The immunoregulatory role of T cell-derived CD73 in the context of inflammation

Dissertation

zur Erlangung des naturwissenschaftlichen Doktorgrades (Dr. rer. nat.)
eingereicht am Fachbereich Chemie der Fakultät für
Mathematik, Informatik und Naturwissenschaften

Universität Hamburg

vorgelegt von
Enja Schneider
aus Hamburg

Hamburg, 2020

Erstgutachterin:	Prof. Dr. Eva Tolosa
Zweitgutachter:	Prof. Dr. Henning Tidow
Vorsitzender der Prüfungskommission:	Prof. Dr. Chris Meier
Tag der Disputation:	04.12.2020
Datum der Druckfreigabe:	04.12.2020

Die vorliegende Arbeit wurde im Zeitraum Mai 2016 bis September 2020 im Institut für Immunologie am Universitätsklinikum Hamburg-Eppendorf in der Arbeitsgruppe von Prof. Dr. Eva Tolosa angefertigt.

Table of contents

Abstract

The ability of self-regulation is central for the immune system to terminate immune responses and to prevent excessive collateral tissue damage. Purinergic signaling controls effector and regulatory responses of the immune system, mainly through ATP and adenosine. Upon inflammation, immune cells release ATP into the extracellular space, where it acts as pro-inflammatory molecule. Degradation of ATP by the ecto-enzyme CD39 yields AMP, the substrate for the ecto-nucleotidase CD73. The enzymatic activity of CD73 leads to the generation of adenosine, a potent suppressor of effector T cells, indicating a crucial function for CD73 in the regulation of T cell responses. The aim of my thesis is to elucidate the immunoregulatory role of T cell-derived CD73 in the context of inflammation.

Most naïve CD8 T cells, a small proportion of memory CD4 T cells and very few regulatory T cells express CD73 in the human T cell compartment. We found that CD8 and CD4 T cells lose CD73 from the plasma membrane after activation. Neither artificial removal of CD73, nor blockade of its enzymatic activity affects activation or proliferation kinetics. We therefore concluded that loss of CD73 surface expression does not actively trigger T cell activation, but it is a consequence of it, possibly to protect effector T cells from adenosine-mediated suppression. Concomitant with the loss of surface CD73 expression, we measured increased AMPase activity in cell culture supernatants of activated T cells. We observed that CD73 co-localizes with vesicle markers in activated CD8 T cells, and showed that extracellular vesicles (EVs) released from activated effector T cells contain CD73 and mediate most of the AMPase activity in T cell culture supernatants. We successfully established an *in vitro* assay to measure the immunoregulatory potential of these EVs, and found that they provide the necessary AMPase activity to suppress effector T cells through the purinergic pathway. Moreover, CD8 T cell-derived EVs cooperate with regulatory T cells, which, due to low CD73 expression, depend on an exogenous source of AMPase activity to induce adenosine-mediated T cell suppression. We further showed that EVs from the synovial fluid of inflamed joints possess CD73-specific AMPase activity and can acquire immunosuppressive function under conditions that favor adenosine signaling.

In conclusion, I showed in this thesis that activated human CD8 T cells release $CD73^+$ EVs with immunosuppressive function. We propose that CD73-mediated adenosine production by

effector T cell-derived EVs constitutes an intrinsic mechanism of immune suppression in humans.

Zusammenfassung

Die Fähigkeit zur Selbstregulierung ist für das Immunsystem von zentraler Bedeutung, um Immunreaktionen zu kontrollieren und somit übermäßige Schädigungen am umliegenden Gewebe zu verhindern. Die Immunantwort wird unter anderem durch purinerge Signalmoleküle, insbesondere ATP und Adenosin, gesteuert. Im Verlauf einer Entzündung setzen aktivierte Immunzellen ATP in den extrazellulären Raum frei, wo es entzündungsfördernd wirkt. Durch das Ekto-Enzym CD39 wird ATP zu AMP, dem Substrat der Ekto-Nukleotidase CD73, abgebaut. Die enzymatische Aktivität von CD73 führt zur Bildung von Adenosin, welches die Immunantwort unterdrückt und somit einen entzündungshemmenden Effekt hat. Aufgrund dieser enzymatischen Aktivität ist es naheliegend, dass CD73 eine entscheidende Funktion bei der Regulation von T-Zellantworten hat. Das Ziel dieser Dissertation ist, die immunregulatorische Rolle des von T-Zellen stammenden CD73 im Zusammenhang mit Entzündungen zu untersuchen.

Im humanen T-Zellkompartiment wird CD73 von den meisten naiven CD8 T-Zellen, einem kleinen Teil der CD4 Gedächtnis-T-Zellen und sehr wenigen regulatorischen T-Zellen exprimiert. Sowohl CD8 als auch CD4 T-Zellen verlieren CD73 nach Aktivierung von der Plasmamembran. Allerdings wurden Aktivierung und Proliferation der T-Zellen weder durch die künstliche Entfernung von CD73 von der Zelloberfläche noch durch die Inhibition der enzymatischen Aktivität beeinflusst. Diese Ergebnisse lassen darauf schließen, dass der Verlust der CD73-Oberflächenexpression nicht die T-Zellaktivierung auslöst, sondern möglicherweise vor allem dazu dient, die aktivierte Effektor-T-Zelle vor Adenosin-vermittelter Suppression zu schützen. Der Verlust der CD73-Oberflächenexpression geht mit einem Anstieg der AMPase-Aktivität in Zellkulturüberständen aktivierter T-Zellen einher. Die Analysen ergaben, dass CD73 mit Vesikelmarkern in aktivierten CD8 T-Zellen ko-lokalisiert und in extrazellulären Vesikeln (EVs) freigesetzt wird. Diese von aktivierten Effektor-T-Zellen freigesetzten EVs vermitteln den größten Teil der AMPase-Aktivität in T-Zellkulturüberständen. Nach erfolgreicher Etablierung eines *in-vitro*-Tests zur Messung des immunregulatorischen Potenzials dieser EVs zeigte sich, dass diese die erforderliche AMPase-Aktivität besitzen, um Effektor-T-Zellen über den purinergen Signalweg zu inhibieren. Darüber hinaus kooperieren EVs von CD8 T-Zellen mit regulatorischen T-Zellen, die aufgrund ihrer geringen CD73-Oberflächenexpression auf eine exogene AMPase-Quelle angewiesen sind,

um die Adenosin-vermittelte Suppression von T-Zellen auszuführen. Zudem weisen EVs, die aus der Synovialflüssigkeit entzündeter Gelenke isoliert wurden, eine CD73-spezifische AMPase-Aktivität auf und können unter Bedingungen, die die purinergen Signalwege begünstigen, eine immunsuppressive Funktion erlangen.

Zusammenfassend zeigt diese Arbeit, dass aktivierte humane CD8 T-Zellen $CD73^+$ EVs mit immunsuppressiver Funktion freisetzen. Aus den Ergebnissen lässt sich schlussfolgern, dass die durch CD73 vermittelte Adenosinproduktion von Effektor-T-Zell-EVs einen intrinsischen Mechanismus der Immunsuppression beim Menschen darstellt.

1. Introduction

1.1 T cells mediate effector and regulatory responses in the immune system

The immune system is the host's defense machinery against invading pathogens and toxins. It is a multi-layered network of immune cells and signaling molecules that, in order to protect the body against disease and damage, has to distinguish foreign from self and harmful from harmless. A proper immune reaction comprises the coordinated action of effector and regulatory mechanisms. In the effector phase, immune cells recognize and fight against the pathogen. After elimination of the potential threat, immunoregulatory responses limit further tissue damage. The ability of self-regulation is central for the immune system and different levels of regulation ensure that the balance between immune cell activation and suppression is tightly controlled.

1.1.1 T cell activation and differentiation

T cells are part of the adaptive immune system. They arise from common lymphoid progenitor cells in the bone marrow and mature in the thymus. Mature thymocytes express a unique T cell receptor (TCR) and are immunologically naïve. Once they have left the thymus as CD4 or CD8 T cells, they circulate in the periphery or home to secondary lymphoid organs. Upon recognition of their specific antigen, T cells become activated and elicit cell-specific effector functions. CD4 T cells were initially discovered as helper T cells that support B cell function, whereas CD8 T cells are cytotoxic and able to kill infected cells. Appropriate T cell activation requires two signals provided by antigen-presenting cells (APCs). The interaction of the TCR with small peptides bound to major histocompatibility complex (MHC) molecules on APCs provides the first signal. CD4 T cells bind to MHC class II, CD8 T cells interact with MHC class I molecules. CD4 and CD8 act as co-receptors that stabilize the interaction of the TCR with the MHC molecules on APCs and together with CD3, which is expressed on all T cells, are essential for TCR signal transduction. Apart from the recognition of the antigen bound to the MHC molecule, T cells need a costimulatory signal to start proliferation and differentiation into effector and memory T cells. CD28 is the best-characterized costimulatory molecule. Binding of CD28 to CD80 and CD86 on APCs promotes a variety of signaling processes in the T cell resulting in production of cytokines such as IL-2, cytoskeletal remodeling and increased

glucose uptake and glycolysis. The lack of costimulatory signals during T cell activation results in T cell anergy or death.

Antigenic stimulation of naïve T cells results in rapid T cell proliferation and differentiation into effector T cells. Depending on the cytokine environment, CD4 T cells differentiate into different T helper (Th) subsets that express characteristic transcription factors and produce a specialized set of effector cytokines. Th1 cells mainly produce IFNγ and increase cellular immunity by promoting macrophages to eliminate intracellular pathogens. Th2 cells produce IL-5 and IL-13 and are crucial for the protection against extracellular parasites. A third subset, Th17 cells, produces IL-17 and is involved in the clearance of extracellular pathogens and fungi (Paul and Zhu, 2010; Sallusto, 2016). Antigen-stimulated CD8 T cells produce IFNγ and TNFα and acquire cytotoxic function to kill virus-infected cells (Zhang and Bevan, 2011).

The induction of memory cells is an essential feature of the adaptive immune system to establish protective immunity. Memory T cells are antigen-experienced T cells that have a lower activation threshold than naïve T cells and quickly respond when they re-encounter their antigen. While naïve T cells express CD45RA, memory T cells are characterized by the expression of CD45RO. Based on the expression of CCR7 (CD197) or CD27, memory T cells are divided into central memory (CM, $CCR7^+$) and effector memory (EM, $CCR7^-$) T cells. A proportion of EM T cells within the CD8 memory compartment termed terminal effector memory RA^+ (TEMRA) T cells expresses CD45RA instead of CD45RO (Mahnke *et al.*, 2013).

1.1.2 Regulatory T cells play a pivotal role in effector T cell suppression

After elimination of the pathogen, termination of immune responses and reconstitution of immune homeostasis are essential to prevent inflammation-induced damage of healthy tissue. Constitutive and high expression of inhibitory molecules phenotypically marks exhausted T cells; however, these molecules exert important functions in controlling T cell activation by negatively regulating T cell function. CTLA-4 and PD-1 are found on the surface of activated T cells and studies in mice revealed that loss of CTLA-4 or PD-1 results in uncontrolled proliferation of T cells that ultimately leads to organ damage (Tivol *et al.*, 1995; Nishimura *et al.*, 1999). In humans, the level of PD-1 expression and thus PD-1 signaling strength determines the degree of T cell suppression: low levels of PD-1 already result in effective inhibition of T cell proliferation, while higher levels are required to block cytokine

production (Wei *et al.*, 2013). CTLA-4 is not only expressed on activated or exhausted T cells but also intracellularly in a specialized subset of T cells called regulatory T cells (Tregs). Tregs play a pivotal role in the maintenance of peripheral tolerance and in the termination of T cell immune responses. They are either generated in the thymus or develop in the periphery from non-regulatory (conventional) CD4 T cells (Sakaguchi *et al.*, 2010). *In vitro*, the combination of retinoic acid and TGF-β or the addition of D-mannose converts conventional CD4 (CD4con) T cells into Tregs (Wang, Huizinga and Toes, 2009; Zhang *et al.*, 2017). High levels of CD25, low levels of CD127 and intracellular expression of the transcription factor FOXP3 distinguish Tregs from CD4con T cells in peripheral blood (Roncador *et al.*, 2005; Liu *et al.*, 2006), where they constitute approximately 10% of CD4 T cells (Hartigan-O'Connor *et al.*, 2007). FOXP3 is essential for the suppressive capacity of Tregs and mutations in the *FOXP3* gene result in dysfunctional Tregs, consequently leading to the immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. Depending on the mutation, patients suffer from various types of autoimmune diseases, revealing the unique role of Tregs in the control of immune responses (Sakaguchi *et al.*, 2020).

Tregs use a variety of mechanisms to suppress effector T cell function (Figure 1). Murine and human Tregs constitutively express CTLA-4 and TCR stimulation promotes its expression on the cell surface (Dieckmann *et al.*, 2001). CTLA-4, a homolog of CD28, binds stronger to CD80 and CD86 on APCs than CD28 and this competition between Tregs and effector T cells for the same ligand on APCs impairs APC-mediated activation of effector T cells. The CTLA-4-dependent interaction of murine and human Tregs with APCs also downregulates expression of these costimulatory molecules in APCs by trans-endocytosis, leading to functionally impaired APCs (Wing *et al.*, 2008; Qureshi *et al.*, 2011).

Besides the modulation of APC function, Tregs themselves produce anti-inflammatory cytokines such as IL-10, IL-35 and TGF-β that exert suppressive function on effector T cells. Although *in vitro* data suggest a minor contribution of each of these cytokines in Treg-mediated suppression, *in vivo* data using mouse models show that Tregs deficient for one of these cytokines are impaired in their suppressive function (Collison *et al.*, 2007; Li, Wan and Flavell, 2007; Rubtsov *et al.*, 2008; Choi *et al.*, 2020). A recent study describes IL-10$^+$ and IL-35$^+$ Tregs in the tumor microenvironment in humans and proposes a non-redundant role of these two Treg subpopulations in the suppression of tumor-infiltrating T cells (Sawant *et al.*, 2019).

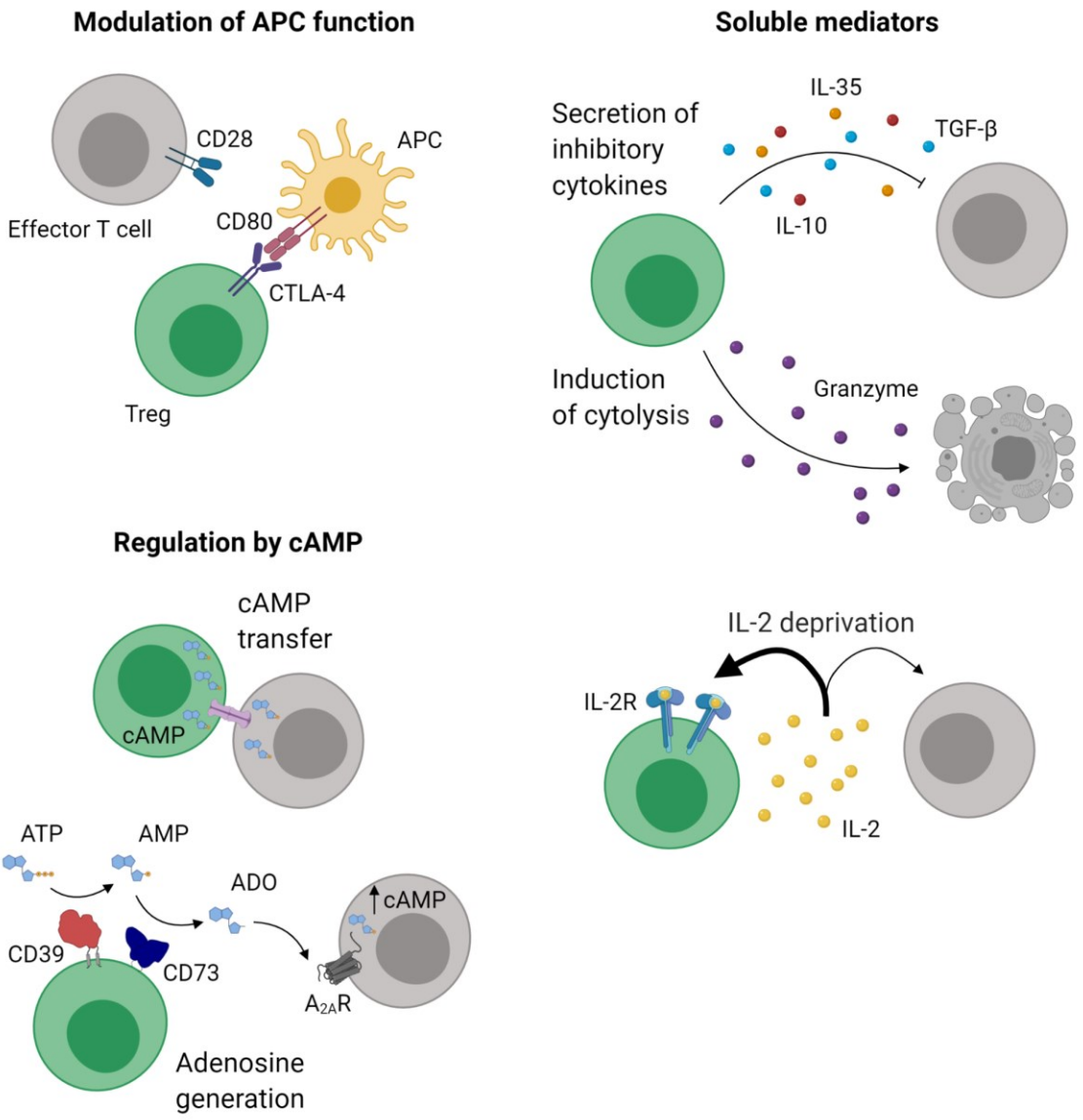

Figure 1 | Tregs use a variety of mechanisms to suppress effector T cell function. Modulation of APC function: Binding of CTLA-4 on Tregs to CD80 on APCs prevents interaction with CD28 expressed on effector T cells and reduces the stimulatory capacity of APCs. Soluble mediators: Tregs secrete IL-10, IL-35 and TGF-β to suppress effector T cells or induce cytolysis by the release of granzyme. Tregs constitutively express the high-affinity IL-2 receptor (IL-2R) and suppress conventional T cells by depriving them of IL-2. Regulation by cAMP: Tregs increase the intracellular concentration of cAMP in effector T cells by the transfer cAMP into effector T cells through gap junctions. Alternatively, CD39 and CD73 on Tregs degrade extracellular ATP to adenosine that binds to A_{2A} receptors ($A_{2A}R$) on effector T cells and induces the generation of cAMP. Image created with BioRender.com.

In mice, the special milieu in the tumor microenvironment endows murine Tregs with cytotoxic function. In contrast to naïve Tregs, a proportion of murine tumor-derived Tregs expresses granzyme B and lyses target cells in a granzyme B- and perforin-dependent manner (Cao *et al.*, 2007). Human natural Tregs produce granzyme A and exhibit perforin-dependent cytotoxicity (Grossman *et al.*, 2004).

For survival, Tregs depend on exogenously derived IL-2 because their intrinsic production is low. Activated effector T cells produce IL-2 to promote their expansion and differentiation. Tregs constitutively express the high-affinity IL-2 receptor and are able to deprive IL-2 from effector T cells, which leads to T cell suppression and ultimately cell death of effector T cells (Pandiyan *et al.*, 2007).

Further mechanisms by which Tregs induce effector T cell suppression involve adenine nucleotides. Murine and human Tregs harbor high intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP) that is a potent suppressor of TCR-mediated signaling and indispensable for Treg function (Bopp *et al.*, 2007; Klein *et al.*, 2012). An elevation of intracellular cAMP in effector T cells leads to T cell suppression and murine Tregs induce this increase using two distinct pathways. In a cell contact-dependent process, Tregs directly transfer cAMP through gap junctions into effector T cells (Bopp *et al.*, 2007). Alternatively, two ecto-enzymes on the Treg plasma membrane, CD39 and CD73, metabolize extracellular ATP to adenosine that subsequently increases cAMP in effector T cells through the activation of the adenosine receptor 2A ($A_{2A}R$) (Deaglio *et al.*, 2007). Peripheral human Tregs rarely co-express CD39 and CD73; however, a substantial amount of double-positive Tregs is present in different types of tumors and might contribute adenosine-mediated T cell suppression in this context (Maj *et al.*, 2017; Di Gennaro *et al.*, 2018).

Of note, it is debatable whether *in vitro* results reflect the *in vivo* situation and whether mechanisms proven to be relevant in mouse models are important in the human system.

1.2 Purinergic signaling

Purinergic signaling describes signaling processes in the extracellular space that involve nucleotides and nucleosides as mediators. In 1971, Burnstock introduced the term “purinergic” to characterize non-adrenergic, non-cholinergic signaling in the nervous system (Burnstock, 1971) and since then evidence accumulated that signaling by purines and also pyrimidines is essential in the regulation of a variety of physiological processes. In the immune system, purinergic signaling promotes pro- and anti-inflammatory reactions and the magnitude of the immune response highly depends on purine-metabolizing enzymes and receptors expressed on the immune cells. ATP and adenosine are key molecules of purinergic

signaling cascades and the conversion of pro-inflammatory ATP to anti-inflammatory adenosine receives special attention in the context of immune regulation.

1.2.1 The basic concept of purinergic signaling

The concentration of ATP inside the cell is 3 mM (Johnsen *et al.*, 2019). Physiological amounts of ATP in the extracellular space are much lower, e.g. under resting conditions, ATP concentrations are around 30 nM in human plasma (Gorman, Feigl and Buffington, 2007). Immune cells constitutively release ATP in the extracellular space and thus maintain low micromolar levels of ATP in close proximity to their cell surface (Lazarowski, Boucher and Harden, 2000; Yegutkin *et al.*, 2006).

Extracellular ATP is a potent signaling molecule and its concentration increases upon cell damage or under inflammatory conditions. Mechanisms of controlled cellular release of ATP include exocytosis and efflux through pore-forming channels such as pannexin or connexin hemichannels (Eltzschig *et al.*, 2006; Praetorius and Leipziger, 2009; Woehrle *et al.*, 2010). In the extracellular space, ATP can activate purinergic receptors to elicit mainly pro-inflammatory reactions or it serves as substrate for several types of ecto-nucleotidases. ATP degradation generates adenosine, a potent anti-inflammatory mediator. CD39 and CD73 are the major nucleotide-metabolizing enzymes expressed on immune cells and the concerted action of these enzymes ensures rapid dephosphorylation of ATP to adenosine (Figure 2). Adenosine can bind to adenosine receptors, e.g. the A_{2A} receptor, and induce immunosuppressive signaling processes or it is further converted to inosine by adenosine deaminase (ADA), a soluble enzyme that attaches to the plasma membrane by binding to CD26. Under resting physiological conditions, plasma adenosine concentrations are between 10 – 300 nM (Ontyd and Schrader, 1984; Yoneyama *et al.*, 2005; Löfgren *et al.*, 2018). ATP release and subsequent degradation are the primary source for extracellular adenosine; however, cells also release adenosine under ischemic conditions (Yegutkin, 2008; Dale and Frenguelli, 2009).

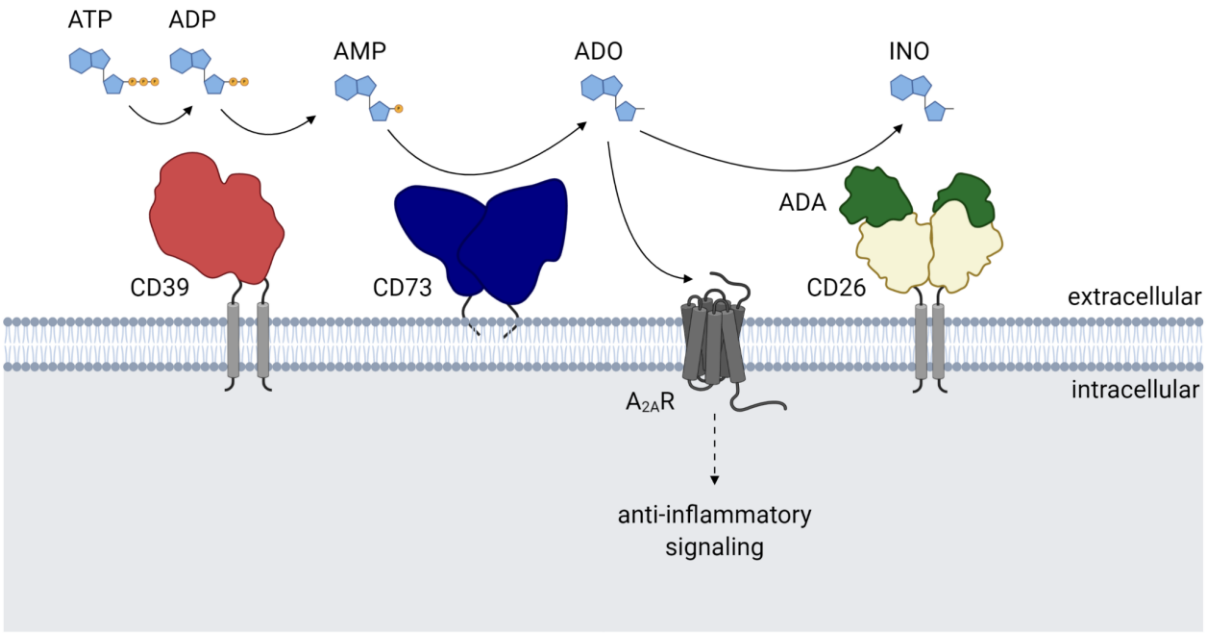

Figure 2 | CD39 and CD73 mediate extracellular degradation of ATP to adenosine. In the extracellular space, CD39 degrades ATP and ADP to AMP. CD73 dephosphorylates AMP to adenosine (ADO) which is further metabolized to inosine (INO) by adenosine deaminase (ADA) bound to CD26. Adenosine-mediated activation of the A_{2A} receptor ($A_{2A}R$) promotes anti-inflammatory signaling. Image created with BioRender.com.

1.2.2 CD39 and CD73 are the major enzymes involved in adenosine generation

CD39, also called NTPDase1, belongs to the family of nucleoside triphosphate diphosphohydrolases (NTPDase) and is encoded by the gene *ENTPD1*. All eight members of this family hydrolyze nucleoside tri- and diphosphates to the corresponding monophosphates in a calcium- or magnesium-dependent manner. CD39 favors adenine nucleotides over uracil nucleotides and dephosphorylates ATP more rapidly than ADP (Zimmermann, Zebisch and Sträter, 2012). The K_m values of CD39 are 17 µM for ATP and 22 µM for ADP (Kukulski *et al.*, 2005). A feature that distinguishes CD39 from other ecto-nucleotidases of the NTPDase family is that CD39 hydrolyzes ATP to AMP and two inorganic phosphates without significant accumulation of ADP as intermediate product (Kukulski *et al.*, 2005; Zimmermann, Zebisch and Sträter, 2012). In the human immune system, the majority of B cells, monocytes, neutrophils and activated T cells expresses CD39 on the cell surface, while less than 10% of resting T cells and NK cells are $CD39^+$ (Pulte *et al.*, 2007). The expression level of CD39 in Tregs and in conventional T cells depends on single nucleotide polymorphisms (SNPs) and, in the case of Tregs, positively correlates with their suppressive capacity (Rissiek *et al.*, 2015; Roederer *et al.*, 2015). CD39 is a highly glycosylated protein with short intracellular N- and C-

termini, two transmembrane spanning regions and a large extracellular loop that contains the catalytic domain (Maliszewski *et al.*, 1994; Zimmermann, Zebisch and Sträter, 2012). Even though the transmembrane domains influence substrate specificity and are important for the catalytic activity, soluble forms of CD39 exist in human plasma (Yegutkin *et al.*, 2012; Zimmermann, Zebisch and Sträter, 2012).

CD73, or ecto-5'-nucleotidase, is a glycosylphosphatidylinositol (GPI)-anchored, cell surface protein encoded by the gene *NT5E* (Misumi *et al.*, 1990). Structurally, the enzyme is a homodimer and each of the two subunits has an N-terminal and a C-terminal domain that are connected by an α-helix. The interface of these two domains forms the active site. The N-terminal domain binds two Zn^{2+} ions that are necessary for the enzymatic function and the C-terminal domain provides the binding pocket for the substrate. Dimerization of CD73 molecules occurs through hydrogen bonds and hydrophobic interactions between the C-terminal domains. The enzyme exists in an open and a closed form and undergoes large domain movement which is thought to control catalytic activity and substrate specificity (Heuts *et al.*, 2012; Knapp *et al.*, 2012). CD73 hydrolyzes nucleoside 5'-monophosphates to the corresponding nucleoside and inorganic phosphate. AMP is the preferred substrate with K_m values between 4 and 55 µM (Dornand, Bonnafous and Mani, 1978; Lehto and Sharom, 1998; Heuts *et al.*, 2012). ATP and ADP function as competitive inhibitors and their inhibition constants are in the low micromolar range (Sträter, 2006). CD73 is predominantly expressed on endothelial cells and lymphocytes (Yegutkin, 2014). Of note, expression on immune cells is species-specific: In the human immune system, most B cells, the majority of naïve CD8 T cells, a small proportion of CD4 T cells and few Tregs express CD73 (Dianzani *et al.*, 1993; Schena *et al.*, 2013; Rissiek *et al.*, 2015; Raczkowski *et al.*, 2018). In mice, CD73 is abundantly present on the plasma membrane of all T cells, and especially on Tregs (Kobie *et al.*, 2006; Raczkowski *et al.*, 2018). Most murine B cells do not express CD73; however, its expression is upregulated after isotype-switching (Yamashita *et al.*, 1998). As a GPI-anchored protein, CD73 is susceptible to enzymatic cleavage by phospholipases C and D (Low and Prasad, 1988; Thomson *et al.*, 1990). In contrast to intracellular phospholipase C, a phospholipase D with specificity for GPI-anchored proteins was found in human serum, suggesting a possible role of this enzyme in the release of GPI-anchored proteins from the cell membrane (Davitz *et al.*, 1987). Indeed, human serum and plasma exhibit CD73-specific AMPase activity, indicating the

presence of soluble and enzymatically active forms of CD73 (Yegutkin, Samburski and Jalkanen, 2003; Pettengill *et al.*, 2013).

While SNPs in the *ENTPD1* gene regulate CD39 expression in different subsets of T cells including Tregs, genetic regulation of the $CD73^+$ T cell frequency is only observed in CD4 T cells and associated with a SNP in the *NT5E* locus (Roederer *et al.*, 2015). In Tregs, CD73 expression is mainly influenced by environmental factors (Mangino *et al.*, 2017). The NT5E gene is located on chromosome 6 and consists of nine coding exons (Ferrero, Faini and Malavasi, 2019). The absence of exon 7 due to alternative splicing, encodes a short version of the CD73 protein that lacks 50 amino acids in the C-terminal domain. This short isoform is primarily located inside the cell, catalytically inactive and unable to dimerize, which is well in line with the observation that the C-terminal domain provides the substrate binding pocket and mediates dimerization (Knapp *et al.*, 2012; Snider *et al.*, 2014). The alternative splicing product of CD73 is less abundant in different human tissues than the long isoform, but it negatively regulates CD73 expression by the formation of intracellular complexes leading to increased proteasome-dependent degradation of CD73 (Snider *et al.*, 2014). Another mechanism of transcriptional regulation of CD73 and CD39 involves hypoxia inducible factor 1 (HIF-1) and Sp1, two transcription factors that are upregulated under hypoxic conditions. Both act as transcriptional activators by binding to the promoter regions of CD73 and CD39, respectively (Synnestvedt *et al.*, 2002; Eltzschig *et al.*, 2009). Mutations in the *NT5E* gene leading to non-functional CD73 cause the rare genetic disease arterial calcification due to deficiency of CD73 (ACDC) (St. Hilaire *et al.*, 2011).

Apart from CD39 and CD73, other purinergic enzymes such as alkaline phosphatases, ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), adenosine deaminase or nucleotide-regenerating enzymes are involved in the regulation of extracellular nucleotides and nucleosides. Like CD39 and CD73, they are expressed on specific subsets of human immune cells or circulate as soluble enzymes in the bloodstream (Yegutkin *et al.*, 2002; Yegutkin, Samburski and Jalkanen, 2003). This network of membrane-bound and soluble purinergic enzymes tightly controls the availability of ATP and adenosine and thus regulates duration and magnitude of purinergic signaling.

1.2.3 ATP and adenosine signal through different types of purinergic receptors

Adenosine signals through P1 receptors and this family consists of four members named A_1, A_{2A}, A_{2B} and A_3. All four types are G protein-coupled receptors and regulate adenylate cyclase activity (Burnstock, 2007). A_{2A} and A_{2B} activate stimulatory G proteins (G_s), increase the activity of adenylate cyclase and rise the cAMP concentration inside the cell. Elevated levels of cyclic AMP lead to T cell suppression in a protein kinase A-dependent manner (Mosenden and Taskén, 2011). Although A_{2A} and A_{2B} receptors have a similar function, A_{2B} receptors are low affinity receptors and their activation requires higher concentrations of adenosine that probably only occur under pathophysiological conditions (Fredholm *et al.*, 2001; Bours *et al.*, 2006). Human A_{2B} receptors can also couple $G_{q/11}$ and regulate phospholipase C activity and calcium mobilization (Linden *et al.*, 1999). In contrast to A_2 receptors, A_1 and A_3 receptors couple to inhibitory $G_{i/o}$ proteins, negatively regulate adenylate cyclase and reduce intracellular cAMP levels (Burnstock, 2007). Human T cells predominantly express A_{2A} receptors, low amounts of A_{2B} and A_3 receptors, while A_1 receptor expression was not detected (Uhlen *et al.*, 2019). T cell activation increases expression of the three adenosine receptor subtypes existing in T cells (Koshiba *et al.*, 1999; Mirabet *et al.*, 1999; Gessi *et al.*, 2004).

Based on pharmacological data, ATP-activated P2 receptors were divided in two families, P2X and P2Y (Burnstock and Kennedy, 1985). P2X receptors are ATP-gated ion channels and seven subtypes ($P2X_{1\text{-}7}$) exist in humans. All members have cytoplasmic N- and C-termini, two transmembrane domains and a large extracellular loop (Burnstock, 2006). P2Y receptors are G protein-coupled receptors with seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. Eight functional subtypes ($P2Y_{1,2,4,6,11\text{-}14}$) have been identified. In general, P2Y receptors couple to $G_{q/11}$ or $G_{i/o}$ and inhibit adenylate cyclases or activate phospholipase Cβ to mobilize intracellular calcium. Depending on the subtype, P2Y receptors are activated by purines like ATP and ADP or by pyrimidines such as UTP, UDP and UDP-glucose. $P2Y_{11}$ does not only regulate phospholipase Cβ through $G_{q/11}$, but can also couple to G_s proteins and increase cAMP production and has a low affinity for its ligand ATP (Burnstock, 2007; Junger, 2011). T cells express $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_7$ and all types of P2Y receptors (Woehrle *et al.*, 2010; Junger, 2011).

1.2.4 Purinergic signaling controls effector and regulatory phases of immune responses

Purinergic signaling coordinates effector and regulatory mechanisms of immune cells to ensure appropriate immune responses while simultaneously limiting excessive tissue damage. In the acute initial phase, rapid release of ATP from immune cells initiates inflammatory reactions mainly by direct signaling of ATP through P2 receptors (Cekic and Linden, 2016). Activated human T cells release ATP that acts as autocrine co-stimulatory molecule on P2X receptors and provides signal amplification during T cell activation (Yip *et al.*, 2009; Woehrle *et al.*, 2010). Similarly, human neutrophils require autocrine ATP signaling through $P2Y_2$ for appropriate activation (Chen *et al.*, 2010) and for the amplification of chemotaxis signals (Chen *et al.*, 2006). Conversion of ATP to adenosine and activation of A_3 receptors further promotes neutrophil migration (Chen *et al.*, 2006). ATP-mediated gating of the $P2X_7$ receptor in LPS-primed monocytes and macrophages leads to the activation of the NLRP3 inflammasome and subsequent release of pro-inflammatory IL-1β (Danquah *et al.*, 2016).

In the second, subacute phase, immune cells release less ATP, and increased activity of ecto-nucleotidases converts extracellular ATP to adenosine. In this phase, P1 receptor signaling predominates over P2 receptor signaling, leading to the resolution of the inflammatory response (Cekic and Linden, 2016). The A_{2A} receptor is the main adenosine receptor in human T cells and its activation by adenosine stimulates adenylate cyclase to generate cAMP. Elevated intracellular levels of cAMP inhibit T cell activation, cytokine production, and proliferation (Linden and Cekic, 2012). Moreover, adenosine signaling through A_{2A} receptors expands murine Tregs and increases their suppressive capacity (Ohta *et al.*, 2012). In monocytes and macrophages, adenosine signaling inhibits the production of pro-inflammatory mediators and increases production of anti-inflammatory cytokines (Haskó and Cronstein, 2004).

In the late phase of immune responses, released ATP is rapidly converted to adenosine, which predominantly supports tissue protection and regeneration. Chronically elevated levels of adenosine, however, favor signaling through low affinity A_{2B} receptors. A_{2B} receptors are probably involved in pathological tissue remodeling processes during wound healing and can initiate pro-inflammatory reactions of immune cells because of their coupling to $G_{q/11}$ (Linden, 2005; Cekic and Linden, 2016).

1.3 Extracellular vesicles

Extracellular vesicle (EV) is a generic term for cell-derived lipid bilayer particles and due to the lack of markers that uniquely identify EVs, many approaches are discussed to classify EV subtypes (Yáñez-Mó *et al.*, 2015). Based on their generation, two main populations of EVs are distinguished (Figure 3). Microvesicles (or ectosomes) bud directly from the plasma membrane and are between 50 nm and 1 µm in size. The second type, exosomes, is of endosomal origin and their sizes ranges from 50 – 150 nm. Exosome generation requires inward budding of the endosomal membrane and formation of the multivesicular endosome (MVE). Fusion of the vesicle-containing MVE with the plasma membrane releases exosomes from the intracellular compartment (van Niel, D'Angelo and Raposo, 2018). EVs have the same membrane orientation as the plasma membrane of their parental cell, thus membrane proteins facing the extracellular space are detectable on the EV surface.

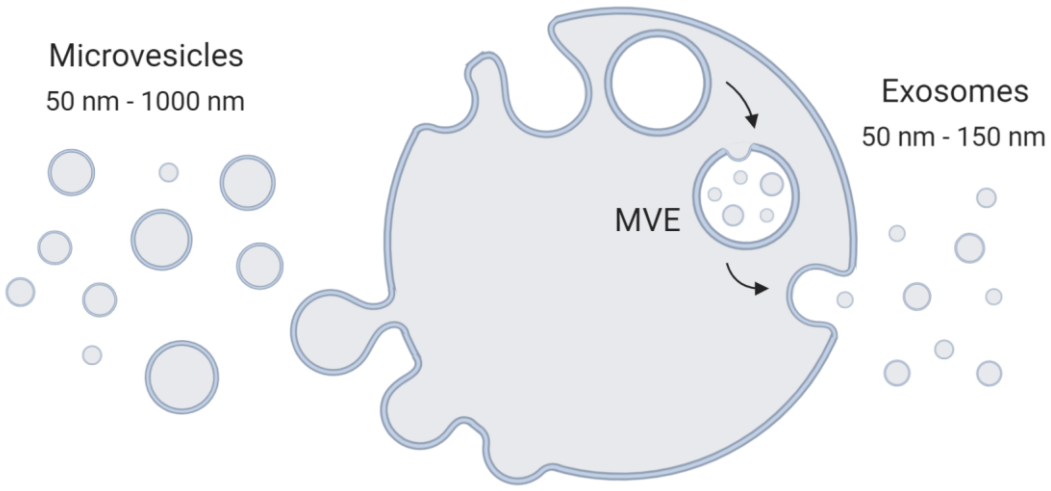

Figure 3 | Generation of extracellular vesicles. Cells release two main types of EVs, microvesicles and exosomes. Microvesicles bud directly from the plasma membrane and their sizes ranges from 50 nm – 1000 nm. Exosomes are between 50 and 150 nm in size and have endocytotic origin. Inward budding of the limiting membrane of endosomes leads to the generation of the multivesicular endosomes (MVE) and fusion of the MVE with the plasma membrane releases exosomes from the cell. Image created with BioRender.com.

Nearly all cell types release EVs and these EVs can serve as vehicles to communicate with other cells and to modulate the cell's microenvironment. EVs deliver cargo in form of membrane and cytosolic proteins, lipids and nucleic acids to recipient cells (Maas, Breakefield and Weaver, 2017). The exact mechanisms by which cargo is selected are not completely

understood, but a crucial step is the targeting of the cargo to site of EV formation. GPI-anchored proteins are enriched in specific membrane microdomains, termed lipid rafts, which participate in EV formation and this might facilitate the sorting of GPI-anchored proteins into EV membranes (de Gassart *et al.*, 2003; Skryabin *et al.*, 2020).

Receptors and ligands in the EV membrane enable interaction of EVs with target cells, induce signaling processes in these cells or mediate EV uptake by phagocytosis or fusion (Yáñez-Mó *et al.*, 2015; van Niel, D'Angelo and Raposo, 2018). For example, B cell-derived EVs can stimulate T cells through peptide-MHC class II complexes present on the surface of EVs (Raposo *et al.*, 1996). Analysis of cancer cell-derived EVs revealed that they are equipped with enzymes involved in purinergic signaling including CD39 and CD73 and that they are able to produce adenosine. EV-generated adenosine has immunosuppressive function on T cells and represents a mechanism by which EVs can modulate target cells (e.g. tumor-infiltrating T cells) in a cell contact- and uptake-independent manner (Clayton *et al.*, 2011; Morandi *et al.*, 2018). Deciphering the various immunomodulatory roles of EVs under physiological and pathological conditions is still an active field of research. Clearly, the immunomodulatory function of EVs highly depends on the cell type that produces the EVs as well as on the target cell and the microenvironment that EVs encounter during interaction with the recipient cells.

