Adenosine and its Impact on Neonatal Immune Effector Cells

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Abbreviations

$A_{1; 2A; 2B; 3}$	purinergic adenosine receptors
AC	adenylate cyclase
AKAP	A-kinase-anchoring protein
ArgI	Arginase I
Ax	anoxia
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CFDA SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
Csk	c-terminal Src kinase
DAPI	4´,6-diamidino-2-phenylindole
DFO	Deferoxamine
FACS	Fluorescence-activated cell sorter
FCS	fetal calf serum
$G_{i/s}$	G protein (inhibitory, stimulatory)
IDO	Indoleamine 2,3-dioxygenase
IL-2	interleukin-2

iNOS	inducible Nitric Oxide Synthase
Kyn	Kynurenine
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
L-Glu	L-Glutamine
LPS	lipopolysaccharides
LTA	lipoteichoic acid
MDSC	Myeloid-derived suppressor cell
MNC	mononuclear cells
NFATc	Nuclear factor of activated T-cells, cytosolic
Nx	normoxia
P2X receptors	family of ligand-gated ion channels
P2Y receptors	family of purinergic G protein-coupled receptors
Pam3Cys	((S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH. trihydrochloride
PBS	phosphate buffered saline
PKA	protein kinase A
P/S	penicillin / streptomycin
PMN cells	polymorphonuclear cells
RPMI-1640 Medium	Roswell Park Memorial Institute 1640 Medium
TCR	T cell receptor

TLR	Toll-like receptor
TNFα	tumor necrosis factor α
Trp	tryptophan
ZAP 70	Zeta-chain associated protein kinase of 70 kDa

Abstract

Neonatal sepsis is a leading cause of neonatal morbidity and mortality. This is partially due to immaturity of the neonatal immune system as a remnant of prenatal development when the activity of neonatal immune system has to be downregulated and feto-maternal tolerance has to be maintained. Postnatally, the switch from immunosuppression to immunocompetence may be delayed because of persisting cellular mechanisms acting inhibitory such as presence of myeloid-derived suppressor cells (MDSC), as well as soluble factors. In adults extracellular adenosine has been shown to act immunosuppressive. In this project, the role of adenosine for neonatal immunosuppression was analyzed. The role of adenosine in neonates is only incompletely understood.

My results show that adenosine is produced by neonatal immune effector cells and that it can inhibit neonatal immune reactions either directly through T cell proliferation inhibition or indirectly via MDSC induction and their activation by induction of indoleamine-2,3-dioxygenase (IDO) production. Pleiotropism of adenosine may therefore be one of the key players in the maintaining of feto-maternal tolerance and neonatal immunosuppression. According to the results of my investigations, adenosine may be a promising target for immunotherapy in the neonate.

Zusammenfassung

Die neonatale Sepsis is eine der Hauptursachen der Morbidität und Mortalität bei Neugeborenen. Das zugrundeliegende Problem scheint das noch nicht an das postnatale Leben adaptierte Immunsystem zu sein. Dieser Funktionszustand ist ein Überbleibsel der pränatalen Entwicklung, während der die Aktivität runterreguliert werden muss um die feto-maternale Toleranz zu erhalten. Der zögerlichen postpartalen Umstellung des Immunsystems liegen wahrscheinlich zelluläre Mechanismen zugrunde, wie die Anwesenheit von immunsuppressiv wirkenden myeloiden Suppressorzellen (MDSC) sowie lösliche Faktoren. Beim Erwachsenen wurde gezeigt, dass extrazelluläres Adenosine immunsuppressiv wirkt. Welche Rolle Adenosin beim Neugeborenen spielt, ist nur unvollständig verstanden.

Meine Ergebnisse zeigen, dass Adenosin von neonatalen Immuzellen gebildet werden kann und neonatale Immunreaktionen hemmen kann, entweder direkt über Hemmung der T-Zellproliferation oder indirekt durch Induktion von MDSC und deren Aktivierung durch Hochregulation von indolamine-2,3-dioxygenase (IDO).

Dieser Pleiotropismus von Adenosin könnte eine wichtige Rolle in der Aufrechterhaltung der feto-maternalen Toleranz und der postnatalen Immunsuppression spielen. Adenosin könnte damit ein vielversprechendes Ziel für eine immunmodulierende Therapie beim Neugeborenen sein.

1. Introduction

The neonatal sepsis is one of the greatest risc factors for neonatal morbidity and mortality [1]. The incidence varies depending on gestational age with about 30% of low birth weight infants, i.e. neonates < 1500g being affected [2]. Neonatal sepsis is the second most reason for death of preterm infants in the neonatal period in Germany [3]. Pathophysiology of the sepsis is based on the delayed switch of immune system from the suppressive status to immunocompetent [4]. Myeloid-derived suppressor cells (MDSC) acting immunosuppressive [5] are present in higher amounts in both, cord blood and neonatal blood [4]. These cells provide the feto-maternal tolerance during pregnancy. The regulatory mechanisms of MDSC function in foetus and neonates are not completely understood. Adenosine is a metabolite with an immunoregulatory effect and its concentration in blood of a neonate is elevated. It is formed in two-step cleavage of extracellular ATP done by two distinct ectonucleotidases (CD73 and CD39). In healthy individuals, the extracellular ATP level is within the range 28 to 64 nmol/l [6]. The adenosine production is an interplay of at least 2 immune cell types. Does adenosine have an influence on the immune cell function?

1.1 Neonatal sepsis

Term- and preterm newborns, particularly those born before 32 week of completed gestation [7], [8], are more susceptible to infections in comparison to the children and adults. Every third preterm newborn with birth weight lower than 1500 gram is affected by systemic infection (sepsis) [9]. According to Shane et al. there are two major risk factors predisposing to infection which may lead to sepsis. The first is prematurity of the infant and the second one is the low birthweight. Comparing the preterm and full-term normal birthweight infants, the incidence of infection was 3- to 10-times higher in preterm infants of low birthweight. Further, immune system dysfunction as consequence of prematurity in combination with absence of maternal IgG, acquired transplacentally, increased the risk of infection [10].The neonatal sepsis is worldwide the main cause of perinatal morbidity and mortality. The

lethality of the Early-Onset Sepsis is 35 – 37% among the newborns with birth weight lower than 1500 gram [11], [12]. Leading causes are group B streptococci, *Staphylococcus epidermidi*s and *E.coli* [13], [14], [15], [16], [17] According to Wynn et al. early-onset sepsis is characterized by appearance of clinical manifestations within the first 72 hours of life [18]. Early onset infections are usually vertically transmitted via mother-to-infant-transmission. In contrast, late-onset infections appears after 3 to 7 days postnatal as a consequence of interaction with hospital environment or community, respectively [10]. As postulated by Adams-Chapman, there is a clear relationship between gestational age and susceptibility to infection – those born earliest are most vulnerable [19]. Furthermore, systemic infectious diseases during perinatal and neonatal period pose a risk to secondary diseases, e.g. bronchopulmonary dysplasia (BPD) or periventricular leukomalacia (PVL) affecting newborns and preterm newborns, in particular. Clinical data show that the risk of BPD or PVL development is more than 2-fold higher in case of intrauterine or perinatal infection, [20], [21], [22], [23]. Pathophysiology of both, BPD and PVL, seems to be based on the prolonged activity of pro-inflammatory cytokines [24], [25]. Molecular mechanisms as well as the immune reaction regulation on the cellular basis still remain elusive.

1.2. Fetal and neonatal immune system

Previous paradigm that embryo develops under sterile conditions has changed over time. Already in 2013, Stout et al. postulated that endometrium of nonpregnant uterus might harbor microbes [26]. As further showed by Stout et al. placentas are quite often colonized by bacteria (27% of 195 investigated placentas). Moreover, intracellular bacteria were detected within the placental basal plate [26]. Aagard et al. showed that uterus and the developing fetus are colonized by low number of microorganisms showing large variety. These bacterial communities are formed by nonpathogenic commensals – *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Fusobacteria* and *Bacteroidetes*. The composition of these communities is unique for each individual and depends on the gestational age. As shown by Aagard et al.

there are strong dissimilarities in bacterial composition of stool and vagina thus bacterial colonization of uterus and placenta cannot be a consequence of contamination. As further showed by Aagard et al. placental microbiome resembles that of oral cavity [27].

Despite of the bacterial load fetal immune system needs to be subdued to prevent any undesirable interaction with maternal immune system. To perform this task several mechanisms of fetal immune system are available – T_H2 polarisation – even an inhibition of T_H1 responses through T_H2 cytokines secretion [28], production of anti-inflammatory cytokine (IL-10), locally restricted expression of tolerogenic molecules (HLA-G) – e.g. extravillous trophoblasts [29], elevated adenosine plasma concentration and presence of immunosuppressive cells – Myeloid-derived suppressor cells (MDSC) [30], [4]. Last mentioned are highly enriched in the cord blood providing T cell proliferation inhibition and control of NK cell cytotoxicity and thus contributing to inhibition of rejection and tolerance induction [4]. Additionally, granulocytic MDSC express receptors for HLA-G. Interaction of MDSC with HLA-G enhances their suppressive character. Moreover, HLA-G does exist in soluble form not only increasing suppressive activity of granulocytic MDSC already present, but inducing MDSC from peripheral blood mononuclear cells (PBMC) [31].

The switch from immunosuppressive to immunocompetent status is challenging. Previous studies have shown that immune system is impaired during first postnatal days and thus neonates may prone to invasive infections [32],[33],[34]. Their ability to produce Ig seems to be diminished, according to Gathings et al. [35]. This was already shown by in vitro experiments showing reduced functionality of both T and B cells [36],[37]. Experiments done by Splawski et al., showed that anti-CD3 stimulation of cord blood T and B cells resulted in minimal number of Ig-producing cells. As further shown by Splawski et al. treatment with *Staphylococcus aureus* or addition of factors from mitogen-stimulated adult T cells enhanced the Ig production [38]. Additional studies have confirmed that neonatal B cells are not capable of antibody production [38]. First stimulation with CD3 and exogenous cytokines occurring simultaneously has led to induction of antibody production. Based on these

observations, the conclusion was done that neonatal T and B cells are either not capable of cytokine production or less responsive to cytokines [39]. Experiments done by Yu et al. also pointed out the immaturity of neonatal immune system. Possible explanation for this phenomenon may be cell population constitution distinct from that of adults, altered cytokine production as well as alteration in expression of distinct genes playing role in the immune response [40]. Reduced expression of adhesion molecules by neonatal neutrophils leads to reduced adhesion ability of these cells [41]. Additionally, elevated IL-6 levels in neonatal blood plasma also contribute to downregulation of neutrophil recruitment [42].

Studies done by Ueno et al. showed that neonatal monocyte population is comparable to that of adults [43] The difference is stronger production of IL-6, IL-8, IL-10 and TNFα by neonatal monocytes in response to different stimuli [44]. Several studies showed that monocyte production starts early in gestation (before 20 week) and that monocytes are highly abundant immune cell fraction (7 – 38%) of cord blood mononuclear cells of term infants [45], [46]. Investigations about the marker expression revealed three distinct monocytes – classic ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$) and nonclassic ($CD14^{+}/CD16^{+}$) [47]. Classic monocytes are the major population (>80%) in humans. Further, nonclassic and intermediate monocytes are closely related on the molecular basis, but nonclassic monocytes are capable of stronger proinflammatory cytokine (TNFα, IL1β) production [48]. According to the studies done by Quinello et al (2014) and Sharma et al. (2015) total amount of monocytes in preterm infants are similar to those in term infants [49], [50]. Experiments focusing on the pattern recognition receptor (PRR) expression revealed decreased amount of TLR 2 and 4 on preterm monocytes relative to monocytes from term infants [51]. Proinflammatory cytokines (TNFα, IL-6, IL-8) production in monocytes of preterm infants seem to be impaired [52]. It needs to be said that studies investigating monocyte cytokine release were performed using purified bacterial products, e.g. LPS and thus the results may not reflect the situation upon in vivo infection.

