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Modulation of the immune response by purinergic molecules: The ATP to adenosine axis in T cells

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I Summary

Purinergic enzymes and receptors are expressed on many immune cell types and are involved in the metabolism of extracellular adenine nucleotides. Under physiological conditions, the concentration of adenosine triphosphate (ATP) in the extracellular space is low, but it can drastically increase after ATP release from cells during inflammation, cell stress or cell lysis. Extracellular ATP induces mainly pro-inflammatory signaling pathways by activating P2 receptors, including the ionotropic P2X7 receptor. ATP can be degraded to adenosine by the concerted action of the ectonucleotidases CD39 and CD73, and adenosine then induces anti-inflammatory effects by activating P1 receptors. During T lymphocyte activation, ATP and adenosine have opposing effects, and the balance between both molecules is critical for the outcome of an immune response. Manipulating purinergic pathways could be used as therapy for inflammatory diseases. This requires a detailed analysis of the expression and complex interplay of the involved enzymes and receptors on human T cells.

In the work presented in this thesis, I assessed the effects of ATP signaling in human T cells, and characterized the function and regulation of enzymes involved in ATP degradation and adenosine generation. We analyzed the expression of the ATP receptor P2X7 in the human T cell compartment, and could show that P2X7 is mainly expressed on innate-like T cells. P2X7 expression renders these cells susceptible to ATP-induced Ca^{2+} influx and cell death. The rapid degradation of ATP to adenosine by CD39 and CD73 is a well described immune suppression mechanism, but co-expression of both ectonucleotidases on human T cells is rare. We thoroughly characterized the expression and function of both enzymes on human T cells, and found that CD73 is released on extracellular vesicles (EVs) from effector T cells upon T cell activation. EV-bound CD73 retains its enzymatic activity and mediates, especially in combination with regulatory T cells, immune suppression. Finally, we have identified murine CD73 as target for adenosine diphosphate (ADP)-ribosylation, a post-translational modification already known from the P2X7 receptor and other targets. Both the release of CD73 in EVs and the decrease of CD73 enzymatic activity through ADP-ribosylation constitute mechanisms to regulate CD73 function, and ultimately the outcome of an immune response.

In summary, in my thesis I show expression patterns, regulation mechanisms, and interactions of purinergic proteins involved in ATP-induced signaling pathways and in the degradation of ATP to adenosine.

II Zusammenfassung

Purinerge Enzyme und Rezeptoren werden auf vielen unterschiedlichen Typen von Immunzellen exprimiert und sind an dem Metabolismus von extrazellulären Adenin-Nukleotiden beteiligt. Unter physiologischen Bedingungen ist die Konzentration von extrazellulärem Adenosintriphosphat (ATP) gering. Während Entzündungsreaktionen, Zellstress oder Zelllyse wird ATP jedoch von Zellen freigesetzt, was zu einem starken Anstieg der extrazellulären Konzentration führen kann. Extrazelluläres ATP induziert vor allem entzündungsfördernde Signalwege durch die Aktivierung von P2-Rezeptoren, einschließlich des ionotropen P2X7-Rezeptors. ATP kann durch die Ektonukleotidasen CD39 und CD73 zu Adenosin abgebaut werden, welches durch die Aktivierung von P1-Rezeptoren entzündungshemmende Signalwege einleitet. Während der Aktivierung von T-Lymphozyten haben ATP und Adenosin gegensätzliche Effekte, und das Gleichgewicht zwischen beiden Molekülen ist entscheidend für den Ausgang einer Immunantwort. Eine gezielte Modulation von purinergen Signalwegen könnte zu der Therapie von entzündlichen Erkrankungen beitragen. Dies erfordert eine detaillierte Analyse der Expression und des komplexen Zusammenspiels der beteiligten Enzyme und Rezeptoren auf humanen T-Zellen.

In dieser Arbeit habe ich die Effekte von ATP-Rezeptor-vermittelten Signalwegen sowie die Funktion und Regulation von Enzymen, die an dem Abbau von ATP und der Bildung von Adenosin beteiligt sind, untersucht. Wir haben die Expression des ATP-Rezeptors P2X7 auf humanen T-Zellen analysiert und konnten zeigen, dass P2X7 vor allem auf „unkonventionellen“ T-Zellen, die Charakteristika von Zellen des angeborenen Immunsystems aufweisen, exprimiert wird. Diese Zellen werden dadurch empfänglich für den ATP-induzierten Einstrom von Ca^{2+}-Ionen und Zelltod. Der Abbau von ATP zu Adenosin durch CD39 und CD73 ist ein Mechanismus, der zu Immunsuppression führt und in diesem Kontext gut charakterisiert ist. Auf humanen T-Zellen ist die Co-Expression beider Ektonukleotidasen allerdings selten. Wir haben die Expression und Funktion von CD39 und CD73 auf humanen T-Zellen umfassend charakterisiert und aufgezeigt, dass CD73 von aktivierten Effektor-T-Zellen auf extrazellulären Vesikeln (EVs) freigesetzt wird. Das EV-gebundene CD73 ist enzymatisch aktiv und führt, vor allem in Kombination mit regulatorischen T-Zellen, zu Immunsuppression. Abschließend haben wir herausgefunden, dass CD73 durch ADP-Ribosylierung modifiziert wird. ADP-Ribosylierung ist eine posttranslationale Modifikation, die bereits von dem P2X7-

Rezeptor und anderen Proteinen bekannt ist. Sowohl die Freisetzung von CD73 auf EVs als auch die Verringerung der enzymatischen Aktivität von CD73 durch ADP-Ribosylierung stellen Mechanismen dar, die die Funktion von CD73 und letztlich den Ausgang einer Immunantwort beeinflussen.

Zusammenfassend zeige ich in dieser Arbeit Expressionsmuster, Regulationsmechanismen und Interaktionen verschiedener Proteine des purinergen Systems, die an ATP-induzierten Signalwegen und an dem Abbau von ATP zu Adenosin beteiligt sind.

1 Introduction

1.1 The immune system

The immune system is critical to protect the host from pathogens. The first line of defense is the innate immune response, which is rapidly activated and recognizes broad classes of pathogens. The innate immune system relies on antimicrobial effectors, the complement system, and the recognition of conserved pathogen-associated molecular pattern (PAMPs) and subsequent phagocytosis of pathogens by innate immune cells such as macrophages and neutrophils. The adaptive immune system consists of B and T lymphocytes recognizing a huge variety of antigens through their highly diverse and specific antigen receptors. Dendritic cells (DCs) continuously transport antigens from the site of infection to lymphoid organs where they are recognized by naïve T cells specific for the presented antigen. After encountering their cognate antigen, naïve T cells proliferate and differentiate into effector T cells, which then either kill their target cells or produce cytokines to support other immune cells. Naïve B cells are activated after antigen recognition and interaction with their cognate T helper (Th) cells. They proliferate in the germinal center of secondary lymphoid organs and differentiate into antibody-secreting plasma cells. Antibodies contribute to pathogen clearance by neutralization, opsonization and complement activation, summarized as humoral immunity. The immune response must be tightly regulated to enable the efficient elimination of pathogens while preventing immune pathologies. Regulatory T cells (Tregs) and T cell-intrinsic mechanisms contribute to maintain the balance between activation and suppression of immune cells. Most B and T cells die after clearance of the infection, but a small pool of antigen-specific lymphocytes persists and, after encountering the same antigen again, expand rapidly. This long-lasting immunological memory contributes to the fast and effective immune response after reinfection and is a hallmark of the adaptive immune system (Murphy et al. 2012).

1.1.1 Activation of T lymphocytes

T lymphocytes are main players of the cell-mediated adaptive immune response. Through their T cell receptor (TCR), T cells recognize foreign antigen peptides presented by the major histocompatibility complex (MHC) of antigen-presenting cells (APC). CD4 helper T cells

recognize peptides presented by MHC II, and, upon activation, produce cytokines to support/activate e.g. B cells or macrophages. Cytotoxic CD8 T cells recognize peptides presented by MHC I and contribute to the clearance of infected cells by releasing granzymes and perforins (Murphy et al. 2012).

The TCR of conventional T lymphocytes consists of two chains, an α- and a β-chain. Less than 10% of peripheral blood T cells have instead a γ- and δ-chain and belong to the so-called unconventional or innate-like T cells (Godfrey et al. 2015). The TCR chains belong to the immunoglobulin protein family and each chain consists of a transmembrane domain, a constant region, and a highly variable region to recognize antigens. The TCR is associated to CD3, forming together the TCR complex (Dong et al. 2019). T cell activation of naïve T cells requires several signals, specifically i) MHC-antigen recognition through the TCR, stabilized by co-receptors CD4 or CD8, ii) activation of co-stimulatory receptor CD28 on the T cells by CD80/CD86 on the APC, and iii) sensing of APC-produced cytokines by the T cell. TCR activation leads to the phosphorylation of immunoreceptor tyrosin activating motifs (ITAM) at the intracellular domain of the ζ-chain of CD3 by the lymphocyte-specific protein tyrosine kinase (Lck). Proteins with Src homology 2 (SH2) domain, e.g. zeta-associated protein-70 (ZAP-70), bind to phosphorylated ITAMs and become phosphorylated themselves. ZAP-70 activates linker of activated T cells (LAT), a protein with several adapter proteins. Then, phospholipase C (PLC), associated to LAT, is activated and cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) in diacylglycerol (DAG) and inositol trisphosphate (IP_3). These molecules induce several signaling pathways, which finally result in the activation and nuclear translocation of the transcription factors nuclear factor of activated T cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1), and subsequent expression of genes responsible for T cell activation, proliferation and cytokine production (Hwang et al. 2020; Murphy et al. 2012; Brownlie and Zamoyska 2013).

Several factors influence the extent of T cell activation, including the strength of MHC-peptide-TCR interaction, co-stimulatory and co-inhibitory receptors, adhesion molecules within the immunological synapse, and the amount of presented antigen peptide by the APC. Additionally, pathways influencing TCR-mediated downstream signaling modify T cell activation. For example, protein kinase A (PKA) prevents the phosphorylation of Lck, inhibiting ZAP-70 activation and subsequent downstream signaling (Tasken and Ruppelt 2006). In

contrast, plasma membrane ion channels that enable Ca^{2+} influx from the extracellular space to the cytoplasm, e.g. P2X, transient receptor potential (TRP) and ORAI channels, increase intracellular Ca^{2+} concentrations and enhance NFAT signaling and T cell activation (Trebak and Kinet 2019).

1.2 Purinergic signaling

Extracellular purine nucleotides and nucleosides induce a variety of cell signaling pathways, commonly referred to as purinergic signaling. Besides purinergic receptors, the purinergic system includes enzymes and transporters that equilibrate the concentration of extracellular nucleotides. Purinergic receptors and enzymes are broadly expressed in almost all tissues of the body and on different cell types, including immune cells. By engaging their receptors, the adenine nucleotides adenosine triphosphate (ATP) and adenosine have immunomodulatory effects, and the balance between both molecules influences the outcome of an immune response.

Under physiological conditions, the concentration of ATP in the extracellular space lays in the low nanomolar range. Inflammation, cellular stress, or cell death induce ATP release by cells, leading to a pericellular increase of the ATP concentration (Trabanelli et al. 2012). ATP can be released in a controlled way through exocytosis and pannexin or connexin channels (Woehrle et al. 2010), or uncontrolled during cell lysis. ATP induces pro-inflammatory signaling by activating P2X or P2Y receptors on immune cells. Alternatively, ATP can be degraded to adenosine, an anti-inflammatory mediator. The best described enzymes for this stepwise hydrolysis of ATP to adenosine are CD39 and CD73, also referred to as the canonical purinergic pathway for adenosine generation (Figure 1).

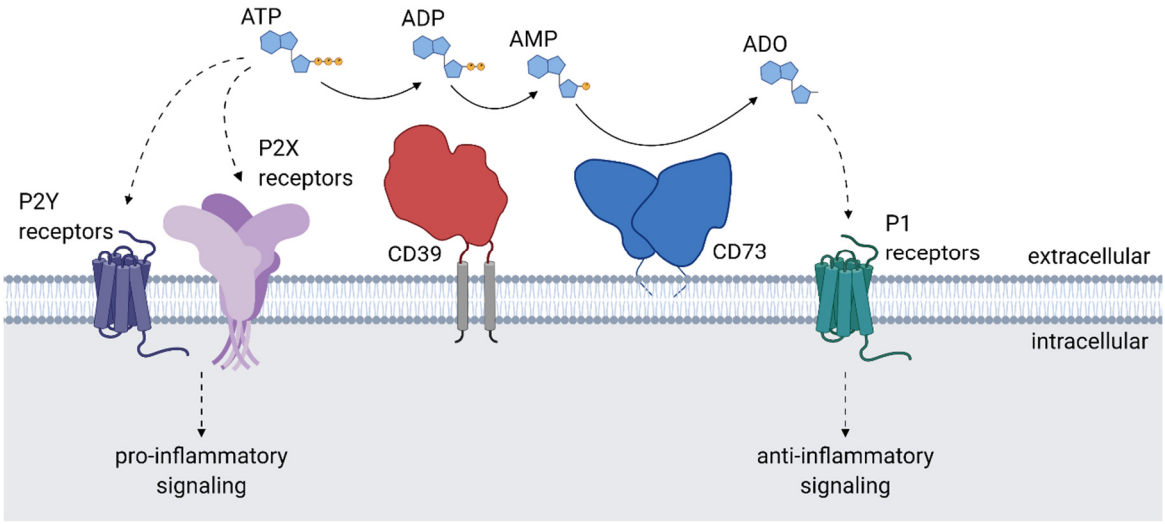

Figure 1. Canonical purinergic pathway. ATP is released into the extracellular space under inflammatory conditions, cellular stress or by dying cells. ATP can activate P2X or P2Y receptors, or is degraded to adenosine by the ectonucleotidases CD39 and CD73. Adenosine mediates mainly anti-inflammatory effects by inducing P1 receptor signaling. Figure created with BioRender.com.

1.3 Purinergic receptor signaling modulates the immune response

The receptors mainly involved in purinergic signaling are P2X and P2Y receptors for ATP, and P1 receptors for adenosine.

ATP receptors comprise the ion channels of the P2X family and the G protein-coupled receptors (GPCR) of the P2Y family. The P2X family consists of seven members (P2X1 through P2X7), which are all ATP-gated transmembrane cation channels (North 2002). Among the P2X receptors, the P2X7 receptor is the best studied subtype. The P2Y family consists of eight members. ATP is the ligand for P2Y1, P2Y2 and P2Y11, while the other members of this family are activated by adenosine diphosphate (ADP), uridine triphosphate (UTP), uridine diphosphate (UDP), or UDP-glucose (Lovászi et al. 2021). P2 receptors are expressed on immune cells, such as DCs, monocytes, and lymphocytes. The effects of P2 receptor signaling are overall described as pro-inflammatory, and include the release of pro-inflammatory cytokines by monocytes, degranulation of neutrophils, and activation of T cells.

The family of adenosine receptors consists of A1, A2A, A2B and A3 receptors. They are all GPCRs coupled to different G proteins. The expression of the different receptor subtypes is highly cell type-specific and the downstream receptor signaling varies according to the subtype that is engaged (Haskó et al. 2008). T cells express A2A and A2B receptors, which induce anti-inflammatory signaling upon receptor engagement.

1.3.1 The ATP receptor and ion channel P2X7

The P2X7 receptor is a ligand-gated transmembrane cation channel, and its only physiological ligand is ATP. P2X7 is a homotrimeric receptor, with each subunit having one extracellular ATP binding site. A special characteristic of the structure of P2X7 is a large cytoplasmic domain, which includes a cysteine-rich region (Illes et al. 2021; McCarthy, Yoshioka, and Mansoor 2019). Palmitoylation of this region restricts the movement of the transmembrane domain of P2X7, and prevents receptor desensitization. The lack of desensitization is an exclusive feature of P2X7 compared to other P2X receptors (McCarthy, Yoshioka, and Mansoor 2019). ATP binding to P2X7 and receptor activation induce channel opening and allow the influx of Ca^{2+} and Na^{+} ions and the efflux of K^{+} ions. Compared to all other members of the P2X receptor family, P2X7 has a low affinity for ATP, and requires ATP concentrations in the micromolar range to become activated (>100 µM, compared to EC_{50} values of 0.5 – 10 µM for the other P2X receptors) (Illes et al. 2021). Importantly, there are differences between the human and rodent receptor in regard of the ATP sensitivity, with rodent P2X7 being around ten times more sensitive than the human receptor (Xing et al. 2016). In mice, P2X7 is not only activated by ATP, but also by ADP-ribosylation of the receptor. ADP-ribosylation is performed by the mono-ADP-ribosyltransferase ARTC2.2, which uses nicotinamide adenine dinucleotide (NAD^{+}) as substrate and transfers the ADP-ribose (ADPR) residue to arginine 125 at the interface of two subunits of the receptor, close to an ATP binding site (Adriouch et al. 2008). ADP ribosylation of the P2X7 receptor mimics ATP binding, and leads to the activation of the receptor. Of note, only the splice variant P2X7(k), expressed mainly in T cells, is susceptible for this ADP-ribosylation, while variant P2X7(a) is less sensitive (Schwarz et al. 2012). There is no human orthologue of the ART2.2 enzyme (Haag et al. 1994), and it is not yet known if the human receptor can be modified to enhance its sensitivity towards ATP.

1.3.1.1 Expression of P2X7

In mice, *P2rx7* is expressed in a variety of tissues, including brain, spleen, intestine, and liver, with the highest expression on mast cells and macrophages. Within the murine T cell compartment, Tregs and natural killer T (NKT) cells have the highest expression of P2X7 (B. Rissiek et al. 2015).

In humans, P2X7 is expressed in different tissues, with the highest expression on neuronal and glial cells (neurons, oligodendrocytes and microglia) in the brain. For immune cells, RNA data shows the highest gene expression of *P2RX7* on monocytes and myeloid DCs. Within the T cell compartment, Tγδ cells and mucosal-associated invariant T (MAIT) cells show a slightly higher expression than other T cell subsets (Manohar et al. 2012; Gutierrez-Arcelus et al. 2019; Uhlen et al. 2019), although still three to four times lower than on monocytes. Protein expression of P2X7 was analyzed by staining of peripheral blood mononuclear cells (PBMCs) with the monoclonal antibody L4, confirming high expression on monocytes, and natural killer (NK) cells, while expression on B and T cells was low (Gu et al. 2000). Protein expression data for different T cell subsets is not yet available. The *P2RX7* gene contains several single nucleotide polymorphisms (SNPs), and the genotype of the donor results in interindividual differences in P2X7 receptor expression and activity. The SNPs lead either to a gain-of-function or loss-of-function (LOF), and the overall P2X7 activity depends on the combination of different SNPs (Adhikary et al. 2019; Fuller et al. 2009; Cabrini et al. 2005; Gu et al. 2001). Some of the *P2RX7* SNPs are associated to disease, e.g. the LOF variant rs28360457 is protective against neuroinflammation in multiple sclerosis (Gu et al. 2015) and the LOF variant rs3751143 is associated with reduced risk of ischemic heart disease and stroke in smokers (Gidlöf et al. 2012). P2X7 antagonists are protective in a mouse model of multiple sclerosis (Matute et al. 2007) and prevent streptozotocin-induced type 1 diabetes in mice (Vieira et al. 2016; Di Virgilio et al. 2017). In a phase II clinical study, P2X7 antagonist CE-224,535 was given to patients with rheumatoid arthritis, it had, however, no beneficial effect (Stock et al. 2012). Other clinical studies e.g. using P2X7 inhibitors for treating depressive disorders or pain conditions, are ongoing (Mishra et al. 2021).

1.3.1.2 Function of P2X7

Function of P2X7 on monocytes

The effects of P2X7 receptor activation are well studied in monocytes. Here, a combination of toll-like receptor (TLR) and P2X7 receptor signaling induce NLRP3 inflammasome activation and subsequent release of pro-inflammatory cytokines IL-1β and IL-18 (Figure 2). Specifically, lipopolysaccharide (LPS) binds to TLRs, inducing NF-kB-mediated expression of inflammasome proteins (inflammasome priming) and cytokine precursors. ATP-induced activation of P2X7

and the resulting decrease in cytosolic K^+ concentration lead to the assembly and activation of the inflammasome. The NLRP3 inflammasome contains the sensory protein NLRP3, the adaptor protein ASC and the effector protein caspase-1 (Pelegrin 2021). During inflammasome activation, caspase-1 becomes activated and cleaves pro-IL-1β and pro-IL-18, as well as gasdermin D (GSDMD) into biologically active forms. The N-terminal part of GSDMD (N-GSDMD) multimerizes and forms large protein complexes in the plasma membrane, which allows the efflux of active IL-1β and IL-18 and other cellular components to the extracellular space. If not repaired by membrane repair mechanisms, large N-GSDMD pores lead to cell death (Rühl et al. 2018; Shi et al. 2015; X. Liu et al. 2016). The release of IL-1β after P2X7 receptor activation is also shown for DCs (Pizzirani et al. 2007).

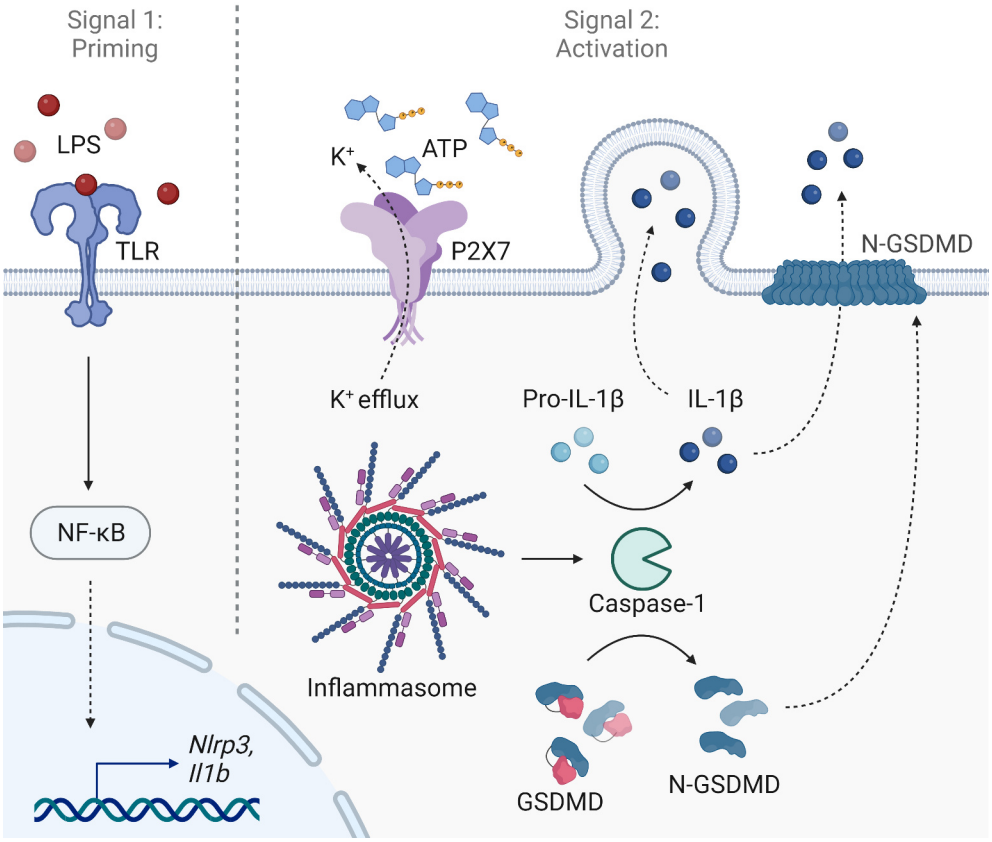

Figure 2. P2X7 activation leads to the release of pro-inflammatory cytokines in monocytes. The combination of TLR and P2X7 activation leads to the assembly and activation of the NLRP3 inflammasome. Activated caspase-1 generates mature IL-1β, which is released from the cell through N-GSDMD pores. Figure created with BioRender.com

Function of P2X7 on T cells

The effects of P2X7 signaling on T cells include T cell activation, differentiation, and cell death (Grassi 2020; Rivas-Yáñez et al. 2020). Early studies showed that low to intermediate ATP

concentrations (10 - 100 µM) increase the Ca^{2+} influx and proliferation of human T cells. This effect was inhibited with the P2X antagonist oxidized ATP (oATP) (Baricordi et al. 1996). Similarly, oATP inhibited TCR-dependent mitogen-activated protein kinase (MAPK) activation, proliferation and IL-2 secretion in CD4 T cells (Schenk et al. 2008). Yip et al. reported >60 µM ATP close to the cell surface, and addition of ATP in a similar concentration (100 µM) promoted T cell activation by enhancing NFAT activation and IL-2 release (Yip et al. 2009). P2X7 is additionally involved in the first steps of T cell activation by contributing to the generation of intracellular Ca^{2+} microdomains. Within milliseconds after TCR stimulation, Ca^{2+} is released from intracellular Ca^{2+} stores, inducing the opening of the pannexin channel PANX1 and the release of ATP to the extracellular space. ATP activates P2X7 in an autocrine manner, resulting in further increase of intracellular Ca^{2+} and amplification of Ca^{2+} microdomains (Brock et al. 2022). Beyond T cell activation, P2X7 is involved in the thymic T cell development by contributing to γδ lineage commitment (Frascoli et al. 2012), in reducing the suppressive function of Tregs and inducing their conversion to Th17 cells in the presence of IL-6 (Schenk et al. 2011), and in promoting the polarization of naïve CD4 T cells into Th17 cells by P2X7 receptor activation on DCs and subsequent IL-6 and IL-23 secretion (Atarashi et al. 2008). Additionally, the activation of P2X7 mediates T cell death (B. Rissiek et al. 2014).

P2X7-induced cell death

Prolonged P2X7 receptor activation leads to the formation of large pores in the cell membrane, leading to cell death if not tightly regulated. The P2X7 receptor, pannexin channels and gasdermin proteins are all able to form pores, and it is not yet known which pore is ultimately responsible for P2X7-mediated cell death. In monocytes, P2X7 engagement leads to the activation of the NLRP3 inflammasome, resulting in the caspase-1-dependent cleavage of GSDMD. N-GSDMD multimerizes and forms large pores in the cell membrane (Figure 2). If the pores are not repaired, the release of cellular material and loss of cell integrity results in cell death (Shi et al. 2015; X. Liu et al. 2016; Rühl et al. 2018). This form of inflammatory, programmed cell death is called pyroptosis (Cookson and Brennan 2001). T cells also contain different inflammasome components, including NLRP1, NLRP3, CARD8, caspases-1/3/8, and GSDMD (C. Zhang et al. 2021; Linder et al. 2020). NLRP3 inflammasome-dependent pyroptosis contributes to the reduced number of CD4 T cells in HIV-infected patients. The process is driven by increased reactive oxygen species (ROS) production and involves caspase-1 and

GSDMD (C. Zhang et al. 2021). During inflammation of the central nervous system, Th17 cells produce IL-1β in response to ATP, through ASC- and NLRP3-dependent caspase-8 activation (Martin et al. 2016). Whether P2X7 activation can induce a T cell inflammasome is not yet known.

In contrast to human cells, where millimolar ATP concentrations are needed to induce P2X7-mediated cell death, murine cells that express both P2X7 and ART2.2 (e.g. Tregs) are very sensitive to P2X7-mediated cell death because low NAD^+ concentrations (1 - 30 µM) can already induce receptor activation by ADP-ribosylation of P2X7 (Seman et al. 2003). Cell death induced by NAD^+ is referred to as NAD^+-induced cell death (NICD) (Seman et al. 2003). NAD^+ is released e.g. during cell preparations from tissue, depleting specifically ATP-sensitive cells (Borges da Silva et al. 2019). Indeed, treating mice with the ARTC2.2-blocking nanobody s+16a before organ harvest significantly increases cell numbers (Koch-Nolte et al. 2007; B. Rissiek et al. 2018). P2X7-mediated cell death contributes to the control of follicular helper T cell (Tfh) numbers in Peyer's Patches (Proietti et al. 2014; Faliti et al. 2019), NKT cell regulation in the liver and mucosal tissues (Kawamura et al. 2006; Q. Liu and Kim 2019), tissue-resident T cells (Trm) maintenance (Fernandez-Ruiz et al. 2016; Stark et al. 2018; Borges da Silva et al. 2019, 2020; Heiss et al. 2008), and Treg depletion for promoting antitumor responses (B. Rissiek et al. 2014; Hubert et al. 2010).

1.3.2 Adenosine receptors A2A and A2B

The adenosine receptor family consists of the A1, A2A, A2B and A3 receptors, summarized as P1 receptors. They are all GPCRs and, dependent on the associated G protein, adenosine receptor activation induces different intracellular signaling pathways. T cells express only the A2A and A2B receptors, and adenosine induces mainly anti-inflammatory effects. The expression of A2A and A2B under physiological conditions is low, but it is upregulated after T cell activation and under hypoxic conditions (Koshiba et al. 1999; Sitkovsky and Lukashev 2005; Alam, Kurtz, Wilson, et al. 2009).

The A2A receptor has a stimulatory G protein ($G\alpha_S$) attached, and receptor activation leads to $G\alpha_S$-dependent activation of the adenylate cyclase, generating intracellular cyclic adenosine monophosphate (cAMP). cAMP activates PKA which then affects several signaling pathways. For example, it phosphorylates the C-terminal Src kinase (Csk), which is then unable to activate

the tyrosine kinase Lck. This prevents the phosphorylation of the TCR-attached protein ZAP-70, and consequently inhibits TCR downstream signaling (Figure 3A, see chapter 1.1.1 for TCR signaling) (Linden and Cekic 2012). Overall, A2A receptor signaling leads to a decreased activation, proliferation and cytokine production of T cells. Additionally, it maintains naïve T cells in a quiescent state by preventing IL-7 receptor downregulation and apoptosis of naïve T cells upon TCR stimulation (Cekic et al. 2013), and enhances the development and immunosuppressive capacity of Tregs (Ohta et al. 2012).

The expression of the A2B receptor on human T cells is very low, but it can be, at least in endothelial cells, upregulated under hypoxic conditions by hypoxia-inducible factor (HIF) binding to the *ADORA2B* promoter (Kong et al. 2006). A2B is a low affinity receptor, needing higher adenosine concentrations for activation than the A2A receptor. Besides the generation of cAMP through the $G\alpha_s$ protein and subsequent pathways as described above, the A2B receptor is also linked to a $G\alpha_q$ protein. $G\alpha_q$ activates PLC, leading to the generation of the second messengers IP_3 and DAG, which induce Ca^{2+} signaling (Figure 3B). It is not yet known which of these pathways is induced under which cellular conditions. In A2A/A2B-transfected cell lines, the two receptors build heteromers, resulting in a decreased accumulation of cAMP, suggesting an inhibition of A2A by A2B (Hinz et al. 2018; Franco et al. 2021).

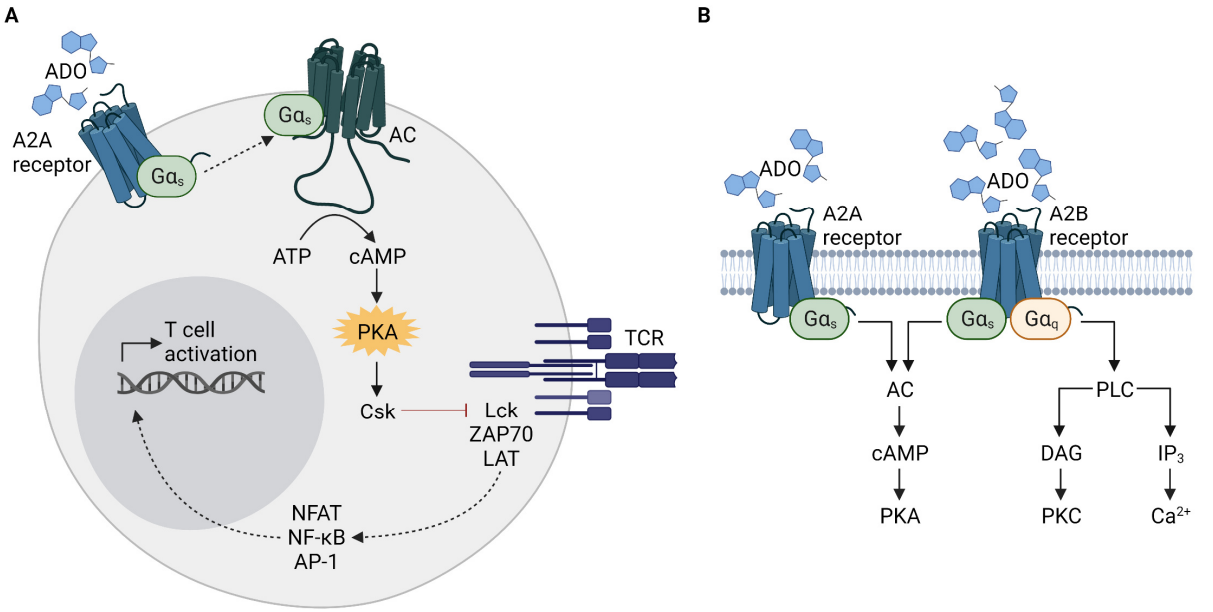

Figure 3. Adenosine receptor signaling in T cells. (A) A2A receptor signaling reduces T cell activation through a signaling pathway involving adenylate cyclase (AC), cAMP, PKA, and Csk. (B) The A2B receptor is associated to $G\alpha_s$ and $G\alpha_q$ proteins, inducing different signaling pathways. When expressed on the same cell, A2A and A2B can interact and form heteromers. Figure created with BioRender.com.

Beyond direct signaling through A2A and A2B receptors, T cells are indirectly affected by adenosine receptor signaling in APCs and other immune cells (Haskó et al. 2008). DCs release less IL-12 and TNF-α after adenosine treatment and have a reduced capacity to induce Th1 polarization (Panther et al. 2003). LPS-stimulated macrophages treated with an A2B receptor agonist produce IL-10 (Németh et al. 2005), which in turn suppresses effector cell responses.

Adenosine is rapidly degraded by adenosine deaminase (ADA) or taken up into the cell by nucleoside transporters, resulting in a very low extracellular half-life of less than ten seconds in blood (Klabunde 1983). Enhancing the half-life of adenosine by blocking equilibrative nucleoside transporters (ENT) is protective against colitis (Aherne et al. 2018).

1.4 Purinergic enzymes metabolize adenine nucleotides

Several ectonucleotidases are involved in the hydrolysis of pro-inflammatory ATP to anti-inflammatory adenosine. The 'canonical' ATP to adenosine pathway involves the enzymes CD39 and CD73, with CD39 degrading ATP to ADP and further to adenosine monophosphate (AMP), and CD73 hydrolyzing AMP to adenosine (see Figure 1 in chapter 1.2). The concerted action of these two enzymes is one of the immunosuppressive pathways of murine Tregs (Deaglio et al. 2007). Further, cancer cells upregulate CD73 to produce adenosine and inhibit surrounding inflammation and activation of immune cells (de Lourdes Mora-García et al. 2016). In addition to CD39 and CD73, other enzymes such as ENPP1, CD38, CD157, CD26, and alkaline phosphatases (AP) are involved in the metabolism of extracellular adenine nucleotides.

1.4.1 CD39 degrades pro-inflammatory ATP to AMP

CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1/ENTPD1) stepwise hydrolyzes ATP to AMP, releasing a single phosphate in each reaction. CD39 is a highly glycosylated enzyme with a size of 70 – 100 kDa (Maliszewski et al. 1994). CD39 has two transmembrane domains, cytoplasmic N- and C-terminal domains, and an extracellular hydrophobic loop. The extracellular loop contains five apyrase-conserved regions with phosphate-binding motifs, critical for the enzymatic activity of CD39 (Mateo, Harden, and Boyer 1999). On immune cells, CD39 is expressed on monocytes, B cells and regulatory T cells (Pulte et al. 2007; Antonioli et al. 2013; Deaglio et al. 2007). CD39 is not expressed on resting CD8 and conventional CD4

T cells, but its expression increases after activation (Raczkowski et al. 2018; Maliszewski et al. 1994), and is high at sites of inflammation (Moncrieffe et al. 2010). The expression of CD39 on Tregs and other T cell populations is genetically determined based on SNPs in the *ENTPD1* gene (A. Rissiek et al. 2015; Roederer et al. 2015). Low CD39 expression on T cells is associated with an increased risk of inflammatory bowel disease (Friedman et al. 2009; Gibson et al. 2015), poor response to methotrexate treatment in rheumatoid arthritis patients (Gupta et al. 2018; Peres et al. 2015), and lower viral loads and slower disease progression in HIV-infected patients (Schulze Zur Wiesch et al. 2011; Nikolova et al. 2011), underscoring the role of CD39 in immune regulation.

