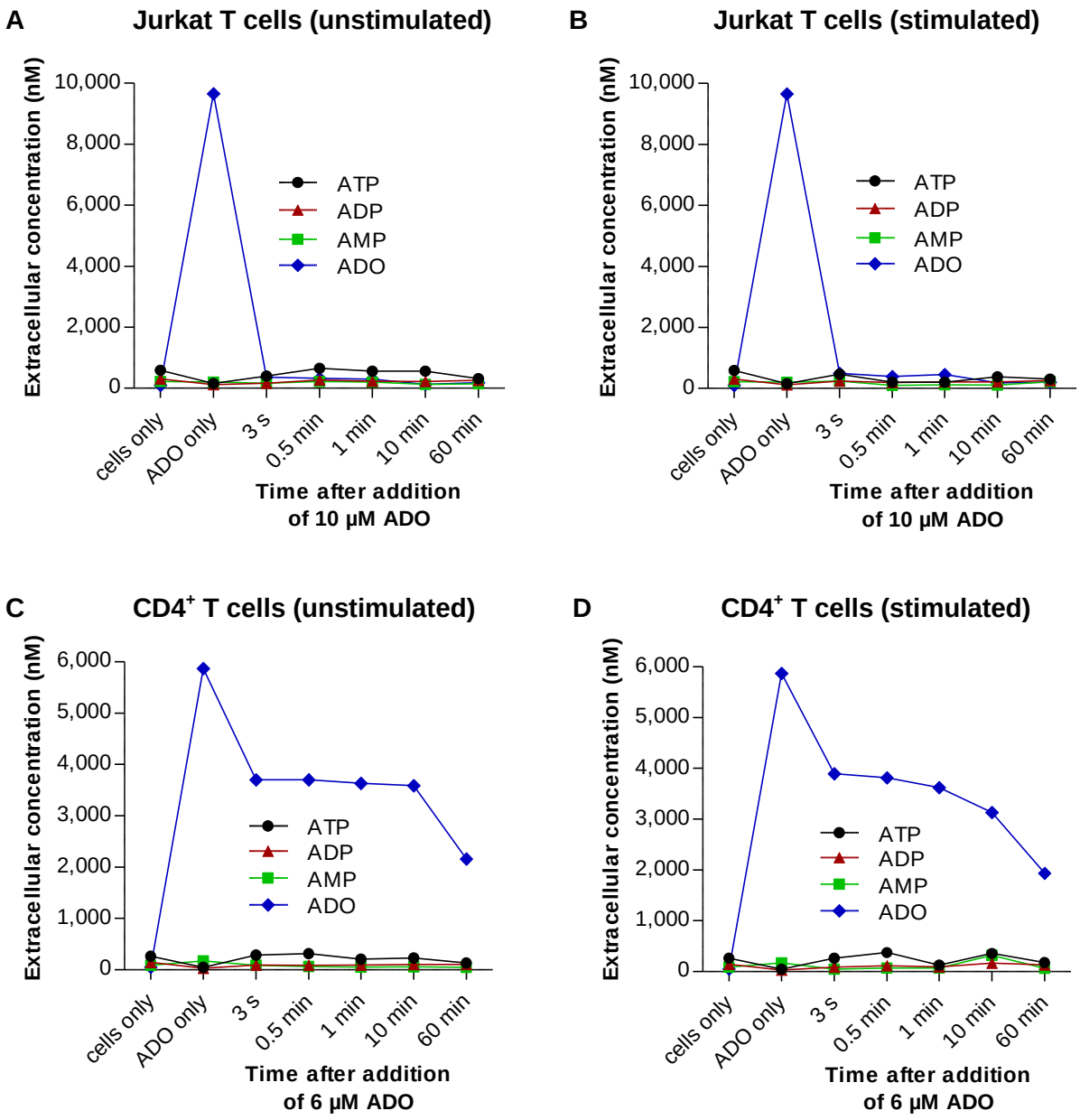


Figure 31: T cells rapidly remove exogenously added adenosine.

(A) Unstimulated Jurkat cells (3.3 x 10^6 cells/ml) and (B) Jurkat cells stimulated for 10 min with anti-CD3/CD28 coated beads were incubated with exogenous adenosine (10 µM) for the time points indicated. The cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) concentrations remaining in supernatant were measured with HPLC. (C) Unstimulated $CD4^+$ T cells (3.34 x 10^6 cells/ml) or (D) $CD4^+$ T cells stimulated for 10 min with anti-CD3/CD28 coated beads were incubated with exogenous adenosine (6 µM) for the indicated time points. The cells were placed on ice and ATP, ADP, AMP and adenosine concentrations remaining in supernatant were measured with HPLC. Data of a single experiment are shown.