Further, neonatal NK cells also count to the affected cell population – their function in antibody-dependent cellular cytotoxicity is diminished in individuals younger than 1 year [53] Additionally, Ivarsson et al. have shown in their experiments that neonatal NK cells are more susceptible to the TGFβ-mediated suppression [54]. Another innate immune cell population are dendritic cells (DCs) sensing pathogen-associated molecular pattern via pattern recognition receptors and activating adaptive immune system. In comparison to the adults, neonatal DCs express lower amount of HLA-DR, ICAM and CD80/CD86 [55]. Further, the ability of neonatal DCs to produce IL-12 and IFNα upon stimulation is reduced [56]. To the adaptive immune system belong CD4- and CD8-positive T cells as well as B cells. According to Hyward (1984) and Burchett (1992) neonatal virus-specific $CD4^+$ T cells showed delayed IFNγ production upon infection with varicella-zoster virus or herpes simplex virus, respectively [57], [58] In contrast to $CD4^+$ T cells, neonatal cytotoxic T cells start to proliferate immediately as a response to primary infection but they are not capable of memory cell forming [59]. Further, humoral immunity of neonates within first months of life is provided by immunoglobulins acquired from mother during the intrauterine development. Last but not least, plasma components may play important role in the modulation of neonatal immune response. Neonatal plasma shows elevated adenosine concentration in comparison to adults (150.3 ± 64.5 vs. 52.0 ± 10.8 nM, $p<0.001$) [60]. Simultaneously, adenosine deaminase plasma concentration is diminished [61]. Another soluble component is L-arginine – an amino acid necessary for both innate and adaptive immunity regulating T cell proliferation and B cell development [62]. According to Yu et al. L-arginine levels in cord blood plasma are lower than those in adult blood plasma [63]. Additionally, Yu et al. have reported about higher abundance of arginase I in neonatal PMN and monocytes, an enzyme cleaving arginine to ornithine and urea [63], [64].

1.3. Myeloid-derived suppressor cells

Investigations on murine ovarian carcinoma done by Yang R et al. led to discovery of specific immunosuppressive $Gr1^+$ $CD11b^+$ myeloid cells [65]. As a result of extensive investigations, the term myeloid-derived suppressor cells (MDSCs) was coined in 2007 to describe non-lymphoid immune suppressor cell population of myeloid origin that was enriched in the cancer patients [66]. The first mentioning of MDSC in context of pregnancy appeared in 2011 in experiments with pregnant mice. Although, authors did not fully understand the function of MDSC in context of pregnancy at that time, they propose a shared mechanism of immune suppressiong occurring during gestation and tumor growth [67]. From today´s view, MDSCs are a subset of heterogeneous innate immune cells exerting suppressive activity on other immune cells [5] forming about 5% of all cord blood mononuclear cells [68]. They can be subdivided into two subgroups – monocytic (Mo-MDSC) and granulocytic (Gr-MDSC). The granulocytic (also known as neutrophilic) MDSC are characterized as cells expressing high amounts of CD66b and CD33, whereas monocytic marker CD14 and HLA-DR are not present [4]. Condamine et al. have described Lectin-type oxidized LDL receptor-1 (LOX-1) as another surface marker enabling distinguishing Gr-MDSC from neutrophils [69]. Moreover, Condamine et al. suggested that endoplasmic reticulum stress induction stimulates LOX-1 expression and conversion of neutrophils to Gr-MDSC [69]. Experiments, done by Sangaletti et al., revealed a novel marker - secreted protein acidic and rich in cysteine (SPARC) - enabling characterization of MDSC in both humans and mice. Genetic deletion of SPARC led to reduced immune suppression, Experiments with $Sparc^{-/-}$ mice unravel the molecular mechanism - translocation of NFκB p50 subunit into nucleus was reduced and thus formation of p65:p50 inflammatory heterodimers became enhanced. As one of the consequences of this process, high amount of TNFα were produced skewing the MDSC phenotype toward an inflammatory, anti-tumor phenotype [70]. As reported by Gantt et al. Mo-MDSCs are able to develop into mature monocytes and dendritic cells [68]. The number of granulocytic MDSC is elevated in the cord blood independent of the gestational age and remains elevated in the

peripheral blood of neonates for next 4 weeks after birth [71]. Gr-MDSC, quantitatively dominant over Mo-MDSC [72], are present in the cord blood and induce tolerogenic state by skewing the immune response towards T_H2 cell response and regulatory T cells induction [73, 74]. Additionally, Gr-MDSC are able to modulate the NK cell activity in cell contact-dependent manner [4]. Gr-MDSC precede the occurence of the regulatory T cells, which are recruited via CCR5 receptor in form of naïve CD4-positive cells [75], development of which is influenced by the presence of Gr-MDSC. Taken together, Gr-MDSC orchestrate the establishment of an immunocompromised state during the peri- and postnatal period.

1.4. Determinants of perinatal immune adaptation

The fetal´s environment radically changes with the event of birth, affecting parameters, which are well known to have an impact on immunological functions. Besides cessation of stimulation by gestational hormones such as estrogen and progesterone produced by the placenta after cord clamping, one major immunological important factor changes, .i.e. oxygen pressure (pO_2). While pO_2 is estimated to 10-30mm Hg in utero, it raises to up to over 90mm Hg immediately after birth [76]. Changing pO_2 is sensed by eukaryotic cells by a highly conserved mechanism via the transcription factor hypoxia-inducible factor 1 (HIF-1). The oxygen sensing subunit is HIF-1-α, which is constitutively expressed and immediately degraded in the presence of oxygen, while accumulates during hypoxia. This process is iron-dependent, thus iron-depletion by chelation may lead to HIF-1-α accumulation. The impact of raising pO_2 for the immunological switch from fetal to neonatal state of function is still unclear.

1.5. Adenosine

As already mentioned in the chapter fetal and neonatal immune system, adenosine is a soluble component playing an important role in the immune response modulation. Its concentration in neonatal plasma is 3-fold higher than in plasma of adults (150.3 ± 64.5 vs. 52.0 ± 10.8 nM, $p<0.001$) [60]. This may be due to diminished concentration of adenosine

deaminase (ADA) in neonatal blood plasma, as discovered by Pettengill [61]. Adenosine as an endogenous metabolite with immunomodulatory properties is formed by placenta acting as a vasodilator [60]. Further, it plays an important role in the fetal and neonatal development. Levy et al. postulated that adenosine can cross the placenta and enter the fetal circulation [60]. To form adenosine from extracellular adenosine triphosphate, two enzymes – ectonucleotidases – are necessary. Synnestvedt et al. found that CD73 expression is enhanced under hypoxic conditions in time-dependent manner [77]. Both ectonucleotidases are ubiquitously expressed on the surface of somatic cells and its enhanced expression can be induced under distinct circumstances. Through their activity adenosine will be produced and thus an immunosuppressive microenvironment formed [78]. The extracellular adenosine concentration varies within the range <1μM in healthy adult persons up to 100μM detected in the patients suffering under rheumatoid arthritis. [79], [80]. As a metabolite, it could influence the function of adjacent cells by binding to the corresponding receptors [81]. It has a negative effect on the activation, proliferation and expansion of T cells [78], [82]. Adenosine binds to corresponding adenosine receptors belonging to the group of G protein coupled receptors, e.g. A_{2A} and A_3, whereas the A_3 is the only adenosine receptor overexpressed on the inflammatory cells [83]. Bono et al. postulated that A_{2A} receptor inhibits the T_H1 / T_H2 differentiation by decreasing naïve T cell proliferation as well as IL-2 production [78].

1.6. Adenosine receptors

Nowadays, we distinguish 4 purinergic P1 receptors (A_1, A_{2A}, A_{2B}, A_3) belonging to the class A (rhodopsin-like) G-protein coupled receptor superfamily [84], differentially expressed on the cell surface of monocytes, dendritic cells and cells [85]. A_1, A_{2A} and A_{2B} receptors are highly conserved across mammalian species (>80% identity) [86]. A_1 and A_3 receptors are preferentially coupled with G_i/G_o proteins inhibiting adenylyl cyclase and cAMP production [87]. Contrary, A_{2A} and A_{2B} are generally coupled with Gs proteins inducing the adenylate cyclase activity leading to cAMP accumulation. Accumulation of cAMP results in the

activation of protein kinase A (PKA) [88], [89]. Through A_{2A} receptor - adenylate cyclase – cAMP – PKA axis the CREB-mediated inhibition of NFκB takes place [90]. Simultaneously, activated CREB induces the transcription of CEBPβ gene [91]. The CEBPβ gene product may influence the production and release of anti-inflammatory IL-10 which in turn suppresses cytokine secretion, antigen presentation and $CD4^+$ T cell activation [92].

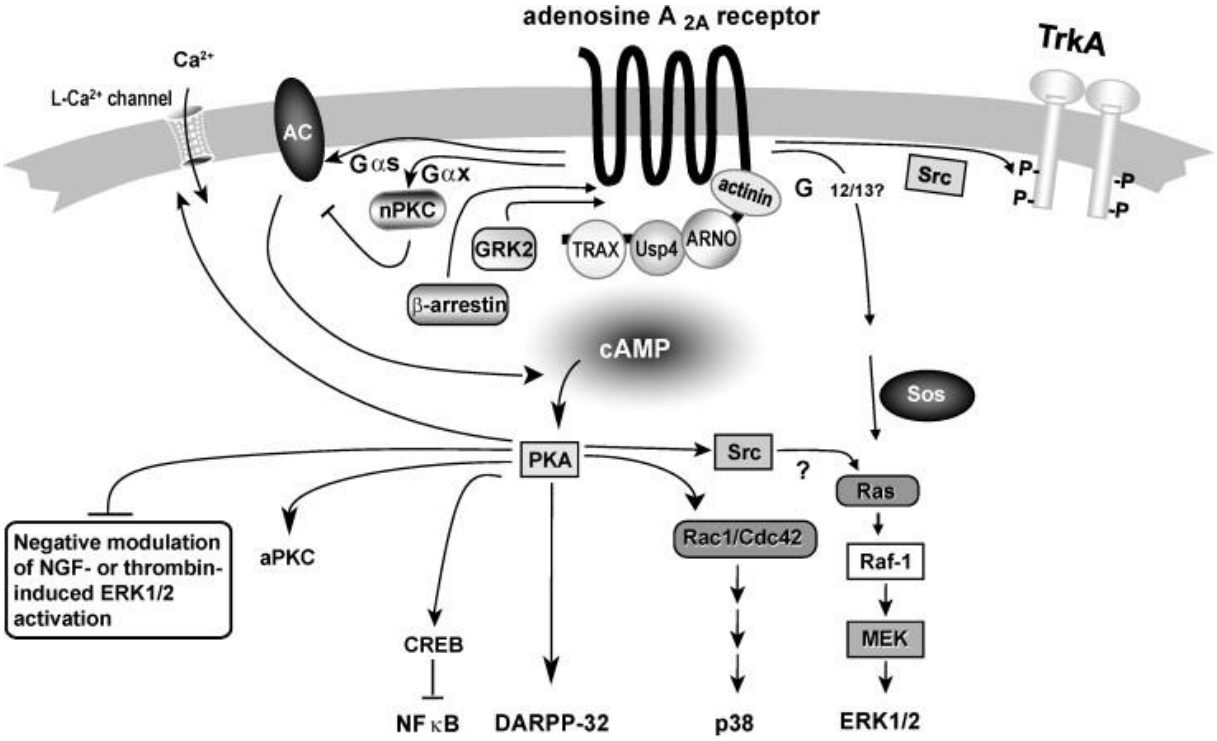


Figure 1. Signaling pathway of A_{2A} receptor. According to Fredholm et al. [90]

A_{2B} receptors, additionally, can also couple with Gq proteins leading to activation of PLC pathway [93], [89], [94]. Further, it was shown that all four adenosine receptors are involved in MAPK and JNK pathway [94]. Adenosine as an end product of ATP metabolism can bind to each of these receptors inducing stimulatory (A_{2A} and A_{2B}) or inhibitory (A_1, A_3) signal within the cells. Adenosine binding to A_1 or A_3 receptor inhibits adenylyl cyclase which in turn leads to decrease of intracellular cAMP [78]. Contrary, A_{2A} and A_{2B} stimulate cAMP production upon adenosine binding. Higher cAMP concentration leads to protein kinase A (PKA) activation and induction of suppressive character of distinct immune cells, e.g. monocytes, dendritic cells, T cells [78]. Appropriate agonist, e.g. adenosine, binds to A_{2A} receptor during T cell activation resulting in regulatory T cells expansion exerting strong

regulatory activity [78]. Additionally, adenosine-based stimulation of A_{2A} receptor led to cessation of T cell receptor-mediated activation of Akt pathway and downregulation of T cell differentiation [78]. According to Bono et al. A_{2A} receptors are expressed by readily activated T cells (both, CD4 and CD8). If the A_{2A} receptors are missing, T cells are lacking in the peripheral tissue and they are deficient in the differentiation into effector / memory cells [78].