1.4.2 CD73 generates anti-inflammatory adenosine from AMP

The ectonucleotidase CD73 (ecto-5'-nucleotidase/NT5E) hydrolyzes AMP to adenosine. CD73 is a glycosylphosphatidylinositol (GPI)-anchored protein with a size of 71 kDa, and forms homodimers connected by non-covalent bonds (Martínez-Martínez et al. 2000; Heuts et al. 2012). The crystal structure of CD73 revealed that the N-terminal domain contains a metal ion binding site, while the C-terminal domain contains the substrate binding site and forms the dimerization interface (Heuts et al. 2012; Knapp et al. 2012). On immune cells, CD73 is expressed on B and T cell subsets. In the murine T cell compartment, CD73 is expressed on both CD4 and CD8 T cells, with the highest expression on Tregs. On human T cells, it is primarily expressed on naïve CD8 T cells, however, also some memory CD8 and CD4 T cell subsets express CD73 (Raczkowski et al. 2018). Of note, the expression of CD73 is very different between mice and humans, as it is e.g. highly expressed on murine Tregs, but almost absent on human Tregs (Schuler et al. 2014). CD73 expression is regulated under certain cellular conditions: it is downregulated on lymphocytes during the course of inflammation (Botta Gordon-Smith et al. 2015) and upregulated under hypoxic conditions due to a HIF-1α binding site in the *NT5E* promoter region (Synnestvedt et al. 2002), and in the presence of TGF-β or activin-A (Regateiro et al. 2011; Morianos et al. 2020). The SNP rs9444346 in *NT5E*, the gene encoding CD73, affects the frequency of $CD73^+$ CD4 T cells (Roederer et al. 2015), but CD73 expression is also influenced by environmental factors (Mangino et al. 2017). Mutations in *NT5E* that lead to a non-functional enzyme can lead to rare cases of arterial calcification, referred to as arterial calcification due to deficiency of CD73 (ACDC), with rheumatic

symptoms like joint pain and early-onset osteoarthritis (St. Hilaire et al. 2011; Ichikawa et al. 2015). There is no immune phenotype described in patients with CD73 deficiency.

The product of CD73 enzymatic activity is adenosine, an anti-inflammatory mediator that restricts T cell activation and function (see chapter 1.3.2). Because of its adenosine-generating function, CD73 plays a protective role in animal models of arthritis and colitis (Chrobak et al. 2015; Bynoe et al. 2012). The potent immunosuppressive role of CD73 is underlined by its upregulation in several types of cancer, establishing an anti-inflammatory milieu for intratumoral T lymphocytes, which enables tumor growth. Blocking CD73 enzymatic activity is promising as cancer therapy. Several small molecule inhibitors of CD73 such as α,β-methylene-ADP (AOPCP), a structural analogue of ADP, are already available for in vitro experiments, and are further refined for potential drug development (Bhattarai et al. 2020, 2019). Clinical trials with inhibitors or monoclonal antibodies against CD73 are ongoing (Leone and Emens 2018).

1.4.2.1 Soluble and vesicle-associated CD73

Bodily fluids such as serum, plasma, and synovial fluid (SF) contain non-cell-bound CD73 and/or CD73-dependent AMPase activity (Pettengill et al. 2013; Yegutkin, Samburski, and Jalkanen 2003; Morello et al. 2019; Schneider et al. 2019). Non-cell-bound CD73 widens the range of action of the enzyme and could transfer AMPase activity to cells lacking CD73.

As GPI-anchored protein, proteolytic cleavage or hydrolytic shedding of the membrane anchor of CD73 leads to the release of the soluble protein. Which enzyme is responsible for the release of CD73 is not yet clear, but potential mechanisms involve PLC, phospholipase D (PLD), or metalloproteases (Lehto and Sharom 1998; Low and Prasad 1988; W. Zhang et al. 2018; Schneider et al. 2019). The enzymatic activity of PLC-released soluble CD73 is higher than for the membrane-bound form (Lehto and Sharom 1998), therefore, the presence of soluble CD73 does not only widen its range of action, but also enhances the enzymatic activity.

Next to its presence as soluble protein, CD73 was detected on extracellular vesicles (EVs) (Clayton et al. 2011; Morandi et al. 2018; F. Zhang et al. 2019; Smyth et al. 2013). GPI-anchored proteins are not evenly distributed on the cell surface, but enriched in lipid rafts, a specific region of the cell membrane rich in cell signaling proteins. EV formation is associated with lipid rafts, and lipid raft-associated molecules are preferentially found in EVs (López-Cobo, Campos-

Silva, and Valés-Gómez 2016; Skryabin et al. 2020; De Gassart et al. 2003). EVs are small particles with a lipid bilayer membrane, released by almost all cell types under physiological or pathological conditions. The term 'EV' comprises exosomes and microvesicles, distinguishable mainly by their way of generation and their size (Raposo and Stoorvogel 2013; Buzas 2022). Exosomes are smaller vesicles (50 – 150 nm), generated through the endosomal pathway, which involves the formation of multivesicular bodies (MVB), and exosome release by MVB-fusion with the plasma membrane. Microvesicles are larger vesicles (50 – 1000 nm), and are released from cells by membrane budding. Typical surface markers of EVs are the tetraspanins CD9, CD63 and CD81, and flotillin, however, these markers are not specific for a certain type of EVs and depend on the cell of origin. Because neither the size nor the surface marker expression are exclusive for one type of EVs, it is difficult to clearly distinguish both types of vesicles. EVs carry membrane proteins, micro RNA (miRNA), cytokines, and other cellular content, in general reflecting the phenotype of their cell of origin (Fitzgerald et al. 2018; Garcia-Martin et al. 2022). By transporting this content to other cells, EVs enable cell-to-cell communication. CD73 was detected on EVs derived from different cell types, including cancer-, B cell-, and T cell-derived EVs (Clayton et al. 2011; Morandi et al. 2018; F. Zhang et al. 2019; Smyth et al. 2013). CD73 is not the only purinergic enzyme released in EVs, also CD39 has been detected on EVs from e.g. Tregs, tumor cells and mesenchymal stromal cells (Tung et al. 2020; Clayton et al. 2011; Carotti et al. 2022).

2 Aims of the study

Purinergic signaling involves various enzymes and receptors, leading to a dynamic metabolism of adenine nucleotides and the induction of different intracellular signaling pathways. In T cells, the balance between pro- and anti-inflammatory signals is crucial for immune homeostasis, and the outcome can be shifted by adenine nucleotides towards immune pathology or immune suppression. The expression and function of purinergic molecules is already well characterized in mice or under certain conditions as e.g. in the tumor microenvironment. The goal of this thesis is to analyze the expression of the ATP receptor P2X7 and the adenosine-generating enzyme CD73 on human T cells, as well as mechanisms regulating CD73 function. Specifically, the aims of this project are:

1.) To investigate the effects of ATP signaling in human T cells, specifically the expression of the P2X7 receptor on human T cell subsets and implications in the immune response

2.) To characterize enzymes involved in ATP degradation and adenosine generation, focusing on the regulation of CD73 expression and activity, and the role of non-cell bound CD73 as important switch for the outcome of an immune response

3 Publications

3.1 P2X7 is expressed on human innate-like T lymphocytes and mediates susceptibility to ATP-induced cell death

The publication "P2X7 is expressed on human innate-like T lymphocytes and mediates susceptibility to ATP-induced cell death" was published in September 2022 in the European Journal of Immunology (EJI, Impact Factor 6.688 [2021]). The paper was selected for the "In this Issue" section of the November 2022 issue of the EJI.

> *P2X7 is expressed on human innate-like T lymphocytes and mediates susceptibility to ATP-induced cell death*
>
> R. Winzer, A. Serracant-Prat, V. J. Brock, C. Pinto-Espinoza, B. Rissiek, M. Amadi, N. Eich, A. Rissiek, E. Schneider, T. Magnus, A. H. Guse, B.-P. Diercks, F. Koch-Nolte, E. Tolosa
>
> *Eur. J. Immunol.* 52, 11 (2022), doi: https://doi.org/10.1002/eji.202249932

In this work, we analyzed the expression and function of the ATP receptor P2X7 on human T cell subsets using nanobodies for cell surface staining and blockade of P2X7. We found that Tγδ cells and other innate-like T lymphocytes have the highest P2X7 expression within the T cell compartment. P2X7 expression correlates with the susceptibility to ATP, and we could show that Tγδ cells have a higher ATP-induced Ca^{2+} influx compared to CD4 T cells, and are prone to P2X7-mediated cell death.

My part of this project is a continuation of the work started by Dr. Arnau Serracant Prat (Serracant Prat 2018). While Arnau Serracant Prat focused on the expression pattern of P2X7 on immune cell populations and the immediate ATP effects, I analyzed the consequences of ATP stimulation on the function and survival of Tγδ cells. For this publication, I performed T cell assays (T cell isolation, sorting and stimulation), and measured cell death, activation and proliferation by flow cytometry (experiments shown in Figure 5B-C, Figure 6, Supplementary Figure 5 and Supplementary Figure 6 of the paper). I also performed immunofluorescence P2X7 staining of peripheral blood T cells (shown in Supplementary Figure 2E-F). I prepared the cells and was involved in establishing the method to measure the Ca^{2+} influx in primary human T cells (Figure 4, measurement was performed by Valerie Brock, Department of Biochemistry and Molecular Cell Biology, UKE). I was involved in conceptually developing the manuscript,

as well as performing all experiments for the revision process. I prepared all figures and wrote the first version of the manuscript, and was involved in all correction steps. As corresponding author, I communicated with the editors and was responsible for preparing and submitting files during the submission and revision processes.

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Place, date	Eva Tolosa (Supervisor)

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Research Article

P2X7 is expressed on human innate-like T lymphocytes and mediates susceptibility to ATP-induced cell death

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Extracellular ATP activates the P2X7 receptor, leading to inflammasome activation and release of pro-inflammatory cytokines in monocytes. However, a detailed analysis of P2X7 receptor expression and function in the human T cell compartment has not been reported. Here, we used a P2X7-specific nanobody to assess cell membrane expression and function of P2X7 on peripheral T lymphocyte subsets. The results show that innate-like T cells, which effectively react to innate stimuli by secreting high amounts of pro-inflammatory cytokines, have the highest expression of P2X7 in the human T cell compartment. Using Tγδ cells as example for an innate-like lymphocyte population, we demonstrate that these cells are more sensitive to P2X7 receptor activation than conventional T cells, affecting fundamental cellular mechanisms like calcium signaling and ATP-induced cell death. The increased susceptibility of innate-like T cells to P2X7-mediated cell death provides a mechanism to control their homeostasis under inflammatory conditions. Understanding the expression and function of P2X7 on human immune cells is essential to assume the benefits and consequences of newly developed P2X7-based therapeutic approaches.

Keywords: P2X7 · innate-like T cells · ATP · cell death · nanobody

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Purinergic P2X receptors are ligand-gated ion channels that open within milliseconds of adenosine triphosphate (ATP) binding, and are widely expressed in different tissues and in immune cells. Among the seven members of the P2X family, P2X7 is the most studied subtype because of its association with autoimmune and inflammatory diseases [1–4]. The physiological ligand for all P2X receptors is ATP, but the affinity to ATP is highly variable among the different family members. Compared to the other P2X receptors (EC_{50} values of 0.5–10 μM), P2X7 has a low affinity to ATP

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and needs concentrations of >100 μM ATP to be activated [5–7]. Under physiological conditions, the concentration of ATP in the extracellular space is in the nanomolar range, but it increases dramatically upon inflammation and cell stress by the release of ATP through pannexin channels [8, 9]. In mice, but not in humans, nicotinamide adenine dinucleotide (NAD^+) can also activate P2X7 by means of NAD^+-induced ADP-ribosylation of the receptor [10, 11].

In the immune system, P2X7 is expressed on monocytes, macrophages, and dendritic cells [12–14] both in humans and mice. In the murine T cell compartment, regulatory T cells (Tregs), invariant natural killer T (NKT) cells, follicular helper T cells (Tfh), and tissue-resident memory T cells (Trm) have a high P2X7 receptor expression [15–22]. Although early studies and gene expression data indicate that P2X7 is expressed by human T cells [12, 14], the protein expression of this receptor on different T cell subsets has not yet been reported.

One of the best-studied effects of P2X7 receptor activation is the release of the pro-inflammatory cytokines IL-1β and IL-18 by monocytes and dendritic cells [23–25]. In this process, toll-like receptor (TLR)-dependent LPS signaling induces the assembly of the NLRP3 inflammasome. In turn, P2X7 receptor activation by ATP activates the inflammasome, leading to the cleavage of caspase-1 into its active form and subsequent release of IL-1β and IL-18. In T cells, intermediate amounts of extracellular ATP (50–300 μM) induce NFAT and MAPK activation and IL-2 production in a P2X7-dependent manner [9, 26, 27]. In contrast, high concentrations of ATP (millimolar range) induce the formation of large pores in the cell membrane and finally lead to cell death [28].

Conventional CD4 and CD8 T cells recognize antigens presented by the major histocompatibility complex (MHC) through highly specific T cell receptors (TCR). However, between 5% and 20% of peripheral T cells with a restricted TCR chain expression present features of innate immunity, including rapid effector responses and TCR-independent activation [29, 30]. These T cells are known as “unconventional” or “innate-like” T cells, and include invariant NKT cells, mucosal-associated invariant T (MAIT) cells and Tγδ cells. They recognize non-protein antigens, including lipids, phosphoantigens, and small-molecule metabolites, presented by non-MHC-encoded molecules. Most innate-like T cells reside in mucosal tissues, where they are crucial to fight against pathogens. Tγδ cells, especially Vδ2 cells, are commonly found in peripheral blood in humans.

The analysis of P2X7 receptor expression on human T cells would help to further understand the role of P2X7 in the immune response and the effects of ATP signaling. Therefore, our aim was to perform a comprehensive analysis of P2X7 cell surface expression on human T cells and to link its expression to receptor function. Our data reveal that, in the human T cell compartment, P2X7 is expressed at the highest levels on Tγδ and MAIT cells, both innate-like T cells. The elevated expression of P2X7 correlates with a higher sensitivity to ATP and, consequently, with ATP-induced cell death.

Results

Expression of the P2X7 receptor on human lymphocyte subsets

The P2X7 receptor is highly expressed on murine Tregs and monocytes [20]. A high expression of P2X7 on monocytes is also reported in humans [12], however, little is known about the expression on human lymphocytes. RNA sequencing data from the Immunological Genome Project (ImmGen) [19] and the Human Cell Atlas [31] reveal differences in *P2RX7* gene expression in lymphocytes between mice and humans, especially in the T cell compartment (Fig. 1A). Using multicolor flow cytometry, we measured the expression of P2X7 on subsets of peripheral blood mononuclear cells (PBMCs) in healthy donors (Fig. 1B and Supplementary Fig. 1) using nanobody Dano1 [23] (Fig. 1C-D). As expected, we found a high cell surface expression of P2X7 on human monocytes [12]. Due to the expected low expression of P2X7 on lymphocytes, we used a nanobody against murine ARTC2.2 (a molecule with no human orthologue) [32], labeled with the same fluorophore as Dano1 as negative control. In order to quantify the specific P2X7 signal for each cell type, we subtracted the median fluorescence intensity (MFI) of the control nanobody from the MFI of Dano1, and found significant differences between negative control and P2X7 staining in all immune cell populations (Fig. 1C). In the lymphocyte compartment, NK cells have the highest expression of P2X7, whereas the expression on B cells is negligible. T cells show a low, but consistent expression of P2X7 on the cell surface in all donors (Fig. 1D).

P2X7 is marginally expressed on the cell surface of CD4 and CD8 T cells

T lymphocytes encompass conventional CD4 helper and CD8 cytotoxic T lymphocytes, as well as Tregs, the immunomodulatory CD4 T cell subset characterized by the constitutive expression of CD25. Because these cell types harbor very different functions, we wondered whether they differ in P2X7 expression. Therefore, we measured P2X7 on human CD4 and CD8 T cells (Fig. 2A), and found that both CD4 and CD8 T cells showed only low levels of P2X7 on the cell surface (Fig. 2B). We also found low expression of P2X7 on Tregs, in contrast to the high expression reported on murine Tregs [20]. Within CD4 T cells, effector memory cells showed higher expression of P2X7 than central memory and naïve cells. The expression on CD8 subpopulations was not significantly different (Supplementary Fig. 2A-B). Among T helper cell subsets, we found a trend toward a higher P2X7 expression on Th17 cells, but the differences were not significant (Supplementary Fig. 2C-D). Although Tfh cells in human Peyer’s Patches show high ATP sensitivity [21], we did not find increased expression of P2X7 on Tfh cells in peripheral blood (Supplementary Fig. 2E-F). Interestingly, CD3 T cells lacking the expression of CD4 and CD8 (double negative, DN) showed a significantly higher cell surface level of

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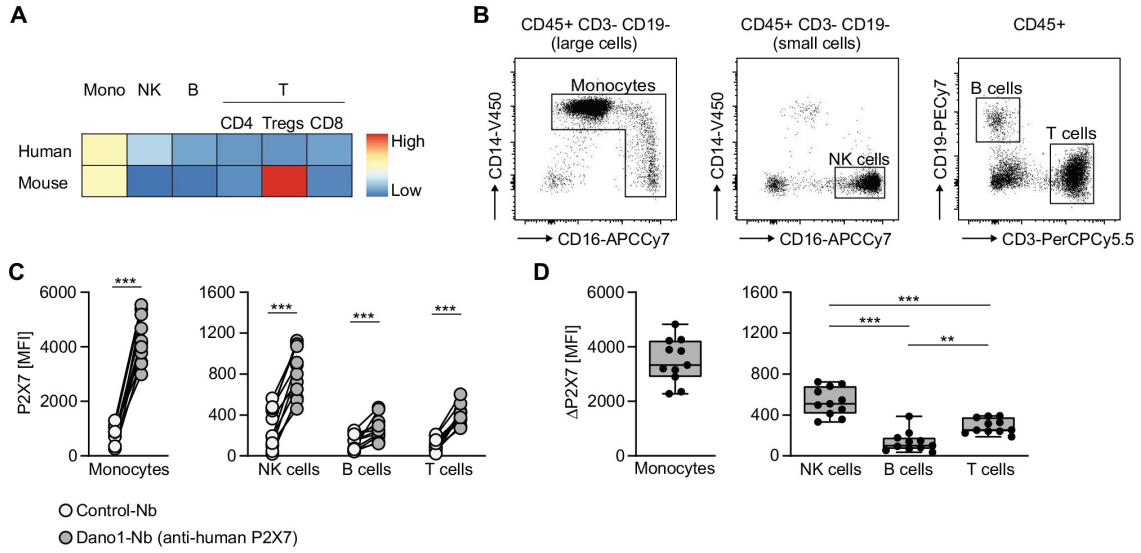

Figure 1. Expression of P2X7 on human peripheral blood mononuclear cells. (A) Gene expression profile of *P2RX7* in selected human and murine immune cell populations (normalized RNA sequencing data obtained from the ImmGen database [19] and the Human Cell Atlas [31]). (B) Representative dot plots for the identification of monocytes and lymphocyte subsets. (C) Median fluorescence intensity (MFI) of P2X7-specific nanobody Dano1 (dark grey) or control nanobody (light grey) staining on different PBMC subsets (n = 11). (D) Cell surface expression of P2X7 on different immune cell subsets, determined by subtracting the MFI of the control nanobody staining from the P2X7-specific staining (ΔP2X7) (n = 11, center line: median, box limits: 25th to 75th percentiles, whiskers: min to max). Paired t test was used to compare the MFI of cells stained with the P2X7-specific and the control nanobody in (C), ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the expression of P2X7 among different immune cell subsets in (D) ($^{**}p < 0.01$, $^{***}p < 0.001$). (B–D) Data were obtained from eleven donors measured in seven independent experiments. Mono, Monocytes; Nb, Nanobody; NK, Natural killer; Treg, Regulatory T cell.

P2X7 than conventional CD4 or CD8 T cells (Fig. 2B). These DN cells contain innate-like T cells, such as Tγδ and MAIT cells.

Innate-like lymphocytes have a higher expression of P2X7 than conventional T cells

We have shown that NK lymphocytes, belonging to the innate immune cell compartment, have a high expression of the P2X7 receptor (Fig. 1D), and we speculated that T cells with functional properties of innate lymphocytes, the innate-like T cells, could also have higher P2X7 cell surface expression than conventional T cells. Both Tγδ and MAIT cells belong to this category, and they are mostly $CD4^{-}CD8^{-}$ or express intermediate levels of CD8 [29, 33]. Using flow cytometry antibody panels designed to identify Tγδ subpopulations and MAIT cells in peripheral blood (Fig. 2C), we found that cell surface expression of P2X7 was significantly higher on MAIT and Tγδ cells than on conventional CD3 T cells, with Tγδ cells almost reaching the levels of P2X7 found on NK cells (Fig. 2D). When comparing different Tγδ subsets, we detected a slightly higher P2X7 expression on Tγδ cells harboring the Vδ2 chain ($V\delta2^{+}$ Tγδ cells) than on $V\delta2^{-}$ cells (containing mainly $V\delta1^{+}$ cells) (Fig. 2E). In line with the protein expression data, quantitative real-time PCR of sorted T cell subsets showed significantly higher *P2RX7* mRNA expression by Tγδ than by conventional T cells (Fig. 2F), as previously shown [14, 34]. Transcriptomic profiling of peripheral lymphocyte populations revealed an 'innateness gradient' with Vδ2 cells being the T cell subset with the most innate properties [30], in contrast to the very little innateness shown by conventional CD4 T cells. Interestingly, gene expression of *P2RX7*, but not of the other P2X receptors expressed on T cells, correlates with the innateness gradient (Supplementary Fig. 3) [30]. In conclusion, innate cells (NK cells) and innate-like lymphocytes (MAIT cells and Tγδ cells) have a higher cell surface expression of P2X7 than conventional T cells, indicating that higher expression of P2X7 is a trait of lymphocytes with innate properties.

Tγδ cells show higher sensitivity to shedding of CD62L and pore formation than conventional T cells

The higher expression levels of P2X7 on innate-like lymphocytes prompted us to investigate whether these cells are more sensitive to ATP stimulation. Gating of P2X7 by extracellular ATP results in activation of ADAM metalloproteinases and subsequent shedding of membrane proteins, such as the cell adhesion molecule L-selectin/CD62L [35–37] (Fig. 3A). Addition of 1.5 mM ATP led to shedding of CD62L on most Tγδ cells, while only half of CD4 and CD8 T cells lost CD62L from the cell surface

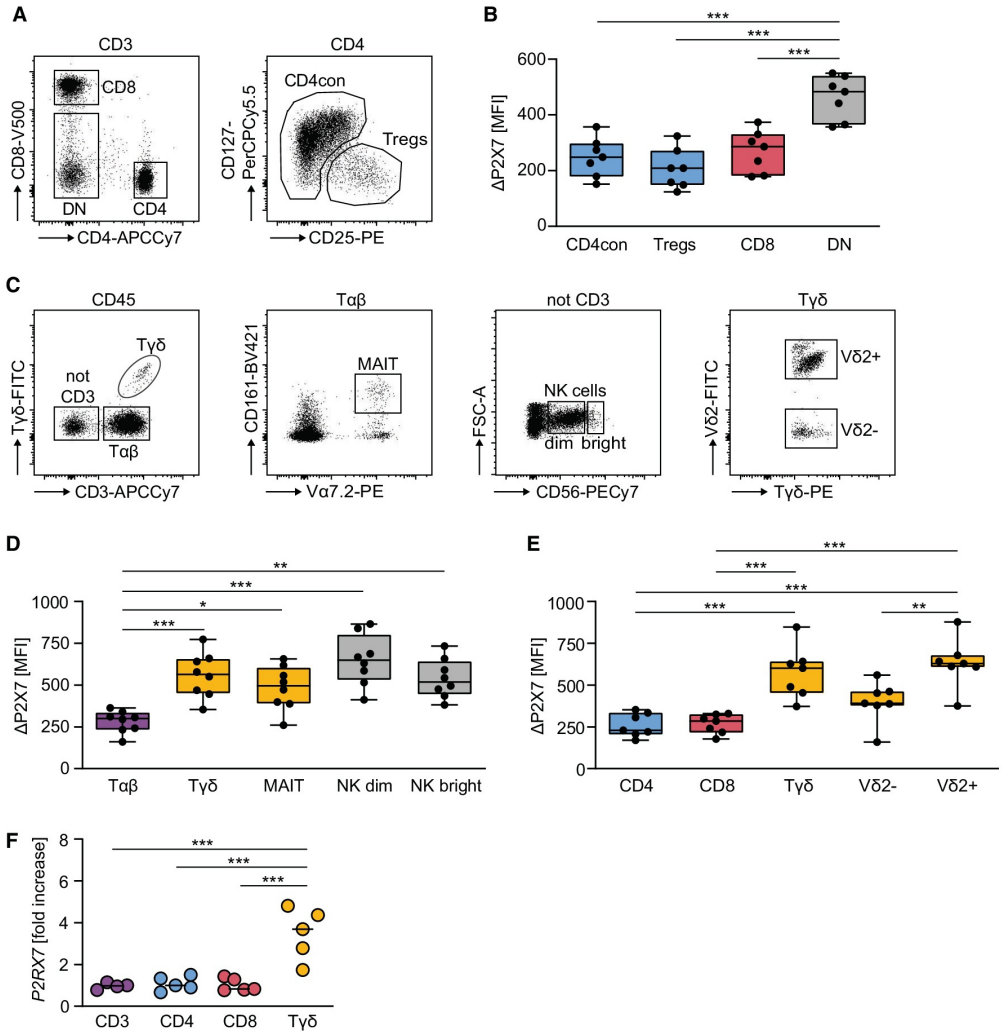

Figure 2. Innate-like T cells have the highest P2X7 expression within the T cell compartment. (A) Representative dot plots for the identification of CD4 and CD8 T cells. (B) P2X7 cell surface expression on different T cell subsets after subtraction of the control (ΔP2X7) (n = 7, center line: median, box limits: 25th to 75th percentiles, whiskers: min to max). (C) Representative gating strategy for the identification of innate cells and innate-like T cell subsets. (D-E) P2X7 expression on NK cells and different innate-like T cell subsets after subtraction of the control (ΔP2X7) (n = 7–8, center line: median, box limits: 25th to 75th percentiles, whiskers: min to max). (F) *P2RX7* gene expression in sorted T cells subsets calculated as $2^{-\Delta\Delta Ct}$, using *RPL13A* as endogenous control and CD3 T cells from one donor as calibrator (n = 4–5, median). Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the expression of P2X7 among multiple immune cell subsets in (B) and (D-F) ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (A-E) Data were obtained from seven or eight donors, measured independently upon blood draw. (F) Gene expression was measured in cell subsets isolated from four or five independent donors. CD4con, Conventional CD4 T cell; DN, Double negative; MAIT, Mucosal-associated invariant T cell; NK, Natural killer; Treg, Regulatory T cell.

(Fig. 3B-C), even though the expression of *ADAM10* is very similar in the different T cell subpopulations [14]. A complete loss of CD62L from conventional T cells could not even be reached with a very high concentration of ATP (4.5 mM). Notably, the CD62L shedding of Tregs was comparable to that of conventional CD4 T cells (data not shown). CD62L shedding was completely abolished by treatment of cells with the P2X7-blocking nanobody Dano1 or by depleting ATP with apyrase, confirming that this process is mediated by P2X7 (Fig. 3D). Furthermore, P2X7 activation triggers the opening of non-selective pores permeable to molecules up to 900 Dalton [38, 39] (Fig. 3A). We compared the capacity of innate-like T cells and conventional CD4 and CD8 T cells to take up DAPI upon treatment of cells with ATP. Stimulation with 1.5 mM and 4.5 mM ATP increased the uptake of DAPI in all T cell subsets; however, the effect was significantly stronger in Tγδ cells and MAIT cells compared to CD4 and CD8 T cells (Fig. 3E

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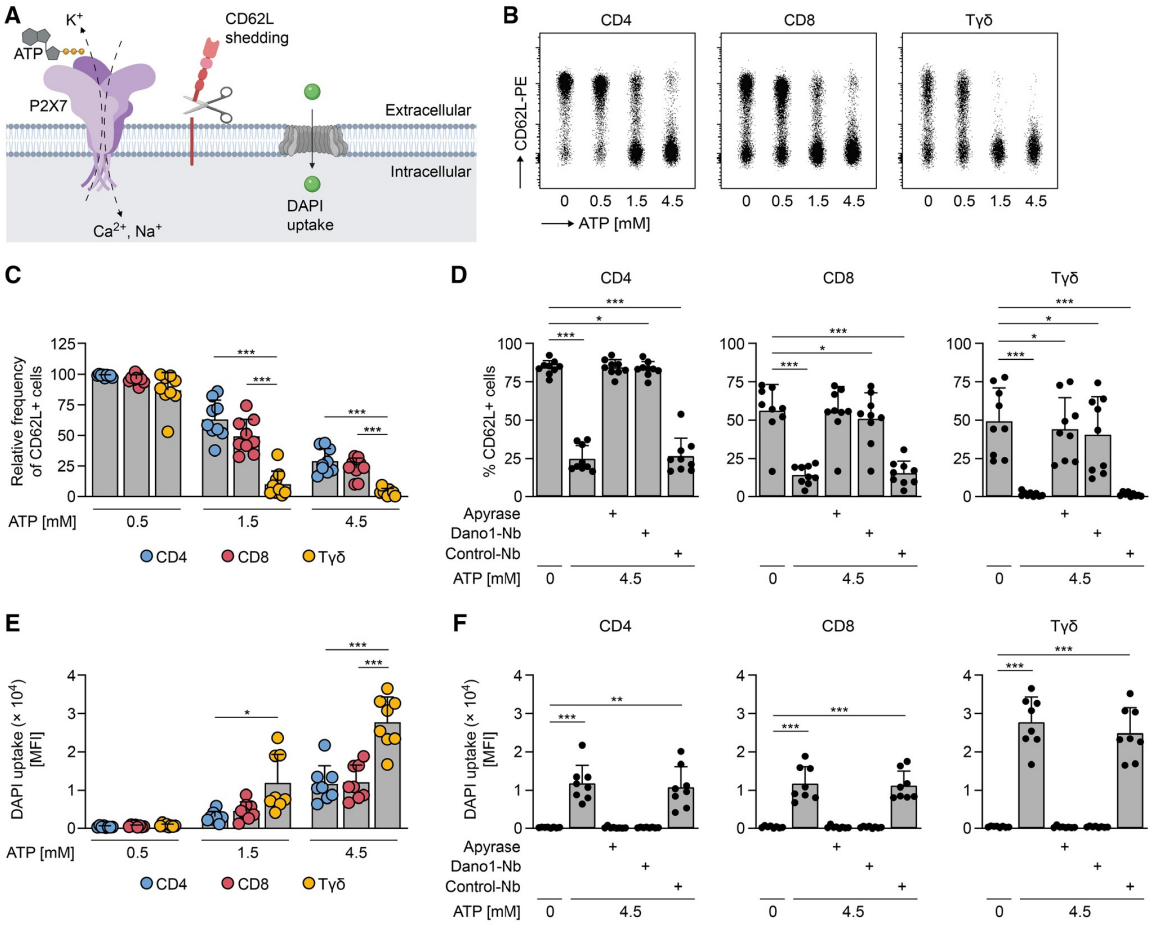

Figure 3. High P2X7 receptor expression on Tγδ cells correlates with a higher sensitivity to ATP-induced shedding of CD62L and pore formation. (A) Schematic representation of the P2X7-induced CD62L shedding and pore formation. (B-F) Human PBMCs were stimulated with different concentrations of ATP for 30 min and the shedding of CD62L (B-D) and uptake of DAPI (E-F) by different T cell subsets were measured by flow cytometry. (B) Representative concatenated dot plots of CD62L expression in CD4 T cells, CD8 T cells and Tγδ cells after treatment with ATP. (C) Relative frequency of $CD62L^+$ cells upon treatment with ATP (relative to the basal frequency of $CD62L^+$ cells without exogenous ATP) (n = 9, mean ± SD). (E) Uptake of DAPI (MFI) upon treatment of cells with ATP (n = 8, mean ± SD). (D, F) Human PBMCs were preincubated with Dano1 (100 nM), or a control nanobody (100 nM), or apyrase (10 U/mL) prior to treatment with ATP (n = 9 in (D) and n = 8 in (F), mean ± SD). Two-way ANOVA with Tukey's multiple comparison test was used to compare all samples treated with one ATP concentration in (C) and (E), RM one-way ANOVA with Dunnett's multiple comparisons test was used to compare all conditions to samples without ATP treatment (first bar) in (D) and (F) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B-F) Data were obtained from eight or nine donors, measured in six (B-D) or five (E-F) independent experiments. Nb, Nanobody.

and Supplementary Fig. 4). Also here, preincubation of the samples with Dano1 or apyrase inhibited the uptake of DAPI (Fig. 3F). These data demonstrate that the higher expression of P2X7 on Tγδ cells results in a higher sensitivity of these cells to ATP compared to conventional T cells.

ATP elicits higher calcium signals in Tγδ cells than in CD4 T cells

The increase of cytosolic calcium ions (Ca^{2+}) is one of the immediate events in T cell activation. It is mediated by different second messengers, which lead to the opening of Ca^{2+} channels in the endoplasmic reticulum, followed by the activation of Ca^{2+} channels in the plasma membrane [40, 41]. After T cell activation, ATP is released from the cell to the extracellular space, and by activating P2X7, it can further increase intracellular Ca^{2+} concentrations [27]. We wondered whether the differences observed in the expression of P2X7 on T cell subsets affect the extent or kinetics of Ca^{2+} influx after ATP treatment. To measure intracellular Ca^{2+} concentrations, we performed global Ca^{2+} imaging with CD4 T cells and Tγδ cells (purity of negatively isolated cell populations is shown in Supplementary Fig. 5). We observed that the intracellular Ca^{2+} concentration after

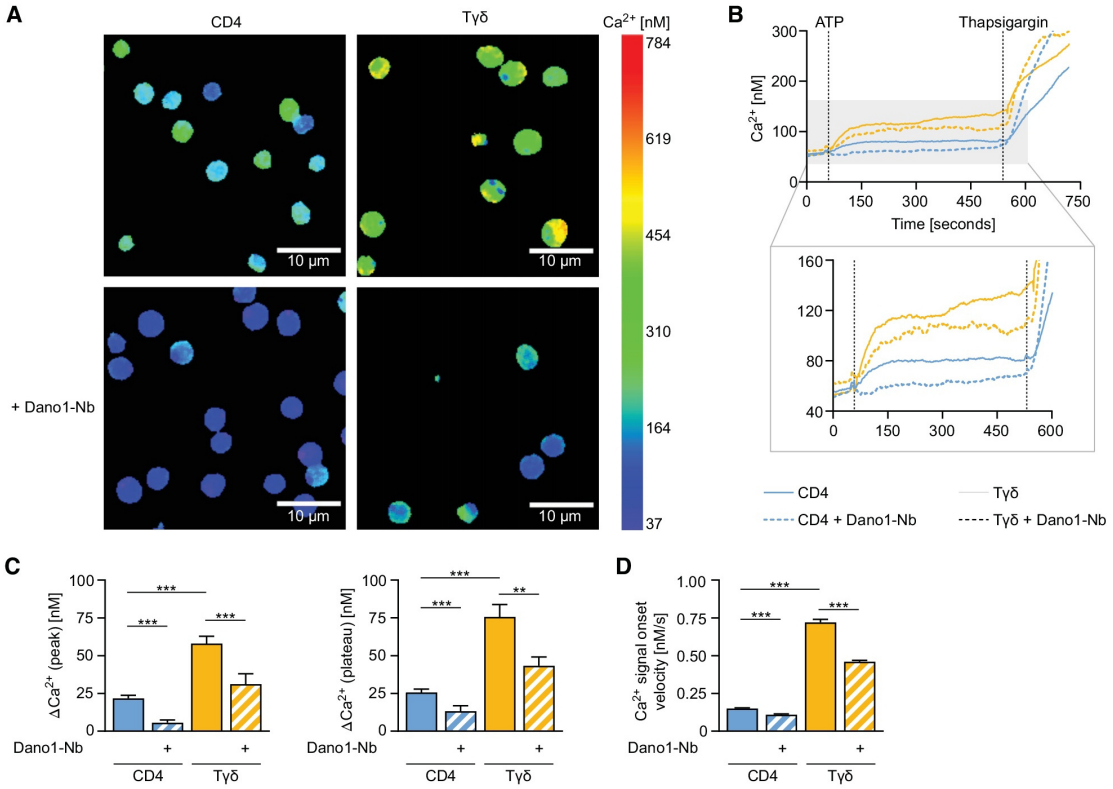

Figure 4. ATP-induced calcium influx is higher in Tγδ cells than in CD4 T cells. (A-D) CD4 T cells and Tγδ cells were treated with 1.5 mM ATP, and Ca^{2+} influx was detected by microscopy using the Ca^{2+} indicator Fura-2. Where indicated, cells were pre-treated with the P2X7-inhibiting nanobody Dano1 (100 nM). (A) Representative microscopy images of CD4 T cells and Tγδ cells 90 s after ATP treatment. The colors of the cells reflect intracellular Ca^{2+} concentrations. (B) Ca^{2+} concentration in CD4 T cells (n = 212 cells, from six donors) and Tγδ cells (n = 273 cells, from six donors) after treatment with ATP. Cells pre-treated with Dano1 prior to stimulation with ATP are shown with dotted lines (n = 188 cells for CD4 T cells and n = 98 cells for Tγδ cells, from four donors). Data are shown as mean. (C) ΔCa^{2+}, calculated by subtracting the basal Ca^{2+} concentration from the Ca^{2+} concentration after ATP treatment, was determined at 150 s (peak) and 450 s (plateau) after the start of the measurement (mean ± SEM). (D) The signal onset velocity was calculated from the mean Ca^{2+} kinetics, and a linear regression from the initial slope was performed. Kruskal-Wallis test with Dunn's multiple comparison test was used to compare samples in (C) (** $p < 0.01$, *** $p < 0.001$). (C-D) summarize data obtained in (B). Data were obtained from four or six donors measured in independent experiments. Nb, Nanobody.

ATP treatment was higher in Tγδ cells than in CD4 T cells, as shown in representative images (Fig. 4A and Supplementary Videos 1 and 2) and as a summary of several measurements (Fig. 4B). By subtracting the basal Ca^{2+} concentration from the Ca^{2+} concentration after ATP treatment, we determined the ΔCa^{2+} for the peak (150 s after start of the measurement) and plateau phase (450 s after start of the measurement). For both time points, the Ca^{2+} response was significantly higher in Tγδ cells compared to CD4 T cells. Blocking of P2X7 by the P2X7-inhibiting nanobody Dano1 decreased the Ca^{2+} influx in both cell types, verifying the role of P2X7 in this process (Fig. 4C). In addition, the signal onset velocity was significantly higher in Tγδ cells than in CD4 T cells, which again could be reduced by Dano1 (Fig. 4D). These data show that a higher cell surface expression of P2X7 on Tγδ cells, compared to CD4 T cells, correlates with increased intracellular Ca^{2+} concentration after ATP stimulation.