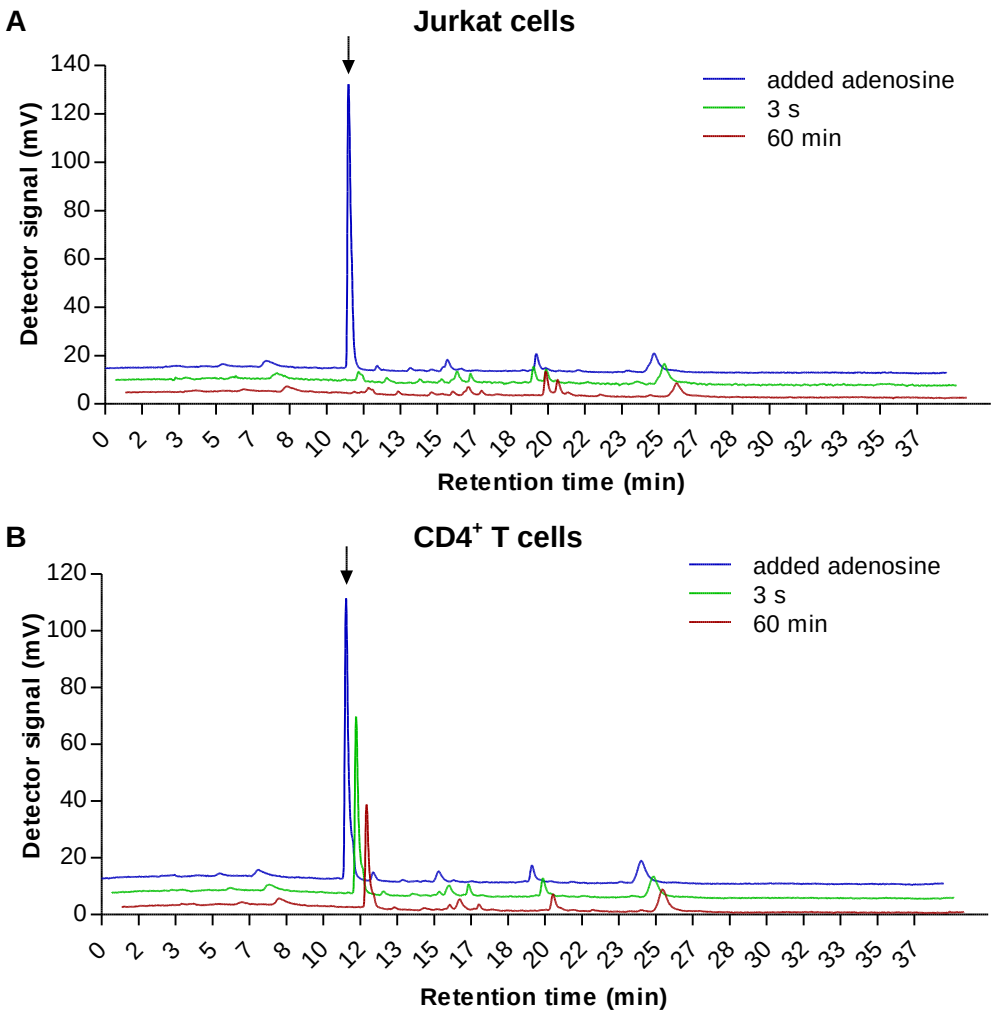


Figure 32: HPLC chromatograms of T cell supernatants showing rapid exogenous adenosine removal.

(A) Unstimulated Jurkat cells and (B) unstimulated $CD4^+$ T cells (3.3×10^6 cells/ml) were incubated with adenosine (A 10 µM, B 6 µM, arrows) for the time points indicated. The cells were placed on ice and the supernatants were analyzed with HPLC. Data shown are representative chromatograms.

After 10 min incubation of unstimulated and stimulated Jurkat cells with adenosine (8 µM) no adenosine was left in the supernatant (Figure 33 A). In $CD4^+$ T cells about 5 µM of 8 µM added adenosine was still present after 10 min (Figure 33 B). In both cell types no difference was observed between unstimulated and stimulated cells, or in ATP, AMP and ADP levels.

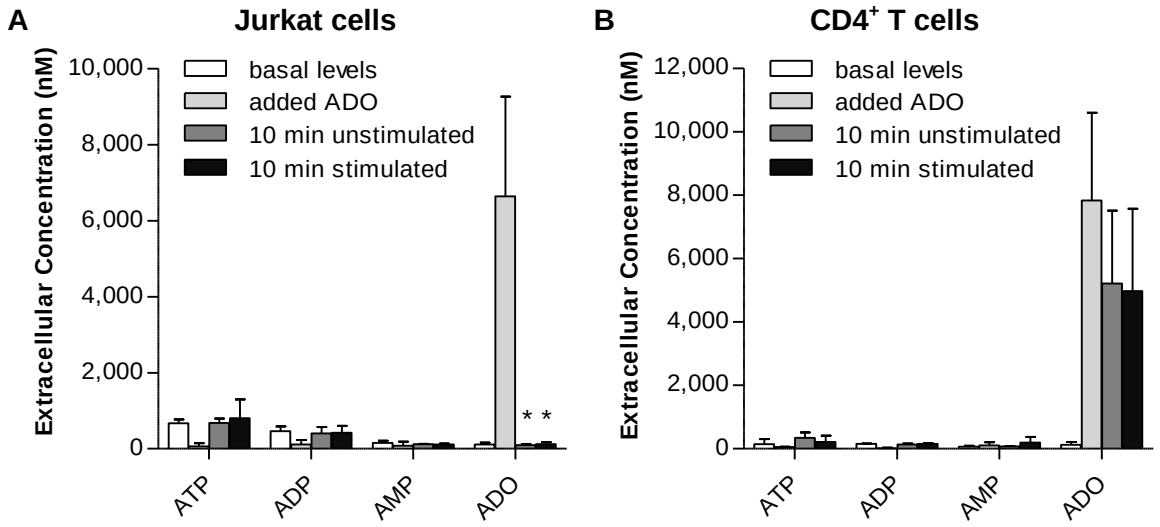


Figure 33: Adenosine rapidly decreases in unstimulated and stimulated T cells after 10 min.
(A) Jurkat cells and (B) human primary $CD4^+$ T cells (3.3×10^6 cells/ml) were suspended in RPMI and stimulated for 10 min with anti-CD3/28 coated beads (1–2 beads/cell) at 37°C. Unstimulated control samples were treated with RPMI instead of beads. Then adenosine (8 µM) was added and the cells were incubated for another 10 min. After these 10 min cells were placed on ice and ATP, ADP, AMP and adenosine (ADO) levels in the supernatant were measured using HPLC. Basal levels represent the ATP, ADP, AMP and adenosine concentration in the supernatant of untreated cells. Data shown are mean values + SD of (A) n = 3, (B) n = 2 independent experiments; * $p < 0.05$ compared to calculated 0 min (calculated as the sum of basal levels and added ADO), Student's t-test)

The adenosine levels were also assessed after 4 h stimulation with anti-CD3/CD28 coated beads to show if a longer stimulation time reveals differences in the adenosine metabolism compared to 10 min stimulation. Unstimulated and stimulated $CD4^+$ T cells showed decreasing adenosine levels, while ATP, AMP, and ADP levels did not change (Figure 34). No difference was found between unstimulated and stimulated $CD4^+$ T cells.