1.7. Ectonucleotidases – CD73 and CD39

Extracellular ATP is cleaved in a two-step process by ubiquitously expressed membrane-bound ectonucleotidases. Orchestrated action of CD39 and CD73 leads to adenosine production and formation of immunosuppressive microenvironment [78] Further, as postulated by Bono et al. variable expression levels of both ectonucleotidases may be detected in several organ, e.g. kidney, colon, liver, lung, placenta, brain [78].

CD39 is a prototype NTPDase, with high affinity to both ATP and ADP [84]. It is formed by four highly conserved domains involved in the active site formation [84]. Interestingly, two transmembrane domains anchoring the ectonucleotidase in the plasma membrane also play an important role in the maintaining of catalytic activity and substrate specificity [95], [96]. It cleaves ATP and ADP to adenosine and thus contributes to the immune response balance [84],[97], [98].

In human peripheral blood, CD39 is constitutively expressed on B cells (>90%), monocytes (>90%), $CD4^+$ T cells (20 – 30%, including memory and Tregs), $CD8^+$ T cells (<5%) and NK cells (2.5%) [84].

Expression of CD39 can be induced upon exposure to proinflammatory cytokines, oxidative stress and hypoxia (Longhi, unpublished). Interestingly, Borsellino et al. detected strong CD39 expression in human $CD4^+$ $Foxp3^+$ T cells. Thus, CD39 might be used as a marker for detection and purification of regulatory T cells from peripheral blood of healthy adults and patients [99]: Additionally, further investigations indicate that the proportion of $CD4^+$ $Foxp3^+$

$CD39^+$ T cells changes during the life [100] and is strongly dependent on physiological status of an individual [99], [101].

Further, CD39 may be used as an indicator of immune cell fitness. As reported by Gupta in chronic viral infections terminally exhausted $CD8^+$ T cells expressed high levels of CD39 [102]. Additionally, ectonucleotidase CD39 may be expressed in various tissues – its mRNA was detected in placenta, lung, skeletal muscle and heart [103].

CD73, a zinc-dependent ectonucleotidase [104] cleaving AMP to adenosine and inorganic phosphate [105], [106] is organized in 3 domains – N-terminal domain with metal (Zn^{2+}) binding site, C-terminal domain with catalytic site and one short alpha-helix bridging the N- and C-terminal domains [84]. Ectonucleotidase CD73 also exists in a soluble form upon shedding of the GPI anchor, whereas the functionality remains unchanged [107],[108]. For correct function, CD73 needs to form a homodimer stabilized by hydrophobic interactions between C-terminal domains. Correct catalytical activity is provided upon binding of two divalent Zinc-ions. Interestingly, zinc ions are not only necessary for correct function of CD73, but even zinc deficiency may be the cause of altered cellular immune responses, e. g. T-helper cell activity, cytotoxic T cell development [109], [110]. Moreover, zinc deficiency was shown to lead to downregulation of CD73 on $CD8^+$ T cells [111].

To cleave AMP to adenosine and inorganic phosphate, the conformation has to cycle through open and closed conformational states [84].

Ectonucleotidase CD73 is less frequently present on the immune cell surface – 75% of B cells, 50% of $CD8^+$ T cells, 10% of $CD4^+$ T cells and 2 – 5% of NK cells [112].

Its expression on $CD4^+$ T cells in human PBMCs can be dose-dependently increased with endogenous TNFα [113]. Additionally, TGFβ as anti-inflammatory cytokine induce strong upregulation of CD73 on conventional T helper cells and cytotoxic T cells [114].

Moreover, presence of functional CD73 on cytotoxic T cells is required for correct antigen recognition, proliferation and cytolytic programm induction upon antigen recognition [115].

According to Synnestvedt et al. (2002) environmental factors such as hypoxia may also have an effect on the CD73 expression. As Synnestvedt further showed hypoxia induced the CD73 expression on both – mRNA and protein level showing time-dependent increase with the maximum protein level after 48 hours [77].

1.8. Adenosine triphosphate

Adenosine triphosphate (ATP) as a molecular unit of currency is retained by distinct cellular mechanisms within the cytoplasma, thus its concentration lies within millimolar range [78], [116]. Contrary, extracellular ATP (eATP) concentration is ~10nM [116]. Despite of very low concentration, eATP is an important mediator of cell-cell communication in physical setttings, e.g. low concentration of eATP indicates presence of living cells in the neighbourhood [117]. Accurate measurement of ATP concentration in human plasma was done by Gorman et al. in 2007. They showed how necessary it is to prevent ATP release from platelets and red blood cells as well as to stop the catabolism of extracellular ATP by ectonucleotidases. For this purpose, Gorman et al. developed stabilizing solution used in the process of blood processing. ATP concentration of red blood cell lies within the millimolar range, thus hemolysis is another factor influencing the measurement [6]. Nanomolar range of the extracellular ATP is recognized as a danger signal indicating an inflammation. The extracellular ATP can be released into extracellular space as a consequence of a stress (hypoxia, necrosis, inflammation) [118] and its concentration can rise up to 3 – 5 mM at the site of damage [119]. Moreover, Elliot et al. showed in their experiments that ATP can be recognized by highly motile monocytes and macrophages as long-range “find me” signal released by apoptotic cells [120]. As Bono et al. postulated ATP can be released from immune effector cells at the site of damage upon activation via Pannexin 1 channel [78]. Upon release, ATP and related nucleotides bind to appropriate purinergic P2 receptors inducing wide range of physiological responses, e.g. induction of IL-2 production, upregulation of cytotoxicity of T cells as well as skewing the differentiation towards pro-

inflammatory T_H17 cells and simultaneous dampening of Tregs differentiation [121]. Low eATP concentration can be detected by P2 receptors of high or intermediate affinity (EC50 < 20μM), whereas high eATP concentrations can be detected by P2X7 (EC50 >100μM) [116]. Purinergic P2 receptors are present on the surface of almost all cell types [122]. P2 receptors are subdivided into two families: P2Y receptors belonging to the group of G protein-coupled receptors comprise 8 distinct subtypes [123]. The group of ligand-gated ion channels comprises 7 P2X subunits which can form homo- and heterodimers [124]. Activity of P2X7 can be modulated by ENTPDase 1 (CD39) expressed on the cell surface of macrophages and degrading ATP to AMP. This might contribute to the fact that P2X7 is activated by higher ATP concentration than the other P2X receptors [125]. According to Barankiewicz et al. extracellular ATP catabolism may serve as a mean of communication between T and B cells in lymphoid organs [126].

1.9. Aim of the study

The aim of the present study was to determine whether immune cells from cord blood are able to express ectonucleotidases and thus form adenosine. Especially, it was focused on the cord blood MDSC. Further, effect of adenosine on other immune cells was analysed. Furthermore perinatal determinants of adenosine-pathway-function were evaluated, focusing on the influence on changing pO_2. We hypothesized that (1) ectonucleotidases would be expressed by neonatal immune cells, (2) anoxia may stimulated ectonucleotidase expression, (3) PAMPs may upregulate ectonucleotidase expression, (4) adenosine receptors are enhanced expressed on neonatal immune cells and (5) adenosine is capable to induce and activate MDSC.

To answer these questions, cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) from adult healthy non-pregnant individuals were taken and an in-vitro Anoxia culture modell was exploited.

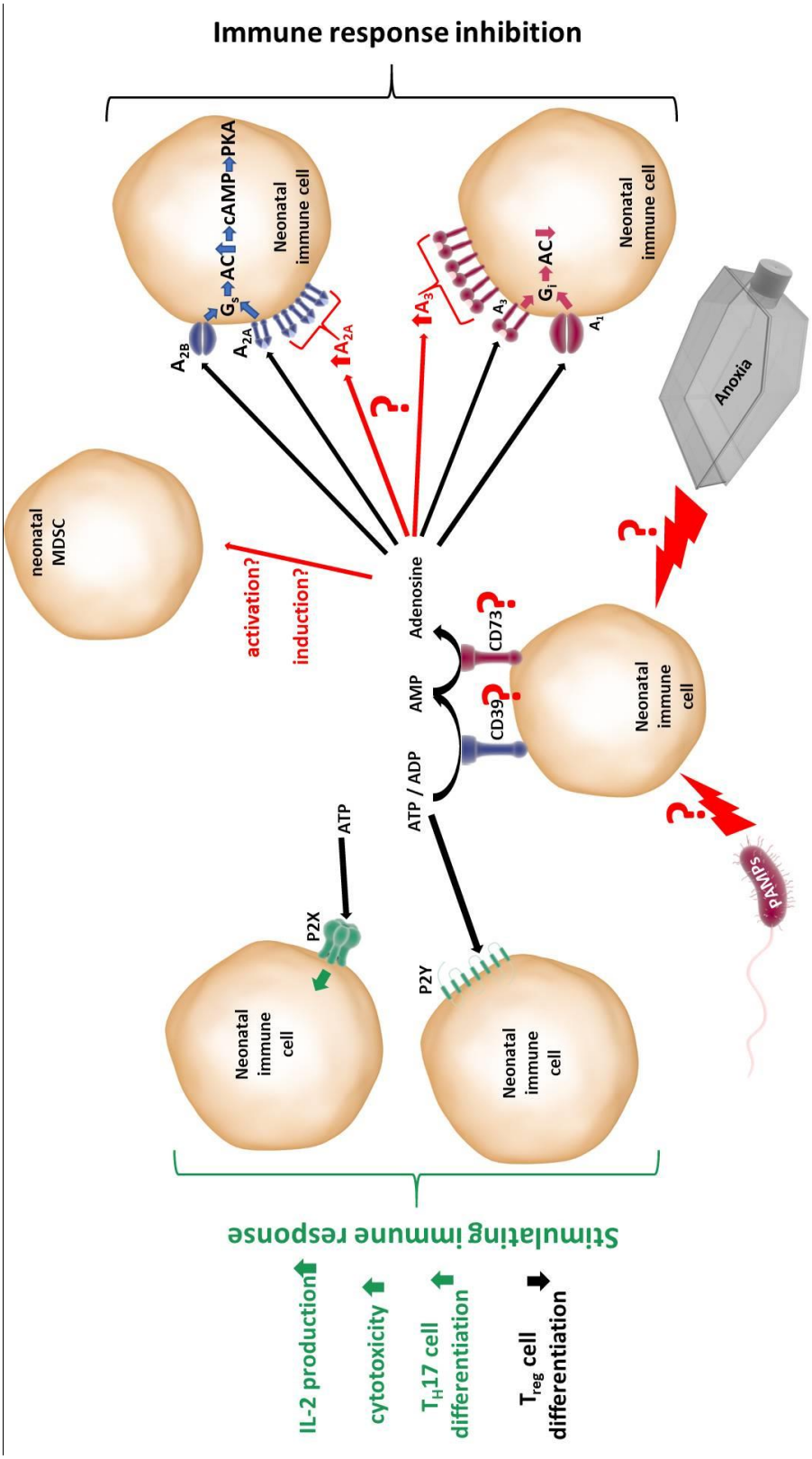


Figure 2. Mode of action of adenosine and ATP - proposed scheme

2. Material

2.1. Antibodies

A_{2A} adenosine receptor	AF647	(clone 7F6-G5-A2)	Santa Cruz Biotechnology, Heidelberg, Germany
A_3 adenosine receptor	PE	(polyclonal)	Biozol, Eching, Germany
ArgI	PE	(clone 658922)	R&D, Wiesbaden,Germany
CD3	FITC	(clone UCHT-1)	BD Biosciences,Heidelberg, Germany
CD3	FITC	(clone REA613)	Miltenyi Biotec, Bergisch Gladbach, Germany
CD3	PerCP	(clone SK7)	BD Biosciences, Heidelberg, Germany
CD4	APC	(clone RPA-T4)	BD Biosciences, Heidelberg, Germany
CD8	PE	(clone SK1)	BD Biosciences, Heidelberg, Germany
CD14	APC	(clone MϕP9)	BD Biosciences, Heidelberg, Germany
CD14	FITC	(clone MϕP9)	BD Biosciences, Heidelberg, Germany
CD14	FITC	(clone TÜK4)	Miltenyi Biotec, Bergisch Gladbach,Germany
CD33	PE	(clone WM53)	BD Biosciences, Heidelberg, Germany
CD39	PE Vio770	(clone REA739)	Miltenyi Biotec, Bergisch Gladbach, Germany