Inhibition of the P2X7 receptor does not impair T cell activation and proliferation

Because we saw that P2X7 cell surface levels affect calcium signaling, an early event of T cell activation, we wondered whether cell proliferation after TCR stimulation is affected by P2X7. First, we stimulated sorted CD4 T cells, CD8 T cells, and Tγδ cells and measured changes in *P2RX7* gene expression. *P2RX7* mRNA levels were upregulated in all T cell subsets over time (Fig. 5A). In line with cell surface expression, the basal gene expression of *P2RX7* was higher in Tγδ cells than in CD4 and CD8 T cells, and this differential expression was maintained over time. Interestingly, we observed a common pattern in the upregulation of *P2RX7*, characterized by a peak one day after activation, followed by a decrease on day three and a further increase thereafter. To determine the effect of P2X7 blockade during activation, we stimulated PBMCs with the combination of αCD3 and the metabolite

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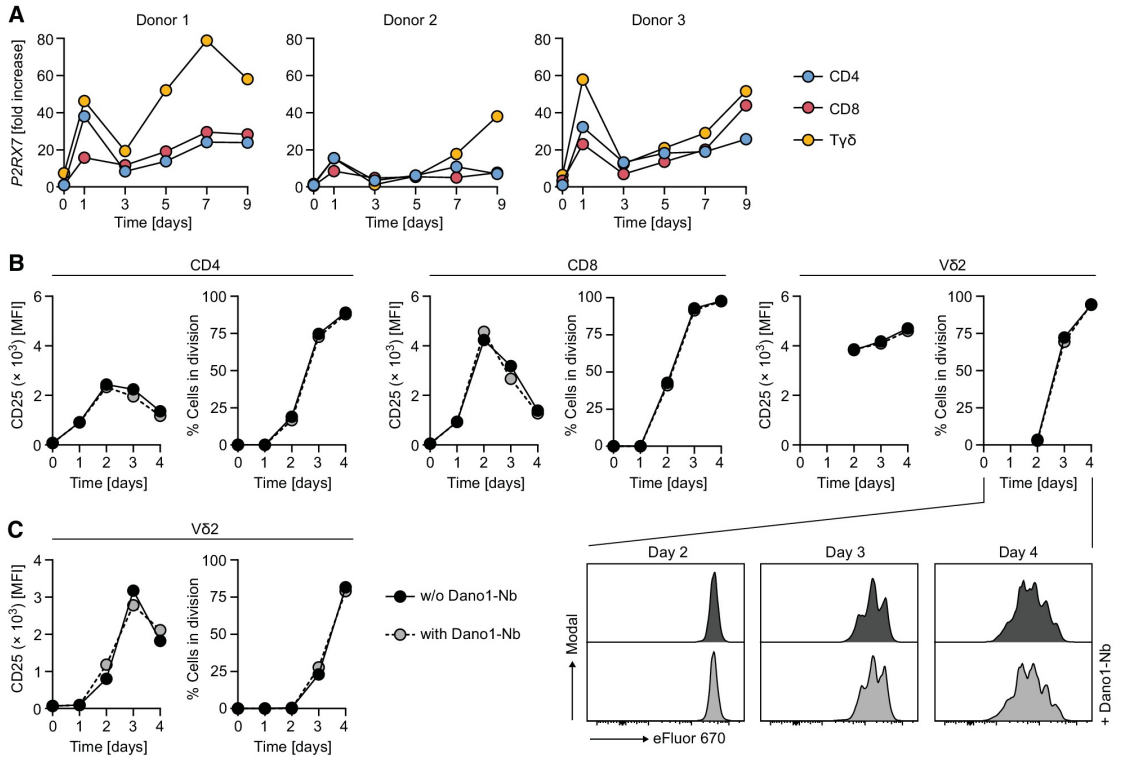

Figure 5. Blockade of P2X7 does not affect T cell receptor-mediated activation and proliferation. (A) Sorted CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$ T cells and CD3$^+$TCRγδ$^+$ T cells were stimulated with αCD3/αCD28 and expression of the *P2RX7* gene was determined by qPCR at different time points using CD4 T cells as calibrator (n = 3). (B-C) PBMCs were stimulated with (B) αCD3/HDMAPP or (C) HDMAPP in the absence or presence of Dano1 (added every day to the cell culture). CD25 expression and proliferation (dilution of the cell staining dye eFluor 670) were measured by flow cytometry. Data are shown for three donors measured in three independent experiments (A) or for one representative donor out of three independent experiments (B-C). Nb, Nanobody.

HDMAPP (to specifically stimulate Tγδ cells) [42] in the presence or absence of the P2X7-blocking nanobody Dano1, and followed activation and proliferation over four days. Daily treatment of cells with the P2X7-blocking nanobody Dano1 had no effect on CD25 expression or eFluor 670 dye dilution (Fig. 5B). Similarly, HDMAPP-induced activation and proliferation of Vδ2 cells (Fig. 5C), and the response of memory cells to TCR stimulation (Supplementary Fig. 6) remained unchanged by Dano1 treatment.

Tγδ cells have a high susceptibility to P2X7-mediated cell death

Activation of P2X7 induces cell death in T cells with high P2X7 cell surface levels, including murine Tregs and NKT cells [11, 20, 43]. For Trm, another population with high P2X7 expression, it was suggested that P2X7-mediated cell death is crucial to control this immune cell population to prevent immune pathology [22, 44]. We hypothesized that cell surface levels of P2X7 on human T cell subsets correlates with their susceptibility to ATP-induced cell death. Therefore, we treated human T cells for 18 hours with ATP and measured the viability of Tγδ cells and conventional CD4 and CD8 T cells. After treatment with ATP, only 25% of Tγδ and Vδ2 cells were alive (defined as live/dead$^-$ and Annexin V$^-$), compared to 75% in the untreated cells. In CD4 and CD8 T cells, 70% of the cells were alive after ATP treatment (compared to 90% in the untreated cells) (Fig. 6A), demonstrating that Tγδ cells are more susceptible to ATP-mediated death than conventional T cells (Fig. 6B). This ATP-induced decrease in viability was clearly dependent on P2X7, because it could be prevented by the P2X7-inhibiting nanobody Dano1. Tγδ cells, as an innate-like T cell population, do not only rely on TCR stimulation, but can be activated also by cytokines. Indeed, stimulation with IL-12/IL-18 induced activation and IFNγ production by Vδ2 cells, but not by conventional CD4 and CD8 T cells (Fig. 6C). The antigen-independent (innate) activation of Vδ2 cells leads to high production of pro-inflammatory cytokines, contributing to pathologic immune responses if not controlled. Therefore, we hypothesized that Tγδ cells are regulated at sites of inflammation by way of ATP-induced, P2X7-mediated cell death. Indeed, also in the presence of

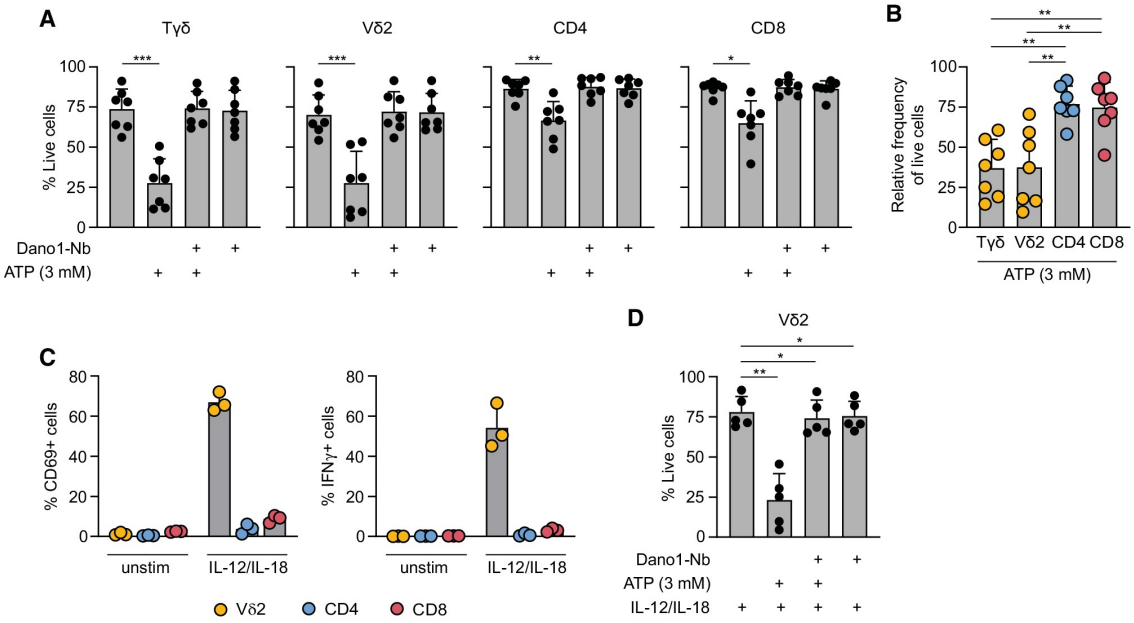

Figure 6. Tγδ cells are susceptible to P2X7-mediated cell death. (A-B) CD3 T cells were treated with ATP (3 mM) for 18 hours in the absence or presence of the P2X7-inhibiting nanobody Dano1. Live cells were defined as live/dead and Annexin V negative and are shown (A) as frequency for each cell type analyzed or (B) as relative frequency (relative to the basal frequency of live cells without exogenous ATP for each cell type) (n = 7, mean ± SD). (C) CD3 T cells were stimulated with IL-12 and IL-18 for 18 hours. CD69 and IFNγ expression were measured by flow cytometry (n = 3, mean ± SD). (D) CD3 T cells were stimulated with IL-12 and IL-18, and treated with ATP (3 mM) for 18 hours in the absence or presence of the P2X7-inhibiting nanobody Dano1. Live Vδ2 cells were defined as live/dead and Annexin V negative (n = 5, mean ± SD). RM one-way ANOVA with Dunnett's multiple comparisons test was used to compare all conditions to samples without ATP treatment (first bar) in (A) and (D), ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the different cell types in (B) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data were obtained from five or seven donors, measured in four (A-B) or three (D) independent experiments, or from three donors measured in one experiment (C). Nb, Nanobody.

IL-12/IL-18, the frequency of live Vδ2 cells was significantly reduced after ATP treatment in a P2X7-dependent manner (Fig. 6D). From these data, we conclude that Tγδ cells are more susceptible to P2X7-mediated cell death than conventional CD4 and CD8 T cells. Under inflammatory conditions, this could dampen tissue damage mediated by cytokines released from Tγδ cells.

Discussion

Extracellular ATP activates the human P2X7 receptor, leading to a variety of cell-specific outcomes including the release of pro-inflammatory cytokines, T cell activation, and cell death. The expression and function of P2X7 has been well studied on human monocytes; however, a comprehensive analysis of its expression in the human T cell compartment has not yet been done. Here, we used a P2X7-specific nanobody to measure the cell surface expression of P2X7 on a broad variety of immune cells by multicolor flow cytometry, and we could show that innate-like lymphocytes have the highest expression of P2X7 in the human T cell compartment. Using Tγδ cells as an example of an innate-like T lymphocyte population, we demonstrated that P2X7 expression and related function affect fundamental cellular mechanisms like calcium signaling and cell death.

The first generated monoclonal antibody against P2X7 was used to stain human P2X7 on macrophages [45], and it was further used to describe P2X7 receptor expression on different immune cell subsets. Beyond a high protein expression on monocytes, a low expression was also described on lymphocytes [12], but no further characterization of P2X7 protein expression on the cell surface of T cell subsets was reported. Using the recently described nanobody Dano1 [23], we found higher cell surface level of P2X7 on innate and innate-like T cells than on conventional CD4 and CD8 T cells. Human Tregs, in contrast to their murine counterparts, express relatively low levels of P2X7. Importantly, the observed differences in P2X7 cell surface levels between conventional and innate-like T cells correlate with ATP sensitivity, as demonstrated by increased shedding of CD62L and DAPI incorporation, Ca^{2+} influx, and ATP-induced cell death in the innate-like T cells.

A common characteristic of all human immune cell types with high P2X7 expression is that they react to non-antigen-specific, innate stimuli. Innate-like T cells share properties from both the innate and the adaptive immunity and include Tγδ cells, MAIT cells and NKT cells. Their rapid activation upon TCR-independent

stimulation, e.g. cytokine stimulation, may require further control mechanisms to prevent tissue damage, and we hypothesize that P2X7 signaling is one of these. *P2RX7* ranges among the top 5% of genes in the innateness score developed by Gutierrez-Arcelus et al., underscoring a possible common role for P2X7 in all innate lymphocytes that are at risk of bystander activation. Interestingly, adaptive effector memory cells score higher than naïve and central memory cells in the innateness ranking [30]. These cells promptly unleash their effector functions in response to TCR activation and they express higher levels of P2X7 than naïve cells. This suggests that similar control programs, including those involving P2X7, are shared in innate-like and adaptive effector memory T cells. For our functional analyses, we focused on Tγδ cells as the model for innate-like T cell population because they constitute a sizeable population (2–8%) of peripheral blood lymphocytes in all donors, while the frequency of MAIT cells is lower (0.5–4%). In all assays in which we tested downstream effects of P2X7 activation, we observed a higher sensitivity of Tγδ cells to extracellular ATP compared to P2X7 low-expressing CD4 T cells. Even Ca^{2+} influx, which is not exclusively associated to P2X7, was higher in Tγδ cells, and was partially blocked by the P2X7-inhibiting nanobody Dano1. Similar to Tγδ cells, MAIT cells also showed higher sensitivity to ATP-induced pore formation compared to conventional T cells.

Murine cells do not only depend on ATP for gating of P2X7, but are also sensitive to NAD^+-induced, ARTC2.2-catalyzed ADP-ribosylation of P2X7 [46, 47]. This covalent modification allows P2X7 activation at low concentrations of extracellular NAD^+ (3–30 μM) [10, 11]. NAD^+-induced cell death by ADP-ribosylation of the P2X7 receptor is described for several murine cell populations with high expression of P2X7 and ARTC2.2, namely Tregs, NKT, Tfh, and Trm cells [20–22, 43, 44, 48]. The P2X7-mediated depletion of murine Trm cells limits Trm-associated immune pathology and destructive immune responses [22, 44], and we hypothesize that a similar mechanism occurs in the human immune system. Tγδ and MAIT cells respond to IL-12/IL-15/IL-18 cytokine stimulation with IFNγ production [49, 50]. A high effector potential of Tγδ cells upon cytokine or TLR stimulation, or recognition of phosphoantigen metabolites, may be a crucial immediate reaction to microbial pathogens before the adaptive immune system is activated [29, 51], however, a prolonged release of IFNγ at sites of inflammation may lead to tissue damage and immune pathology. Therefore, we propose that high ATP concentrations, as they can be present pericellularly at sites of inflammation, may act as an emergency danger signal, leading to the selective death of Tγδ cells. Indeed, compared to conventional T cells, the frequency of live Tγδ cells after ATP treatment *in vitro* was strongly reduced. The mechanism behind ATP-induced cell death in human T cells is not fully understood. In monocytes, caspase-1 mediates cleavage of gasdermin D. The N-terminal domain of gasdermin D multimerizes and forms large channels in the plasma membrane, facilitating the efflux of IL-1β and IL-18, but also leading to cell death if pore formation is not reversed, e.g. by shedding of pores [52–54]. Inflammasome activation followed by gasdermin cleavage and subsequent pore formation has been recently reported in T cells [55, 56], however, whether this can be triggered by ATP-mediated activation of P2X7, or if this could apply to Tγδ cells has not been shown.

A tight regulation of P2X7 activation is necessary to prevent uncontrolled cell death. ATP-induced cell death depends on the expression of P2X7, the concentration and availability of ATP, and potential factors modulating the conformation of P2X7. In this paper, we thoroughly analyzed the expression of P2X7 on different T cell subsets and showed that high P2X7 expression on the cell surface is associated with higher susceptibility to ATP-induced calcium influx and cell death. Importantly, ATP concentrations inducing calcium influx were not able to induce cell death, suggesting a concentration-dependent P2X7 receptor activation with different functional outcomes. Consistently, a broad array of P2X7-dependent effects on T cells are reported, including differentiation of DN cells in the thymus, IL-2 release and T cell activation, Th17 polarization, generation and maintenance of T cell memory, and cell death [16, 17, 22, 27, 57–59]. In our experiments, we did not observe any effects of P2X7 blockade on T cell activation and proliferation, probably because of low ATP concentrations in the *in vitro* experiments, far from the suspected high pericellular concentrations at inflamed sites. This indicates that the effects of P2X7 activation are highly dependent on the cell types analyzed, the concentration of ATP (or NAD^+ in mice), and the metabolic stage of the cells.

Millimolar concentrations of extracellular ATP are needed to activate human P2X7, but under which circumstances these concentrations are present *in vivo* is an intensely debated topic. The low sensitivity of P2X7 to ATP and the low extracellular concentrations of ATP under basal conditions require a vast increase of extracellular ATP to activate the receptor. A common view is that millimolar ATP concentrations can be transiently reached at the pericellular space under inflammatory conditions, however, to measure pericellular ATP in a physiological setting in humans is still challenging. The availability of high concentrations of extracellular ATP for P2X7 activation is not only dependent on ATP release from the cells upon cell stress or inflammation, but also on its extracellular degradation. The ectonucleotidases CD39 (ectonucleoside triphosphate diphosphohydrolase-1, ENTPD1) and CD203a (ectonucleotide pyrophosphatase/phosphodiesterase 1, ENPP1) hydrolyze ATP to AMP, which can then be further degraded by CD73 (ecto-5'-nucleotidase, NT5E) to the anti-inflammatory mediator adenosine [60]. In the human T cell compartment, CD39 is mainly expressed on Tregs [61]. Peripheral blood Tγδ cells do not express CD39 [62], a further indication that P2X7-mediated cell death in these cells is plausible because they are not able to quickly degrade pericellular ATP. While ATP in the periphery is an immediate danger signal, commensal bacteria in the intestine constitutively release ATP [63, 64], making the intestine an ATP-rich environment. Interestingly, Tγδ cells in the intestine express CD39 [62]. High ATP concentrations in the intestine increase the severity of T cell-mediated colitis [63], while removal of ATP suppresses intestinal inflammation and could potentially be used for therapy of inflammatory bowel disease [65]. It is plausible that P2X7

activation is fine-tuned by the tissue environment, and that high CD39 expression on Tγδ cells in the ATP-rich environment of the intestine mediates ATP degradation, thereby preventing ATP-induced, P2X7-mediated cell death and intestinal inflammation. In addition, the affinity of P2X7 for ATP might be modulated by co-factors, as reported for the natural peptides polymyxin B and LL-37 [66, 67], small chemicals like ivermectin and compound K [5, 68], and the mouse P2X7-specific nanobody 14D5 [23]. Furthermore, other P2 receptors may influence the activation of P2X7: a negative regulation of P2X7-mediated cell death has been reported for P2Y11 [69], an ATP-gated G protein-coupled receptor that exists in humans, but not in mice, underscoring again major differences in the sensing of ATP in the two species.

We conclude that innate-like T lymphocytes have the highest expression of P2X7 in the human T cell compartment and that P2X7 renders these cells susceptible to ATP-mediated effects. This includes the influx of Ca^{2+} as an early step of T cell activation, but also the induction of cell death at high ATP concentrations. The induction of cell death could be an 'emergency brake' to deplete Tγδ cells under inflammatory conditions because their high effector potential promotes tissue damage. Analysis of the expression and function of P2X7 on human immune cells is essential to estimate the benefits and consequences of newly developed P2X7-based therapeutic approaches. Our comprehensive analysis of P2X7 expression on human T cells using a highly specific P2X7 nanobody with blocking capacity constitutes the basis for a better understanding of the P2X7 receptor and its complex functions.

Material and methods

Human samples and ethics approval

Peripheral blood was freshly drawn from healthy volunteers in EDTA or heparin collection tubes at the University Medical Center Hamburg-Eppendorf (UKE). Buffy coats were obtained from the blood bank of the UKE. All donors were of legal age, and informed consent was obtained from all volunteers. Blood samples were handled according to corresponding ethics protocol (Ethics Committee of the Hamburg Chamber of Physicians, PV5139).

Isolation of peripheral blood mononuclear cells (PBMCs) and T cell subsets

PBMCs from peripheral blood or buffy coats were isolated by Biocoll (Merck) density gradient centrifugation. Blood was diluted with PBS (Thermo Fisher Scientific) and carefully layered on Biocoll. After centrifugation (25 min, 800 × g, RT, without brake), the lymphocyte layer was collected and washed twice with cold PBS (650 × g, 10 min, 4°C, and 450 × g, 5 min, 4°C). T cells, CD4 T cells, and Tγδ cells were enriched from PBMCs by negative selection using the EasySep™ Human T Cell Enrichment Kit, CD4+ T Cell Enrichment Kit, and Gamma/Delta T Cell Isolation Kit (Stemcell), respectively, according to the manufacturer's protocol. The purity of the isolated cells was assessed by flow cytometry.

Flow cytometry

Cells (0.2 – 1 × 10^6 cells) or 50 μL blood were used for staining of cell surface antigens. To block unspecific binding of the antibodies, cells were pre-incubated with human immunoglobulins (5 min, RT). Cells were subsequently stained with fluorochrome-labeled antibodies or nanobodies against cell surface markers of interest for 30 min at 4°C. Mouse ARTC2.2-specific nanobody s-14 [70] was included as negative control to ensure the specificity of the P2X7 signal. ΔP2X7 was calculated by subtracting the median fluorescence intensity (MFI) of the control nanobody staining from the P2X7-specific staining. For intracellular cytokine staining, cells were fixed and permeabilized (both buffers from Thermo Fisher Scientific) after staining of surface markers, and subsequently stained with antibodies against intracellular targets. A table with all used antibodies is provided in the (Supplementary Table 1). Dead cells were excluded using amine-reacting fluorescent live/dead dyes (Pacific Orange or Alexa Fluor 750 succinimidyl ester, 1 μg/mL, Thermo Fisher Scientific). Apoptotic cells were stained with Annexin V (2 μL/staining, BioLegend). When the staining was performed directly on blood, erythrocytes were lysed after staining by addition of 1 mL lysis buffer (BioLegend) for 10 min at RT. Samples were measured at a FACSCanto II or FACSCelesta (BD Biosciences) and data were analyzed with FlowJo (BD). Fluorescence-activated cell sorting (FACS) to isolate T cell subpopulations was performed on a FACSAria IIIU or FACSAria Fusion (BD Biosciences).

Real-time polymerase chain reaction (real-time PCR)

RNA extraction from sorted T cells was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was used as template for the synthesis of cDNA in a reverse transcription PCR. In the first step, 10 μL RNA was incubated with 1 μL random hexamers (100 ng/μL) and 1 μL dNTPs (10 mM) for 5 min at 65°C. Then, 4 μL 5X first strand buffer, 2 μL DTT (0.1 M), and 1 μL MLV-RT (200 U/μL), and 1 μL H_2O were added for 50 min at 37°C and 15 min at 70°C. Real-time quantitative PCR (qPCR) to measure gene expression of the P2X7 receptor (*P2RX7*) on sorted T cells was performed using SYBR Green assay. cDNA (5 μL) was mixed with 10 μL 2X Maxima SYBR Green/ROX qPCR Master Mix, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM) and filled up with nuclease-free water to a final volume of 20 μL. The samples were measured on a StepOnePlus Real-Time PCR System (Applied Biosystems) with denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 40 s. Melting curve analyses were performed to verify the amplification specificity. The relative gene expression was calculated with the $\Delta\Delta C_T$ method using ribosomal protein L13a

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(*RPL13A*) as endogenous control, and samples were calibrated to CD3 or CD4 T cells.

Measurement of P2X7-mediated effects (uptake of DAPI and shedding of CD62L)

PBMCs (1×10^6 cells) were treated with 0.5, 1.5, or 4.5 mM ATP (Sigma-Aldrich) for 30 min at 37°C to activate P2X7. To specifically assess the role of P2X7, cells were preincubated with 100 nM P2X7-blocking nanobody Dano1 or a control nanobody against mouse ARTC2.2 (30 min, 4°C) or with 10 U/mL apyrase (20 min, 5% CO_2, 37°C, Sigma-Aldrich) before addition of ATP. Readouts for P2X7 activation were the uptake of 4′,6-diamidino-2-phenylindole (DAPI) to assess P2X7-dependent pore formation and the shedding of CD62L from the cell surface. For the uptake of DAPI, PBMCs were incubated with 1.43 μM DAPI (Merck) during treatment with ATP. DAPI uptake was measured by flow cytometry in the BV421 channel of the violet (405 nm) laser. CD62L expression was assessed by flow cytometry using an anti-CD62L PE-labeled antibody.

Calcium imaging

Negatively selected CD4 T cells and Tγδ cells ($1 – 5 \times 10^6$ cells) were incubated with 4 μM Fura-2 acetoxymethyl (AM) ester (Life Technologies) for 35 min at 37°C. Cells were washed and resuspended in Ca^{2+} buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 20 mM HEPES, 5.5 mM glucose, pH 7.4), and 0.125×10^6 cells were transferred to glass coverslips coated with BSA (5 mg/mL) and poly-L-lysine (0.1 mg/mL). Ratiometric Ca^{2+} imaging was performed for 12 min with an acquisition rate of one frame per 2 s. The measurements were performed at 37°C. After 60 s, ATP (final concentration of 1.5 mM) was added to the cell suspension. After 9 min, thapsigargin (final concentration of 1.67 μM) was added to release intracellularly stored Ca^{2+}. To determine the role of P2X7 in Ca^{2+} influx, cells were preincubated for 10 min with the P2X7-inhibiting nanobody Dano1 (100 nM) before treatment with ATP. Imaging was performed on a Leica IRBE microscope (40-fold magnification) with a Sutter DG-4 as light source and an electron multiplying charge-coupled device (CCD) camera (C9100-13, Hamamatsu). Acquisition of images (512×512 pixels) was done in 16-bit mode with Volocity software (PerkinElmer). Exposure time was set to 25 ms for 340 nm and 380 nm. The following filters were used (excitation (ex), beamsplitter (bs), and emission (em), all in nanometers): ex, HC 340/26, HC 387/11; bs, 400DCLP; em, 510/84. Post-processing of the data, including background correction, splitting of the fluorescence channels and selection of the regions of interest (ROI), was performed with Fiji software.

In vitro stimulation of human T cells

PBMCs were stimulated with soluble αCD3 (0.5 μg/mL, clone OKT3, BioLegend) and/or 1-Hydroxy-2-methyl-2-butenyl 4-pyrophosphate (HDMAPP, 10 nM) in X-VIVO 15 medium (Lonza) for up to four days (5% CO_2, 37°C) at a cell density of 2×10^5 cells/well. To assess the influence of P2X7 blockade on T cell activation, 100 nM Dano1 was added daily to the cell culture. T cell activation and proliferation was monitored by flow cytometry measuring cell membrane expression of the activation marker CD25 and dilution of eFluor 670, respectively. FACS-isolated CD4 and CD8 (memory and naïve) T cells, and Tγδ cells were stimulated with plate-bound αCD3 (1 μg/mL, clone OKT3, BioLegend) and soluble αCD28 (5 μg/mL, clone CD28.2, BioLegend) in RPMI 1640 containing 10% FBS (Biochrom), 1% penicillin-streptomycin, and 1% L-glutamine (Thermo Fisher Scientific) for four or nine days (5% CO_2, 37°C) at a cell density of 1×10^5 cells/well. In some experiments, T cells were stimulated with IL-12 (10 ng/mL, Peprotech) and IL-18 (100 ng/mL, MBL) for 18 hours, with Brefeldin A (Thermo Fisher Scientific) added for the last four hours. Activation and cytokine production were monitored by flow cytometry, measuring CD69 and IFNγ expression, respectively.

Statistical analysis

Data representation and statistical analysis were performed using Prism 9 (GraphPad). For comparisons between two groups, two-tailed Student's t test was used. For comparisons among multiple groups, repeated measures (RM) one-way ANOVA was used for paired data and ordinary one-way ANOVA (for normally distributed data) or Kruskal-Wallis test (for non-parametric data) were used for unpaired data. Two-way ANOVA was performed when two variables were analyzed. For multiple comparisons, post hoc tests were performed as stated in the figure legends. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conflict of interest: FKN is coinventor of a patent on P2X7-specific nanobodies (WO2013/178783). The other authors declare that they have no conflict of interest.

Author contributions: Idea and design of research project: ET, FKN, RW, ASP. Writing manuscript: RW, ET. Establishment of methods: ASP, RW, VJB, CPE, BR, AR, ES, BPD. Experimental work: ASP, RW, VJB, CPE, MA, NE. Data analysis and interpretation: RW, ASP, VJB, ET. Scientific input and manuscript revision: all authors.

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Data availability statement: All data generated or analyzed during this study are included in this published article and its supplementary information files or are available from the corresponding author on request. This study includes no data deposited in external repositories. Data from publicly available sources shown in this paper can be obtained from https://immunogenomics.io/itc or Gene Expression Omnibus (GEO) (transcriptomic characterization of human innate T cells, accession number GSE124731), ImmGen database (gene expression of murine *P2rx7*, immgen.org), and the Human Cell Atlas (gene expression of human *P2RX7*, immunecellatlas.net).

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Abbreviations: **ATP:** adenosine triphosphate · **DAPI**: 4′,6-diamidino-2-phenylindole · **DN:** double negative · **FACS:** fluorescence-activated cell sorting · **HDMAPP:** hydroxy-2-methyl-2-butenyl 4-pyrophosphate · **MAIT:** mucosal-associated invariant T cells · **MFI:** median fluorescence intensity · **NAD^+:** nicotinamide adenine dinucleotide · **NKT:** natural killer T cells · **PBMCs:** peripheral blood mononuclear cells · **TCR:** T cell receptor · **Tfh:** T follicular helper cells · **TLR:** Toll-like receptor · **Tregs:** regulatory T cells · **Trm:** tissue-resident memory cells

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3.2 CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression

The publication "CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression" was published in October 2021 in Nature Communications (Impact Factor 17.694 [2021]).

> *CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression*
>
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In this publication, we describe that the ectonucleotidase CD73 is released from the plasma membrane of effector T cells on EVs. CD73 on EVs remains enzymatically active and conducts, especially in cooperation with regulatory T cells, anti-inflammatory effects. This publication is a shared first authorship together with Dr. Enja Schneider.

For this publication, I established a sensitive method to measure adenine nucleotides (with the help of Andreas Bauche and Dr. Ralf Fliegert, Department of Biochemistry and Molecular Cell Biology, UKE). This method is based on the incubation of samples (e.g. cells, EVs, or serum) with fluorescent adenine nucleotide derivatives. Separation and quantification by high-performance liquid chromatography (HPLC) allowed us to follow the stepwise degradation of these nucleotides, from which we can draw conclusions about the activity of enzymes involved (brief schematic overview of the method in Fig. 3b of the publication). This method was fundamental for the project, as it allowed us to assess the enzymatic activity of CD73 on EVs, which in turn was the basis for functional T cell assays. In addition, I performed various assays with primary human T cells for the publication and analyzed them by flow cytometry and ELISA. I am responsible for the performance, analysis and presentation of the experiments shown in Fig. 1b-c, Fig. 3c-d, Fig. 4f, Fig. 6a, Fig. 7d and Supplementary Fig. 1c. Additionally, I performed and analyzed the experiments in Response letter Fig. 1, Response letter Fig. 3 and Response letter Fig. 5 (first resubmission), and Response letter Fig. 1 (second resubmission).

Together with Enja Schneider, I was involved in performing the experiments shown in Fig. 1e-f, Fig. 4a-b, Fig. 6b, Supplementary Fig. 7, Supplementary Fig. 8a, and Supplementary Fig. 9e as well as in preparing samples for experiments performed by co-authors. I was in involved in conceptually developing the manuscript. I wrote the first version of the results section including figure legends, the methods section (except for "Characterization of EVs", "Fluorescence microscopy" and "Statistical analysis"), and parts of the discussion, and I was involved in the revision of all parts of the paper. As corresponding author, I communicated with the editors and was responsible for preparing and submitting files during the submission process.

This publication was awarded as UKE Paper of the Month October 2021.

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Place, date	Eva Tolosa (Supervisor)

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CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression

Enja Schneider[1,15], Riekje Winzer[1,15✉], Anne Rissiek[1], Isabell Ricklefs[2,3], Catherine Meyer-Schwesinger[4], Franz L. Ricklefs[5], Andreas Bauche[6], Jochen Behrends[7], Rudolph Reimer[8], Santra Brenna[9], Hauke Wasielewski[1], Melchior Lauten[10], Björn Rissiek[9], Berta Puig[9], Filippo Cortesi[11,12], Tim Magnus[9], Ralf Fliegert[6], Christa E. Müller[13], Nicola Gagliani[11,12,14] & Eva Tolosa[1✉]

Immune cells at sites of inflammation are continuously activated by local antigens and cytokines, and regulatory mechanisms must be enacted to control inflammation. The stepwise hydrolysis of extracellular ATP by ectonucleotidases CD39 and CD73 generates adenosine, a potent immune suppressor. Here we report that human effector CD8 T cells contribute to adenosine production by releasing CD73-containing extracellular vesicles upon activation. These extracellular vesicles have AMPase activity, and the resulting adenosine mediates immune suppression independently of regulatory T cells. In addition, we show that extracellular vesicles isolated from the synovial fluid of patients with juvenile idiopathic arthritis contribute to T cell suppression in a CD73-dependent manner. Our results suggest that the generation of adenosine upon T cell activation is an intrinsic mechanism of human effector T cells that complements regulatory T cell-mediated suppression in the inflamed tissue. Finally, our data underscore the role of immune cell-derived extracellular vesicles in the control of immune responses.

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Immune cell activation, cellular stress, or metabolic changes during inflammation favor the release of ATP into the extracellular space. High extracellular ATP is a danger signal for immune cells and is swiftly metabolized by ATP-degrading enzymes. Among them, the ectonucleotidase CD39 dephosphorylates ATP and ADP to AMP, which is subsequently converted to adenosine by Ecto-5′-nucleotidase (CD73)[1]. The amount of available extracellular adenosine is further determined by the rate of adenosine deaminase (ADA)-mediated degradation, and by cellular uptake through nucleoside transporters. Activation of the adenosine receptor subtype A_{2A}, the predominantly expressed adenosine receptor in T cells (ImmGen database consortium[2]), results in a rise of intracellular cAMP, leading to decreased T cell activation and effector function[3,4].

Increased adenosine signaling limits mucosal inflammation[5] and improves disease in several animal models of autoimmunity[6,7]. Deletion of the A_{2A} receptor enhances gastritis in *Helicobacter*-infected mice[8] and exacerbates inflammation in the early stages of experimental autoimmune encephalomyelitis[9]. In humans, high ADA activity has been documented in the serum of patients with autoimmune diseases[7,10]. Consequently, the adenosine-generating enzyme CD73 plays a protective role in the animal models of arthritis[11] and colitis[12].

The control of immune responses is crucial to prevent inflammation-induced damage to healthy tissue. $FOXP3^+$ regulatory T cells (Tregs) are essential to maintain peripheral tolerance to self-antigens and play a pivotal role in terminating an immune response by inhibiting T cell proliferation and effector function. Tregs use an array of suppressive mechanisms to restore immune homeostasis, including the production of anti-inflammatory cytokines, engagement of co-inhibitory receptors, and the modulation of effector T cell metabolism[13]. Murine Tregs express CD39 and high levels of CD73 on the cell surface to degrade ATP and produce adenosine, which in turn has a dual effect; inhibiting effector T cells[14] and enhancing the suppressive capacity of Tregs[15]. In the human T cell compartment, however, CD73 is expressed on the surface of most naïve CD8 T cells and in a small proportion of mature CD4 and CD8 memory T cells, but it is almost absent on Tregs[16–18]. Therefore, co-expression of CD73 with CD39 on Tregs is a rare event[17,19], challenging the concept of adenosine generation as a suppression mechanism used by Tregs in humans. While several studies demonstrate the importance of CD39 expression on human Tregs for their suppressive capacity[17,20,21], the evidence for an essential role of CD73 on Tregs is controversial[22–24].

Considering the low expression of CD73 on human Tregs, the question arises of how immunosuppressive adenosine is generated in the human system under conditions of inflammation. Our data reveal that CD73 contained in extracellular vesicles (EVs) derived from activated CD8 T cells is sufficient to degrade AMP and dampen T cell proliferation and function. This T cell-intrinsic mechanism, in concerted action with a high ATPase activity of Tregs, mediates the production of adenosine from ATP, and warrants sufficient immune suppression. Moreover, we find that EVs isolated from the synovial fluid (SF) of patients with juvenile idiopathic arthritis (JIA) induce T cell suppression in a CD73-dependent manner, underscoring the relevance of CD73 on EVs in the control of inflammation.