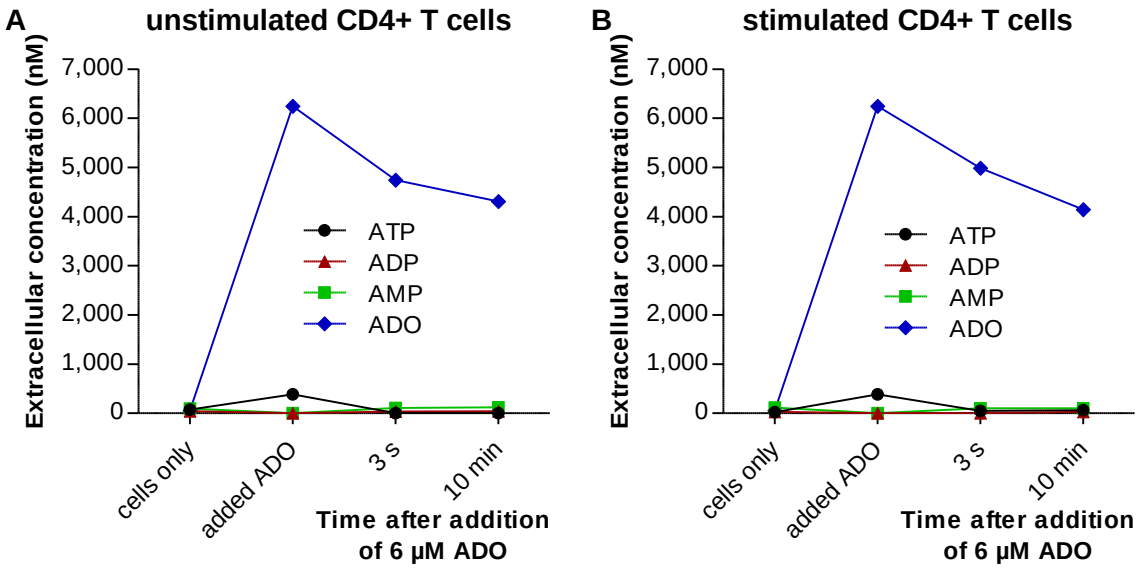


Figure 34: Unstimulated and 4 h stimulated CD4$^+$ T cells display no difference in adenosine metabolism.

CD4$^+$ T cells were isolated from human blood, suspended in RPMI supplemented with 10 % FBS (3.3 x 10^6 cells/ml) and incubated for 4h in the (A) absence or (B) presence of anti-CD3/CD28 coated beads (1–2 beads/cell). Then adenosine (ADO, 6 µM) was added and ATP, ADP, AMP and adenosine concentrations in the supernatant were measured using HPLC. Data of a single experiment are shown.

To assess if adenosine is broken down by ADA or taken up by the cell, EHNA and dipyridamole were added to the cells 10 min prior to adenosine addition. EHNA blocks ADA and prevents adenosine degradation, while dipyridamole blocks the adenosine uptake into the cell. After 3 s incubation with exogenously added adenosine, extracellular adenosine levels dropped in Jurkat cells in the absence and presence of dipyridamole, but not in the presence of the ADA inhibitor EHNA (Figure 35 A). After 10 min adenosine levels had partly decreased also when cells were treated with EHNA. At this time point, treatment with a combination of EHNA and dipyridamole resulted in higher adenosine levels compared to EHNA alone, suggesting that uptake contributes to the elimination of extracellular adenosine. Adenosine metabolism of CD4$^+$ T cells differed from that of Jurkat cells. CD4$^+$ T cells eliminated extracellular adenosine at a markedly slower rate than Jurkat cells. In addition, dipyridamole seemed to block adenosine removal after 3 s but not after 10 min and the presence of EHNA played a greater role in blocking adenosine scavenging at 10 min compared to 3 s after addition of adenosine (Figure 35 B).

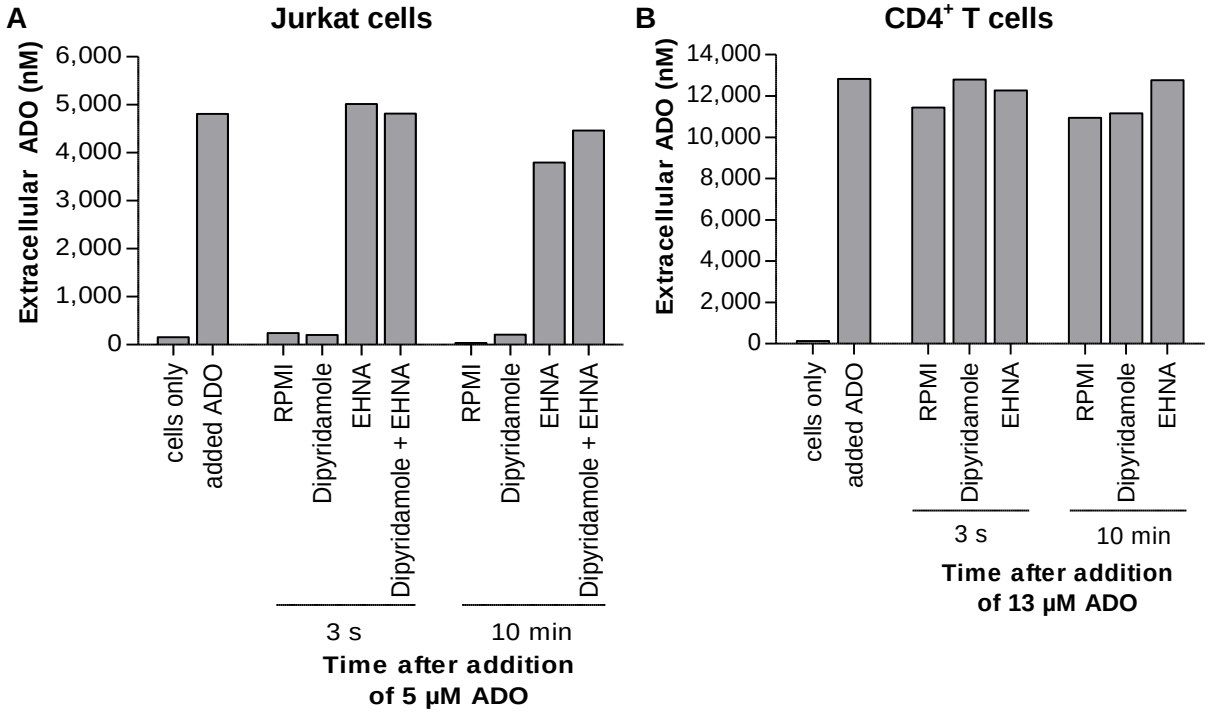


Figure 35: ADA is responsible for the rapid degradation of adenosine in Jurkat cells.
(A) Jurkat cells (3.3 x 10^6 cells/ml) were pretreated for 10 min with RPMI, dipyridamole (10 µM), EHNA (10 µM) or both. Then adenosine (5 µM) was added to all cells and incubated for 3 s or 10 min. Cells were placed on ice and ATP concentrations in the supernatant were measured using HPLC. (B) $CD4^+$ T cells (3.3 x 10^6 cells/ml) were incubated for 10 min with RPMI, dipyridamole (10 µM) or EHNA (10 µM). After that adenosine (13 µM) was added and incubated for 3 s or 10 min. Cells were placed on ice and ATP concentrations in the supernatant were measured using HPLC. Data of a single experiment are shown.