CD66b	FITC	(clone G10F5)	BD Biosciences, Heidelberg, Germany
CD66b	FITC	(clone REA306)	Miltenyi Biotec, Bergisch Gladbach, Germany
CD73	PE	(clone AD2)	BD Biosciences, Heidelberg, Germany
IDO	PE	(clone 700838)	R&D, Wiesbaden, Germany
iNOS	AF647	(clone C-11)	Santa Cruz Biotechnology, Heidelberg, Germany
antibody against CD3	***	(clone OKT3)	ThermoFischer Scientific, Darmstadt, Germany
ZAP70	R-PE	(clone 1E7.2)	ThermoFischer Scientific, Darmstadt, Germany
CD33 MicroBeads	***	***	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-FITC MicroBeads	***	***	Miltenyi Biotec, Bergisch Gladbach, Germany

2.2. Biological Material

Adenosine	Sigma, Steinheim, Germany
Bovine Serum Albumin	Sigma, Steinheim, Germany
Brefeldin A	BD Biosciences, Heidelberg, Germany
Detachin	Genlantis, San Diego, USA
FCS	Biochrom, Berlin, Germany
L-Glutamine	Biochrom, Berlin, Germany
LPS	Sigma, Steinheim, Germany
LTA	Sigma, Steinheim, Germany
Pam3Cys	EMC microcollections, Tübingen, Germany
Penicillin / Streptomycin	Biochrom, Berlin, Germany
recombinant IL-2	R&D Systems, Wiesbaden, Germany
recombinant GM-CSF	PeproTech, Hamburg, Germany
RPMI-1640	PAN Biotech, Aidenbach, Germany

2.3. Solutions

Ampuwa	Fresenius Kabi, Bad Homburg, Germany
Cell pack 20l	Sysmex, Norderstedt, Germany
Cytofix / Cytoperm	BD Biosciences, Heidelberg, Germany
DAPI (4´,6-diamidino-2-phenylindole)	ThermoFischer Scientific, Darmstadt, Germany
Descosept AF	Dr. Schumacher, Malsfeld, Germany
DPBS	PAN Biotech, Aidenbach, Germany

Ethanol (96%)	Sigma, Steinheim, Germany
FACSFlow™, sheath fluid 20l	BD Biosciences, Heidelberg, Germany
Heparin-Natrium-5000 I.E./0,2ml	Ratiopharm, Ulm, Germany
Paraformaldehyde (4%)	ThermoFischer Scientific, Darmstadt, Germany
Pancoll human	PAN Biotech, Aidenbach, Germany
Perm / Wash solution	BD Biosciences, Heidelberg, Germany
Pro Long Antifade Mountant for fixed cells	ThermoFischer Scientific, Darmstadt, Germany
Softasept N	B.Braun Melsungen AG, Melsungen, Germany
sterile isotonic NaCl solution 0.9	Fresenius Kabi, Bad Homburg, Germany
Trypan Blue solution T8154-20ml	Merck KGaA, Darmstadt, Germany
Tween 20	Sigma, Steinheim, Germany

2.4. Machines

AccuJet	Brand, Wertheim, Germany
AutoMACS Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Benchtop centrifuge Biofuge fresco	Heraeus Instruments, Stuttgart, Germany
Binocular	Olympus, Hamburg, Germany
Cell-Quest software	BD Biosciences, Heidelberg, Germany
Cytospin 2 Shandon	Marshall Scientific, Hampton, New Hampshire, USA
Centrifuge Rotanta 460 RS	Hettich, Bäch, Schweiz

Digital camera DXM 1200	Nikon Instruments Europe BV, Amsterdam, Netherlands
FACS Calibur	BD Biosciences, Heidelberg, Germany
Freezer -80C Telstar Igloo	Telstar, Élancourt, France
Fridge Comfort NoFrost	Bosch, Stuttgart, Germany
Incubator	Heraeus Instruments, Stuttgart, Germany
Incubator	Binder GmbH, Tuttlingen, Germany
Inverted microscope Olympus IX83	Olympus, Hamburg, Germany
Light microscope Olympus BX60	Olympus, Hamburg, Germany
MS1 Minishaker	Ika, Staufen, Germany
Sterile bench	Heraeus Instruments, Stuttgart, Germany
Sysmex KX-21N	Sysmex GmbH, Norderstedt, Germany
Waterbath	GFL GmbH, Burgwedel, Germany

2.5. Non-biological material

50 ml Conical Centrifuge Tube	Greiner bio-one, Frickenhausen, Germany
15ml Conical Centrifuge tube	Greiner bio-one, Frickenhausen, Germany
1,5ml Microcentrifuge Tube	Eppendorf Hamburg, Germany
2ml Microcentrifuge Tube	Eppendorf Hamburg, Germany
5ml Strippette	Corning Inc., Wiesbaden, Germany
10ml Stripette	Corning Inc., Wiesbaden, Germany

25ml Strippette	Corning Inc., Wiesbaden, Germany
6-well-plate	Corning Inc., Wiesbaden, Germany
12-well-plate	Corning Inc., Wiesbaden, Germany
24-well-plate	Corning Inc., Wiesbaden, Germany
96-well-plate (flat bottom)	Corning Inc., Wiesbaden, Germany
96-well-plate (round bottom)	Corning Inc., Wiesbaden, Germany
Cell Scraper	Corning Inc., Wiesbaden, Germany
Disposal Bags, PP autoclavable	Brand, Wertheim, Germany
FACS Tubes	Corning Inc., Wiesbaden, Germany
Gilson Pipette	Abimed, Langenfeld, Germany
Medical Examination Gloves (Nitrile, powder-free)	Abena, Aabenraa, Denmark
Pasteur Capillary Pipettes (long size 230mm, glas)	ISG Intermed Service GmbH & Co. KG, Geesthacht, Germany
Pippette Tips 10µl (colourless)	Biozym Biotech, Vienna, Austria
Pippette Tips 100µl (yellow)	Greiner bio-one, Frickenhausen, Germany
Pippette Tips 1000µl (blue)	Greiner bio-one, Frickenhausen, Germany
Pur-Zellin	Hartmann, Wiener Neudorf, Austria
Safety Multifly 21G 200mm long	Sarstedt, Nürnbrecht, Germany
Single Use Syringes	B.Braun Melsungen AG, Melsungen, Germany

3. Methods

3.1. Blood collecting and PBMC isolation

Blood samples from healthy adult individuals as well as a cord blood were collected in tubes pre-filled with sterile phosphate buffered saline and heparin (100 U/ml). Mononuclear cells were isolated applying Biocoll density gradient centrifugation according to Boyum [127], [128]. Briefly, blood (diluted with sterile 1xPBS in ratio 2:1) was stacked in 50 ml tubes pre-filled with Biocoll and spinned down for 25 minutes, 400 g at room temperature without brake. After the first centrifugation step has finished, blood plasma was taken up by sterile 5ml strippette and stored for later use. The cloudy ring, containing mononuclear cells, was taken up gently with a sterile 5 ml stripette and transferred into a new sterile 50 ml tube followed by adding sterile 1x PBS up to 50 ml. Second centrifugation step ran for 10 minutes at 10°C and 500 g with brake to remove residual Biocoll solution completely. After the second centrifugation step has finished, supernatant was discarded and pellet resuspended in a sterile 1x PBS and filled up to 10 ml. An aliquot (100 µl) was taken to count the cells on Sysmex, whereas the rest was spinned down for 10 minutes at 400 g and 4°C. The total cell count was calculated for 10ml based on the results shown by Sysmex.

Value according to Sysmex (cells/µl)	Cell count for 1ml	Cell count for 10ml
$5,6*10^3$	$5,6*10^6$	$5,6*10^7$

After the third centrifugation step the supernatant was discarded and the pellet resuspended in complete medium (RPMI + 10% FCS + 1% P/S + 1% L-glutamine) or in a sterile 1x PBS and diluted to $1*10^7$ cells/ml.

3.2. Detection of CD73 and CD39

PBMCs and CBMCs were isolated and diluted with complete medium (RPMI + 10% FCS + 1% P/S + 1% L-glutamine) to $2*10^6$ cells per ml were divided into two aliquots. One aliquot

was used for the immediate detection of both ectonucleotidases (t=0h). Other aliquot was put in a cell culture and let incubate at 37°C; 5% CO_2 under normoxy, anoxy or treated with deferoxamine (an iron ions chelating agens, $c_{end}=10^{-4}$ M, stock conc. 10^{-2} M), lipoteichoic acid (c_{end}=10 μg/ml), lipopolysaccharides (c_{end}=1 μg/ml) and Pam3Cys (c_{end}=10; 1; 0.1 μg/ml) for 24 or 48 hours, respectively. At given time points, cells were harvested and the expression of both ectonucleotidases (CD73, CD39) was examined by flow cytometry.

3.3. Detection of adenosine receptors

Mononuclear cells isolated from both adult and cord blood samples and diluted with a complete medium (RPMI + 10% FCS + 1% P/S + 1% L-glutamine) to $2*10^6$ cells per ml were divided into two aliquots. One aliquot was used for the immediate detection of adenosine receptors (t=0h). Other aliquot was put in a cell culture and let incubate at 37°C; 5% CO_2 under normoxy, normoxy with adenosine (100 μM) and normoxy with Pam3Cys (10 μg/ml). The adenosine receptor expression was examined by flow cytometry at two distinct time points - 24 and 48 hours, respectively.

3.4. T cell proliferation

PBMCs were resuspended in a sterile 1x PBS and diluted to $1*10^7$ cells per ml and stained with CFDA SE, prepared as follows: 2 μL CFDA SE diluted in 100 μl sterile 1x PBS. Diluted CFDA SE (25 μl) was added to 1 ml cells suspension ($1*10^7$ / ml) and let incubate for 10 minutes in a darkness at room temperature followed by adding FCS (2 ml) to bind a residual free CFDA SE. After that the cell suspension was filled up to 6 ml with modified medium (RPMI + 10% autologous plasma + 1% P/S + 1% L-glutamine) and spinned down for 5 minutes at 310 g, with brake, 4°C. After the centrifugation step has finished the medium was discarded and cell pellet resuspended in modified medium (RPMI + 10% autologous plasma + 1% P/S + 1% L-glutamine). Cells were counted using Sysmex (automated hemocytometer) and diluted with modified medium to $1*10^6$ cells/ml. One aliquot of CFSE-stained cells was treated with OKT3 (c_{end} 10 μg/ml; stock 1 mg/ml) and IL-2 (c_{end}=100 U/ml) to stimulate the T

cell proliferation. Other aliquot remained untreated as a negative control. Both, unstimulated cells as well as stimulated cells were incubated in presence of adenosine of ascending concentration (0 - 1000 µM) at 37°C; 5% CO_2 for 4 days. On day 4, T cell proliferation was examined by flow cytometry.