2. Aims of this thesis

Purinergic signaling controls effector and regulatory responses in the human immune system. The ecto-nucleotidase CD73 has a central role in generation of an anti-inflammatory milieu because its enzymatic activity yields adenosine, a potent suppressor of effector T cell function. CD73 is differentially expressed in subsets of human T cells and little is known about the fate of CD73 in activated T cells and its potential role in immune regulation.

The overarching goal of this thesis is to elucidate the immunoregulatory role of T cell-derived CD73 in the context of inflammation.

The specific aims are

I. To determine the regulation of CD73 expression in human T cells

II. To investigate the consequences of the loss of CD73 expression in activated T cells

III. To assess the immunomodulatory potential of non-cell-bound CD73

3. Results

The results of this thesis will be presented in three parts. In the first part, I will analyze the regulation of CD73 expression in human T cells. Next, I will investigate how CD73 is lost from the T cell plasma membrane after activation. Finally, I will assess the functional role of non-cell-bound CD73 in the context of inflammation.

3.1 Regulation of CD73 expression in human T cell subsets

During an immune response, leukocytes release ATP into the extracellular space, where it acts as pro-inflammatory molecule (Junger, 2011). In concert with CD39, CD73 generates immunosuppressive adenosine from ATP, indicating a crucial role for this ecto-nucleotidase in switching from a pro-inflammatory to an anti-inflammatory milieu.

3.1.1 Co-expression of CD73 and CD39 is a rare event in human T cells

To gain insight in the regulation of CD73, we first assessed *ex vivo* expression of ecto-nucleotidases CD73 and CD39 in different human leukocyte subsets by flow cytometry (Figures 4 and 5). We used the UMAP algorithm to generate a dimensionality-reduced representation of the data set, which allowed us to get an overview of marker expression at single cell level and visualizes CD73 and CD39 expression in human immune cells (Figure 4). The UMAP algorithm represents cells, which are similar to each other in all dimensions, in clusters in a two-dimensional plot. The characteristic expression of lineage markers (see Figure 36 in chapter 5.2.5) allowed assigning the clusters to different immune cell populations (Figure 4 A). We identified B cells, NK cells, monocytes and different T cell subsets, specifically CD8 T cells, CD4 T cells, regulatory T cells (Tregs), mucosal-associated invariant T cells (MAIT cells) and Tγδ cells. The density of cells within a cluster represents the frequency of these cells in the sample (Figure 4 B). A color-coded version of the UMAP plot for CD73 (Figure 4 C) and CD39 (Figure 4 D) revealed a cell type-specific expression of these ecto-nucleotidases in human immune cells. In the donor shown here, B cells were the most prominent cell type expressing CD73 and also a small proportion of CD8 and CD4 T cells were $CD73^+$ (Figure 4 C). In the Treg compartment, only very few cells expressed CD73; however, CD39 was abundantly present (Figure 4 C, D). CD39 was also highly expressed in monocytes and B cells. Further, the

data show that co-expression of CD73 and CD39 in human leukocytes is an exclusive feature of B cells.

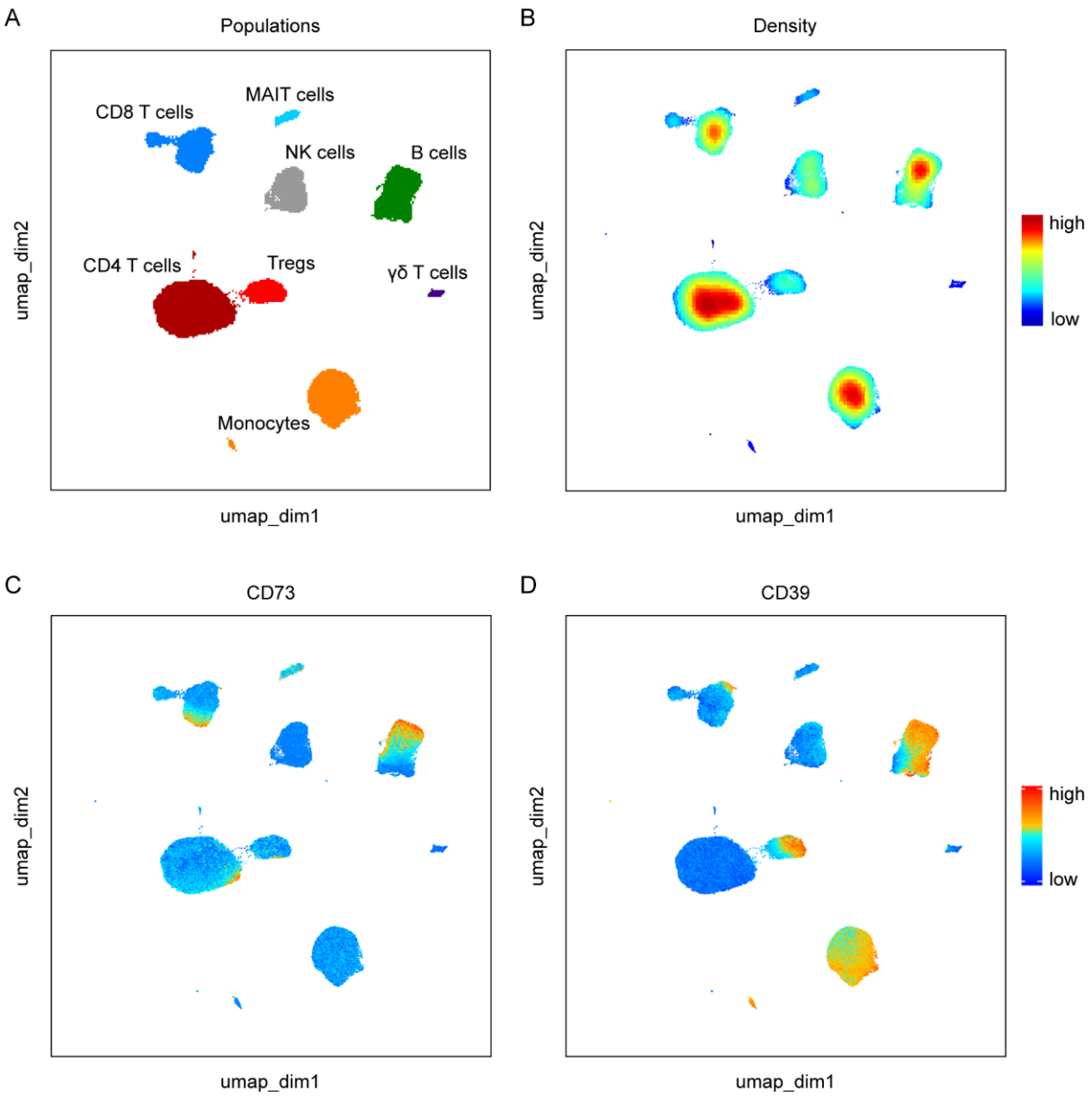

Figure 4 | Visualization of CD73 and CD39 expression in human immune cells using UMAP. A compensated FCS file was exported from FlowJo and analyzed in R with the UMAP algorithm (Laura Glau). (A) Based on lineage marker expression, clusters were assigned to immune cell populations. Detailed information on lineage marker expression in the different clusters are provided in Figure 36 in chapter 5.2.5. (B-D) Information on cell density (B) and expression levels of CD73 (C) and CD39 (D) are color-coded in the UMAP plot. Blue indicates low cell density or low marker expression, red represents high cell density or strong marker expression.

We detected CD39 and CD73 expression in different immune cell populations and aimed to quantify their expression in these cell types for several donors.

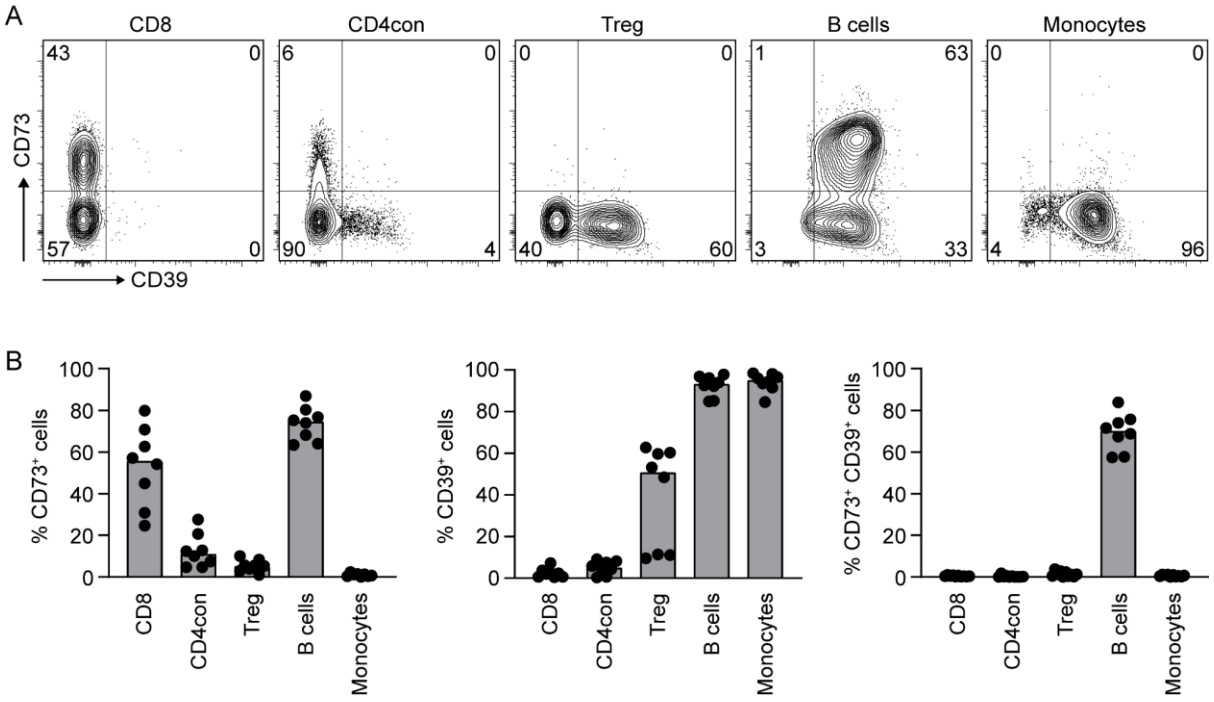

Figure 5 | Expression of CD73 and CD39 on human immune cells. CD73 and CD39 expression on human PBMCs from healthy donors were determined by flow cytometry (see Figure 34 in chapter 5.2.4 for the gating strategy). Monocytes and lymphocytes were gated based on FSC vs. SSC. Lymphocytes were further subdivided in B cells ($CD19^+$), CD8 T cells ($CD8^+$), Tregs ($CD4^+$ $CD25^{high}$ $CD127^{low}$) and conventional CD4 T cells (CD4con, defined as non-Treg $CD4^+$ T cells). (A) Representative contour plots show expression of CD73 and CD39 in different T cell subsets, B cells and monocytes. (B) Quantification of CD73 and CD39 expression in human leukocyte subsets for eight donors. Bar graphs show median percentage of CD73 and CD39 single or double positive cells for the different leukocyte subsets.

Representative contour plots show the gating strategy to determine expression of CD73 and CD39 in CD8 and conventional CD4 (CD4con) T cells, Tregs, B cells, and monocytes (Figure 5 A). Within the T cell compartment, 20 – 80% of CD8 T cells expressed CD73 (Figure 5 B, left). In CD4con T cells, CD73 expression varied between 4% and 25%, and less than 10% of Tregs were $CD73^+$. The majority of B cells was $CD73^+$, while monocytes did not express CD73. Regarding CD39, nearly all B cells and monocytes were $CD39^+$, whereas there was no or low expression in CD8 and CD4 T cells (Figure 5 B, middle). In Tregs, where CD39 expression is genetically driven, the percentage of $CD39^+$ Tregs ranges from 2% to 70% of cells (Rissiek *et al.*, 2015; Roederer *et al.*, 2015). In the donors tested here, the frequency of CD39-expressing Tregs was either around 10% or 60% of cells. Co-expression of CD73 and CD39 is a hallmark of murine Tregs (Deaglio *et al.*, 2007). In contrast to mice, human Tregs did not co-express both ecto-nucleotidases (Figure 5 B, right). In the human immune system, B cells were the only immune cell type co-expressing CD73 and CD39 with a decent frequency. In summary, our data show

that CD8 T cells are the major subset in the human T cell compartment expressing CD73 and that co-expression of CD73 and CD39 is a rare event in human conventional and regulatory T cells.

3.1.2 CD73 is highly expressed in naïve CD8 T cells

Considering the variety of CD73 expression in CD8 T cells, we further investigated whether CD73 expression depends on the differentiation status of T cells. Naïve T cells and a subset of effector memory T cells express CD45RA. In CD8 T cells, $CD45RA^+$ $CD73^+$ cells appeared as dense population, whereas $CD45RA^-$ cells showed a wide spread of CD73 expression levels, indicating variation in the amount of CD73 molecules per cell (Figure 6 A). In the CD4 compartment, we observed a strong difference in CD73 staining intensity between $CD45RA^+$ and $CD45RA^-$ T cells. $CD45RA^+$ CD4 T cells were $CD73^{dim}$ revealing that CD73 expression levels were much lower compared to $CD45RA^-$ CD4 T cells. Based on the expression of CD45RA and CD27, we defined T cells as naïve ($CD45RA^+$ $CD27^+$), central memory (CM, $CD45RA^-$ $CD27^+$), effector memory (EM, $CD45RA^-$ $CD27^-$) and terminal effector memory RA^+ (TEMRA, $CD45RA^+$ $CD27^-$) (Figure 6 B). In all donors tested here, the TEMRA population was not detected in CD4 T cells. Quantification of CD73 expression in these differentiation states showed that almost all (> 90%) naïve CD8 T cells expressed CD73, whereas less than 50% of memory (CM, EM, TEMRA) CD8 T cells were $CD73^+$ (Figure 6 C, D). The median fluorescence intensity (MFI) of CD73 revealed that naïve $CD73^+$ CD8 T cells have a higher density of CD73 at the plasma membrane than memory CD8 T cells. In CD4 T cells, the frequency of $CD73^+$ cells was not significantly different in naïve, CM and EM cells; however, CD73 MFI was clearly higher in memory compared with naïve CD4 T cells (Figure 6 C, D). In summary, the majority of naïve CD8 T cells express CD73, and CD73 density is higher in naïve than in memory CD8 T cells. In CD4 T cells, however, cells with high CD73 MFI harbor a memory phenotype.

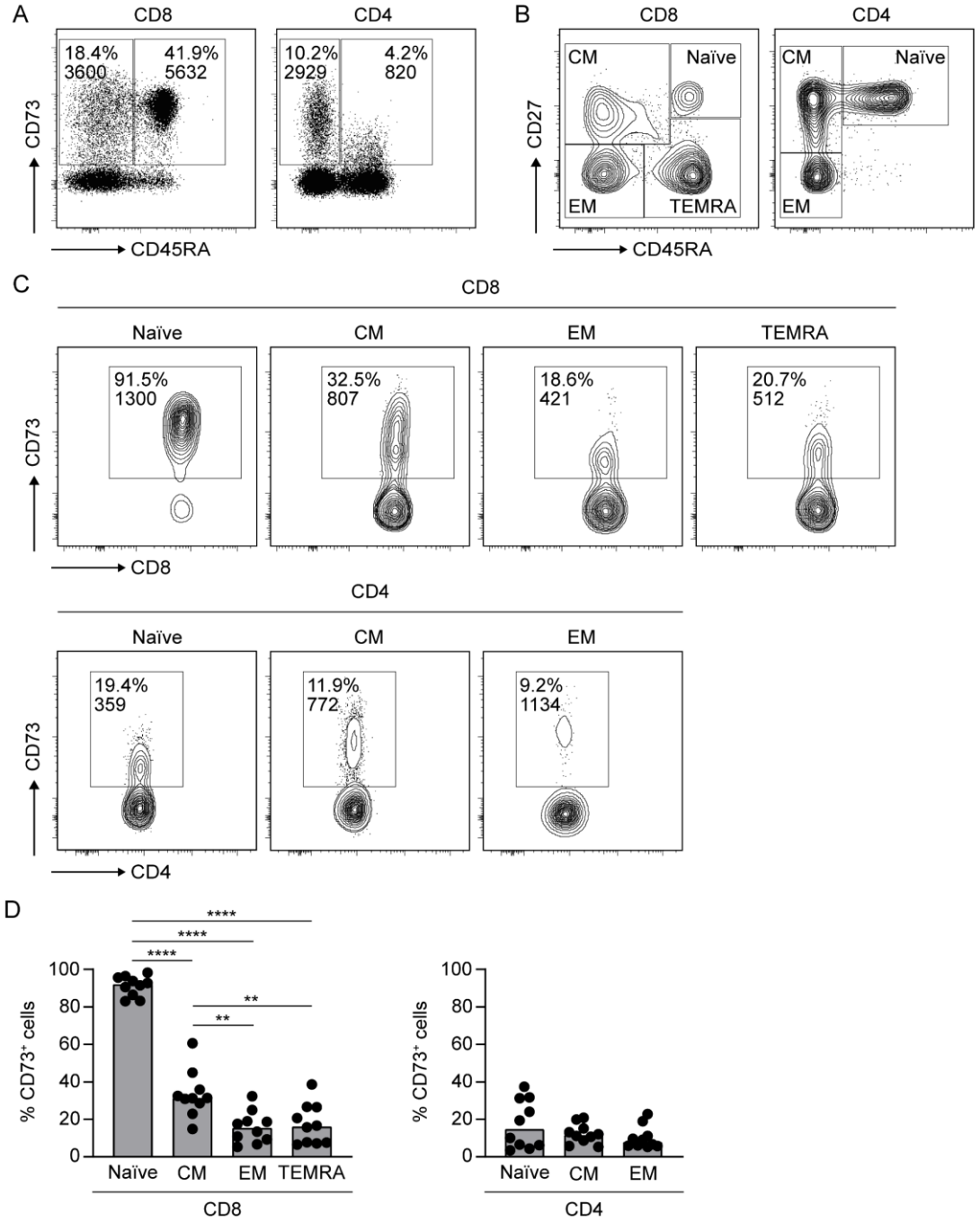

Figure 6 | CD73 is highly expressed in naïve CD8 T cells. CD8 and CD4 T cells were gated in human PBMCs from healthy donors. (A) Representative dot plots show CD73 versus CD45RA expression in CD8 and CD4 T cells, frequency and median fluorescence intensity (MFI) is indicated for the $CD73^+$ T cells. (B) Representative contour plots show the gating strategy to determine the differentiation status of CD8 and CD4 T cells. Based on CD45RA and CD27 expression, T cells were defined as naïve ($CD45RA^+$ $CD27^+$), central memory (CM, $CD45RA^-$ $CD27^+$), effector memory (EM, $CD45RA^-$ $CD27^-$) or terminal effector memory RA^+ (TEMRA, $CD45RA^+$ $CD27^-$). (C) Representative contour plots show frequency and MFI of $CD73^+$ CD8 or CD4 T cells according to the differentiation status. (D) Bars represent median frequency of $CD73^+$ CD8 or CD4 T cells in ten healthy individuals with respect to the T cell's differentiation status. RM one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all groups, ** $p < 0.01$, **** $p < 0.0001$.

3.1.3 T cell activation results in a reduction of CD73$^+$ cells and decreases the number of CD73 molecules on the cell surface

CD73 expression is less frequent in memory than in naïve CD8 T cells (Figure 6 C). Moreover, T cells from sites of inflammation show low expression of CD73 and increased expression of CD39 when compared to peripheral blood lymphocytes (Moncrieffe *et al.*, 2010; Botta Gordon-Smith *et al.*, 2015). To investigate the influence of T cell activation on CD73 and CD39 expression, we stimulated PBMCs or enriched T cells *in vitro* and monitored changes in ecto-nucleotidase expression by flow cytometry (Figure 7). In all three donors, the percentage of CD73$^+$ CD8 T cells increased within 24 hours after T cell activation followed by a drastic reduction that reached its minimum at day four in most donors (Figure 7 A, C). The MFI of CD73$^+$ CD8 T cells mirrored the initial increase, but dropped back to initial levels 24 hours after maximum intensity and was then stable for the following days (Figure 7 C). In contrast to the frequency of CD73$^+$ CD8 T cells that decreased from day two onwards, the percentage of CD39-expressing CD8 T cells increased after 48 hours of T cell activation and this was also reflected in the MFI of CD39$^+$ CD8 T cells (Figure 7 A, C). Furthermore, we observed the same kinetics of CD39 expression and signal intensity in CD4 T cells, although upregulation of CD39 expression was marginal in one donor (Figure 7 B, D). In terms of CD73, CD4 T cells of two donors showed an initial increase in the percentage and MFI of CD73$^+$ T cells as observed in CD8 T cells. Even though the decrease in CD73-expressing cells was not as prominent as in the CD8 T cells, we could measure a reduction in the frequency of CD73$^+$ CD4 T cells in two donors (Figure 7 B, D). In summary, T cell activation results in an upregulation of CD39 expression in CD8 and CD4 T cells. The frequency of CD73-expressing T cells increases within one day of activation, followed by a continuous reduction over the next days. The ecto-nucleotidase expression pattern of TCR-stimulated T cells reflects the phenotype of T cells isolated from sites of inflammation and reveals that low CD73 and elevated CD39 expression are characteristic for activated T cells.

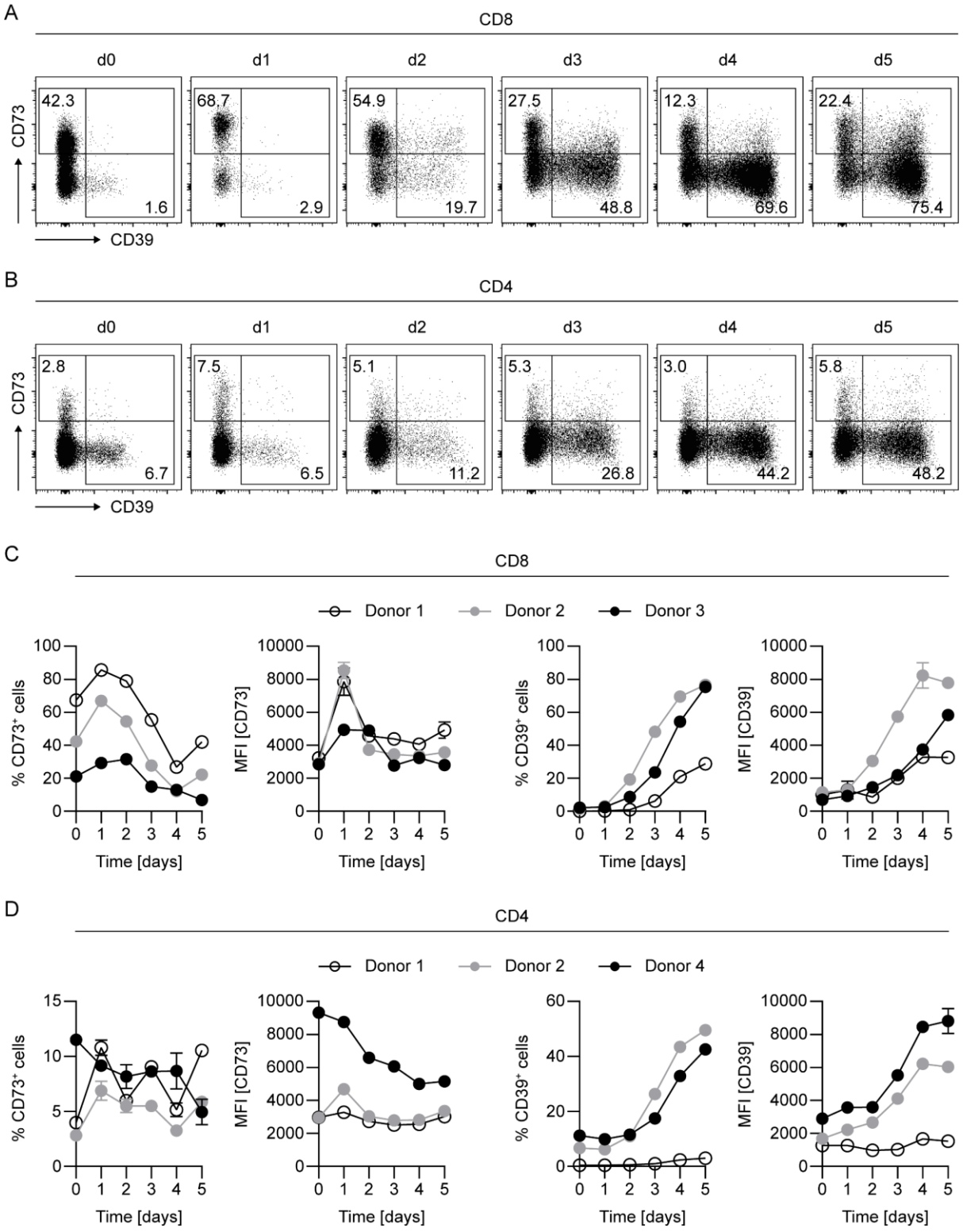

Figure 7 | Different kinetics of CD73 and CD39 expression in activated T cells. CD73 and CD39 expression were analyzed by flow cytometry in CD8 and CD4 T cells before and during *in vitro* T cell activation. PBMCs (donors 1 and 2) or enriched CD8 or CD4 T cells (donors 3 and 4) were stimulated with αCD3 or αCD3 and αCD28. (A-B) Dot plots show the gating strategy to determine the percentage of CD73- and CD39-expressing cells in CD8 (A) and CD4 (B) T cells during T cell activation for donor 2. (C-D) Frequency and MFI (mean ± SD) of $CD73^+$ or $CD39^+$ CD8 (C) or CD4 (D) T cells were determined for the different donors.

Within five days of culture, the net result of T cell activation is a decrease in the percentage of CD73$^+$ T cells and an upregulation of CD39 expression. We wondered if this low frequency of CD73-expressing T cells is stable for a longer period of culture and followed CD73 expression in CD8 and CD4 T cells for up to 21 days after PBMC stimulation (Figure 8).

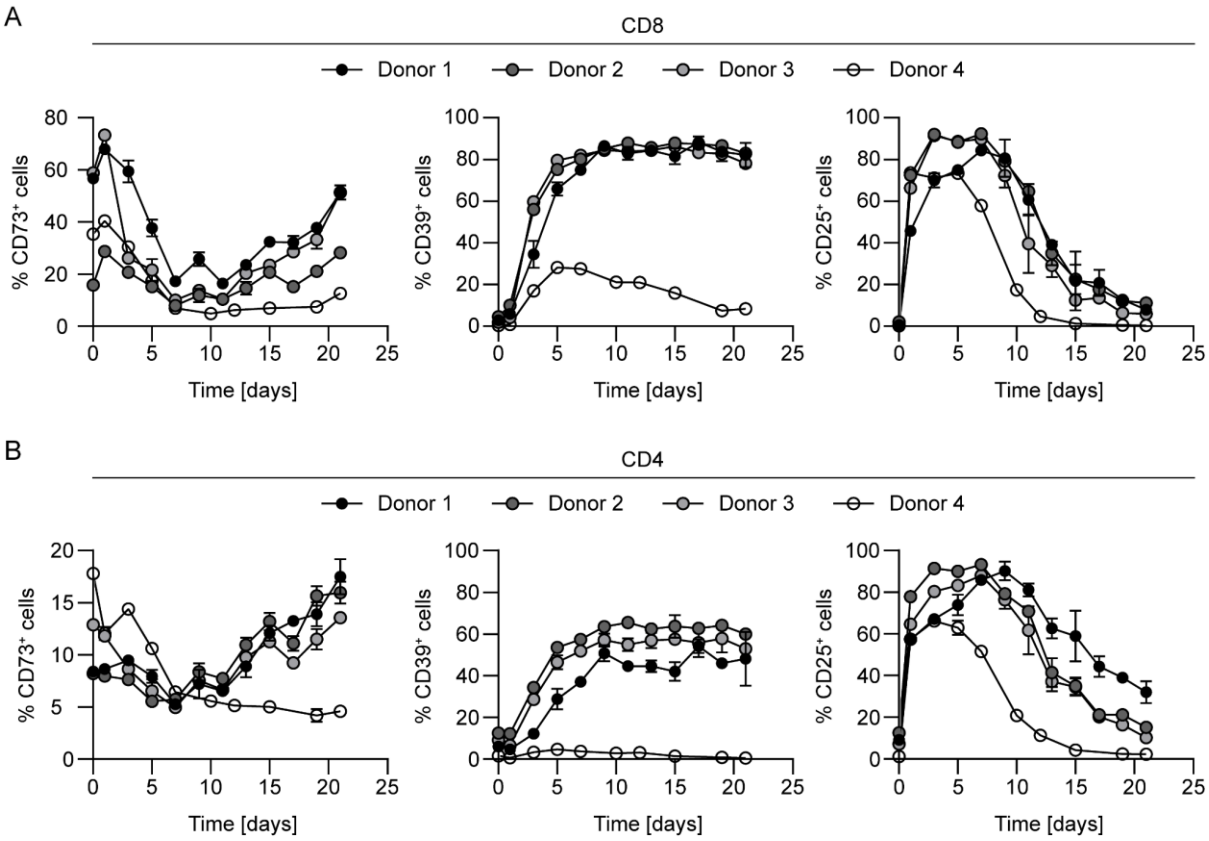

Figure 8 | CD8 and CD4 T cells re-express CD73 after three weeks of activation. PBMCs of four donors were stimulated with αCD3 and cultured for three weeks. From day four onwards, fresh medium with recombinant IL-2 was added every four days. At days seven and fifteen, half of the cells were transferred to a new plate to prevent overgrowing of the culture. Expression of CD73, CD39 and CD25 (mean ± SD) was assessed regularly by flow cytometry in CD8 (A) and CD4 (B) T cells.

Although flow cytometric analysis revealed interindividual differences in the timing of CD73 loss from the cell membrane, we observed a commonly shared pattern of changes in CD73 expression for CD8 T cells (Figure 8 A). In all four donors, the percentage of CD73$^+$ CD8 T cells increased within 24 hours after activation and then decreased until day seven. At this time point, the amount of CD73$^+$ CD8 T cells was around 10% in three of four donors, independent of the frequency at day zero. After day seven, most donors showed partial re-expression of CD73 in CD8 T cells. In CD4 T cells (Figure 8 B), all donors showed a decrease of CD73$^+$ T cells until day seven of activation that was followed by a steady increase in the majority of donors.

The percentage of $CD39^+$ CD8 and CD4 T cells increased in three of four donors, reached maximum levels around day nine after activation and stayed high until the end of culture (Figure 8 A, B). In the fourth donor, the highest amount of CD39-expressing CD8 T cells was 30% and the percentage of $CD39^+$ cells decreased after one week of culture. CD4 T cells from this donor showed only a marginal increase in CD39-expressing T cells. In general, the maximum frequency of CD39-expressing cells was lower in CD4 compared to CD8 T cells. The expression of activation marker CD25 strongly increased within 24 hours of activation and stayed high for one week (Figure 8 A, B). At around day ten of culture, T cells progressively downregulated CD25, and this downregulation occurred earlier in the donor with low CD39 expression. Overall, the data show a dynamic change in CD73 expression during T cell activation. After one week of activation, the percentage of $CD73^+$ T cells is minimal. In the following days, most donors show partial re-expression of CD73.

After one to two days of T cell activation, we observed a steady loss in the frequency of $CD73^+$ CD8 T cells (Figures 7 and 8). Notably, human T cells start to proliferate at day three, indicating that T cells might require the loss of cell surface CD73 to induce proliferation. To investigate if CD73 expression is linked to the capacity of T cells to proliferate, we labeled human T cells with eFluor 670 prior to activation and analyzed CD73 expression and proliferation by flow cytometry (Figure 9).

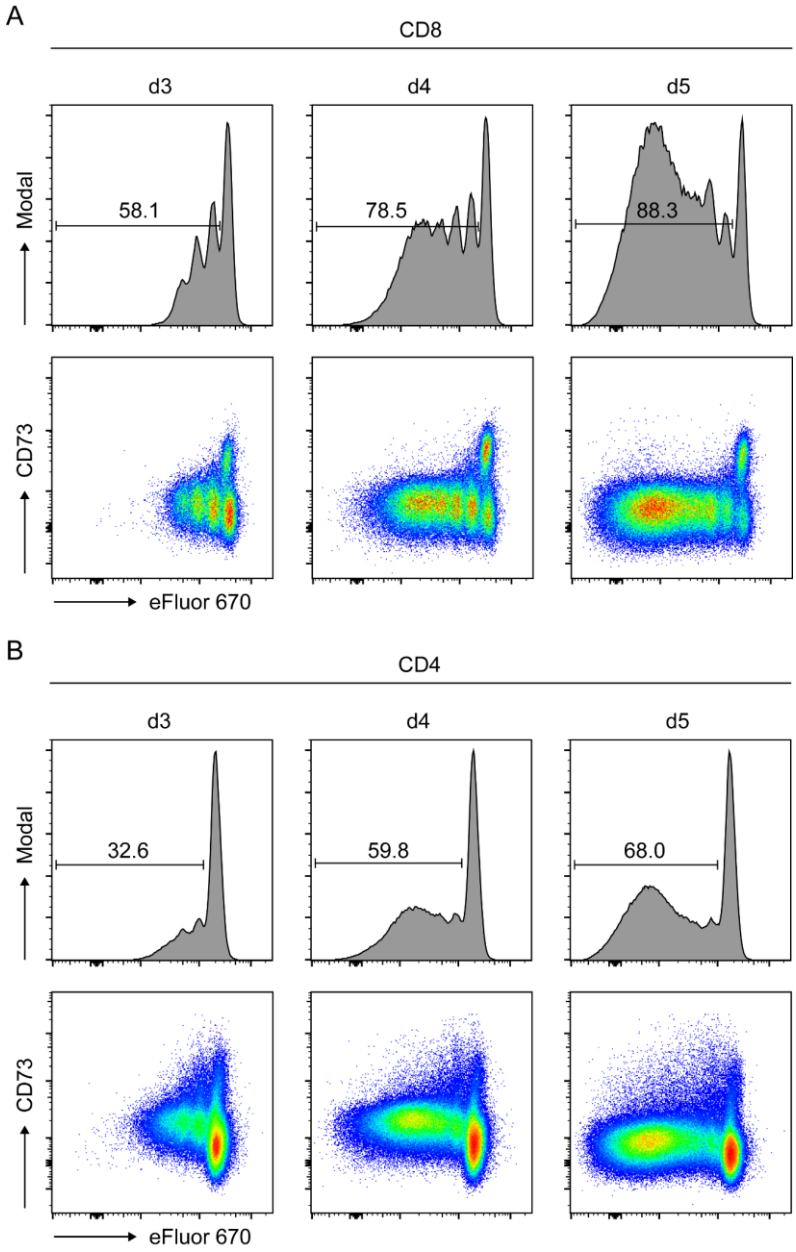

Figure 9 | Proliferating T cells do not express CD73. CD8 (A) and CD4 (B) T cells were labeled with eFluor 670, stimulated with αCD3 and αCD28, and proliferation was analyzed by flow cytometry on days three to five after stimulation. Data correspond to donors 3 and 4 from Figure 7. (A-B) Histograms (upper row) show dilution of proliferation dye eFluor 670 and percentage of cells in division in CD8 and CD4 T cells. Pseudocolor dot plots (lower row) display CD73 expression versus proliferation in CD8 and CD4 T cells.

In line with published data (Mishima *et al.*, 2017), we observed that CD8 T cells showed a higher degree of proliferation compared to CD4 T cells (Figure 9). Proliferating CD8 and CD4 T cells were $CD73^{-}$, supporting the hypothesis that loss of CD73 is necessary to induce proliferation. Pseudocolor plots indicated that CD4 T cells lose their CD73 expression with each division step. Proliferating $CD73^{-}$ CD8 T cells may originate from two phenomena: either only $CD73^{-}$ T cells proliferate and thus overgrow the $CD73^{+}$ population, or an active regulatory

process eliminates CD73 expression in former CD73$^+$ T cells during T cell activation. To answer the question whether CD73$^+$ CD8 T cells proliferate, we repeated the experiment with sorted CD73$^+$ (blue) and CD73$^-$ (red) CD8 T cells (Figure 10).

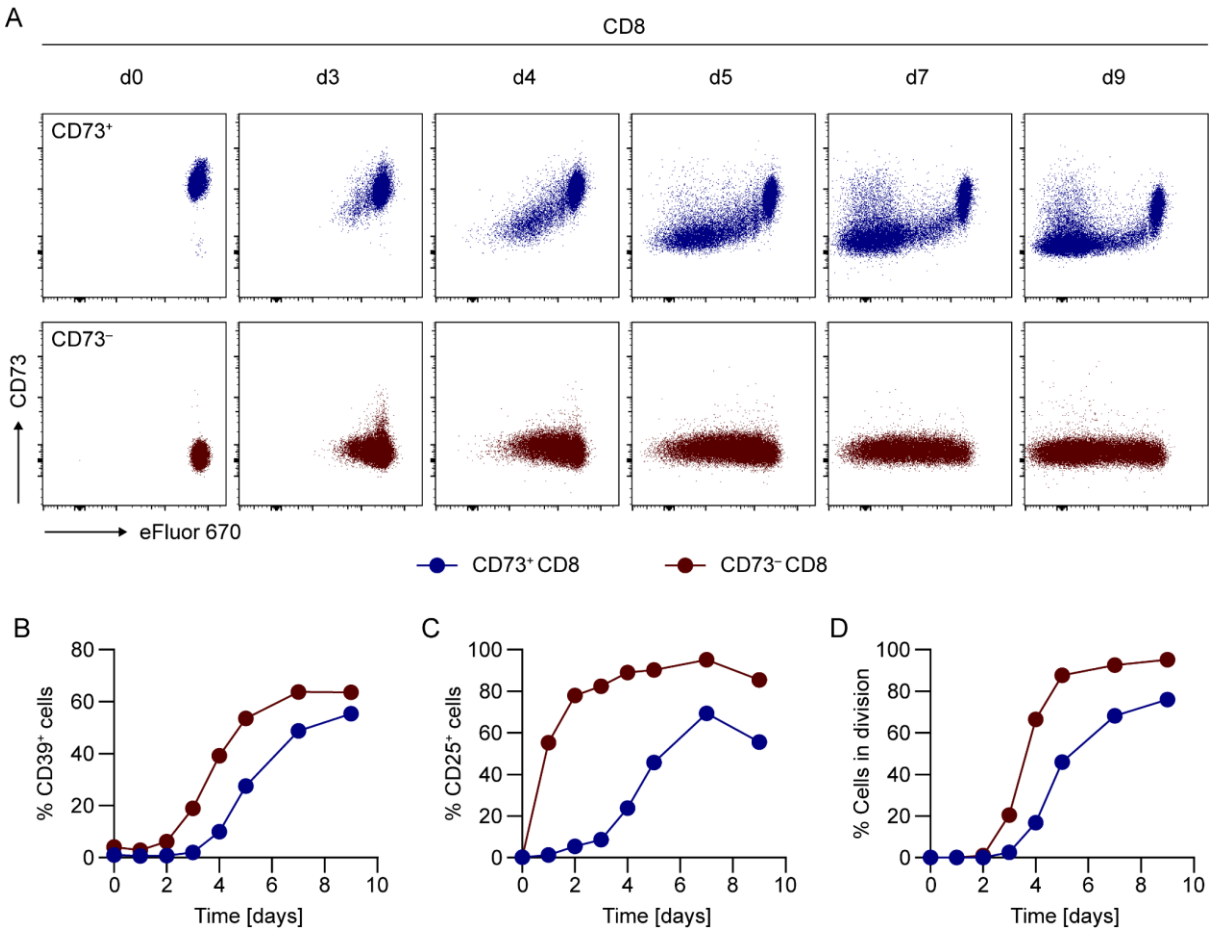

Figure 10 | Activation induces loss of surface CD73 expression in CD8 T cells. PBMCs were labeled with eFluor 670 and sorted into CD73$^+$ (blue) and CD73$^-$ (red) CD8 T cells prior to stimulation with αCD3 and αCD28. (A) Representative dot plots show CD73 expression versus proliferation dye eFluor 670 in CD73$^+$- and CD73$^-$-sorted CD8 T cells during T cell activation. (B-C) Frequency (mean ± SD) of CD73$^+$- and CD73$^-$-sorted CD8 T cells expressing CD39 (B) or CD25 (C) up to nine days after activation. (D) Percentage (mean ± SD) of CD73$^+$- and CD73$^-$-sorted CD8 T cells in division. (A-D) Data are shown for one donor representative for three independent experiments.

Flow cytometric analysis revealed that both CD73$^+$- and CD73$^-$-sorted CD8 T cells proliferated in response to stimulation with αCD3 and αCD28 (Figure 10 A, D). Moreover, CD73$^+$ cells lost surface CD73 expression with proliferation while CD73$^-$ sorted cells stayed negative for CD73 after activation. After one week of activation, solely former CD73$^+$ cells showed partial re-expression of CD73. CD8 CD73$^-$ T cells exhibited more pronounced upregulation of activation markers CD39 and CD25 and showed stronger proliferation compared to CD73$^+$-sorted CD8

T cells (Figure 10 B-D). In summary, activation-dependent loss of surface CD73 expression from CD73$^+$ cells as well as proliferating CD73$^-$ cells contribute to a decreased proportion of CD73$^+$ cells after CD8 T cell activation.

Naïve and memory CD8 T cells are functionally different and vary in their CD73 expression levels (Figure 6). To determine if the loss of CD73 is influenced by the differentiation status, we performed a further experiment using sorted naïve (mostly CD73$^+$) and memory (mostly CD73$^-$) CD8 T cells and measured CD73 expression after T cell activation (Figure 11).