These data demonstrate that adenosine is removed very rapidly from the extracellular environment surrounding Jurkat T cells but not $CD4^+$ primary T cells. ADA degrades adenosine in Jurkat cells within seconds. $CD4^+$ T cells are less efficient in removing adenosine. Deamination by ADA and uptake by adenosine transporters seem to contribute to the removal of adenosine by primary $CD4^+$ T cells. However, due to the different amounts of added adenosine, which possibly resulted from different stock solutions used, other behaviors of the cells might be conceivable. In addition these data represent single experiments, thus repetitions are needed to confirm these data.

4 Discussion

There is growing evidence that the release of ATP and autocrine feedback signaling through purinergic receptors are essential for proper T cell activation and function [9,76,77]. However, the mechanisms that regulate the extracellular concentration of ATP and other ligands of purinergic receptors are incompletely understood. To address this question, in this study T cells were examined for their ATP, ADP, AMP and adenosine metabolism using HPLC and a luciferase assay. Further, mRNA expression levels of enzymes capable of hydrolyzing these molecules were analyzed using qPCR. In addition, mechanisms that are involved in the release of ATP from stimulated T cells were studied in more detail. The human Jurkat T cell line was compared to primary isolated human $CD4^+$ T cells, which were either analyzed unstimulated or stimulated with beads or plates coated with anti-CD3/CD28 antibodies.

4.1 ATP levels in resting and stimulated T cells

As the nucleotide metabolism was studied in this work, it was necessary to determine the basal ATP levels in the cell supernatant and the conditions under which ATP is released by T cells. It has been demonstrated that intact mammalian cells release ATP and a number of possible mechanisms for this have been proposed [7,102]. In T cells ATP release might be facilitated by various mechanisms like channels such as pannexin 1 hemichannels [8,76,77] or via vesicular exocytosis as shown in murine and Jurkat T cells [83]. However, for human T cells these mechanisms are still incompletely understood. Thus, ATP release of T cells and involved mechanisms were observed in this work.

In order to be able to observe increasing extracellular ATP levels the basal ATP concentrations in the cell supernatant had to be assessed. In various resting cells extracellular ATP is balanced at low levels via an equal ATP hydrolysis and release rate [102,103]. Jurkat cells, as has been reported previously [104], as well as isolated $CD4^+$ T cells revealed certain basal ATP levels in the supernatant. However, Jurkat cells showed higher basal ATP levels than $CD4^+$ T cells. These higher basal ATP levels in Jurkat cells might be due to the slower ATP degradation compared to $CD4^+$ T cells.

Not only resting cells are able to release ATP, but also stimulated cells. Stimulation with beads was unreliable during short incubation times. This might be due to a synapse formation of the cells with the beads and a local ATP increase within this immune synapse that is not released and can therefore not be measured in the supernatant. Further, cells are not activated simultaneously with beads. As a consequence and to guarantee uniform activation, antibodies against CD3 and CD28 were coated onto a 96-well plate for short term stimulation to avoid the use of beads. After stimulation of 5×10^5 Jurkat cells

(3.3×10^6 cells/ml) with plate-bound anit-CD3/CD28 extracellular ATP levels increased within the low nanomolar range measured via luciferase.
An ATP increase after stimulating Jurkat cells with anti-CD3/CD28 coated beads has been reported previously [77]. In that study ATP concentrations in the supernatant of 5×10^6 cells reached 3 µM in 30 s and remained elevated for over 5 min. This concentration is obviously higher than the one found in the present work. However, ATP values are hardly comparable, because of the different cell concentrations used. It was further reported that the extracellular ATP concentrations close to the cell surface reached > 60 µM within 2 min after cell stimulation [77]. This was observed using microscopic analysis. The luciferase assay used in the present study is not suitable to determine concentrations close to the cell surface [105]. Thus, ATP might be kept close to the cell surface and just a small amount of the released ATP diffuses further into the supernatant. Proteins on the cell surface like receptors and enzymes might be tightly arranged to allow only a small amount of nucleotides to leave this close cell surface area. This would mean that the used methods, luciferase and HPLC, detected not all of the released ATP. Thus, it might not be possible to examine the ATP concentration that the cell itself uses in the close environment. However, with HPLC and a luciferase assay it is possible to assess the ATP concentration that reaches cells within a short distance to the ATP releasing cell.

As previously reported pannexin 1 hemichannels are involved in the ATP release of stimulated mouse naive $CD4^+$ [8] and stimulated human T cells [76,106]. Consistent with these reports, the results of the current study indicate that pannexin 1 is involved in the stimulation-induced ATP increase in the supernatant of Jurkat cells. In activated T cells, mitochondria accumulate at the immune synapse that T cell form with APCs where they are thought to contribute to the regulation of intracellular Ca^{2+} levels [107]. In addition, recent evidence indicates that mitochondria also provide the ATP that is released into the synaptic cleft after T cell stimulation [101]. In agreement, blocking mitochondrial translocation with latrunculin B, an inhibitor of actin polymerization, inhibited the release of ATP. Taken together, our results suggest that after T cell stimulation mitochondria move to the membrane and produce ATP that is in turn released via pannexin 1 hemichannels (Figure 36).