3.5. MDSC induction and inhibition assay

Freshly isolated PBMCs were resuspended either in complete medium (containing 10% FCS) or in modified medium containing 10% adult plasma (autologous or heterologous) or 10% cord blood plasma, respectively. PBMCs were diluted to $5*10^5$ cells per ml. Cell culture ($2.5*10^6$ cells per well) was incubated one week long at 37°C; 5% CO_2 in presence of adenosine (100 µM), GM-CSF (1 ng/ml) or left untreated, respectively. Medium was changed on day 4. On day 7, cells were harvested applying prewarmed (37°C) Detachin™ (500 µl per well, incubation for 10 minutes at 37°C, 5% CO_2) and using a cell scraper to detach the cells from the plastic dish bottom and wash with sterile 1x PBS to wash out residual cells. The collected cells were examined for the CD33 expression (a marker of induced adult MDSC). The CD33-positive cells were isolated by AutoMACS using CD33-positive magnetic beads. An aliquot (10 µl) from each collected population was taken and stained with Trypan Blue and the cells were counted using hemacytometer and binocular. The cell number of each population was adjusted to $1*10^3$ per µl and put into a co-culture with freshly isolated CFSE-stained OKT3/IL-2-stimulated autologous PBMC in distinct ratios (2:1, 4:1, 8:1) and let incubate for 4 day in modified medium containing autologous plasma (RPMI + 10% autologous plasma + 1% P/S + 1% L-glutamine). On day 4, the T cell proliferation was examined by flow cytometry.

3.6. Detection of effector enzymes

PBMCs and CBMCs were diluted with complete medium (RPMI + 10% FCS + 1% P/S + 1% L-glutamine) to $2*10^6$ cells per ml and put in cell culture with or without adenosine (100 µM) and let incubate at 37°C; 5% CO_2 for 24, 48 and 72 hours, respectively. At given time points,

the cells were harvested and intracellular staining of effector enzymes was done according to the protocol. In brief, harvested cells were split into two aliquots (unstained negative control, lineage marker stained sample). The aliquot for lineage marker staining ($4*10^6$ cells) was treated with antibodies against CD3 (PerCP, 2 µl), CD14 (APC, 4 µl) and CD66b (FITC, 2 µl), respectively, and let incubate for 10 minutes at 4°C. After that a washing step was conducted by adding FACSFlow™ and spinning down (5 minutes, 310 g, 4°C), supernatant was discarded and pellet resuspended. In next step, the cells (together with the unstained negative control) were fixed and plasma membrane was permeabilized by adding Cytofix/Cytoperm solution and incubating the cell suspension in darkness at 37°C for 10 minutes. After that a Cytoperm/Cytowash solution was added and the cell suspensions were spinned down (5 minutes, 310 g, 4°C). After the centrifugation step was completed the supernatant was discarded and the pellet was resuspended in Cytoperm/Cytowash to preserve the plasma membrane permeabilized. The unstained negative control was put aside and the lineage marker stained sample was aliquoted into prepared FACS tubes containing antibodies against Arginase I (PE-conjugated, 3 µl), IDO (PE-conjugated, 5 µl) and iNOS (APC, 2 µl). As a positive control, one aliquot of lineage marker stained cell suspension was stained with antibody against Zap70 protein (PE-conjugated, 1:10 diluted, 5 µl). The tubes were incubated at 37°C for 10 minutes followed by washing step and centrifugation (5 minutes, 310 g, 4°C). After the centrifugation step was completed, supernatant was discarded and pellet resuspended and cell suspension investigated by flow cytometry.

3.7. Detection of adenosine A_{2A} receptors by immunofluorescence

Purified CBMC and PBMC were cultivated for 24 hours under following conditions – untreated or in presence of adenosine [100µM]. After 24 hours, cells were harvested by centrifugation (5 minutes, 310 g), supernatant was discarded and cell pellet resuspended. To do immunofluorescence cell suspension (10 µl) was resuspended with 1x PBS (195 µl) and

the cytospin was done (120 x 10 g, 3 minutes) using Cytospin 2 Shandon and cover slips 9mm. On next day, cells on cover slip were fixed with 4% paraformaldehyde for 10 minutes, washed 3-times with 1x PBS + Tween (0.1 %). In next step, blocking agent (4% bovine serum albumin in 1x PBS + 0.1% Tween) was added and samples were incubated for 30 minutes. Antibodies against CD3 FITC and A_{2A} receptor AlexaFluor 647 (each antibody – diluted in 4% bovine serum albumin in 1x PBS + 0.1% Tween, dilution rate 1:50) were applied and samples were incubated for 2 hours. DAPI (4´,6-diamidino-2-phenylindole) was used for nucleus staining. After that, samples were washed with Et-OH briefly and embedded with Pro Long. Prepared samples were investigated under microscope using following parameters - Light: DAPI – 0.06, FITC – 0.8, AlexaFluor 647 – 0.18; Exposure: DAPI – 0.03 sec., FITC – 0.4 sec., AlexaFluor 647 – 0.1 sec., Gain – DAPI, FITC, AlexaFluor – 1.0.

3.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Group comparisons were done by student´s t-test. A p-value of <0.05 was considered statistically significant,

4. Results

4.1. CD39 is dominantly expressed by monocytes and CD73 by T cells

To evaluate the possibility that neonatal immune effector cells may autonomously produce adenosine, we first asked for their ectonucleotidase expression. CD39 expression on T cells from cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) was low (Figure 3a). However, 85 ± 5% of cord blood and 86 ± 5% of adult monocytes expressed CD39 with moderate densities (22 ± 10 MFI vs. 23 ± 10 MFI, n=7-11, p=0.48, Figure 3b). Cord blood MDSC expressed CD39 at low percentages (2 ± 0.5%) and at low levels (7 ± 2.5 MFI, Figure 3c) while adult MDSC showed stronger expression (15 ± 5%, 10 ± 2.5 MFI, n=9, $p<0.01$, Figure 3c). CD73 was expressed mainly by T cells (Figure 4). Cord blood T cells showed stronger expression (30 ± 10%; 7 ± 2.5 MFI) than adult T cells (22 ± 2.5%; 5 ± 1.0 MFI; n=7-11, $p<0.05$, Figure 4a). Cord blood and adult blood monocytes both showed low CD73 expression (8 ± 5.0%, 3 ± 1 MFI and 2 ± 0.5%, 3 ± 0.5 MFI, n=7-10, $p<0.05$ Figure 4b). The CD73 expression by cord blood MDSC was weaker (2 ± 0.5%, 5 ± 0.5 MFI, n=7) than that of adult MDSC (10 ± 8%, 5 ± 1 MFI, n=10, $p<0.01$, Figure 4c). Taken together the results showed a differential expression of ectonucleotidases in neonatal immune effector cells hinting towards a co-operation of at least 2 different immune cell types to synthetize adenosine, i.e. CD39-positive monocytes cleaving ATP and ADP to AMP and CD73-positive T cells cleaving AMP to adenosine. Myeloid-derived suppressor cells (MDSC) express both ectonucleotidases at low level and their contribution to the adenosine production may rather be low. (Ďurčo et al. submitted).

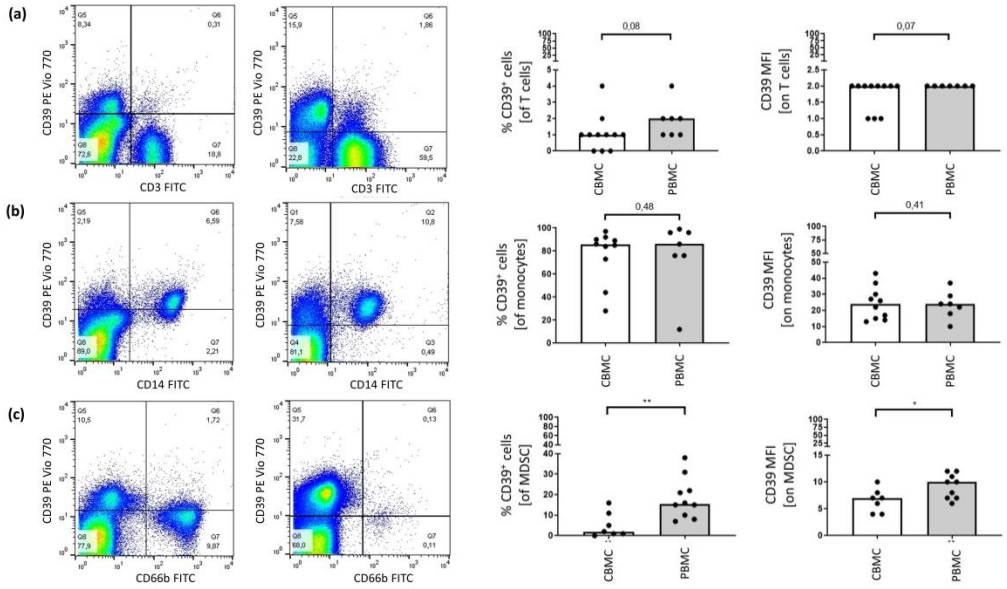


Figure 3. CD39 expression on freshly isolated cord blood and peripheral blood immune effector cells. *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated followed by staining for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Given are representative density plots, mean fluorescence intensities (MFI) and percentage of positive cells (%). CBMC n=11, PBMC n=7, * = p<0.05, ** = p<0,01 (Ďurčo et al. submitted)*

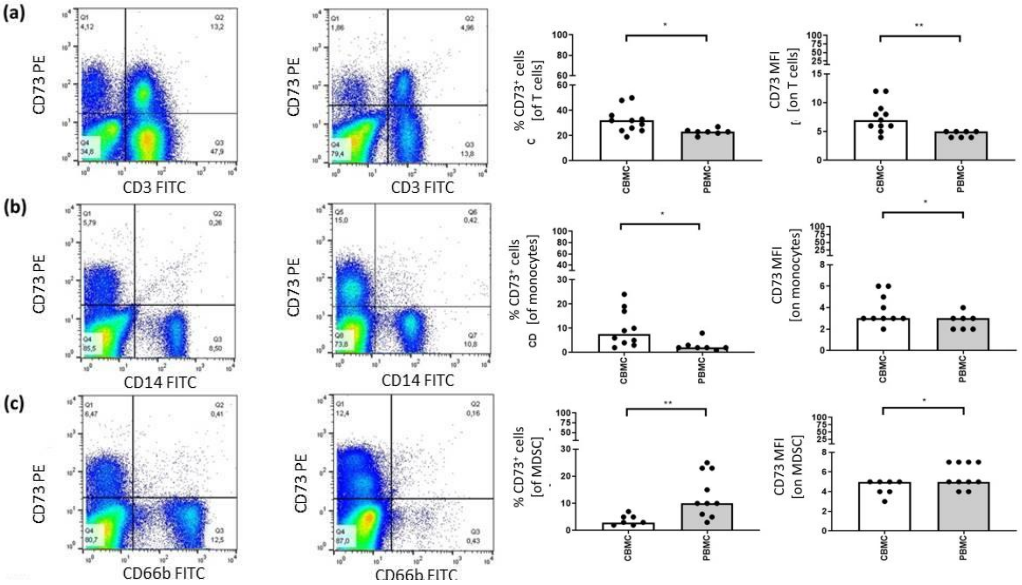


Figure 4. CD73 expression on freshly isolated cord blood and peripheral blood immune effector cells. *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated followed by staining for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Given are representative density plots, mean fluorescence intensities (MFI) and percentage of positive cells (%). CB n=7-11, PB n=7-10, * = p < 0.05; ** = p < 0.01, (Ďurčo et al. submitted)*

4.2. Anoxia downregulated the CD39 expression on adult monocytes, contrary DFO upregulated CD39 expression by cord blood MDSC

Influence of low oxygen pressure (<1% O_2) or presence of bivalent iron ions chelator (deferoxamine) as HIF-1-α inductor was investigated by incubation of CBMC and PBMC for 24 and 48 hours. The expression of CD39 by T cells (both cord blood and adult blood) remained low under normoxic as well as anoxic conditions (4 ± 2.5% vs. 4.5 ± 3%, n=11-18, p=0.48, Figure 5.2a) at very low densities (2 ± 4 MFI, n=11-18, p=0.11, Figure 5.2a). Similarly, DFO had no great impact on the expression of CD39 by T cells (4 ± 4%, 2 ± 1 MFI, n=11-18).