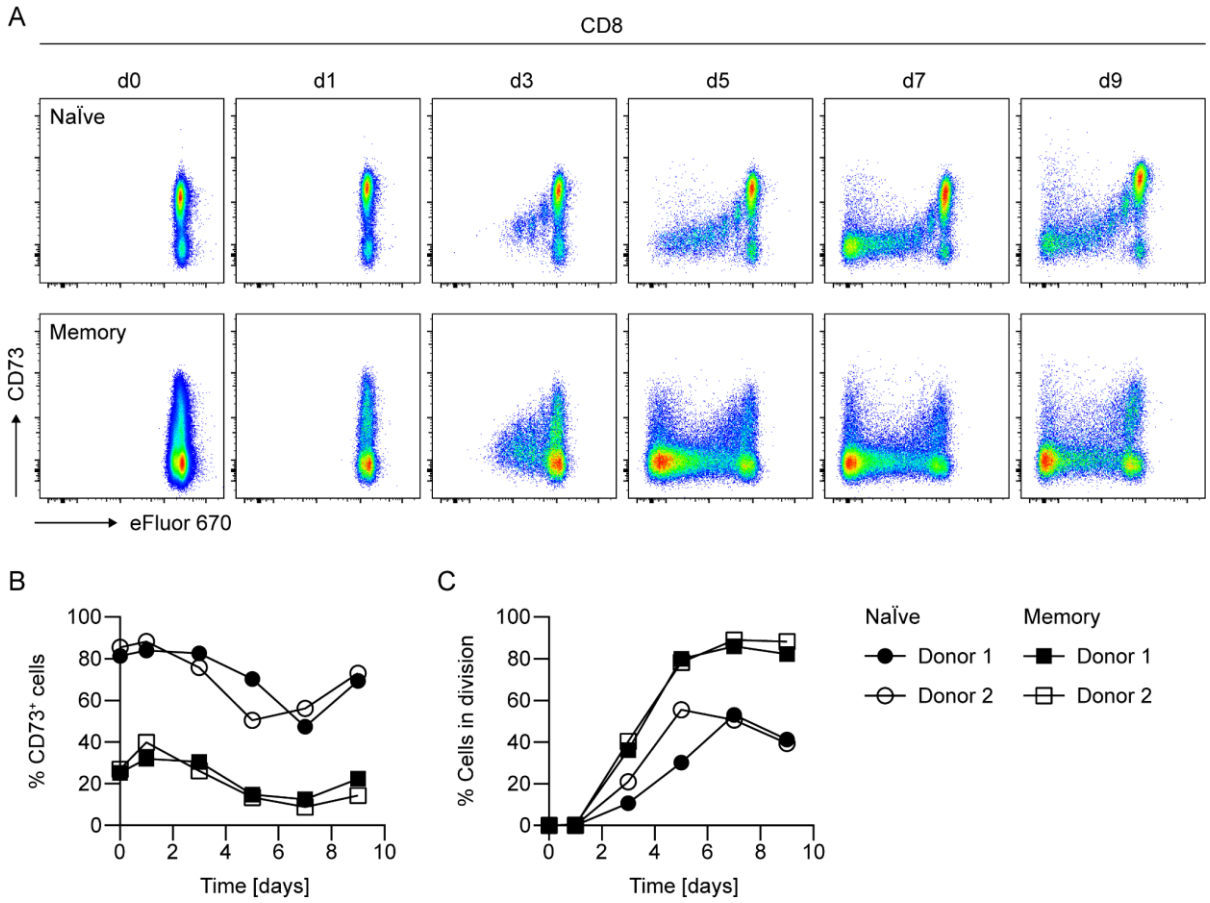

Figure 11 | Proliferating CD8 T cells in the naïve compartment are almost exclusively CD73$^+$. PBMCs were labeled with eFluor 670, sorted into naïve (CD45RA$^+$ CD27$^+$) and memory (CD45RA$^-$) CD8 T cells and stimulated with αCD3 and αCD28. (A) Pseudocolor plots show changes of eFluor 670 and CD73 expression during activation of naïve and memory CD8 T cells from one representative donor out of four donors. (B-C) Graphs display the frequency of CD73$^+$ cells (B) and the percentage of cells in division (C) in naïve (circles) and memory (squares) CD8 T cells in two out of four donors during T cell activation.

As expected, memory cells proliferated stronger than naïve cells (Figure 11 A, C) and T cell activation resulted in a reduction of CD73$^+$ T cells (Figure 11 B). Remarkably, in the naïve

compartment, primarily $CD73^+$ CD8 T cells went into division, while only very few $CD73^-$ naïve CD8 T cells proliferated (Figure 11 A). In contrast, $CD73^-$ and $CD73^+$ memory CD8 T cells had the capacity to proliferate. Naïve and memory $CD73^+$ CD8 T cells lost CD73 expression with proliferation and cells that have undergone multiple divisions partially re-expressed the ecto-nucleotidase.

Altogether, T cell activation results in a decreased percentage of $CD73^+$ T cells because proliferating T cells lose CD73 surface expression and this loss is independent of the T cell differentiation status.

3.2 Mechanisms of CD73 loss from the T cell membrane

We have observed that proliferating T cells are $CD73^-$, linking loss of CD73 expression from the T cell surface to proliferation. In this second part, I will investigate the role of CD73 during T cell activation by manipulating CD73 expression in human T cells. Further, I will examine how CD73 is lost from the cell membrane.

3.2.1 Artificial removal of cell surface CD73 or blockade of CD73 enzymatic activity does not influence T cell activation kinetics

The A_{2A} receptor is the predominant adenosine receptor in human T cells and its expression is further upregulated after T cell activation (Koshiba *et al.*, 1999; Uhlen *et al.*, 2019), which renders them susceptible towards adenosine-mediated suppression. Activation-induced loss of plasma membrane CD73, the enzyme that generates adenosine, might thus be a T cell-intrinsic mechanism to prevent autocrine T cell suppression. We wondered if elimination of CD73 function prior to T cell activation would abrogate early autocrine adenosine-mediated suppression and result in accelerated T cell activation. To investigate this, we artificially removed the GPI-anchored CD73 from the T cell plasma membrane using phospholipase C (PI-PLC) or blocked its enzymatic activity and measured T cell activation in untreated and treated CD8 T cells (Figure 12).

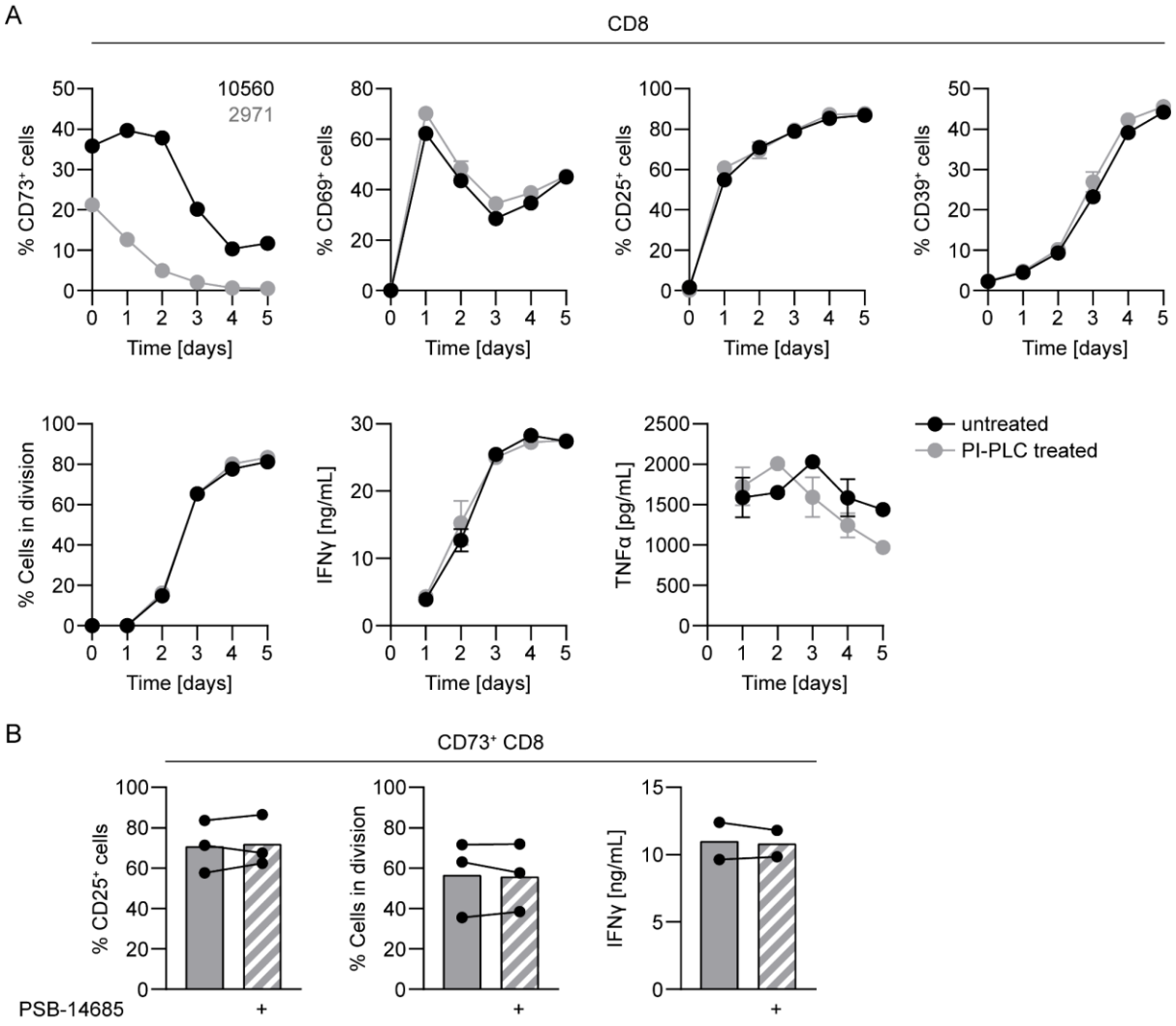

Figure 12 | Artificial removal of CD73 or blockade of CD73-enzymatic activity does not accelerate T cell activation. (A) eFluor 670-labeled CD8 T cells were incubated with PI-PLC (0.5 U/mL) for 1 hour at 37°C and 5% CO_2 (grey circles) or left untreated (black circles) prior to stimulation with αCD3 and αCD28. Cells were analyzed daily by flow cytometry and cytokines in the cell culture supernatant were measured by ELISA. Numbers in the upper left plot indicate MFI of CD73⁺ CD8 T cells in untreated and PI-PLC-treated cells at day zero. All graphs show data (mean ± SD) for one donor. (B) CD73⁺ CD8 T cells were sorted from eFluor 670-labeled PBMCs and stimulated for four days with αCD3 and αCD28 in the presence of ADA inhibitor EHNA (10 µM). When indicated, the CD73-specific inhibitor PSB-14685 (10 µM) was added to the T cells at the beginning of the culture. Percentage of CD25⁺ and proliferating T cells were measured by flow cytometry and IFNγ production was assessed by ELISA. Bars show mean values for three or two donors.

Even though the incubation of CD8 T cells with PI-PLC did not completely remove CD73 from the T cell plasma membrane, we measured a strong reduction in CD73 MFI after PI-PLC treatment, indicating that the remaining CD73⁺ CD8 T cells carried considerably fewer CD73 proteins on their cell membrane than untreated CD8 T cells (Figure 12 A). The characteristic increase in CD73⁺ CD8 T cells at day one after activation was only detected in the untreated CD8 T cells. T cell activation further decreased CD73 expression in both conditions, and

resulted in a complete removal of cell surface CD73 expression in PI-PLC-treated CD8 T cells. When we compared expression of the activation markers CD69, CD25 and CD39 between PI-PLC-treated and untreated CD8 T cells, we observed that the marker-specific pattern of upregulation was similar in both conditions (Figure 12 A). Moreover, proliferation and IFNγ production were identical irrespective of PI-PLC treatment. Only TNFα production showed a slight shift indicating earlier production in PI-PLC-treated CD8 T cells. In summary, these data lead to the conclusion that artificial removal of CD73 does not change CD8 T cell activation kinetics.

PI-PLC treatment does not exclusively remove CD73 but all GPI-linked proteins from the T cell plasma membrane. To explicitly investigate the role of CD73-mediated AMPase activity in the T cell activation process, we blocked CD73 enzymatic activity with the specific inhibitor PSB-14685 (Bhattarai *et al.*, 2019). We used $CD73^+$ CD8 T cells in the assay to exclude any influence of $CD73^-$ CD8 T cells on the activation process. The addition of adenosine deaminase (ADA) inhibitor EHNA ensured that endogenously produced adenosine is not degraded to inosine and can function as immunosuppressive mediator through the activation of A_{2A} receptors.

Flow cytometric analysis of T cell activation and proliferation revealed that treatment with PSB-14685 did not change the frequency of $CD25^+$ T cells or the percentage of cells in division compared to untreated control T cells (Figure 12 B). Likewise, IFNγ production was similar in in both groups. These data show that inhibition of CD73-mediated AMPase activity does not alter the dynamics of T cell activation in this *in vitro* setting. Elimination of CD73 enzymatic function does not seem to result in substantially reduced adenosine levels, probably because adenosine generation is low during this phase of activation. In summary, we conclude that elimination of membrane-bound CD73 does not actively trigger T cell activation, but it is rather one of its consequences.

3.2.2 Constant exposure of T cells to CD73 does not inhibit T cell activation

Premature elimination of cell surface CD73 does not expedite T cell activation. Nevertheless, proliferating T cells lose CD73 expression during T cell activation. We wondered how T cells would react when they cannot escape CD73 activity during T cell activation. To test this, we co-cultured PBMCs with human CD73-transfected HEK293 cells to constantly expose T cells to enzymatically active CD73 (Figure 13). Co-culture with CD73-transfected HEK293 cells is

advantageous compared to addition of recombinant protein because the HEK293 cells constitutively express high levels of CD73 and ensure that CD73 is present throughout the culture.

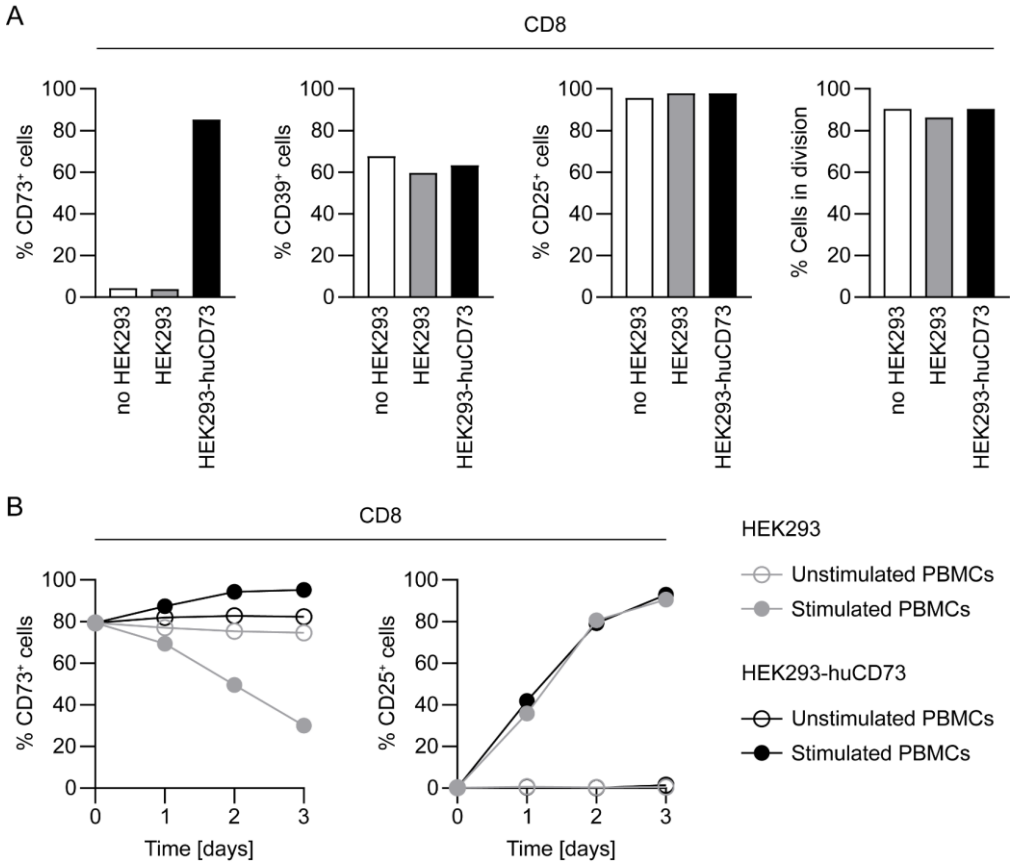

Figure 13 | Constant exposure of CD8 T cells to CD73 does not abrogate T cell activation. (A) PBMCs were labeled with eFluor 670, stimulated with αCD3 and cultured in the absence or presence of HEK293 cells. Frequency of $CD73^+$, $CD39^+$, $CD25^+$ and proliferating CD8 T cells were determined after three days of activation. White bars indicate absence of HEK293 cells, grey bars indicate co-culture with untransfected HEK293 cells and black bars indicate co-culture with human CD73-transfected HEK293 cells. Graphs show data from one donor. (B) PBMCs were co-cultured with untransfected (grey) or human CD73-transfected (black) HEK293 cells. Stimulation of PBMCs with αCD3 is indicated by filled circles, open circles represent unstimulated PBMCs. Graphs show data from one donor.

After three days of activation, we measured a striking increase in the frequency of $CD73^+$ CD8 T cells when PBMCs were co-cultured with human CD73-transfected HEK293 cells. We did not observe this effect in the presence of untransfected HEK293 or in the control condition without HEK293 cells (Figure 13 A). However, co-culture of PBMCs with human CD73-transfected HEK293 cells had no influence on the upregulation of CD39 and CD25 or on proliferation. In further experiments, we determined that human T cells acquired proteins

from transfected HEK293 cells in a contact-dependent manner and that protein acquisition was not limited to human or GPI-anchored proteins (data not shown). In the presence of human CD73-transfected HEK293 cells, acquisition of CD73 was already detectable after 24 hours of activation and promoted by T cell stimulation (Figure 13 B). In contrast to CD8 T cells co-cultured with human CD73-transfected HEK293 cells, CD8 T cells that were co-cultured with untransfected HEK293 cells showed activation-induced loss of surface CD73 expression. The frequency of $CD25^+$ CD8 T cells increased progressively over three days when PBMCs were stimulated with αCD3, and this increase was independent of the type of HEK293 cells added to the PBMCs. Altogether, co-culture of PBMCs with human CD73-transfected HEK293 cells revealed that constant exposure to CD73 did not abrogate T cell activation. Moreover, T cells acquired CD73 from transfected HEK293 cells and these increased levels of CD73 on the plasma membranes of CD8 T cells did not have any influence on the activation process or on proliferation. From these data we again conclude that loss of membrane-bound CD73 is not a prerequisite for, but a consequence of T cell activation.

3.2.3 A T cell-intrinsic mechanism controls loss of CD73 surface expression during T cell activation

The percentage of $CD73^+$ T cells decreases as a consequence of T cell activation, and we wondered how the loss of CD73 from the plasma membrane is regulated. CD73 is a GPI-anchored protein that is sensitive to phospholipases (Thomson *et al.*, 1990), and several studies reported CD73-specific AMPase activity in human body fluids, suggesting the presence of CD73 in a soluble form (Pettengill *et al.*, 2013; Zeiner *et al.*, 2019). Further, we could show that the cell culture supernatant of activated T cells displays AMPase activity, indicating the release of soluble CD73 (Winzer, 2017). In humans, phospholipase C and D were reported to shed CD73 (Low and Prasad, 1988; Kalsi *et al.*, 2002); however, previous data generated in our lab revealed that they are not involved in activation-induced loss of lymphocyte CD73. A recent publication proposed a role for matrix metalloproteinase-9 (MMP-9) in the shedding of CD73 from murine retinal pigment epithelium (Zhang *et al.*, 2018). We blocked MMP-9 activity during T cell activation, but MMP-9 did not regulate activation-induced loss of CD73 from the T cell plasma membrane (data not shown).

Enzymatic shedding of CD73 during T cell activation would require that the responsible enzyme is expressed on T cells where it faces the extracellular space or that it is released into

the cell culture supernatant. Consequently, contact to activated T cells or their cell culture supernatant should reduce CD73 surface expression in unstimulated T cells.

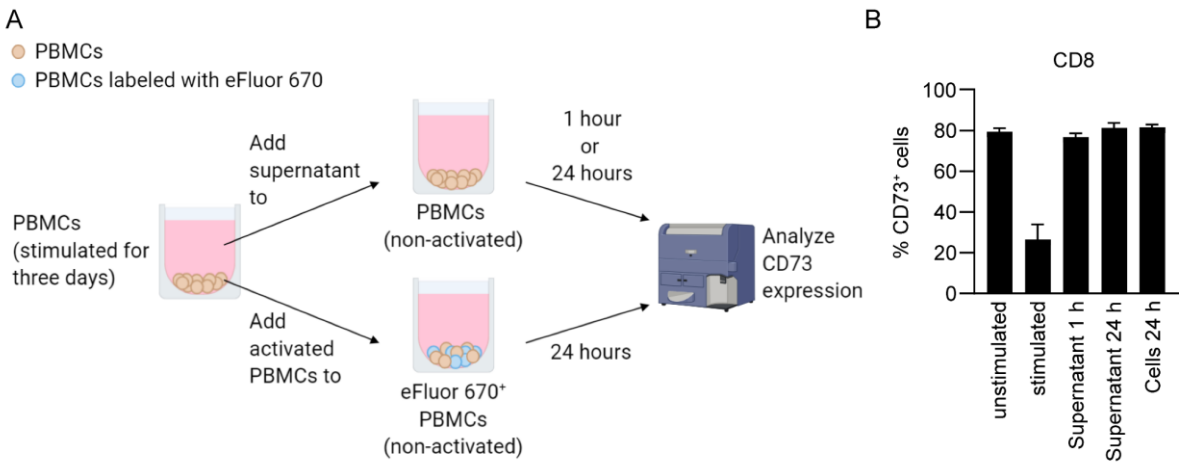

Figure 14 | CD73 is not enzymatically shed from the cell surface during T cell activation. (A) Schematic representation (image created with BioRender.com) of the experiment conducted to test whether CD73 is enzymatically shed from the plasma membrane during T cell activation. After three days of stimulation with αCD3, the cell culture supernatant of activated PBMCs or the cells themselves were added to unstimulated PBMCs. In the latter case, unstimulated PBMCs were labeled with eFluor 670 to distinguish activated and non-activated cells. After one hour or 24 hours, CD73 expression of the treated cells was analyzed by flow cytometry (B). The percentages of $CD73^+$ CD8 T cells in unstimulated and stimulated PBMCs served as reference values to estimate if shedding was induced in treated cells. Bars show data (mean ± SD) for one representative donor out of two donors.

Figure 14 A shows a schematic representation of the experiment performed to test whether lymphocyte CD73 is enzymatically shed during T cell activation. Activated PBMCs or their cell culture supernatant were added to unstimulated PBMCs and after incubation for one hour or 24 hours, the percentage of CD73-expressing CD8 T cells was determined by flow cytometry. In unstimulated PBMCs, 80% of CD8 T cells expressed CD73 on their cell surface (Figure 14 B). After stimulation with αCD3, approximately 25% of CD8 T cells remained $CD73^+$. Neither incubation with cell culture supernatant of activated PBMCs, nor contact to activated PBMCs reduced the frequency of $CD73^+$ CD8 T cells (Figure 14 B). In summary, the data indicate that CD73 is not enzymatically shed from the plasma membrane during T cell activation. We further conclude that a T cell-intrinsic signal controls the activation-induced loss of CD73 from the T cell plasma membrane.

3.2.4 T cell activation does not induce strong downregulation of CD73 mRNA

We considered downregulation of CD73 mRNA as a possible intrinsic mechanism that results in decreased CD73 surface expression, and performed qPCR of CD8 T cells at different time points of activation to monitor changes in *NT5E* gene expression. To correlate gene expression to protein expression, a part of the CD8 T cells were analyzed by flow cytometry while the rest was used for RNA isolation and subsequent qPCR. In parallel to CD73 and *NT5E* expression, we measured CD39 and *ENTPD1* expression because CD39 is known to be upregulated upon T cell activation (Rissiek *et al.*, 2015). After three days of T cell activation, the percentage of CD39-expressing cells strongly increased in $CD73^+$- (blue) and $CD73^-$-sorted (red) CD8 T cells (Figure 15 A, B, see also Figure 10). *ENTPD1*, the gene encoding CD39, was upregulated in both groups (Figure 15 C). In comparison with $CD73^+$-sorted CD8 T cells, $CD73^-$-sorted CD8 T cells showed earlier upregulation of *ENTPD1* and CD39 surface expression. In $CD73^+$-sorted CD8 T cells, the frequency of CD73-expressing cells decreased by 50% between days three and five after activation (Figure 15 A, D); however, *NT5E* gene expression at day five was not different compared to day zero levels and even slightly increased at days seven and nine (Figure 15 E). Dot plots displaying CD73 and CD39 expression revealed a detectable increase in $CD39^+$ $CD73^+$ double positive cells after one week of activation that did not change the overall percentage of CD73-expressing cells (Figure 15 A, D). $CD73^-$-sorted cells stayed negative for CD73 surface expression during the course of activation (Figure 15 A, D). Of note, the pattern of *NT5E* expression during T cell activation was similar between $CD73^+$- and $CD73^-$-sorted CD8 T cells (Figure 15 E). In summary, there was a strong correlation between *ENTPD1* upregulation and CD39 surface expression, whereas changes in CD73 surface expression were not matching *NT5E* gene expression.

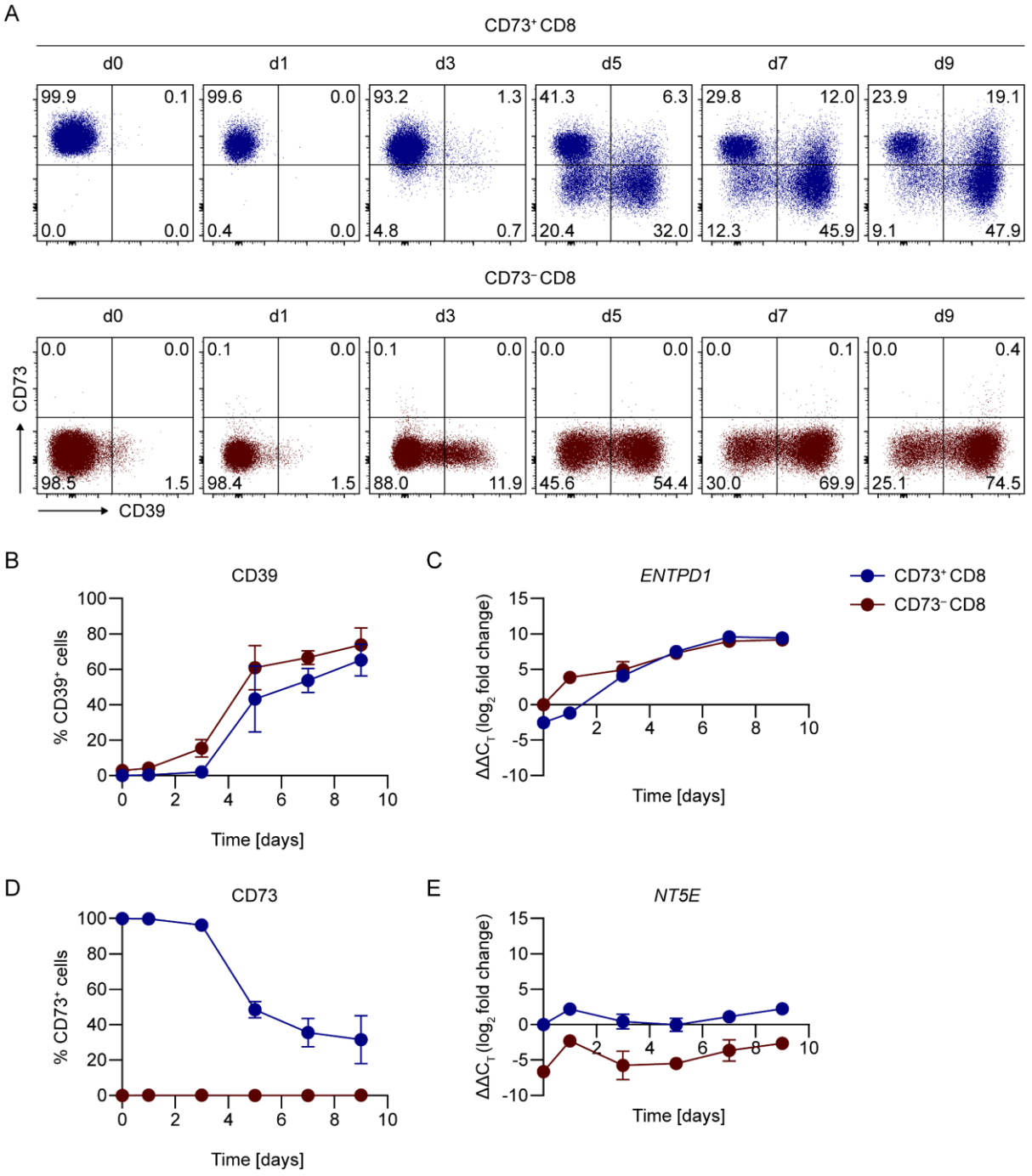

Figure 15 | T cell activation does not induce a strong downregulation in *NT5E* gene expression. PBMCs were labeled with eFluor 670 and sorted into CD73$^+$ (blue) and CD73$^-$ (red) CD8 T cells prior to stimulation with αCD3 and αCD28. (A, B, D) Flow cytometric analysis of CD73 and CD39 expression in CD73$^+$- and CD73$^-$-sorted CD8 T cells at different time points during activation shown as dot plots (A) for one representative donor or as graphic summary (B, D) for three donors (mean ± SD). (C, E) $\Delta\Delta C_T$ values (Log_2 fold change; mean ± SD, n = 3) of *ENTPD1* (C) and *NT5E* (E) gene expression in CD73$^+$- and CD73$^-$-sorted CD8 T cells during T cell activation. *RPL13A* served as endogenous control. Data were calibrated to day zero samples of CD73$^+$-sorted cells (E) or CD73$^-$-sorted cells (C).

3.2.5 CD73 co-localizes with a marker of extracellular vesicles after T cell activation

Protein internalization is another intrinsically regulated mechanism that down-modulates cell surface protein expression. For example, T cells internalize the TCR upon activation (Monjas, Alcover and Alarcón, 2004) and we hypothesized that CD73 is likewise endocytosed during T cell activation. To investigate changes in intracellular CD73 content during T cell activation, we stained surface and intracellular CD73 in CD8 T cells before and after activation using the same αCD73 antibody clone coupled to two different fluorochromes.

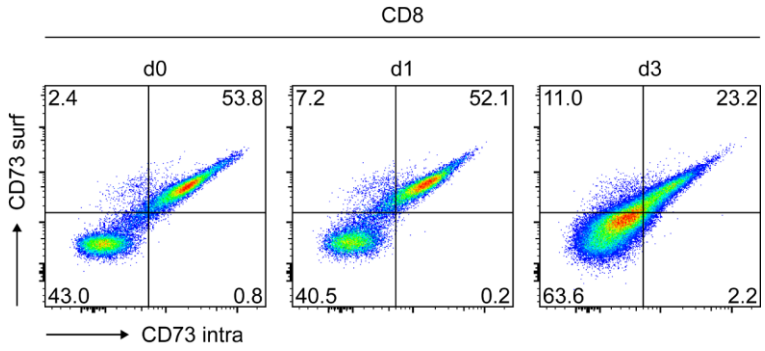

Figure 16 | CD73 surface expression correlates with intracellular CD73 protein expression. PBMCs were stimulated with αCD3 for one or three days. Surface (αCD73-PE) and intracellular CD73 (αCD73-APC) expression in CD8 T cells was monitored by flow cytometry before and after T cell activation.

Flow cytometric analysis revealed that CD8 T cells that expressed CD73 on their plasma membrane were also positive for intracellular CD73 (Figure 16). At day zero and day one, approximately 50% of CD8 T cells of this donor expressed CD73. On day three, the percentage of $CD73^+$ cells was reduced to 20%, and surface and intracellular expression levels were similarly affected. We conclude that CD73 does not accumulate in the cytoplasm of CD8 T cells that had lost CD73 surface expression, but we cannot rule out that CD73 had been internalized prior to removal from the cell surface.

To pinpoint the cellular localization of CD73 in non-activated and activated CD8 T cells, we established a cooperation with Prof. Dr. Catherine Meyer-Schwesinger (Department of Cellular and Integrative Physiology, UKE), who performed high-resolution fluorescence microscopy on our CD8 T cell samples.

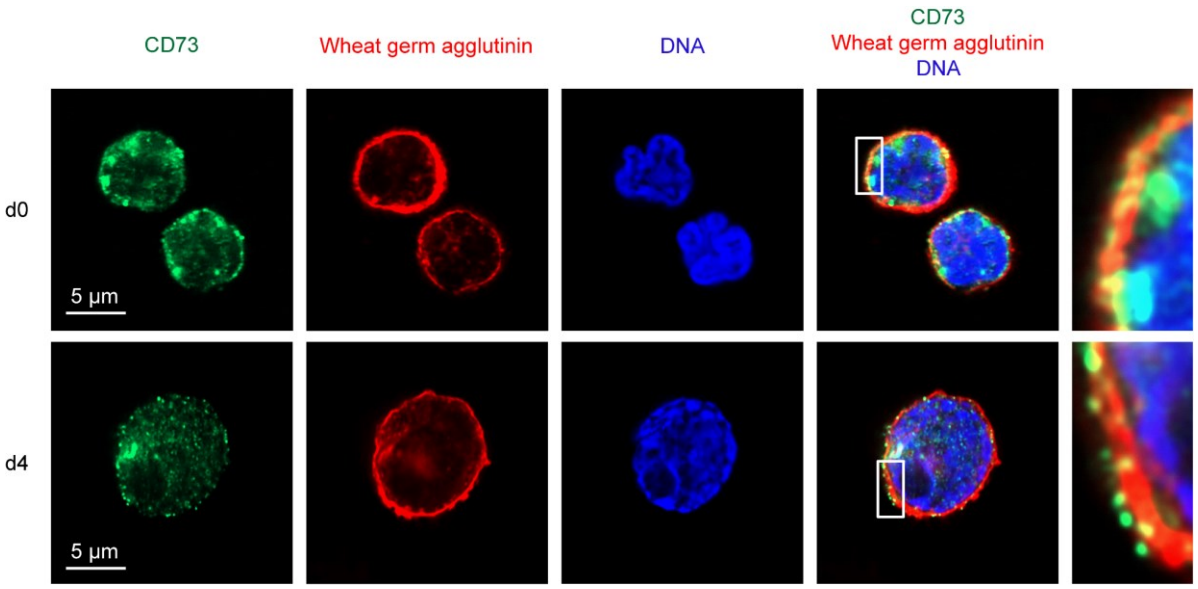

Figure 17 | CD73 localizes in discrete spots on the plasma membrane of activated CD8 T cells four days after activation. CD8 T cells, non-activated or activated for four days in the presence of αCD3 and αCD28, were spun on cytoslides and fixed with PFA. Prof. Dr. Catherine Meyer-Schwesinger stained the cytoslides for CD73 (green), wheat germ agglutinin (red) that demarcates the glycocalix, and DNA (blue) and measured the samples at a LSM800 confocal microscope.

In non-activated CD8 T cells, CD73 localizes in clusters at the cell membrane (Figure 17). After four days of activation, staining intensity of CD73 at the plasma membrane was reduced and CD73 appeared in discrete spots intracellular and attached to the cell membrane. The images suggest that CD73 localizes in vesicular structures probably because of previous internalization. We further speculated that the CD73 spots at the plasma membrane on day four (Figure 17, bottom right) are vesicles containing CD73 that are released from the cell into the cell culture supernatant. To test if CD73 is present in vesicles, Prof. Dr. Catherine Meyer-Schwesinger performed confocal microscopy on CD8 T cells before and after activation and determined co-localization of CD73 and tetraspanin CD9, a marker of vesicles with endocytic origin (Zöller, 2009).

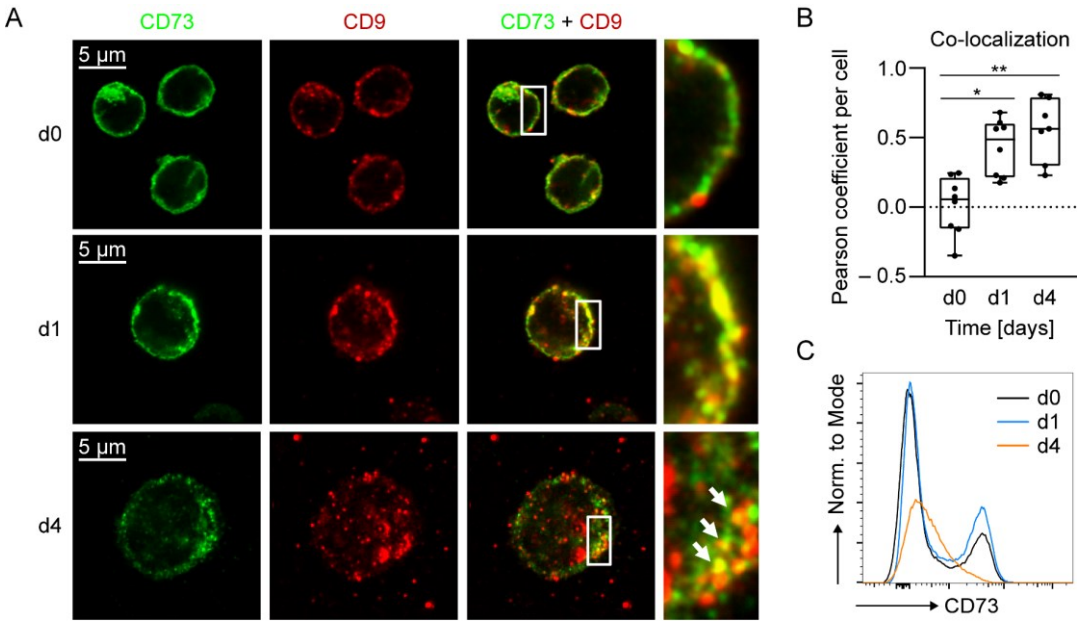

Figure 18 | T cell activation leads to co-localization of CD73 with tetraspanin CD9. (A) Confocal microscopy, performed by Prof. Dr. Catherine Meyer-Schwesinger, of CD8 T cells before and after stimulation with αCD3 and αCD28 for one or four days. Samples were stained for CD73 (green) and CD9 (red), co-localization of both markers is indicated in yellow. (B) Pearson coefficient was calculated to quantify co-localization of CD73 and CD9; the higher, the more the proteins co-localize, the lower, the more the proteins are completely separated. (C) Flow cytometric analysis of CD73 expression in non-activated and activated CD8 T cells. (B) Kruskal-Wallis test with Dunn's multiple comparisons test was used to compare the mean ranks of all three groups, * $p < 0.05$, ** $p < 0.01$.

High-resolution fluorescence microscopy revealed the presence of CD73 in the cell membrane at day zero (Figure 18 A). Tetraspanin CD9 was also present in the plasma membrane; however, co-localization of CD73 and CD9 was a rare event in non-activated CD8 T cells (Figure 18 B). At day one after activation, CD73 and CD9 significantly co-localized at the cell membrane, and at day four, co-localization of CD73 and CD9 was also detected in the cytoplasm (Figure 18 A, B). Flow cytometric analysis confirmed a strong reduction of surface CD73 expression four days after activation (Figure 18 C). Altogether, the data indicate that CD73 appears in vesicular structures inside of activated T cells and that CD73 is (at least partly) removed from the membrane by internalization and packaging into vesicles.

3.2.6 Activated CD8 T cells release CD73 in small extracellular vesicles

We could previously show that CD8 T cell activation led to an increase in CD73-specific AMPase activity in CD8 T cell culture supernatants that we attributed to the presence of soluble CD73 shed from the T cell plasma membrane by a yet unknown mechanism (Winzer,

2017). Given the fact that we did not find evidence for enzymatic shedding of surface CD73 and we observed co-localization of CD73 with vesicle markers, we hypothesized that activated CD8 T cells release CD73 primarily in extracellular vesicles (EVs) and not as a soluble protein. We further assumed that the formation of CD73-containing vesicles (and their subsequent release from activated CD8 T cells) is the dominant mechanism for activation-induced loss of surface CD73 expression. To test our hypotheses, we first assessed if vesicular CD73 is the source of AMPase activity in cell culture supernatants derived from activated CD8 T cells.