Results

Human regulatory T cells do not generate sufficient adenosine to suppress T cell proliferation and function. The concentration of pericellular ATP increases upon T cell activation[25] and ATP is quickly metabolized to adenosine by the ectonucleotidases CD39 and CD73. Activation of A_2 receptors on immune cells increases intracellular cAMP levels, resulting in decreased T cell activation, and effector function[4] (Fig. 1a). To assess the effect of adenosine receptor activation in human primary T cells, we first added EHNA, an inhibitor of ADA that prevents the degradation of adenosine. In the presence of EHNA, we observed a 20% reduction in T cell proliferation compared to the untreated control (Fig. 1b). Similarly, the metabolically stable, nonselective adenosine analogs 5′-*N*-ethylcarboxamidoadenosine (NECA) and 2-chloroadenosine (CADO) led to a concentration-dependent decrease in activation (measured as a percentage of $CD25^+$ cells out of CD4 T cells) and proliferation (Fig. 1c, d), demonstrating the suppressive effect of adenosine receptor activation on human T cells.

Adenosine is generated from ATP by the concerted action of CD39 and CD73, and co-expression of these ectonucleotidases is a hallmark of murine $Foxp3^+$ Tregs and allows them to generate immunosuppressive adenosine[14]. Even though this pathway is widely accepted to be valid also in the human system, gene expression data from the Human Protein Atlas[26,27] and the ImmGen project[2,28] show that the expression of *NT5E*, the gene encoding CD73, is divergent in different immune cell compartments of the two species. According to gene expression data, all murine T cell subpopulations express *Nt5e*, while in the human peripheral T cell compartment CD8 T cells have a high *NT5E* expression and the expression in Tregs is much lower. Our data confirm that nearly all peripheral Tregs in mice express high levels of CD73 on the cell surface, and two-thirds of the cells co-express CD39 (Supplementary Fig. 1), fulfilling the enzymatic requirements for adenosine generation. To systematically explore CD39 and CD73 expression on human peripheral T cells, we measured cell surface expression of these two ectonucleotidases by flow cytometry (Fig. 1e, f). We found that only a minimal frequency of human peripheral Tregs express CD73 (average of 3%, ranging from 0.8 to 6%, Fig. 1f). CD39 is expressed on 10 to 70% of Tregs, depending on the genotype of the donor[17,29], and on around 5% of nonactivated conventional CD4 (CD4con, defined as non-Treg CD4 T cells) and CD8 T cells. CD73, in contrast, is expressed on ~20 to 60% of the CD8 T cells, and on less than 20% of CD4con T cells. Given the low frequency of Tregs expressing CD73, co-expression of both ectoenzymes is a rare event even in donors with high CD39 expression (Fig. 1e, f), questioning the relevance of Treg-derived adenosine for immune suppression in the human system. To address this point experimentally, we performed an in vitro suppression assay using different ratios of CD4con T cells:Treg (Fig. 1g), considering that the physiological proportion of Tregs is around 10% of CD4 T cells[30]. As responder cells, we used sorted $CD73^-$ CD4con T cells to prevent the production of adenosine by $CD73^+$ cells other than Tregs (Supplementary Fig. 2). In these specific conditions, we did not observe an effect of Tregs on suppression at any ratio. When we added ATP to mimic the inflammatory milieu, we observed maximal suppression of T cell activation, proliferation, and IFNγ production at a high Treg ratio (1:0.5 CD4con T cells to Tregs, fivefold the physiological concentration). The addition of recombinant CD73 had no further suppressive effect, indicating that there was enough CD73 in the system (in this donor in particular 2% of Tregs were $CD73^+$) to produce adenosine. At a low Treg ratio (1:0.125 CD4con T cells to Tregs, similar to physiological conditions), Tregs could only induce partial suppression in the presence of ATP. In combination with recombinant CD73, though, expression of CD25 was reduced to the minimum, and proliferation and IFNγ production were completely abolished (Fig. 1g). These data indicate that at a physiological CD4con:Treg ratio, there is not enough Treg-derived AMPase activity to generate adenosine that mediates significant suppression.

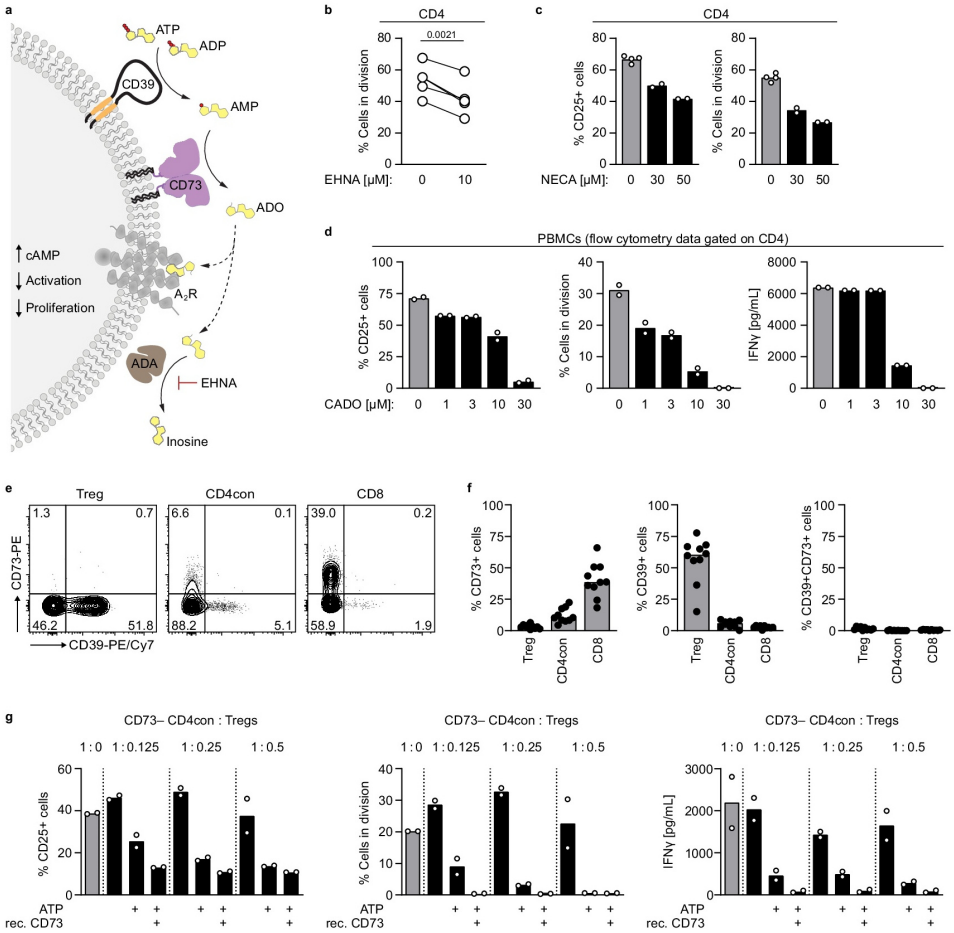


Fig. 1 Adenosine generated in the course of T cell activation prevents an exacerbated T cell response. a Schematic representation for the degradation of ATP to adenosine by the ectonucleotidases CD39 and CD73. **b**–**d** PBMCs or CD4 T cells were stimulated with αCD3/αCD28 and treated with **b** the ADA inhibitor EHNA or different concentrations of adenosine receptor agonists **c** NECA, and **d** CADO. CD25 expression and proliferation were measured after 3 to 4 days by flow cytometry. Data were shown for **b** five donors or **c**, **d** one representative donor (mean of technical replicates). **e**, **f** CD73 and CD39 expression on human T cell subsets in **e** representative dot plots and **f** as a summary for ten donors (median). **g** CD73$^-$ CD4con T cells were stimulated with αCD3/αCD28 in the presence of the ADA inhibitor EHNA (10 μM) and incubated with Tregs, ATP (50 μM) and recombinant CD73 (15 ng/mL) as indicated. The ratios of CD4con T cells to Tregs were 1:0.125 to 1:0.5. CD25 expression and proliferation were measured after 4 days by flow cytometry. IFNγ production was determined by ELISA in the cell culture supernatant on day 4. Data were shown for one representative donor out of four analyzed (mean of technical duplicates). A two-tailed paired *t*-test was used to compare untreated and treated samples in **b**.

CD73-mediated AMPase activity by Tregs is dispensable for the control of CD4 T cell proliferation and function. In humans, CD73 expression is less frequent in Tregs than in CD4con and CD8 T cells (Fig. 1f). We hypothesized that AMPase activity derived from nonregulatory T cells contributes to adenosine production and immune suppression. To test this, we stimulated CD4con T cells and added Tregs at different ratios. As expected, the addition of Tregs at a very high CD4con:Treg ratio (1:2) resulted in a decrease of T cell activation, and proliferation by 30 to 50%, respectively. We observed a dramatic reduction when exogenous AMP was added to the cell culture to ensure an equal amount of substrate for CD73 in all conditions. Importantly, this effect was independent of the Treg-derived AMPase activity (Fig. 2a). We reasoned that the likely source of AMPase activity in our system could be CD73 from the responder T cells themselves. To test this, we sorted CD4con T cells into CD73$^-$ and CD73$^+$ and stimulated them in the presence of AMP, but without Tregs (Fig. 2b). As predicted, CD73$^+$-sorted cells were less activated and proliferated at lower levels after incubation with AMP, and this effect was reversed by adding the specific CD73 inhibitor PSB-14685. Importantly, the addition of AMP to CD73$^-$-sorted CD4con T cells did not have any suppressive effect on activation or proliferation (Fig. 2b), indicating that CD73 on the responder T cells is necessary for the production of adenosine. Addition of recombinant CD73 to AMP-treated CD73$^-$ CD4con T cells reestablished the suppressive effect, demonstrating that exogenous CD73 functionally compensates the lack of CD73 in the responder cells (Fig. 2c). Of note, recombinant CD73 was able to induce T cell suppression of CD73$^-$ CD4con T cells at a concentration as low as 0.15 ng/mL of the enzyme when the substrate AMP was provided (Fig. 2d). IFNγ production followed the same pattern as CD25 expression and proliferation in the

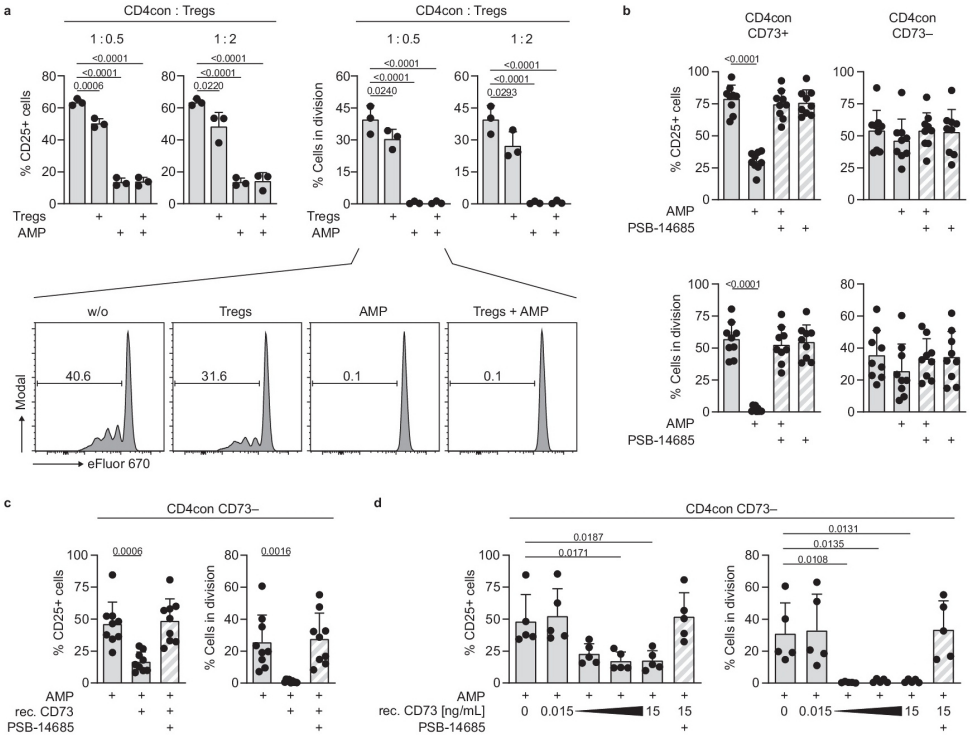


Fig. 2 Treg-derived CD73 is not essential for adenosine-mediated suppression of conventional CD4 T cells. a–d CD4 responder T cells were stimulated with αCD3/αCD28 in the presence of the ADA inhibitor EHNA (10 μM). CD25 expression and proliferation were measured after 4 days by flow cytometry. **a** CD4con T cells were stimulated and incubated with AMP (50 μM) and Tregs in the indicated ratio. **b** CD4con T cells were sorted into CD73$^-$ and CD73$^+$, the cells were incubated with AMP (50 μM) and the specific CD73 inhibitor PSB-14685 (10 μM). **c** CD4con CD73$^-$ T cells were incubated with AMP (50 μM), the specific CD73 inhibitor PSB-14685 (10 μM), and recombinant CD73 (15 ng/mL). **d** CD4con CD73$^-$ T cells were incubated with AMP (50 μM) and different concentrations of soluble recombinant CD73 (three tenfold serial dilutions starting with 15 ng/mL). PSB-14685 was used to block the highest concentration of recombinant CD73. Data were shown for **a** three, **b**, **c** nine and **d** five donors (mean ± SD). Ordinary one-way ANOVA with Dunnett's multiple comparisons test was used to compare all conditions to cells treated with EHNA or EHNA and AMP (first bar).

experiments shown in Fig. 2a–d (Supplementary Fig. 3a–d). We conclude that CD73-mediated production of adenosine constitutes an intrinsic mechanism of conventional T cells to control ongoing activation.

Enzymatically active CD73 is released from the T cell membrane upon activation. In human peripheral blood (PB) T cells, CD73 is predominantly expressed on CD8 T cells (Fig. 1f), and we have previously shown that activated T cells lose the membrane expression of CD73[31]. We hypothesized that the CD73 released from activated cells plays a role in adenosine generation. To address this point, we first investigated the time point of release in peripheral blood mononuclear cells (PBMCs) during activation. We observed a peak of CD73 expression on CD8 T cells 1 day after stimulation, followed by a marked decrease 2 or 3 days after stimulation (Fig. 3a). The loss of CD73 from the cell membrane was also detected in CD4 T cells. CD39 was upregulated in both T cell populations. Even though the loss of CD73 from the cell membrane was a general phenomenon, we observed interindividual variability both in the timing and extend of CD73 loss, and also in the level of CD39 upregulation.

Next, we investigated the fate of the released form of CD73. CD73 is a GPI-anchored protein that can be found as an enzymatically active soluble form, and CD73-specific AMPase activity is present in human plasma[32,33]. We implemented a sensitive, HPLC-based assay with improved signal-to-noise sensitivity using fluorescent 1,N^6-etheno-AMP (eAMP) as a substrate to measure AMPase activity in cells and cell culture supernatants (Fig. 3b). As a proof of principle, we forced shedding of cell surface CD73 on CD8 T cells using phosphatidylinositol-specific phospholipase C (PI-PLC) (Fig. 3c, left) and observed lower AMPase activity of the PI-PLC-treated cells compared to untreated cells (Fig. 3c, middle). In contrast, the supernatants from PI-PLC treated cells converted more eAMP to 1,N^6-etheno-adenosine (eADO), in line with a higher amount of CD73 present in the supernatant (Fig. 3c, right). The CD73-specific inhibitor PSB-14685 completely blocked eAMP degradation, verifying the specificity of the AMPase activity. Activated T cells that had lost CD73 expression (Fig. 3d, left), showed lower AMPase activity than unstimulated cells (Fig. 3d, middle). This decrease in activity in the cell-bound compartment was paralleled by increased generation of eADO in the cell culture supernatant of activated cells and accompanied by an increase in the concentration of soluble CD73 detected by ELISA (Fig. 3d, right). These data demonstrate that CD73 is released in the cell culture supernatant upon T cell activation and retains its enzymatic activity.

EVs are the major source of AMPase activity in CD8 T cell culture supernatants and mediate suppression of responder T cells. We next sought to decipher the mechanism of CD73 release from the membrane. We first considered enzyme-

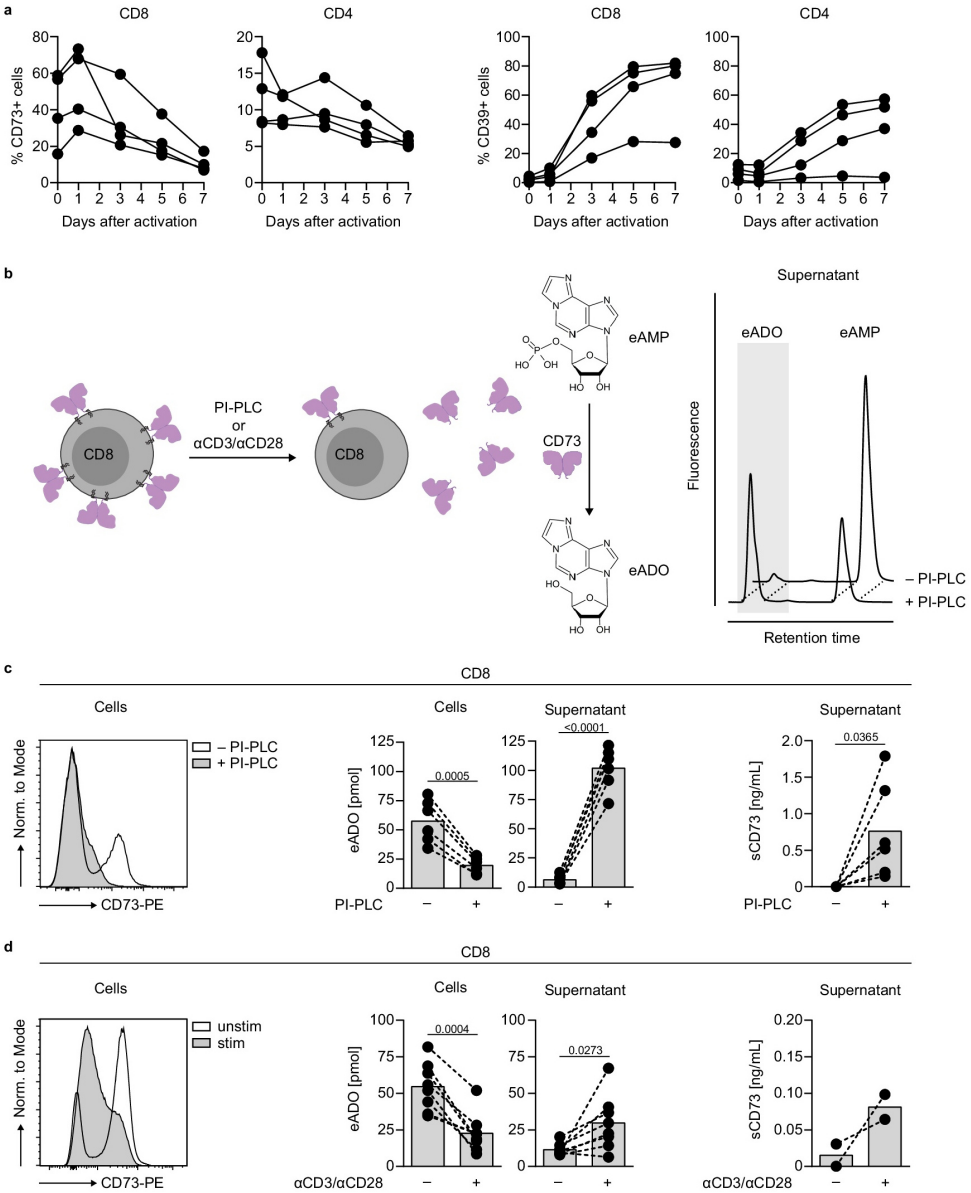
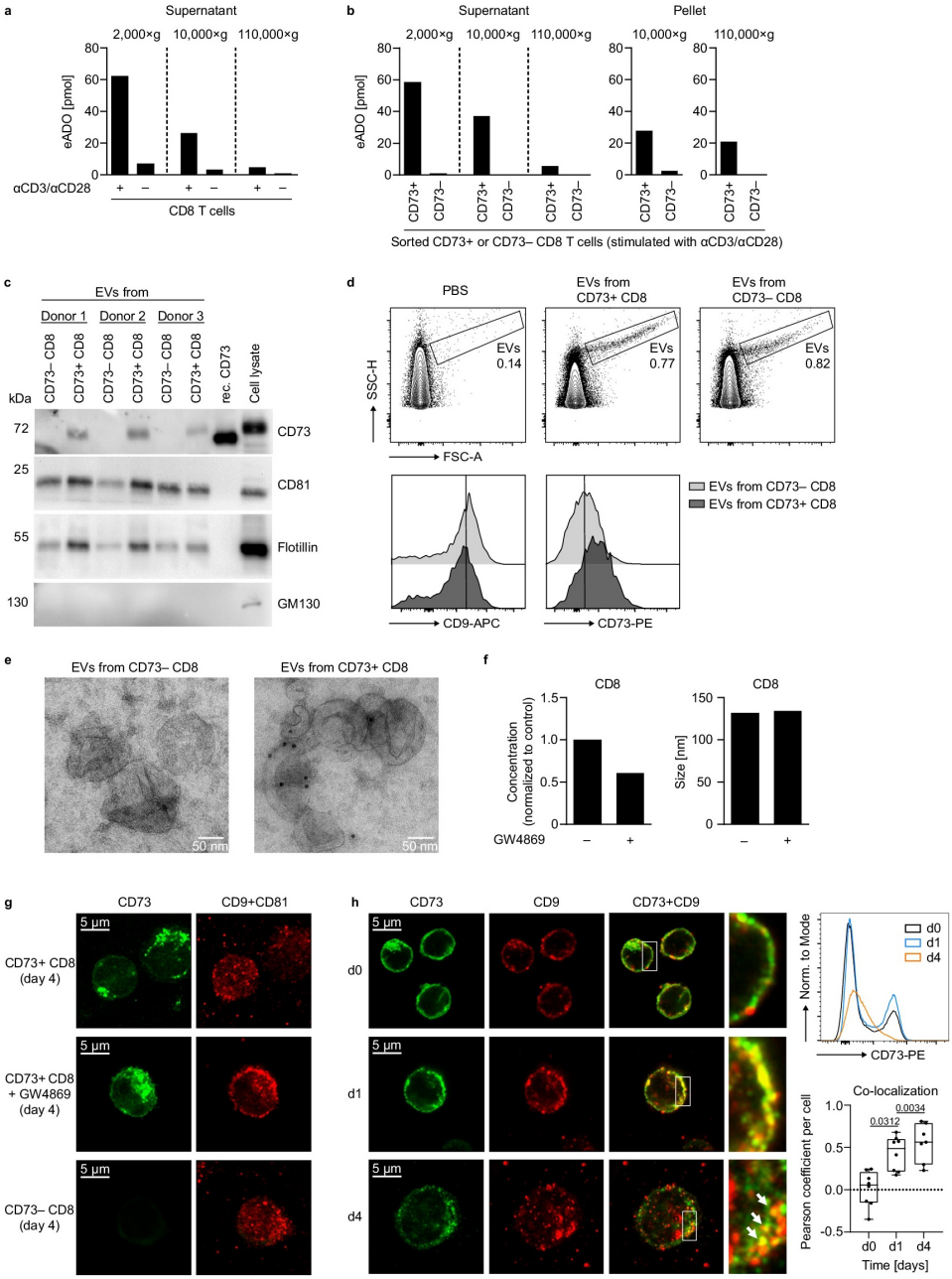


Fig. 3 CD8 T cells release enzymatically active CD73 upon activation. a Flow cytometric analysis of CD73 and CD39 expression on CD8 and CD4 T cells after activation of PBMCs (measurement on five time points from day 0 to day 7). Data were shown for four donors. **b** Schematic representation of CD73 released from CD8 T cells after PI-PLC treatment or activation. AMPase activity is measured by the conversion of 1,N^6-etheno-AMP (eAMP) to 1,N^6-etheno-ADO (eADO). **c**, **d** Determination of the AMPase activity of CD8 T cells and supernatants. 0.2×10^6 CD8 T cells were treated with **c** PI-PLC (0.5 U/mL) or **d** stimulated with αCD3/αCD28 for 4 days. Histograms show CD73 expression of the cells at the time of eAMP incubation for one selected donor. The cells and supernatants were incubated with eAMP and degradation of eAMP to eADO was determined by HPLC (middle panel, mean of **c** six donors and **d** eight donors). The amount of soluble CD73 (sCD73) was measured by ELISA (right panel, mean of **c** six donors and **d** two donors). A two-tailed paired *t*-test was used to compare untreated and treated samples in **c**, **d**.

mediated shedding; however, neither specific inhibition of phospholipase D nor inhibition of metalloproteinases prevented loss of CD73 from the cell surface (Supplementary Fig. 4). CD73 is enriched in lipid rafts of T cells after activation, suggesting that the release might occur in form of vesicles, as it has been described in tumor cells[34]. Using differential centrifugation of cell culture supernatants, we observed a stepwise reduction in the AMPase activity in the supernatants of stimulated CD8 T cells after $10{,}000 \times g$ (large vesicles and apoptotic bodies are pelleted) and $110{,}000 \times g$ centrifugation (small vesicles are pelleted), indicating that CD73 is, indeed, present on vesicles (Fig. 4a). To verify the specificity of the CD73-mediated enzymatic activity on these vesicles, supernatants from activated $CD73^-$ and $CD73^+$-sorted CD8 T cells were subjected to differential centrifugation and



AMPase activity was measured in the obtained fractions. The cell culture supernatants of CD73$^+$ CD8 T cells displayed AMPase activity after 2000 × *g* centrifugation (removal of cell debris), but ultracentrifugation heavily reduced eADO generation. Remarkably, the EV-enriched pellets derived from 110,000×*g* centrifugation (hereinafter named EVs) showed substantial AMPase activity (Fig. 4b). Supernatants from CD73$^-$ CD8 T cells did not have AMPase activity, demonstrating the specificity of CD73 for the enzymatic degradation of AMP in these samples.

Nanoparticle tracking analysis (NTA) of CD8 T cell EVs revealed a size of around 100 nm for the majority of the isolated CD8 T cell vesicles (Supplementary Fig. 5). The presence of EV markers CD81 and flotillin, as well as the absence of the Golgi protein GM130 confirmed the EV nature and purity of our samples. In addition, CD73 protein was detected in EVs derived from CD73$^+$ CD8 T cells, but not in those from CD73$^-$ CD8 T cells (Fig. 4c). Even though the small size of the EVs limits precise analysis by conventional flow cytometry, particles gated by forward vs. side scatter showed increased staining intensity of CD73 in vesicles derived from CD73$^+$ CD8 T cells compared to those derived from CD73$^-$ CD8 T cells, while expression of tetraspanin CD9 was comparable (Fig. 4d). Electron microscopy

Fig. 4 Extracellular vesicles are the major source of AMPase activity in human CD8 T cell culture supernatants. a, **b** CD8 T cells were stimulated with αCD3/αCD28 and kept in culture for 4 days. Analysis of AMPase activity in **a** cell culture supernatants of stimulated or unstimulated total CD8 T cells and **b** supernatants and EVs of sorted CD8 CD73$^+$ or CD8 CD73$^-$ T cells after differential centrifugation. The conversion of eAMP to eADO was measured by HPLC. **c**–**e** Analysis of CD73 and EV markers on EVs derived from stimulated cell culture supernatants of CD73$^+$ or CD73$^-$ CD8 T cells by **c** western blot, **d** flow cytometry, and **e** electron microscopy with immunogold labeling. Recombinant CD73 or cell lysate from stimulated CD8 T cells served as positive controls. **f** EVs were isolated from CD8 T cells stimulated with αCD3/αCD28 for 3 days in the presence or absence of GW4869 (10 μM), and particle concentration and size were measured by NTA. **g** Microscopy analysis of CD73, CD9, and CD81 expression in sorted CD73$^+$ or CD73$^-$ CD8 T cells after 4 days of stimulation with αCD3/αCD28. GW4869 (10 μM) was added at day 0. **h** Microscopy analysis of CD73 and CD9 expression in CD8 T cells before and after activation. Pearson coefficient was determined to quantify the co-localization of CD73 and CD9. Data were analyzed from seven (d4) or eight (d0, d1) high power fields (center line: median, box limits: 25th to 75th percentiles, whiskers: min to max). Donors analyzed: **a**, **b** three donors in three independent experiments; **c** six donors in three independent experiments; **d**–**f** three donors; **g**, **h** samples from three donors processed independently. Each figure panel shows a representative donor/experiment. Kruskal–Wallis test with Dunn's multiple comparisons test was used to compare Pearson coefficients of co-localization in **h**.

of the purified vesicles revealed the characteristic cup-shaped morphology with sizes between 50 to 200 nm. Immunogold labeling further confirmed the presence of CD73 in EVs derived from CD73$^+$ CD8 T cells (Fig. 4e). To investigate the vesicular release of CD73, we treated T cells with GW4869, a pharmacological compound that blocks the generation of a specific type of EVs called exosomes[35]. The addition of GW4869 to the cell culture reduced the amount of generated EVs whereas the size was not affected (Fig. 4f). It also prevented the loss of tetraspanins CD9 and CD81 from the cell (Fig. 4g). High-resolution fluorescence microscopy of CD8 T cells showed the reduction of CD73 surface expression after 4 days of stimulation (Fig. 4h) consistent with the results obtained by flow cytometry (Fig. 4h upper right panel and Fig. 3a). Overlay of CD73 with tetraspanins CD9 and CD81 revealed significant co-localization of CD73 with vesicle markers at day 1 and day 4 after activation (Fig. 4h lower right panel and Supplementary Fig. 6). Altogether, these data show that CD73 is released with EVs upon T cell activation and that these EVs comprise the majority of AMPase activity in T cell culture supernatants.

We wondered if CD73-containing EVs isolated from T cell culture supernatants functionally compensate the lack of membrane-bound CD73 in the control of T cell activation and proliferation as we have shown for recombinant CD73 (Fig. 2c). To test if EVs, a natural source of non-cell-bound CD73, can suppress T cell function, we activated CD73$^-$ CD4con T cells (to prevent any effect of residual membrane-bound CD73 on the responder T cells) in the presence of AMP. Addition of recombinant CD73 or EVs isolated from the supernatant of CD73$^+$ CD8 T cells induced strong suppression, which was dose-dependent and could be blocked by the CD73 inhibitor PSB-14685 (Fig. 5a, b and Supplementary Fig. 7). Importantly, EVs isolated from the supernatant of CD73$^-$ CD8 T cells did not suppress activation and proliferation of the responder T cells, and neither did the pelleted material after ultracentrifugation of cell culture medium (Fig. 5 and Supplementary Fig. 8), indicating that the observed suppressive effect is CD73-specific and not due to contaminants in the EV preparation. In summary, we show that enzymatically active CD73 is released from activated CD8 T cells with EVs, and that EVs derived from activated CD73$^+$ T cells are highly suppressive.

T cell-derived EVs released during immune cell activation cooperate with regulatory T cells for the efficient suppression of effector T cells. In our previous experiments, we exogenously provided AMP, the substrate for CD73. AMP is generated from ATP by the enzymatic activity of CD39. In contrast to conventional T cells, a substantial proportion of Tregs constitutively express CD39 in humans (Fig. 1f). Therefore, Tregs are likely to be superior at degrading ATP than conventional T cells. We used the sensitive HPLC-based assay to compare the ATPase activity of purified Tregs, CD4con, and CD8 T cells after activation. Using 1,N^6-etheno-ATP (eATP) as substrate, we found that Tregs had the highest ATPase activity of all tested T cell subsets (Fig. 6a), and this was more pronounced in donors with substantial basal CD39 expression on Tregs (Fig. 6a, left panel) than in donors with low frequency of CD39-expressing Tregs (Fig. 6a, right panel). Notably, CD8 T cells were the second most efficient cell type in degrading ATP, while CD4con T cells showed the least ATPase activity, in agreement with the percentage of cells upregulating CD39 after activation (Fig. 3a).

We suspected that the high ATPase activity of Tregs combines with CD73-containing EVs to mediate maximum immune suppression. We performed a suppression assay, using CD73$^-$ CD4con T cells as responder T cells, and added different ratios of Tregs. With a ratio of CD4con T cells to Tregs close to the physiological situation (1:0.125), Tregs could only induce partial suppression. The addition of EVs from CD73$^+$ CD8 T cells, a natural source of non-cell-bound CD73, reduced CD25 expression, and completely suppressed proliferation and IFNγ production of effector T cells (Fig. 6b). Importantly, EVs derived from CD73$^-$ CD8 T cells had no additional effect on the suppression of responder T cells, revealing that they do not cooperate with Tregs to generate immunosuppressive adenosine. We conclude that Tregs are the main providers of ATPase activity in our system, and the concerted action between Treg-derived ATPase activity and effector cell-derived AMPase activity in form of EVs ensures the best conditions for immune suppression.

EVs isolated from the synovial fluid of patients with juvenile idiopathic arthritis are immunosuppressive. An interesting question to address is the relevance of non-cell-bound CD73 in the context of inflammation. The SF of patients with autoimmune arthritis offers an ideal chance to analyze an inflammatory compartment in the human system because we can access disease-relevant infiltrating immune cells. Flow cytometric analyses of the cellular composition of the SF from eight children with JIA (Fig. 7a) revealed that T cells constitute the main cell population, and that CD8 T cells are enriched in the SF compared to PB in most cases. B cells, the only other prominent CD73-expressing cell type in the human immune compartment[2,28], are clearly underrepresented in the SF compared to PB in all patients. We next compared the expression of CD73 and CD39 on the T cells from PB and SF (Fig. 7b). In agreement with published data[36,37], we found increased expression of CD39 on CD4 T cells and Tregs and decreased CD73 expression on CD8 T cells in the synovial T cell subsets compared to their peripheral counterparts. In analogy to our in vitro findings after activation, we suspected that CD73 has been lost from the cell membrane. Indeed, we detected moderate to high concentrations of soluble CD73 in the

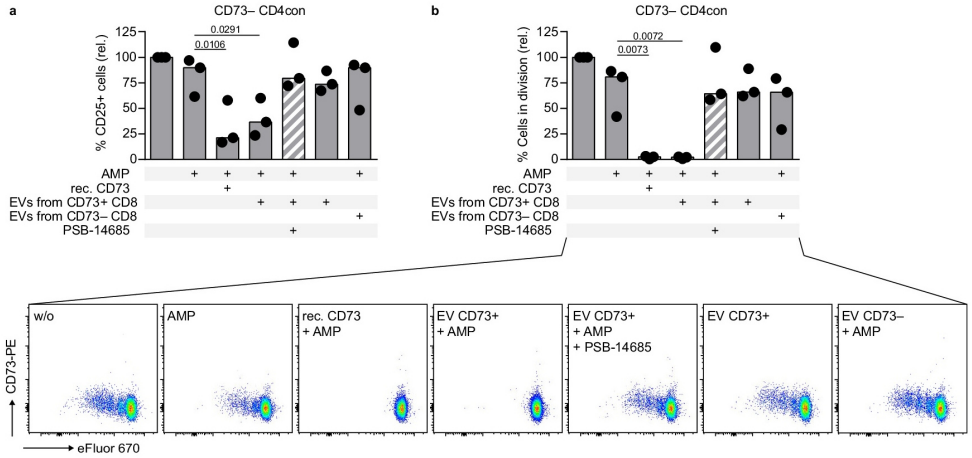


Fig. 5 Extracellular vesicles isolated from the cell culture supernatant of activated CD73$^+$ CD8 T cells are immunosuppressive. CD73$^-$ CD4con T cells were stimulated with αCD3/αCD28 in the presence of the ADA inhibitor EHNA (10 μM) and incubated with AMP (50 μM), recombinant CD73 (15 ng/mL), EVs and PSB-14685 (10 μM) as indicated. **a** CD25 expression and **b** proliferation were measured after 4 days by flow cytometry. Data were shown as a median of three donors from independent experiments. For each donor, CD25 expression and proliferation were set in relation to cells only treated with EHNA. Repeated measures one-way ANOVA with Dunnett's multiple comparisons test was used to compare all conditions to cells treated with EHNA and AMP (second bar).

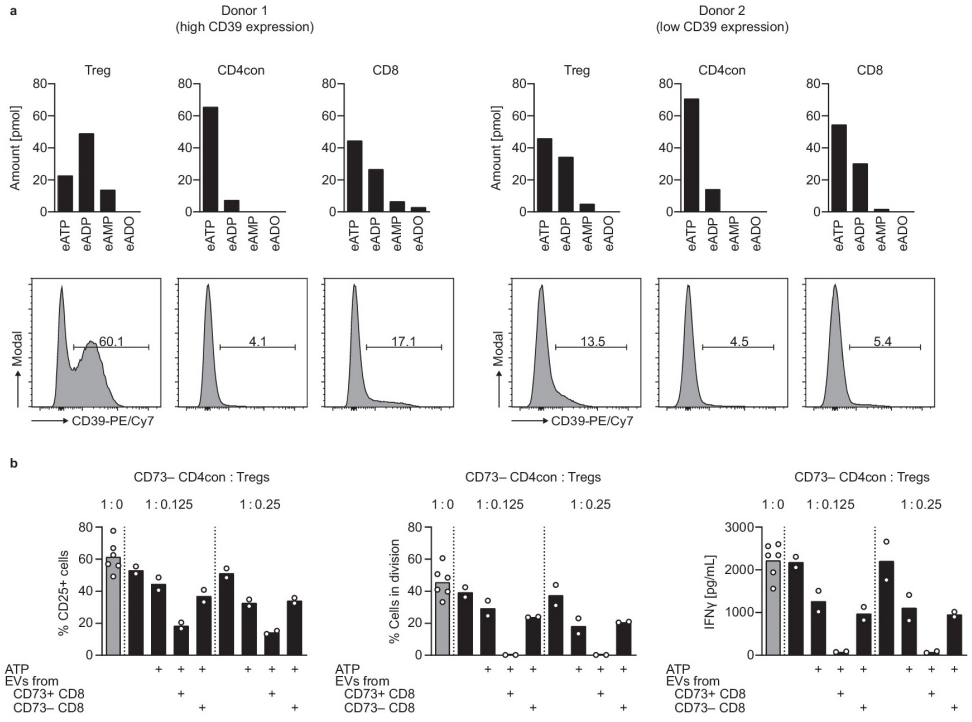


Fig. 6 The combined activities of Treg-derived CD39 and vesicular CD73 result in optimal suppression of T cell function. a Analysis of ATPase activity in sorted T cell subsets of two donors by HPLC. Activated cells (αCD3/αCD28, 3 days) were incubated with 1,N^6-etheno-ATP (eATP) and degradation of etheno-nucleotides was determined by HPLC. Histograms show CD39 expression on the cells at the time of eATP incubation. **b** CD73$^-$ CD4con T cells were stimulated with αCD3/αCD28 in the presence of the ADA inhibitor EHNA (10 μM) and incubated with Tregs, ATP (50 μM), and EVs from cell culture supernatants of sorted and activated CD8 T cells as indicated. CD25 expression and proliferation were measured after 4 days by flow cytometry. IFNγ production was determined by ELISA in the cell culture supernatant harvested on day 4. Data were shown for one representative donor out of four analyzed (mean of technical replicates).