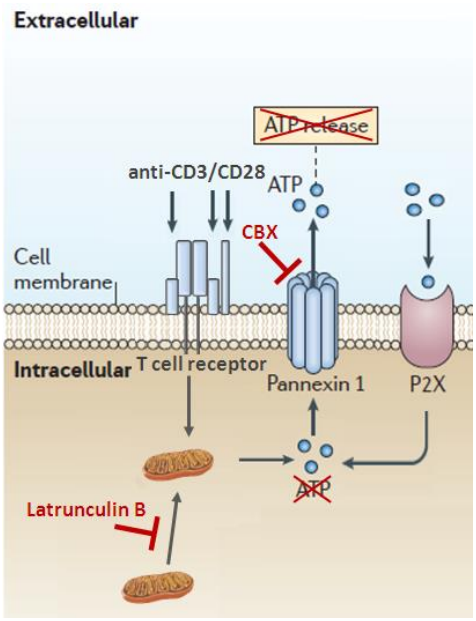


Figure 36: Stimulation-induced ATP release via pannexin 1 (modified from [9]).
Stimulation of the T cell receptor with anti-CD3 and the CD28 co-receptor with anti-CD28 leads to an extracellular ATP increase in T cells. CBX blocks pannexin 1 hemichannels and thereby reduces the extracellular ATP increase. Latrunculin prevents the movement of mitochondria to the membrane and also inhibits the increase of extracellular ATP.

In human gamma delta T cells ATP release was abrogated not only by inhibition of pannexin 1 hemichannels, but also reduced by bafilomycin A, which blocks vesicular exocytosis [106]. Also in murine T cells ATP release was decreased with bafilomycin A [83]. Thus, ATP release after T cell receptor and CD28 stimulation is regulated by various mechanisms, including hemichannels and exocytosis [83]. However, brefeldin A showed no and bafilomycin A only a marginal reduction of the released ATP in Jurkat cells. This indicates that vesicles are not involved in the stimulation-induced ATP release of these cells. In contrast to this data, Tokunaga et al. reported in their study with Jurkat cells and murine T cells that bafilomycin A significantly blocked the ATP release and they also presented pictures of ATP containing vesicles in both T cells. They also reported that the knock down of a vesicular nucleotide transporter reduced T cell receptor dependent ATP release in Jurkat cells. Thus, they illustrated a role of vesicular exocytosis of ATP during T cell activation [83]. A plausible reason for these different results might be the use of another Jurkat cell clone, as the exact clone they used was not stated. Further experiments are needed to elucidate an involvement of vesicular release in purinergic signaling in Jurkat cells. Another point of interest is if $CD4^+$ T cells show an ATP release via vesicles or not.

4.2 ATP-induced ATP increase

Not only TCR stimulation, but also ATP itself can increase extracellular ATP levels. So far an ATP-induced ATP increase has been reported only for a few cell types. Microglia, which are immune cells in the central nervous system, were found to respond to extracellular ATP by releasing ATP through lysosomal exocytosis [108]. Dou et al. state in this study that this provides a positive feedback mechanism for generating a long-range extracellular signal to attract distant microglia to migrate and accumulate at the site of injury [108].

In the present work an ATP-induced ATP release was also found in T cells (Figure14, 16, 17). Exogenously added ATP decreased over time, presumably due to degradation via ectonucleotidases. This ATP decrease could be further reduced with CCCP and CBX. CCCP blocks mitochondrial ATP production and the ATP level was decreased in its presence. This suggests that the decreased part is produced by mitochondria. CBX inhibits pannexin 1 hemichannels and reduced ATP in Jurkat cells, thus it can be assumed that the produced ATP is released via pannexin 1 (Figure 37). Therefore, a part of the added ATP is degraded, but in addition a part is released from the cells.

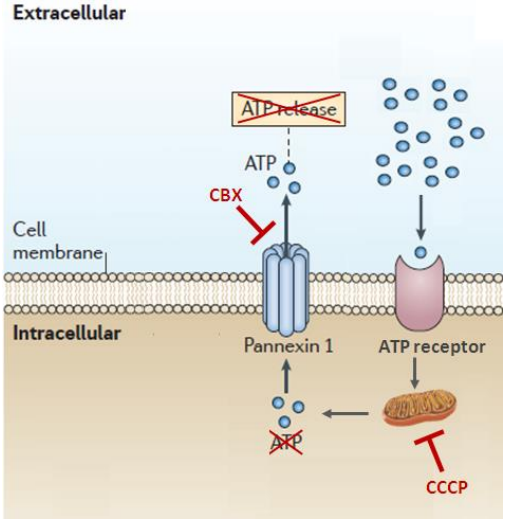


Figure 37: ATP-induced ATP release via pannexin 1 (modified from [9]).
Addition of exogenous ATP to T cells leads to an increase in extracellular ATP. CBX blocks pannexin 1 hemichannels and reduces the extracellular ATP increase. CCCP, a blocker of mitochondrial ATP production, also reduces the ATP increase.

However, three independent experiments differed in terms of the ability to decrease the ATP levels. This might be due to a different behavior of cells from different passages. In some experiments stimulated cells showed lower ATP concentrations; in others the concentration was nearly unchanged. This might suggest a stronger ATP degradation by stimulated cells in some experiments, but not in all. Thus, cells might react differently, depending on the cell passage used.

ATP seems to induce the release of ATP, but it might also be degraded. Therefore, ATPγS, which is non-hydrolysable, was used instead of ATP to further examine the amount of released ATP. In accordance with the prior results, the addition of ATPγS led to an increase in extracellular ATP.

ATP is considered as a danger signal, as it is released from damaged or dying cells and elicits inflammatory responses [9]. However, the reason for the ATP-induced ATP increase in healthy cells is still unclear, but it might describe a signal amplification mechanism to influence or stimulate the cell itself and/or to reach other cells with the released ATP that in turn release ATP and spread the signal.

4.3 ADP-induced ATP increase

Jurkat and $CD4^+$ T cells showed decreasing ADP concentrations after exogenous addition, but interestingly increasing ATP levels in addition to the increase of AMP. This suggests that ADP is broken down to AMP and further to adenosine. In addition, some of the ADP seems to have been converted to ATP. This notion is supported by the findings that inhibition of adenylate kinase (2 ADP ↔ ATP + AMP [93]) reduced the ATP increase. As CCCP and CBX did not block the increase an involvement of an ADP-induced ATP release is unlikely (Figure 38). A conversion of ADP to ATP was previously reported on the cell surface of various cell types [7,109–111], including Jurkat cells [104]. HUVEC cells showed a stepwise dephosphorylation of ATP to ADP, AMP and adenosine if only one nucleotide was present in the extracellular milieu. However, a combination of nucleotides led to the phosphorylation of AMP to ADP and ATP. This suggests a nucleotide kinase activity on the cell surface [109].