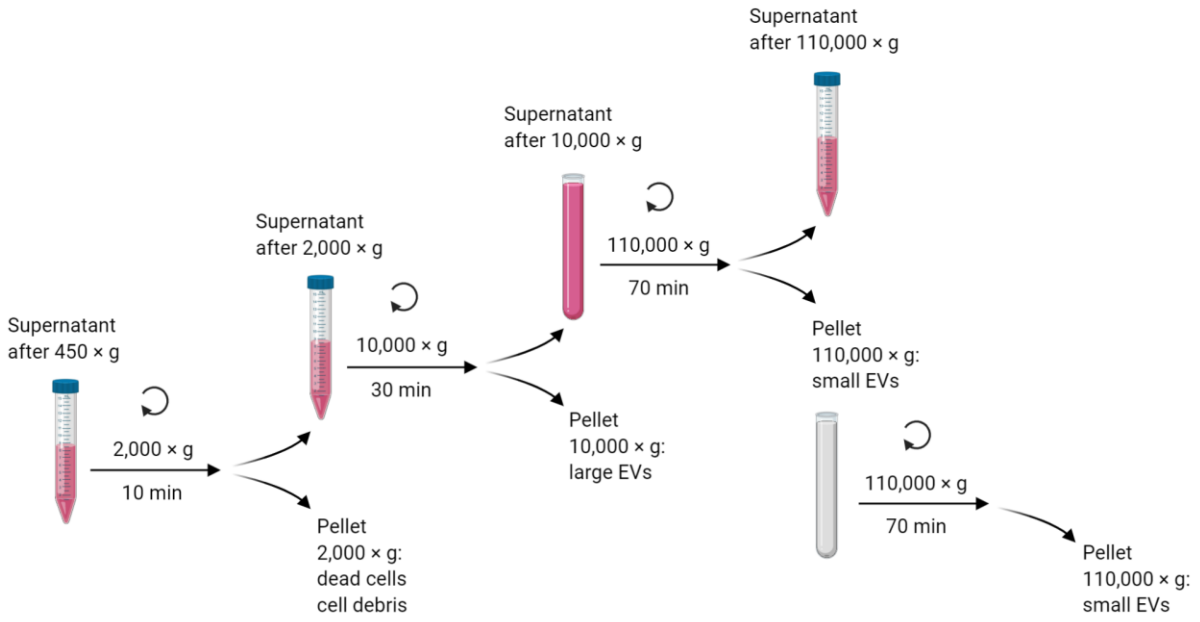

Figure 19 | Differential centrifugation of CD8 T cell culture supernatants. Cell-free CD8 T cell culture supernatant (supernatant after 450 × g) was centrifuged at 2,000 × g for 10 min to remove dead cells and cell debris. The supernatant from this centrifugation step was centrifuged at 10,000 × g for 30 min to pellet large extracellular vesicles (EVs). The supernatant after 10,000 × g was subjected to ultracentrifugation at 110,000 × g for 70 min to isolate small EVs. Small EVs were washed in PBS at 110,000 × g for 70 min. Aliquots of supernatant were taken after each centrifugation step for HPLC analysis. Image created with BioRender.com.

We subjected the cell culture supernatants of sorted $CD73^+$ and $CD73^-$ CD8 T cells to differential centrifugation (Figure 19) for a stepwise removal of dead cells and cell debris, large EVs, and small EVs. Soluble proteins remain in the supernatant after ultracentrifugation at 110,000 × g. An aliquot of supernatant was taken at each centrifugation step and AMPase activity in the sample was measured at the HPLC using a protocol established by Riekje Winzer during her master thesis (Winzer, 2017).

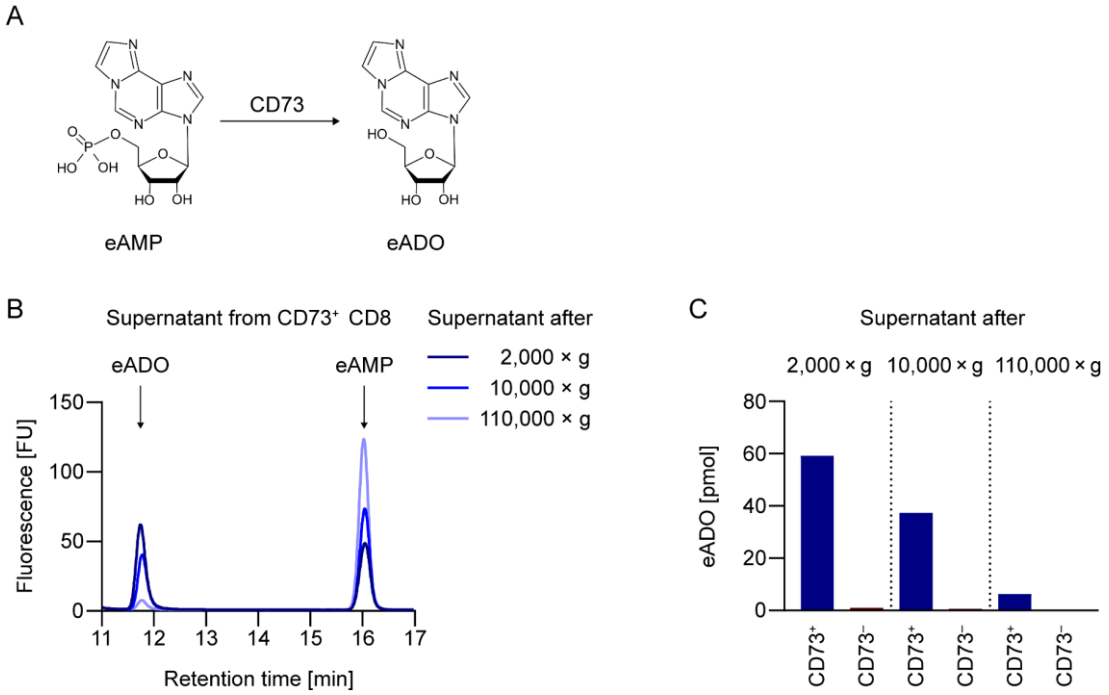

Figure 20 | Extracellular vesicles mediate AMPase activity of CD8 T cell culture supernatants. (A) CD73-mediated AMPase activity leads to the conversion of 1,N^6-etheno-AMP (eAMP) to 1,N^6-etheno-adenosine (eADO). (B) Cell culture supernatant of activated CD8 CD73$^+$ T cells was subjected to differential centrifugation and samples were taken after 2,000 × g, 10,000 × g and 110,000 × g. Aliquots were incubated with eAMP for 60 min and the fluorescence signal of eAMP and eADO was detected by rp-HPLC. The presence of peaks for eADO in the chromatograms indicates AMPase activity in the cell culture supernatants. (C) The amount of eADO in cell culture supernatants of activated CD8 CD73$^+$ and CD8 CD73$^-$ T cells was quantified from the peak for eADO in the recorded chromatograms. (B-C) The chromatogram and the graph show data from one donor who is representative for four donors.

To assess AMPase activity, CD8 T cell culture supernatants were incubated with 1,N^6-etheno-AMP (eAMP) and conversion of eAMP to 1,N^6-etheno-adenosine (eADO) (Figure 20 A) was detected by rp-HPLC. The fluorescent molecules eAMP and eADO have different retention times and the peak area in the chromatograms varies depending on the amount of eAMP or eADO present in the sample. The overlay of the chromatograms from CD8 CD73$^+$ T cell culture supernatants after centrifugation at 2,000 × g, 10,000 × g and 110,000 × g revealed that the area of the peak of eADO got smaller with increasing centrifugation speed, while the area of the peak of eAMP got bigger (Figure 20 B). Quantification of the amount of eADO in CD73$^+$ CD8 T cell culture supernatants confirmed a reduction in eADO production with each centrifugation step (Figure 20 C). After centrifugation at 110,000 × g, a fluorescent signal of eADO was still detectable, but the peak of eADO was much smaller and the amount of eADO was drastically reduced compared to initial amounts (Figure 20 B, C). Cell culture supernatants from CD73$^-$ CD8 T cells did not

produce eADO after incubation with eAMP, indicating the absence of AMPase activity in these cell culture supernatants (Figure 20 C). In summary, these data show that activated CD8 T cells release EVs of different sizes and that these EVs are responsible for the majority of AMPase activity in CD8 T cell culture supernatants.

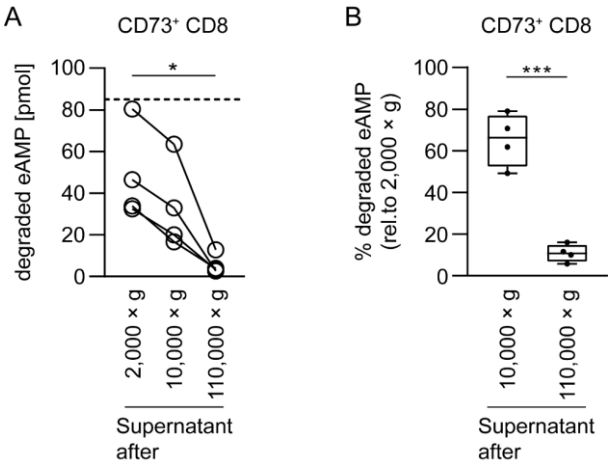

Figure 21 | Small extracellular vesicles are the major source of AMPase activity in CD8 T cell culture supernatants. (A) Cell culture supernatants from activated CD73$^+$ CD8 T cells of four donors were subjected to differential centrifugation and AMPase activity was measured by rp-HPLC in cell culture supernatants after 2,000 × g, 10,000 × g and 110,000 × g. The amount of degraded eAMP was defined as the difference between the amount of eAMP added to sample (measured in the PBS control) and the amount of eAMP detected in the sample. The dashed line represents the value of 85 pmol that is equivalent to complete degradation of the added eAMP. (B) The percentage of degraded eAMP in CD73$^+$ CD8 T cell culture supernatants after centrifugation at 10,000 × g and 110,000 × g was calculated relative to the amount of degraded eAMP in cell culture supernatant centrifuged at 2,000 × g (n = 4). (A) Friedman test with Dunn's multiple comparisons test was used to compare the mean ranks of all three groups, * $p < 0.05$. (B) Unpaired *t*-test, *** $p < 0.001$.

To investigate the individual contribution of large and small EVs to total AMPase activity in CD8 T cell culture supernatants, we analyzed the absolute and relative reduction of eAMP degradation after each centrifugation step (Figure 21). For this, we added eAMP to CD73$^+$ CD8 T cell culture supernatants after centrifugation at 2,000 × g, 10,000 × g and 110,000 × g and to PBS, and measured the amount of eAMP in the sample after 60 min of incubation. The amount of degraded eAMP was calculated as difference between the amount of eAMP added to the sample (measured in the PBS control) and the amount of eAMP that was detected by rp-HPLC after the incubation time of 60 min. The cell culture supernatants from the four donors measured showed individual differences in the capacity to degrade eAMP (Figure 21 A). The donor close to the dashed line degraded nearly all of the added eAMP. In the other donors, we calculated 30 – 45 pmol of degraded eAMP. Centrifugation at 10,000 × g

decreased the amount of degraded eAMP in average by one third and the removal of small EVs (centrifugation at 110,000 × g) resulted in a nearly complete loss of eAMP degradation (Figure 21 A, B). Without small EVs, we observed only 10% of the initially measured AMPase activity (Figure 21 B). In relation to the 10,000 × g sample, the removal of small EVs reduces AMPase activity by 80%. These data underline that small EVs are the major source of AMPase activity in cell culture supernatants from activated CD8 T cells.

As mentioned above, we hypothesized that activated CD8 T cells eliminate surface CD73 through vesicular release and we showed that small EVs (the 110,000 × g pellet, hereinafter referred to as EVs) contained the majority of AMPase activity in CD8 T cell culture supernatants. To investigate if T cell activation promotes release of CD73 in EVs, we isolated EVs from unstimulated and stimulated $CD73^+$ CD8 T cells, and from stimulated $CD73^-$ CD8 T cells and compared their size, concentration and AMPase activity (Figure 22). Nanoparticle tracking analysis revealed that all three cell types released EVs and the majority of these EVs were around 100 – 200 nm in size (Figure 22 A). The size profile of the EVs was similar and there were no differences in the particle size (Figure 22 A, B). $CD73^+$ and $CD73^-$ CD8 T cells produced a comparable amount of EVs after activation, whereas unstimulated $CD73^+$ CD8 T cells released six times less vesicles compared with stimulated $CD73^+$ CD8 T cells (Figure 22 B). These data show that CD8 T cell activation promotes the release of EVs. To measure AMPase activity, we incubated equal amounts of EVs from unstimulated and stimulated $CD73^+$ and stimulated $CD73^-$ CD8 T cells with eAMP and compared production of eADO after 60 min (Figure 22 C). EVs from unstimulated $CD73^+$ CD8 T cells produced 5 pmol of eADO from eAMP. The same amount of EVs derived from stimulated $CD73^+$ CD8 T cells generated four times more eADO. The presence of the CD73-specific inhibitor PSB-14685 completely abrogated eADO production. Likewise, EVs from $CD73^-$ CD8 T cells did not degrade eAMP. These data reveal that CD8 T cell activation boosts AMPase activity of EVs and that this AMPase activity is CD73-specific.

In summary, we show that *in vitro* CD8 T cell activation leads to an increased production of EVs, and these EVs are the major source CD73-specific AMPase activity in the cell culture supernatants. Because CD8 T cell activation increased AMPase activity of EVs, we conclude that CD73 is enriched in EVs from stimulated CD8 T cells. These findings support our

hypothesis that the formation and release of EVs substantially contribute to activation-induced loss of surface CD73 expression in CD8 T cells.

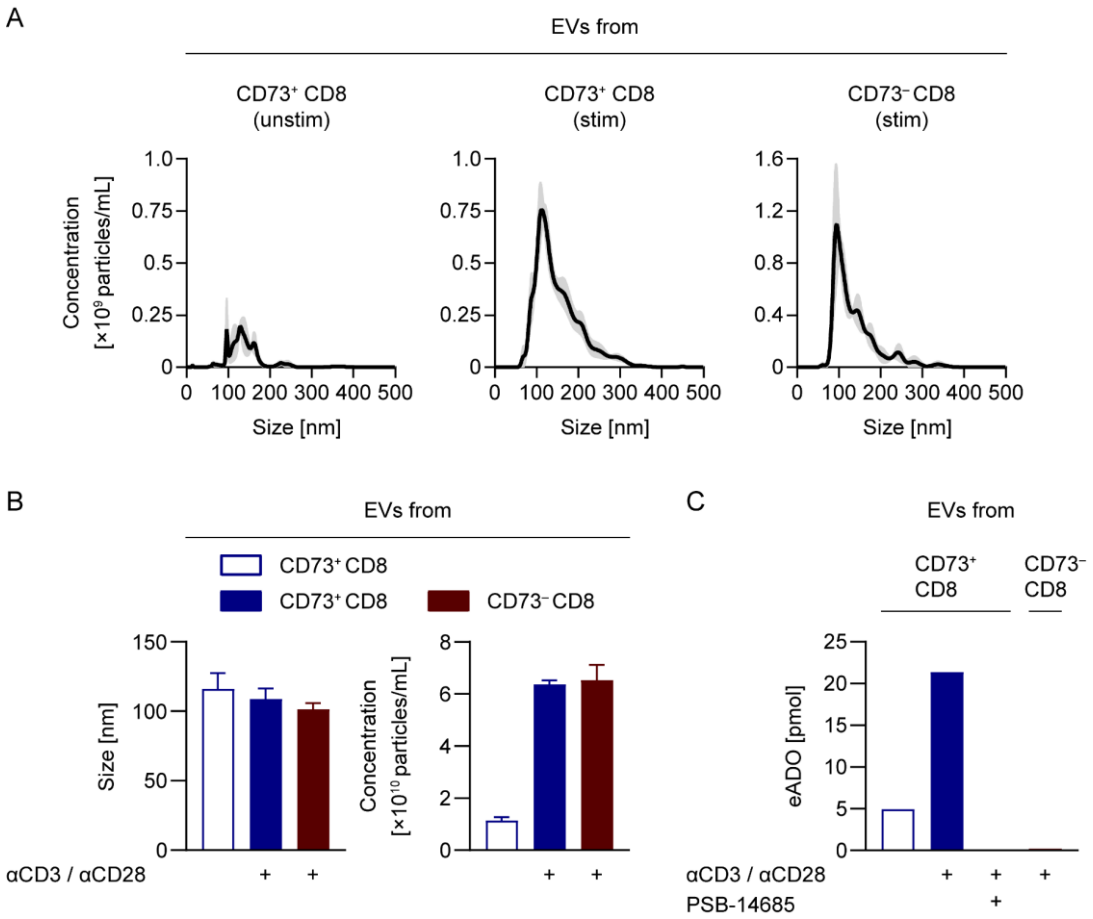

Figure 22 | CD8 T cell activation promotes the release of extracellular vesicles and boosts their AMPase activity. (A-B) Nanoparticle tracking analysis was performed to determine particle size and concentration of EVs (110,000 × g pellet) isolated from the cell culture supernatant of unstimulated and stimulated CD73$^+$ and stimulated CD73$^-$ CD8 T cells. (A) Size profile of EVs averaged from three measurements ± SEM. (B) Size (mode) and concentration (both mean ± SD, calculated from three consecutive measurements) of EVs from unstimulated CD73$^+$ CD8 T cells (empty blue bars), stimulated CD73$^+$ CD8 T cells (filled blue bars) and stimulated CD73$^-$ CD8 T cells (filled red bars). (C) Equal amounts of EVs (1.3 × 10^8 EVs) from unstimulated (empty blue bar) and stimulated (filled blue bar) CD73$^+$ CD8 T cells and stimulated CD73$^-$ CD8 T cells (filled red bar) were incubated with eAMP, and eADO production was measured after 60 min of incubation. AMPase activity of EVs from stimulated CD73$^+$ CD8 T cells was quantified in the presence of the CD73-specific inhibitor PSB-14685 (10 µM).

3.2.7 Extracellular vesicles from CD73$^+$ CD8 T cells carry CD73

To confirm the presence of CD73 in EVs, we performed flow cytometry and western blot (Figure 23).

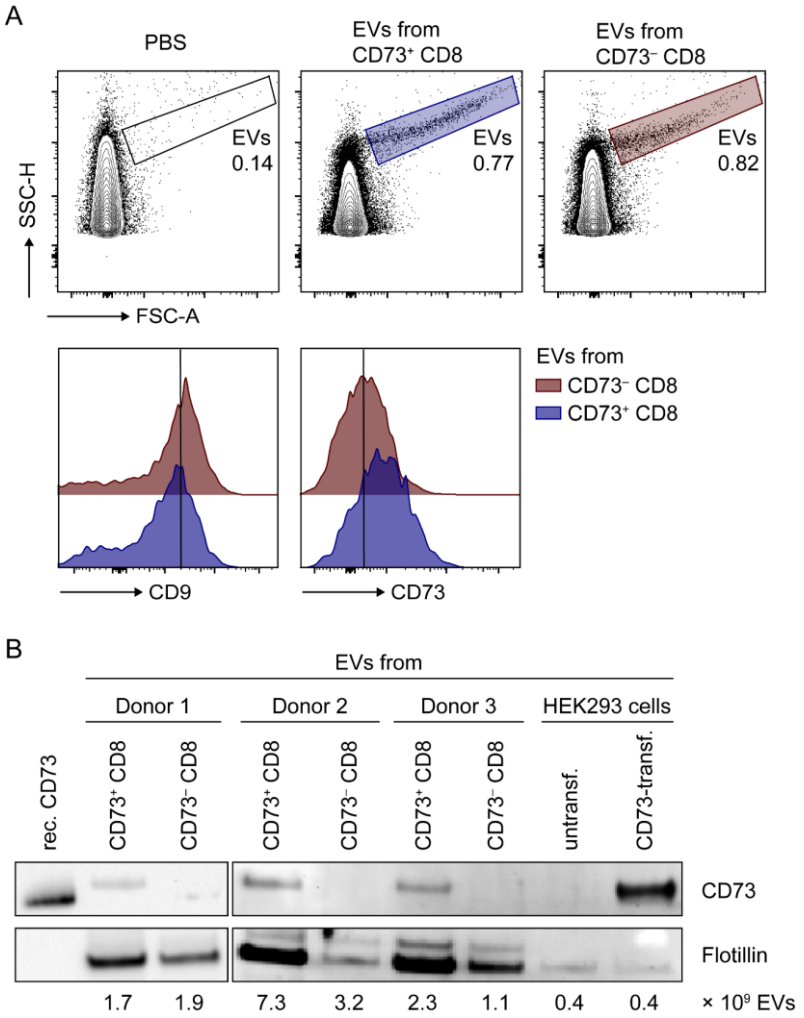

Figure 23 | Extracellular vesicles from CD73⁺ CD8 T cells carry CD73. (A) EVs from CD73⁺ (blue) and CD73⁻ (red) CD8 T cells were stained for CD9 and CD73 and measured at a FACSAria IIIU. Histograms show staining intensity for CD9 and CD73 in EVs gated by forward scatter (FSC) vs. side scatter (SSC). PBS without EVs but containing the antibodies for staining was used as control to set the EV gate. (B) CD73 and Flotillin were analyzed in EVs from CD73⁺ and CD73⁻ CD8 T cells by western blot. Recombinant CD73 (15 ng) or EVs from human CD73-transfected HEK293 cells served as positive control. Numbers below the blot indicate the amount of vesicles in the respective sample.

The small size (100 – 200 nm) of EVs isolated by ultracentrifugation at 110,000 × g limits analysis of single EVs by conventional flow cytometry (Nolan and Duggan, 2018). In cooperation with Dr. Jochen Behrends (Core Facility Fluorescence Cytometry, Research Center Borstel), we were able to detect EVs by forward vs. side scatter using a sensitive FACSAria IIIU (Figure 23 A). EVs from CD73⁺ CD8 T cells showed a higher staining intensity for CD73 than EVs from CD73⁻ CD8 T cells, while levels of CD9 expression were similar in both EV populations. These data indicate that CD73 is present in the membrane of EVs derived from CD73⁺ CD8 T cells.

To ascertain if only EVs from CD73$^+$ CD8 T cells carry CD73, we performed western blot of EVs from CD73$^+$ and CD73$^-$ CD8 T cells. In all three donors, we detected CD73 only in those EVs that derived from CD73$^+$ CD8 T cells (Figure 23 B). Flotillin, a vesicle marker, was present in all six samples. Altogether, we confirmed that EVs reflect the phenotype of their parental cells in terms of CD73 expression.

3.3 Functional relevance of non-cell-bound CD73

In the previous parts, I showed that CD8 T cell activation induced the loss of CD73 surface expression in proliferating CD8 T cells and that loss of CD73 is mediated by a T cell-intrinsic mechanism. Co-localization of CD73 with vesicle markers after CD8 T cell activation and increased CD73-specific AMPase activity in EVs from activated CD8 T cells reveal that formation and release of CD73-containing vesicles are involved in the activation-induced loss of membrane-bound CD73. In this part, I will investigate the functional relevance of CD8 T cell-derived EVs as natural source of non-cell-bound CD73 in the context of inflammation.

3.3.1 Extracellular vesicles from CD73$^+$ CD8 T cells generate adenosine from ATP

EVs from CD73$^+$ CD8 T cells metabolize AMP and generate the anti-inflammatory mediator adenosine (Figure 22). AMP is a degradation product of ATP, which is present in elevated concentrations at sites of inflammation. To further evaluate the immunomodulatory potential of EVs, we investigated if EVs are able to produce adenosine from ATP. We incubated EVs and CD8 T cell culture supernatants from different centrifugation steps with 1,N^6-etheno-ATP (eATP) and measured degradation products as well as non-metabolized eATP by rp-HPLC (Figure 24). After centrifugation at 2,000 × g, CD8 T cell culture supernatants from CD73$^+$ and CD73$^-$ CD8 T cells degraded eATP almost completely (Figure 24 A, B). We observed a small reduction in ATPase activity after centrifugation at 10,000 × g, but the majority was lost after ultracentrifugation at 110,000 × g. We detected eADO only in cell culture supernatants from CD73$^+$ CD8 T cells. eAMP and eADO were not detected in CD8 T cell culture supernatants after ultracentrifugation (Figure 24 A, B). EVs from CD73$^+$ CD8 T cells degraded slightly more eATP than EVs from CD73$^-$ CD8 T cells and were able to generate detectable amounts of eADO (Figure 24 C). The ability of EVs from CD8 T cells to degrade eATP was strictly dependent on T cell activation, because EVs from cell culture supernatants of unstimulated CD73$^+$ CD8

T cells did not dephosphorylate eATP (Figure 24 D). In summary, these data show that EVs are the major source of ATPase activity in cell culture supernatants of activated CD8 T cells and that EVs from CD73$^+$ CD8 T cells possess the whole machinery for adenosine production from ATP.

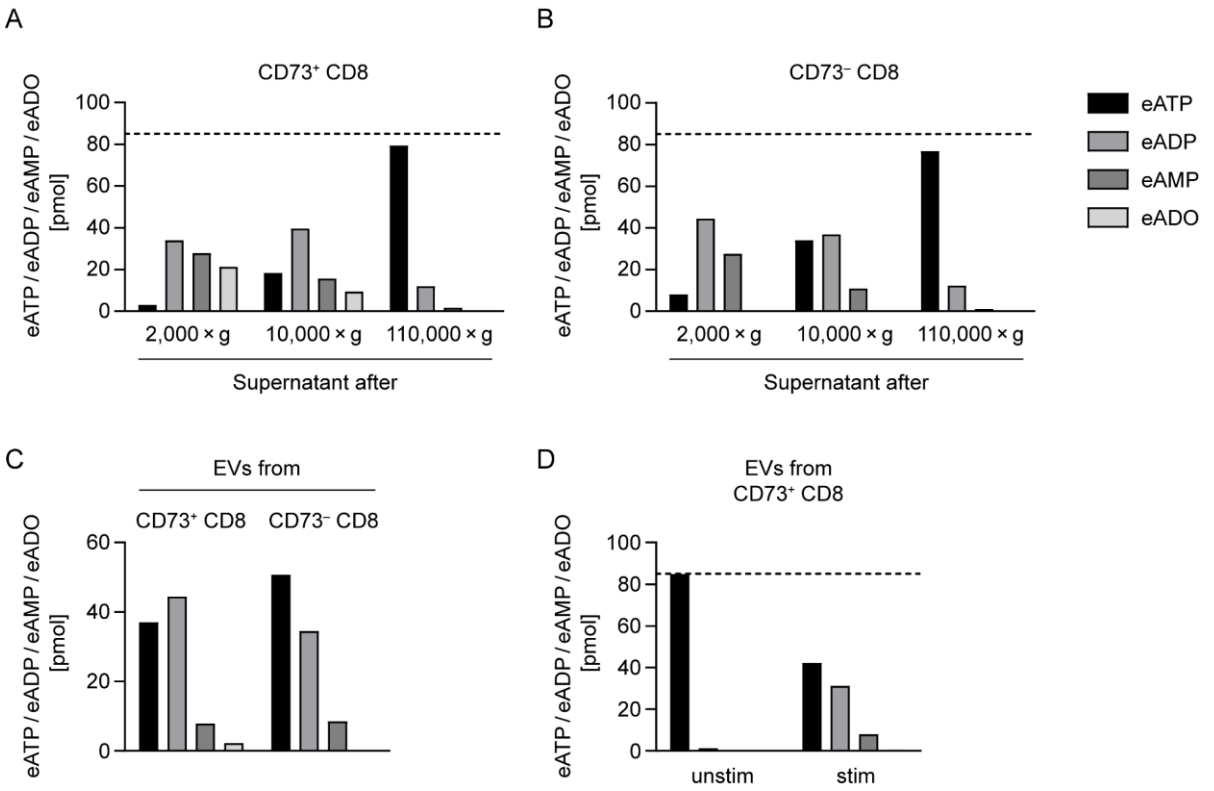

Figure 24 | Extracellular vesicles are the major source of ATPase activity in CD8 T cell culture supernatants. (A-D) Cell culture supernatants from activated CD73$^+$ (A) or CD73$^-$ (B) CD8 T cells after centrifugation at 2,000 × g, 10,000 × g and 110,000 × g or EVs (C-D) were incubated with 1,N^6-etheno-ATP (eATP) for 60 min. Degradation products 1,N^6-etheno-ADP (eADP), eAMP and eADO as well as non-metabolized eATP were detected by rp-HPLC. The dashed line at 85 pmol corresponds to the amount of eATP that is measured when ATPase activity is absent. (C) EVs were isolated from cell culture supernatants shown in (A) and (B) and the amount of EVs measured in (C) corresponds to the amount of cell culture supernatant analyzed in (A) and (B). (D) EVs were isolated from cell culture supernatants of unstimulated and stimulated CD73$^+$ CD8 T cells and equal amount of EVs (1.3×10^8 EVs) was used in the assay.

3.3.2 Extracellular vesicles from CD73$^+$ CD8 T cells are immunosuppressive

EVs are important mediators in cell-to-cell communication and we showed that EVs from activated CD73$^+$ CD8 T cells are equipped with enzymatically active ecto-nucleotidases, which generate adenosine. Therefore, we wanted to investigate the role of vesicular CD73 under inflammatory conditions. We speculated that adenosine generated by EVs from activated T cells suppresses T cell activation and proliferation. To test this, we developed a sensitive *in*

vitro T cell assay. In this assay, we cultured T cells in the presence of ADA inhibitor EHNA to ensure the detection of adenosine-mediated immunosuppressive effects. Inflammation leads to a massive increase in extracellular ATP that is swiftly converted to AMP, which is the substrate for CD73. To mimic this setting, we added AMP to the T cell culture and first evaluated the influence of AMP on activation and proliferation of $CD73^+$ and $CD73^-$ CD4con T cells (Figure 25).

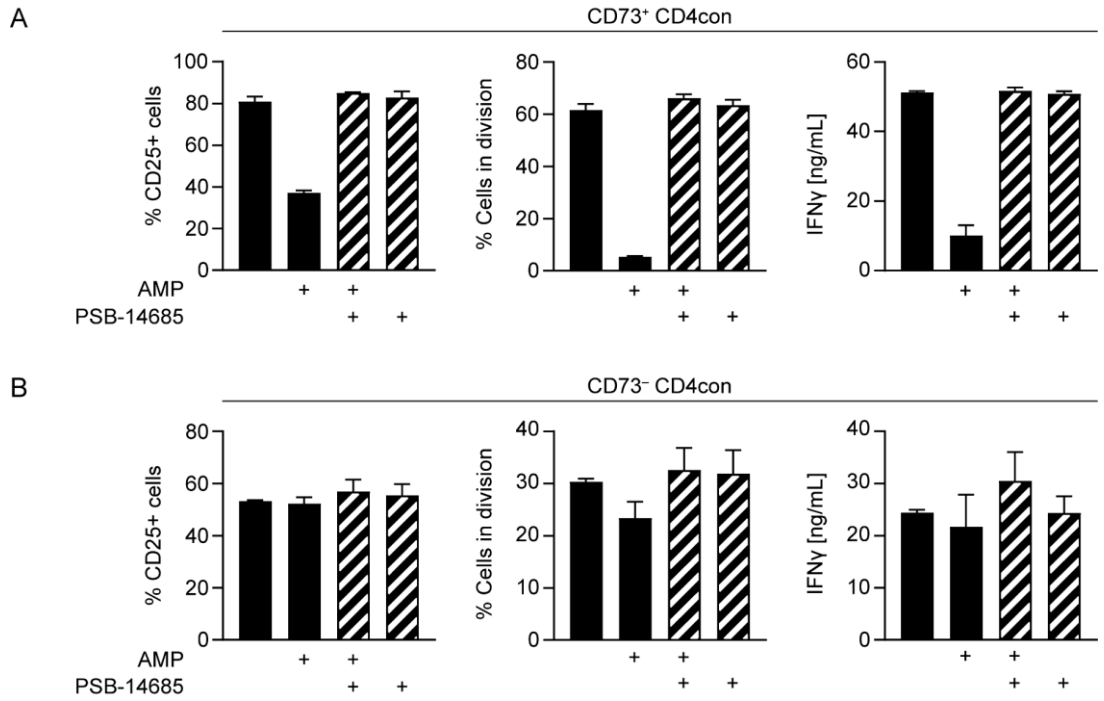

Figure 25 | Suppressive effects of exogenous AMP are dependent on CD73-mediated AMPase activity. PBMCs were labeled with eFluor 670, sorted into $CD73^+$ (A) and $CD73^-$ (B) CD4con T cells and stimulated for four days with αCD3 and αCD28 in the presence of ADA inhibitor EHNA (10 µM). When indicated, AMP (50 µM) or the CD73-specific inhibitor PSB-14685 (10 µM) were added. Frequency of $CD25^+$ T cells and proliferation were assessed by flow cytometry. IFNγ was measured in T cell culture supernatants by ELISA. Graphs show data (mean ± SD) from one donor who is representative for six donors.

Addition of AMP to $CD73^+$ CD4con T cells in the presence of EHNA decreased the frequency of $CD25^+$ T cells and strongly reduced proliferation and IFNγ production of these cells (Figure 25 A). In contrast, AMP did not affect activation and proliferation of $CD73^-$ CD4con T cells (Figure 25 B). When we added the CD73-specific inhibitor PSB-14685 to $CD73^+$ CD4con T cells, we prevented AMP-induced suppression (Figure 25 A). In summary, these data underline the relevance of CD73-specific AMPase activity in T cell-intrinsic immune suppression. Absence or blocking the enzymatic activity of CD73 protects CD4con T cells from

adenosine-mediated immunosuppressive effects. We further conclude that CD73⁻ CD4 T cells do not express another AMPase that is able to compensate for the lack of CD73 expression.

The majority of CD4 T cells does not express CD73 (Figure 5) and we wondered if non-cell-bound forms of CD73 generate immunosuppressive adenosine able to suppress CD73⁻ CD4con T cells. To investigate this, we added recombinant CD73 to CD73⁻ CD4con T cells treated with AMP (Figure 26).

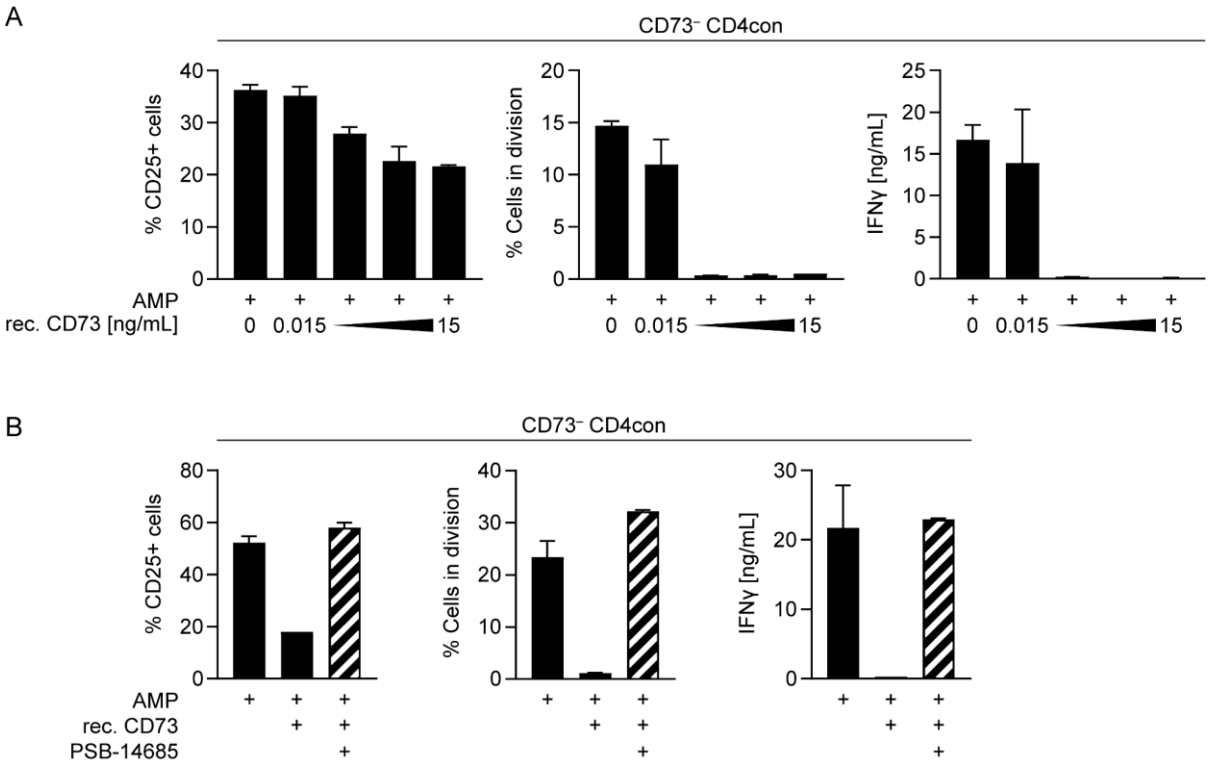

Figure 26 | Enzymatic activity of recombinant CD73 mediates immune suppression in CD73⁻ CD4 T cells. CD73⁻ CD4 T cells were sorted from eFluor 670-labeled PBMCs, stimulated with αCD3 and αCD28 and cultured for four days in the presence of EHNA (10 µM). Frequency of $CD25^+$ T cells and proliferation were measured by flow cytometry, and IFNγ production was detected in T cell culture supernatants by ELISA. (A) CD73⁻ CD4con T cells were incubated with AMP (50 µM) and four different concentrations of recombinant CD73 (rec. CD73, 15 ng/mL and three ten-fold serial dilutions). (B) CD73⁻ CD4con T cells were treated with 15 ng/mL recombinant CD73 and 50 µM AMP. When indicated, the CD73-specific inhibitor PSB-14685 was added to the cell culture. Graphs show data (mean ± SD) from one donor who is representative for two donors (A) or six donors (B).

In a previous test at the HPLC, we established that 15 ng/mL recombinant CD73 showed an optimal conversion of eAMP to eADO in a cell-free system (data not shown). To determine the best concentration of recombinant CD73 for our T cell assay, we tested 15 ng/mL and three ten-fold serial dilutions of the protein. We observed a stepwise reduction in the

frequency of $CD25^+$ T cells with increasing amounts of recombinant CD73 (Figure 26 A). The difference between the highest and the second highest concentration was minimal, indicating that we achieved maximum reduction of $CD25^+$ T cells using 15 ng/mL recombinant CD73. In all conditions except for the lowest concentration tested (0.015 ng/mL), proliferation and IFNγ production were completely abrogated (Figure 26 A). Based on these results, we defined 15 ng/mL recombinant CD73 as optimal concentration for *in vitro* T cell assays. To prove that the observed suppression was due to the AMPase activity of CD73, we blocked CD73-mediated enzymatic activity with PSB-14685. Addition of the CD73 inhibitor prevented T cell suppression when $CD73^-$ CD4con T cells were treated with the combination of recombinant CD73 and AMP (Figure 26 B). These data show that we successfully set up an *in vitro* T cell assay to measure the effect of non-cell-bound CD73 and we could show that the AMPase activity of non-cell-bound CD73 contributes to immune suppression of CD4con T cells in the absence of cellular CD73 expression.

EVs from $CD73^+$ CD8 T cells represent a natural source of non-cell-bound CD73 in human body fluids. T cell activation leads to an increased release of these vesicles and boosts their AMPase activity (Figure 22). Using our sensitive *in vitro* T cell assay, we evaluated the immunosuppressive potential of EVs (Figure 27). We used $CD73^-$ CD4con T cells in this assay to prevent any effect of membrane-bound CD73. In the presence of AMP, EVs from activated $CD73^+$ CD8 T cells mediated strong suppression of $CD73^-$ CD4con T cells (Figure 27). We measured a reduction in the percentage of $CD25^+$ T cells comparable to the positive control recombinant CD73 (Figure 27 A), and IFNγ production and proliferation were completely blocked by EVs from $CD73^+$ CD8 T cells (Figure 27 B, C). In contrast, EVs from $CD73^-$ CD8 T cells did not suppress T cell activation or proliferation (Figure 27). Addition of the CD73-specific inhibitor PSB-14685 prevented the suppressive effect of EVs from $CD73^+$ CD8 T cells. In summary, the data show that EVs from $CD73^+$ CD8 T cells mediate T cell suppression and that the suppressive function depends on CD73-specific AMPase activity.