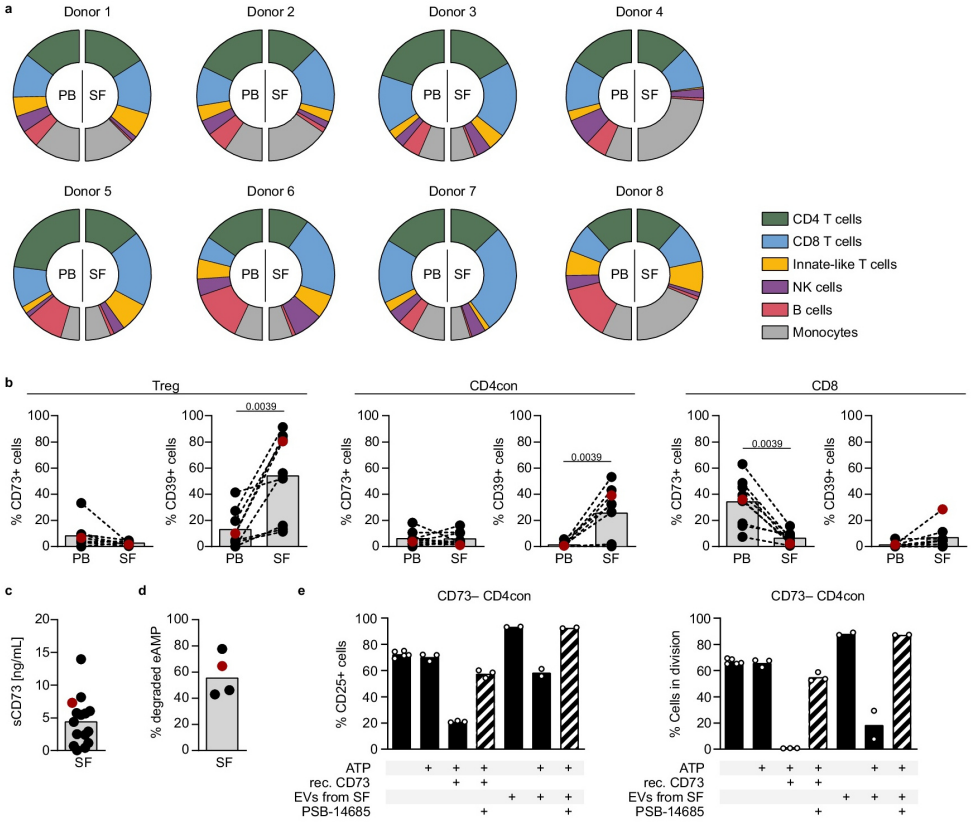


Fig. 7 Extracellular vesicles isolated from synovial fluid of patients with juvenile idiopathic arthritis are immunosuppressive. a, **b** Flow cytometric analysis of **a** immune cell subsets and **b** CD73 and CD39 expression in SF and PB of JIA patients. **c** CD73 in the cell-free moiety of SF was measured by ELISA. **d** SF was incubated with eAMP and degradation of eAMP was determined by HPLC. **e** $CD73^-$ CD4con T cells were stimulated with αCD3/αCD28 in the presence of the ADA inhibitor EHNA (10 μM) and incubated with ATP (50 μM), recombinant CD73 (15 ng/mL), EVs (3.1×10^8 particles), and PSB-14685 (10 μM) as indicated. CD25 expression and proliferation were measured after 4 days by flow cytometry. Data were shown for **a** eight individual donors, as mean of **b** nine, **c** fifteen, and **d** four donors indicated by dots, or for **e** a representative donor out of three analyzed (mean of technical replicates), marked as a red dot in **b**–**d**. A two-tailed Wilcoxon test was used to compare ectonucleotidase expression in PB and SF in **b**.

SF of nearly all patients tested (Fig. 7c) and confirmed AMPase activity in the samples measured (Fig. 7d). We next isolated EVs from the SF and verified their EV nature by electron microscopy and western blot. The EV markers flotillin and CD81 could be detected in all EV samples along with CD73 (Supplementary Fig. 9a, b). We also assessed for the presence of protein contamination and found rests of albumin, as previously reported when using differential ultracentrifugation for EV isolation[38], but not of apolipoproteins (Supplementary Fig. 8b).

Among other cell types, T cells clearly contributed to the cellular sources of EVs in the SF (Supplementary Fig. 9c), and we could show co-expression of CD8 and CD73 on these EVs by conventional flow cytometry (Supplementary Fig. 9d). We assessed if SF-derived EVs suppress $CD73^-$ CD4con T cells. As observed before, the addition of recombinant CD73 and ATP to the responder T cells resulted in reduced T cell activation and completely abolished proliferation. When SF-derived EVs were added to the culture in the absence of ATP, we observed an increase in activation and cell proliferation. Importantly, in the presence of ATP, this increase was abrogated, and a clear reduction in cell proliferation was induced, which was reversed by the addition of the CD73-specific inhibitor PSB-14685 (Fig. 7e). Of note, SF-derived EVs decreased the activation and proliferation of responder T cells in a dose-dependent manner (Supplementary Fig. 9e). In summary, we show here that EVs from the SF of JIA patients degrade AMP, generating adenosine and suppressing T cell proliferation and function.

Discussion

Adenosine is a potent regulator of inflammation generated in the extracellular space by the sequential hydrolysis of ATP by ectonucleotidases CD39 and CD73. We show here that activated human CD8 T cells release CD73-containing EVs that generate adenosine, contributing substantially to immune suppression.

The degradation of ATP and generation of adenosine is a well-described mechanism of suppression in murine $Foxp3^+$ Tregs, which express both CD39 and CD73 on their cell surface[14]. However, very few human $FOXP3^+$ Tregs express CD73, and they are not particularly adept at adenosine generation. We describe here how CD73-mediated generation of adenosine is mostly independent from Tregs in the human system, and propose a coordinated effort involving CD39 on Tregs for the degradation of ATP to AMP, and CD73 on T cell-derived EVs that provide the necessary AMPase activity to generate adenosine. We have shown the release of CD73-containing EVs from activated CD8 T cells. However, CD4 T cells also lose CD73 from the

cell membrane upon activation, suggesting a parallel mechanism for CD4 T cells.

Intracellular ATP functions as an essential energy source. Once released into the extracellular space, ATP becomes a biologically active signaling molecule[39]. Conditions such as cellular activation and stress, inflammation, ischemia, or hypoxia promote ATP release and raise the pericellular concentration of ATP sufficiently to activate P2 receptors and support inflammation[25,40,41]. Extracellular ATP is rapidly hydrolyzed by ectonucleotidases in a stepwise manner to yield adenosine, a potent immune suppressor upon binding to P1 receptors A_{2A} and A_{2B} on immune cells. The degradation of adenosine by ADA further regulates the availability of adenosine for signaling. Thus, a complex network of purinergic enzymes and receptors controls the duration and magnitude of purinergic signaling and regulates the immune response[42]. Remarkably, the affinity of purinergic molecules for its receptors and the outcome after receptor activation are cell- and species-specific[43]. Adenosine signaling supports the expansion and suppressive function of murine Tregs[15] while inhibiting effector T cells. In humans, adenosine inhibits both Tregs and effector T cells[44]. Not only the response to extracellular adenine nucleotides is different between mice and humans, but also the expression and regulation of ectonucleotidases CD39 and CD73. The co-expression of both enzymes on murine Tregs secures the production of adenosine, and this metabolic path is important for their suppressive function[14,45]. In contrast, very few circulating human Tregs express CD73 and they are still suppressive. This poses the question of whether human Tregs rely on adenosine generation or rather favor other mechanisms of immune suppression. The absence of CD73 on the membrane of human Tregs could be an evolutionary advantage to avoid the production of pericellular adenosine that inhibits their function[44]. Further, human Tregs do not express CD26, a docking site for ADA at the cell membrane[46], lacking a mechanism for efficient degradation of pericellular adenosine.

CD73, as a GPI-anchored protein, clusters at the plasma membrane in lipid rafts that are poised for EV formation[47,48]. We found that CD73 diminished drastically from the surface of T cells 3 to 4 days after activation, concomitant to the loss of AMPase activity in the cellular compartment. In parallel, enzymatic activity increased in the cell culture supernatants. Importantly, the capacity to produce adenosine found in the cell culture supernatants was lost after ultracentrifugation, indicating that it was contained in EVs and not present as a soluble protein. Part of the AMPase activity was already lost after 10,000 × *g* centrifugation, which we attribute to CD73 on apoptotic bodies or larger EVs. Even though CD73 has higher enzymatic activity in its soluble form compared to the membrane-bound variant[49], we speculate that the vesicular form has advantages, such as an extended half-life, and better distribution through body fluids. It is not yet known how far EVs can travel in human body fluids, but murine EVs can rapidly traffic to the spleen and liver before elimination 6 h after intravenous injection[50]. The generation of EVs poses the question of how the cells recover the membrane loss. A possibility would be the fusion with foreign EVs, which would permit a very dynamic exchange of membrane components between cells and the acquisition of new functions[51,52].

The exact contribution of EV- and T cell-derived adenosine to immune suppression in vivo is difficult to predict due to the multiple levels of regulation that influence the availability of adenine nucleotides during an immune response. Contributing factors include the rapid generation and degradation of signaling-relevant molecules, local differences in nucleotide concentrations, potential feed-forward inhibition mechanisms, and alternative degrading enzymes. In our in vitro experiments with human T cells, we used different substrates and specific inhibitors to overcome these difficulties. By blocking the degradation of adenosine with an ADA inhibitor, we emphasized the role of the purinergic cascade in immune suppression. The addition of exogenous ATP at a moderate concentration (50 µM) served to mimic local increases of ATP as it has been reported in different settings of inflammation and ischemia[53]. In the absence of Tregs, we added AMP to ensure the availability of enough substrate for CD73, because conventional T cells are much less efficient than Tregs in producing AMP. Furthermore, adding AMP as substrate bypasses a potential feed-forward inhibition of CD73 by ATP or ADP[54]. Although CD73 is the main AMP-degrading enzyme, adenosine is still generated by tissue-nonspecific alkaline phosphatase (TNAP) when CD73 is absent, as shown in CD73 knockout mice[55,56] and in cells from patients with CD73 deficiency[57]. In our experiments, we could completely abolish AMPase activity with a specific CD73 inhibitor[58,59], indicating that TNAP activity is negligible in our system. The observed ATPase activity is likely CD39-mediated, but we cannot exclude a contribution of other enzymes, such as ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase-1) or other members of this family of pyrophosphatases[60]. Of note, the expression, regulation, and activity of ENPP1 on T cells has not been explored. The detection of ADP as an intermediate product in our assays indicates a stepwise degradation of ATP, favoring the role of ectonucleoside triphosphate diphosphohydrolases (ENTPDases) like CD39, but not pyrophosphatases.

Both CD39 and CD73 are expressed in distinct immune cell types. In humans, apart from T cells, B cells express both ectonucleotidases, while monocytes and dendritic cells express mostly CD39. Also, endothelial cells and mesenchymal cells express CD73, and all these cell types contribute to ATP metabolism in vivo. EVs containing CD39 or CD73 have been isolated from mesenchymal stem cells, B cells, and Tregs[24,61,62]. These EVs have immunoregulatory properties[63] and their modulatory role has been reported in cancer[34,62]. We have not addressed here the contribution of other ectonucleotidases present in T cell-derived EVs to ATP metabolism, but it has been shown that Treg-derived EVs contain CD39 and inhibit T cell proliferation[24]. Further work will be required to dissect the role of cellular and EV-associated ectonucleotidases in immune suppression.

We had the unique chance to isolate EVs from a human site of inflammation, the SF of JIA patients. The SF contains predominantly monocytes and memory T cells that produce inflammatory cytokines, sustaining inflammation in the affected joints[64,65]. In agreement with previous reports, we found increased CD39 and negligible expression of CD73 in the T cells of the SF. While SF-infiltrating cells show ATPase activity, AMPase activity is decreased in the cellular fraction[36,37]. Our analysis revealed that CD73 is present in the cell-free fraction of the SF, including EVs. The cellular source for these EVs are T cells, monocytes/macrophages, and endothelial and mesenchymal stem cells. Because human monocytes do not express CD73[26,27], we concluded that activated T cells are a relevant source of vesicular CD73. This was confirmed by the co-expression of CD73 and CD8 on SF-derived EVs. The endothelial cells in the synovial lining and mesenchymal stem cells, both expressing CD73, are also plausible sources for $CD73^+$ SF EVs. This finding deserves further investigation. Our in vitro experiments showed that SF-derived EVs promote T cell activation and proliferation, probably due to cytokines and growth factors contained in these EVs[66]. However, by strengthening the purinergic signaling cascade with the addition of ADA inhibitor EHNA and ATP, we turned the outcome around, and EVs became immunosuppressive, as it has been shown for CD73-containing EVs produced by tumor cells[34]. Therefore, in an ATP-rich environment, as it is at sites of inflammation and ischemia or

in the intestine, the purinergic signaling cascade becomes highly relevant for immune regulation[67].

We conclude that abundant CD39 expression on Tregs secures the hydrolysis of ATP to ADP and AMP, but that the AMPase activity of CD73 is provided by CD8 T cells, and mostly contained in EVs. We propose that adenosine generation by conventional (nonregulatory) T cells is a built-in mechanism of immune suppression necessary to restrain ongoing inflammation, contesting the common paradigm that Tregs must provide the whole machinery for adenosine production. Finally, our results highlight the role of EVs in the control of immune responses and support the prospect of modulating the purinergic axis for the treatment of local inflammation.

Methods

Origin of samples and isolation of human mononuclear cells. Buffy coats were obtained from the blood bank of the University Medical Center Hamburg-Eppendorf (UKE). PB was drawn from healthy volunteers visiting the UKE. Blood and SF of patients with JIA, specifically oligo- and polyarthritis, were obtained from children visiting the UKE, the Altona Children's Hospital, the University Medical Center Schleswig-Holstein (campus Lübeck), or the Medical Center Bad Bramstedt. SF was obtained from joint puncture for diagnostic or therapeutic reasons. All samples were handled according to corresponding ethics protocols (Ethics Committee of the Hamburg Chamber of Physicians, protocols PV5139 for samples from healthy donors, and PV3746 for samples from JIA patients), and informed consent was obtained from all donors. Mononuclear cells (MC) were isolated from blood and SF by Biocoll density gradient centrifugation (Merck). Isolated immune cells were used for flow cytometric analyses or further isolation of T cell subsets.

Isolation of murine splenocytes. All mouse experiments were performed in accordance with national and institutional guidelines on animal care (Hamburg Authority for Health and Consumer Protection, Veterinary Affairs/Food Safety, protocol ORG983). Lymphocytes were isolated from the spleen of C57BL/6 mice housed in the animal facility at the UKE. Single-cell suspensions were prepared by processing the spleen through a 70 µm strainer. After erythrocyte lysis, splenocytes were used for the analysis of surface markers by flow cytometry.

Flow cytometry and fluorescence-activated cell sorting (FACS). Human and murine immune cells were preincubated with immunoglobulins to block unspecific binding and stained with fluorescence-labeled antibodies for 30 min at 4 °C. All antibodies were titrated prior to use. The following fluorochrome-conjugated anti-human antibodies were used: anti-CD3 (clone UCHT1 and clone OKT3), anti-CD4 (clone RPA-T4, clone SK3, and clone OKT4), anti-CD8α (clone RPA-T8 and clone HIT8a), anti-CD9 (clone HI9a), anti-CD16 (clone 3G8), anti-CD19 (clone HIB19), anti-CD25 (clone BC96), anti-CD39 (clone A1), anti-CD73 (clone AD2), anti-CD127 (clone HCD127 and clone A019D5) (all BioLegend), anti-CD14 (clone M5E2), anti-CD25 (clone 2A3), anti-CD45 (clone HI30), anti-TCRγδ (clone 11F2) (all BD Biosciences), anti-CD56 (clone N901) (Beckman Coulter). The following fluorochrome-conjugated anti-mouse antibodies were used: anti-CD3 (clone 17A2), anti-CD4 (clone RM4-5), anti-CD25 (clone PC61), anti-CD73 (clone TY/11.8) (all BioLegend), anti-CD8 (clone 53-6.7), anti-CD19 (clone 1D3), and anti-CD39 (clone 24DMS1) (all eBioscience). For dead cell exclusion, a live/dead dye (Thermo Fisher Scientific) was included. The staining cocktails were designed to minimize the effects of spectral overlap. Prior to analysis, a compensation matrix was calculated after single color staining of human PBMCs, as described[68]. Samples were measured at FACSCanto II, FACSCelesta, or LSR-Fortessa (BD Biosciences) using FACSDiva software for data acquisition (BD Biosciences) and analyzed using FlowJo software (BD). For isolation of specific T cell subpopulations by FACS, cells were stained as described above and sorted at FACSAria IIIU (BD Biosciences).

Preparation and stimulation of human T cells. CD8 or CD4 T cells were isolated from PBMCs by negative selection using the EasySep Human T Cell Enrichment Kit (Stemcell Technologies). The purity of the isolated cells was assessed by flow cytometry. For indicated assays, Tregs (defined as $CD4^+$ $CD25^{high}$ $CD127^{low}$) and conventional CD4 T cells (CD4con, defined as non-Treg CD4 T cells) were obtained by FACS. CD4con T cells were additionally sorted with regard to their CD73 expression if indicated. To assess proliferation in T cell assays, responder cells were labeled with 2 µM eFluor 670 (Thermo Fisher Scientific).

Purified T cells were stimulated with 1 µg/mL coated αCD3 (OKT3) and 5 µg/mL soluble αCD28 (CD28.2) (both BioLegend) and cultured in serum-free X-VIVO 15 medium (Lonza). PBMCs were activated with 0.5 µg/mL soluble αCD3 in RPMI containing 1% penicillin-streptomycin, 1% L-glutamine (all Thermo Fisher Scientific), and 10% FBS (Biochrom). If not otherwise stated, cells were seeded at a density of 1×10^6 cells/mL.

Isolation of EVs. EVs were isolated from cell culture supernatants, X-VIVO 15 medium, and SF by differential centrifugation at 4 °C. Samples were centrifuged at $450 \times g$ for 5 min, followed by centrifugation at $2000 \times g$ for 10 min. After centrifugation at $10{,}000 \times g$ for 30 min, the supernatant was subjected to ultracentrifugation ($110{,}000 \times g$, 70 min, in an SW 60 Ti swinging-bucket rotor, Beckman Coulter). The EV-enriched pellet (hereinafter named EVs) was washed and resuspended in PBS. Particle concentration and size were determined using the NTA instrument NanoSight LM14 (Malvern Panalytical) equipped with a 638 nm laser and a Marlin F-033B IRF camera (Allied Vision Technologies), operated with NTA 3.0 software.

Characterization of EVs

Western blot. The EVs were characterized according to the ISEV guidelines[69]. For western blot analysis, EVs were incubated with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, and 0.1% SDS) in the presence of protease and phosphatase inhibitors (Roche). The protein content of EVs was assessed with a Micro BCA Protein assay kit (Thermo Scientific). Samples (3.5 µg per EV sample, 15 ng recombinant CD73, 10 µg cell lysate, 2 µL pure SF, 15 µL X-VIVO 15 medium, or 15 µL of X-VIVO ultracentrifugation pellet corresponding to 2 mL X-VIVO 15 medium) were mixed with 4X loading buffer (250 mM Tris-HCl, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.008% Bromophenol Blue, pH 6.8), boiled for 5 min at 95 °C and subjected to electrophoresis on a 10% Bis-Tris gel (Invitrogen) under denaturing conditions. The gel was electroblotted onto a nitrocellulose membrane (LI-COR) and stained with the Revert Total Protein Stain (LI-COR). After blocking for 60 min with Roti-Block (Carl Roth), membranes were incubated overnight with the following primary antibodies diluted in Roti-Block: anti-CD73 (clone D7F9A, Cell Signaling, 1:1000), anti-CD81 (clone D3N2D, Cell Signaling, 1:1000), anti-Flotillin-1 (clone 18/Flotillin-1, BD Biosciences, 1:1000), anti-GM130 (clone 35/GM130, BD Biosciences, 1:500), anti-Albumin (clone F-10, Santa Cruz, 1:1000), anti-apoA-1 (clone E-20, Santa Cruz, 1:500), or anti-apoB (polyclonal, Acris, 1:1000). After washing with TBST, the membranes were incubated for 60 min with anti-rabbit-HRP-conjugated secondary antibody (Cell Signaling, 1:1000), or anti-mouse-HRP-conjugated secondary antibody (Cell Signaling, 1:1000), or anti-goat-HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, 1:5000). Membranes were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and images were taken on a ChemiDoc Imaging System (Bio-Rad) using the Quantity One software.

Electron microscopy. For electron microscopy with immunogold labeling, EVs were adsorbed to carbon-coated grids for 20 min, washed with PBS, quenched in glycine, and blocked with blocking solution for goat gold conjugates (Aurion)[70]. After 30 min incubation with the primary anti-CD73 antibody (clone AD2, BioLegend, 5 µg/mL), the grids were incubated for 20 min with the anti-mouse IgG 10 nm gold conjugate (Sigma, 1:20), fixed in 2.5% glutaraldehyde, washed with water, and transferred onto methyl cellulose/uranyl acetate mixture drops on ice for 10 min. Grids were looped out, air-dried, and analyzed by transmission electron microscopy. Electron microscopy was performed at 80 kV in an FEI Tecnai G20 microscope equipped with an SIS Veleta camera.

Flow cytometry. For phenotypical single EV analysis by conventional flow cytometry, the EV suspension was extensively diluted in 400 µL filtered PBS and stained for 30 min with anti-CD8-BV421 (clone RPA-T8, 1:500), anti-CD73-PE (clone AD2, 1:300), and anti-CD9-APC (clone HI9a, 1:200) antibodies as indicated. After extensive washing of the fluidics system of the cytometer, ApogeeMix (Apogee Flow Systems, silica and latex beads) and Megamix-Plus SSC (BioCytex, latex/polystyrene beads) heterogenous fluorescent beads were used to set up the system. Samples were measured at the FACSAria IIIU (BD Biosciences) for 1 min at a flow rate of 1.0 at 4 °C. Controls included buffer alone, buffer with single and combined antibodies, EVs with buffer, and EVs with fluorescence minus one (FMO) stainings for each fluorochrome. To confirm the nature of the EVs, the samples were incubated with 0.5% NP40 for 45 min and reanalyzed for 1 min (lysis control).

For the phenotypical characterization of SF EVs, we used the MACSPlex Exosome kit (Miltenyi). This bead-based assay allows the simultaneous detection of 37 surface markers to determine the cellular origin of the EVs. In brief, EVs were incubated overnight with antibody-coated capture beads, washed, and incubated with tetraspanin CD9/CD81/CD63 antibodies provided in the kit. The measurements were done at FACSCanto II (BD Biosciences).

T cell assays. T cells were stimulated as described above and cultured at a density of $0.25–0.5 \times 10^6$ cells/mL in serum-free X-VIVO 15 medium (Lonza) for 3 to 4 days at 37 °C, 5% CO_2 in the presence of ADA inhibitor EHNA (10 µM, Tocris). In Treg suppression assays, responder T cells were cocultured with Tregs at different ratios. When indicated, AMP (50 µM, Sigma-Aldrich), ATP (50 µM, Sigma-Aldrich), the CD73-specific inhibitor 2-chloro-N^6-*o*-chlorobenzyl-α,β-methylene-ADP (PSB-14685, 10 µM)[59], recombinant CD73 (15 ng/mL, unless otherwise noted, Sino Biological) or EVs (equivalent to 150 µL of cell culture supernatant of activated CD8 T cells, if not stated otherwise) were added. At the time of harvest, the cell membrane expression of the activation marker CD25, and the dilution of

eFluor 670 as a measure of proliferation were assessed by flow cytometry. IFNγ was determined in cell culture supernatants by ELISA (BioLegend), measured with a Victor^3 1240 plate reader equipped with Wallac 1240 Manager software (PerkinElmer).

Phospholipase C treatment. Forced shedding of CD73 was achieved by incubating 0.2×10^6 CD8 T cells with 0.5 U/mL bacterial phosphatidylinositol-specific phospholipase C (PI-PLC, Thermo Fisher Scientific) for 30 min at 37 °C. Cells and supernatants after PI-PLC treatment were subsequently used for the determination of AMPase activity by high-performance liquid chromatography (HPLC).

Analysis of ATPase and AMPase activities by HPLC. AMPase and ATPase activity of cells, cell culture supernatants, EVs, and SF were analyzed by assessing the degradation of 1,N^6-etheno-AMP (eAMP) or 1,N^6-etheno-ATP (eATP), respectively. For this, 0.2×10^6 T cells, 150 µL cell culture supernatant, EVs (equivalent to 150 µL of supernatant), or 20 µL SF were incubated with 1 µM eAMP or eATP (Biolog) for 30 min (cells) or 60 min (cell culture supernatants, EVs and SF) at 37 °C. After the incubation, cells were removed by centrifugation ($450 \times g$, 5 min, 4 °C) and all samples were directly frozen and stored at −20 °C until HPLC analysis. The analysis of etheno-nucleotides was conducted by ion pair reversed-phase HPLC on a 1260 Infinity system (Agilent Technologies). Before measurement, samples were passed through 10 kDa size exclusion filters to remove proteins (10 min, $10{,}000 \times g$, 4 °C, Sartorius). A volume corresponding to starting amount of 85 pmol eAMP or eATP was loaded on the HPLC. For quantification of nucleotides in the sample, different amounts of commercially available etheno-nucleotides (Biolog) were analyzed under the same conditions. The separation was performed on a 250 mm × 4.6 mm C-18 BDS Multohyp 5 µm column (CS Chromatographie Service) with a C-18 security guard cartridge (Phenomenex). The mobile phase was composed of HPLC buffer A (20 mM KH_2PO_4, 5 mM TBAHP, pH 6.0) and HPLC buffer B (50% buffer A and 50% methanol) with the following gradient: 0.0 min (30.0% buffer B), 3.5 min (30.0% buffer B), 11.0 min (62.5% buffer B), 15.0 min (62.5% buffer B), 25.0 min (100.0% buffer B), 27.0 min (100.0% buffer B), 29.0 min (30.0% buffer B), and 38.0 min (30.0% buffer B). The injection volume was 100 µL and the flow rate was 0.8 mL/min. The temperature of the column compartment was 20 °C and the autosampler was kept at 8 °C. The signals were detected by the fluorescence detector (excitation 230 nm and emission 410 nm) of the system. Peak integration was performed using ChemStation Software (Agilent Technologies).

Detection of non-cell-bound CD73. Non-cell-bound CD73 was determined in cell culture supernatants and in SF by ELISA (Abcam).

Fluorescence microscopy. Human CD73$^+$ and CD73$^-$ CD8 T cells or total CD8 T cells were stimulated as described above. At the day of harvest, cells were plated on poly-L-lysine coated cytoslides (Thermo Fisher Scientific) at a density of 0.2×10^6 cells/cytoslide using a cytospin centrifuge (Shandon Elliott). In some experiments, exocytosis was blocked with 10 µM GW4869 (Cayman Chemical). Cells were fixed for 10 min at room temperature (RT) with 4% PFA (EMSciences) and washed with PBS. For immunofluorescent localization of CD73, unspecific binding was blocked with 5% normal horse serum (Vector) diluted in 0.05% Triton X-100 (Merck) in PBS. The cytoslides were incubated overnight with unconjugated anti-CD73 (clone AD2, 1:10, Biolegend) in blocking buffer at 4 °C and detected using a Cy2 anti-mouse antibody (1:200, Jackson ImmunoResearch Laboratories) for 30 min at RT. After washing, cells were further incubated with APC anti-CD9 (clone HI9A, 1:10) or/and anti-CD81 (clone 5A6, 1:10) (both BioLegend) for 60 min at RT. An LSM800 confocal microscope with airyscan and the ZENblue software (all ZEISS) were used for analysis. Pearson's coefficient of co-localization was determined in seven to ten randomly chosen high power fields (630X) per condition (three individual cells/high power field).

Statistical analysis. Prism 8 (GraphPad) was used to perform statistical analyses, detailed information are provided in the figure legends. In brief, data were analyzed for normal distribution. When they passed the normality test, they were analyzed by two-tailed Student's *t*-test (two groups, paired), ordinary one-way ANOVA (multiple groups, unpaired), or repeated measures (RM) one-way ANOVA (multiple groups, paired). When data did not pass the normality test, they were analyzed by a two-tailed Wilcoxon test (two groups, paired) or Kruskal–Wallis test (multiple groups, unpaired). When multiple groups were compared, post hoc tests were performed to correct for multiple comparisons. Dunnett's multiple comparisons test was used when all groups were compared to the same control condition. Dunn's multiple comparisons test was performed to compare the mean ranks of not normally distributed data. p values < 0.05 were considered to indicate statistical significance. Non-significant differences were not annotated.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files, or in the source data file. Data from publicly available sources shown in this paper can be obtained from the Human Protein Atlas (https://www.proteinatlas.org/ENSG00000135318-NT5E/blood, *NT5E* expression in human blood). Source data are provided with this paper.

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Author contributions

Idea and design of research project: E.T., E.S., R.W., and N.G. Writing manuscript: R.W., E.S., and E.T. Supply of patient material: I.R. and M.L. Establishment of methods: E.S., R.W., A.R., J.B., A.B., R.F., B.P., B.R., and F.L.R. Experimental work: E.S., R.W., A.R., C.M.-S., R.R., J.B., S.B., and H.W. Data analysis and interpretation: E.S., R.W., A.R., and E.T. Scientific input and manuscript revision: A.R., C.M.-S., F.L.R., J.B., B.R., B.P., F.C., T.M., R.F., C.E.M., N.G.

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3.3 Generation and function of non-cell-bound CD73 in inflammation [Review]

The review “Generation and Function of Non-cell-bound CD73 in Inflammation” was published in July 2019 in Frontiers in Immunology (Impact Factor 8.787 [2021]).

Generation and Function of Non-cell-bound CD73 in Inflammation

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In this publication, we summarize what is known about the presence of CD73 as non-cell-bound protein (as soluble protein and in EVs) and its relevance in inflammation.

I contributed to this review article with a section on the release of CD73 from the cell membrane. This included the literature search as well as writing the first version of the corresponding section (page 3, "Evidence for non-cell bound and functionally active CD73", from paragraph 3). In addition, I was involved in the revision of the entire article.

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Place, date Eva Tolosa (Supervisor)

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MINI REVIEW
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Generation and Function of Non-cell-bound CD73 in Inflammation

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Extracellular adenine nucleotides participate in cell-to-cell communication and modulate the immune response. The concerted action of ectonucleotidases CD39 and CD73 plays a major role in the local production of anti-inflammatory adenosine, but both ectonucleotidases are rarely co-expressed by human T cells. The expression of CD39 on T cells increases upon T cell activation and is high at sites of inflammation. CD73, in contrast, disappears from the cellular membrane after activation. The possibility that CD73 could act in *trans* would resolve the conundrum of both enzymes being co-expressed for the degradation of ATP and the generation of adenosine. An enzymatically active soluble form of CD73 has been reported, and AMPase activity has been detected in body fluids of patients with inflammation and cancer. It is not yet clear how CD73, a glycosylphosphatidylinositol (GPI)-anchored protein, is released from the cell membrane, but plausible mechanisms include cleavage by metalloproteinases and shedding mediated by cell-associated phospholipases. Importantly, like many other GPI-anchored proteins, CD73 at the cell membrane is preferentially localized in detergent-resistant domains or lipid rafts, which often contribute to extracellular vesicles (EVs). Indeed, CD73-containing vesicles of different size and origin and with immunomodulatory function have been found in the tumor microenvironment. The occurrence of CD73 as non-cell-bound molecule widens the range of action of this enzyme at sites of inflammation. In this review, we will discuss the generation of non-cell-bound CD73 and its physiological role in inflammation.

Keywords: soluble CD73, shedding, extracellular vesicles, adenosine, immune regulation

INTRODUCTION

Under physiological conditions, the concentration of the purine nucleotides adenosine triphosphate (ATP), ADP and AMP in biological fluids and extracellular space is low (30–100 nM), while the intracellular concentration of ATP is in the millimolar range. Upon cell activation and tissue damage, ATP is readily released from the cells, resulting in a surge of pericellular ATP (1–3). Indeed, the concentration of ATP measured in close proximity to the cell using imaging methods reached 60 μM a few minutes after T cell stimulation (4), and this concentration is

sufficient to stimulate P2 receptors and elicit pro-inflammatory signals. The excess of extracellular ATP is rapidly hydrolyzed by ectonucleotidases such as CD39 or ectonucleotide pyrophosphatase/phosphodiesterases (i.e., ENPP1, also known as CD203a or PC-1) to generate ADP and finally AMP, the substrate for the ecto-5′-nucleotidase CD73 (NT5E) (**Figure 1**). AMP is converted to adenosine primarily by CD73 and, less efficiently, by alkaline phosphatase. AMP can likewise be generated from extracellular nicotinamide adenine dinucleotide (NAD^+) by the coordinated activity of the ecto-NAD-glycohydrolase CD38, which metabolizes NAD^+ to ADPR, and the pyrophosphatase/phosphodiesterase CD203a (ENPP1), responsible for the conversion of ADPR to AMP (5, 6) (**Figure 1**). ENPP1 can also convert ATP directly to AMP. Similar to ATP, the concentration of NAD^+ is higher inside the cells (high micromolar to millimolar range) than in the extracellular space, where it has been measured at 20–60 nM (7), and is released upon cell damage.

Adenosine, by binding to P1 receptors on immune cells elicits predominantly anti-inflammatory signals (8). As the rate-limiting enzyme for adenosine production, CD73 plays a key role in the balance between inflammation and immune suppression (9, 10). Independently of its enzymatic activity, CD73 has been reported to provide a costimulatory signal for lymphocyte activation (11, 12), and mediate lymphocyte adhesion to the endothelium (13). More recent data, however, suggest that CD73-mediated production of adenosine and subsequent signaling through adenosine receptors restricts leukocyte adhesion to endothelium (14–16). Surprisingly, CD73-deficiency protected animals from experimental autoimmune encephalomyelitis by reducing migration of pathogenic immune cells into the brain (17), but resulted in larger cerebral infarct volumes and increased local leukocyte infiltration in a model of stroke (18). These apparently contradictory results on the role of CD73 in brain inflammation might be explained by adenosine availability and differential adenosine receptor engagement in chronic and acute settings, underscoring the multifaceted role of CD73 and adenosine in inflammation (19). Expression of CD73 and adenosine signaling have also been involved in the regulation of vascular permeability by promoting endothelial barrier function (20).

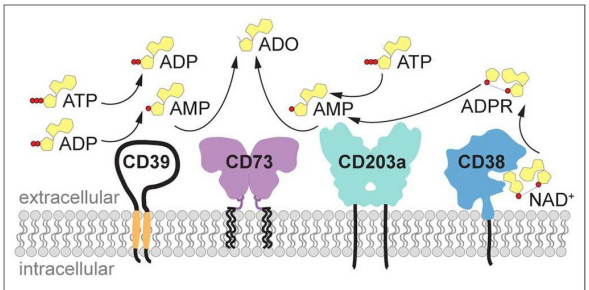

FIGURE 1 | Generation of extracellular adenosine by purinergic ectoenzymes. Extracellular ATP is stepwise dephosphorylated by the ectonucleotidase CD39 (ENTPDase1) to produce ADP and AMP. Alternatively, AMP can be generated from NAD^+ by the coordinated activity of the ecto-NAD-glycohydrolase CD38, which metabolizes NAD^+ to ADPR, and the pyrophosphatase/phosphodiesterase CD203a (ENPP1), responsible for the conversion of ADPR to AMP. ENPP1/CD203a is also capable of generating AMP directly from ATP. Finally, the hydrolysis of AMP by ecto-5′-nucleotidase (NT5E) CD73 yields adenosine (ADO). ADO, adenosine; ADPR, adenosine diphosphate ribose; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1 (CD203a, PC-1); ENTPDase1, ectonucleoside triphosphate diphosphohydrolase 1 (CD39); NAD^+, nicotinamide adenine dinucleotide; NT5E, ecto-5′-nucleotidase (CD73).