The CD39 expression by cord blood monocytes showed very slight changes dependent on conditions. Under normoxic conditions and in presence of DFO 95 ± 5% of all cord blood monocytes were positive for CD39 (n=11-18, p<0.01, Figure 5.2b), expressing moderate densities (40 ± 15 MFI, n=11-18, p=0.17, Figure 5.2b). Comparing normoxic and anoxic conditions, CD39 expression is slightly higher than under normoxia (97 ± 3% vs. 95 ± 5%, n=11-18, p=0.08, Figure 5.2b) showing moderate densities (40 ± 15 MFI vs. 38 ±15, n=11-18, p=0.24, Figure 5.2b). The influence of normoxia, anoxia and deferoxamine on the CD39 expression is strongly pronounced in adult monocytes. An decrease in CD39 density may be observed after 24 hours incubation in presence of DFO (38 ± 8 MFI vs. 42 ± 10 MFI, n=11-18, p<0.01, Figure 5.2b). If cells were incubated under anoxic conditions, decrease in CD39 density became greater in comparison to normoxic conditions (30 ± 8 MFI vs. 42 ± 10 MFI, n=11-18, p<0.001, Figure 5.2b). Whereas, percentage of CD39-positive monocytes remained stable comparing normoxia and deferoxamine (96 ± 3% vs. 96 ± 3%, n=11-18, p=0.31, Figure 5.2b). Decrease in CD39-positive adult monocytes was detected under anoxic conditions (90 ± 10% vs. 96 ± 3%, n=11-18, p=0.05, Figure 5.2b).

Comparing cord blood and adult MDSC, it may be said cord blood MDSC are more susceptible against deferoxamine (DFO) and anoxic conditions than the MDSC from adult blood. Treatment with DFO induced the CD39 expression in approximately 50% of cord

blood monocytes (50 ± 45%, vs. 8 ±6%, n=11, p=0.05, Figure 5.2c). Similarly, anoxic conditions contributed to enhanced expression of CD39 by cord blood MDSC (30 ± 20% vs. 8 ± 6%, n=11, p=0.05, Figure 5.2c). Even CD39 densities became higher in presence of DFO in comparison to normoxic conditions (20 ± 10 MFI vs. 10 ± 8 MFI, n=11, p=0.001, Figure 5.2c). Similarly, anoxic conditions contributed to enhanced CD39 densities (15 ±10 MFI vs. 10 ± 8 MFI, n=11, p=0.001, Figure 5.2c). Evaluating the influence of distinct conditions on adult MDSC, the conclusion can be done adult MDSC are less responsive against these three stimuli than the cord blood MDSC. Despite of this fact, slight increase in percentage of CD39-positive MDSC could be observed upon treatment with DFO or under anoxic conditions, respectively. After DFO-treatment arose the percentage of CD39-positive MDSC in comparison to the treatment under normoxic conditions (15 ± 10% vs. 10 ± 10, n=11-18, p=0.05, Figure 5.2c). Stronger CD39 expression was observed upon treatment under anoxic conditions (18 ± 10 MFI vs 10 ± 10, n=11-18, p=0.05, Figure 5.2c). Contrary, CD39 densities remained unchanged independent from conditions (5 ± 3 MFI, n=11-18, Figure 5.2c).

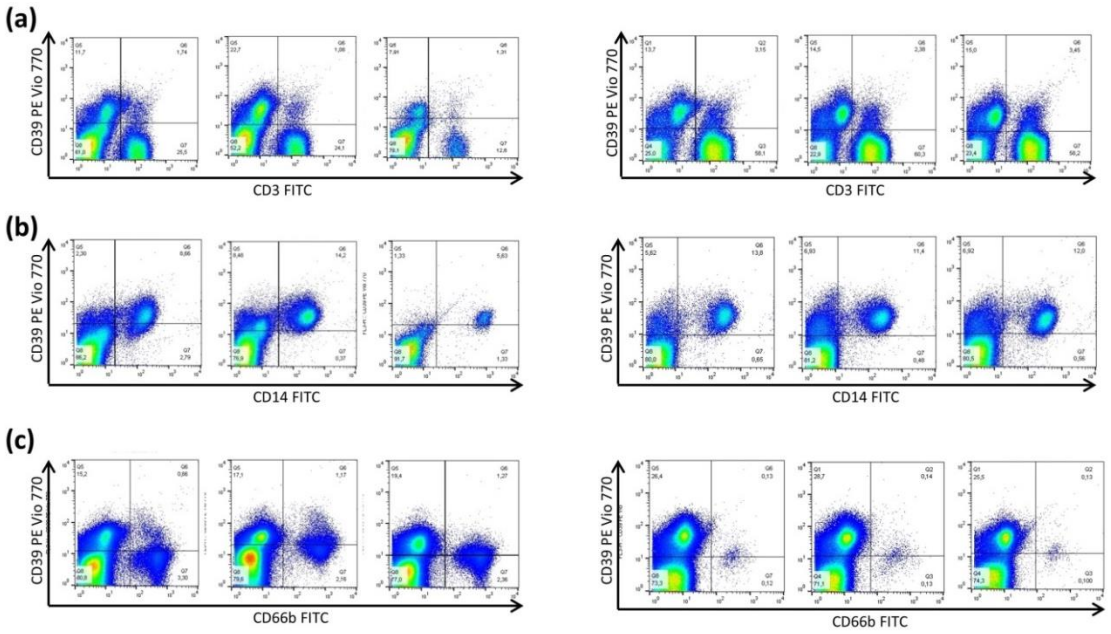


Figure 5.1. CD39 expression on cord blood and adult blood immune effector cells after 24-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubate for 24 hours under following conditions – anoxia (<1% O_2), DFO [10^{-4} M], normoxia. After 24 hours, cells were harvested, washed and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Ax – anoxia (<1% O_2), DFO – deferoxamine (Fe^{2+} chelator), Nx – normoxia. Given are representative density plots (from left to the right: CBMC: Nx, DFO, Ax, PBMC: Nx, DFO, Ax).*

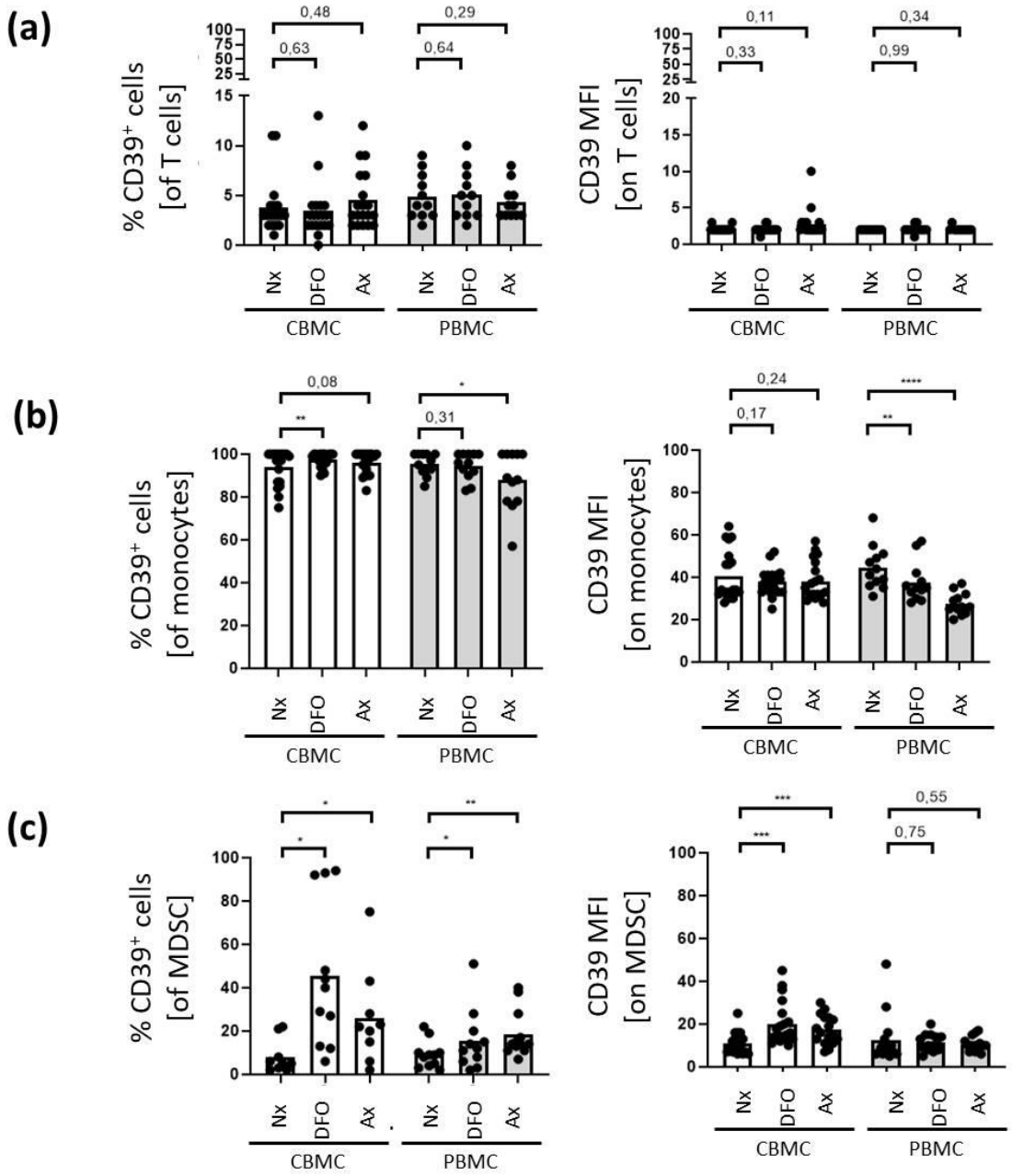


Figure 5.2. CD39 expression on cord blood and adult blood immune effector cells after 24-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubate for 24hours under following conditions – anoxia (<1% O_2), DFO [10^{-4} M], normoxia. After 24 hours, cells were harvested, washed and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Ax – anoxia (<1% O_2), DFO – deferoxamine (Fe^{2+} chelator), Nx – normoxia. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%). CBMC n=11-18, PBMC n=11-18,* = p<0.05, ** = p<0.01, *** = p<0.001*

4.3. Percentage of CD39-positive monocytes was diminished under anoxic conditions and in presence of DFO

Even upon 48 hours CD39 expression by T cells remained low independent from stimuli: 2 – 3% of T cells (both, cord blood and adult blood) did express CD39 of very low densities (2 ± 1 MFI, Figure 6.2a).

Percentage of CD39-positive cord blood monocytes remained stable independent from stimuli (90 ± 10%, n=8-11, Figure 6.2b). CD39 densities became lower upon treatment with DFO, or under anoxic conditions: DFO vs. Normoxia (38 ± 2 MFI vs. 58 ± 15 MFI, n=11, $p<0.01$), Anoxia vs. Normoxia (35 ± 5 MFI vs. 58 ± 15 MFI, n=11, $p<0.01$, Figure 6.2b).

Contrary, the percentage of CD39-positive adult monocytes declined upon treatment. In normoxia, the expression remained unchanged. Up to 95 ± 5% of all adult monocytes did express CD39. In presence of DFO, the CD39 expression during next 48 hours was downregulated leading to diminished CD39-positive population (78 ± 10%) expressing CD39 at lower densities in comparison to normoxia (40 ± 12 vs. 58 ± 20, n=7, $p<0.05$, Figure 6.2b). Under anoxic conditions, CD39 expression was diminished – approximately 65% of all adult monocytes expressed CD39. Simultaneously, expression densities became smaller in comparison to normoxic conditions (30 ± 10 MFI vs. 58 ± 20, n=6-7, $p<0.05$, Figure 6.2b).

CD39 expression by cord blood MDSC became stronger in presence of DFO in comparison to normoxia (10 ± 9% vs. 8 ± 6%, n=8, p=0.31, Figure 6.2c). Under anoxic conditions, CD39 expression became lower (5 ± 4% vs. 8 ± 6%, n=8, 0.89, Figure 6.2c). Contrary, CD39 expression by adult MDSC became stronger under anoxic conditions in comparison to normoxia (12 ± 10% vs. 5 ± 3%, n=6-7, p=0.21, Figure 6.2c). CD39 densities became higher upon treatment with DFO in comparison to normoxia (12 ± 4% vs. 7.5 ± 4, n=9, $p<0.05$, Figure 6.2c). Anoxic conditions did not have an influence on the CD39 densities (7.5 ± 4 vs. 7.5 ± 4, n=6, p=0.42, Figure 6.2c).