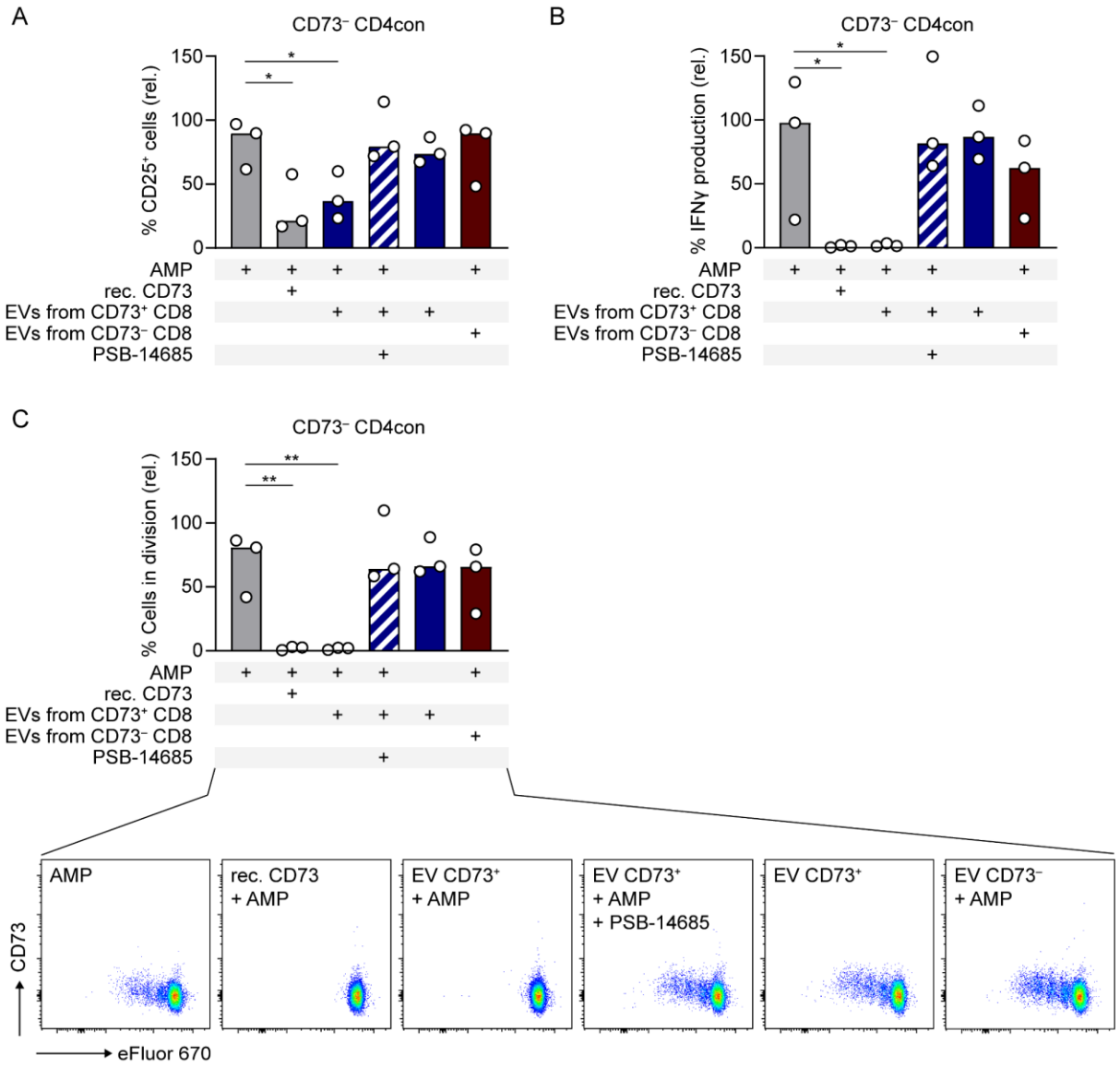

Figure 27 | AMPase activity in extracellular vesicles from CD73⁺ CD8 T cells mediates immune suppression. CD73⁻ CD4con T cells were stimulated with αCD3 and αCD28 and cultured for four days in the presence of EHNA (10 µM). Frequency of CD25⁺ T cells (A), IFNγ production (B) and proliferation (C) were measured by flow cytometry (A, C) or ELISA (B) to assess T cell suppression in three donors. For each donor, data were normalized to CD73⁻ CD4 T cells treated with EHNA. Open circles show mean data for each donor, bars represent the median based on three donors. Pseudocolor plots show CD73 versus eFluor 670 to illustrate proliferation for one representative donor from (C). When indicated, AMP (50 µM), recombinant CD73 (15 ng/mL), EVs from CD73⁺ or CD73⁻ CD8 T cells (1.3×10^8 EVs, equivalent to 150 µL cell culture supernatant of activated CD8 T cells) or PSB-14685 (10 µM) were added. RM one-way ANOVA with Dunnett's multiple comparisons test was used to compare the means of all conditions to CD73⁻ CD4 T cells treated with EHNA and AMP * $p < 0.05$, ** $p < 0.01$.

We next asked if EVs contribute to immune suppression under inflammatory conditions mimicking the *in vivo* situation. In these assays, we provided 50 µM ATP instead of AMP. ATP is released from immune cells at the site of inflammation and is the ligand for different types of P2 receptors. Several groups investigated the influence of exogenously added ATP on

human T cell proliferation. While some report that 50 µM ATP or ATP analogues partially inhibit T cell proliferation (Duhant *et al.*, 2002; Sueyoshi *et al.*, 2019) others measure an inhibitory effect only at higher concentrations of 200 µM or 1 mM ATP (Trabanelli *et al.*, 2012; Weiler *et al.*, 2016). To evaluate the effect of 50 µM ATP in our T cell assay, we measured activation and proliferation of EHNA-treated CD73$^+$ and CD73$^-$ CD4con T cells in the presence of exogenously added ATP (Figure 28).

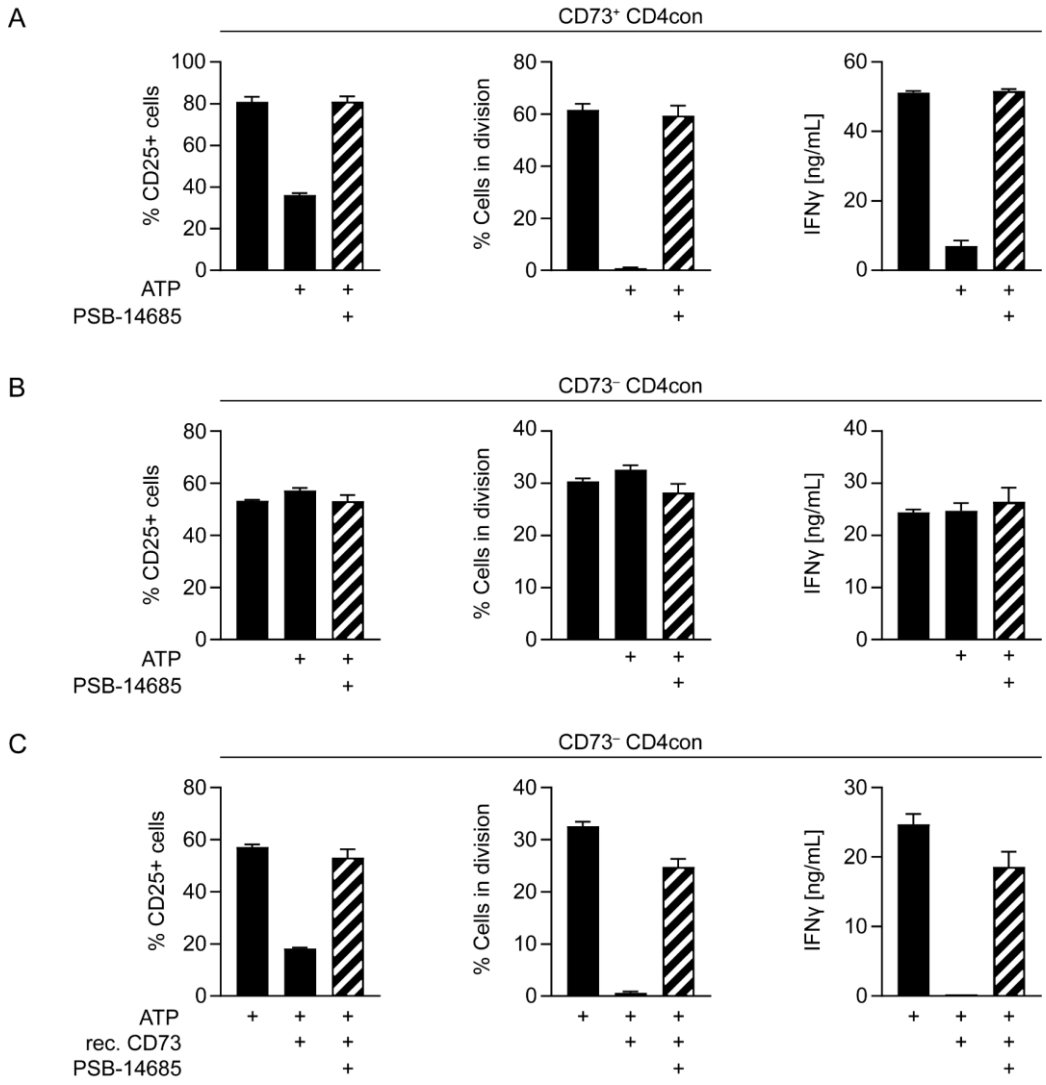

Figure 28 | Suppressive effects of exogenous ATP are dependent on CD73-mediated AMPase activity. PBMCs were labeled with eFluor 670, sorted into CD73$^+$ (A) and CD73$^-$ (B+C) CD4con T cells and stimulated with αCD3 and αCD28 in the presence of ADA inhibitor EHNA (10 µM). When indicated, ATP (50 µM), the CD73-specific inhibitor PSB-14685 (10 µM) or recombinant CD73 (15 ng/mL) were added. Frequency of CD25$^+$ T cells and proliferation were assessed by flow cytometry. IFNγ production was measured in T cell culture supernatants by ELISA. Graphs show data (mean ± SD) from one donor who is representative for five donors.

Incubation of $CD73^+$ CD4con T cells with ATP decreased the frequency of $CD25^+$ T cells and completely blocked proliferation and IFNγ production (Figure 28 A). We did not measure T cell suppression in $CD73^-$ CD4con T cells by ATP unless we provided recombinant CD73 (Figure 28 B, C). Blocking of CD73 AMPase activity with PSB-14685 prevented the suppressive effects of ATP, indicating that adenosine formation is involved in this process (Figure 28 A, C). From these data, we conclude that we can measure CD73-mediated effects in T cell suppression even when ATP is provided and substrate availability for CD73 depends on upstream ATPase activity.

Next, we tested the immunosuppressive potential of EVs when we mimicked the inflammatory environment by addition of ATP (Figure 29). $CD73^-$ CD4 T cells treated with the combination of ATP and EVs from $CD73^+$ CD8 T cells showed significantly reduced upregulation of CD25, proliferation and IFNγ production compared with $CD73^-$ CD4 T cells that were incubated only with ATP (Figure 29). EVs from $CD73^+$ CD8 T cells showed the same degree of T cell suppression as recombinant CD73 which served as positive control. The presence of the CD73-specific inhibitor PSB-14685 prevented suppressive effects of EVs from $CD73^+$ CD8 T cells. EVs from $CD73^-$ CD8 T cells did not reduce T cell activation, proliferation or cytokine production (Figure 29). Altogether, these data reveal that T cell suppression was due to AMPase activity of CD73 and thus mediated by adenosine. We show here that EVs that carry CD73 can intrinsically suppress activated T cells.

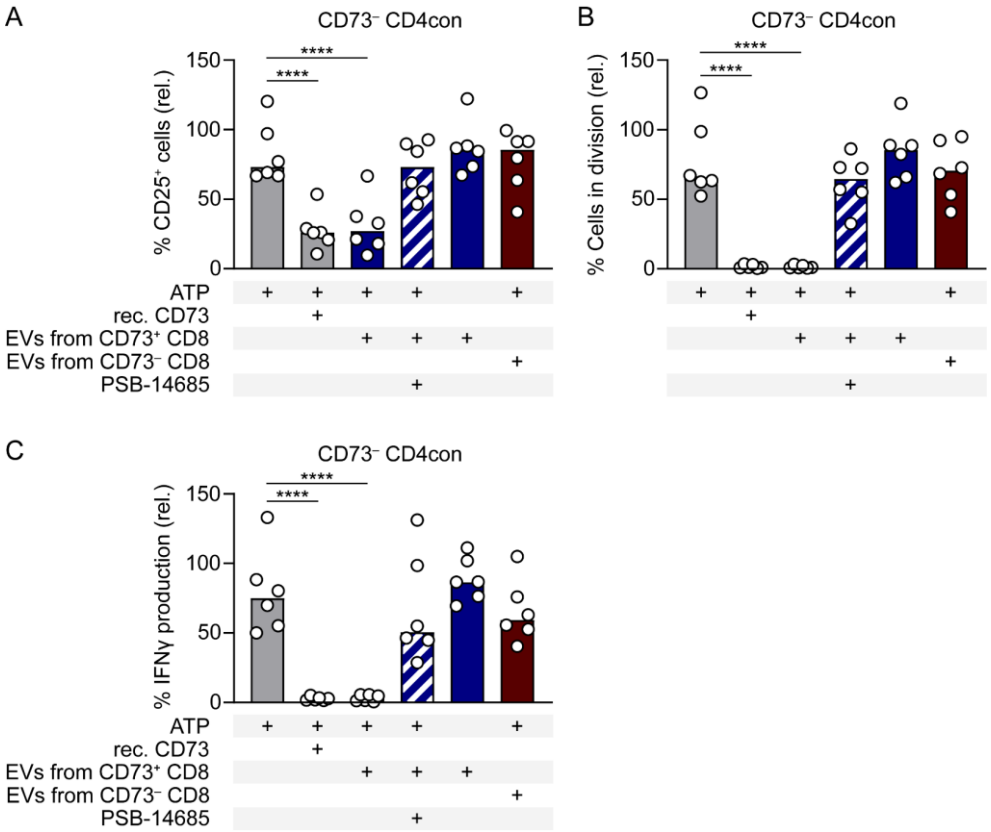

Figure 29 | Extracellular vesicles from CD73⁺ CD8 T cells are immunosuppressive under inflammatory conditions. CD73⁻ CD4con T cells were sorted from eFluor 670-labeled PBMCs, stimulated with αCD3 and αCD28 and cultured for four days in the presence of EHNA (10 µM). Frequency of CD25⁺ T cells (A), proliferation (B) and IFNγ production (C) were measured by flow cytometry (A, B) or ELISA (C) to assess T cell suppression in six donors. For each donor, data were normalized to CD73⁻ CD4 T cells treated with EHNA. Open circles show mean values for each donor, bars represent the median based on six donors. When indicated, ATP (50 µM), recombinant CD73 (15 ng/mL), EVs from CD73⁺ or CD73⁻ CD8 T cells (equivalent to 150 µL cell culture supernatant of activated CD8 T cells) or PSB-14685 (10 µM) were added. RM one-way ANOVA with Dunnett's multiple comparisons test was used to compare the means of all conditions to CD73⁻ CD4 T cells treated with EHNA and ATP, **** $p < 0.0001$.

Next, we investigated whether immunosuppressive EVs are generated *in vivo* at sites of inflammation. We had the chance to isolate EVs from the synovial fluid (SF) of a patient with juvenile idiopathic arthritis (JIA). In these patients, the SF is removed routinely by joint puncture for therapeutic reasons. This allowed us to purify EVs directly from the inflammatory site and to analyze their immunoregulatory potential (Figure 30).

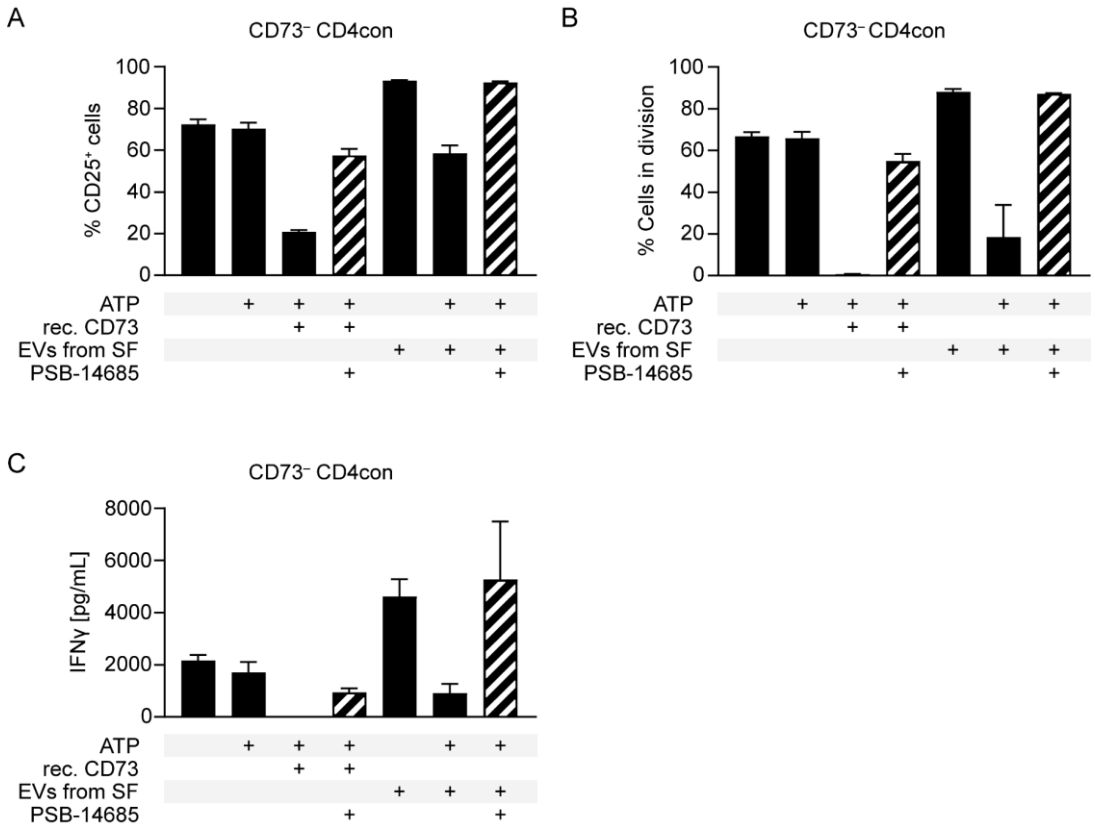

Figure 30 | Synovial fluid-derived extracellular vesicles can acquire immunosuppressive properties. CD73⁻ CD4con T cells were sorted from enriched CD4 T cells, labeled with eFluor 670 and stimulated with αCD3 and αCD28 for four days in the presence of EHNA (10 µM). Frequency of CD25⁺ T cells (A), proliferation (B) and IFNγ production (C) were measured by flow cytometry (A, B) or ELISA (C) to assess T cell suppression. In some conditions, ATP (50 µM), recombinant CD73 (15 ng/mL), EVs (3.1×10^8 particles) or the CD73 inhibitor PSB-14685 (10 µM) were added. All graphs show data (mean ± SD) from one donor who is representative for three donors.

Without exogenously added ATP, SF-derived EVs increased the proportion of CD25-expressing T cells, induced proliferation and promoted IFNγ production (Figure 30). In combination with ATP, we observed a lower frequency of CD25⁺ T cells in the range of the non-suppressed control conditions (Figure 30 A), and reduction in proliferation and IFNγ production revealed T cell suppression (Figure 30 B, C). However, SF-derived EVs failed to achieve the suppressive potential of recombinant CD73 (Figure 30). Blocking of CD73-mediated AMPase activity restored the pro-inflammatory phenotype of SF-derived EVs. From these data, we conclude that EVs from a local site of inflammation possess CD73-specific AMPase activity and can acquire immunosuppressive properties under conditions that favor the generation and retention of adenosine.

3.3.3 Extracellular vesicles from CD73$^+$ CD8 T cells complement Treg function

Regulatory T cells are crucial to maintain peripheral tolerance and terminate immune responses to prevent inflammation-induced damage of healthy tissue (Sakaguchi *et al.*, 2020). Murine Tregs co-express CD39 and CD73, are able to generate adenosine and use this pathway to suppress conventional T cells (Deaglio *et al.*, 2007). In human Tregs, CD39 expression is genetically determined (Rissiek *et al.*, 2015) and CD73 expression is a rare event (Figure 5), challenging the concept that metabolic disruption of effector T cells by Treg-derived adenosine is valid in the human system.

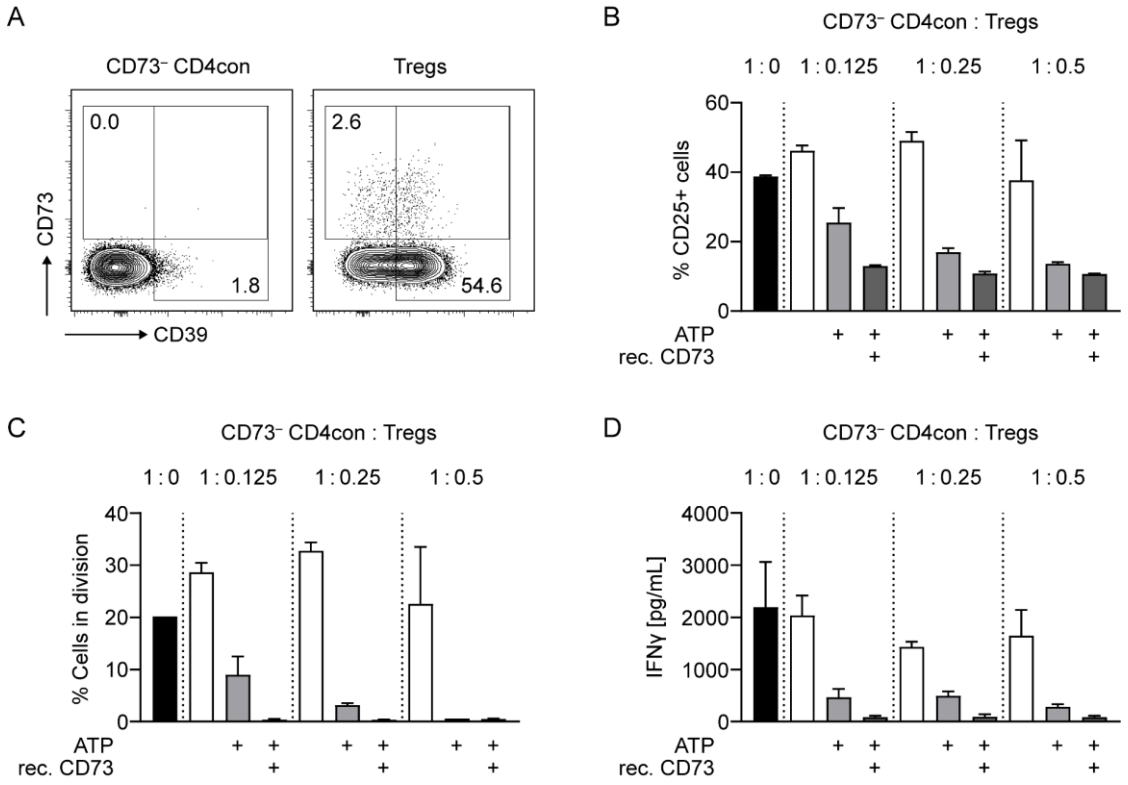

Figure 31 | Recombinant CD73 cooperates with human Tregs in T cell suppression. CD73$^-$ CD4con T cells and Tregs were sorted from enriched CD4 T cells and CD73$^-$ CD4con T cells were labeled with eFluor 670 after sorting. (A) Contour plots show expression of CD73 and CD39 in CD73$^-$ CD4con T cells and Tregs at the beginning of the co-culture. (B-D) CD73$^-$ CD4con T cells were co-cultured with different ratios of autologous Tregs and stimulated with αCD3 and αCD28 for four days in the presence of ADA inhibitor EHNA (10 µM). The black bars represent responder T cells without Tregs. When indicated, ATP (50 µM, light grey bars) or ATP and recombinant CD73 (15 ng/mL, dark grey bars) were added to the cell culture. Frequency of CD25$^+$ T cells (B), proliferation (C) and IFNγ production (D) were measured by flow cytometry (B, C) or ELISA (D) to determine suppression of responder T cells. Graphs show data (mean ± SD) from one donor who is representative for four donors.

To investigate if non-cell-bound CD73 can cooperate with human Tregs in T cell suppression by providing AMPase activity, we set up a Treg suppression assay using CD73$^-$ CD4con T cells

as responder cells (Figure 31). Eliminating CD73 from responder T cells allowed us to specifically measure the contribution of CD73 on Tregs and beneficial effects of additional non-cell-bound CD73 in the system. Flow cytometric analysis of sorted responder T cells and Tregs verified the absence of CD73 from responder T cells and showed that only few Tregs expressed CD73 (Figure 31 A). CD39 was present on 55% of Tregs. Human Tregs constitute approximately 10% of CD4 T cells in peripheral blood (Hartigan-O'Connor *et al.*, 2007) and a similar frequency of Tregs was found in the SF of JIA patients, representing a local site of inflammation (Ruprecht *et al.*, 2005). We co-cultured $CD73^-$ CD4con T cells with different amounts of autologous Tregs, including a ratio close to physiological conditions (1 : 0.125). Without ATP, we did not observe suppression of responder T cells by Tregs at any ratio (Figure 31 B-D). Addition of ATP enabled Treg-mediated suppression, revealing a ratio-dependent reduction in the frequency of $CD25^+$ T cells and proliferation (Figure 31 B, C). At the highest ratio of responder T cells to Tregs (1 : 0.5, five-fold physiological amounts of Tregs), we measured the strongest reduction in CD25-expressing responder T cells and proliferation was completely abrogated. IFNγ production was similarly suppressed independent of the amount of Tregs (Figure 31 D). The addition of recombinant CD73 and ATP led to maximal suppression of responder T cells at all ratios and reduced IFNγ production to the minimum (Figure 31 B-D). In summary, these data show that ATP is indispensable for efficient Treg-mediated suppression of effector T cells. Abundant CD39 expression on Tregs ensured ATP degradation; however, maximal T cell suppression by physiological amounts of Tregs required cooperation with an exogenous source of CD73.

Our data suggest that human Tregs do not express enough CD73 for efficient adenosine-mediated suppression of responder T cells. However, exogenously provided CD73 can compensate for the lack of Treg-intrinsic AMPase activity. EVs represent a natural source of non-cell-bound CD73, and human Tregs were shown to produce adenosine when they are co-incubated with plasma-derived EVs that carry CD73 (Schuler *et al.*, 2014). Until now, the functional consequences of this cooperation have not been addressed. The observed immunosuppressive potential of EVs from $CD73^+$ CD8 T cells (Figures 27 and 29) prompted us to investigate if these effector T cell-derived EVs cooperate with human Tregs to generate adenosine and thus complement Treg function. To test this, we repeated the suppression assay using EVs from activated CD8 T cells instead of recombinant CD73 (Figure 32).

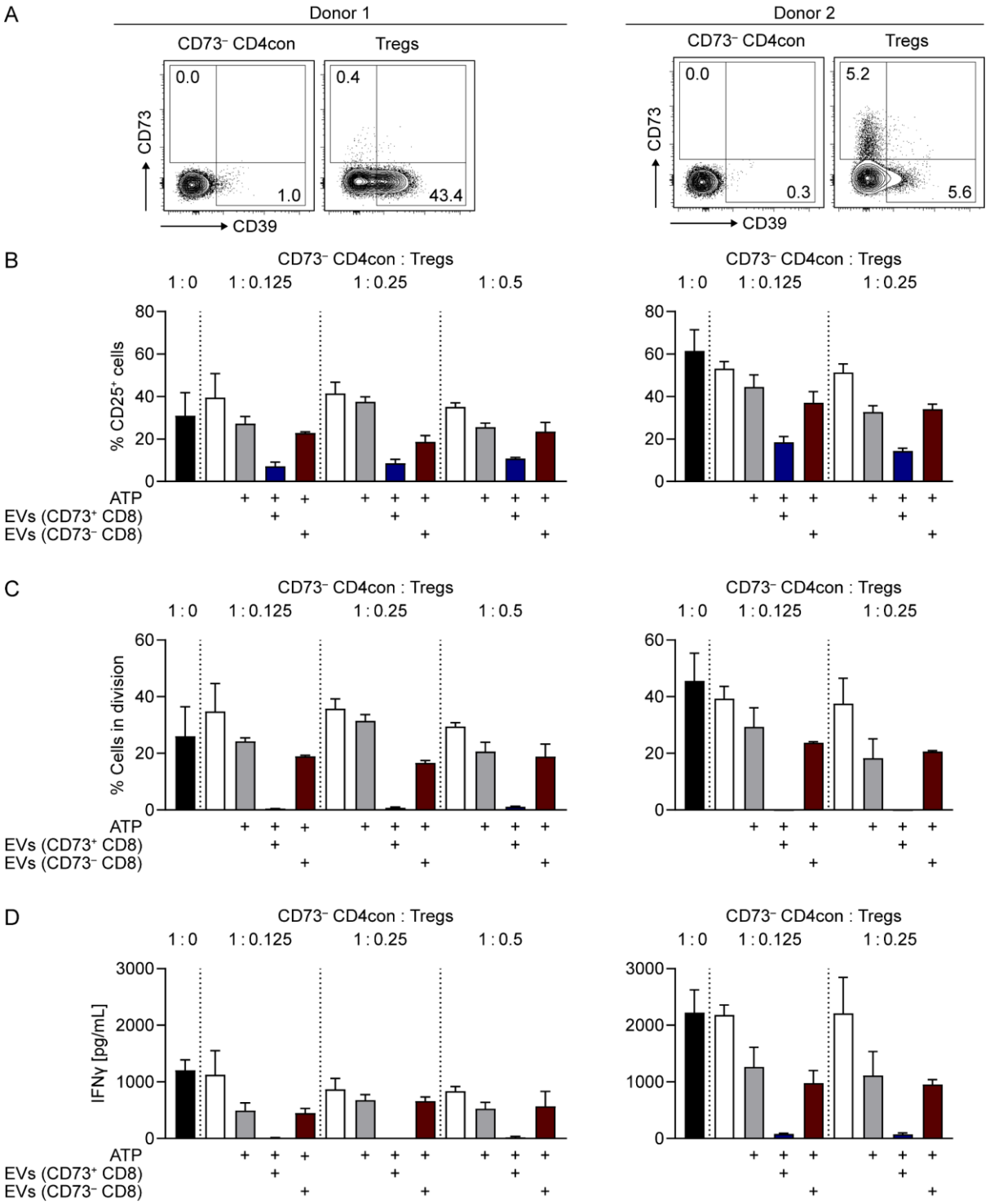

Figure 32 | Extracellular vesicles from CD73⁺ CD8 T cells synergize with Tregs in T cell suppression. CD73⁻ CD4con T cells and Tregs were sorted from enriched CD4 T cells and CD73⁻ CD4con T cells were labeled with eFluor 670 after sorting. (A) Contour plots show expression of CD73 and CD39 in CD73⁻ CD4con T cells and Tregs at the beginning of the co-culture. (B-D) CD73⁻ CD4con T cells were co-cultured with different ratios of autologous Tregs and stimulated with αCD3 and αCD28 for four days in the presence of ADA inhibitor EHNA (10 µM). The black bars represent responder T cells without Tregs. When indicated, ATP (50 µM, light grey bars), ATP and EVs from CD73⁺ CD8 T cells (blue bars) or ATP and EVs from CD73⁻ CD8 T cells (red bars) were added to the culture. The amount of EVs corresponded to 75 µL of CD8 T cell culture supernatant. Frequency of CD25⁺ T cells (B), proliferation (C) and IFNγ production (D) were measured by flow cytometry (B, C) or ELISA (D) to determine suppression of responder T cells. For each of the two donors, graphs show data as mean ± SD.

We hypothesized that the frequency of CD73-expressing Tregs might influence the outcome of the suppression assay and considered this point in the selection of the donors. In one donor, 0.4% of Tregs expressed CD73, in the other donor we detected CD73 expression in 5.2% of Tregs (Figure 32 A). In both donors and at all ratios tested, EVs from $CD73^+$ CD8 T cells cooperated with Tregs in immune suppression, while EVs from $CD73^-$ CD8 T cells did not complement Treg function (Figure 32 B-D). The combination of Tregs, ATP and EVs from $CD73^+$ CD8 T cells strongly decreased the percentage of $CD25^+$ T cells (Figure 32 B), completely abrogated proliferation (Figure 32 C) and reduced IFNγ production of responder T cells to background levels (Figure 32 D). In contrast, addition of EVs from $CD73^-$ CD8 T cells did not enhance T cell suppression. Both types of EVs have the same capacity of ATP degradation, but only EVs from $CD73^+$ CD8 exhibit AMPase activity (see Figure 24 C). Even though donor 2 had a low frequency of CD39-expressing Tregs, we only observed beneficial effect of EVs when they provided AMPase activity, suggesting that Tregs were not dependent on ATPase activity of EVs. In summary, these data show that CD73-containing EVs from effector T cells cooperate with human Tregs by providing AMPase activity and complement Treg-mediated T cell suppression using the purinergic pathway.

4. Discussion

The activity of purinergic ecto-enzymes regulates the concentration of extracellular nucleotides and nucleosides, and the engagement of these molecules to P1 and P2 receptors determines the magnitude of purinergic signaling in immune cells. Degradation of ATP and ADP by CD39 terminates P2 receptor signaling and yields AMP, the substrate for CD73. The enzymatic activity of CD73 leads to the generation of adenosine, thus enabling anti-inflammatory signaling through P1 receptors. Therefore, CD73 is the central enzyme that controls purinergic suppression of effector T cells. While the adenosine-mediated inhibition of T cell immune responses has great potential in the therapy of inflammatory diseases, it is disadvantageous in the setting of cancer, where anti-tumor T cell responses are desirable. Understanding the regulation of CD73 expression in T cells will offer new options to modulate effector T cell function and to shift the balance between pro- and anti-inflammatory signaling in immune-mediated diseases.

In this work, I could show that activated human CD8 T cells release enzymatically active CD73 in extracellular vesicles (EVs). These EVs mediate immune suppression, complementing the function of $FOXP3^+$ regulatory T cells. We propose that effector T cell-derived EVs constitute an intrinsic immunoregulatory mechanism in the human T cell compartment and offer new opportunities for the treatment of inflammatory diseases.

4.1 Regulation of extracellular nucleotide and nucleoside concentrations

CD39 and CD73 play an exclusive role in the regulation of extracellular nucleotide concentrations. Two other families of ecto-nucleotidases, ecto-nucleotide pyrophosphatase/ phosphodiesterases (E-NPPs) and alkaline phosphatases exist, but their role in purinergic immune regulation is not well characterized. Both enzyme families show optimal activity at alkaline pH of 9 – 10, exhibiting reduced activity at neutral pH (Zimmermann, Zebisch and Sträter, 2012). E-NPP1, also known as CD203a or PC-1, metabolizes ATP to AMP and pyrophosphate, but it can also use NAD^+ or ADP-ribose as substrates for AMP generation. Even though E-NPP1 was found on Jurkat T cells, a leukemic human T cell line often used as a model for human T cells, there is no evidence of E-NPP1 expression and function in T cells from healthy donors (Deterre *et al.*, 1996; Horenstein *et al.*, 2013). Alkaline phosphatases can degrade ATP, ADP and AMP, revealing that these enzymes can inactivate ligands for P2

receptors and simultaneously generate adenosine, a P1 receptor agonist (Zimmermann, Zebisch and Sträter, 2012). As soluble enzyme in human plasma, tissue-nonspecific alkaline phosphatase (TNAP) activity is irrelevant for adenosine generation at low micromolar levels of AMP, but contributes significantly to adenosine production in the presence of high pathophysiogical levels (100 µM) of AMP (Pettengill *et al.*, 2013). TNAP also generates adenosine in mesenchymal stromal cells from patients with a loss-of-function mutation in *NT5E* (the gene coding for CD73) but not in cells from healthy donors that possess CD73 (Jin *et al.*, 2016). Of note, alkaline phosphatase gene expression or activity is not detected in resting or stimulated human peripheral blood lymphocytes (Latheef *et al.*, 2016). The latter observation is in line with our data showing that blocking CD73-mediated AMPase activity or the absence of cellular CD73 expression prevents degradation of AMP, indicating that no other nucleotidase can compensate CD73 function in CD4 T cells. These data underline the outstanding role of CD73 in the context of T cell immune suppression.

Given the immunosuppressive role of adenosine, tight control of CD39 and CD73 expression is essential for adequate T cell activation. Low CD73 expression has been previously associated with activated T cells (Botta Gordon-Smith *et al.*, 2015) and CD39 expression is characteristic for cytokine-producing, non-regulatory T cells at sites of inflammation (Moncrieffe *et al.*, 2010). We confirmed previous reports that CD73 is expressed in resting human T cells on the majority of naïve CD8 T cells and on a small proportion of memory CD4 T cells, while less than 10% of CD8 and CD4 T cells are $CD39^+$ (Dianzani *et al.*, 1993; Pulte *et al.*, 2007; Doherty *et al.*, 2012). In general, co-expression of CD73 and CD39 is a rare event in human T cells (Tóth *et al.*, 2013) - under resting conditions and during the first days of activation - suggesting that T cells protect themselves from adenosine-mediated suppression. In agreement with published data (Raczkowski *et al.*, 2018), we observed a progressive increase in the frequency of CD39-expressing T cells after two to three days of TCR stimulation. We could show that increasing levels of CD39 expression resulted from upregulation of *ENTPD1* gene expression that was already elevated 24 hours post stimulation. Concomitant with CD39 upregulation after T cell activation, human T cells lost CD73 expression and this loss did not correlate with *NT5E* expression. Despite differences in initial CD73 expression, the progressive decrease of CD73 expression at the cell membrane was obvious in both CD8 and CD4 T cells. We concluded that loss of CD73 expression is a consequence of T cell activation, probably to prevent pericellular adenosine generation in

activated T cells and thus protect them from inhibitory signaling through A_{2A} receptors. Indeed, we could show that the absence of cellular CD73 expression protected CD4 T cells from adenosine-mediated suppression when T cells were cultured with exogenously added ATP or AMP to mimic the inflammatory milieu. In the absence of exogenously added nucleotides, T cell activation was not altered when T cells were constantly exposed to CD73 provided by human CD73-transfected HEK293 cells, indicating that activated T cells themselves do not release massive amounts of ATP or AMP that serve as substrate for adenosine generation. Moreover, CD73 protein acquisition from human CD73-transfected HEK293 cells suggests that the expression of CD73 on the T cell plasma membrane does not interfere with T cell activation unless the cells are exposed to pathophysiological levels of adenine nucleotides. Thus, activated T cells might indeed downmodulate CD73 expression to achieve protection at sites of inflammation. In most donors, we observed that the frequency of $CD73^+$ T cells increased after one week of culture due to re-expression of CD73. We could show that T cells re-expressing CD73 have undergone several rounds of cell division and arose from $CD39^+$ T cells, thus creating a population of $CD39^+$ $CD73^+$ T cells. In CD8 T cells that were sorted according to CD73 expression prior to activation, we observed re-expression of CD73 and generation of double positive cells almost exclusively after activation of initially $CD73^+$ T cells. In the face of this, it would be interesting to investigate how re-expression of CD73 is regulated and if these $CD39^+$ $CD73^+$ T cells represent a subset of exhausted cells or if they are necessary for the termination of the T cell response due to their ability to generate adenosine.

Why human naïve CD8 T cells express high amounts of CD73 is not clear. Adenosine signaling has an essential role in the survival of naïve murine T cells. Tonic activation of the A_{2A} receptor protects murine naïve T cells from apoptosis by maintaining their responsiveness to IL-7 (Cekic *et al.*, 2013). In line with these data, high CD73 expression in human naïve CD8 T cells might ensure the generation of sufficient amounts of adenosine for the maintenance of the naïve CD8 T cell pool. Apart from its enzymatic activity, CD73 cross-linking was found to deliver co-stimulatory signals during T cell activation, thereby increasing the sensitivity of naïve CD8 T cells to TCR stimulation (Thompson *et al.*, 1989; Massaia *et al.*, 1990; Dianzani *et al.*, 1993). It is unclear how CD73 transmits the co-stimulatory signal, because neither the GPI-anchor, nor its enzymatic activity are required for CD73-mediated T cell activation (Resta *et al.*, 1994; Gutensohn *et al.*, 1995). Interestingly, we observed that CD73 marks the subset of naïve CD8

T cells that proliferated in response to T cell stimulation. No antibody against CD73 was present during the culture, so we excluded that the observed effect was due to a co-stimulatory signal delivered by CD73 cross-linking. In summary, CD73 expression identifies naïve T cells with proliferative capacity, but why this is the case remains an open question.

CD39 hydrolyzes nucleoside tri- and diphosphates to the corresponding monophosphate and inorganic phosphate. Thus, the activity of CD39 controls extracellular ATP and ADP concentrations and regulates ligand availability for all P2X receptors and most P2Y receptors (Kukulski *et al.*, 2005). Under homeostatic conditions, extracellular ATP concentrations in human plasma are in the low nanomolar range (Gorman, Feigl and Buffington, 2007). Increasing levels of extracellular ATP due to cell damage or inflammatory reactions initiate the acute phase of purinergic signaling that is characterized by the activation of P2 receptors to promote inflammation (Cekic and Linden, 2016). In this phase, T cells express little or no CD39, and ATP has a dual role in effector T cell activation. Within seconds after TCR stimulation, human CD4 T cells release ATP (> 60 µM) at sufficient concentrations to activate P2X receptors (Yip *et al.*, 2009). Pannexin-1, $P2X_1$ and $P2X_4$, but not $P2X_7$, translocate to the immune synapse within five to ten minutes, where pannexin-1-mediated ATP release activates $P2X_1$ and $P2X_4$ and this autocrine signaling is essential for TCR signal amplification and IL-2 production (Woehrle *et al.*, 2010). Low affinity $P2X_7$ receptors are uniformly distributed on the plasma membrane of recently activated T cells and might allow T cells to remain sensitive to ATP in their environment (Woehrle *et al.*, 2010; Junger, 2011). Indeed, $P2X_7$ receptors are involved in apoptosis of human CD4 T cells that were exposed to 1 mM ATP (Trabanelli *et al.*, 2012). Inhibition of human T cell activation and function by extracellular ATP is dose-dependent. Purified CD4 T cells show reduced proliferation in the presence of 200 µM ATP (Weiler *et al.*, 2016), while low micromolar (1 – 10 µM) ATP concentrations are sufficient to impair proliferation and cytokine production of CD4 T cells that were not separated from other PBMCs (Duhant *et al.*, 2002; Sueyoshi *et al.*, 2019). ATP itself induces ATP release from human leukocytes (De Ita *et al.*, 2016), which leads to elevated extracellular ATP levels and might explain discrepancies in the amounts of ATP reported to induce T cell suppression. The observed suppressive effect of extracellular ATP is independent of its degradation to adenosine and mediated by activation of the $P2Y_{11}$ receptor, the only P2 receptor that couples to stimulatory G proteins and elevates intracellular cAMP

concentrations (Sueyoshi *et al.*, 2019). In our assays, we used purified conventional CD4 T cells and circumvented ATP-induced ATP release from non-T cells. Moreover, $CD73^-$ CD4con T cells were unable to generate adenosine from extracellular ATP, and allowed us to measure adenosine-independent suppressive effects of ATP. Interestingly, we observed T cell suppression in the presence of 50 µM ATP in some donors, while others showed no signs of suppression after addition of ATP. T cells express all types of P2Y receptors (Bours *et al.*, 2006), but a recent gene expression analysis in different types of human blood cells revealed that $P2Y_{11}$ is the predominant P2Y receptor in human T cells and expression of other P2Y receptors is negligible (Uhlen *et al.*, 2019). $P2Y_{11}$ receptor-mediated upregulation of cAMP possibly occurred in all donors, but the strength and duration of ATP signaling might have differed between the individuals. Because upregulation of CD25, proliferation and cytokine production are rather late read-out parameters to assess T cell suppression, we might have missed early suppressive effects of ATP in some donors. We did not analyze individual $P2Y_{11}$ receptor expression levels that might account for the different sensitivity to ATP. Likewise, we did not genotype the donors for single nucleotide polymorphisms (SNPs) in the CD39 gene that control the frequency of CD39-expressing Tregs and influence basal CD39 expression levels in conventional T cells (Rissiek *et al.*, 2015; Roederer *et al.*, 2015). Thus, a higher basal capacity to eliminate extracellular ATP might be beneficial for T cell activation as long as autocrine ATP signaling is not affected.