Considering that the ATP increase was not completely blocked by inhibiting the adenylate kinase, other enzymes or mechanisms might be involved in this process. In hepatocytes, ADP also induces an extracellular ATP production [110]. Besides adenylate kinase, nucleoside diphosphokinase (ADP + nucleoside triphosphate (NTP) ↔ ATP + nucleoside diphosphate (NDP)) was found to contribute to the generation of ATP. Mitochondrial ATP synthase (ADP + Pi ↔ ATP) is not involved in these cells [110]. On the other hand keratinocytes showed an involvement of adenylate kinase, ATP synthase, and nucleoside diphosphokinase in ADP to ATP conversion [111].

This data suggests the involvement of other kinases in the conversion of ADP to ATP in T cells. In addition to extracellular nucleotide degradation, an ATP generation pathway seems to be involved in the purinergic signaling of T cells.

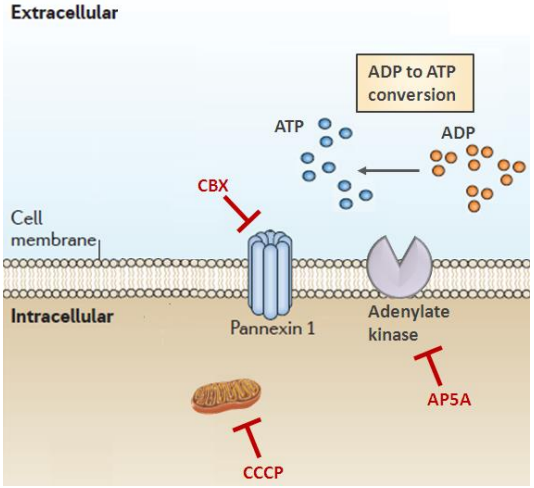


Figure 38: ADP-induced ATP increase (modified from [9]).
In T cells addition of exogenous ADP leads to an increase in extracellular ATP. Adenylate kinase plays a major role in the conversion of ADP to ATP. Inhibition of adenylate kinase with AP5A largely reduces, but does not fully block the increase in ATP. CBX, which inhibits ATP release via pannexin 1 and CCCP, which blocks mitochondrial ATP production, do not affect the ADP-induced ATP increase.

4.4 Adenosine metabolism

T cells degraded exogenous ATP, ADP and AMP to various extends. Adenosine was the fastest of these molecules to be degraded. Jurkat cells degraded all of the adenosine that

was added in low micromolar concentrations within a few seconds. The degradation of adenosine was fully prevented in the presence of EHNA indicating that deamination of adenosine by ADA was responsible for the observed adenosine removal. A high adenosine deamination by Jurkat cells and an inhibited adenosine deamination by approximately 90 % with EHNA was already previously reported [104]. However, this group incubated cells for 1 h with adenosine, while the current work focused on the short term metabolism. Degradation within milliseconds would be conceivable, but due to sample handling reasons the shortest possible observation time is 3 s. After 10 min EHNA alone was not able to fully block the degradation, but the complete inhibition was achieved when combining EHNA and dipyridamol, which suggests that a small amount of adenosine might also be taken up by the cells. On the other hand, $CD4^+$ T cells showed a slower adenosine removal than Jurkat cells. The findings of this work indicate that adenosine is taken up by the cell after 3 s and degraded after 10 min via ADA. However, to clearly disclose the adenosine removal especially in $CD4^+$ T cells, more experiments need to be done.

The current data suggest that Jurkat cells do not degrade ATP as fast as $CD4^+$ T cells. Thus, in Jurkat cells the conversion of AMP to adenosine by CD73 is inhibited. Adenosine is considered as anti-inflammatory and as Jurkat cells show faster adenosine degradation than $CD4^+$ T cells and higher remaining ATP levels on the cell surface they tend to be surrounded by pro-inflammatory ATP rather than by anti-inflammatory adenosine. This might be preferred by the cells to support their malignant nature.

4.5 Correlation of mRNA levels with ATP, AMP, ADP and adenosine degradation

The breakdown profiles for ATP, AMP, ADP and adenosine were compared with the expression patterns of the ectoenzymes that are able to degrade these molecules. Jurkat cells and $CD4^+$ T cells differ in their mRNA expression of ectonucleotidases. While in Jurkat cells the mRNA levels increased after stimulation, in $CD4^+$ T cells they rather decreased except for 5NTE.

As discussed in the prior chapter adenosine was rapidly degraded by T cells. ADA was the enzyme showing the highest expression with Jurkat cells expressing even more ADA mRNA than $CD4^+$ T cells. In accordance with the mRNA data of this study, an abundant expression of adenosine deaminase on the surface of Jurkat has been reported before [104]. This explains the rapid adenosine degradation in both cell types, but the even faster degradation in Jurkat cells.

In $CD4^+$ T cells AMP was degraded to adenosine. In agreement with this finding, both Jurkat and $CD4^+$ T cells expressed 5NTE mRNA that encodes for CD73, which degrades

AMP to adenosine. In Jurkat cells no adenosine increase was observed after addition of AMP, probably due to the rapid degradation of adenosine by these cells. Therefore, it seems likely that CD73 is also involved in the degradation of AMP in Jurkat cells.