While the expression of CD73 on endothelia has been documented across species, the expression of CD73 on immune cells is species-specific. In humans, CD73 is expressed on most B cells (certainly on all mature naïve B cells) and on some T cell subsets, namely naïve cells in the CD8 compartment [ImmGen database consortium Heng et al. (21)], innate-like T cells (22), and a small subset of memory CD4 T cells (23, 24). In mice, CD73 is expressed on peritoneal macrophages, most T cells, including Tregs, NK cells, and in the B cell compartment it is preferentially expressed in mature class-switched and germinal center B cells (25–28). Remarkably, CD39 and CD73 are rarely co-expressed on human conventional T cells in the periphery, and the expression of CD73 is a seldom event on human Tregs (29–31), even though murine Tregs do express these ectoenzymes constitutively (32). In the inflamed joints of patients with arthritis CD39 is upregulated on T cells, while the expression of CD73 is low (25, 29, 33, 34). Accordingly, *in vitro* activation of human conventional T cells results in the upregulation of CD39 and loss of CD73 from the cell membrane (11, 25). Interestingly, CD39 and CD73 are co-expressed in a subset of Th17 cells with suppressive features (24, 35). These cells are prominent in the lamina propria, and were found decreased in patients with inflammatory bowel disease, underscoring their relevance for the control of inflammation in the gut (24).

The enzymatic activities of CD39 and CD73 are complementary for the generation of adenosine and subsequent control of the inflammatory response, but since their co-expression in T cells is rare, it is plausible that the AMPase activity of CD73 is provided in *trans* by neighboring cells of different lineages. How CD39-expressing T cells are endowed with AMPase activity in the context of inflammation is not completely understood, but we have learned from the tumor microenvironment that CD73-positive extracellular vesicles (EVs) contribute to the dampening of anti-tumor immune responses (36, 37), and EVs derived from murine regulatory T cells display AMPase activity (38). Vesicular release is facilitated upon cell activation (39) and the loss of cell surface CD73 in T cells of the synovial fluid in patients with arthritis or after *in vitro* stimulation (25, 33) suggests that CD73 may be shed from the cell surface, either as a soluble molecule or in form of vesicles. As a consequence, non-cell-bound and enzymatically active CD73 spreads at sites of inflammation and modulates the immune response by generating an adenosine-rich anti-inflammatory environment. In this review we will discuss the generation of non-cell-bound CD73 and its physiological role in inflammation.

EVIDENCE FOR NON-CELL BOUND AND FUNCTIONALLY ACTIVE CD73

CD73 is a 71 kDa homodimer attached to the plasma membrane by a GPI-anchor (40, 41). It can be found as a membrane-bound phospholipase C-sensitive form, a membrane-bound phospholipase C-resistant form, and as a soluble variant of the protein deriving from the GPI-anchored form (42, 43). Soluble and enzymatically active CD73 could be purified from the supernatant of human placental extracts, showing similar affinity for AMP as the membrane-bound form (42). Moreover, human plasma and serum (44–46) as well as vitreous fluid (47) exhibit AMPase activity that can be specifically blocked by ecto-5′-nucleotidase inhibitor adenosine 5′-(α,β-methylene)-diphosphate (APCP), indicating the presence of soluble CD73 (sCD73).

CD73 is not the only ectoenzyme that exists in soluble form in peripheral blood. Other purine-metabolizing enzymes, namely alkaline phosphatase, adenosine deaminase (ADA), CD38, ATP-degrading enzymes like ENPPs and CD39, as well as ATP-regenerating kinases are also present in human plasma (46, 48–51), indicating that there is a complex network of enzymatically active molecules shifting the balance of purinergic signaling and thereby modulating the immune response.

Shedding of CD73 can occur through hydrolysis of the GPI-anchor by endogenous phospholipases (42, 43, 52) or by proteolytic cleavage (53). Soluble CD73 from human placenta contained myo-inositol, a part of the GPI-anchor linked to the protein after phospholipase shedding, confirming release from the membrane by endogenous phospholipase C or D (42). These two phospholipases cleave the GPI-anchor at different sites, and only cleavage by phospholipase C leaves the cross-reacting determinant (CRD) epitope intact (54). Using an antibody that recognizes the CRD, this epitope was detected in purified bovine sCD73, pointing at phospholipase C-mediated shedding of CD73 (43). Partial resistance of placental and lymphocytic CD73 to phospholipase C cleavage suggested the existence of a non-GPI-linked version of CD73 (42, 55). However, none of the cloned CD73 cDNAs from different species was found to encode for a variant with a conventional membrane domain (56). Interestingly, there is evidence that the observed resistance to phospholipase C-mediated shedding is due to palmitoylation of the inositol-group (57). Therefore, palmitoylation of the GPI-anchor may represent a regulatory mechanism to control shedding of CD73. Of note, palmitoylated GPI-anchored proteins remain sensitive to phospholipase D (57, 58), and extracellular phospholipase D capable of shedding CD73 is present in mammalian plasma (59). Interestingly, alkaline phosphatase, which can also generate adenosine from AMP, is also GPI-anchored (6), indicating that phospholipase-mediated cleavage may be a common mechanism by which immune cells can prevent autocrine adenosine-mediated inhibition.

In addition to phospholipase-mediated shedding, proteolytic cleavage of CD73 can also generate a soluble form of the enzyme. Matrix metalloproteinase 9 (MMP-9) has been shown to cleave lipid raft-associated CD73 from the membrane of activated mouse retinal pigment epithelial cells. The generated sCD73, however, was enzymatically inactive (60). In contrast, an active form of sCD73 generated by proteolytic cleavage and lacking the GPI-anchor was found in bull seminal plasma (53). This soluble protein differs from the GPI-anchored form in its posttranslational modifications, aggregation patterns and in enzymatic activity, since it has a lower affinity for AMP compared to the membrane-bound version (53).

Direct comparison of AMPase activity among lymphocytic membrane-bound CD73, GPI-anchored CD73 inserted into an artificial lipid bilayer and sCD73 revealed lower catalytic efficiency of the membrane-bound forms. Further, phospholipase C-mediated release of CD73 from the membrane results in enhanced ectonucleotidase activity (61, 62). Thus, phospholipase-mediated release of CD73 from the cell membrane does not only increase its range of action, but also boosts its enzymatic activity.

CD73 IN EXTRACELLULAR VESICLES

A characteristic feature of GPI-anchored proteins is that they are not evenly distributed on the cell surface, but rather enriched in specific domains, the so-called lipid rafts or detergent-resistant membranes, which serve as platforms for signal transduction (2, 63). GPI-anchored proteins, probably by the fact that they are residents of these specific domains, are also present in EVs (64). EVs are lipid bilayer vesicles released by most cell types that can transport different kinds of cargos such as proteins, lipids, mRNA, non-coding RNA and DNA (65). According to their origin, EVs can be differentiated into exosomes (with an endosomal origin) and ectosomes or microvesicles/microparticles (originated by vesicle shedding at the plasma membrane) (66), including apoptotic bodies (originated from the plasma membrane of cells undergoing apoptosis) (67) or large oncosomes (originated from the plasma membrane of cancer cells) (68, 69). Depending on their origin, EVs vary in size from 30 nm to various μm (70). CD73 protein and AMPase activity have been detected in EVs, in particular exosomes, derived from cancer cells (36, 71), regulatory T cells (38), mesenchymal stem cells (72), and also from human plasma (30). Moreover, it is unclear if the soluble CD73 (or its enzymatic activity) in human body fluids reported in other studies is truly soluble or vesicle-associated, or both, since the presence of EVs was not addressed in those studies.

Extracellular vesicles derived from different cancer cell lines co-express CD39 and CD73 and are capable of hydrolyzing ATP to adenosine, modulating the tumor microenvironment and T cell function independently of direct contact to immune cells (36). Moreover, B cell-derived $CD39^{+}CD73^{+}$ EVs are elevated in the serum of colon cancer patients and metabolize tumor-derived ATP to adenosine, impairing anti-tumor responses of CD8 T cells (37). In both studies the inhibition of CD73 had a major impact on T cell function. Vesicles isolated from plasma of healthy donors or patients with neck squamous cell carcinoma exhibited AMPase activity and converted ATP to adenosine in co-cultures with $CD39^{+}$ Tregs, demonstrating that co-expression of CD39 and CD73 on the same cell is not necessary to endow Tregs with adenosine-mediated suppressive function (30). Thus, the enzymatic activity of CD73 in EVs contributes significantly to impair anti-tumor immune responses.

PHYSIOLOGICAL RELEVANCE OF NON-CELL BOUND CD73 IN INFLAMMATION

Murine Tregs express both CD39 and CD73, and EVs derived from activated murine Tregs are CD73-positive, convert AMP to adenosine, and mediate immune suppression (38). In contrast, activated human T cells (conventional or regulatory) express CD39, but not CD73. The analysis of the enzymatic activity responsible for ATP degradation in human blood revealed that while ATPase and ADPase activities are primarily mediated by cell-associated enzymes, it is the enzymes present in body fluids, e.g., plasma, that perform the last step and convert AMP to adenosine (44, 48, 73). Indeed, CD73-specific AMPase activity can be measured in the supernatant of synovial fluid in patients with arthritis (74, 75). In contrast to the cell-free moiety, synovial fluid T cells from patients with arthritis show high expression of CD39 and robust production of AMP, but low levels of CD73 and, consequently, poor generation of adenosine (33). Mechanistically, soluble CD73 or CD73-containing EVs

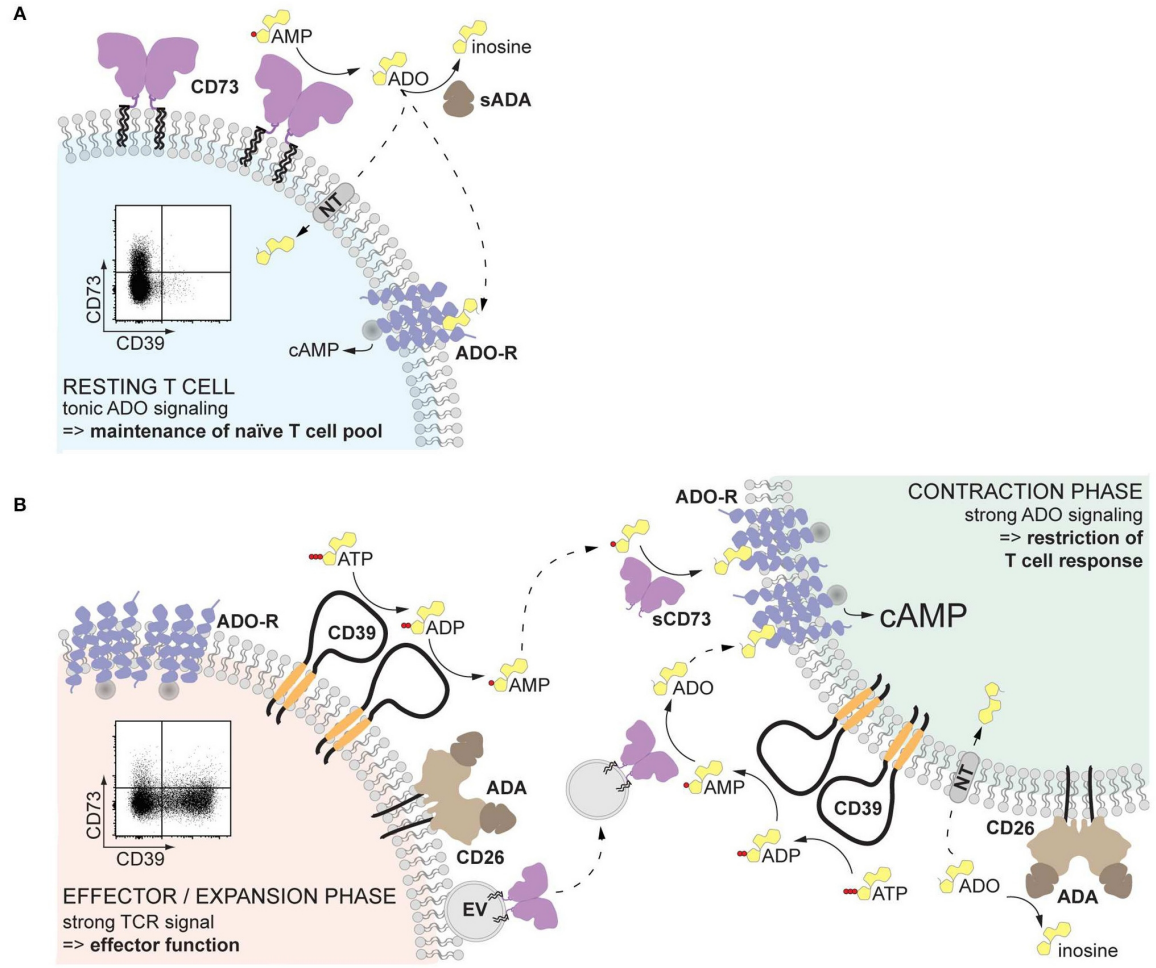

FIGURE 2 | Proposed role of non-cell-bound CD73 in inflammation. **(A)** Human resting T cells, especially naïve $CD8^+$ T cells, express CD73 on the cell surface. The pericellularly generated adenosine binds to adenosine receptors, providing a tonic signal that serves to maintain the naïve T cell pool. **(B)** Upon T cell receptor activation, T cells upregulate CD39, which hydrolyzes local extracellular ATP to AMP. At the same time, CD73 is released from the cell membrane and diffuses away, maintaining the local adenosine production low and therefore preserving T cell effector function (left). Soluble or vesicular CD73 can reach other activated T cells and act in a paracrine way generating adenosine. Engagement of high affinity adenosine receptors will result in elevated cAMP and restriction of the T cell response, inducing the contraction phase (right). The availability of adenosine is further regulated by the activity of adenosine deaminase, which converts adenosine into inosine, and transport into the cell by nucleoside transporters. Solid-lined arrows: enzymatic activities; dotted-lined arrows: diffusion. ADA, adenosine deaminase; ADO, adenosine; ADO-R, adenosine receptor; cAMP, cyclic AMP; NT, nucleoside transporter; sADA, soluble ADA; EV, extracellular vesicle.

locally released upon activation could provide the missing AMPase activity, thus completing the ATP degradation cascade to adenosine (30, 72, 75). Moreover, it is plausible that the AMP generated pericellulary diffuses short distances within the extracellular space from the generating cell to a CD73-expressing cell to be metabolized. Hence, production of adenosine does not necessarily require the co-expression of the ATP- and AMP-degrading enzymes in the same cell, and since the AMPase activity can be provided in *trans*, differences in CD73 expression on immune cells between humans and mice may be of limited relevance. Of note, the contribution of non-cell-bound CD38 and ENPPs for the generation of AMP is much less explored, and only recently the enzymatic activities for these enzymes have been described in EVs from multiple myeloma cells (76).

ATP and NAD^+ are released from activated or dying cells at sites of inflammation. In parallel, activated immune cells upscale the expression of purinergic enzymes CD39 and CD38 and lose CD73 from the cell membrane, either as a soluble molecule or contained in EVs. Local stromal cells, such as endothelial or mesenchymal stem cells, can also release CD73-containing vesicles. ATP and NAD^+ will be degraded either pericellularly by cell bound enzymes, or by soluble or vesicular enzymes to produce adenosine, which by engaging P1 receptors on immune cells will dampen the immune response. Compared to cell-associated CD73, the non-cell-bound form presents two advantages: first, it increases the distance range of enzymatic activity; second, the activated 'donor' cell that loses membrane CD73 is protected from pericellular adenosine that could terminate its effector function prematurely. Even though CD73 in soluble form is enzymatically more active than the membrane-bound variant (61), the release of membrane-bound enzymes in form of vesicles has several advantages: (i) first, extended half-life, since degradation is circumvented; (ii) improved efficiency of enzymatic activity, due to the concentration of the enzyme in microdomains; (iii) facilitation of distant transport, permitting systemic modulation; (iv) finally, the fusion of the EVs with the plasma membrane of target cells can provide them with enzymatic activities that were lacking.

The adenosinergic pathway is controlled at several checkpoints: First, the regulation of CD73 expression and shedding dictates the rate of extracellular adenosine production at the inflammation site. Importantly, higher concentrations of non-cell-bound CD73 and of its substrate AMP do not necessarily result in high extracellular adenosine, since the availability of adenosine is further modulated by its degradation to inosine mediated by adenosine deaminase, and by cellular uptake facilitated by nucleoside transporters (2, 77). Moreover, high concentrations of ATP and ADP can inhibit CD73 enzymatic activity, as it was demonstrated in the supernatants of inflamed ileum organ culture in a model of postinflammatory ileitis (77). Finally, the concentration of available adenosine will determine if the high (A_{2A}) or low (A_{2B}) affinity receptors are engaged in the target cells (78), and further modulation of adenosine signaling occurs in hypoxic conditions, when A_{2B} receptors are specifically upregulated (79) and silence A_{2A} receptor signaling (80).

When is AMPase activity desirable? In resting T cells, since tonic adenosine signaling is necessary for the maintenance of the naïve T cell pool (81) (**Figure 2A**). After infection or sterile inflammation, immune regulation is necessary for the contraction of the immune response and for limiting immunopathology (**Figure 2B**). Beyond $FoxP3^+$ Treg cells, many other immune cell types can acquire regulatory properties, but too much control can lead to insufficient immune responses and subsequent pathogen expansion or impaired tumor control. Therefore, a mechanism that can be quickly modulated and acts on different cell types represents a substantial advantage. Adenosine-mediated immune suppression fulfills these requirements, since inhibitory P1 receptors are widely expressed on immune cells, and the enzymatic activities for adenosine generation and degradation are quickly modulated. The finding that the enzymes responsible for the generation of adenosine are readily shed from the cell membrane and can be transported to act on other cell types further endorse adenosine as an ideal regulator of the immune response (**Figure 2**). Indeed, after myocardial infarction, T cell-mediated production of adenosine was found enhanced and beneficial for recovery (82). Similarly, a transient surge of systemic adenosine is detected after stroke in humans (83), and high AMPase activity can be measured in the plasma of neonates, probably ensuring tolerance to the new microbial environment (44). Understanding how systemic AMPase activity is regulated will provide clues for therapeutic intervention. While in cancer immunotherapy the blockade of CD73 seems a promising strategy (84), the systemic usage of CD73 to dampen inflammation is hampered by vasodilation and subsequent decrease in blood pressure in response to adenosine receptor engagement in the vasculature. Therefore, strategies that restrict adenosine signaling to the site of inflammation must be arranged, as elegantly shown by Flögel et al. (85) in a model of arthritis. The use of locally delivered CD73-containing EVs may provide a further therapeutic option.

AUTHOR CONTRIBUTIONS

ES, AR, and ET conceived the structure of the review and wrote the manuscript. RW and BR contributed with the part related to shedding of CD73. BP and TM contributed with the part on extracellular vesicles. H-WM and FH revised and corrected the manuscript. All authors read the final version of the manuscript and approved it for submission.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.4 Identification of the mouse T cell ADP-ribosylome uncovers ARTC2.2 mediated regulation of CD73 by ADP-ribosylation

The publication "Identification of the Mouse T Cell ADP-Ribosylome Uncovers ARTC2.2 Mediated Regulation of CD73 by ADP-Ribosylation" was published in August 2021 in Frontiers in Immunology (Impact Factor 8.787 [2021]).

> *Identification of the Mouse T Cell ADP-Ribosylome Uncovers ARTC2.2 Mediated Regulation of CD73 by ADP-Ribosylation*
>
> M. Leutert, Y. Duan, R. Winzer, S. Menzel, E. Tolosa, T. Magnus, M. O. Hottiger, F. Koch-Nolte, B. Rissiek
>
> *Front. Immunol.* 12 (2021), doi: https://doi.org/10.3389/fimmu.2021.703719

In this publication, we describe that the ADP-ribosylation of murine CD73, a post-translational modification mediated by the mono-ADP-ribosyltransferase ARTC2.2, decreases the enzymatic activity of CD73.

For this publication, I performed experiments by HPLC to measure the enzymatic activity of CD73 in different immune cell populations of different mouse lines (Figure 3D and Figure 3E). For this, I adapted the method described in Schneider, Winzer et al. (see chapter 3.2), and incubated murine cells with etheno-AMP. The sensitive detection of etheno-AMP and its degradation products by HPLC allowed us to see small differences between different conditions tested. I was responsible for the analysis of these experiments, and wrote the method to this part.

_____	_____
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Identification of the Mouse T Cell ADP-Ribosylome Uncovers ARTC2.2 Mediated Regulation of CD73 by ADP-Ribosylation

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Mouse T cells express the ecto-ADP-ribosyltransferase ARTC2.2, which can transfer the ADP-ribose group of extracellular nicotinamide adenine dinucleotide (NAD^+) to arginine residues of various cell surface proteins thereby influencing their function. Several targets of ARTC2.2, such as P2X7, CD8a and CD25 have been identified, however a comprehensive mouse T cell surface ADP-ribosylome analysis is currently missing. Using the Af1521 macrodomain-based enrichment of ADP-ribosylated peptides and mass spectrometry, we identified 93 ADP-ribsoylated peptides corresponding to 67 distinct T cell proteins, including known targets such as CD8a and CD25 but also previously unknown targets such as CD73. We evaluated the impact of ADP-ribosylation on the capability of CD73 to generate adenosine from adenosine monophosphate. Our results show that extracellular NAD^+ reduces the enzymatic activity of CD73 HEK cells co-transfected with CD73/ARTC2.2. Importantly, NAD^+ significantly reduced CD73 activity on WT CD8 T cells compared to ARTC2ko CD8 T cells or WT CD8 T cells treated with an ARTC2.2-blocking nanobody. Our study provides a comprehensive list of T cell membrane proteins that serve as targets for ADP-ribosylation by ARTC2.2 and whose function may be therefore affected by ADP-ribosylation.

Keywords: NAD, CD73, ADP-ribosylation, T cells, ARTC2.2

INTRODUCTION

Ecto-ADP-ribosyltransferases (ARTCs) are cell surface enzymes that utilize extracellular nicotinamide adenine dinucleotide (NAD^+) to covalently attach the ADP-ribose group of NAD^+ to arginine residues of various cell surface proteins under the release of nicotinamide (1, 2). The mouse ARTC family consist of six members: GPI-anchored ARTC1, ARTC2.1 and ARTC2.2, ARTC3, ARTC4 and the soluble ARTC5 (3). ARTC2.1 and ARTC2.2 are the ARTCs predominantly expressed by cells of the murine immune system (4). ARTC2.1 is highly expressed on the cell surface

of innate immune cells such as macrophages and microglia (5) and to some extent on T cells (6). In contrast, ARTC2.2 is highly expressed on most T cell populations. Further, it is worth noting that the ARTC2.1 encoding gene, *Art2a*, is inactivated by a premature stop codon in the C57BL/6 (B6) mouse strain, whereas other strains such as Balb/c carry an intact *Art2a* gene (7). Therefore, in B6 mice, ecto-ARTC activity in the immune system is limited to the T cell compartment.

Results from ADP-ribosylation assays using ^{32}P-NAD$^+$ or ethcno-NAD$^+$ as substratc, rcvcalcd that ARTC2.2 ADP-ribosylatcs a broad spectrum of membrane proteins (8–11). So far, a limited number of ARTC2.2 targets have been characterized. Among them are cell surface receptors such as the interleukin 2 (IL-2) receptor alpha subunit (CD25) (12) and the alpha chain of CD8 (CD8a) (13) molecule, both chains of the integrin LFA1 (11) and the ATP-gated ion channel P2X7 (14).

The functional impact of ADP-ribosylation on the target protein has been extensively studied in case of P2X7. ADP-ribosylation of P2X7 mediates NAD$^+$-induced cell death of T cells co-expressing ARTC2.2 and high levels of P2X7, such as regulatory T cells (Tregs), natural killer T cells, T follicular helper cells and tissue-resident memory T cells (14–19). Consistently, injection of NAD$^+$ induces temporary depletion of Tregs, thereby favoring anti-tumor responses (15). Cells expressing both ARTC2.2 and P2X7 are particularly affected by NAD$^+$ released during cell preparation procedures, i.e. isolation of T cells from spleen, resulting in extensive cell death in subsequent *in vitro* assays or upon adoptive cell transfer (20). Further, it has been shown that ADP-ribosylation of CD25 dampens IL-2 signalling by regulatory T cells, as the presence of NAD$^+$ reduced STAT1 phosphorylation in response to IL-2 stimulation (12). ADP-ribosylation of CD8a inhibits binding to MHCI and ADP-ribosylation of LFA-1 inhibits homotypic binding to LFA1 on other cells (13, 21)

Apart from interference with target protein function, ADP-ribosylation can also affect the binding of monoclonal antibodies. For example, binding of clone 53-5.8 to CD8a is inhibited by ADP-ribosylation whereas clone H35-17.2 is unaffected (13). Similarly, ADP-ribosylation of P2X7 affects binding of clone Hano43, whereas clone Hano44 is unaffected (22).

The functional and technical consequences of ADP-ribosylation of cell surface proteins warrant proteomic investigation of the tissue- or cell-specific ADP-ribosylome. A comprehensive list of ADP-ribosylted target proteins opens the perspective to investigate the potential impact of this post-translational modification on the target protein function. For this, we recently developed a method combining Af1521 macrodomain-based enrichment of ADP-ribosylated peptides with mass spectrometry analyses to identify ADP-ribosylation sites across the proteome (23). Using this approach we previously generated ADP-ribosylomes of HeLa cells and mouse liver (23), mouse skeletal muscle and heart (24), mouse embryonic fibroblasts (25) and mouse microglia (26). The goal of this study was to subject mouse spleen T cells to a comprehensive ADP-ribsylome analyses in order to identify new targets of ARTC2.2-mediated cell surface protein ADP-ribosylation. From T cells incubated with NAD$^+$, we identified 67 ADP-ribosylated target proteins, including 48 plasma membrane and 16 Golgi/ER proteins.

MATERIAL AND METHODS

Mice

C57BL/6 mice were used for all experiments. ARTC2ko mice (Art2b^{tm1Fkn}, MGI#2388827) (27) were backcrossed onto the C57BL/6J background for at least 12 generations. All mice were bred at the animal facility of the University Medical Center (UKE). All experiments involving tissue derived from animals were performed with approval of the responsible regulatory committee (Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/Lebensmittelsicherheit, ORG722, N18/006). All methods were performed in accordance with the relevant guidelines and regulations.

Preparation of Immune Cells

Spleen and liver tissue were mashed through a cell strainer (50 mL falcon strainer, 70 µm, GBO) using a syringe piston. Additionally, liver leukocytes were purified by running a percoll gradient. Cells were resuspended in 5 mL 33% percoll/PBS in a 15 mL Falcon tube, and centrifuged at 1600 rpm, 12°C, for 20 min. The pellet was washed once in PBS (ThermoFisher). Single cell suspensions were kept in FACS buffer containing 1 mM EDTA (Sigma) and 0.1% bovine serum albumin (Sigma). Erythrocytes were lyzed using an ACK lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). Peritoneal macrophages were harvested from the peritoneal cavity by lavage with 5 mL cold PBS + 1 mM EDTA. In order to prevent T cell surface ADP-ribosylation during cell preparation, some mice were i.v. injected with 30 µg of the ARTC2.2-blocking nanobody s+16a (28) 30 min prior to sacrificing.

Antibodies and Flow Cytometry

The following monoclonal antibodies were used for flow cytometric analyses: anti-CD3e-PE (clone 17A2, Biolegend), anti-ARTC2.2-AF647 [clone A109, Prof. Koch-Nolte (29)], anti-CD73-PE (clone TY/11.8, Biolegend), anti-CD8a-FITC (clone 53-6.7, Biolegend), anti-CD11b-FITC (clone M1/70, Biolegend). For protein harvesting, CD3$^+$ T cells from spleen and liver were isolated by fluorescence activated cell sorting (FACS) on a BD FACS Aria III.

T Cell Protein Harvesting

FACS-sorted spleen T cells where subjected to *ex vivo* treatment with 50 µM NAD$^+$ (Sigma) whereas a second preparation of spleen T cells and the liver T cells were left untreated in order to identify targets that were ADP-ribosylated during cell preparation (30). NAD$^+$ was washed away after 15 min of incubation at 4°C and cells were subsequently treated with ARTC2.2-blocking nanobody s+16a for 15 min to avoid ADP-ribosylation of cell surface proteins by intracellular ADP-ribosyltransferases during lysis with denaturing RIPA buffer (Sigma).

Proteomic Sample Preparation and ADP-Ribosylated Peptide Enrichment

For buffer exchange, protein reduction, alkylation, poly to mono-ADP-ribose reduction by PARG (Poly(ADP-Ribose) Glycohydrolase) and tryptic digestion a modified FASP (filter-aided sample preparation) protocol (31) was applied. For each sample 100-200 µg protein extracts were reduced in 1 mM DTT for 30 min and subsequently transferred to a 0.5 mL molecular weight cut off centrifugal filter unit (Microcon-30kDa Milipore, Sigma) and centrifuged until all buffer was passed through the filter. Samples were alkylated for 15 min using urea buffer containing 20 mM chloroacetamide and washed once with 100 µL urea buffer (8 M Urea, 0.1 M Tris-HCl pH 8) and once with 100 µL PARG buffer (50 mM Tris-HCL pH 8, 10 mM $MgCl_2$, 250 µM DTT, 50 mM NaCl). 0.5 µg recombinant PARG enzyme (in-house) in 100 µL PARG buffer was added on to the filter and incubated for 1 h. Filter was subsequently washed with 100 µL 50 mM ammonium bicarbonate buffer. On filter digestion was performed in 100 µL 50 mM ammonium bicarbonate using 5 µg sequencing grade modified trypsin (Promega) at room temperature overnight.

ADP-ribosylated peptide enrichment was performed as previously described (23). The peptide mixture was diluted in PARG buffer (50 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, 250 µM DTT and 50 mM NaCl) and binding was carried out for 2 h at 4°C using the Af1521 macrodomain GST-fusion protein coupled to glutathione-Sepharose beads. Beads were washed three times with PARG buffer and bound peptides were eluted three times with 0.15% TFA. The resulting mixture was desalted using stage tips packed with C18 filters.

Mass Spectrometry Data Acquisition

Samples were analyzed using an Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled to a nano EasyLC 1000 (Thermo Fisher Scientific). Peptides were loaded onto a reverse-phase C18 (ReproSil-Pur 120 C18-AQ, 1.9 µm, Dr. Maisch GmbH) packed self-made column (75 µm × 150 mm) that was connected to an empty Picotip emitter (New Objective, Woburn, MA). Solvent compositions in buffers A and B were 0.1% formic acid in H2O and 0.1% formic acid in acetonitrile, respectively. Peptides were injected into the mass spectrometer at a flow rate of 300 nL/min and were separated using a 90 min gradient of 2% to 25% buffer B. The mass spectrometer was operated in data-dependent acquisition mode and was set to acquire full MS scans from 300–1700 m/z at 60,000 resolution with an automated gain control (AGC) target value of 3×10^6 or a maximum injection time of 110 ms. Charge state screening was enabled, and unassigned charge states and single charged precursors were excluded. The 12 most abundant ions on the full scan were selected for fragmentation using 2 m/z precursor isolation window and beam-type collisional-activation dissociation (HCD) with 28% normalized collision energy. MS/MS spectra were collected with AGC target value of 1×10^6 or a maximum injection time of 240 ms. Fragmented precursors were dynamically excluded from selection for 20 s.

Mass Spectrometry Data Analysis

MS and MS/MS spectra were converted to Mascot generic format (MGF) by use of Proteome Discoverer, v2.1 (Thermo Fisher Scientific). The MGFs were searched against the UniProtKB mouse database (taxonomy 10090, version 20160902), which included 24'905 Swiss-Prot, 34'616 TrEMBL entries, 59'783 reverse sequences, and 262 common contaminants. Mascot 2.5.1.3 (Matrix Science) was used for peptide sequence identification with previously described search settings (32). Enzyme specificity was set to trypsin, allowing up to four missed cleavages. The ADP-ribose variable modification was set to a mass shift of 541.0611, with scoring of the neutral losses equal to 347.0631 and 249.0862. The marker ions at m/z 428.0372, 348.0709, 250.0940, 136.0623 were ignored for scoring. S, R, T, K, E, D and Y residues were set as variable ADP-ribose acceptor sites. Carbamidomethylation was set as a fixed modification on C and oxidation as a variable modification on M. Peptides are considered correctly identified when a mascot score > 20 and an expectation value < 0.05 are obtained. ADP-ribosylation sites were considered correctly localized with a localization probability of > 70%.

Bioinformatic Analyses

For protein network visualization and GO enrichment analyses cytoscape (33), STRING database (v. 11) (34) and the cytoscape string app (35) were used. For the network visualization only highest confidence interactions are shown (≥0.9) and proteins were clustered using the cytoscape string app.

HEK Cell Transfection

Human embryonic kidney (HEK) 293T cells were transfected with a pCMVSport6.1 plasmid encoding mouse *Nt5e* (CD73) using jetPEI transfection reagent (Polysciences Europe). Transfected cells were FACS-isolated every 3 - 4 days for high CD73 expression in order to generate stably transfected HEK cells. The stably transfected $CD73^+$ HEK cells were then co-transfected with pME plasmid encoding for *Art2b* (ARTC2.2) in order to evaluate the impact of ADP-ribosylation on CD73 enzymatic activity.

AMP Degradation Assay

1×10^4 HEK 293 T cells were incubated with 50 µM NAD^+ on ice for 30 min. Cells were washed with FACS buffer twice (1410 rpm, 5 min, 4°C). Cells were resuspended in 100 µL FACS buffer, subsequently 100 µL AMP were added to a final concentration of 10 µM and incubated at room temperature for 40 min. Cells were spin down (1410 rpm, 5 min, 4°C) and 25 µL supernatant was transferred to a solid white plate. 25 µL AMP-Glo Reagent I were added per well, mixed and incubated at room temperature for 30 min. This was followed by addition of 50 µL AMP-Glo Detection Solution per well and incubation for 60 min at room temperature. Plate was read with a plate-reading luminometer.

HPLC CD73 Enzymatic Activity Assay

To determine the AMPase activity by high performance liquid chromatography (HPLC), 0.2×10^6 $CD8^+$ T cells or peritoneal

macrophages were incubated with 1 µM 1,N^6-etheno-AMP (eAMP, Biolog) for 30 min at 37°C. After the incubation, cells were removed (450 × g, 5 min, 4°C) and all samples were passed through 10 kDa size exclusion filters (10,000 × g, 10 min, 4°C, Pall Corporation) and stored at -20°C until analyses. The analyses was performed on reversed-phase HPLC system (Agilent Technologies) with a 250 mm × 4.6 mm C8 Luna column (5 µm particle size, Phenomenex) as stationary phase. The mobile phase consisted of different compositions of HPLC buffer A (20 mM KH_2PO_4, pH 6.0) and B (50% buffer A, 50% methanol), and elution of the nucleotides from the column resulted from an increasing methanol content in the mobile phase [0.0 min (0.0% buffer B), 5.0 min (0.0% buffer B), 27.5 min (100.0% buffer B), 30.0 min (100.0% buffer B), 32.0 min (0.0% buffer B), 43.0 min (0.0% buffer B)]. The signals in both systems were detected by fluorescence detection (230 nm excitation wavelength, 410 nm emission wavelength). Different amounts of etheno-nucleotides (Biolog) were measured to quantify eAMP and the degradation product etheno-adenosine (eADO).

Statistics and Software

For statistical analyses, GraphPad Prism 8 was used. Two groups were compared using the student's t test. Multiple groups were compared using oneway ANOVA in combination with Dunnett's multiple comparison test. Analysis of flow cytometric data was performed using FlowJo (Treestar). The structure model of mouse CD73 (Q61503) was analyzed using Pymol software.

RESULTS

Identification of Potential ARTC2.2 ADP-Ribosylation Targets on T Cells

The aim of this study was to reveal potential ARTC2.2 ADP-ribosylation targets of mouse T cells. These proteins are most likely ADP-ribosylated on arginine residues facing the extracellular environment. Based on our previous studies (24, 26) we hypothesized that it is possible to detect ARTC2.2 mediated ADP-ribosylation by mass spectrometry even on proteins extracted from relatively small numbers of FACS-sorted T cells. We aimed to map ADP-ribosylated proteins of T cells isolated from mice under basal conditions [i.e. after encounter with endogenous extracellular NAD^+ *in vivo* or during cell preparation (30)] and after *ex vivo* treatment with exogenous NAD^+. For this we applied our established mass spectrometry-based strategy with modifications to make it applicable to low sample input (23, 26).

$CD3^+$ T cells were FACS sorted from seven spleens and livers of C57BL/6 mice. Aliquots of cells were incubated for 15min in the absence or presence of exogenous NAD^+. To prevent ADP-ribosylation of intracellular proteins after cell lysis, cells were incubated with the ARTC2.2-blocking nanobody s+16a for 15 min before lysis with RIPA buffer. Proteins were subjected to filter-aided digestion and ADP-ribosylated peptides were enriched using the Af5121 macrodomain (**Figure 1A**). Samples were subsequently analyzed by mass spectrometry to identify peptides and to localize ADP-ribosyl modification sites. Importantly, we used higher-energy collisional dissociation (HCD) for peptide fragmentation, since this allows efficient identification of arginine ADP-ribosylated peptides due to the stability of ADP-ribosyl-arginine but is less effective in localizing serine and other O-linked ADP-ribosylations due to the lability of this modification type in HCD (24, 32, 36).

We identified 93 unique ADP-ribosylated peptides corresponding to 67 proteins (**Supplementary Table 1**). 49 ADP-ribosylated proteins were exclusively identified in T cells treated with NAD^+ (**Figures 1B, C**), 12 ADP-ribosylated proteins were found in both untreated and NAD^+ treated cells, 6 ADP-ribosylated proteins were only identified in the untreated conditions. We obtained confident ADP-ribose site localizations (localization probability >70%, considering R, S, T, Y, E, D, K as variable ADP-ribose amino acid acceptor sites) for 35 unique R- and 1 S-ADP-ribosylation sites (**Figure 1C**).