CD39 enzymatic activity degrades ATP to AMP and two molecules of inorganic phosphate without significant release of ADP as intermediate product. This unique pattern of nucleotide hydrolysis distinguishes CD39 from other members of the NTPDase family, and is thought to favor CD73-mediated adenosine generation by preventing the accumulation of extracellular ADP (Kukulski *et al.*, 2005). ATP and ADP are strong competitive inhibitors of CD73 function, with ADP being more potent than ATP (Burger and Lowenstein, 1975). Studies with rat hippocampal nerve terminals suggest that the concentration of ATP plus ADP needs to be lower than 5 µM to enable adenosine generation by CD73 (Cunha, 2001). We observed that the activation-induced increase of CD39 expression in T cells is donor-dependent. CD39 can form oligomers with increased catalytic activity (Wang, Ou and Guidotti, 1998). It is therefore well possible that the strength of CD39 upregulation represents a mechanism to control ATP and ADP levels and consequently CD73 activity.

CD73-mediated AMP degradation is the major source for extracellular adenosine (Möser, Schrader and Deussen, 1989) and is therefore particularly relevant for the activation of P1 receptors. Under physiological conditions, intracellular adenosine concentrations are around 100 nM and the activity of ubiquitously expressed equilibrative nucleoside transporters (ENTs) ensures that extracellular concentration are approximately the same (Fredholm, 2014). Nanomolar (300 – 700 nM) concentrations of adenosine are sufficient to activate adenosine receptors with exception of A_{2B}, which requires low micromolar (20 µM) levels of extracellular adenosine (Fredholm *et al.*, 2001). Adenosine deaminase (ADA) activity controls the availability of extracellular adenosine. The enzyme converts adenosine to inosine, thus eliminating the agonist for P1 receptor signaling. ADA is a cytosolic protein, but the secreted form can attach to the cell surface of lymphocytes by binding to CD26 or the A_{2B} receptor (Kameoka *et al.*, 1993; Herrera *et al.*, 2001). CD26 and ADA are both upregulated after T cell activation (Martin *et al.*, 1995) and pharmacological inhibition of ADA increases the sensitivity of T cells towards adenosine-mediated T cell suppression (Dong *et al.*, 1996). These data suggest that activated T cells use the CD26-ADA complex as further regulatory mechanism to protect themselves from adenosine-mediated suppressive signaling especially in the presence of CD73. In our assays, we blocked the enzymatic activity of ADA by the addition of EHNA to prevent adenosine degradation. This allowed us to measure adenosine-mediated suppressive effects and to investigate the influence of various sources of CD73 during T cell activation. We showed that blockade of ADA did not influence T cell activation in the absence of exogenously added ATP or AMP independently of cell surface CD73 expression. This reveals that extracellular adenosine levels were low despite inhibition of ADA and indicates that activated T cells do not release sufficient amounts of endogenous ATP for adenosine generation. The lack of substrate for adenosine generation in purified T cell cultures explains our observations that constant exposure to CD73 (e.g. during co-culture of T cells with human CD73-transfected HEK293 cells) does not interfere with T cell activation. However, the combination of CD73 and EHNA led to accumulation of extracellular adenosine under inflammatory conditions (mimicked in *in vitro* assays by the addition of AMP or ATP) and resulted in T cell suppression. The A_{2A} receptor is the predominant receptor in human T cells (Uhlen *et al.*, 2019) and its activation interferes with TCR signaling, resulting in reduced upregulation of activation markers and decreased cytokine production (Huang *et al.*, 1997; Raskovalova *et al.*, 2007). Human T cells also express A_{2B} receptors and both types of adenosine receptors are

upregulated in activated T cells (Koshiba *et al.*, 1999; Mirabet *et al.*, 1999). A_{2A} and A_{2B} receptors both couple to stimulatory G proteins, increase intracellular cAMP levels and induce T cell suppression. However, A_{2B} receptors can also promote pro-inflammatory reactions when they couple to $G_{q/11}$ (Linden *et al.*, 1999). Moreover, A_{2B} receptors can dimerize with A_{2A} receptors and reduce their ligand affinity (Hinz *et al.*, 2018). Because the A_{2B} receptor is a low affinity adenosine receptor, we might favor its activation by EHNA-induced accumulation of adenosine. However, anti-suppressive effects of A_{2B} receptors seem to play only a minor role, if any, in our assays because we observed strong T cell suppression by adenosine.

Membrane-bound ecto-nucleotidases expressed on immune and endothelial cells as well as soluble forms of nucleotide-metabolizing enzymes control extracellular nucleotide and nucleoside levels in human blood. The cellular fraction, consisting of lymphocytes and granulocytes, predominantly controls ATPase and ADPase activities, while soluble enzymes, in particular CD73, are responsible for the majority of AMPase activity in blood (Coade and Pearson, 1989; Heptinstall *et al.*, 2005; Pettengill *et al.*, 2013). Endothelial cells exhibit comparable ecto-nucleotidase activities to white blood cells and to soluble enzymes circulating in the blood, suggesting an equal contribution of the flowing blood and endothelial cells to adenine nucleotide metabolism in large vessels. In microvessels with reduced blood flow, however, endothelial adenine nucleotide turnover will predominate (Coade and Pearson, 1989). Endothelial cells and lymphocytes also express enzymes involved in ATP-regeneration such as adenylate kinase and nucleoside diphosphate kinase (Yegutkin *et al.*, 2002). By counteracting the enzymatic activity of CD39, these enzymes directly regulate the amount of AMP and thus have an indirect influence on the adenosine concentration.

4.2 The role of non-cell-bound CD73 and T cell-derived extracellular vesicles in immune regulation

The majority of AMPase activity in human blood is associated with non-cell-bound enzymes, in particular with CD73. Several studies reported CD73-specific AMPase activity in human body fluids, but not all of them address the question whether this activity results from vesicular CD73 or a truly soluble form of the enzyme (Pettengill *et al.*, 2013; Morello *et al.*, 2017; Zeiner *et al.*, 2019). We observed a gradual loss of CD73 surface expression in activated

T cells with a concomitant increase in AMPase activity in the T cell culture supernatant, and aimed to elucidate the underlying mechanism. As a GPI-anchored protein, CD73 is susceptible to cleavage by phospholipases. Soluble CD73 from human placental extracts carried myo-inositol, suggesting that it derived from the GPI-anchored form through cleavage by phospholipase C or D (Klemens *et al.*, 1990). TNFα-treatment induced shedding of CD73 from endothelial cells and this effect was blocked by an unspecific phospholipase C inhibitor (Kalsi *et al.*, 2002). Despite this observation, a direct involvement of cytosolic phospholipase C in CD73 shedding remains questionable because it would require the release of phospholipase C into the extracellular space. Of note, human serum contains a phospholipase D capable of cleaving the GPI-anchor of purified proteins, but it failed to shed GPI-anchored proteins from intact cells (Davitz *et al.*, 1987). Recently, matrix metalloproteinase-9 was reported to shed an enzymatically inactive form of CD73 from retinal pigment epithelium (Zhang *et al.*, 2018). Our data do not support a role of phospholipases or other soluble proteases in the activation-induced loss of CD73 from the T cell plasma membrane. Specific blockade of phospholipases or matrix metalloproteinases did not prevent loss of cell surface CD73 expression in activated T cells. Likewise, cell culture supernatant from activated T cells did not induce shedding of CD73 in non-activated T cells, indicating that it did not contain secreted phospholipases or proteases capable of removing CD73 from the plasma membrane. Thus, removal of T cell plasma membrane CD73 is a T cell-intrinsically regulated mechanism.

We then hypothesized that CD73 might be shed contained in extracellular vesicles. Indeed, we observed co-localization of CD73 with the EV marker CD9 in activated CD8 T cells, and showed that EVs are the major source of AMPase activity in cell culture supernatants from activated CD8 T cells. GPI-anchored proteins such as CD73 are enriched in lipid rafts and preferentially sorted in EVs because these specific membrane microdomains contribute to EV formation (de Gassart *et al.*, 2003). TCR stimulation strongly increases EV release from T cells and co-stimulatory signals further promote this process (Blanchard *et al.*, 2002; van der Vlist *et al.*, 2012). We did not only detect elevated numbers of EVs in cell culture supernatants from stimulated CD8 T cells, but also increased AMPase activity per EV. These observations suggest that formation and release of EVs substantially contribute to activation-induced loss of surface CD73 expression in CD8 T cells. Because T cells release CD73 into the extracellular space, we speculated that loss of cell membrane CD73 expression is not only necessary to protect T cells from adenosine generation, but represents a mechanism by which T cells can

control the function of other immune cells. We found that T cell-derived EVs possess substantial immunomodulatory potential. It is known that murine Tregs release EVs with immunosuppressive capacity upon activation, and these EVs suppress effector T cell proliferation and cytokine production by the transfer of miRNAs (Okoye *et al.*, 2014). Another study reported the expression of CD73 in murine Treg-derived EVs leading to T cell suppression through the production of immunosuppressive adenosine (Smyth *et al.*, 2013). Human Treg-derived EVs are also capable of T cell suppression; however, this effect is CD73-independent because these EVs lack the expression of CD73 (Tung *et al.*, 2020). EVs from stimulated, non-regulatory T cells can have pro- and anti-inflammatory effects. While T cell-derived EVs dampen the immune response by the downmodulation of the T cell stimulatory capacity of antigen-presenting cells (APCs), they induce proliferation of resting T cells when combined with IL-2 (Xie *et al.*, 2010; Wahlgren *et al.*, 2012). Our results reveal a previously unknown function of EVs from effector T cells (Figure 33). EVs from activated $CD73^+$ CD8 T cells provide sufficient AMPase activity to induce T cell suppression through the purinergic pathway. This AMPase activity is due to CD73 present in the membrane of these EVs. We detected CD73 expression by flow cytometry and western blot, and the specific CD73 inhibitor PSB-14685 completely blocked adenosine generation and EV mediated T cell suppression. Our *in vitro* assays also showed that CD8 T cell-derived EVs with CD73-specific AMPase activity cooperate with Tregs in T cell suppression (Figure 33). Tregs are indispensable for the maintenance of peripheral tolerance and the termination of T cell immune responses and use a variety of suppressive mechanisms. These pathways are usually discovered in mice and *in vitro* assays indicate a relevance for some of these mechanisms in the human system (Sakaguchi *et al.*, 2010). A striking difference between human and murine immune cells is the expression pattern of CD39 and CD73: While the majority of murine Tregs express both ecto-nucleotidases and produce immunosuppressive adenosine (Deaglio *et al.*, 2007), few human Tregs are $CD73^+$ and co-expression with CD39 is thus a rare event, challenging the concept of effector T cell suppression through purinergic signaling in humans. A cooperation between plasma-derived EVs and Tregs to produce adenosine has been previously reported; however, a functional relevance of this mechanism was not demonstrated (Schuler *et al.*, 2014). The use of $CD73^-$ CD4 T cells as responder cells allowed us to determine the contribution of Treg-derived and exogenous CD73 in effector T cell suppression. We showed that physiological amounts of Tregs do not harbor enough AMPase activity to induce maximal effector T cell

suppression through the CD39/CD73/adenosine-axis. Even though we blocked ADA activity and thereby enhanced adenosine signaling, we needed to provide CD73 in form of EVs to completely inhibit effector T cell proliferation and cytokine production.

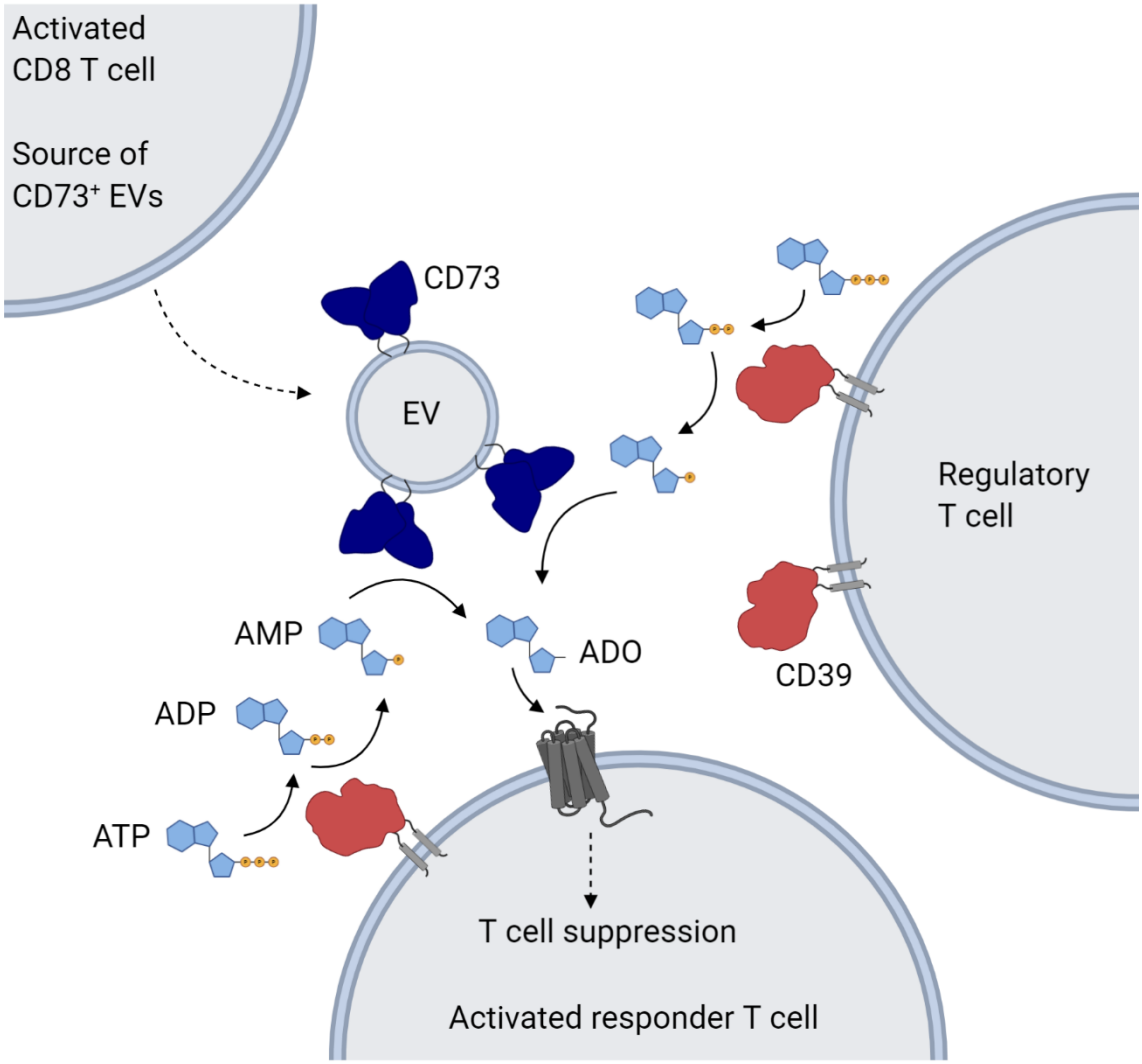

Figure 33 | Immunosuppressive function of effector T cell-derived extracellular vesicles. Activated CD73$^+$ CD8 T cells release EVs that carry enzymatically active CD73. Regulatory T cells and activated responder T cells express CD39 and degrade ATP and ADP to AMP. Vesicular CD73 hydrolyzes AMP to adenosine (ADO), which activates the A2A receptor and suppresses responder T cells. Image created with BioRender.com.

Due to our clean assay system that lacks APCs, we only measured suppressive functions of Tregs that acted directly on T cells and excluded indirect effects that involve the modulation of APC function. The suppressive capacity of Tregs clearly depended on the presence of ATP or its metabolites under these conditions because effector T cell suppression did not occur in the absence of ATP. Strong T cell stimulation, as we use it in our assays to mimic inflammatory conditions, impairs Treg function and renders responder T cells resistant to suppression

(Baecher-Allan, Viglietta and Hafler, 2002). Addition of ATP could overcome the unresponsiveness of effector T cells to suppression, underlining the importance of purinergic signaling in the inhibition of effector T cell responses. Adenosine, which results from ATP degradation, might not only act on effector T cells and induce suppression, but also promote Treg function. Murine Tregs expand in the presence of adenosine and show increased suppressive capacity (Ohta *et al.*, 2012). Even though adenosine inhibits proliferation of human Tregs, it does not reduce their suppressive capacity (Baroja-Mazo *et al.*, 2019).

ATP and adenosine have a short half-life of less than one second in human blood due to rapid metabolism and uptake of adenosine into blood cells through nucleoside transporters (Ontyd and Schrader, 1984; Möser, Schrader and Deussen, 1989; Mortensen *et al.*, 2011). Thus, the range of action of ATP and adenosine is likely limited to the production site and the immediate vicinity that might cover a few hundred microns (Fitz, 2007). In this context, the release of ecto-nucleotidases from the cell membrane as enzymatically active molecules represents a powerful mechanism to widen the range of action of purinergic signaling.
CD73 is catalytically activated after shedding from the membrane (Lehto and Sharom, 1998). We speculate that deployment of CD73 in enriched form, as it occurs in EVs, compensates for the reduced activity of the membrane bound form compared with the soluble form. Moreover, EVs offer the possibility to provide CD73 in combination with CD39, and thus the whole machinery for ATP degradation, at distant sites. It is unclear how far EVs travel *in vivo* especially in the tissue. The injection of platelet-derived EVs into the bloodstream of rabbits suggest a rather rapid clearance from the circulation by cellular uptake within ten minutes (Rand *et al.*, 2006). In a study with humans, transfused platelet-derived EVs were still detected after three hours (Rank *et al.*, 2011).

We had the chance to isolate EVs from a local site of inflammation, specifically the synovial fluid (SF) of patients with juvenile idiopathic arthritis (JIA). JIA is an autoimmune disorder of unknown etiology and characterized by local inflammation in the joints. Joint-infiltrating T cells and the balance between effector and regulatory T cells are involved in disease pathology and outcome (de Kleer *et al.*, 2004; Nistala *et al.*, 2008). Extracellular vesicles in the SF originate from various cell types including synovial fibroblasts and infiltrating immune cells such T cells and monocytes (Calvo and Izquierdo, 2020). B cells are less frequent in SF than in

peripheral blood of JIA patients and B cell-derived vesicles, a potent source of CD73 in the human immune system, are underrepresented in the SF of JIA patients compared with the SF from patients with other forms of arthritis, e.g. rheumatoid arthritis (RA) (Hunter *et al.*, 2010; György *et al.*, 2012). EVs from RA patients show a pro-inflammatory phenotype and might contribute to disease progression. These EVs carry a membrane-bound form of TNFα and induce resistance to apoptosis in CD4 T cells (Zhang *et al.*, 2006). Of note, a higher frequency of CD4 T cell-derived SF EVs co-expressing CD39 and CD73 is associated with reduced disease severity in RA (Fan *et al.*, 2017). We showed that SF-derived EVs from JIA patients have pro-inflammatory effects on T cells derived from healthy donors. However, these EVs exhibit CD73-specific AMPase activity and can acquire immunosuppressive function under conditions that favor adenosine signaling. Adenosine is a potent vasodilator, which limits its use as anti-inflammatory drug. The prodrug chet-AMP, that is activated by CD73 and acts then as A_{2A} receptor agonist, exhibits negligible vasodilatory activity and ameliorates disease in a mouse model of arthritis in a CD73-dependent manner (Flögel *et al.*, 2012). Application of chet-AMP in combination with CD73-containing vesicles might therefore be a promising therapeutic strategy to treat JIA.

4.3 Conclusion and perspectives

This thesis provides new insights in the regulation of CD73 expression in human T cells and postulates a role of effector T cell-derived EVs containing CD73 in the context of immune suppression. We show that conventional T cells lose CD73 expression from the plasma membrane as a consequence of T cell activation and that this process involves the formation and release of EVs in CD8 T cells. These EVs generate adenosine in a CD73-dependent manner and exhibit potent immunosuppressive function. We also provide evidence that the EV-based modulation of purinergic signaling can shift the balance from pro-inflammatory to anti-inflammatory signaling and might ameliorate the course of inflammatory diseases.

Most research studies in the field of immune regulation use mice, and translation of study results to the human system can be challenging. The huge difference regarding CD73 expression on immune cells, in particular Tregs, between mice and humans will affect immune regulation pathways and might account for some of these difficulties. Our data offer new insights in the purinergic regulation of effector T cells in the human system and underscore

the role of EVs as immune regulators. We have shown the *in vivo* relevance of this process using EVs isolated from the inflamed joints. This works paves the way to a possible intervention using CD73-containing nanoparticles for the treatment of local inflammation.

5. Material and methods

5.1 Material

5.1.1 Antibodies

Human antibodies for flow cytometry

Specificity	Fluorochrome	Clone	Company
CD3	BV650	OKT3	BioLegend, San Diego (CA), USA
CD4	V500	RPA-T4	Becton Dickinson, Franklin Lakes (NJ), USA
CD4	AF488	RPA-T4	BioLegend, San Diego (CA), USA
CD4	FITC	RPA-T4	BioLegend, San Diego (CA), USA
CD4	PE/Dazzle 594	RPA-T4	BioLegend, San Diego (CA), USA
CD4	PerCP/Cy5.5	RPA-T4	BioLegend, San Diego (CA), USA
CD4	APC	SK3	BioLegend, San Diego (CA), USA
CD4	AF700	OKT4	BioLegend, San Diego (CA), USA
CD8	BV510	RPA-T8	BioLegend, San Diego (CA), USA
CD8	BV605	RPA-T8	BioLegend, San Diego (CA), USA
CD8	AF700	HIT8a	BioLegend, San Diego (CA), USA
CD9	APC	HI9A	BioLegend, San Diego (CA), USA
CD14	PE	M5E2	BioLegend, San Diego (CA), USA
CD14	BV711	M5E2	BioLegend, San Diego (CA), USA
CD16	APC/Cy7	3G8	BioLegend, San Diego (CA), USA
CD19	BV570	HIB19	BioLegend, San Diego (CA), USA
CD20	V450	L27	Becton Dickinson, Franklin Lakes (NJ), USA
CD20	AF700	2H7	BioLegend, San Diego (CA), USA
CD25	BV421	BC96	BioLegend, San Diego (CA), USA
CD25	BV785	BC96	BioLegend, San Diego (CA), USA
CD26	FITC	M-A261	Becton Dickinson, Franklin Lakes (NJ), USA
CD27	FITC	O323	Thermo Fisher Scientific, Waltham (MA), USA
CD27	APC/Cy7	O323	BioLegend, San Diego (CA), USA
CD38	BV605	HIT2	BioLegend, San Diego (CA), USA
CD39	PE/Cy7	A1	BioLegend, San Diego (CA), USA
CD45	BV510	HI30	BioLegend, San Diego (CA), USA
CD45RA	BV421	HI100	BioLegend, San Diego (CA), USA
CD45RA	BV785	HI100	BioLegend, San Diego (CA), USA
CD56	BV421	HCD56	BioLegend, San Diego (CA), USA
CD69	AF488	FN50	BioLegend, San Diego (CA), USA
CD73	PE	AD2	BioLegend, San Diego (CA), USA
CD73	PerCP/Cy5.5	AD2	BioLegend, San Diego (CA), USA
CD73	APC	AD2	BioLegend, San Diego (CA), USA
CD127 (IL-7Rα)	PerCP/Cy5.5	A019D5	BioLegend, San Diego (CA), USA

CD157	APC	SY11B5	Thermo Fisher Scientific, Waltham (MA), USA
CD197 (CCR7)	AF647	G043H7	BioLegend, San Diego (CA), USA
TCRγδ	PE	11F2	Becton Dickinson, Franklin Lakes (NJ), USA

Human antibodies for T cell stimulation

Specificity	Conjugate	Clone	Company
CD3	unconjugated	OKT3	BioLegend, San Diego (CA), USA
CD28	unconjugated	CD28.2	BioLegend, San Diego (CA), USA

Antibodies for western blot

Specificity	Host	Conjugate	Clone	Company
CD73 (human)	rabbit	unconjugated	D7F9A	Cell Signaling Technology, Danvers (MA), USA
IgG (rabbit)	goat	HRP-linked	polyclonal	Cell Signaling Technology, Danvers (MA), USA
Flotillin-1	mouse	unconjugated	18/Flotillin-1	Becton Dickinson, Franklin Lakes (NJ), USA
IgG (mouse)	horse	HRP-linked	polyclonal	Cell Signaling Technology, Danvers (MA), USA

Antibodies for fluorescence microscopy

Specificity	Host	Conjugate	Clone	Company
CD73 (human)	mouse	unconjugated	AD2	BioLegend, San Diego (CA), USA
IgG (mouse)	donkey	Cy2	polyclonal	Jackson ImmunoResearch, West Grove (PA) USA
CD9	mouse	APC	HI9A	BioLegend, San Diego (CA), USA

5.1.2 Buffers and media

Buffer	Composition		
ELISA wash buffer	1x PBS (-/-) with	0.05%	Tween 20
FACS buffer	1x PBS (-/-) with	0.1%	BSA
		0.02%	NaN_3
Full RPMI	RPMI with	10%	FCS
		1%	Penicillin-streptomycin
		2 mM	L-glutamine
HPLC buffer A	ddH_2O with	20 mM	KH_2PO_4
		5 mM	TBAHP
			pH 6.0

HPLC buffer B	HPLC buffer A with	50%	Methanol
MACS buffer	1x PBS (-/-) with	0.5%	BSA
		2 mM	EDTA
RIPA buffer	ddH_2O with	50 mM	Tris-HCl (pH 7.4)
		150 mM	NaCl
		1%	Nonidet P 40 substitute
		0.5%	Na-deoxycholate
		0.1%	SDS
Sandwich buffer (10 X)	ddH_2O with	250 mM	Tris base
		1.92 M	Glycine
			pH 8.3
TBS (10 X)	ddH_2O with	80 g	NaCl
		2 g	KCl
		30 g	Tris-HCl (pH 7.4)
Transfer buffer	ddH_2O with	10%	Sandwich buffer
		10%	Methanol
Western Blot wash buffer	ddH_2O with	10%	TBS (10 X)
		0.05%	Tween 20

5.1.3 Cell lines

Human CD73-transfected HEK293 cells as well as untransfected control HEK293 cells were obtained from the Department of Neurology, University Medical Center Hamburg-Eppendorf.

5.1.4 Chemicals, reagents and solutions

Material	**Company**
1,N^6-etheno-nucleotides (eATP, eADP, eAMP, eADO)	Biolog, Bremen, Germany
2-Mercaptoethanol	Sigma-Aldrich, St. Louis (MO), USA
Adenosine monophosphate (AMP)	Sigma-Aldrich, St. Louis (MO), USA
Adenosine triphosphate (ATP)	Sigma-Aldrich, St. Louis (MO), USA
Alexa Fluor 750 NHS ester (live/dead dye)	Thermo Fisher Scientific, Waltham (MA), USA
BD FACS Clean solution	Becton Dickinson, Franklin Lakes (NJ), USA
BD FACS Rinse solution	Becton Dickinson, Franklin Lakes (NJ), USA
BD FACS Flow sheath fluid	Becton Dickinson, Franklin Lakes (NJ), USA
Biocoll cell separating solution	Merck, Darmstadt, Germany
Bovine serum albumin (BSA)	Thermo Fisher Scientific, Waltham (MA), USA
CD73 inhibitor 2-chloro-N^6-*o*-chlorobenzyl-α,β-methylene-ADP (PSB-14685)	Kindly provided by Prof. Dr. Christa E. Müller

Cell Proliferation Dye eFluor® 670	Thermo Fisher Scientific, Waltham (MA), USA
cOmplete Protease Inhibitor Tablets	Roche, Basel, Switzerland
Dimethyl sulfoxide (DMSO)	AppliChem, Darmstadt, Germany
Dithiothreitol (DTT, 0.1 M)	Thermo Fisher Scientific, Waltham (MA), USA
DNase (RNase-free)	Qiagen, Hilden, Germany
dNTP-Mix	Thermo Fisher Scientific, Waltham (MA), USA
Dulbecco's phosphate-buffered saline (PBS)	Thermo Fisher Scientific, Waltham (MA), USA
Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA)	Cayman Chemical, Ann Arbor (MI), USA
Ethanol	Th. Geyer, Renningen, Germany
Ethylenediaminetetraacetic acid (EDTA)	AppliChem, Darmstadt, Germany
Fetal calf serum (FCS)	Merck, Darmstadt, Germany
First Strand buffer (5 X)	Thermo Fisher Scientific, Waltham (MA), USA
Glycine	Carl Roth, Karlsruhe, Germany
Hoechst	Thermo Fisher Scientific, Waltham (MA), USA
Human IgG	Jackson ImmunoResearch, West Grove (PA) USA
Human recombinant CD73	Sino Biological, Beijing, China
Human recombinant IL-2	Thermo Fisher Scientific, Waltham (MA), USA
L-glutamine	Thermo Fisher Scientific, Waltham (MA), USA
Methanol	Merck, Darmstadt, Germany
Maxima Probe/ROX qPCR Master Mix	Thermo Fisher Scientific, Waltham (MA), USA
M-MLV reverse transcriptase (200 U/µL)	Thermo Fisher Scientific, Waltham (MA), USA
Nonidet P 40 substitute	Sigma-Aldrich, St. Louis (MO), USA
Normal horse serum	Vector Laboratories, Burlingame (CA), USA
NuPAGE antioxidant	Thermo Fisher Scientific, Waltham (MA), USA
NuPAGE LDS sample buffer (4 X)	Thermo Fisher Scientific, Waltham (MA), USA
NuPAGE MES SDS running buffer (20 X)	Thermo Fisher Scientific, Waltham (MA), USA
NuPAGE sample reducing agent (10 X)	Thermo Fisher Scientific, Waltham (MA), USA
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher Scientific, Waltham (MA), USA
Permeabilization buffer	Thermo Fisher Scientific, Waltham (MA), USA
Paraformaldehyde aqueous solution (16%), EM grade	Electron Microscopy Sciences, Hatfield (PA), USA
Phosphatidylinositol-specific phospholipase C (PI-PLC)	Thermo Fisher Scientific, Waltham (MA), USA
PhosSTOP tablets	Roche, Basel, Switzerland
Poly-L-lysine solution	Sigma-Aldrich, St. Louis (MO), USA
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma-Aldrich, St. Louis (MO), USA

Precision Plus Protein Kaleidoscope prestained protein standard	Bio-Rad, Hercules (CA), USA
Random hexamer primer	Thermo Fisher Scientific, Waltham (MA), USA
RNase-free water	Thermo Fisher Scientific, Waltham (MA), USA
RNaseZAP	Sigma-Aldrich, St. Louis (MO), USA
RPMI 1640 + GlutaMAX, with phenol red	Thermo Fisher Scientific, Waltham (MA), USA
Roti-Block	Carl Roth, Karlsruhe, Germany
Sodium azide (NaN_3)	Sigma-Aldrich, St. Louis (MO), USA
Sodium chloride (NaCl)	Avantor, Radnor (PA), USA
Sodium deoxycholate	Sigma-Aldrich, St. Louis (MO), USA
Sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe, Germany
Streptavidin-AF647	Vector Laboratories, Burlingame (CA), USA
Sulfuric acid (H_2SO_4)	Carl Roth, Karlsruhe, Germany
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Fisher Scientific, Waltham (MA), USA
SuperSignal West Pico PLUS chemiluminescent substrate	Thermo Fisher Scientific, Waltham (MA), USA
Tetrabutylammonium dihydrogen phosphate (TBAHP)	Sigma-Aldrich, St. Louis (MO), USA
Tris base	Sigma-Aldrich, St. Louis (MO), USA
Tris-HCl	Sigma-Aldrich, St. Louis (MO), USA
Triton X-100	Sigma-Aldrich, St. Louis (MO), USA
Trypan blue	Sigma-Aldrich, St. Louis (MO), USA
Tuerks solution	Sigma-Aldrich, St. Louis (MO), USA
Tween 20	AkzoNobel, Amsterdam, Netherlands
Wheat germ agglutinin coupled to biotin	Vector Laboratories, Burlingame (CA), USA
X-VIVO 15 medium	Lonza, Basel, Switzerland

5.1.5 Consumables

Material	**Company**
5 mL polystyrene round-bottom tubes	Corning, Corning (NY), USA
14 mL polystyrene round-bottom tubes	Corning, Corning (NY), USA
48-well cell culture plates	Greiner Bio-One, Kremsmünster, Austria
96-well cell culture plates, u-bottom	Thermo Fisher Scientific, Waltham (MA), USA
96-well plates for qPCR	Sarstedt, Nümbrecht, Germany
CellTrics cell strainer, 30 µm	Sysmex, Kobe, Japan
ep Dualfilter T.I.P.S (10/200/1000 µL)	Eppendorf, Hamburg, Germany
Micro tubes (0.5/1.5/2 mL)	Sarstedt, Nümbrecht, Germany
Nunc MaxiSorp ELISA plates, uncoated	BioLegend, San Diego (CA), USA
NuPAGE 10% Bis-Tris protein gels	Thermo Fisher Scientific, Waltham (MA), USA

Odyssey nitrocellulose membrane (pore size 0.22 µm)	LI-COR Biosciences, Lincoln, NE, USA
Pipette tips (10/200/1000 µL)	Sarstedt, Nümbrecht, Germany
Plate sealers	Sarstedt, Nümbrecht, Germany
Polypropylene tubes (15/50 mL)	Greiner Bio-One, Kremsmünster, Austria
Reagent reservoir	Starlab, Hamburg, Germany
Serological pipets	Corning, Corning (NY), USA
Shandon single cytofunnels with white filtercards	Thermo Fisher Scientific, Waltham (MA), USA
Shandon single cytoslides	Thermo Fisher Scientific, Waltham (MA), USA
Stericup filtration system (0.22 µm)	Merck, Darmstadt, Germany
Ultracentrifugation tubes, polypropylene (11 × 60 mm)	Beckman Coulter, Brea (CA), USA
Verex Vial	Phenomenex, Torrance (CA), USA
Via1-Cassette	ChemoMetec, Allerød, Denmark
VivaSpin 500 (10 kDa size exclusion filters)	Sartorius, Göttingen, Germany

5.1.6 Human samples

Human buffy coats were obtained from blood donations of healthy volunteers and provided by the blood bank of the UKE. Peripheral blood from healthy volunteers was drawn at the institute of immunology and handled according to the ethics protocol PV5139 (Ethikkommission der Ärztekammer Hamburg).

Synovial fluid (SF) was collected from inflamed joints of patients with juvenile idiopathic arthritis. Joint puncture was performed at the UKE, the Altona Children's Hospital or the Medical Center Bad Bramstedt. Samples were handled according to the ethics protocol PV3746 (Ethikkommission der Ärztekammer Hamburg).

5.1.7 Kits

Kit	Company
Anti-Human Foxp3 Staining Set	Thermo Fisher Scientific, Waltham (MA), USA
EasySep Human $CD8^+$ T cell Enrichment Kit	Stemcell Technologies, Vancouver, Canada
EasySep Human $CD4^+$ T cell Enrichment Kit	Stemcell Technologies, Vancouver, Canada
Human IFNγ ELISA MAX Deluxe Set	BioLegend, San Diego (CA), USA
Human TNFα ELISA MAX Deluxe Set	BioLegend, San Diego (CA), USA
QIAShredder	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany

5.1.8 Laboratory equipment

Equipment	Company
Analytical balance LA 124i	VWR, Radnor (PA), USA
Balance PC 440 DeltaRange	Mettler-Toledo, Columbus (OH), USA
Centrifuge 5424R	Eppendorf, Hamburg, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Centrifuge Allegra X-30R	Beckman Coulter, Brea (CA), USA
Centrifuge Biofuge fresco	Heraeus, Hanau, Germany
ChemiDoc Imaging System	Bio-Rad, Hercules (CA), USA
Clean bench MSC-Advantage	Thermo Fisher Scientific, Waltham (MA), USA
Clean bench ScanLaf MARS	LaboGene, Allerød, Denmark
CO_2 incubator MCO-20AIC	Sanyo (Panasonic), Kadoma, Japan
CO_2 incubator Heracell VIOS 160i	Thermo Fisher Scientific, Waltham (MA), USA
EasySep magnets (silver and purple)	Stemcell Technologies, Vancouver, Canada
FACSAria IIIU	Becton Dickinson, Franklin Lakes (NJ), USA
FACSAria Fusion	Becton Dickinson, Franklin Lakes (NJ), USA
FACSCanto II	Becton Dickinson, Franklin Lakes (NJ), USA
FACSCelesta	Becton Dickinson, Franklin Lakes (NJ), USA
Freezer -20°C	Liebherr, Bulle, Switzerland
Freezer -80°C	Thermo Fisher Scientific, Waltham (MA), USA
HPLC system 1260 Infinity	Agilent Technologies, Santa Clara (CA), USA
HPLC column C18 BDS Multohyp 250 mm × 4.6 mm (5 µm particle size)	CS Chromatographie Service, Langerwehe, Germany
HPLC column C18 security guard cartridge	Phenomenex, Torrance (CA), USA
LSM800 with airyscan	ZEISS, Oberkochen, Germany
LSRFortessa	Becton Dickinson, Franklin Lakes (NJ), USA
Microcentrifuge Galaxy MiniStar	VWR, Radnor (PA), USA
Microscope CKX41	Olympus, Shinjuku, Japan
Microscope Standard 20	ZEISS, Oberkochen, Germany
Multi-channel pipettes 10/100/300 µL	Eppendorf, Hamburg, Germany
Multi-channel adjustable spacer pipette 300 µL	Mettler-Toledo, Columbus (OH), USA
NanoSight LM14	Malvern Panalytical, Malvern, United Kingdom
Neubauer counting chamber	Marienfeld-Superior, Lauda-Königshofen, Germany
NucleoCounter NC-200	ChemoMetec, Allerød, Denmark
pH meter CG 822	Schott, Mainz, Germany
PIPETBOY acu 2	Integra Biosciences, Biebertal, Germany
Pipettes 2.5/10/20/200/1000 µL	Eppendorf, Hamburg, Germany

Pipettes 20/200 µL	Gilson, Villiers le Bel, France
Plate reader Victor^3 1240	PerkinElmer, Waltham (MA), USA
Plate shaker MTS 4	IKA, Staufen, Germany
Refrigerator/freezer	Liebherr, Bulle, Switzerland
Shandon Elliott cytospin centrifuge	Thermo Fisher Scientific, Waltham (MA), USA
StepOne Plus Real Time PCR system	Thermo Fisher Scientific, Waltham (MA), USA
Thermal cycler T3	Biometra, Göttingen, Germany
Ultracentrifuge Optima L-100 XP	Beckman Coulter, Brea (CA), USA
Vacuum pump Vacusafe	Integra Biosciences, Biebertal, Germany
Vortex mixer REAX 2000	Heidolph, Schwabach, Germany
Vortex mixer Vortex-Genie 2	Scientific Industries, Bohemia (NY), USA
Waterbath SW22	Julabo, Seelbach, Germany

5.1.9 Primer and Probes for qPCR

Gene	TaqMan assay ID	Company
NT5E	Hs00159686_m1	Thermo Fisher Scientific, Waltham (MA), USA
ENTPD1	Hs00969559_m1	Thermo Fisher Scientific, Waltham (MA), USA
RPL13A	Hs04194366_g1	Thermo Fisher Scientific, Waltham (MA), USA

5.1.10 Software

Software	Company
Adobe Illustrator CS2	Adobe System, San José (CA), USA
BD FACSDiva	Becton Dickinson, Franklin Lakes (NJ), USA
BioRender	BioRender.com
ChemStation	Agilent Technologies, Santa Clara (CA), USA
FlowJo 10.7.1	Becton Dickinson, Franklin Lakes (NJ), USA
GraphPad Prism 8	GraphPad Software, San Diego (CA), USA
Mendeley Desktop 1.19.4	RELX Group, London, United Kingdom
Microsoft Office 2016	Microsoft, Redmond (WA), USA
Nanodrop 2000/2000c	Thermo Fisher Scientific, Waltham (MA), USA
NTA software 2.3 Build 0033	Malvern Instruments, Malvern, United Kingdom
Optima L-XP Expert Software	Beckman Coulter, Brea (CA), USA
Quantity One	Bio-Rad, Hercules (CA), USA
R version 3.6.1	The R Foundation for Statistical Analysis
RStudio version 1.2.5019	RStudio, Inc. Boston (MA), USA
StepOne Software 2.3	Thermo Fisher Scientific, Waltham (MA), USA
Wallac 1420 Manager 3.0	PerkinElmer, Waltham (MA), USA
ZENblue	ZEISS, Oberkochen, Germany

5.2 Methods

5.2.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by density gradient centrifugation. Buffy coats were diluted two- to three-fold with PBS, layered on Biocoll cell separating solution and centrifuged at 800 × g for 25 min at RT with slow acceleration and slow brake. The PBMC layer was collected and washed with cold PBS by centrifugation at 650 × g for 10 min, 4°C and at 450 × g for 5 min, 4°C. Remaining erythrocytes were lysed by incubation with ddH_2O for 20 sec and reaction was stopped by washing with PBS (450 × g, 5 min, 4°C). Cell number was determined either with a Neubauer counting chamber or an automated cell counter. If not used immediately, cells were kept over night in full RPMI at 4°C.