Jurkat cells further expressed ENPP1, which was only marginally expressed in $CD4^+$ T cells. In addition $CD4^+$ T cells express mRNAs of ENTPD1, ENPP2 and ENPP3, which are not or only marginally expressed in Jurkat cells. However, it is hard to distinguish ENPPs from ENTPDs due to similar substrate and degradation patterns. Nevertheless, the different expression of these enzymes in Jurkat and $CD4^+$ T cells might contribute to the differential nucleotide degradation. As already mentioned a main difference between Jurkat and $CD4^+$ T cells is the ATP metabolism. In $CD4^+$ T cells ATP decreased and AMP increased with only a slight increase in ADP. This profile fits to NTPD1, which hydrolyzes ATP to AMP with producing only small amounts of ADP [6]. The mRNA of this enzyme was also expressed in $CD4^+$, but not in Jurkat cells. In Jurkat cells ATP was degraded mainly to ADP. This suggests that another enzyme is involved in the ATP hydrolysis in Jurkat cells, which might be ENTPD2. This enzyme is also expressed in $CD4^+$ T cells and could also contribute to the ATP degradation, but might be the main actor in Jurkat cells.
The ratio in which the nucleotides are present is another important factor. ADP and ATP inhibit CD73 [61], which converts AMP to adenosine. Thus, the ratios of ATP, ADP and AMP determine if nucleotides are degraded or synthesized [104].

Overall, $CD4^+$ T cells showed higher NTPDase and CD73 mRNA levels than Jurkat cells suggesting that these cells tend to counteract pro-inflammatory effects of ATP by the generation of adenosine. In contrast, Jurkat cells showed lower ectonucleotidase levels, but higher adenosine deaminase mRNA levels, which might help them to keep ATP levels elevated and to eliminate anti-inflammatory adenosine. By doing so they might avoid the lymphotoxic effect of adenosine and further mediate ATP signaling to other cells [104]. This might also contribute to their malignant phenotype.

A summary of the findings of this work can be seen in figure 39. Extracellular ATP is kept at a steady level in T cells. However, surplus of extracellular ATP leads to the release of ATP, but it is also degraded to ADP and AMP. Although ADP is degraded to AMP, it can also be converted back to ATP via kinase activity. Further, AMP is degraded to adenosine, which is rapidly removed extracellularly by cellular uptake or deamination via adenosine deaminase.

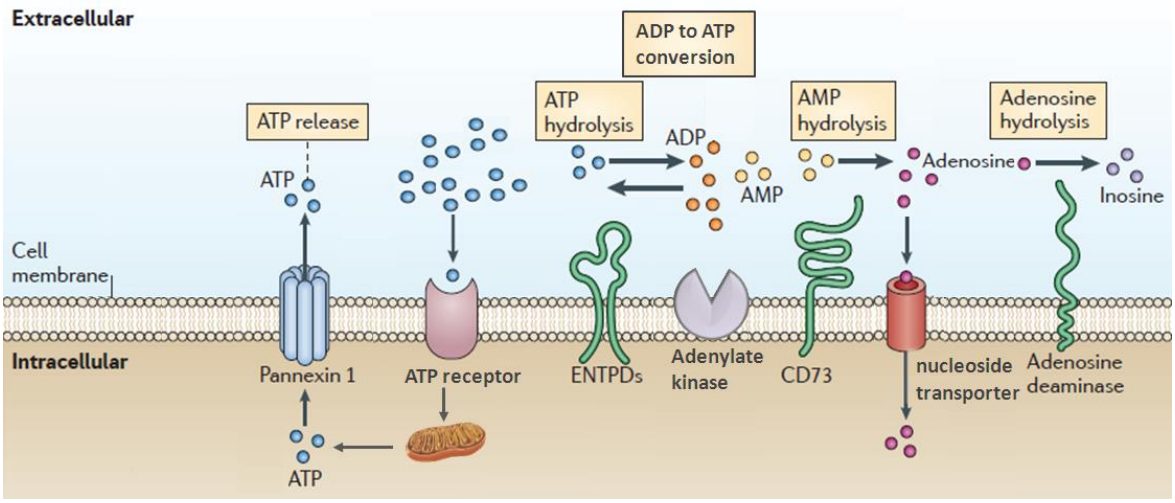


Figure 39: Extracellular ATP, ADP, AMP and adenosine metabolism (modified from [9]).
Extracellular ATP is kept at a certain basal level. Exogenous nucleotides are degraded from ATP to ADP, AMP and adenosine via ectonucleotidases like ectonucleoside triphosphate diphosphohydrolases (ENTPDs) and CD73. ADP in return can be converted to ATP via adenylate kinase activity. Adenosine is further degraded via adenosine deaminase or can be taken up by the cell.

In conclusion, ATP degradation and ATP release and/or resynthesis were found to coexist in human T cells. ATP, ADP, AMP and adenosine were broken down and thus inactivated. Even so, ATP can also be generated from ADP. The leukemia cell line, Jurkat cells, and isolated human $CD4^+$ T cells differ in the expression of ectoenzymes and the ability to degrade and form ATP and adenosine, which might explain in part their malignant and benign phenotypes. Thus, purinergic signaling might be differently regulated in health and disease and can be considered as future therapeutic target. However, a lot of questions remain to be elucidated concerning purinergic signaling in human T cells.

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List of Figures

List of Abbreviations

ADO	adenosine
ADP	adenosine diphosphate
ALP	alkaline phosphatase
AMP	adenosine monophosphate
AP5A	diadenosine pentaphosphate
APC	antigen presenting cell
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPγS	Adenosine 5'-[γ-thio]-Triphosphate
CBX	carbenoxolone
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CD	clusters of differentiation
CD73/5NTE	ecto-5′-nucleotidase (protein/gene)
CTLs	cytotoxic T lymphocytes
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EHNA	erythrohydroxynonyladenine
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
IL-2	Interleukin-2
KOH	potassium hydroxide
NDP	nucleoside diphosphate
NPP/ENPP	ecto-nucleotide pyrophosphatase/phosphodiesterase (protein/gene)
NTP	nucleoside triphosphate
NTPDase/ENTPD	ecto-nucleoside triphosphate diphosphohydrolase (protein/gene)
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCA	perchloric acid
PCR	polymerase chain reaction
qPCR	quantitative PCR
RPMI	Roswell Park Memorial Institute
TBP	TATA-box-binding protein
TCR	T cell receptor