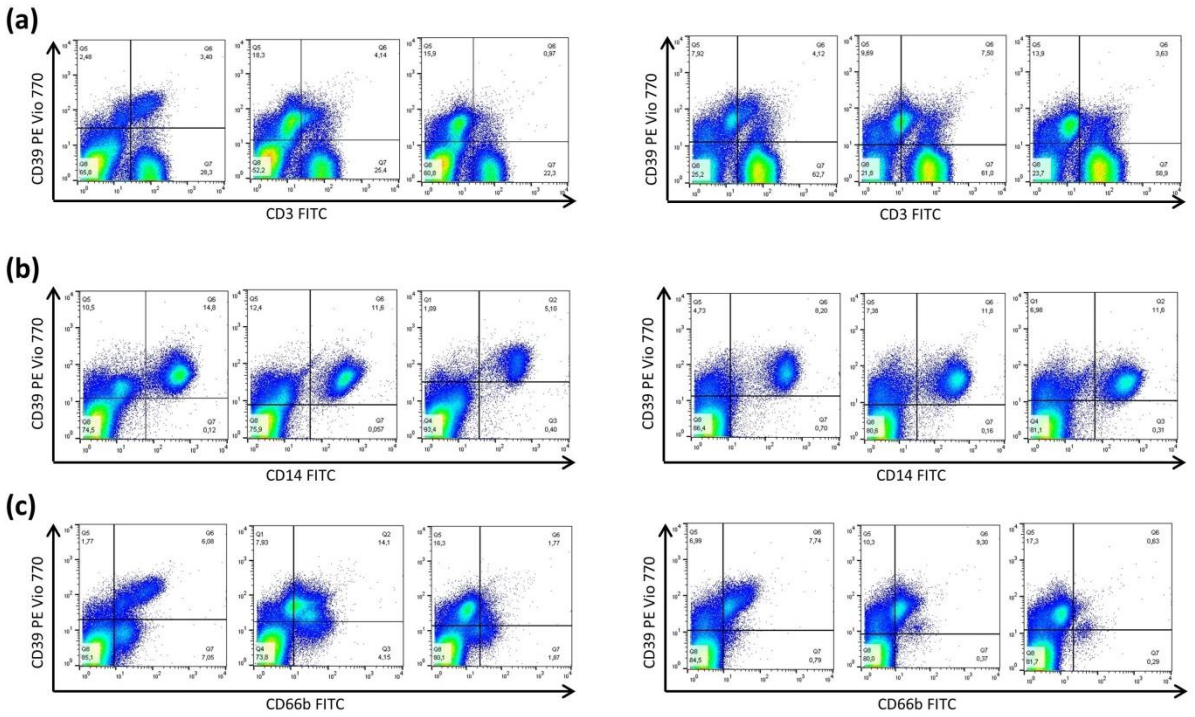


Figure 6.1. CD39 expression on cord blood and adult blood immune effector cells after 48-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to $2*10^6$ cells per ml and let incubate for 48 hours under following conditions – anoxia (<1% O_2), DFO [10^{-4} M], normoxia. After 48 hours, cells were harvested, washed and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Ax – anoxia (<1% O_2), DFO – deferoxamine (Fe^{2+} chelator), Nx – normoxia. Given are representative density plots (from left to the right: CBMC: Nx, DFO, Ax, PBMC: Nx, DFO, Ax).*

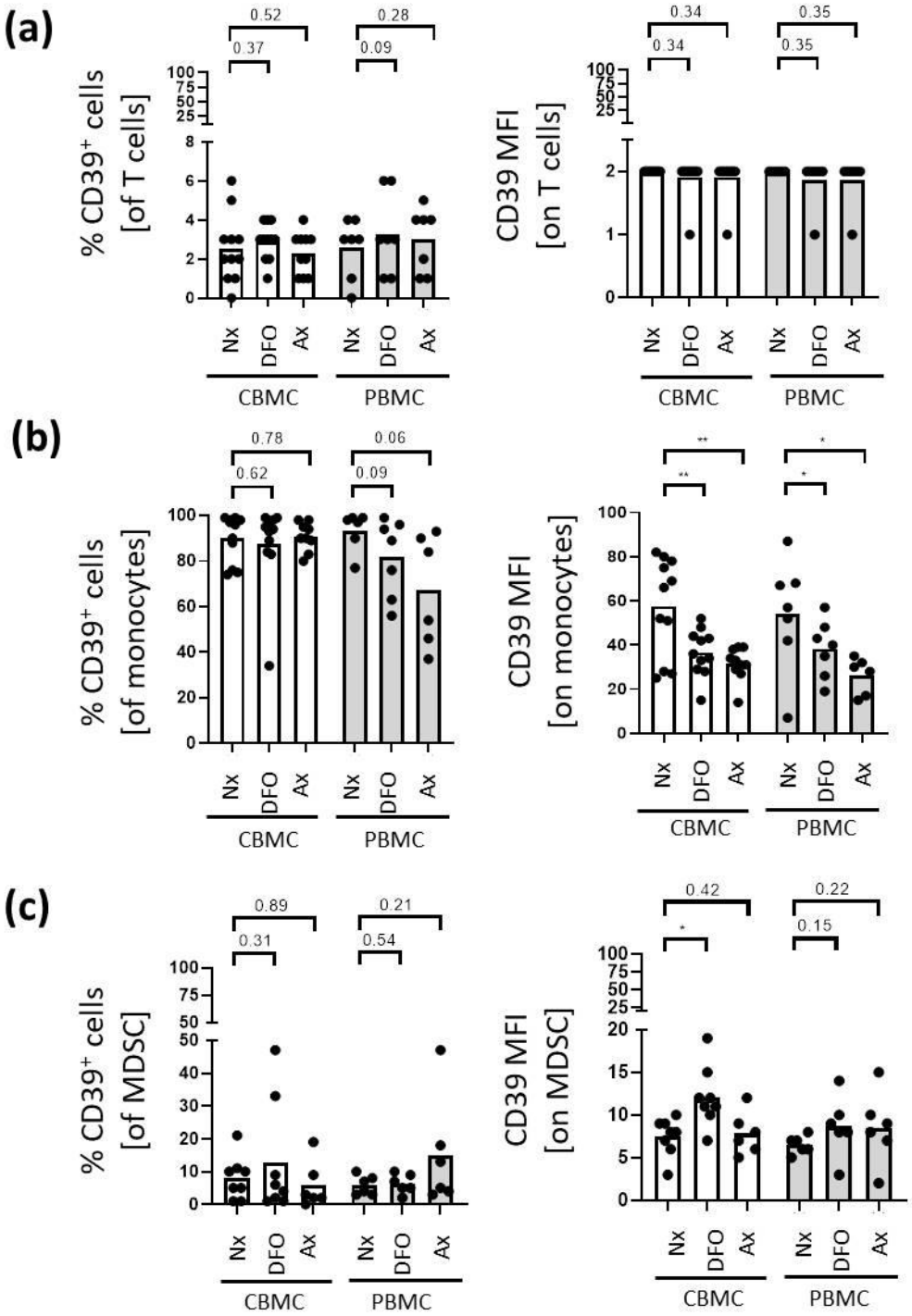


Figure 6.2. CD39 expression on cord blood and adult blood immune effector cells after 48-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to $2*10^6$ cells per ml and let incubate for 48 hours under following conditions – anoxia (<1% O_2), DFO [10^{-4} M], normoxia. After 48 hours, cells were harvested, washed and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Ax – anoxia (<1% O_2), DFO – deferoxamine (Fe^{2+} chelator), Nx – normoxia. Given mean fluorescence intensities (MFI) and percentage of positive cells (%). CBMC n=6-11, PBMC n=6-7, * = p<0.05, ** = p<0.01*

4.4. CD73 expression pattern remained unchanged after 24 hours independent from shortage of oxygen and presence of iron chelator

CD73 expression by T cells (both, cord blood and adult) did not change over time independent from stimuli. Approximately 35 ± 15% of all cord blood T cells expressed CD73 at low densities (8 ± 4 MFI, n=11-18, Figure 7.2a). Similarly, CD73 expression by adult T cells did not change over time independent from stimuli. In comparison to cord blood T cells, smaller fraction (25%) of all adult T cells were CD73-positive expressing CD73 at low densities (5 ± 2 MFI, n=11-18, Figure 7.2a).

Both, cord blood and adult blood monocytes became CD73-positive upon 24 hours incubation independent from stimuli. In case of cord blood monocytes, slight increase in percentage of CD73-positive cells may be observed upon treatment with DFO (35 ± 30% vs. 30 ± 25%, n=11-18, p=0.23, Figure 7.2b), or under anoxic conditions (38 ± 30% vs. 30 ± 25%, n=11-18, p=0.05, Figure 7.2b), respectively. Cord blood monocytes expressed CD73 at low densities (<10 MFI), whereas slight elevation is observed upon treatment with DFO (8 ± 5 MFI), or under anoxic conditions (10 ± 9 MFI), respectively.

Adult blood monocytes showed slightly different expression pattern. CD73 expression by adult blood monocytes became stronger upon treatment with DFO in comparison to normoxia (32 ± 15% vs. 25 ± 10%, n=11-18, p=.016, Figure 7.2b) and anoxia (32 ± 15% vs. 26 ± 20, n=11-18, p=0.78, Figure 7.2b). Adult blood monocytes expressed CD73 at very low densities (<5 ± 2 MFI). No enhancement has been observed independent from stimuli.

Only a minority of cord blood MDSC (up to 10%) upregulated CD73 expression upon 24 hours incubation under given conditions (normoxia, DFO, anoxia) at low densities (6 ± 2.5 MFI). In comparison to normoxic conditions, slight increase in percentage of CD73-positive MDSC was observed upon treatment with DFO (8 ± 5% vs. 5 ± 2.5%, n=11, p=0.14, Figure 7.2c) and under anoxic conditions (7 ± 6 vs. 5 ± 2.5%, n=9, p=0.18, Figure 7.2c). In contrast to percentage, CD73 densities remained unchanged (6 ± 3) independently from stimuli.

In case of adult blood MDSC, an increase in percentage of CD73-positive MDSC may be observed upon treatment with DFO (12 ± 10% vs. 10 ± 8%, n=11-18, p=0.47, Figure 7.2c), or under normoxic conditions (15 ± 12% vs. 10 ± 8%, n=11-18, p<0.05, Figure 7.2c). Despite of the slightly increased percentages of CD73-positive adult MDSC, CD73 density remained unchanged (6 ± 2.5 MFI) independent from stimuli.