5.2.2 Labeling with eFluor 670

To assess proliferation, mononuclear cells were labeled with eFluor 670 proliferation dye. The fluorescent dye binds to cellular proteins and is distributed equally to the daughter cells at each cell division resulting in halved dye intensity. PBMCs or isolated T cells were labeled with 2 µM eFluor 670 according to the manufacturer's protocol. In brief, cells were resuspended in diluted eFluor 670 solution and incubated for 10 min at 37°C. Labeling reaction was stopped by addition of 4-5 volumes of full RPMI and incubation on ice for 5 min. Cells were centrifuged (450 × g, 5 min, 4°C) and washed with full RPMI (450 × g, 5 min, 4°C).

5.2.3 Enrichment of T cells by magnetic separation

CD4 and CD8 T cells were enriched by magnetic bead-based isolation using Stemcell negative selection kits according to the manufacturer's protocol. In brief, PBMCs were resuspended in MACS buffer to adjust a cell concentration of 5×10^7 cells/mL. Cells were incubated with enrichment cocktail (50 µL/mL of sample) for 10 min at RT, followed by incubation with magnetic particles (100 or 150 µL/mL of sample) for 5 min at RT. The required amount of MACS buffer was added and after further 5 min incubation in the magnet, the enriched cells were poured off into a new tube.

5.2.4 Flow cytometry and fluorescence-activated cell sorting

Flow cytometry allows characterization of cells and, with limitations, smaller particles regarding size, granularity and expression of specific markers. Cell surface and intracellular molecules are stained by fluorochrome-conjugated antibodies and based on marker expression, distinct populations of cells can be separated (fluorescence-activated cell sorting, FACS).

Staining of cell surface markers

To determine cell surface marker expression, 0.1 – 1 × 10^6 cells were resuspended in 100 µL PBS, pre-treated with human IgG to block unspecific binding and incubated with titrated, fluorochrome-conjugated antibodies for 30 min at 4°C in the dark. For dead cell exclusion, an Alexa Fluor 750-conjugated NHS ester was added 10 min after starting the surface marker staining and thus incubated for 20 min. This dye binds to primary amines, resulting in an intensive staining of cells without intact cell membrane. Cells were then washed with PBS (450 × g, 5 min) and resuspended in FACS buffer. Measurements were performed at FACSCanto II, FACSCelesta or LSRFortessa. Figure 34 shows an exemplary gating strategy of a multicolor panel to determine the frequency of $CD73^+$ and $CD39^+$ cells in different leukocytes subsets (see Figure 5).

Intracellular staining

For intracellular staining, cells were fixed and permeabilized after cell surface marker staining. Instead of FACS buffer, cells were resuspended in 1 mL freshly prepared Fix/Perm buffer (FoxP3 staining kit, Thermo Fisher Scientific) after washing and incubated at 4°C for 45 - 60 min. After two washing steps with 1 mL permeabilization buffer (450 × g, 5 min), cells were incubated with antibodies against intracellular molecules for 30 min at 4°C in the dark. Cells were washed with permeabilization buffer (450 × g, 5 min) and resuspended in FACS buffer for measuring.

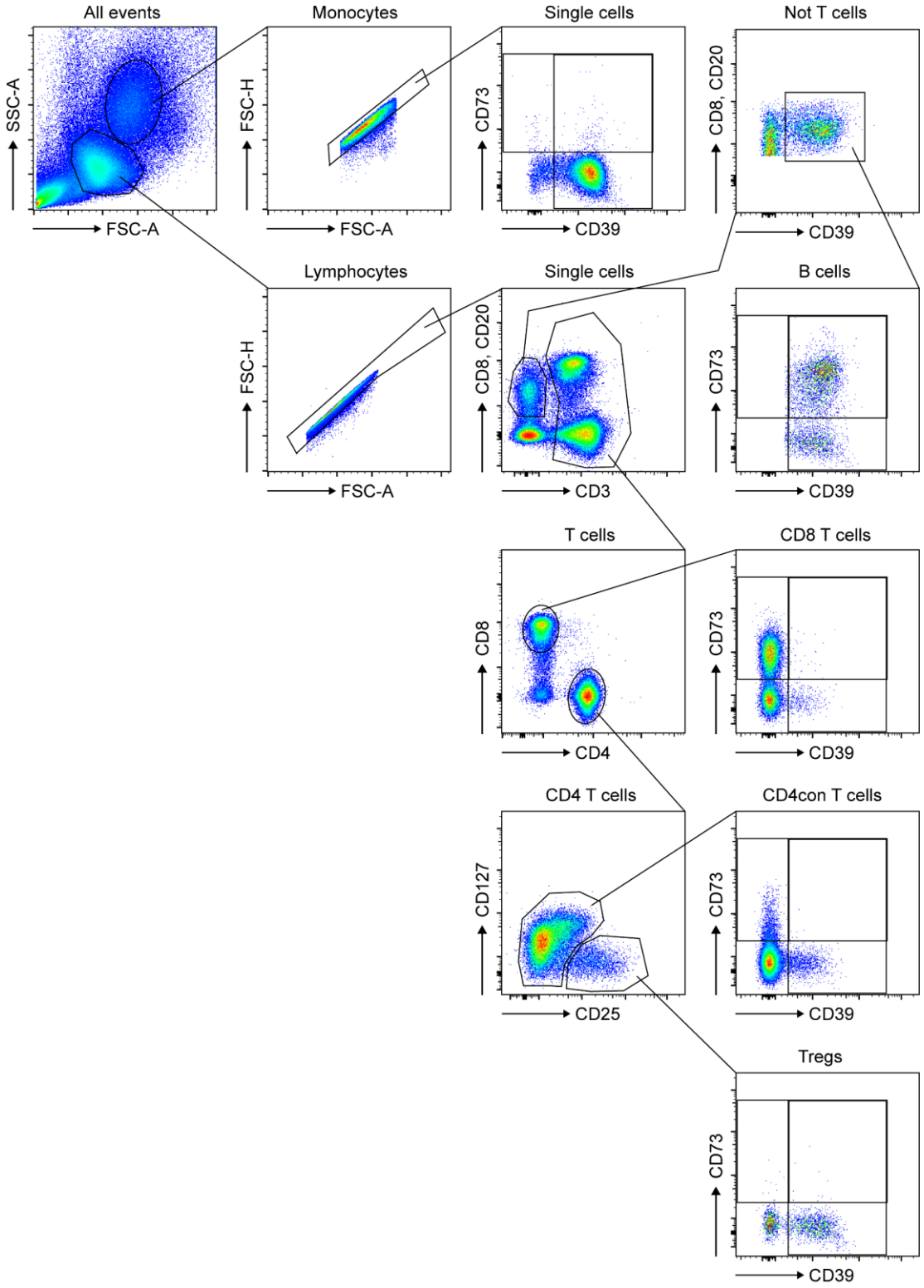

Figure 34 | Gating strategy to determine CD73 and CD39 expression in leukocyte subsets. Lymphocytes and monocytes were gated by forward scatter (FSC) vs. side scatter (SSC) and FSC was used to define single cells. B cells ($CD20^+$ $CD39^+$) and T cells ($CD3^+$) were gated and T cells were further subdivided into CD8 and CD4 T cells. CD4 T cells were split into regulatory T cells (Tregs, $CD25^{high}$ and $CD127^{low}$) and non-regulatory, conventional CD4 T cells (CD4con). CD73 and CD39 expression were then determined in monocytes, B cells, CD8 T cells, CD4con T cells and Tregs.

Sorting of human lymphocytes by fluorescence-activated cell sorting (FACS)

PBMCs or enriched CD4 or CD8 T cells were stained with antibodies against cell surface markers as described above (10×10^6 cells/mL PBS). After washing, the cell suspension was filtered over a 30 µm cell strainer. Sorting into FCS-coated collection tubes was performed at FACSAria IIIU or FACSAria Fusion in the Cytometry and Sorting Core Unit at the UKE. After sorting, cells were centrifuged at 450 × g for 10 min and resuspended in full RPMI or serum-free X-VIVO 15 medium. Table 1 gives an overview of the T cell populations sorted for *in vitro* T cell assays. Figure 35 shows how the gates were set to sort these T cell populations of interest.

Table 1 | Overview of human T cell populations sorted for *in vitro* T cell assays.

Population (phenotype)	**Starting material**	**Experimental setup**
$CD73^-$ CD4con ($CD4^+$ $CD73^-$)	PBMCs or enriched CD4 T cells	Activation and proliferation assay; Suppression assay
$CD73^+$ CD4con ($CD4^+$ $CD73^+$)	PBMCs or enriched CD4 T cells	Activation and proliferation assay
Tregs ($CD4^+$ $CD25^{hi}$ $CD127^{lo}$)	PBMCs or enriched CD4 T cells	Suppression assay
$CD73^-$ CD8 ($CD8^+$ $CD73^-$)	PBMCs or enriched CD8 T cells	Activation and proliferation assay; Production of EVs
$CD73^+$ CD8 ($CD8^+$ $CD73^+$)	PBMCs or enriched CD8 T cells	Activation and proliferation assay; Production of EVs
Naïve CD8 ($CD8^+$ $CD45RA^+$ $CD27^+$)	PBMCs	Activation and proliferation assay
Memory CD8 ($CD8^+$ $CD45RA^-$)	PBMCs	Activation and proliferation assay

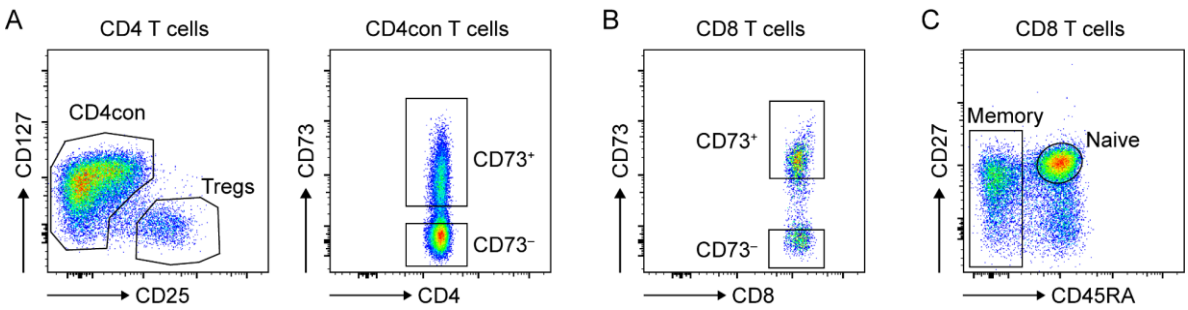

Figure 35 | Gating strategy for the sorting of T cell populations used in *in vitro* T cell assays. Lymphocytes and single cells were defined using FSC and SSC. Populations of interest (listed in Table 1) were then gated in CD4 (A) and CD8 (B+C) T cells. Tight gating ensured that only T cells with the desired phenotype were sorted.

Staining of extracellular vesicles

For flow cytometric analysis of extracellular vesicles (EVs), we established a cooperation with Dr. Jochen Behrends from the Research Center in Borstel. EVs isolated from cell culture supernatants of $CD73^+$ or $CD73^-$ CD8 T cells (see chapter 5.2.12) were diluted with PBS (1:100) and stained for 15 min with antibodies against CD9 and CD73 (1:150). Without washing, samples were measured at a FACSAria IIIU at flow rate 1 for 4 min. PBS containing antibodies but no vesicles was used as control.

5.2.5 Visualization of high-dimensional flow cytometry data using Uniform Manifold Approximation and Projection (UMAP)

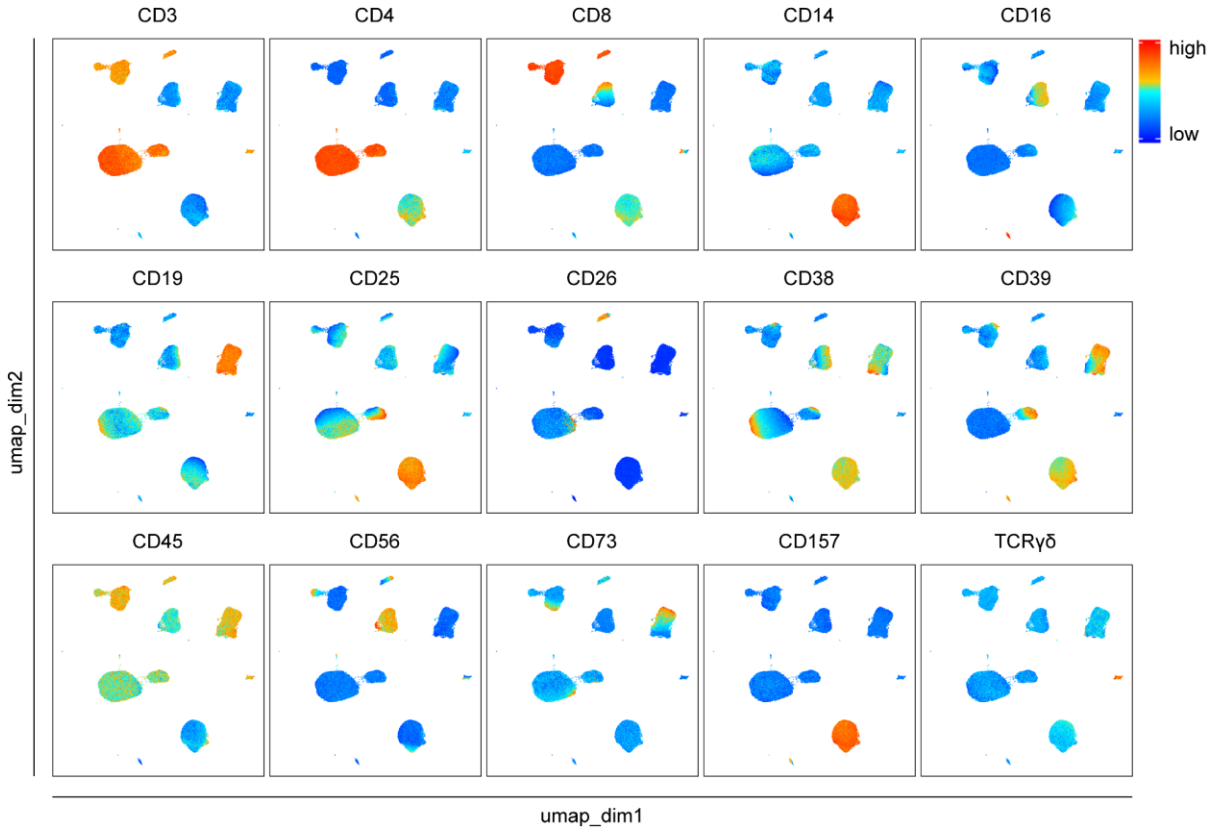

Figure 36 | UMAP analysis of human leukocyte subsets. Human peripheral blood was stained with antibodies to analyze the expression of molecules involved in purinergic signaling in different immune cell subsets (data acquired by Anne Rissiek). For analysis, $CD45^+$ cells without granulocytes were exported from FlowJo and all markers shown above were used in the UMAP algorithm to cluster immune cells (Laura Glau). Based on lineage markers, the following subsets were identified: CD4 T cells ($CD3^+$ $CD4^+$), Tregs ($CD3^+$ $CD4^+$ $CD25^+$), CD8 T cells ($CD3^+$ $CD8^+$), mucosal-associated invariant T cells (MAIT cells, $CD3^+$ $CD8^+$ $CD26^+$), Tγδ cells ($CD3^+$ $TCR\gamma\delta^+$), monocytes ($CD14^+$ $CD157^+$ and some cells were also $CD16^+$), B cells ($CD19^+$) and NK cells ($CD56^+$ $CD16^+$). The signal for CD25 expression on monocytes is a staining artifact.

Compensated FCS files were exported from FlowJo and analyzed in R (R Core Team, 2019) by Laura Glau. R Package flowCore (Ellis *et al.*, 2019) was used for reading and handling of the FCS files. To represent the multidimensional data in a two-dimensional plot, a dimensionality reduction was performed. The 2D UMAP representation of the compensated and arcsinh-scaled data (with cofactor 450) was calculated using the R package umap (Konopka, 2019) with default parameters. The graphical representation was created using the R package ggplot2 (Wickham, 2016). Figure 36 shows an exemplary UMAP analysis of human leukocyte subsets.

5.2.6 *In vitro* T cell assays

Unless otherwise specified, PBMCs or isolated T cells were cultured in 96-well u-bottom plates at 37°C and 5% CO_2. To assess proliferation of activated T cells, PBMCs were labeled with eFluor 670. Full RPMI was used as standard culture medium. For the generation of EVs, in suppression assays, and in T cell assays to investigate the role of adenine nucleotides and non-cell-bound CD73 on T cell activation, T cells were cultured in serum-free X-VIVO 15 medium.

Activation and proliferation assays with human PBMCs

Human PBMCs were seeded at a density of 1×10^6 cells/mL in full RPMI and stimulated with 0.5 µg/mL soluble αCD3. Analysis of cell surface marker expression and proliferation by flow cytometry was performed every 24 – 48 hours. In long-term cultures, half of the culture medium was replaced by fresh medium containing recombinant IL-2 at a final concentration of 100 U/mL from day four onwards every four days. At days seven and fifteen, half of the cells were transferred to a new plate to prevent overgrowing of the culture.

Co-Culture of human PBMCs with transfected HEK293 cells

In a 48-well plate, 0.05×10^6 HEK293 cells were seeded in full RPMI. Once HEK293 cells have attached to the plate, 0.2×10^6 PBMCs were seeded on top, stimulated with 0.5 µg/mL soluble αCD3 and cultured for up to three days.

Activation and proliferation assays with enriched CD4 and CD8 T cells

Enriched CD4 or CD8 T cells were seeded at a density of 1×10^6 cells/mL in full RPMI and cultured for up to one week. Two hours prior to seeding, plates were coated with 100 µL of a 1 µg/mL αCD3 solution and washed twice with PBS immediately before cell seeding. Together with the T cells, soluble αCD28 was added at a final concentration of 5 µg/mL. Activation marker expression and proliferation were assessed by flow cytometry every 24 - 48 hours.

Activation and proliferation assays with sorted CD4 and CD8 T cells

For some assays, CD4 or CD8 T cells were sorted with regard to their CD73 expression or their differentiation status (see Table 1). In all assays, T cells were stimulated with αCD3 (coated) and αCD28 (soluble) as described above for the activation of CD4 and CD8 T cells enriched by negative selection.

In standard activation or proliferation assays, $0.1 – 0.2 \times 10^6$ T cells were seeded in full RPMI and cultured for up to nine days. To correlate CD73 and CD39 surface expression with gene expression, 0.4×10^6 T cells were cultured in the presence of soluble αCD28 (5 µg/mL) in 48-well plates coated with 200 µL αCD3 solution (1 µg/mL). At the day of harvest, one fourth of the T cells was stained for flow cytometric analysis and residual cells were used for RNA isolation.

To assess the influence of adenine nucleotides and non-cell-bound CD73 on T cell activation and proliferation, 0.1×10^6 $CD73^-$ CD4con T cells were stimulated for three to four days in X-VIVO 15 medium in the presence of adenosine deaminase (ADA) inhibitor EHNA (10 µM). The same amount of $CD73^+$ CD4con T cells was used as control population. When indicated, AMP (50 µM) or ATP (50 µM), recombinant CD73 (15 ng/mL, unless otherwise stated), the specific CD73 inhibitor 2-chloro-*N*^{6}-*o*-chlorobenzyl-α,β-methylene-ADP (PSB-14685, 10 µM) or EVs (equivalent to 150 µL of cell culture supernatant from activated CD8 T cells, unless otherwise stated) were added to the cells. Expression of activation marker CD25 and proliferation were measured by flow cytometry, T cell culture supernatants were analyzed for IFNγ production by ELISA.

To generate T cell culture supernatants for EV isolation, $CD73^+$ or $CD73^-$ CD8 T cells were cultured at a density of 1×10^6 cells/mL in X-VIVO 15 medium in 48-well plates. After four to five days, supernatants of wells belonging to the same condition were pooled and subjected to differential centrifugation to isolate EVs.

T cell suppression assays

In T cell suppression assays, 0.1×10^6 eFluor 670-labeled $CD73^-$ CD4con T cells (responder T cells) were stimulated with αCD3 (coated) and αCD28 (soluble) as described above and co-cultured with different ratios of unlabeled Tregs in the presence of EHNA (10 µM). When responder T cells were co-cultured with autologous Tregs, eFluor 670-labeling of responder T cells was performed, unlike the usual procedure, after sorting of responder T cells. AMP (50 µM) or ATP (50 µM), recombinant CD73 (15 ng/mL), the specific CD73 inhibitor PSB-14685 (10 µM) or EVs (equivalent to 150 µL of cell culture supernatant of activated CD8 T cells, unless otherwise stated) were added to the culture, when indicated. After three to four days, T cells and their cell culture supernatants were harvested to assess expression of activation marker CD25 and proliferation by flow cytometry and IFNγ production by ELISA.

5.2.7 Phospholipase C treatment

To shed CD73 artificially from the plasma membrane, enriched CD8 T cells (2×10^6 cells/mL) were incubated with 0.5 U/mL bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) for 60 min at 37°C. Cells were washed with PBS (450 × g, 5 min) and activated as described above.

5.2.8 Determination of cytokine concentrations in T cell culture supernatants

IFNγ and TNFα concentrations in T cell culture supernatants were detected using the Human ELISA MAX Deluxe Sets according to the manufacturer's protocol. In brief, 96-well plates were coated with capture antibody and incubated over night at 4°C. All following incubation steps were performed at RT on an orbital shaker. After each incubation step, plates were washed with PBS-Tween. First, plates were blocked for 60 min with assay diluent and then incubated with samples (1:10 diluted in assay diluent) and standards for 120 min. For detection, plates were incubated with detection antibody (60 min) and streptavidin-HRP (30 min). Incubation with substrate solution was performed in the dark for 20 min and reaction was stopped with H_2SO_4. Signal intensity was measured in a plate reader at 450 nm.

5.2.9 RNA isolation from human lymphocytes

RNA from human lymphocytes was isolated with RNeasy Mini Kit according to the manufacturer's protocol. Cells were centrifuged at 9400 × g and 4°C for 5 min, the pellet was resuspended in 350 µL RLT buffer containing 1% β-mercaptoethanol, frozen in liquid nitrogen and stored at -80°C until the day of RNA isolation. After thawing, the lysate was given over a QIAshredder column and spun at full speed for 2 min. The collected flow-through was mixed with 350 µL 70% ethanol and loaded onto a RNeasy spin column. All following centrifugation steps were carried out at 10,000 × g and RT for 15 sec, if not stated otherwise. After centrifugation, the flow-through was discarded and the column was washed with 350 µL buffer RW1. Next, the column was incubated with DNaseI for 15 min and washed again with 350 µL buffer RW1. The column was washed twice with 500 µL buffer RPE, the second washing step was carried out for 2 min. To remove residual washing buffer, the empty column was washed at full speed for 1 min. RNA was eluted with 30 µL RNase-free water and centrifugation for 1 min at 10,000 × g. The eluate was used for a second round of elution to increase RNA yield. RNA concentration was determined by Nanodrop.

5.2.10 cDNA synthesis

Isolated mRNA was transcribed to cDNA in a two-step process. RNA was diluted to 10 ng/µL and 100 ng of RNA were mixed with random hexamer primers and dNTPs and incubated for 5 min at 65°C. First Strand buffer, DTT, M-MLV reverse transcriptase and ddH_2O were added to the first reaction mix and the mixture was incubated for 50 min at 37°C and for 15 min at 70°C. The cDNA concentration was determined by Nanodrop.

5.2.11 Real-time polymerase chain reaction

Gene expression of *NT5E* and *ENTPD1* was quantified by real-time PCR. *RPL13A* was used as endogenous control (EC). For TaqMan assays, cDNA (1:10 dilution) was mixed with Maxima Probe/ROX qPCR Master Mix and the TaqMan probe and filled up with water to a final volume of 20 µL. Plates were sealed and measured in a StepOne Plus real-time PCR system with protocol for standard run. According to the protocol, samples were kept at 50°C for 2 min, denatured at 95°C for 10 min, and amplified in 40 cycles (denaturation 95°C, 15 sec; signal amplification 60°C, 1 min). The relative gene expression was determined with the $\Delta\Delta C_T$

method. First, ΔC_T values were calculated by subtracting the C_T value of the gene of interest (GOI) from the C_T value of the EC ($\Delta C_T = C_T[EC] - C_T[GOI]$). To obtain the $\Delta\Delta C_T$ values, samples were calibrated to day zero samples of $CD73^+$ (*NT5E*) or $CD73^-$ (*ENTPD1*) CD8 T cells.

5.2.12 Isolation of EVs

EVs were isolated from cell culture supernatants of $CD73^+$ or $CD73^-$ CD8 T cells or synovial fluid (SF) samples by differential centrifugation. All centrifugation steps were performed at 4°C. After four to five days of CD8 T cell activation, T cell culture supernatants were collected and centrifuged at 450 × g for 5 min to remove residual cells. SF samples were centrifuged at 300 × g for 5 min and 450 × g for 5 min to gain a cell-free sample. The T cell culture supernatant or the SF sample was centrifuged at 2,000 × g for 10 min to eliminate dead cells and cell debris and at 10,000 × g for 30 min to pellet larger EVs like apoptotic bodies and microvesicles. To isolate small EVs, the supernatant of the 10,000 × g centrifugation was subjected to ultracentrifugation (110,000 × g, 70 min) using a SW 60 Ti swinging-bucket rotor. The pellet was washed with filtered PBS (110,000 × g, 70 min) and EVs were resuspended in filtered PBS.

5.2.13 Determination of EV size and concentration

EVs were diluted 1:300 - 1:1000 in filtered PBS and measured with a NanoSight LM14 instrument equipped with a 638 nm laser and a Marlin F-033B IRF camera. Roughly half of the 600 µl sample was injected into a chamber that is crossed by a laser beam. Light scattering allowed visualization of particles in the sample moving due to Brownian motion. To determine particle size and concentration, 3 - 10 videos of > 1000 valid particle tracks were recorded. Between the measurements, a small volume of the remaining sample was injected to gain new particles in the area of recording. Based on the tracked particles, the software calculates the size and concentration.

5.2.14 Detection of CD73 in EVs by Western Blot

EVs were lysed in RIPA buffer containing protease and phosphatase inhibitors, mixed with loading dye and incubated for 10 min at 70°C. Samples were loaded onto a 10% Bis-Tris gel in MES running buffer and electrophoresis was performed for 80 min at 150 V. Sponges, filter

papers, the nitrocellulose membrane and the gel were soaked with transfer buffer, built to a blot and proteins were transferred at 500 mA for 70 min. After transfer, the membrane was blocked for 60 min with Roti-Block and incubated with the primary antibody (anti-CD73 or flotillin-1; 1:1000 in Roti-Block) over night at 4°C. The membrane was washed six times with TBS-Tween, incubated with anti-rabbit (CD73) or anti-mouse (flotillin-1) IgG secondary antibody (1:1000 in Roti-Block) for 60 min at RT, washed again six times and incubated for 3 min with SuperSignal West chemiluminescent substrate solution. Signals were detected with a ChemiDoc Imaging System using Quantity One software. CD73 and flotillin-1 were detected on the same membrane in a stepwise process. After staining and detection of CD73, the membrane was washed and staining and detection of flotillin-1 was performed.

5.2.15 Fluorescence microscopy

Enriched CD8 T cells were seeded in a density of 2×10^6 cells/mL in full RPMI in 48-well plates that were pre-coated with 200 µL of αCD3 solution (1 µg/mL) two hours prior to seeding. Soluble αCD28 was added at a final concentration of 5 µg/mL and CD8 T cells were incubated at 37°C and 5% CO_2 for up to four days. At the day of harvest, CD8 T cells were resuspended in PBS at a concentration of 1×10^6 cells/mL and 200 µL cell suspension were spun (1000 rpm, 3 min) on cytoslides pre-coated with poly-L-lysine. CD8 T cells were fixed with 100 µL 4% PFA for 10 min, washed thrice with cold PBS and stored covered with PBS at 4°C until they were stained for fluorescence microscopy. Staining, fluorescence microscopy and image analysis were performed by Prof. Dr. Catherine Meyer-Schwesinger (Department of Cellular and Integrative Physiology, UKE). Samples were blocked with 5% normal horse serum diluted in PBS with 0.05% Trition X-100 and incubated over night at 4°C with unconjugated αCD73 antibody in blocking buffer. For detection of CD73, samples were stained for 30 min at RT with a Cy2-conjugated antibody directed against mouse IgG. Depending on the experiment, cytoslides were further incubated with Hoechst to stain the DNA and biotin-coupled wheat germ agglutinin and streptavidin-AF647 to demarcate the glycocalix (30 min, RT) or with APC-conjugated αCD9 antibody (60 min at RT). Samples were analyzed with a LSM800 with airyscan and ZENblue software. Co-localization of CD73 and CD9 was determined by calculating the Pearson's coefficient from seven or eight randomly chosen high power fields (630X) per condition (three individual cells/high power field).

5.2.16 Analysis of ATPase and AMPase by high performance liquid chromatography

To assess ATPase and AMPase activity of CD8 T cell culture supernatants or EVs, degradation of 1,N^6-etheno-ATP (eATP) or 1,N^6-etheno-AMP (eAMP) was measured by ion pair reversed-phase high performance liquid chromatography (rp-HPLC). This method allowed the separation and quantification of sample components based on their interaction with the hydrophobic stationary phase, a C18 column. Sample components were eluted from the column by increasing the methanol content in the mobile phase. The mobile phase contained the ion pairing reagent tetrabutylammonium dihydrogen phosphate (TBAHP) which interacts with negatively charged nucleotides, enhances their retention on the stationary phase and thus improvs their separation by rp-HPLC.

The basic concept of the following protocol was established by Riekje Winzer during her master thesis (Winzer, 2017). For analysis of ATPase and AMPase activity, 150 µL CD8 T cell culture supernatant or the corresponding amount of EVs in 150 µL PBS were incubated for 60 min at 37°C with 50 µL eATP or eAMP at a final concentration of 1 µM. To block CD73-specific AMPase activity, samples were incubated with 10 µM PSB-14685 for 15 min at 37°C before the addition of eATP or eAMP. After the incubation with the etheno-nucleotides, samples were immediately frozen and stored at -20°C until the day of analysis by HPLC. To measure ATPase and AMPase activity, samples were thawed, passed through a 10 kDa size exclusion filter, and 102 µL of the flow-through were mixed with 18 µL methanol. A volume of 100 µL of this mixture was injected in the HPLC system (flow rate 0.8 mL/min), corresponding to 85 pmol of eATP or eAMP under the assumption that no conversion had occured. The mobile phase was composed of HPLC buffer A and B and the following gradient was applied during the run at the HPLC system: 0.0 min (30.0% buffer B), 3.5 min (30% buffer B), 11.0 min (62.5% buffer B), 15 min (62.5% buffer B), 25 min (100% buffer B), 27 min (100% buffer B), 29.0 min (30.0% buffer B), and 38 min (30.0% buffer B). The column compartment had a temperature of 20°C and the auto sampler was kept at 8°C. The etheno-derivatives of ATP and AMP and their degradation products were detected by fluorescence (excitation at 230 nm, emission at 410 nm), allowing the precise and sensitive measurement of nucleotide conversion in the samples. Different concentrations of commercially available standards for etheno-nucleotides and etheno-nucleosides were analyzed under the same conditions and used for identification and quantification of eATP, eADP, eAMP and eADO in the sample. For

quantification, peak integration was performed with ChemStation software and the area under the peak was correlated with the concentration of the standard.

5.2.17 Statistical analysis

All statistical analyses were performed in GraphPad Prism 8. When data passed the normality test, they were analyzed by Student's *t*-test (two groups) or repeated measures (RM) one-way ANOVA (more than two groups of paired data). Kruskal-Wallis test (unpaired data) and Friedman test (paired data) were used to compare multiple groups of not normally distributed data. 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. Tukey's multiple comparisons test was chosen when the means of all groups of normally distributed data were compared. Dunn's multiple comparisons test was performed to compare the mean ranks of not normally distributed data. Statistical significance was indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$. Non-significant differences were not annotated.

6. References

Baecher-Allan, C., Viglietta, V. and Hafler, D. A. (2002) 'Inhibition of Human CD4+ CD25+high Regulatory T Cell Function', *The Journal of Immunology*, 169(11), pp. 6210–6217. doi: 10.4049/jimmunol.169.11.6210.

Baroja-Mazo, A. *et al.* (2019) 'Extracellular adenosine reversibly inhibits the activation of human regulatory T cells and negatively influences the achievement of the operational tolerance in liver transplantation', *American Journal of Transplantation*, 19(1), pp. 48–61. doi: 10.1111/ajt.15023.

Bhattarai, S. *et al.* (2019) 'X-Ray Co-Crystal Structure Guides the Way to Subnanomolar Competitive Ecto-5'-Nucleotidase (CD73) Inhibitors for Cancer Immunotherapy', *Advanced Therapeutics*, 2(10), p. 1900075. doi: 10.1002/adtp.201900075.

Blanchard, N. *et al.* (2002) 'TCR Activation of Human T Cells Induces the Production of Exosomes Bearing the TCR/CD3/ζ Complex', *The Journal of Immunology*, 168(7), pp. 3235–3241. doi: 10.4049/jimmunol.168.7.3235.

Bopp, T. *et al.* (2007) 'Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression', *Journal of Experimental Medicine*, 204(6), pp. 1303–1310. doi: 10.1084/jem.20062129.

Botta Gordon-Smith, S. *et al.* (2015) 'Correlation of low CD73 expression on synovial lymphocytes with reduced adenosine generation and higher disease severity in juvenile idiopathic arthritis', *Arthritis and Rheumatology*, 67(2), pp. 545–554. doi: 10.1002/art.38959.

Bours, M. J. L. *et al.* (2006) 'Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation', *Pharmacology and Therapeutics*, 112(2), pp. 358–404. doi: 10.1016/j.pharmthera.2005.04.013.

Burger, R. M. and Lowenstein, J. M. (1975) '5'-Nucleotidase from Smooth Muscle of Small Intestine and from Brain. Inhibition by Nucleotides', *Biochemistry*, 14(11), pp. 2362–2366. doi: 10.1021/bi00682a014.

Burnstock, G. (1971) 'Neural Nomenclature', *Nature*, 229(5282), pp. 282–283. doi: 10.1038/229282d0.

Burnstock, G. (2006) 'Purinergic signalling', *British Journal of Pharmacology*, 147(S1), pp. S172–S181. doi: 10.1038/sj.bjp.0706429.

Burnstock, G. (2007) 'Purine and pyrimidine receptors', *Cellular and Molecular Life Sciences*, 64(12), pp. 1471–1483. doi: 10.1007/s00018-007-6497-0.

Burnstock, G. and Kennedy, C. (1985) 'Is there a basis for distinguishing two types of P2-purinoceptor?', *Gen. Pharmacol.*, 16(5), pp. 433–440. doi: 10.1016/0306-3623(85)90001-1.

Calvo, V. and Izquierdo, M. (2020) 'Inducible polarized secretion of exosomes in T and B lymphocytes', *International Journal of Molecular Sciences*, 21(7), p. 2631. doi: 10.3390/ijms21072631.

Cao, X. *et al.* (2007) 'Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance', *Immunity*, 27(4), pp. 635–646. doi: 10.1016/j.immuni.2007.08.014.

Cekic, C. *et al.* (2013) 'Extracellular adenosine regulates naive T cell development and peripheral maintenance', *The Journal of Experimental Medicine*, 210(12), pp. 2693–2706. doi: 10.1084/jem.20130249.

Cekic, C. and Linden, J. (2016) 'Purinergic regulation of the immune system', *Nature Reviews Immunology*, 16(3), pp. 177–192. doi: 10.1038/nri.2016.4.

Chen, Y. *et al.* (2006) 'ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors', *Science*, 314(5806), pp. 1792–1795. doi: 10.1126/science.1132559.

Chen, Y. *et al.* (2010) 'Purinergic signaling: A fundamental mechanism in neutrophil activation', *Science Signaling*, 3(125), p. ra45. doi: 10.1126/scisignal.2000549.

Choi, G. *et al.* (2020) 'Autocrine TGF-β1 Maintains the Stability of Foxp3+ Regulatory T Cells via IL-12Rβ2

Downregulation', *Biomolecules*, 10(6), p. 819. doi: 10.3390/biom10060819.

Clayton, A. *et al.* (2011) 'Cancer Exosomes Express CD39 and CD73, Which Suppress T Cells through Adenosine Production', *The Journal of Immunology*, 187(2), pp. 676–683. doi: 10.4049/jimmunol.1003884.

Coade, S. B. and Pearson, J. D. (1989) 'Metabolism of Adenine Nucleotides in Human Blood', *Circulation Research*, 65(3), pp. 531–537. doi: 10.1161/01.res.65.3.531.

Collison, L. W. *et al.* (2007) 'The inhibitory cytokine IL-35 contributes to regulatory T-cell function', *Nature*, 450(7169), pp. 566–569. doi: 10.1038/nature06306.

Cunha, R. A. (2001) 'Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals', *Neurochemical Research*, 26(8–9), pp. 979–991. doi: 10.1023/A:1012392719601.

Dale, N. and Frenguelli, B. (2009) 'Release of Adenosine and ATP During Ischemia and Epilepsy', *Current Neuropharmacology*, 7(3), pp. 160–179. doi: 10.2174/157015909789152146.

Danquah, W. *et al.* (2016) 'Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation', *Science Translational Medicine*, 8(366), p. 366ra162. doi: 10.1126/scitranslmed.aaf8463.

Davitz, M. A. *et al.* (1987) 'A glycan-phosphatidylinositol-specific phospholipase D in human serum', *Science*, 238(4823), pp. 81–84. doi: 10.1126/science.2443973.

Deaglio, S. *et al.* (2007) 'Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression', *The Journal of Experimental Medicine*, 204(6), pp. 1257–1265. doi: 10.1084/jem.20062512.

Deterre, P. *et al.* (1996) 'Coordinated regulation in human T cells of nucleotide-hydrolyzing ecto-enzymatic activities, including CD38 and PC-1. Possible role in the recycling of nicotinamide adenine dinucleotide metabolites', *Journal of Immunology*, 157(4), pp. 1381–1388.

Dianzani, U. *et al.* (1993) 'Co-stimulatory signal delivered by CD73 molecule to human CD45RAhiCD45ROlo (naive) CD8+ T lymphocytes', *Journal of immunology*, 151(8), pp. 3961–3970.

Dieckmann, D. *et al.* (2001) 'Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood', *Journal of Experimental Medicine*, 193(11), pp. 1303–1310. doi: 10.1084/jem.193.11.1303.

Doherty, G. A. *et al.* (2012) 'CD73 is a phenotypic marker of effector memory Th17 cells in inflammatory bowel disease', *European Journal of Immunology*, 42(11), pp. 3062–3072. doi: 10.1002/eji.201242623.

Dong, R.-P. *et al.* (1996) 'Characterization of adenosine deaminase binding to human CD26 on T cells and its biologic role in immune response', *Journal of Immunology*, 156(4), pp. 1349–1355.

Dornand, J., Bonnafous, J. -C and Mani, J. -C (1978) 'Purification and Properties of 5'-Nucleotidase from Lymphocyte Plasma Membranes', *European Journal of Biochemistry*, 87(3), pp. 459–465. doi: 10.1111/j.1432-1033.1978.tb12396.x.

Duhant, X. *et al.* (2002) 'Extracellular Adenine Nucleotides Inhibit the Activation of Human CD4+ T Lymphocytes', *The Journal of Immunology*, 169(1), pp. 15–21. doi: 10.4049/jimmunol.169.1.15.

Ellis, B. *et al.* (2019) 'flowCore: Basic structures for flow cytometry data.' R package version 1.52.0.

Eltzschig, H. K. *et al.* (2006) 'ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function', *Circulation Research*, 99(10), pp. 1100–1108. doi: 10.1161/01.RES.0000250174.31269.70.

Eltzschig, H. K. *et al.* (2009) 'Central role of Sp1-regulated CD39 in hypoxia/ischemia protection', *Blood*, 113(1), pp. 224–232. doi: 10.1182/blood-2008-06-165746.

Fan, W. *et al.* (2017) 'Identification of CD4+ T-cell-derived CD161+ CD39+ and CD39+ CD73+ microparticles as new biomarkers for rheumatoid arthritis', *Biomarkers in Medicine*, 11(2), pp. 107–116. doi: 10.2217/bmm-2016-0261.

Ferrero, E., Faini, A. C. and Malavasi, F. (2019) 'A phylogenetic view of the leukocyte ectonucleotidases', *Immunology Letters*, 205, pp. 51–58. doi: 10.1016/j.imlet.2018.06.008.

Fitz, J. G. (2007) 'Regulation of cellular ATP release.', *Transactions of the American Clinical and Climatological Association*, 118, pp. 199–208.

Flögel, U. *et al.* (2012) 'Selective activation of adenosine A2A receptors on immune cells by a CD73-dependent prodrug suppresses joint inflammation in experimental rheumatoid arthritis', *Science Translational Medicine*, 4(146), p. 146ra108. doi: 10.1126/scitranslmed.3003717.

Fredholm, B. B. *et al.* (2001) 'Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells', *Biochemical Pharmacology*, 61(4), pp. 443–448. doi: 10.1016/s0006-2952(00)00570-0.

Fredholm, B. B. (2014) 'Adenosine - A physiological or pathophysiological agent?', *Journal of Molecular Medicine*, 92(3), pp. 201–206. doi: 10.1007/s00109-013-1101-6.

de Gassart, A. *et al.* (2003) 'Lipid raft-associated protein sorting in exosomes', *Blood*, 102(13), pp. 4336–4344. doi: 10.1182/blood-2003-03-0871.

Di Gennaro, P. *et al.* (2018) 'T regulatory cells mediate immunosuppresion by adenosine in peripheral blood, sentinel lymph node and TILs from melanoma patients', *Cancer Letters*, 417, pp. 124–130. doi: 10.1016/j.canlet.2017.12.032.