A: Reagents and Equipment

Name	Cat. No.	Company
1,1,2-Trichlorotrifluoroethane	1100-6-08	SynQuest Laboratories (Alachua, FL, USA)
Absolute Ethanol	E7023	Sigma-Aldrich (St. Louis, MO, USA)
Adenosine	A9251	Sigma-Aldrich (St. Louis, MO, USA)
ADP (Adenosine 5′-diphosphate sodium salt)	A2754	Sigma-Aldrich (St. Louis, MO, USA)
AMP (Adenosine 5′-monophosphate sodium salt)	A1752	Sigma-Aldrich (St. Louis, MO, USA)
AP5A (P1,P5-Di(adenosine-5′) pentaphosphate pentasodium salt)	D4022	Sigma-Aldrich (St. Louis, MO, USA)
APC anti-human CD4 Antibody	317416	BioLegend (San Jose, CA, USA)
APC Mouse IgG1, κ	555751	BD Biosciences Pharmingen (San Jose, CA, USA)
ATP (Adenosine 5′-triphosphate disodium salt)	A2383	Sigma-Aldrich (St. Louis, MO, USA)
ATP Determination Kit	A22066	Molecular Probes, Life Technologies (Grand Island, NY, USA)
ATPγS	A1388	Sigma-Aldrich (St. Louis, MO, USA)
Autosampler glass inserts	C4015-643	Thermo Scientific (Rockwood, TN, USA)
Bafilomycin A	B1793	Sigma-Aldrich (St. Louis, MO, USA)
Brefeldin A	B7651	Sigma-Aldrich (St. Louis, MO, USA)
CBX	C4790	Sigma-Aldrich (St. Louis, MO, USA)
CCCP	C2759	Sigma-Aldrich (St. Louis, MO, USA)
CD4 microbeads, human	130-045-101	Miltenyi Biotec Inc. (San Diego, CA, USA)
Chloroacetaldehyde	317276	Sigma-Aldrich (St. Louis, MO, USA)
Dipyridamole	D9766	Sigma-Aldrich (St. Louis, MO, USA)
Dynabeads Pan Mouse IgG	11041	Invitrogen, Life Technologies (Grand Island, NY, USA)
EHNA hydrochloride	E114	Sigma-Aldrich (St. Louis, MO, USA)
Ethanol	2701	Decon Laboratories (King of Prussia, PA, USA)
FBS	10437-028	Gibco, Life Technologies (Grand Island, NY, USA)
Ficoll-Paque Plus	17-1440-02	GE Healthcare Life Sciences (Pittsburgh, PA, USA)

Formaldehyde	F8775	Sigma-Aldrich (St. Louis, MO, USA)
HPLC column: SUPELCOSIL LC-18-T, 3 µm, Column Dimensions: 15 cm x 4.6 mm	58970-U	Sigma-Aldrich (St. Louis, MO, USA)
iQ SYBR Green Supermix	170-8880	Bio-Rad (Hercules, CA, USA)
Latrunculin B	L5288	Sigma-Aldrich (St. Louis, MO, USA)
Methanol	34966	Fluka, Sigma-Aldrich (St. Louis, MO, USA)
mouse anti-human CD28 antibody	555726	BD Biosciences Pharmingen (San Jose, CA, USA)
mouse anti-human CD3 antibody	555337	BD Biosciences Pharmingen (San Jose, CA, USA)
Na_2HPO_4 (Sodium phosphate dibasic)	255793	Sigma-Aldrich (St. Louis, MO, USA)
NH_4HCO_3 (Ammonium bicarbonate)	09830	Fluka, Sigma-Aldrich (St. Louis, MO, USA)
Nylon Membrane Filter, Hydrophilic, 0.20 µm, 47 mm	GNWP04700	EMD Millipore (Billerica, MA, USA)
PE anti-Leu-4	7347	BD(San Jose, CA, USA)
Phosphate Buffered Saline (PBS)	SH3025601	HyClone, Thermo Scientific (Logan, UT, USA)
Potassium hydroxide solution (KOH)	03564	Fluka, Sigma-Aldrich (St. Louis, MO, USA)
Potassium phosphate monobasic (KH_2PO_4)	60221-250G	Fluka, Sigma-Aldrich (St. Louis, MO, USA)
Primer (qPCR) - ENTPD1 - ENTPD2 - ENTPD3 - ENTPD8 - ENPP1 - ENPP2 - ENPP3 - ADA - ALP - IL-2 - TBP - 5NTE	 QT00081473 QT00205779 QT00059507 QT00076188 QT00094787 QT00044387 QT00086744 QT00062370 QT00012957 QT00015435 QT00000721 QT00027279	Qiagen (Valencia, CA, USA)
QuantiTect Reverse Transcription Kit	205311	Qiagen (Valencia, CA, USA)

RNeasy Mini Kit	74104	Qiagen (Valencia, CA, USA)
R-PE-conjugated Mouse IgG2b Monoclonal Isotype Control	66375X	BD Biosciences Pharmingen (San Jose, CA, USA)
RPMI 1640	30-2001	American Type Culture Collection (ATCC) (Manassas, VA, USA)
SYBR Green Dye Spectral Calibration Plate, 96-well	4432346	Applied Biosystems, Life Technologies (Grand Island, NY, USA)
Tetrabutylammonium bisulfate	86853-10G-F	Fluka, Sigma-Aldrich (St. Louis, MO, USA)
Tri-n-octylamine, 97 %, 100 ml	AC14028-1000	Acros Organics, Fisher Scientific (Pittsburgh, PA, USA)
Water, Cell Culture Grade	SH3052902	HyClone, Fisher Scientific (Pittsburgh, PA, USA)
Water, HPLC	MK679510	Macron Fine Chemicals, VWR (Radnor, PA, USA)

Equipment

Centrifuges	for 15 ml and 50 ml tubes: RT6000B (Sorvall) for 1.5 ml and 2 ml tubes: centrifuge 5415 D, 5415 R (Eppendorf)
Flow cytometer	FACSCalibur flow cytometer (BD Biosciences)
Hemacytometer	Bright-Line Hemacytometer, Z359629 (Sigma-Aldrich)
HPLC	Waters 510 HPLC Pump Millipore Waters 717plus Autosampler Waters 474 Scanning Fluorescence Detector Empower2 software (Waters Corporation)
Luminometer	Luminoskan Ascent (Thermo Scientific)
Water baths	for 72°C – HAAKE DC10-B3 (Thermo Scientific) for 37°C – 1083 shaking water bath (GFL) water bath 182 (Precision Scientific)
Water Purification System	Milli-Q Integral Water Purification System (EMD Millipore)