Taken together, our approach allowed us to identify a considerable number of ADP-ribosylated proteins from a low number of T cells. We observed induction of R-ADP-

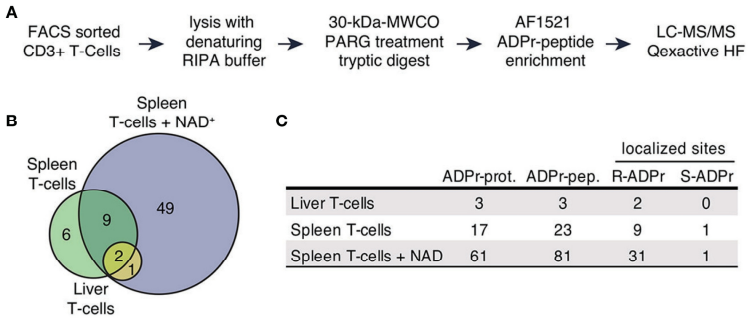

FIGURE 1 | The ADP-ribosylated proteome identified in T cells. **(A)** Schematic workflow of proteomic sample processing, digestion and ADPr-peptide enrichment tailored to the low input protein amount obtained from FACS sorted T cells. **(B)** ADP-ribosylated proteins identified in the three different sample types depicted in a Venn diagram. **(C)** Numbers of uniquely identified ADP-ribosylated proteins, unique ADP-ribosylated peptides and modified amino acids that were confidently localized (localization probability > 70%). Modified arginine and serine sites were found.

ribosylation upon treatment with exogenous NAD^+ indicating active ARTC2.2 on these cells.

Exogenous NAD^+ Induces Extracellular ADP-Ribosylation of Proteins Relevant for the Immune Response

To functionally cathegorize the identified ADP-ribosylation T cell target proteins, we performed gene-ontology (GO) term enrichment analysis, protein-protein interaction network visualization and literature comparisons. GO cellular component (GOCC) term enrichment analyses revealed strong enrichment for ADP-ribosylated proteins to be localized on the cell surface and plasma membrane (**Figure 2A**), providing further evidence that these proteins are most likely targets of ARTC2.2. Other significantly enriched cellular components were the endoplasmic reticulum and Golgi apparatus. GO biological processes (GOBP) were enriched in immune system processes, cell surface receptor signaling, cell adhesion and regulation of T cell activity (**Figure 2A**). Reactome pathway enrichment analysis provided additional separation of ADP-ribosylated proteins into more specific functional terms such as antigen presentation, signaling by interleukins, T cell receptor (TCR) signaling, and integrin cell surface interactions (**Figure 2A**).

Next, we performed protein level visualization of all identified ADP-ribosylated proteins by their association to Reactome pathway terms, relevant protein features, and protein-protein interactions. For this, we plotted the ADP-ribosylated proteins in form of a STRING network (**Figure 2B**) (34). ADP-ribosylated proteins with strong evidence for interactions among each other are connected with a grey line and proteins that had no interaction partners were preserved and shown as unconnected nodes. ADP-ribosylated proteins are color coded based on their affiliation to Reactome pathway terms identified in **Figure 2A**. ADP-ribosylated proteins are additionally marked if they are associated with the plasma membrane or cell surface (GOCC), are an ADP-ribosyltransferase or were already identified under basal conditions. The major hub of interacting proteins targeted by ADP-ribosylation was identified to have a role in TCR signaling, antigen presentation and cell surface integrin interactions. Connected to this cluster was *Nt5e* (5-prime-nucleotidase, CD73), a protein that hydrolyzes extracellular AMP to adenosine (37). An additional hub of interacting proteins consisted of the heteromeric IL-2 receptor complex, including IL2Rα, IL2Rβ and IL2Rγ that were all found to be ADP-ribosylated after addition of NAD^+. We have previously identified IL2Rα as a target of ARTC2.2 and shown that its ADP-ribosylation functionally diminishes IL2 signaling (12). Most ADP-ribosylated proteins present at basal conditions were disconnected from these interaction hubs and less likely localized to the cell surface (**Figure 2B**). Two ADP-ribosyltransferases, ARTC2.2 (*Art2b*) and ARTD8/PARP14 (*Parp14*), were identified to be ADP-ribosylated, potentially by auto modification. Both were found to be ADP-ribosylated under basal and NAD^+ treated conditions. Proteomic identification of ADP-ribosylation sites on ARTCs have previously been observed in mouse liver on ARTC2.2 (23), in mouse microglia cells on ARTC2.1 (26) and on ARTC1 in mouse heart and skeletal muscle tissues (24). ARTC (auto-)ADP-ribosylation can thus serve as a marker for ARTC activity. ADP-ribosylation by ARTD8/PARP14 has previously been associated with immune cell functions (38).

Next, we compared ADP-ribosylation levels of a few selected sites among the different conditions and with our previously published data on mouse liver (23) and microglia cells (26) (**Figure 2C**). ADP-ribosylation of CD73 at R149 was found exclusively on T cells treated with NAD^+. Proteins that were identified in multiple different sample types showed modification on the same site (Pdia3, Slc44a2, Ptprc, Itgb2, Il2rg, H2-K1), or on additional sites (Ptprc, Itgb2, H2-K1) (**Figure 2C**). As exemplified by Pdia3 (a cytosolic protein exclusively modified on R62) and Slc44a2 (a multispan transmembrane protein exclusively modified on extracellular R130), R-ADP-ribosylation showed high site specificity on some proteins. In most of the analyzed cases, the R-ADP-ribosylation sites are located on the extracellular domain of the protein. An exception is the intracellular ARTD8/PARP14, which we found to be modified on S842 under basal and NAD^+ treated conditions, consistent with our recent observation that ARTD8/PARP14 is modified by O-linked ADP-ribosylation (24). In summary, we identified numerous T cell surface proteins with immune system relevant functions that are R-ADP-ribosylated, likely by ARTC2.2, in the presence of exogenous NAD^+.

ADP-Ribosylation of CD73 Reduces the Capability of CD8 T Cells to Generate Adenosine

The majority of the identified T cell surface ADP-ribosylation targets are membrane proteins that act as receptors in cell signalling, antigen presentation or cell-cell adhesion. Apart from ARTC2.2 itself, CD73 was the only identified cell surface enzyme to be ADP-ribosylated. CD73 is expressed on several cell populations of the immune system, including regulatory T cells, $CD8^+$ T cells and macrophages. It converts extracellular adenosine monophosphate (AMP) to adenosine (ADO) (**Figure 3A**), which acts as an immunosuppressant e.g. by inhibiting T cell proliferation (39). While ADP-ribosylation has been shown to impact the function of several cell surface receptors, little is known about the impact of ADP-ribosylation on the enzymatic activity of cell surface enzymes. Therefore, we investigated the impact of ADP-ribosylation on the catalytic activity of CD73. Analyses of the 3D structure model of mouse CD73 (Q61503) revealed that the identified ADP-ribosylation site R149 (red) is distant to the active site (yellow) of CD73 (**Figure 3B**).

We first tested the impact of CD73 ADP-ribosylation in HEK cells stably transfected with mouse CD73 upon transient co-transfection with an expression vector for ARTC2.2. We pretreated these cells with NAD^+ 24 h after transfection, and FACS-sorted equal amounts of $CD73^+ARTC2.2^-$ and $CD73^+ARTC2.2^+$ HEK cells (**Figure 3C**). The gates during cell collections were adjusted for equivalent cell surface levels of CD73 on $ARTC2.2^-$ and $ARTC2.2^+$ HEK cells. We then performed a comparative AMP degradation assay with the

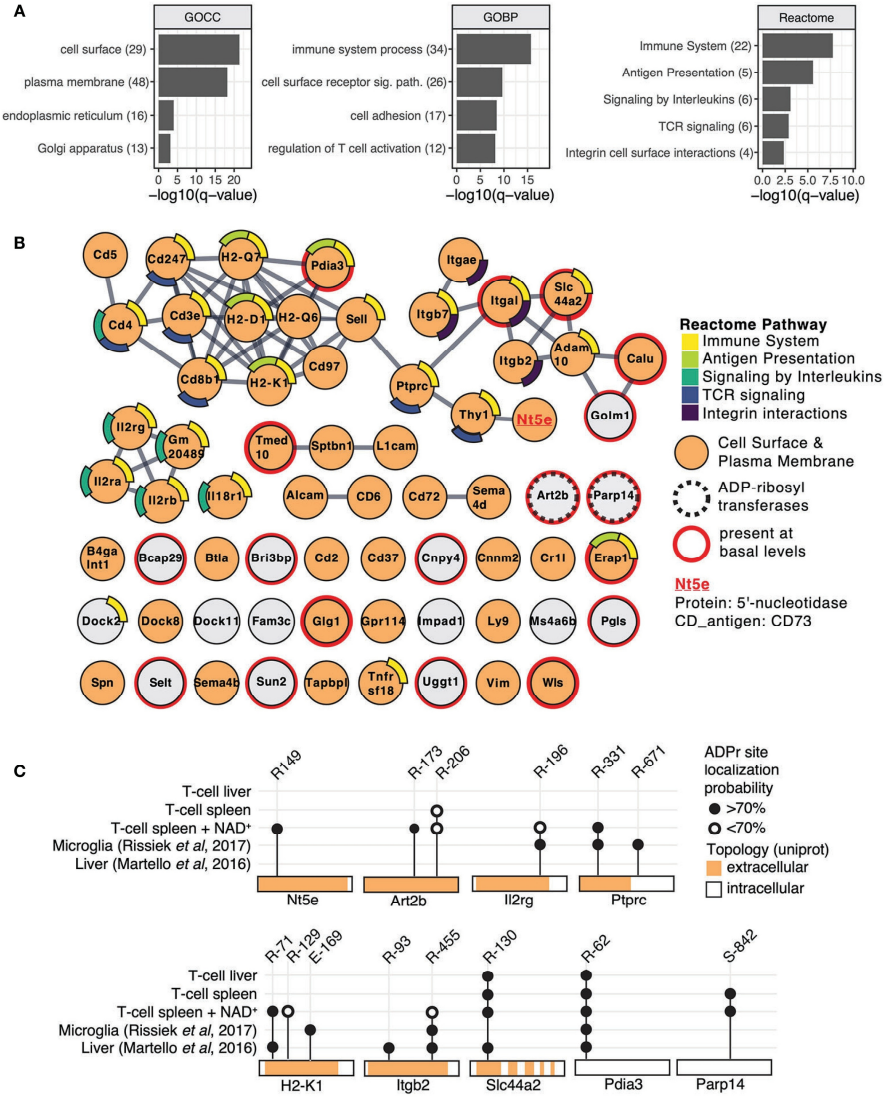

FIGURE 2 | Mouse T cell surface ADP-ribosylome analyses. **(A)** Gene ontology term enrichment was performed for ADP-ribosylated proteins identified in all conditions against the whole mouse genome. Gene ontology biological processes (GOBP), gene ontology cellular components (GOCC) and Reactome pathways were included. The q-values of selected and significantly enriched terms are plotted. Numbers of ADP-ribosylated proteins included in the specific terms are indicated in brackets. **(B)** STRING protein-protein interaction network of ADP-ribosylated proteins identified in all conditions. Gene names of ADP-ribosylated proteins are shown and high confidence protein-protein interactions (STRING interaction score ≥0.9) are indicated with grey lines. Protein nodes are color coded by their affiliation to the Reactome pathways shown in **(A)** Proteins associated with the plasma membrane or cell surface are marked in orange. Proteins that were already identified under untreated conditions are marked with a red line and the two identified ADP-ribosyltransferases with a dashed line. CD73 that was chosen for follow up studies is highlighted. **(C)** ADP-ribosylation sites on selected proteins are plotted and compared to ADP-ribosylation sites identified in (23, 26).

collected CD73$^+$ARTC2.2$^-$ and CD73$^+$ARTC2.2$^+$ HEK cells. We found that NAD$^+$-treated CD73$^+$ARTC2.2$^+$ cells were slightly but significantly less potent in degrading AMP than NAD$^+$-treated CD73$^+$ARTC2.2$^-$ HEK cells (**Figure 3C**), consistent with an inhibitory effect of ADP-ribosylation on CD73 activity.

We next analyzed the impact of NAD$^+$ on the enzymatic activity of CD73 on primary T cells. For this we chose CD8$^+$ T cells, which co-express ARTC2.2 and CD73, but are much less sensitive to NAD$^+$-mediated cell death along the ARTC2.2/P2X7 axis when compared to Tregs (15). We isolated CD8$^+$ T cells

from untreated B6 WT mice, B6 WT mice treated with the ARTC2.2 blocking nanobody s+16a in order to block ARTC2.2 already *in vivo*, or from B6 ARTC2ko mice. Of note, cell surface levels of CD73 were comparable among WT and ARTC2.2ko $CD8^+$ T cells (**Figure 3D**). Since $CD8^+$ T cells express much lower cell surface levels of CD73 than CD73-transfected HEK cells, we used an HPLC-based assay measuring the CD73-dependent generation of etheno-adenosine (eADO) from etheno-adenosine monophosphate (eAMP), as this was a more sensitive approach compared to the AMP degradation assay. We treated all three samples with NAD^+ and quantified the generation of eADO. Here, we observed that NAD^+ treated WT $CD8^+$ T cells generated less eADO compared to $CD8^+$ T cells from s+16a treated WT mice or from ARTC2.2ko mice (**Figure 3D**). Finally, we performed a similar assay with peritoneal macrophages that express high surface level of CD73 but lack ARTC2.2 expression. Here, NAD^+-treated peritoneal macrophages of WT mice generate slightly but significantly more eADO compared to ARTC2ko macrophages, consistent with the fact that a larger fraction of WT peritoneal macrophages expressed high level of CD73 when compared to ARTC2ko peritoneal macrophages (**Figure 3E**). In summary, we could demonstrate that in an NAD^+-rich environment, CD73 enzymatic activity is dampened on cells co-expressing ARTC2.2.

DISCUSSION

In this study we investigated the T cell ADP-ribosylome with a focus on ARTC2.2-mediated ADP-ribosylation of T cell surface proteins. We identified 67 ADP-ribosylated target proteins 48 of which are expressed at the cell surface and 16 of which in the ER or the Golgi apparatus. Of the identified ADP-ribosylation sites 35 were on arginine residues and 1 on a serine residue. Many of the identified target proteins are involved in immune system processes such as signalling, cell adhesion and regulation of T cell

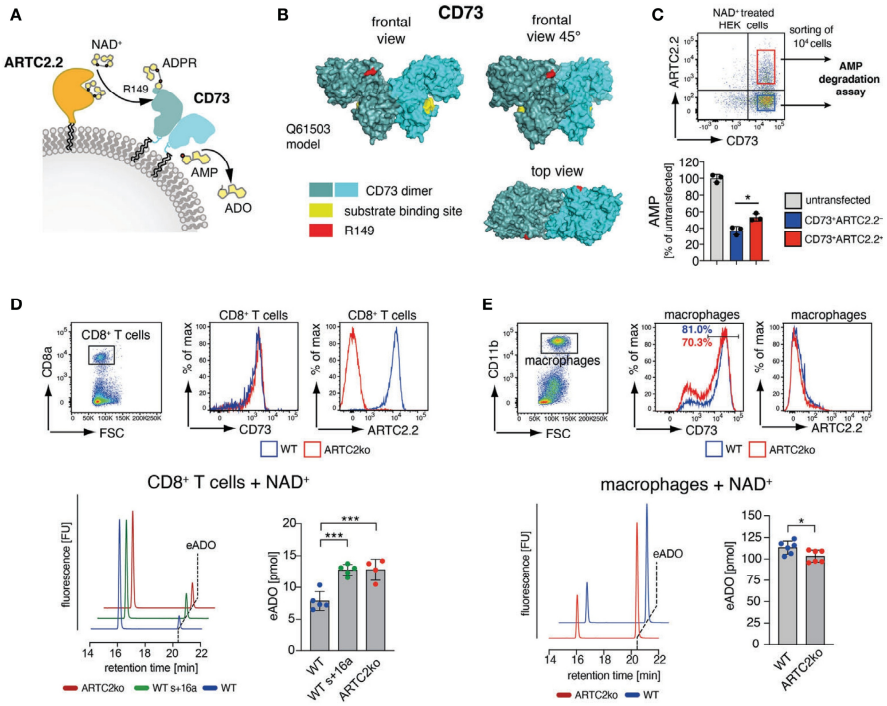

FIGURE 3 | ARTC2.2-dependent regulation of CD73 enzymatic activity. **(A)** CD73 can degrade adenosine monophosphate (AMP) to adenosine ADO. ARTC2.2 ADP-ribosylates CD73 at R149, potentially interfering with enzymatic activity. **(B)** The structure model Q61503 of a mouse CD73 dimer is shown in cyan. ADP-ribosylation site R149 (red) and substrate binding site (yellow) are indicated. **(C)** $ARTC2.2^+CD73^+$ and $ARTC2.2^-CD73^+$ HEK cells (n = 3) were incubated with NAD^+, FACS sorted and compared towards their capacity to degrade AMP in the AMPGlo assay. **(D)** Spleen $CD8^+$ T cells from B6 WT, B6 WT treated with ARTC2.2-blocking nanobody s+16a, and from ARTC2ko mice were analyzed towards CD73 and ARCT2.2 expression. After NAD^+ treatment, FACS sorted cells (n = 4-5 technical replicates) were further subjected to an HPLC-based assay to measure their capacity to generate etheno-ADO (eADO) from etheno-AMP (eAMP). **(E)** Peritoneal macrophages from B6 WT and ARTC2ko mice were analyzed towards CD73 and ARCT2.2 expression. After NAD^+ treatment, FACS sorted cells (n = 6 technical replicates) were analyzed for their capacity to generate eADO. Statistical comparison of the two groups was performed by using the Student's t test, comparison of more than two groups was performed by using oneway ANOVA analyses with Dunnett's multiple comparisons ($p < 0.05$ = */$p < 0.001$ = ***). Data represent results from two **(C, E)** or three **(D)** independent experiments.

activity. It is conceivably that ADP-ribosylation modifies the function of many of these target proteins, thereby fine tuning immune reactions (2, 20). As an example, we analyzed the impact of ADP-ribosylation on the capacity of CD73 to hydrolyze AMP into adenosine. Our results indicate that ADP-ribosylation of CD73 leads to a reduced conversion of AMP to adenosine.

In our study we analyzed the ADP-ribosylome of T cells treated with or without exogenous NAD^+. It is important to note that NAD^+ is also released during the preparation of murine T cells (30), which is sufficient to allow the ADP-ribosylation of T cell surface proteins. Therefore, we can not exclude that at least some of the observed ADP-ribosylated T cell surface proteins detected under basal conditions without addition of exogenous NAD^+, such as *Slc44a2* or *Itgal*, identified in untreated T cells from spleen or liver were modified during cell isolation. To further investigate whether the observed ADP-ribosylation of proteins occurred during the cell isolation or is catalyzed *in vivo*, systemic injection of the ARTC2.2 blocking nanobody s+16a prior to cell harvesting would prevent ADP-ribosylation during preparation (17, 28). However, this would also block ADP-ribosylation *in vivo* during the time between injection and the sacrifice of mice, but would not prevent de-ADP-ribosylation by enzymes. In order to identify further targets that are ADP-ribosylated *in vivo* it might thus be necessary to block de-ADP-ribosylation. Finally, it would also be interesting to compare the ADP-ribosylome of different T cell types such as $CD4^+$ helper T cells, $CD4^+$ regulatory T cells or cytotoxic $CD8^+$ T cells as well as the ADP-ribosylome of the same T cell population from different organs, e.g. spleen and liver. However, this would probably need large amounts of cell material to start with.

Our analyses of proteins derived from NAD^+ treated T cells identified known and new targets of ARTC2.2-mediated ADP-ribosylation. We confirmed already known ARTC2.2 targets such as CD25 (*Il2rg*), CD8β (*Cd8b*) and CD45 (*Ptprc*). For CD25 we confirmed R196 (R178 in the mature protein) as ADP-ribosylation site, as reported in a previous study (12). Similarly, CD45 was found to be ADP-ribosylated on R331 of cells analyzed in the microglia study (26). Of note, the T cells used in this study were isolated from C57BL/6 (B6) mice, whereas the microglia study used cells from Balb/c mice. B6 mice carry a premature stop codon in the gene for the ARTC2.1 (40), and B6 T cells therefore exclusively express ARTC2.2 as cell surface ADP-ribosyltransferase (41). Balbc microglia express the thiol-activated ARTC2.1, but not ARTC2.2 and Balb/c T cells co-express ARTC2.1 and ARTC2.2 (5, 6). The finding of the same ADP-ribosylated targets on microglia and T cells indicates that these two closely related ADP-ribosyltransferases may share common targets and modify common sites.

MHC class I (MHC-I) molecules are yet not well characterized regarding the potential impact of ADP-ribosylation. In this study we identified H2-D, H2-K and the MHC-Ib molecule H2-Q to be ADP-ribosylated on T cells. MHC-I molecules present endogenous peptides to $CD8^+$ T cells. Therefore, it would be interesting to test if MHC-I ADP-ribosylation affects its interaction with the T cell receptor (TCR) or loading of the peptide to form the MHC-I/peptide complex. Indeed, the identified R169 ADP-ribosylation site in H2-D1 is in close proximity to the TCR interaction surface (see PDB file 5m01). Further, a former study showed that ADP-ribosylation of the CD8β T cell coreceptor affects MHC-I/TCR interaction (13). It would thus be interesting to investigate, whether MHC-I ADP-ribosylation diminishes TCR binding in a similar fashion. Moreover, it is tempting to speculate that MHC-I ADP-ribosylation has an impact on TCR binding in an antigen-specific fashion: introduction of an ADP-ribose group at the MHC-I/TCR interaction site could lead to the activation of alternative $CD8^+$ T cell clones that recognize this modified MHC-I/peptide complex. Future studies should address this hypothesis.

The list of ADP-ribosylation targets on T cells identified here is probably underestimated. The ATP gated P2X7 ion channel for example, a prominent and well characterized ARTC2.2 target on T cells (14), was not identified as target for ADP-ribosylation by our MS approach. P2X7 is expressed on regulatory T cells, NKT cells and CD4 effector/memory T cells (15, 17, 42) – together these cells constitute only a minor fraction of the T cells analyzed here. Therefore, the amount of available P2X7 proteins might have been below the detectable threshold. Furthermore, it is possible that ADP-ribosylated peptides are lost during sample preparation or mass spectrometry analysis due to technical circumstances.

In this study, we focused on the functional impact of CD73 ADP-ribosylation. CD73 is a ecto-5′-nucleotidase that generates immunosuppressive adenosine from AMP and thus plays a critical role in balancing the course of an inflammatory reaction (43). From a technical point of view, it is worth noting that adenosine is rapidly degraded to inosine by adenosine deaminase (ADA), both *in vivo* and *in vitro*. The etheno-adenosine (eADO) used in our HPLC-based assay to monitor and quantify CD73 enzymatic activity, however, is not a substrate for ADA (44) and therefore much more stable. Further, it has been recently shown that eADO is not taken up by cells *via* adenosine transporters (45). Therefore, differential degradation or uptake resulting in an experimental bias seems unlikely to explain the impact of NAD^+ on the CD73 enzymatic activity. The identified ADP-ribosylation site at R149 is distant from the active site of CD73 and is therefore likely to act allosterically. When comparing $CD8^+$ T cells and macrophages, NAD^+ only had a dampening impact on the catalytic activity of CD73 on $CD8^+$ T cells that co-express ARTC2.2 but not on macrophages that lack ARTC2.2. Therefore, CD73 ADP-ribosylation might be a T cell-specific mechanism that modulates CD73 activity in an NAD^+ rich microenvironment, such as tumor tissue. Here, NAD^+ could be released along with ATP during tumor cell secondary necrosis. Indeed, prostate cancer cell lines have been reported to actively release intracellular NAD^+ into the culture medium (46). $CD8^+$ T cells play a critical role in anti-tumor immune responses. CD73 on CD8 T cells seems to significantly contribute to the anti-tumor immunity response, since adoptively transferred CD73-deficient ovalbumin-specific OT-I T cells were more potent in killing OVA-expressing B16 melanoma tumors compared to

WT OT-I T cells (47). This was accompanied by lower expression levels of the exhaustion markers programmed cell death protein 1 (PD-1) and CD39, strengthening the role of CD73 as an immune checkpoint and as a potential target in tumor therapy. Vice versa, it would be interesting to evaluate whether ARTC2-deficient OT-I T cells are less potent in killing OVA-expressing B16 melanoma, as CD73 activity would not be dampened by NAD^+ in the tumor environment.

Interestingly, both ARTC2.2 and CD73 can be shed from T cells (48, 49) and it has recently been shown that soluble ARTC2.2 can ADP-ribosylate various cytokines, including IFNγ (50). Therefore, it would be interesting to elucidate whether soluble ARTC2.2 could also ADP-ribosylate the soluble form of CD73 and thereby control the cell-independent generation of adenosine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/ Lebensmittelsicherheit.

AUTHOR CONTRIBUTIONS

BR and SM collected and prepared samples for mass spectrometry. ML performed mass spectrometry experiments, data collection and analyses. YD and RW performed CD73 functional assays. ET and TM assisted with CD73 functional assay data analyses and interpretation. FK-N and MH supervised the experiments and assisted with data interpretation and manuscript preparation. BR and ML assembled the figures and wrote the manuscript, which has been reviewed by all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.703719/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4 Discussion

In the publications presented in this thesis, we show expression patterns, immune-related functions, and regulatory mechanisms of enzymes and receptors of the purinergic system. We could show that human innate-like T cells express higher levels of P2X7 than conventional T cells, which makes them susceptible to ATP-induced cell death. The ectonucleotidases CD39 and CD73 rapidly degrade ATP, and we characterized the expression and function of both enzymes on human T cells. We show that CD73 is released on EVs from effector T cells upon T cell activation. The enzymatic activity of CD73 on EVs is preserved and mediates, in combination with Tregs, immune suppression. Furthermore, we have identified murine CD73 as target for ADP-ribosylation. The release of CD73 in EVs and the decrease of the enzymatic activity of CD73 through ADP-ribosylation constitute mechanisms that alter CD73 function, and affect the outcome of an immune response.

In this discussion, I will focus on the multiplicity of purinergic enzymes and receptors involved in the outcome of purinergic signaling events on T cells, their effect on the immune response, and the relevance of understanding this complex system for translational research. I will also discuss the differences between the expression and function of purinergic molecules in mice and humans. Finally, I will summarize current technologies available for measuring adenine nucleotides as well as techniques that would be useful in the future to further disentangle mechanisms of the purinergic system. The role of adenine nucleotides in inflammatory responses and cancer, and the huge efforts already made to develop therapeutics to intervene in purinergic signaling pathways, underscore the need for understanding the function of purinergic enzymes and receptors under physiological and pathological conditions.

4.1 The conundrum of ATP signaling through the human P2X7 receptor

Although single publications report the release of adenosine from the intra- to the extracellular space through transporters (Almeida et al. 2003), it is commonly accepted that the main source of extracellular adenosine is ATP. This leads to the paradoxical situation that a pro-inflammatory mediator must be released to generate an anti-inflammatory mediator, and raises the question of how the equilibrium between inflammation and tolerance is regulated and homeostasis is restored (Faas, Sáez, and de Vos 2017). Some researchers focus their research on ATP as ligand for P2 receptors without taking into account its degradation

to adenosine. Others see ATP merely as source of adenosine, without considering the presence of P2 receptors and potential ATP-mediated pro-inflammatory signaling (Nascimento et al. 2021; Ingwersen et al. 2016). Whether ATP leads rather to a stimulation of P2 receptors or to the generation of adenosine is most likely cell type- and tissue-specific. Peripheral blood Tγδ cells, for example, do not express CD39, making them presumably poor in degrading ATP, which could favor P2 receptor signaling. In the intestine, an ATP-rich environment, Tγδ cells have a high CD39 expression (Libera et al. 2020), and I would speculate that they are able to degrade ATP from their microenvironment and, consequently, avoid extensive P2 receptor activation.

Activation of the human P2X7 receptor requires concentrations of extracellular ATP in the high micromolar to millimolar range (Jacobson, Jarvis, and Williams 2002). Early studies described an enhancing effect of P2X7 on T cell activation events such as IL-2 release, when the concentration of ATP is moderate (up to 100 µM) (Yip et al. 2009). In our hands, physiological concentrations of ATP released during T cell stimulation did not alter the activation of conventional or innate-like T cells because addition of the specific P2X7-blocking nanobody Dano1 had no effect on the upregulation of CD25 and cell proliferation (Winzer et al. 2022). When adding exogenous ATP to a proliferation assay, we observe a reduction in proliferation. This effect can be blocked with a CD73 inhibitor, demonstrating that the reduced proliferation occurs due to ATP degradation to adenosine and subsequent activation of A2A receptors. The addition of AMP or adenosine in concentrations of 50 µM already reduces T cell activation significantly (Schneider, Winzer et al. 2021). Considering these data, it seems more likely that ATP released during T cell activation is rather degraded to adenosine and acts on A2A receptors than activating P2X7. We observed P2X7-mediated effects in human peripheral blood T cells only after treatment with >1 mM ATP, resulting in cell death particularly of Tγδ cells, which have the highest P2X7 expression in the T cell compartment. We propose that the depletion of Tγδ cells in an ATP-rich environment could avoid Tγδ-mediated tissue damage and immune pathology (Winzer et al. 2022). In vivo, however, there might be factors that facilitate P2X7 signaling already with lower ATP concentrations. For years, researchers working with the human P2X7 receptor have wondered how millimolar ATP concentrations needed for P2X7 activation can be reached in the extracellular space. One explanation could be ATP release of neighboring cells due to cell death or massive inflammation. In this situation,

ATP concentrations in the millimolar range can only be reached pericellulary during a very short time. Additionally, unknown mechanisms may positively modulate the sensitivity of human P2X7 towards ATP. The murine P2X7 receptor can be activated with intermediate ATP concentrations (>100 µM) or low NAD^+ concentrations (<30 µM) upon ARTC2.2-mediated ADP-ribosylation of the receptor (Seman et al. 2003). Humans do not express ARTC2.2, and the isoform ARTC1 is not expressed on immune cells. It is plausible that ARTC1 modulates P2X7 on neighboring cells (Wennerberg et al. 2022), or is released as soluble protein. Moreover, murine P2X7 can be positively modulated by nanobodies (Danquah et al. 2016), and human P2X7 can be positively modulated with the antiparasitic drug ivermectin, the antibiotic polymyxin B, the plant metabolite compound K and the antimicrobial peptide LL-37 (Tomasinsig et al. 2008; Bidula et al. 2019; Ferrari et al. 2004; Nörenberg et al. 2012), altogether suggesting that there may exist physiological modulators of the receptor. A modulation of the P2X7 receptor could facilitate activation at micromolar ATP concentrations available in the pericellular environment during T cell activation.

P2X7 is not the only ATP receptor expressed on human T cells, and all other P2 receptors are activated already with lower ATP concentrations (Figure 4) (Xing et al. 2016). Our work demonstrates that ATP-induced cell death is exclusively mediated by P2X7 because it can be completely blocked with the P2X7-inhibiting nanobody Dano1. The influx of Ca^{2+}, however, occurs through different ATP-gated ion channels, and cannot be completely blocked with Dano1 in Tγδ cells (Winzer et al. 2022). Based on gene expression data, *P2RX4* is expressed on different T cell populations, with the highest expression on CD4 memory T cells (Uhlen et al. 2019). The sensitivity of P2X4 for ATP is around 100-fold higher than that of P2X7, suggesting that P2X4 rather than P2X7 is activated during T cell activation. P2X4 is, however, expressed on lysosomes, and it is not yet clear under which circumstances it is transported to the cell membrane (Kanellopoulos et al. 2021). In our experiments, the combination of the P2X7 inhibitor Dano1 and the P2X4 inhibitor 5-BDBD abolished ATP-induced Ca^{2+} influx in Tγδ cells, indicating that P2X4 is at least to some extent present at the cell surface (data available in the peer review file of Winzer et al. 2022). P2X4 also contributes to NFAT activation and IL-2 expression in P2X4 receptor-overexpressing Jurkat T cells (Woehrle et al. 2010), and to the formation of Ca^{2+} microdomains in murine T cells (Brock et al. 2022). Human CD4 T cells and Jurkat T cells also express *P2RX1* and *P2RX5* (Woehrle et al. 2010). Furthermore, the GPCR

P2RY11 has a similar expression profile as *P2RX7*, with a high gene expression in MAIT and Tγδ cells (Uhlen et al. 2019). P2Y11 is only expressed in humans, and counteracts P2X7-mediated pore formation and cell death (Dreisig et al. 2018), adding one more level of regulation to the activity of P2X7.

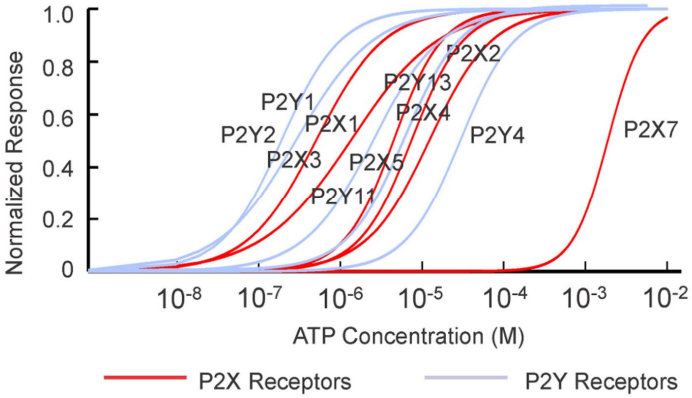

Figure 4. ATP sensitivity of human P2 receptors. P2 receptors differ in their responsiveness to ATP. P2X7 needs much higher ATP concentrations for activation than all other P2 receptors. Figure obtained and adapted from Xing et al. (Xing et al. 2016).

In conclusion, it is almost impossible to predict if extracellular ATP induces P2 receptor signaling or is immediately degraded by CD39 or other ATPases and elicits adenosine receptor signaling. The human P2X7 receptor requires high extracellular ATP concentrations for activation, suggesting that P2X7 is only activated under very specific conditions (e.g. at sites of inflammation or cell death), or that P2X7 may be modulated in vivo to increase its ATP sensitivity. ATP sensors and specific inhibitors for P2 receptors and for ATP-degrading enzymes would help to determine the fate of ATP in the extracellular space (see chapter 4.4).

4.2 Complexity of the purinergic system

The rapid metabolism of extracellular adenine nucleotides by purinergic enzymes, together with the dynamic receptor activation, requires studying the purinergic signaling cascade as a system rather than analyzing the role of individual proteins. The complexity of the system is reflected in additional enzymes involved in ATP degradation beyond the very well studied CD39 and CD73, post-translational modifications of purinergic molecules, and the recently discovered possibility of purinergic enzymes released from cells as soluble molecules or contained in vesicles.

Enzymes involved in the metabolism of adenine nucleotides

The availability of ATP and adenosine in the close cellular environment determines the shift towards P2 or P1 receptor signaling on target cells. CD39 and CD73 are the better known enzymes responsible for ATP degradation and adenosine generation (Figure 5A and chapter 1.4), but also other enzymes are involved in the metabolism of adenine nucleotides, shifting the signaling either towards a P2 or a P1 receptor response. These 'alternative players' are enzymes that either i) 'feed' the canonical pathway by generating substrates for CD39 or CD73, ii) take over the role of CD39 or CD73, or iii) irreversibly remove adenosine from the milieu. The activities of these enzymes are often referred to as the non-canonical purinergic pathway (Figure 5B-D). Further, nucleoside transporters influence the extent of purinergic signaling by shuffling adenosine through the cell membrane.

The ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1, also known as CD203a or PC-1) hydrolyzes the direct cleavage of two phosphates from ATP to yield AMP. ENPP1 has also other substrates, including ADPR. In combination with CD38 or CD157, ENPP1 enables the generation of AMP from NAD^+ (Figure 5B) (Horenstein et al. 2013; Deterre et al. 1996). Our own unpublished data shows that ENPP1 is only marginally expressed on the cell membrane of human T cells, with the exception of MAIT cells, but rather present on two very specific subsets of innate immune cells, $CD141^{high}$ DCs and $CD56^{bright}$ NK cells. A soluble form of ENPP1 is present in plasma and serum (Belli, van Driel, and Goding 1993; Jansen et al. 2012), and it may be possible that soluble ENPP1 contributes to ATP degradation in bodily fluids.

Alkaline phosphatases (AP) constitute a class of enzymes with broad substrate specificity that dephosphorylate a variety of targets. APs are divided into four groups: tissue-nonspecific AP (TNAP), intestinal AP, placental AP, and germ cell AP. The expression of each isoform is tissue-dependent, with TNAP being broadly expressed in e.g. lung, brain, liver, and kidney. Lymphocytes do not express APs, but APs exist as soluble proteins (Pettengill et al. 2013), potentially enabling nucleotide metabolism in proximity to lymphocytes. ATP, ADP, and AMP count among the multiple substrates of APs, so it is plausible that APs take over the role of both CD39 and CD73 (Figure 5C), especially in the context of forced absence of these enzymes. Indeed, APs contribute significantly to the AMPase activity in the kidney and brain of CD73 knockout mice (D. Zhang et al. 2012; Jackson et al. 2014), indicating a compensatory mechanism in the absence of CD73. Also in patients with CD73 deficiency, the activity of TNAP

is increased (Jin et al. 2016). The increased degradation of pyrophosphate (PP_i) to phosphate (P_i) by TNAP in CD73 deficient patients decreases bone mineralization and promotes arterial calcification, leading to severe disease phenotypes (Jin et al. 2016). In the experiments described in this thesis, we used the highly specific CD73 inhibitor PSB-14685 (Schneider, Winzer et al. 2021; Bhattarai et al. 2020) to verify that the observed AMPase activity derives from CD73, or we performed the measurements in phosphate buffer to inhibit AP-mediated activity.