Gessi, S. *et al.* (2004) 'Expression of A3 Adenosine Receptors in Human Lymphocytes: Up-Regulation in T Cell Activation', *Molecular Pharmacology*, 65(3), pp. 711–719. doi: 10.1124/mol.65.3.711.

Gorman, M. W., Feigl, E. O. and Buffington, C. W. (2007) 'Human plasma ATP concentration', *Clinical Chemistry*, 53(2), pp. 318–325. doi: 10.1373/clinchem.2006.076364.

Grossman, W. J. *et al.* (2004) 'Human T regulatory cells can use the perforin pathway to cause autologous target cell death', *Immunity*, 21(4), pp. 589–601. doi: 10.1016/j.immuni.2004.09.002.

Gutensohn, W. *et al.* (1995) 'Ecto-5'-nucleotidase Activity Is Not Required for T Cell Activation through CD73', *Cellular Immunology*, 161(2), pp. 213–217. doi: 10.1006/cimm.1995.1029.

György, B. *et al.* (2012) 'Improved Flow Cytometric Assessment Reveals Distinct Microvesicle (Cell-Derived Microparticle) Signatures in Joint Diseases', *PLoS ONE*, 7(11), p. e49726. doi: 10.1371/journal.pone.0049726.

Hartigan-O'Connor, D. J. *et al.* (2007) 'Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells', *Journal of Immunological Methods*, 319(1–2), pp. 41–52. doi: 10.1016/j.jim.2006.10.008.

Haskó, G. and Cronstein, B. N. (2004) 'Adenosine: an endogenous regulator of innate immunity', *Trends in Immunology*, 25(1), pp. 33–39. doi: 10.1016/j.it.2003.11.003.

Heptinstall, S. *et al.* (2005) 'Adenine nucleotide metabolism in human blood - Important roles for leukocytes and erythrocytes', *Journal of Thrombosis and Haemostasis*, 3(10), pp. 2331–2339. doi: 10.1111/j.1538-7836.2005.01489.x.

Herrera, C. *et al.* (2001) 'Adenosine A2B Receptors Behave as an Alternative Anchoring Protein for Cell Surface Adenosine Deaminase in Lymphocytes and Cultured Cells', *Molecular Pharmacology*, 59(1), pp. 127–134. doi: 10.1124/mol.59.1.127.

Heuts, D. P. H. M. *et al.* (2012) 'Crystal Structure of a Soluble Form of Human CD73 with Ecto-5'-Nucleotidase Activity', *ChemBioChem*, 13(16), pp. 2384–2391. doi: 10.1002/cbic.201200426.

St. Hilaire, C. *et al.* (2011) 'NT5E Mutations and Arterial Calcifications', *New England Journal of Medicine*, 364(5), pp. 432–442. doi: 10.1056/NEJMoa0912923.

Hinz, S. *et al.* (2018) 'Adenosine A2A receptor ligand recognition and signaling is blocked by A2B receptors', *Oncotarget*, 9(17), pp. 13593–13611. doi: 10.18632/oncotarget.24423.

Horenstein, A. L. *et al.* (2013) 'A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes', *OncoImmunology*, 2(9), p. e26246. doi: 10.4161/onci.26246.

Huang, S. *et al.* (1997) 'Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion', *Blood*, 90(4), pp. 1600–1610. doi: 10.1182/blood.v90.4.1600.1600_1600_1610.

Hunter, P. J. *et al.* (2010) 'Biologic predictors of extension of oligoarticular juvenile idiopathic arthritis as determined from synovial fluid cellular composition and gene expression', *Arthritis and Rheumatism*, 62(3), pp. 896–907. doi: 10.1002/art.27284.

De Ita, M. *et al.* (2016) 'ATP releases ATP or other nucleotides from human peripheral blood leukocytes through purinergic P2 receptors', *Life Sciences*, 145, pp. 85–92. doi: 10.1016/j.lfs.2015.12.013.

Jin, H. *et al.* (2016) 'Increased activity of TNAP compensates for reduced adenosine production and promotes ectopic calcification in the genetic disease ACDC', *Science Signaling*, 9(458), p. ra121. doi: 10.1126/scisignal.aaf9109.

Johnsen, B. *et al.* (2019) 'P2X7-mediated ATP secretion is accompanied by depletion of cytosolic ATP', *Purinergic Signalling*, 15(2), pp. 155–166. doi: 10.1007/s11302-019-09654-5.

Junger, W. G. (2011) 'Immune cell regulation by autocrine purinergic signalling', *Nature Reviews Immunology*, 11(3), pp. 201–212. doi: 10.1038/nri2938.

Kalsi, K. *et al.* (2002) 'Regulation of ecto-5 ' -nucleotidase by TNF- α in human endothelial cells', *Molecular and Cellular Biochemistry*, 232(1–2), pp. 113–119. doi: 10.1023/a:1014806916844.

Kameoka, J. *et al.* (1993) 'Direct Association of Adenosine Deaminase with T Cell Activation Antigen , CD26', *Science*, 261(5120), pp. 466–469. doi: 10.1126/science.8101391.

de Kleer, I. M. *et al.* (2004) 'CD4+ CD25 bright Regulatory T Cells Actively Regulate Inflammation in the Joints of Patients with the Remitting Form of Juvenile Idiopathic Arthritis', *The Journal of Immunology*, 172(10), pp. 6435–6443. doi: 10.4049/jimmunol.172.10.6435.

Klein, M. *et al.* (2012) 'Repression of Cyclic Adenosine Monophosphate Upregulation Disarms and Expands Human Regulatory T Cells', *The Journal of Immunology*, 188(3), pp. 1091–1097. doi: 10.4049/jimmunol.1102045.

Klemens, M. R. *et al.* (1990) 'Characterization of soluble vs membrane-bound human placental 5'-nucleotidase', *Biochemical and Biophysical Research Communications*, 172(3), pp. 1371–1377. doi: 10.1016/0006-291X(90)91601-N.

Knapp, K. *et al.* (2012) 'Crystal structure of the human ecto-5'-nucleotidase (CD73): Insights into the regulation of purinergic signaling', *Structure*, 20(12), pp. 2161–2173. doi: 10.1016/j.str.2012.10.001.

Kobie, J. J. *et al.* (2006) 'T Regulatory and Primed Uncommitted CD4 T Cells Express CD73, Which Suppresses Effector CD4 T Cells by Converting 5'-Adenosine Monophosphate to Adenosine', *The Journal of Immunology*, 177(10), pp. 6780–6786. doi: 10.4049/jimmunol.177.10.6780.

Konopka, T. (2019) 'umap: Uniform Manifold Approximation and Projection'. R package version 0.2.4.0.

Koshiba, M. *et al.* (1999) 'Patterns of A2A extracellular adenosine receptor expression in different functional subsets of human peripheral T cells. Flow cytometry studies with anti-A2A receptor monoclonal antibodies',

Molecular Pharmacology, 55(3), pp. 614–624.

Kukulski, F. *et al.* (2005) 'Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8', *Purinergic Signalling*, 1(2), pp. 193–204. doi: 10.1007/s11302-005-6217-x.

Latheef, S. A. A. *et al.* (2016) 'Differential expression of alkaline phosphatase gene in proliferating primary lymphocytes and malignant lymphoid cell lines', *Immunology Letters*, 170, pp. 37–41. doi: 10.1016/j.imlet.2015.12.008.

Lazarowski, E. R., Boucher, R. C. and Harden, T. K. (2000) 'Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations.', *The Journal of Biological Chemistry*, 275(40), pp. 31061–31068. doi: 10.1074/jbc.M003255200.

Lehto, M. T. and Sharom, F. J. (1998) 'Release of the glycosylphosphatidylinositol-anchored enzyme ecto-5'-nucleotidase by phospholipase C: catalytic activation and modulation by the lipid bilayer.', *Biochemical Journal*, 332(Pt 1), pp. 101–109. doi: 10.1042/bj3320101.

Li, M. O., Wan, Y. Y. and Flavell, R. A. (2007) 'T Cell-Produced Transforming Growth Factor-β1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation', *Immunity*, 26(5), pp. 579–591. doi: 10.1016/j.immuni.2007.03.014.

Linden, J. *et al.* (1999) 'Characterization of human A(2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells.', *Molecular Pharmacology*, 56(4), pp. 705–13.

Linden, J. (2005) 'Adenosine in Tissue Protection and Tissue Regeneration', *Molecular Pharmacology*, 67(5), pp. 1385–1387. doi: 10.1124/mol.105.011783.

Linden, J. and Cekic, C. (2012) 'Regulation of lymphocyte function by adenosine', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 32(9), pp. 2097–2103. doi: 10.1161/ATVBAHA.111.226837.

Liu, W. *et al.* (2006) 'CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells', *Journal of Experimental Medicine*, 203(7), pp. 1701–1711. doi: 10.1084/jem.20060772.

Löfgren, L. *et al.* (2018) 'Accurate measurement of endogenous adenosine in human blood', *PLoS ONE*, 13(10), p. e0205707. doi: 10.1371/journal.pone.0205707.

Low, M. G. and Prasad, A. R. S. (1988) 'A phospholipase D specific for the phosphatidylinositol anchor of cell-surface proteins is abundant in plasma', *Proc Natl Acad Sci U S A*, 85(4), pp. 980–984. doi: 10.1073/pnas.85.4.980.

Maas, S. L. N., Breakefield, X. O. and Weaver, A. M. (2017) 'Extracellular Vesicles: Unique Intercellular Delivery Vehicles', *Trends in Cell Biology*, 27(3), pp. 172–188. doi: 10.1016/j.tcb.2016.11.003.

Mahnke, Y. D. *et al.* (2013) 'The who's who of T-cell differentiation: Human memory T-cell subsets', *European Journal of Immunology*, 43(11), pp. 2797–2809. doi: 10.1002/eji.201343751.

Maj, T. *et al.* (2017) 'Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor', *Nature Immunology*, 18(12), pp. 1332–1341. doi: 10.1038/ni.3868.

Maliszewski, C. R. *et al.* (1994) 'The CD39 lymphoid cell activation antigen. Molecular cloning and structural characterization.', *Journal of Immunology*, 153(8), pp. 3574–83.

Mangino, M. *et al.* (2017) 'Innate and adaptive immune traits are differentially affected by genetic and environmental factors', *Nature Communications*, 8, p. 13850. doi: 10.1038/ncomms13850.

Martin, M. *et al.* (1995) 'Expression of ecto-adenosine deaminase and CD26 in human T cells triggered by the TCR-CD3 complex. Possible role of adenosine deaminase as costimulatory molecule.', *Journal of Immunology*, 155(10), pp. 4630–4643.

Massaia, M. *et al.* (1990) 'Human T cell activation. Synergy between CD73 (ecto-5'-nucleotidase) and signals delivered through CD3 and CD2 molecules.', *Journal of Immunology*, 145(6), pp. 1664–1674.

Mirabet, M. *et al.* (1999) 'Expression of A(2B) adenosine receptors in human lymphocytes: Their role in T cell activation', *Journal of Cell Science*, 112(4), pp. 491–502.

Mishima, T. *et al.* (2017) 'Rapid G0/1 transition and cell cycle progression in CD8+ T cells compared to CD4+ T cells following in vitro stimulation', *Microbiology and Immunology*, 61(5), pp. 168–175. doi: 10.1111/1348-0421.12479.

Misumi, Y. *et al.* (1990) 'Primary structure of human placental 5'-nucleotidase and identification of the glycolipid anchor in the mature form', *European Journal of Biochemistry*, 191(3), pp. 563–569. doi: 10.1111/j.1432-1033.1990.tb19158.x.

Moncrieffe, H. *et al.* (2010) 'High Expression of the Ectonucleotidase CD39 on T Cells from the Inflamed Site Identifies Two Distinct Populations, One Regulatory and One Memory T Cell Population', *The Journal of Immunology*, 185(1), pp. 134–143. doi: 10.4049/jimmunol.0803474.

Monjas, A., Alcover, A. and Alarcón, B. (2004) 'Engaged and bystander T cell receptors are down-modulated by different endocytotic pathways', *Journal of Biological Chemistry*, 279(53), pp. 55376–55384. doi: 10.1074/jbc.M409342200.

Morandi, F. *et al.* (2018) *Microvesicles released from multiple myeloma cells are equipped with ectoenzymes belonging to canonical and non-canonical adenosinergic pathways and produce adenosine from ATP and NAD+*, *OncoImmunology*. doi: 10.1080/2162402X.2018.1458809.

Morello, S. *et al.* (2017) 'Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab', *Journal of Translational Medicine*, 15(1), p. 244. doi: 10.1186/s12967-017-1348-8.

Mortensen, S. P. *et al.* (2011) 'Local release of ATP into the arterial inflow and venous drainage of human skeletal muscle : insight from ATP determination with the intravascular microdialysis technique', *The Journal of Physiology*, 589(Pt 7), pp. 1847–1857. doi: 10.1113/jphysiol.2010.203034.

Mosenden, R. and Taskén, K. (2011) 'Cyclic AMP-mediated immune regulation - Overview of mechanisms of action in T cells', *Cellular Signalling*, 23(6), pp. 1009–1016. doi: 10.1016/j.cellsig.2010.11.018.

Möser, G. H., Schrader, J. and Deussen, A. (1989) 'Turnover of adenosine in plasma of human and dog blood', *American Journal of Physiology-Cell Physiology*, 256(4), pp. C799–C806. doi: 10.1152/ajpcell.1989.256.4.C799.

van Niel, G., D'Angelo, G. and Raposo, G. (2018) 'Shedding light on the cell biology of extracellular vesicles', *Nature Reviews Molecular Cell Biology*, 19(4), pp. 213–228. doi: 10.1038/nrm.2017.125.

Nishimura, H. *et al.* (1999) 'Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor', *Immunity*, 11(2), pp. 141–151. doi: 10.1016/S1074-7613(00)80089-8.

Nistala, K. *et al.* (2008) 'Interleukin-17 – Producing T Cells Are Enriched in the Joints of Children With Arthritis , but Have a Reciprocal Relationship to Regulatory T Cell Numbers', *Arthritis and Rheumatism*, 58(3), pp. 875–887. doi: 10.1002/art.23291.

Nolan, J. P. and Duggan, E. (2018) 'Analysis of Individual Extracellular Vesicles by Flow Cytometry', in *Hawley T., Hawley R. (eds) Flow Cytometry Protocols. Methods in Molecular Biology*. doi: 10.1007/978-1-4939-7346-0_5.

Ohta, Akio *et al.* (2012) 'The development and immunosuppressive functions of CD4+ CD25+ FoxP3+ regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway', *Frontiers in Immunology*, 3, p. 190. doi: 10.3389/fimmu.2012.00190.

Okoye, I. S. *et al.* (2014) 'MicroRNA-Containing T-Regulatory-Cell-Derived Exosomes Suppress Pathogenic T Helper 1 Cells', *Immunity*, 41(1), pp. 89–103. doi: 10.1016/j.immuni.2014.05.019.

Ontyd, J. and Schrader, J. (1984) 'Measurement of adenosine, inosine, and hypoxanthine in human plasma', *Journal of Chromatography B: Biomedical Sciences and Applications*, 307(2), pp. 404–409. doi: 10.1016/S0378-4347(00)84113-4.

Pandiyan, P. *et al.* (2007) 'CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells', *Nature Immunology*, 8(12), pp. 1353–1362. doi: 10.1038/ni1536.

Paul, W. E. and Zhu, J. (2010) 'How are TH2-type immune responses initiated and amplified?', *Nature Reviews Immunology*, 10(4), pp. 225–235. doi: 10.1038/nri2735.

Pettengill, M. *et al.* (2013) 'Soluble ecto-5'-nucleotidase (5'-NT), alkaline phosphatase, and adenosine deaminase (ADA1) activities in neonatal blood favor elevated extracellular adenosine', *Journal of Biological Chemistry*, 288(38), pp. 27315–27326. doi: 10.1074/jbc.M113.484212.

Praetorius, H. a. and Leipziger, J. (2009) 'ATP release from non-excitable cells', *Purinergic Signalling*, 5(4), pp. 433–446. doi: 10.1007/s11302-009-9146-2.

Pulte, E. D. *et al.* (2007) 'CD39/NTPDase-1 activity and expression in normal leukocytes', *Thrombosis Research*, 121(3), pp. 309–317. doi: 10.1016/j.thromres.2007.04.008.

Qureshi, O. S. *et al.* (2011) 'Trans-Endocytosis of CD80 and CD86: A Molecular Basis for the Cell-Extrinsic Function of CTLA-4', *Science*, 332(6029), pp. 600–603. doi: 10.1126/science.1202947.

R Core Team (2019) 'R: A language and environment for statistical computing'. Vienna, Austria: R Foundation for Statistical Computing.

Raczkowski, F. *et al.* (2018) 'CD39 is upregulated during activation of mouse and human T cells and attenuates the immune response to Listeria monocytogenes', *Plos One*, 13(5), p. e0197151. doi: 10.1371/journal.pone.0197151.

Rand, M. L. *et al.* (2006) 'Rapid clearance of procoagulant platelet-derived microparticles from the circulation of rabbits', *Journal of Thrombosis and Haemostasis*, 4(7), pp. 1621–1623. doi: 10.1111/j.1538-7836.2006.02011.x.

Rank, A. *et al.* (2011) 'Clearance of platelet microparticles in vivo', *Platelets*, 22(2), pp. 111–116. doi: 10.3109/09537104.2010.520373.

Raposo, G. *et al.* (1996) 'B lymphocytes secrete antigen-presenting vesicles', *The Journal of Experimental Medicine*, 183(3), pp. 1161–1172. doi: 10.1084/jem.183.3.1161.

Raskovalova, T. *et al.* (2007) 'Inhibition of cytokine production and cytotoxic activity of human antimelanoma specific CD8+ and CD4+ T lymphocytes by adenosine-protein kinase A type I signaling', *Cancer Research*, 67(12), pp. 5949–5956. doi: 10.1158/0008-5472.CAN-06-4249.

Resta, R. *et al.* (1994) 'Glycosyl phosphatidylinositol membrane anchor is not required for T cell activation through CD73.', *Journal of Immunology (Baltimore, Md. : 1950)*, 153(3), pp. 1046–53.

Rissiek, A. *et al.* (2015) 'The expression of CD39 on regulatory T cells is genetically driven and further upregulated at sites of inflammation', *Journal of Autoimmunity*, 58, pp. 12–20. doi: 10.1016/j.jaut.2014.12.007.

Roederer, M. *et al.* (2015) 'The Genetic Architecture of the Human Immune System: A Bioresource for Autoimmunity and Disease Pathogenesis', *Cell*, 161(2), pp. 387–403. doi: 10.1016/j.cell.2015.02.046.

Roncador, G. *et al.* (2005) 'Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level', *European Journal of Immunology*, 35(6), pp. 1681–1691. doi: 10.1002/eji.200526189.

Rubtsov, Y. P. *et al.* (2008) 'Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces', *Immunity*, 28(4), pp. 546–558. doi: 10.1016/j.immuni.2008.02.017.

Ruprecht, C. R. *et al.* (2005) 'Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia', *Journal of Experimental Medicine*, 201(11), pp. 1793–1803. doi: 10.1084/jem.20050085.

Sakaguchi, S. *et al.* (2010) 'FOXP3+ regulatory T cells in the human immune system', *Nature Reviews Immunology*, 10(7), pp. 490–500. doi: 10.1038/nri2785.

Sakaguchi, S. *et al.* (2020) 'Regulatory T Cells and Human Disease', *Annual Review of Immunology*, 38(1), pp. 541–566. doi: 10.1146/annurev-immunol-042718-041717.

Sallusto, F. (2016) 'Heterogeneity of Human CD4+ T Cells Against Microbes', *Annual Review of Immunology*, 34, pp. 317–334. doi: 10.1146/annurev-immunol-032414-112056.

Sawant, D. V. *et al.* (2019) 'Adaptive plasticity of IL-10+ and IL-35+ Treg cells cooperatively promotes tumor T cell exhaustion', *Nature Immunology*, 20(6), pp. 724–735. doi: 10.1038/s41590-019-0346-9.

Schena, F. *et al.* (2013) 'Dependence of Immunoglobulin Class Switch Recombination in B Cells on Vesicular Release of ATP and CD73 Ectonucleotidase Activity', *Cell Reports*, 3(6), pp. 1824–1831. doi: 10.1016/j.celrep.2013.05.022.

Schuler, P. J. *et al.* (2014) 'Human CD4+ CD39+ regulatory T cells produce adenosine upon co-expression of surface CD73 or contact with CD73+ exosomes or CD73+ cells', *Clinical & Experimental Immunology*, 177(2), pp. 531–543. doi: 10.1111/cei.12354.

Skryabin, G. O. *et al.* (2020) 'Lipid Rafts in Exosome Biogenesis', *Biochemistry (Moscow)*, 85(2), pp. 177–191. doi: 10.1134/S0006297920020054.

Smyth, L. A. *et al.* (2013) 'CD73 expression on extracellular vesicles derived from CD4+CD25+Foxp3+ T cells contributes to their regulatory function', *European Journal of Immunology*, 43(9), pp. 2430–2440. doi: 10.1002/eji.201242909.

Snider, N. T. *et al.* (2014) 'Alternative splicing of human NT5E in cirrhosis and hepatocellular carcinoma produces a negative regulator of ecto-5'-nucleotidase (CD73)', *Molecular Biology of the Cell*, 25(25), pp. 4024–4033. doi: 10.1091/mbc.E14-06-1167.

Sträter, N. (2006) 'Ecto-5'-nucleotidase: Structure function relationships', *Purinergic Signalling*, 2(2), pp. 343–350. doi: 10.1007/s11302-006-9000-8.

Sueyoshi, K. *et al.* (2019) 'Lipopolysaccharide suppresses T cells by generating extracellular ATP that impairs their mitochondrial function via P2Y11 receptors', *Journal of Biological Chemistry*, 294(16), pp. 6283–6293. doi: 10.1074/jbc.RA118.007188.

Synnestvedt, K. *et al.* (2002) 'Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia', *Journal of Clinical Investigation*, 110(7), pp. 993–1002. doi: 10.1172/JCI15337.

Thompson, L. F. *et al.* (1989) 'Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate', *The Journal of Immunology*, 143(6), pp. 1815–1821.

Thomson, L. F. *et al.* (1990) 'Production and characterization of monoclonal antibodies to the glycosyl phosphatidylinositol-anchored lymphocyte differentiation antigen ecto-5'-nucleotidase (CD73)', *Tissue Antigens*, 35(1), pp. 9–19. doi: 10.1111/j.1399-0039.1990.tb01750.x.

Tivol, E. A. *et al.* (1995) 'Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4', *Immunity*, 3(5), pp. 541–547. doi: 10.1016/1074-7613(95)90125-6.

Tóth, I. *et al.* (2013) 'Decreased frequency of CD73+ CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion', *Journal of Leukocyte Biology*, 94(4), pp. 551–561. doi: 10.1189/jlb.0113018.

Trabanelli, S. *et al.* (2012) 'Extracellular ATP Exerts Opposite Effects on Activated and Regulatory CD4+ T Cells via Purinergic P2 Receptor Activation', *The Journal of Immunology*, 189(3), pp. 1303–1310. doi: 10.4049/jimmunol.1103800.

Tung, S. L. *et al.* (2020) 'Regulatory T Cell Extracellular Vesicles Modify T-Effector Cell Cytokine Production and Protect Against Human Skin Allograft Damage', *Frontiers in Cell and Developmental Biology*, 8, p. 317. doi: 10.3389/fcell.2020.00317.

Uhlen, M. *et al.* (2019) 'A genome-wide transcriptomic analysis of protein-coding genes in human blood cells', *Science*, 366(6472), p. eaax9198. doi: 10.1126/science.aax9198.

van der Vlist, E. J. *et al.* (2012) 'CD4+ T cell activation promotes the differential release of distinct populations of nanosized vesicles', *Journal of Extracellular Vesicles*, 1(1), p. 18364. doi: 10.3402/jev.v1i0.18364.

Wahlgren, J. *et al.* (2012) 'Activated Human T Cells Secrete Exosomes That Participate in IL-2 Mediated Immune Response Signaling', *PLoS ONE*. Edited by P. Busson, 7(11), p. e49723. doi: 10.1371/journal.pone.0049723.

Wang, J., Huizinga, T. W. J. and Toes, R. E. M. (2009) 'De Novo Generation and Enhanced Suppression of Human CD4+ CD25+ Regulatory T Cells by Retinoic Acid', *The Journal of Immunology*, 183(6), pp. 4119–4126. doi: 10.4049/jimmunol.0901065.

Wang, T.-F., Ou, Y. and Guidotti, G. (1998) 'The Transmembrane Domains of Ectoapyrase (CD39) Affect Its Enzymatic Activity and Quaternary Structure', *The Journal of Biological Chemistry*, 273(38), pp. 24814–24821. doi: 10.1074/jbc.273.38.24814.

Wei, F. *et al.* (2013) 'Strength of PD-1 signaling differentially affects T-cell effector functions', *Proceedings of the National Academy of Sciences*, 110(27), pp. E2480–E2489. doi: 10.1073/pnas.1305394110.

Weiler, M. *et al.* (2016) 'Inhibitory effect of extracellular purine nucleotide and nucleoside concentrations on T cell proliferation', *Experimental Cell Research*, 349(1), pp. 1–14. doi: 10.1016/j.yexcr.2016.05.017.

Wickham, H. (2016) 'ggplot2: Elegant Graphics for Data Analysis'. R package version 3.3.2.

Wing, K. *et al.* (2008) 'CTLA-4 Control over Foxp3+ Regulatory T Cell Function', *Science*, 322(5899), pp. 271–275. doi: 10.1126/science.1160062.

Winzer, R. (2017) *Regulation of adenosine production by the ectonucleotidase CD73 in the context of neonatal tolerance*. Master Thesis, University of Hamburg.

Woehrle, T. *et al.* (2010) 'Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse', *Blood*, 116(18), pp. 3475–3484. doi: 10.1182/blood-2010-04-277707.

Xie, Y. *et al.* (2010) 'Dendritic Cells Recruit T Cell Exosomes via Exosomal LFA-1 Leading to Inhibition of CD8+ CTL Responses through Downregulation of Peptide/MHC Class I and Fas Ligand-Mediated Cytotoxicity', *The Journal of Immunology*, 185(9), pp. 5268–5278. doi: 10.4049/jimmunol.1000386.

Yamashita, Y. *et al.* (1998) 'CD73 expression and fyn-dependent signaling on murine lymphocytes', *European Journal of Immunology*, 28(10), pp. 2981–2990. doi: 10.1002/(SICI)1521-4141(199810)28:10<2981::AID-IMMU2981>3.0.CO;2-D.

Yáñez-Mó, M. *et al.* (2015) 'Biological properties of extracellular vesicles and their physiological functions', *Journal of Extracellular Vesicles*, 4(1), p. 27066. doi: 10.3402/jev.v4.27066.

Yegutkin, G. G. *et al.* (2002) 'The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells', *Biochemical Journal*, 367(1), pp. 121–128. doi: 10.1042/BJ20020439.

Yegutkin, G. G. *et al.* (2006) 'The Detection of Micromolar Pericellular ATP Pool on Lymphocyte Surface by

Using Lymphoid Ecto-Adenylate Kinase as Intrinsic ATP Sensor', *Molecular Biology of the Cell*, 17(8), pp. 3378–3385. doi: 10.1091/mbc.E05-10-0993.

Yegutkin, G. G. (2008) 'Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1783(5), pp. 673–694. doi: 10.1016/j.bbamcr.2008.01.024.

Yegutkin, G. G. *et al.* (2012) 'Metabolism of circulating ADP in the bloodstream is mediated via integrated actions of soluble adenylate kinase-1 and NTPDase1/CD39 activities', *The FASEB Journal*, 26(9), pp. 3875–3883. doi: 10.1096/fj.12-205658.

Yegutkin, G. G. (2014) 'Enzymes involved in metabolism of extracellular nucleotides and nucleosides: Functional implications and measurement of activities', *Critical Reviews in Biochemistry and Molecular Biology*, 49(6), pp. 473–497. doi: 10.3109/10409238.2014.953627.

Yegutkin, G. G., Samburski, S. S. and Jalkanen, S. (2003) 'Soluble purine-converting enzymes circulate in human blood and regulate extracellular ATP level via counteracting pyrophosphatase and phosphotransfer reactions', *The FASEB Journal*, 17(10), pp. 1328–1330. doi: 10.1096/fj.02-1136fje.

Yip, L. *et al.* (2009) 'Autocrine regulation of T-cell activation by ATP release and P2X $_7$ receptors', *The FASEB Journal*, 23(6), pp. 1685–1693. doi: 10.1096/fj.08-126458.

Yoneyama, Y. *et al.* (2005) 'Plasma adenosine concentrations increase in women with hyperemesis gravidarum', *Clinica Chimica Acta*, 352(1–2), pp. 75–79. doi: 10.1016/j.cccn.2003.12.026.

Zeiner, J. *et al.* (2019) 'Soluble and membrane-bound adenylate kinase and nucleotidases augment ATP-mediated inflammation in diabetic retinopathy eyes with vitreous hemorrhage', *Journal of Molecular Medicine*, 97(3), pp. 341–354. doi: 10.1007/s00109-018-01734-0.

Zhang, D. *et al.* (2017) 'D-mannose induces regulatory T cells and suppresses immunopathology', *Nature Medicine*, 23(9), pp. 1036–1045. doi: 10.1038/nm.4375.

Zhang, H.-G. *et al.* (2006) 'A Membrane Form of TNF-α Presented by Exosomes Delays T Cell Activation-Induced Cell Death', *The Journal of Immunology*, 176(12), pp. 7385–7393. doi: 10.4049/jimmunol.176.12.7385.

Zhang, N. and Bevan, M. J. (2011) 'CD8+ T Cells: Foot Soldiers of the Immune System', *Immunity*, 35(2), pp. 161–168. doi: 10.1016/j.immuni.2011.07.010.

Zhang, W. *et al.* (2018) 'Multiple steps determine CD73 shedding from RPE: lipid raft localization, ARA1 interaction, and MMP-9 up-regulation', *Purinergic Signalling*, 14(4), pp. 443–457. doi: 10.1007/s11302-018-9628-1.

Zimmermann, H., Zebisch, M. and Sträter, N. (2012) 'Cellular function and molecular structure of ecto-nucleotidases', *Purinergic Signalling*, 8(3), pp. 437–502. doi: 10.1007/s11302-012-9309-4.

Zöller, M. (2009) 'Tetraspanins: Push and pull in suppressing and promoting metastasis', *Nature Reviews Cancer*, 9(1), pp. 40–55. doi: 10.1038/nrc2543.

7. Appendix

7.1 Hazard and precautionary statements

The information originate from the GESTIS substance database or from the manufacturer's safety sheet.

Table 2 | Hazard and precautionary statements of the used reagents.

Reagent	GHS symbol	Hazard statement	Precautionary statement
2-Mercaptoethanol	GHS05, GHS06, GHS08, GHS09	H301+H331, H310, H315, H317, H318, H361d, H373, H410	P201, P262, P280, P301+P310+P330, P302+P352+P310, P305+P351+P338+P310
Dithiothreitol	GHS07	H302, H315, H319, H335	P261, P305+P351+P338
DNase I	GHS08	H317, H334	P261, P280, P284, P304+P340, P342+P311
Ethanol	GHS02, GHS07	H225, H319	P210, P240, P305+P351+P338, P403+P233
Ethylenediaminetetra-acetic acid (EDTA)	GHS07, GHS08	H319, H332, H373	P280, P304+P340, P312, P305+P351+P338, P337+P313
FACS Clean Solution	GHS07	H315, H319, H400	P264, P280, P273, P305+P351+P338, P337+P313, P302+P352, P332+P313, P321, P362, P501b
Methanol	GHS02, GHS06, GHS08	H225, H331, H311, H301, H370	P210, P233, P280, P302+P352, P304+P340, P308+P310, P403+P235
Nonidet P 40 substitute	GHS07, GHS09	H315, H319, H411	P273, P302+P352, P305+P351+P338
NuPAGE antioxidant	GHS07, GHS08	H360, H319	P201, P202, P264, P280, P305+P351+P338, P337+P313, P308+P313
Penicillin-streptomycin	GHS07, GHS08	H317, H334, H360	P201, P202, P261, P280, P284, P272, P302+P352, P333+P313, P342+P311, P308+P313, P362, P304+P340

Paraformaldehyde aqueous solution (16%), EM grade	GHS05, GHS07, GHS08	H302, H312, H315, H317, H332, H335, H319, H341, H350	P201, P261, P264, P270, P280, P301+P310, P302+P352, P304+P340, P305+P351+P338, P308+P313, P403+P233, P501
Poly-L-lysine solution	GHS07	H315, H317, H319	P261, P264, P272, P280, P302+P352, P305+P351+P338
Sodium azide	GHS06, GHS08, GHS09	H300+H310+H330, H373, H410, EUH032	P262, P273, P280, P301+P310+P330, P302+P352+P310, P304+P340+P310
Sodium deoxycholate	GHS07	H302, H412	P273, P301+P312+P330
Sodium dodecyl sulfate (SDS)	GHS02, GHS05, GHS07	H228, H302+H332, H315, H318, H335, H412	P210, P261, P280, P301+P312+P330, P305+P351+P338+P310, P370+P378
Sulfuric acid	GHS05	H290, H314	P280, P301+P330+P331, P303+P361+P353, P305+P351+P338+P310
Tetrabutylammonium dihydrogen phosphate (TBAHP)	GHS07	H302, H315, H319, H335	P261, P305+P351+P338
Triton X-100	GHS05, GHS07, GHS09	H302, H315, H318, H410	P273, P280, P302+P352, P305+P351+P338, P313
Trypan blue	GHS08	H350	P201, P202, P280, P308+P313, P405, P501

7.2 Abbreviations

ACDC	Arterial calcification due to deficiency of CD73
ADA	Adenosine deaminase
ADO	Adenosine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CD4con	Conventional CD4 T cells
cDNA	Complementary deoxyribonucleic acid
CM	Central memory
eADO	Etheno-adenosine
eADP	Etheno-ADP
eAMP	Etheno-AMP
eATP	Etheno-ATP
EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride
EM	Effector memory
E-NPP	ecto-nucleotide pyrophosphatase/phosphodiesterase
ENTPD1	Ecto-nucleoside triphosphate diphosphohydrolase 1; gene encoding CD39
EV	Extracellular vesicle
FACS	Fluorescence-activated cell sorting
FOXP3	Forkhead box P3
GPI	Glycosylphosphatidylinositol
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
INO	Inosine
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
JIA	Juvenile idiopathic arthritis
kDa	Kilodalton
LPS	Lipopolysaccharide
MAIT	Mucosal-associated invariant T cells
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMP-9	Matrix metalloproteinase-9
mRNA	Messenger ribonucleic acid
MVE	Multivesicular endosome
NK cell	Natural killer cell

NT5E	5’-nucleotidase ecto; gene encoding CD73
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI-PLC	Phosphatidylinositol-specific phospholipase C
rec. CD73	Recombinant CD73
rp-HPLC	Reversed-phase high performance liquid chromatography
RPL13A	Ribosomal protein L13a
RT	Room temperature
SF	Synovial fluid
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TEMRA	Terminal effector memory RA^+
TGF	Transforming growth factor
Th cell	T helper cell
TNAP	Tissue-nonspecific alkaline phosphatase
TNF	Tumor necrosis factor
Treg	Regulatory T cell
UMAP	Uniform Manifold Approximation and Projection

7.3 List of figures and tables

List of figures

List of tables

8. Publications and congress contributions

Publications

Schneider, Enja*, Riekje Winzer*, Anne Rissiek, Isabell Ricklefs, Catherine Meyer-Schwesinger, Franz L. Ricklefs, Andreas Bauche, Jochen Behrends, Rudolph Reimer, Santra Brenna, Björn Rissiek, Berta Puig, Filippo Cortesi, Tim Magnus, Ralf Fliegert, Christa E. Müller, Nicola Gagliani, Eva Tolosa. 2020. "CD73-mediated adenosine production by T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune suppression." *Submitted*

Johnsen, Bjarne, Klaus Eric Kaschubowski, Sorush Nader, **Enja Schneider**, Jan Andrei Nicola, Ralf Fliegert, Insa M. A. Wolf, Andreas H. Guse, Viacheslav O. Nikolaev, Friedrich Koch-Nolte, and Friedrich Haag. 2019. "P2X7-Mediated ATP Secretion Is Accompanied by Depletion of Cytosolic ATP." Purinergic Signalling 15(2):155–66.

Reinicke, Anna T., Friederike Raczkowski, Malte Mühlig, Pina Schmucker, Timo Lischke, Julia Reichelt, **Enja Schneider**, Stephanie Zielinski, Marlies Sachs, Elisabeth Jurack, Eva Tolosa, Christian Kurts, Hans-Willi Mittrücker, and Catherine Meyer-Schwesinger. 2019. "Deubiquitinating Enzyme UCH-L1 Promotes Dendritic Cell Antigen Cross-Presentation by Favoring Recycling of MHC Class I Molecules." The Journal of Immunology 203(7):1730–42.

Ricklefs, Franz L., Cecile L. Maire, Rudolph Reimer, Lasse Dührsen, Katharina Kolbe, Mareike Holz, **Enja Schneider**, Anne Rissiek, Anna Babayan, Claudia Hille, Klaus Pantel, Susanne Krasemann, Markus Glatzel, Dieter Henrik Heiland, Jörg Flitsch, Tobias Martens, Nils Ole Schmidt, Sven Peine, Xandra O. Breakefield, Sean Lawler, E. Antonio Chiocca, Boris Fehse, Bernd Giebel, André Görgens, Manfred Westphal, and Katrin Lamszus. 2019. "Imaging Flow Cytometry Facilitates Multiparametric Characterization of Extracellular Vesicles in Malignant Brain Tumours." Journal of Extracellular Vesicles 8(1):1588555.

Schneider, Enja, Anne Rissiek, Riekje Winzer, Berta Puig, Björn Rissiek, Friedrich Haag, Hans-Willi Mittrücker, Tim Magnus, and Eva Tolosa. 2019. "Generation and Function of Non-Cell-Bound CD73 in Inflammation." Frontiers in Immunology 10:1729.

Conferences

2020	World Immune Regulation Meeting (online due to COVID-19) **Talk:** CD73-mediated adenosine production by T cell-derived extracellular vesicles constitutes an intrinsic mechanism of immune regulation in humans
2019	1st European Purine Meeting (Santiago de Compostela) **Short Talk and Poster:** T cell-derived exosomal CD73 mediates immune suppression
2018	12th German Meeting on Immune Regulation (Berlin-Schmöckwitz) **Talk:** Regulation and function of CD73 in inflammation
2017	20th Meeting on T cells – Subsets and Function (Marburg) **Talk:** Regulation of CD73 expression and function in inflammation
2016	46th Annual Meeting of the German Society for Immunology (Hamburg)
2015	4th European Congress of Immunology (Vienna) **Poster:** Using FRET-based sensors to monitor ATP concentrations at the cell surface (Topic of the Master thesis)
2015	6th Joint German-Italian Purine Club Meeting (Hamburg) **Poster:** Using FRET-based sensors to monitor ATP concentrations at the cell surface (Topic of the Master thesis) Best Poster Slam Award

9. Acknowledgements

Special thanks go to my mentor and supervisor Prof. Dr. Eva Tolosa, who gave me the opportunity to do my PhD thesis in her group. Eva, I am thankful for your excellent support and helpful feedback. I have learned so many things in the last years.

Herrn Prof. Dr. Henning Tidow danke ich für die Begutachtung meiner Dissertation.

Many thanks to all IFIs, especially to the current and former members of the Tolosa Lab: Anna, Anne, Annika, Arnau, Dana, Elena, Hauke, Jolan, Kati, Laura, Laurenz, Manu, Nora, Riekje, Romy, Sabine and Tina. You all supported me during my work and created a great (working) atmosphere. I really appreciate to be part of this wonderful team. Ganz besonders bedanken möchte ich mich bei meinen CD73-Mitstreiterinnen Anne und Riekje. Anne, du hast mich an Bord des CD73-Teams geholt, mich wunderbar eingearbeitet und standest mir jederzeit mit Rat und Tat zur Seite. Riekje, ich schätze deine fachliche Meinung und deine Freundschaft sehr und es ist eine Freude mit dir zusammenzuarbeiten.

My thanks also go to our cooperation partners Prof. Dr. Catherine Meyer-Schwesinger, Dr. Jochen Behrends and Santra Brenna. Cathy generated the beautiful microscopy pictures, Jochen helped us a lot with the flow cytometric analysis of vesicles and Santra Brenna showed me how to analyze the vesicles by western blot.

Einen wesentlichen Beitrag zum Gelingen dieser Arbeit haben auch alle Blutspender des UKE und die Mitarbeiterinnen der Cytometry und Sorting Core Unit geleistet; dafür mein aufrichtiger Dank.

Ich bedanke mich bei der Universität Hamburg, die mich im Rahmen eines Stipendiums nach dem Hamburgischen Gesetz zur Förderung des wissenschaftlichen und künstlerischen Nachwuchses während meiner Doktorarbeit finanziell unterstützt hat.

Ganz lieben Dank an alle Freunde, ihr habt dafür gesorgt, dass das Leben auch in der Endspurtphase nicht zu kurz kam.

Abschließend möchte ich mich ganz herzlich bei meiner Familie und meinem Freund bedanken. Ihr wart immer für mich da, habt mich bedingungslos unterstützt, Freude und Leid mit mir geteilt und immer an mich geglaubt.

10. Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Dissertation selbst verfasst und keine anderen als die angegebenen Hilfsmittel benutzt zu haben. Die eingereichte schriftliche Fassung entspricht der auf dem elektronischen Speichermedium. Ich versichere, dass diese Dissertation nicht in einem früheren Promotionsverfahren eingereicht wurde.

Ort, Datum

Unterschrift (Enja Schneider)