With a half-life between five and ten seconds, the turnover of adenosine in bodily fluids and cell culture systems is extremely fast (Klabunde 1983). The reason is a rapid degradation of adenosine to inosine by ADA (Figure 5D). ADA is found as a soluble enzyme or, in humans, bound to the cell surface of T cells through CD26 (dipeptidyl peptidase-4, DPP4) (De Meester et al. 1994; Gao et al. 2018). Conventional T cells upregulate CD26 upon activation, which enables ADA binding and prevents adenosine-mediated auto-inhibition of the cells by rapid pericellular degradation of adenosine. Tregs do not express CD26 and, consequently, do not have ADA bound to the cell surface. They are more effective in suppression, potentially due to increased pericellular adenosine concentrations (Salgado et al. 2012; Mandapathil et al. 2012). ADA deficiency leads to a form of severe combined immunodeficiency (SCID) because of the accumulation of intracellular deoxy-adenosine and deoxy-ATP, and extracellular adenosine (Whitmore and Gaspar 2016). When assessing the effect of adenosine on the activation and proliferation of responder T cells, we always added the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) to the cells to prevent the degradation of adenosine (Schneider, Winzer et al. 2021). This is of course an artificial system and it is possible that we are overestimating the effect of adenosine by this experimental design, however, it is currently not possible to mimic a cellular system in which there is a constant source of ATP and a controlled metabolism of adenine nucleotides.

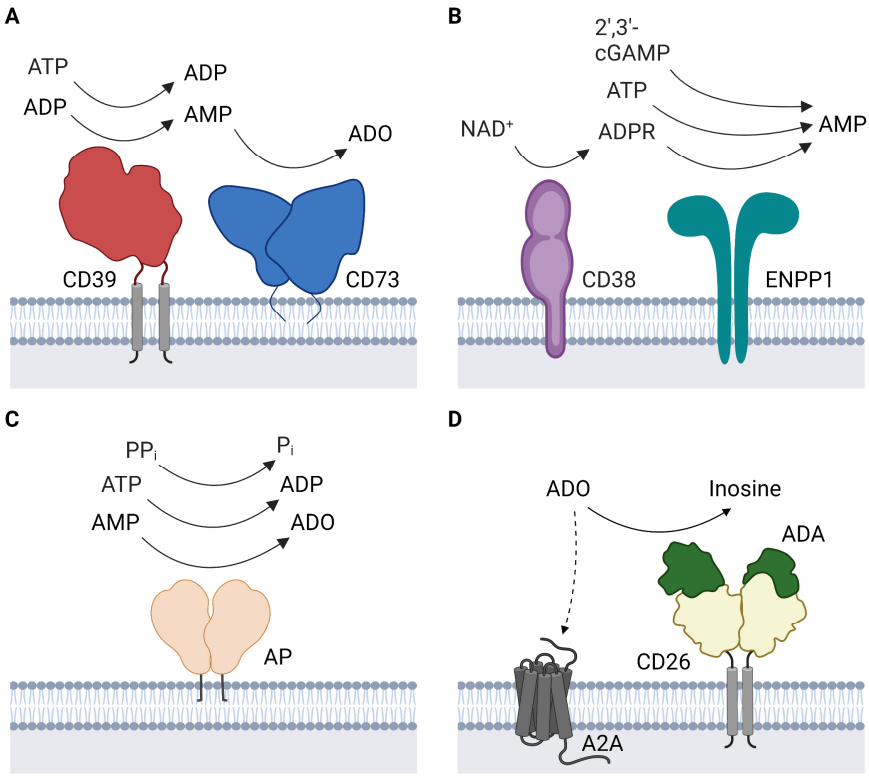

Figure 5. Extracellular adenine nucleotides are metabolized by a variety of enzymes. (A) CD39 and CD73 degrade ATP to adenosine in a concerted action. (B) ENPP1 has a broad substrate specificity and is e.g. able to generate AMP from ATP. (C) APs release P_i from several substrates, including ATP and AMP. (D) ADA rapidly degrades adenosine to inosine, preventing adenosine receptor signaling. Figure created with BioRender.com.

Transcriptional and post-translational regulation of purinergic enzymes and receptors

Purinergic enzymes and receptors can be modified to enhance or block their function. An example for a protein regulated on multiple levels is CD73 (Alcedo, Bowser, and Snider 2021). On the transcriptional level, transcription factors HIF-1α, STAT3, and SP1 promote CD73 expression (Morianos et al. 2020; Synnestvedt et al. 2002; Kordaß, Osen, and Eichmüller 2018; Hansen et al. 1995). miRNAs, e.g. miR-422a and miR-30a, bind to the 3‘-untranslated region of *NT5E* mRNA and prevent translation (Kordaß, Osen, and Eichmüller 2018). Post-transcriptionally, alternative splicing generates a shorter transcript variant of CD73 that lacks AMPase enzymatic activity, is unable to dimerize, and builds a complex with canonical CD73 leading to proteasome-dependent degradation of the functional CD73. The truncated variant is found in low amounts in healthy tissue, but is increased in liver cirrhosis and hepatocellular carcinoma (HCC) (Snider et al. 2014). Post-translationally, the CD73 protein is modified by *N*-linked glycosylation. An altered glycosylation pattern, as found in HCC tumors, decreases CD73 enzymatic activity (Alcedo et al. 2019).

We present here another mechanism how murine CD73 can be post-translationally modified by mono-ADP-ribosylation, a modification already well described for the P2X7 receptor and many other non-purinergic proteins (Seman et al. 2003; Teege et al. 2015; Lischke et al. 2013; Menzel et al. 2021). For murine P2X7, ARTC2.2 transfers the ADPR moiety of NAD^+ to the P2X7 receptor, mimicking ATP binding and leading to receptor activation in an NAD^+-rich environment. The ADP-ribosylation of CD73 on murine T cells, mediated by the same enzyme, reduces the enzymatic activity of CD73 (Leutert et al. 2021). The ADP-ribosylation site of CD73 is distant to its active site, but CD73 undergoes major conformational changes during substrate binding and cleavage (Knapp et al. 2012), and it is plausible that ADP-ribosylation impairs this movement. Recently, ADP-ribosylation has been described for human CD73 and P2X7. Recombinant ARTC1 mono-ADP-ribosylates human CD73 at six arginine residues, some of them at positions likely relevant for CD73 dimerization or substrate binding, and leads to an impaired generation of adenosine (Hesse et al. 2022). Because ARTC1 is not expressed in human immune cells (Uhlen et al. 2019), the relevance of CD73 mono-ADP-ribosylation for adenosine production by T cells is questionable. However, co-expression of ARTs with its targets may not be necessary as tumor cell-derived ART1 ADP-ribosylates P2X7-expressing tumor-infiltrating CD8 T cells, rendering them susceptible to NICD (Wennerberg et al. 2022). The cleavage of ARTs from the cell membrane and their release as soluble proteins would also overcome co-expression with its targets. Membrane cleavage has been shown for murine ARTC2.2, and is predicted for human ARTC1 (Kahl et al. 2000; Menzel et al. 2015).

Extracellular vesicles

Purinergic enzymes and receptors exist as soluble proteins or, as highlighted in this thesis, on EVs. The occurrence of CD73 on T cell-derived EVs has been extensively discussed in the publications (Schneider et al. 2019; Schneider, Winzer et al. 2021; Schneider 2020), however, also other purinergic molecules are contained in EVs derived from cancer cells, stem cells, T cells, or endothelial cells (Figure 6) (Carotti et al. 2022). CD39 and CD73 are both present on tumor-derived EVs (Clayton et al. 2011; Morandi et al. 2018; Schuler et al. 2014). EVs from the bone marrow of patients with multiple myeloma additionally contain CD38 and ENPP1 (Morandi et al. 2018). Serum $CD19^+$ B cell-derived EVs, increased in colon cancer patients, express CD39 and CD73, and decrease post-chemotherapeutic CD8 T cell responses (F. Zhang et al. 2019). Treg-derived EVs contain CD73 and CD39, potentially contributing to their

immunosuppressive capacity (Smyth et al. 2013; Tung et al. 2020). Moreover, purinergic signaling is involved in regulating the secretion of EVs as it has been shown that ATP induces the release of EVs from microglia and tumor cells in a P2X7-dependent way (Bianco et al. 2005; Ruan et al. 2020; Pegoraro et al. 2021; Lombardi et al. 2021). ATP is also contained as cargo in vesicles (Figure 6) (Sakaki et al. 2013; Graner 2018).

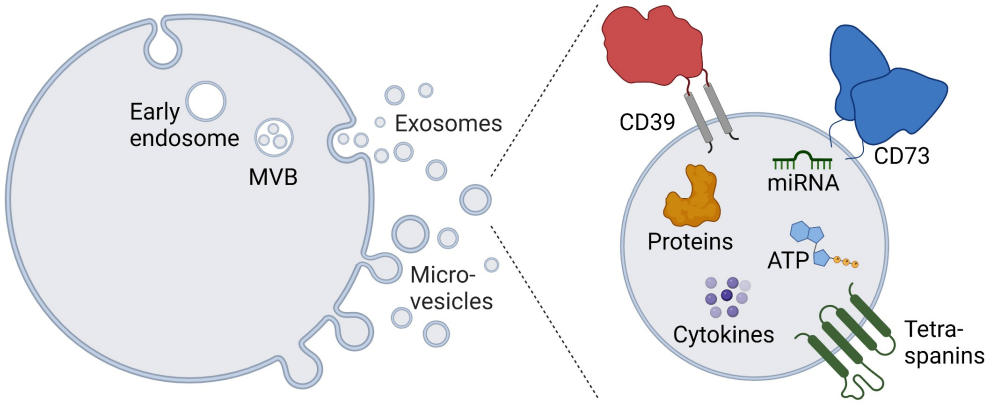

Figure 6. Extracellular vesicles carry purinergic enzymes and other cellular content. EVs include exosomes and microvesicles, which are generated via the endosomal pathway and by membrane budding, respectively. EVs reflect their cell of origin in terms of protein expression and cargo. It has been shown that purinergic enzymes are released on EVs. Figure created with BioRender.com.

The release of purinergic enzymes on EVs increases their spatial range of action by bringing the potential to metabolize adenine nucleotides away from the donor cell. This could as well protect the donor cells for example from the suppressive effect of adenosine, as well as facilitate local adenosine production close to target cells, particularly relevant when taking into account the fast turnover of adenosine. We propose that an advantage of releasing CD73 on EVs is an increased half-life and a better distribution of the enzyme in bodily fluids when compared to the soluble protein (Schneider et al. 2019). There are many open questions related to the function of EVs. For instance, it is not yet known how far EVs can ‘travel’ in vivo or which signals poise them to fuse with a target cell. Most studies analyzing tissue distribution and uptake of EVs rely on injecting pre-labeled EVs in mice, in combination with in vivo imaging (Arifin, Witwer, and Bulte 2022). After injecting luciferase-labeled EVs intravenously in mice, EVs were rapidly distributed in the body and eliminated via hepatic and renal routes within six hours (Lai et al. 2014). Other groups were still able to detect EVs from luciferase-transfected cancer cell lines in the lung, liver and spleen of mice after nine days (Gangadaran et al. 2017). Magnetic resonance imaging (MRI), X-ray, and positron emission tomography (PET) are other methods commonly used for in vivo EV imaging (Arifin, Witwer, and Bulte 2022). Due to the

small size of EVs, most imaging techniques cannot discriminate between EV binding and uptake. Attaching a dual EGFP-Renilla luciferase-split tag to tetraspanins allowed the measurement of EV binding to a cell by EGFP fluorescence and EV uptake by luminescence (Toribio et al. 2019). Injection of exogenously labeled EVs for imaging biases the EV biodistribution depending on the site of administration, and labeled EVs may differ in their properties compared to endogenous EVs. To analyze EVs in vivo under more physiological conditions, prostate cancer cells were transfected with the bioluminescence resonance energy transfer (BRET)-based CD63-Antares2-reporter and transplanted into mice. The EVs released by the tumor cells were followed for up to 35 days, and could be found in blood, with a bioluminescence intensity correlating with tumor size, and in different organs, including lungs, stomach, intestine, and genital glands (Hikita et al. 2020). Incubation of T cells with EVs from a CD73-GFP-overexpressing glioblastoma cell line results in CD73 expression of the T cells, indicating EV uptake (Wang et al. 2021). Whether all and to which extent EVs fuse with target cells and equip them with previously not expressed purinergic enzymes is not yet known (Prada and Meldolesi 2016; Mathieu et al. 2019).

EVs and EV-related cargo are associated with several diseases, making them interesting as diagnostic biomarkers, while their easy administration and bioavailability puts them in the focus for therapy. miRNA or proteins contained in EVs are discussed as biomarkers for e.g. rheumatoid arthritis and diabetes, or to predict the severity of COVID-19 (Fujita et al. 2021; Garcia-Contreras et al. 2017; Buzas 2022). Patients with rheumatoid arthritis show an increased plasma concentration of EVs, and EVs can increase the inflammation by containing e.g. pro-inflammatory cytokines like TNF-α (Withrow et al. 2016; Schioppo et al. 2021). On the other side, we have demonstrated an immune regulatory role for SF-derived EVs (Schneider, Winzer et al. 2021). Cancer cell line-derived EVs produce adenosine through CD39 and CD73, contributing to the suppression of T cells in the tumor environment (Clayton et al. 2011). Compared to cell-based therapy and soluble drugs, EVs may offer advantages including a low immunogenicity, the potential to cross biological barriers, and the cell-specific fusion and internalization (Arifin, Witwer, and Bulte 2022; Noren Hooten et al. 2020). Yet, open questions regarding their in vivo targeting mechanisms, biodistribution, stability, and off-target effects must be answered first.

The role of EVs in intercellular communication and their potential use as therapeutics placed EVs into a main focus of research. The presence of purinergic enzymes in EVs adds complexity and extends the scope of purinergic signaling, and may have major effects on the outcome of an immune response.

4.3 Differences in murine and human purinergic signaling pathways

It is virtually impossible to study complex cellular processes and in vivo effects of treatments in humans, therefore we use mouse models to dissect immunological processes. However, there are obvious differences between human and mouse physiology, and the impact of genetic and environmental factors cannot be detected when studying mice. The purinergic system stands out as an example of difference between mice and humans.

The generation of anti-inflammatory adenosine by the concerted action of CD39 and CD73 is a well described immunosuppressive mechanism of murine Tregs (Deaglio et al. 2007). While murine Tregs express both CD39 and CD73 on the cell surface, human Tregs express CD39 (the frequency is highly dependent on the genotype of the donor, see chapter 1.4.1), but mostly no CD73. This conundrum is known for many years, however, the 'desire to fit' in translational research has pushed researchers to overestimate CD73 in Tregs and overlook data that were discordant to findings in the murine system (Da et al. 2022). The CD73 expression is not only different on Tregs, but also almost all conventional CD4 and CD8 T cells in the mouse express CD73, while CD73 expression in humans is high on naïve CD8 T cells, but rather low on circulating memory CD8 and CD4 T cells (Raczkowski et al. 2018). This opens up the question how adenosine is generated in human T cells. The release of CD73 by effector T cells on EVs offers a possibility how CD73 can interact with Treg-expressed CD39 to enable adenosine production (Schneider, Winzer et al. 2021). A reason why CD73 expression is different in mice and humans could be that adenosine receptor signaling affects Tregs and effector T cells in a different way in each species. Adenosine signaling inhibits the activation of both Tregs and effector T cells in humans (Baroja-Mazo et al. 2018), while it does not block, but even enhances the number of murine Tregs (Ohta et al. 2012; Ohta and Sitkovsky 2014; Zarek et al. 2008). The lack of CD73 on human Tregs could prevent an adenosine-mediated auto-inhibition, similarly to our proposed mechanism for effector T cells, which lose CD73 after activation (Schneider, Winzer et al. 2021; Schneider 2020). Moreover, T cells upregulate the plasma

membrane protein CD26 after activation. In humans, but not in mice, CD26 serves as 'anchor' for the adenosine-degrading enzyme ADA (Richard et al. 2000), suggesting that ADA in close proximity to the cell surface could reduce an autocrine effect of adenosine and auto-inhibition of the cells.

Major differences between mice and humans are also noted for P2 receptors. There are several factors suggesting that humans evolved in a way to avoid P2X7 receptor signaling: Compared to murine P2X7, human P2X7 is i) less sensitive to ATP (Xing et al. 2016), ii) expressed at lower levels (at least on circulating T cells) (Winzer et al. 2022), and iii) not activated by low concentrations of NAD^+ through ARTC2.2-mediated ADP-ribosylation (Haag et al. 1994). As an example, murine Tregs express high levels of P2X7 at the cell surface, and are very sensitive to P2X7-mediated cell death already with micromolar NAD^+ concentrations released during tissue preparation. In contrast, human Tregs have a similar P2X7 expression as conventional CD4 T cells, and millimolar ATP concentrations are needed to induce cell death (Winzer et al. 2022). Of note, P2X7 expression varies between mouse strains with different genetic backgrounds (Er-Lukowiak et al. 2020), posing the question of how P2X7 would be expressed in 'wild mice' with a much higher genetic diversity. Moreover, laboratory mice are housed under controlled specific pathogen-free (SFP) conditions. Especially in the intestine, ATP concentrations are influenced by the microbiota, and it could well be possible that P2X7 function is differentially regulated in the presence of a diverse intestinal flora. P2Y11, another ATP-gated receptor, is expressed in humans, but not in mice. P2Y11 interacts with P2X7, and inhibits P2X7-mediated pore formation and cell death (Dreisig et al. 2018), providing another mechanism how human P2X7 is maintained in an inactive state. Currently, we do not understand why there is such a difference in the ATP sensitivity and ATP response between murine and human T cells.

Based on the differences in purinergic signaling between mice and humans, results from mouse models need to be carefully evaluated and are presumably not directly translatable to humans.

4.4 Experimental approaches: Tools we have and tools we need

As described in the publications presented in this thesis, we are able to measure the AMPase and ATPase activity of cells, EVs, cell culture supernatants, or bodily fluids with a sensitive

method based on the incubation of samples with fluorescent adenine nucleotides paired with HPLC detection (Schneider, Winzer et al. 2021; Leutert et al. 2021). The use of fluorescent etheno-nucleotides as substrates has several advantages. Fluorescence detection increases the sensitivity compared to ultraviolet (UV) detection, permitting the measurement of nucleotides in cell culture medium without disturbing background signals. Additionally, we can use concentrations of the substrate in the low micromolar or even nanomolar range, avoiding feed-forward inhibition of CD73 by ATP or ADP. The resulting product of etheno-AMP degradation is etheno-adenosine, which is not further degraded to inosine, overcoming the major setback of the very short half-life of adenosine. In combination with specific inhibitors, the HPLC-based detection of etheno-nucleotides is a powerful method to disentangle the role of the enzymes involved in adenine nucleotide hydrolysis. We used this method to monitor the ATPase and AMPase activities in different cell types, cell culture supernatants during differential ultracentrifugation, in EVs, and in SF (Schneider, Winzer et al. 2021; Leutert et al. 2021). We could e.g. show a correlation between CD39 expression and ATPase activity in different T cell subsets with only small variances in CD39 expression (Schneider, Winzer et al. 2021). This method can also be used to test newly discovered CD39 inhibitors, as we did for the anticancer drug ceritinib (Schäkel et al. 2022).

Specific and potent inhibitors are crucial tools for assigning enzymatic activity to specific nucleotidases. Prof. Dr. Christa Müller (Department of Pharmaceutical and Medicinal Chemistry, University of Bonn) and colleagues have developed highly specific and stable small molecule inhibitors for CD73, e.g. PSB-14685, which we have used in the presented study (Schneider, Winzer et al. 2021). New inhibitors with improved potency or metabolic stability are continuously generated (Bhattarai et al. 2020; Scortichini et al. 2022). Developing inhibitors against CD39 seems to be more challenging. The most commonly used CD39 inhibitor ARL67156 has a moderate potency for CD39, and a low selectivity against NTPDase3, ENPP1, and CD73 (Schäkel et al. 2020; Lévesque et al. 2007). Consequently, measured ATPase activity in complex biological fluids like serum or SF cannot be clearly assigned to CD39 or other ATPases. Used in many publications, this multi-target inhibitor may have caused conflicting results. Similarly, in our hands, published inhibitors against A2A and A2B receptors do not reliably work in human T cell assays because of a low efficacy and/or high toxicity (unpublished data). For targets for which the development of chemical-based inhibitors is

difficult, blocking nanobodies could be used as highly specific, non-toxic inhibitors of purinergic molecules. Nanobodies are the single variable domains/VHH fragments of camelid heavy chain antibodies. In contrast to conventional antibodies, nanobodies can bind functional pockets of proteins due to their small size. Additionally, they have a high stability, solubility, and tissue penetration, and can be easily coupled e.g. to albumin for an extended half-life for in vivo usage (Eggers et al. 2021; Bannas, Hambach, and Koch-Nolte 2017). Blocking nanobodies are available for both human and murine P2X7 receptors, and the anti-human P2X7 nanobody Dano1, used in this study, displays a very high potency compared to the small-molecule P2X7 antagonists JNJ47965567 and AZ10606120 (Danquah et al. 2016).

Beyond the measurement of enzymatic activities of purinergic enzymes, we aim to measure the nucleotide content in biological samples. Because of the short half-life of nucleotides due to metabolic processes, precautions are to be taken before and during sample processing to preserve the nucleotide content. By preventing the generation and degradation of adenosine during blood draw using a solution containing inhibitors for several purinergic enzymes and transporters, it is possible to ‘snapshot’ endogenous adenosine levels in blood (Löfgren et al. 2018). This method can be adapted using combinations of different enzyme inhibitors. The combination of liquid chromatography and mass spectrometry (LC-MS) offers the possibility to separate, identify and quantify nucleotides with a high sensitivity and can be even used to detect low amounts of ATP contained in vesicles (unpublished data from Dr. Anna Worthmann, Department of Biochemistry and Molecular Cell Biology, UKE).

LC-MS and similar methods are not suitable to quantify ATP with high spatiotemporal resolution. This is though essential to determine pericellular nucleotide concentrations, and to estimate which receptors are possibly activated in resting or stimulated cells. Available methods to detect ATP in cellular proximity of living cells are based on chemical approaches or genetically encoded biosensors (Lobas et al. 2019). A family of fluorescent chemosensors to detect phosphate anions is based on zinc-dipicolylamine (DPA) complexes, which can be further modified to specifically detect ATP (Ojida et al. 2002). To measure pericellular ATP, sensors must be attached to the cell membrane by lipophilic anchors or covalent bonds, which can alter their selectivity. The synthesis of chemosensors specific for ATP, with high sensitivity and membrane anchoring is currently ongoing (Laubach 2021). Most genetically encoded biosensors are based on Förster resonance energy transfer (FRET) or BRET signals (White and

Yang 2022). The ATP sensor ATeam contains the ε subunit of a bacterial ATP synthase linked to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). In the presence of ATP, a conformational change of the ε subunit brings the two fluorescent proteins close to each other, resulting in a FRET signal (Imamura et al. 2009). ATeam was modified with a transmembrane anchor to detect extracellular ATP (Conley et al. 2017). Another version of ATeam, termed BTeam, detects intracellular ATP through BRET (Yoshida, Kakizuka, and Imamura 2016). The drawback of these methods is that they require transfection of the cells with the sensor. This is difficult when using primary cells because transfection leads to cell stress and a reduced viability, accompanied by undesired ATP release. To overcome the problems associated with cell transfection, 'sniffer cells' that detect the desired nucleotide can be used. For this, cell lines are transfected with sensitive ATP-gated Ca^{2+} channels and loaded with a Ca^{2+} sensor. During co-culture with primary cells, ATP release of the primary cells is sensed by the 'sniffer cells', and the resulting Ca^{2+} influx can be measured by fluorescence microscopy. With this method, low nanomolar ATP concentrations were detected using rat P2X2-transfected HEK293 cells loaded with the Ca^{2+} sensor Fura-2, (Eersapah, Hugel, and Schlichter 2019). Another possibility to detect pericellular ATP without the need of transfection is to link a bacteria-produced ATP sensor to the plasma membrane of the cells of interest. A modified, Cy3 fluorophore-labeled ε subunit of a bacterial ATP synthase (ATPOS sensor) and the fluorescent dye Alexa Fluor 488 were coupled through biotin-streptavidin interactions to a protein that binds gangliosides on the cell surface of neurons. ATPOS detects ATP in the nanomolar range and enables e.g. the visualization of extracellular ATP signaling during neuronal excitation (Kitajima et al. 2020). The ATPOS sensor complex could be adapted to bind other cell surface molecules, e.g. CD45 present on T cells (unpublished data from Valerie Brock, Friedrich Haag et al.). Besides detecting pericellular ATP, biosensors are useful to measure adenosine receptor activation by detecting the intracellular A2A downstream second messenger cAMP. This is particularly important because flow cytometry antibodies or nanobodies to measure adenosine receptor protein expression are not available. Binding of adenosine to A2A-expressing reporter HEK293 cells induces cAMP, which can be detected through a cAMP-responsive luciferase construct (Häusler et al. 2010). Additionally, the FRET-based sensor Epac1-camps has been used to measure cAMP in T cells of transgenic mice (Kurelic et al. 2021).

4.5 Conclusion and perspectives

The pro-inflammatory mediator ATP is released under inflammatory conditions and activates P2 receptors such as P2X7. We used the anti-P2X7 nanobody Dano1 to show that, in the human T cell compartment, P2X7 is expressed mainly on innate-like T cells making them susceptible to ATP-induced Ca^{2+} influx and cell death. ATP signaling is restrained by the ectonucleotidases CD39 and CD73, which rapidly degrade ATP to adenosine. We thoroughly characterized the expression and function of these enzymes on human T cells, and showed that enzymatically active CD73 is released on EVs from activated T cells. The presence of non-cell-bound ectonucleotidases has major implications on ATP and adenosine availability, even for cells that do not express the enzymes. We additionally showed that ADP-ribosylation, a post-translational modification already known from the P2X7 receptor, reduces the activity of CD73. The release of CD73 in EVs and the decrease of CD73 enzymatic activity through ADP-ribosylation regulate the function of CD73, and affect the outcome of an immune response.

The data on human P2X7 and CD73 presented in this study derive from peripheral blood T cells. There are, however, many indications that the expression and function of these proteins and other purinergic molecules may be different in tissue. Interestingly, a subset of memory $CD73^+$ CD8 T cells in the blood have elevated expression of Trm markers (Fang et al. 2021) and show enriched gene expression of the gut homing marker *CCR9* (Figure 7A). We had the chance to measure CD73 expression in lymphocytes from the small intestine of patients undergoing bariatric surgery. Our preliminary results show that CD73 is much higher expressed on intestinal T cells compared to the periphery (Figure 7B-C), in line with existing literature (Alam, Kurtz, Rowlett, et al. 2009). The intestine is an environment rich in antigens, of which some derive from commensal bacteria or other non-pathogenic sources. Adenosine production by CD73-expressing intraepithelial or lamina propria lymphocytes may contribute to establish tolerance to intestinal antigens and prevent inflammation. Supporting this idea, CD73 was found protective in a mouse model of colitis (Bynoe et al. 2012). Using functional T cell assays, we plan to elaborate the function of CD73 and its interplay with other purinergic enzymes in the human intestine.

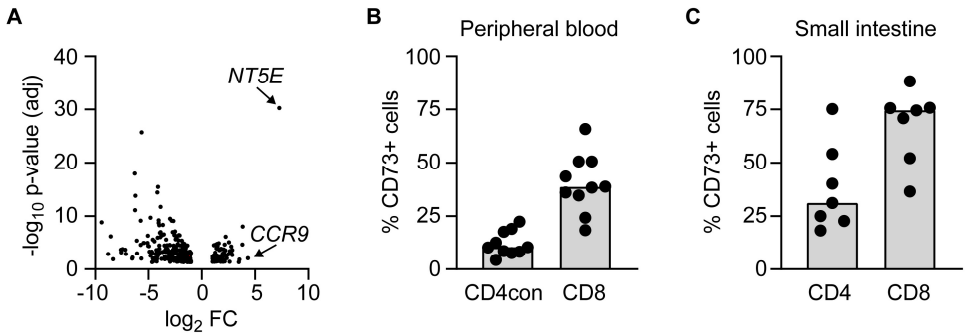

Figure 7. CD73 is highly expressed on intestinal T cells. (A) Bulk RNA sequencing analysis of peripheral blood CD73$^+$ versus CD73$^-$ CD8 memory T cells (n = 3). (B-C) CD73 expression on CD4 and CD8 T cells in (B) peripheral blood of healthy donors (data from Schneider, Winzer et al. 2021) and (C) intestinal lymphocytes from patients who underwent bariatric surgery (samples kindly provided by Jonas Wagner, UKE).

P2X7 is highly expressed on hepatic and intestinal murine Trm cells (Stark et al. 2018; Borges da Silva et al. 2019). There is not much known about the expression of P2X7 in human tissue. Tfh cells from the Peyer's Patches (PP) have a higher ATP sensitivity compared to other cells from the PPs, indicating a higher P2X7 expression (Proietti et al. 2014). When staining P2X7 on peripheral blood cells with a Tfh-like phenotype (CD4$^+$CXCR5$^+$PD-1$^+$), we measured a P2X7 expression even lower than in total CD4 T cells (Winzer et al. 2022). This points out that the expression of P2X7 is highly dependent on the cellular environment. We plan to measure P2X7 expression e.g. in the liver and in the intestine. Using the anti-P2X7 nanobody Dano1 for flow cytometry analysis and cellular assays, we have a good tool to measure differences in P2X7 expression and function between peripheral blood and tissue. This will help us to determine if there is a tissue-specific expression of P2X7, in addition to the already known species-specific expression. The development of new biosensors suitable for measuring pericellular ATP in primary human T lymphocytes hopefully will give us the opportunity to better understand the cellular environment in which P2 receptors become activated.

III List of abbreviations

µM	Micromoles/liter
AC	Adenylate cyclase
ACDC	Arterial calcification due to deficiency of CD73
ADA	Adenosine deaminase
ADO	Adenosine
ADP	Adenosine diphosphate
ADPR	ADP-ribose
AMP	Adenosine monophosphate
AOPCP	α,β-methylene ADP
AP	Alkaline phosphatase
AP-1	Activator protein 1
APC	Antigen-presenting cell
ART	ADP-ribosyltransferase
ATP	Adenosine triphosphate
BRET	Bioluminescence resonance energy transfer
cAMP	Cyclic AMP
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
CFP	Cyan fluorescent protein
Csk	C-terminal Src kinase
DAG	Diacylglycerol
DC	Dendritic cell
doi	Digital object identifier
DPA	Dipicolylamine
DPP4	Dipeptidyl peptidase-4
e.g.	*Exempli gratia*, for example
EC_{50}	Effective concentration (half maximal)
EGFP	Enhanced GFP
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride
EJI	European Journal of Immunology
ELISA	Enzyme-linked immunosorbent assay
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1

ENT	Equilibrative nucleoside transporter
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase-1
et al	*Et alii,* and others
Eur. J. Immunol.	European Journal of Immunology
EV	Extracellular vesicle
FRET	Förster resonance energy transfer
Front. Immunol.	Frontiers in Immunology
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GSDMD	Gasdermin D
HCC	Hepatocellular carcinoma
HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
IL	Interleukin
IP_3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	Kilo Dalton
LAT	Linker of activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LC-MS	Liquid chromatography-mass spectrometry
LOF	Loss of function
LPS	Lipopolysaccharide
LRR	Leucin-rich repeat
MAIT	Mucosal-associated invariant T cell
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
miRNA	Micro RNA
MRI	Magnetic resonance imaging
MVB	Multivesicular bodies
NAD^+	Nicotinamide adenine dinucleotide
Nat. Commun.	Nature communications
NFAT	Nuclear factor of activated T cells
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NICD	NAD^+-induced cell death
NK	Natural killer
NKT	Natural killer T
NLRP	NOD-, LRR- and pyrin domain-containing protein
nm	Nanometer
NOD	Nucleotide-binding oligomerization domain
NT5E	Ecto-5'-nucleotidase
oATP	Oxidized ATP
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PC-1	Plasma cell glycoprotein 1
PET	Positron emission tomography
P_i	Inorganic phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PLC	Phospholipase C
PLD	Phospholipase D
PP	Peyer's Patches
PP_i	Inorganic pyrophosphate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SF	Synovial fluid
SH2	Src Homology 2
SNP	Single nucleotide polymorphism
SPF	Specific pathogen-free
TCR	T cell receptor
Tfh	T follicular helper
Th	T helper
TLR	Toll-like receptor
TNAP	Tissue-nonspecific AP
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
Trm	Tissue resident memory
TRP	Transient receptor potential

UDP	Uridine diphosphate
UKE	University Medical Center Hamburg-Eppendorf
UTP	Uridine triphosphate
UV	Ultraviolet
YFP	Yellow fluorescent protein
ZAP-70	Zeta-associated protein 70

IV List of figures

V References

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VI Publications, awards, conferences

Publications

R. Winzer, A. Serracant-Prat, V. J. Brock, C. Pinto-Espinoza, B. Rissiek, M. Amadi, N. Eich, A. Rissiek, E. Schneider, T. Magnus, A. H. Guse, B.-P. Diercks, F. Koch-Nolte, E. Tolosa, P2X7 is expressed on human innate-like T lymphocytes and mediates susceptibility to ATP-induced cell death. *Eur. J. Immunol.* 52, 11 (2022).

L. Schäkel, S. Mirza, **R. Winzer**, V. Lopez, R. Idris, H. Al-Hroub, J. Pelletier, J. Sévigny, E. Tolosa, C. E. Müller, Protein kinase inhibitor ceritinib blocks ectonucleotidase CD39 - a promising target for cancer immunotherapy. *J. Immunother. Cancer*. 10, 8 (2022).

E. Schneider*, **R. Winzer***, A. Rissiek, I. Ricklefs, C. Meyer-Schwesinger, F. L. Ricklefs, A. Bauche, J. Behrends, R. Reimer, S. Brenna, H. Wasielewski, M. Lauten, B. Rissiek, B. Puig, F. Cortesi, T. Magnus, R. Fliegert, C. E. Müller, N. Gagliani, E. Tolosa, CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression. *Nat. Commun.* 12 (2021). *equal contribution

M. Leutert, Y. Duan, **R. Winzer**, S. Menzel, E. Tolosa, T. Magnus, M. O. Hottiger, F. Koch-Nolte, B. Rissiek, Identification of the Mouse T Cell ADP-Ribosylome Uncovers ARTC2.2 Mediated Regulation of CD73 by ADP-Ribosylation. *Front. Immunol.* 12 (2021).

A. Löhndorf, L. Hosang, W. Dohle, F. Odoardi, S. A. Waschkowski, A. Rosche, A. Bauche, **R. Winzer**, E. Tolosa, S. Windhorst, S. Marry, A. Flügel, B. V. L. Potter, B. P. Diercks, A. H. Guse, 2-Methoxyestradiol and its derivatives inhibit store-operated Ca^{2+} entry in T cells: Identification of a new and potent inhibitor. *Biochim. Biophys. Acta. Mol. Cell Res.* 1868, 6 (2021).

E. Schneider, A. Rissiek, **R. Winzer**, B. Puig, B. Rissiek, F. Haag, H. W. Mittrücker, T. Magnus, E. Tolosa, Generation and Function of Non-cell-bound CD73 in Inflammation. *Front. Immunol.* 10 (2019).

R. Fliegert, A. Bauche, A. M. Wolf Pérez, J. M. Watt, M. D. Rozewitz, **R. Winzer**, M. Janus, F. Gu, A. Rosche, A. Harneit, M. Flato, C. Moreau, T. Kirchberger, V. Wolters, B. V. L. Potter, A. H. Guse, 2'-Deoxyadenosine 5'-diphosphoribose is an endogenous TRPM2 superagonist. *Nat. Chem. Biol.* 13, 9 (2017).

Awards

AAI Bright Sparks Award for the presentation "CD73-mediated adenosine production by T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression" during the Joint Meeting of the German Society for Immunology (DGfI) and the Austrian Society for Allergology & Immunology (ÖGAI) (September 2022)

UKE Paper of the Month for the publication "CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression" (October 2021)

Best master's degree in the study program Molecular Life Sciences at the University of Hamburg with the title of the master thesis "Regulation of adenosine production by the ectonucleotidase CD73 in the context of neonatal tolerance" (December 2018)

Conferences

51st Annual Meeting of the German Society for Immunology (DGfI) and 50th Annual Meeting of the Austrian Society for Allergology and Immunology (ÖGAI) (Hannover, 2022)

> CD73-mediated adenosine production by T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression (oral presentation)

16th World Immune Regulation Meeting (Davos, 2022)

> Human effector T cell-derived extracellular vesicles convey immune suppression by CD73-mediated adenosine generation (oral presentation and poster)

24th Meeting on T Cells – Subsets and Functions (online, 2021)

> CD73-mediated adenosine production by CD8 T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression (oral presentation)

1st European Purine Meeting (Santiago de Compostela, 2019)

20th Meeting on T Cells – Subsets and Functions (Marburg, 2017)

Further accomplishments

Mentor in the P4M mentoring program (Postdocs/PhD students for Medical students) of the SFB 1328 (since October 2019)

Associated member of the executive board of the SFB 1328 (since November 2021)

VII Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Ich versichere, dass dieses gebundene Exemplar der Dissertation und das in elektronischer Form eingereichte Dissertationsexemplar (über den Docata-Upload) und das bei der Fakultät (Studienbüro Biologie) zur Archivierung eingereichte gedruckte Exemplar der Dissertationsschrift identisch sind.

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Ort, Datum	Unterschrift (Riekje Winzer)

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