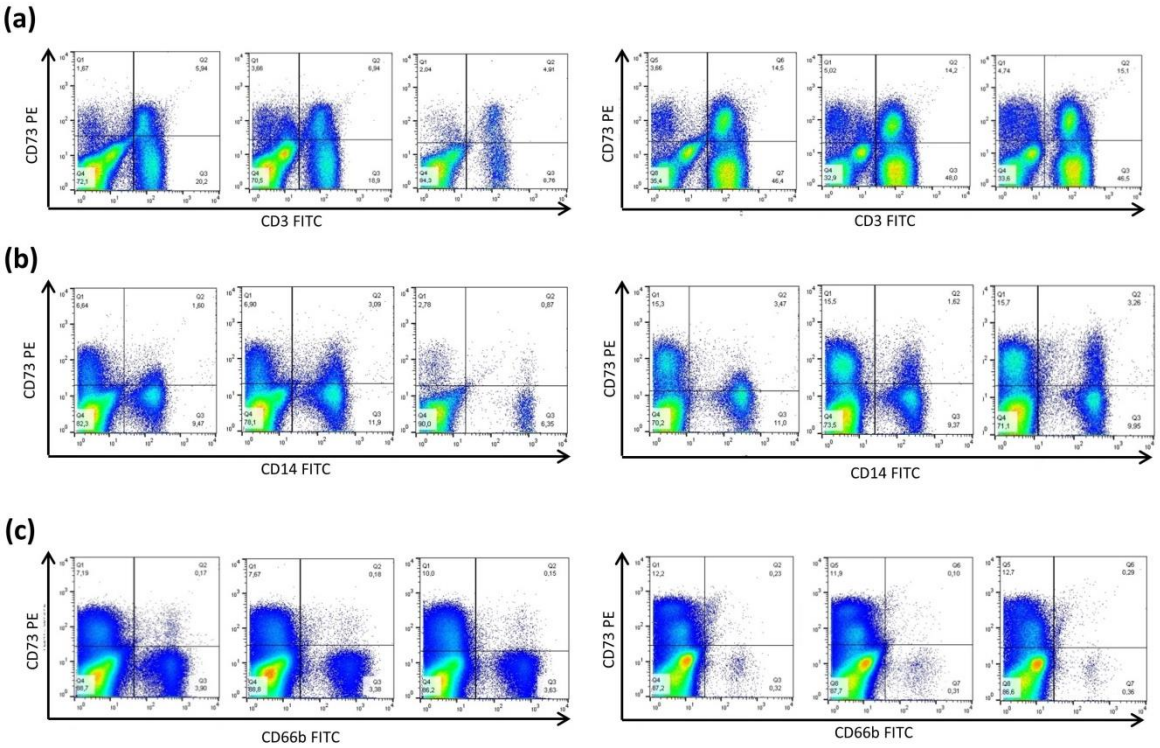


Figure 7.1. CD73 expression on cord blood and adult blood immune effector cells after 24-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubate for 24 hours under following conditions – anoxia (<1% O_2), DFO [10^{-4} M], normoxia. After 24 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Ax – anoxia (<1% O_2), DFO – deferoxamine (Fe^{2+} chelator), Nx – normoxia. Given are representative density plots (from left to the right: CBMC: Nx, DFO, Ax, PBMC: Nx, DFO, Ax).*

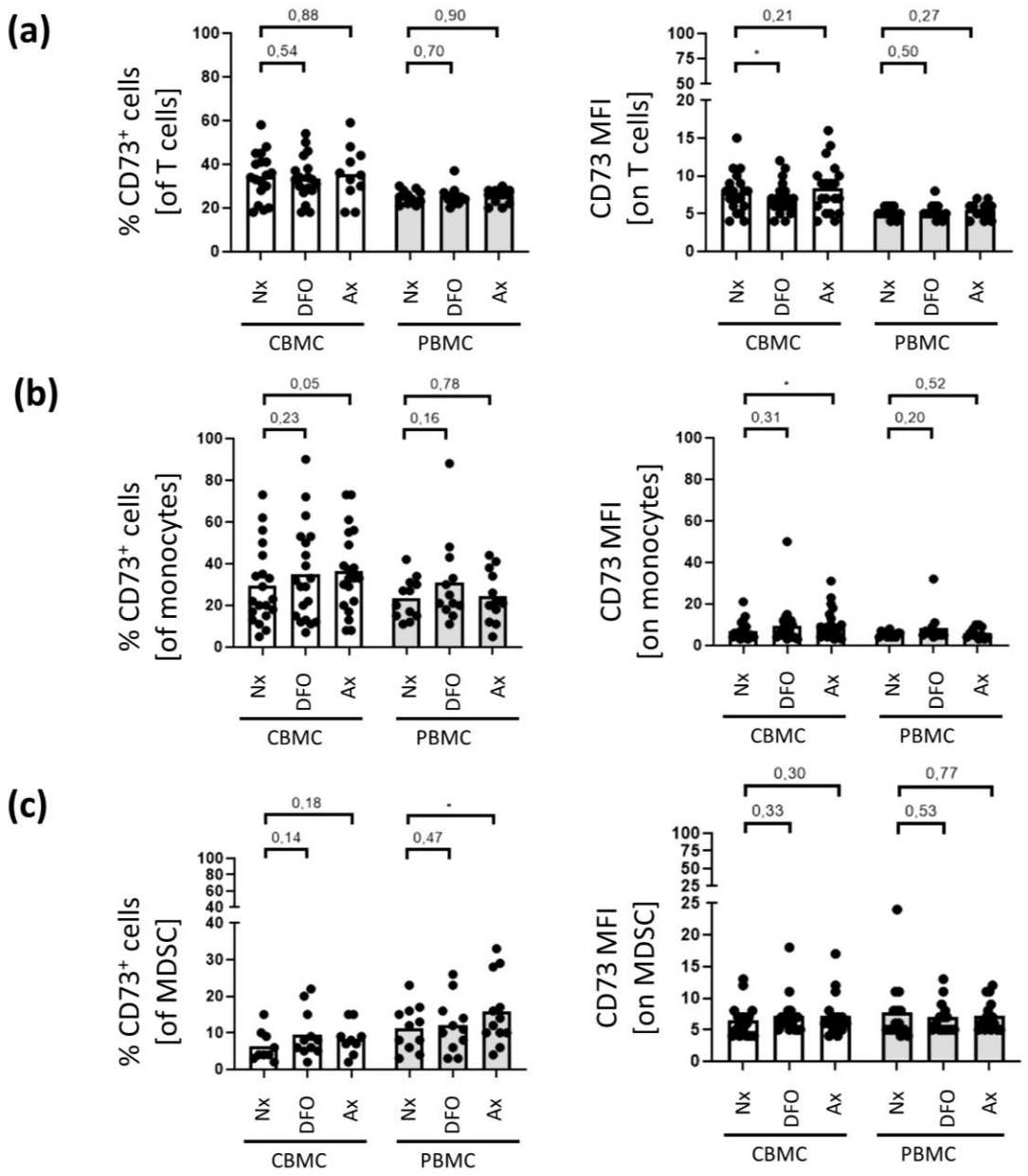


Figure 7.2. CD73 expression on cord blood and adult blood immune effector cells after 24-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubate for 24 hours under following conditions – anoxia (<1% O_2), DFO [10^{-4} M], normoxia. After 24 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Ax – anoxia (<1% O_2), DFO – deferoxamine (Fe^{2+} chelator), Nx – normoxia. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%). CBMC n=11-18, PBMC n=11-18, * = p<0.05*

4.5. DFO enhanced CD73 expression on cord blood monocytes and adult MDSC during 48 hours incubation

Comparing the results after 24 hours and 48 hours incubation, it could be said that CD73 expression pattern by T cells did not change. The percentage of CD73-positive cord blood T cells was 35 ± 10% independent from stimuli. CD73 densities on cord blood T cells incubated under normoxic conditions or in presence of DFO remained low (7.5 ± 3 MFI, n=10-11, p=0.84, Figure 8.2a). Slight increase in CD73 densities could be observed upon incubation under anoxic conditions (8 ± 4, n=10, p=0.60, Figure 8.2a).

Comparing the expression patterns of adult T cells after 24 and 48 hours, it may be said that smaller percentage (25 ± 7%) of adult T cells express CD73 in comparison to cord blood T cells. The percentage of CD73-positive adult T cells remained constant independent from conditions. Also the CD73 densities were lower (5 ± 2.5 MFI) in comparison to the cord blood T cells. Additionally, CD73 densities remained stable independent from conditions.

Incubation for 48 hours led to enhanced expression of CD73 by cord blood monocytes in presence of DFO (62 ± 25% vs. 50 ± 30%, n=11, p=0.33, Figure 8.2b). Even anoxia contributed to enhanced CD73 expression by cord blood monocytes (58 ± 18% vs. 50 ± 30%, n=10, p=0.47, Figure 8.2b). CD73 densities corresponded with the percentages of CD73-positive monocytes. Monocytes incubated in presence of DFO showed the highest density (25 ± 20 MFI, n=10, p=0.13, Figure 8.2b). Second strongest density was detected after incubation under anoxic conditions (20 ± 10 MFI, n=10, $p<0.05$, Figure 8.2b).

Similarly to incubation for 24 hours, adult monocytes express CD73 to a lesser extent. Also in this case, treatment with DFO led to slight increase in the percentage of CD73-positive cells in comparison to incubation under normoxic conditions (55 ± 20%, vs. 50 ± 20%, n=7, p=0.28, Figure 8.2b). Interestingly, in contrast to cord blood monocytes, anoxia had an opposite effect on the expression of CD73 by adult monocytes – CD73 expression was downregulated (38 ± 18%, n=6, p=0.24, Figure 8.2b). CD73 densities by adult monocytes became higher after treatment with DFO in comparison to normoxic conditions

(25 ± 20 MFI vs. 15 ± 10 MFI, n=7, p=0.17, Figure 8.2b). Under anoxic conditions, CD73 densities were comparable with those of normoxia (17 ± 12 MFI vs. 15 ± 10 MFI, n=6, p=0.83).

Pecentage of CD73-positive cord blood MDSC remained low (6 ± 6%) independent from stimuli and only slight changes were observed in comparison to the measurement after 24 hours. CD73 densities on cord blood MDSC remained low (~5 ± 2 MFI).Contrary, the adult MDSC upregulated CD73 expression as a response to the presence of DFO in comparison to normoxic conditions (13 ± 12% vs. 6 ± 5%, n=7, p<0.05, Figure 8.2c). Also the CD73 density on adult MDSC became slightly higher in presence of DFO in comparison to normoxic conditions (7.5 ± 3 MFI vs. 5.5 ± 2 MFI, n=6, p=0.07, Figure 8.2c). CD73 densities under anoxic conditions were comparable with those under normoxic conditions (5.5 ± 2 MF vs. 6 ± 3 MFI, n=6, p=0.82, Figure 8.2c).

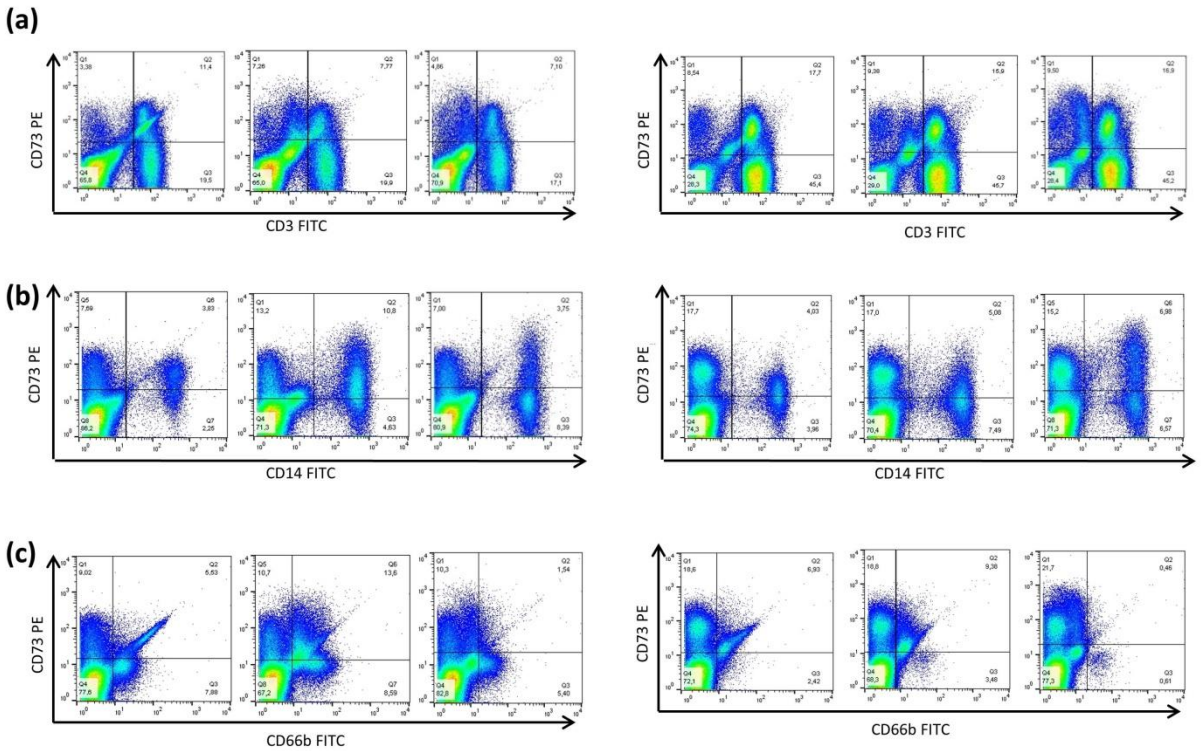


Figure 8.1. CD73 expression on cord blood and adult blood immune effector cells after 48-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *(For details – see Figure 8.2 on page 56)*

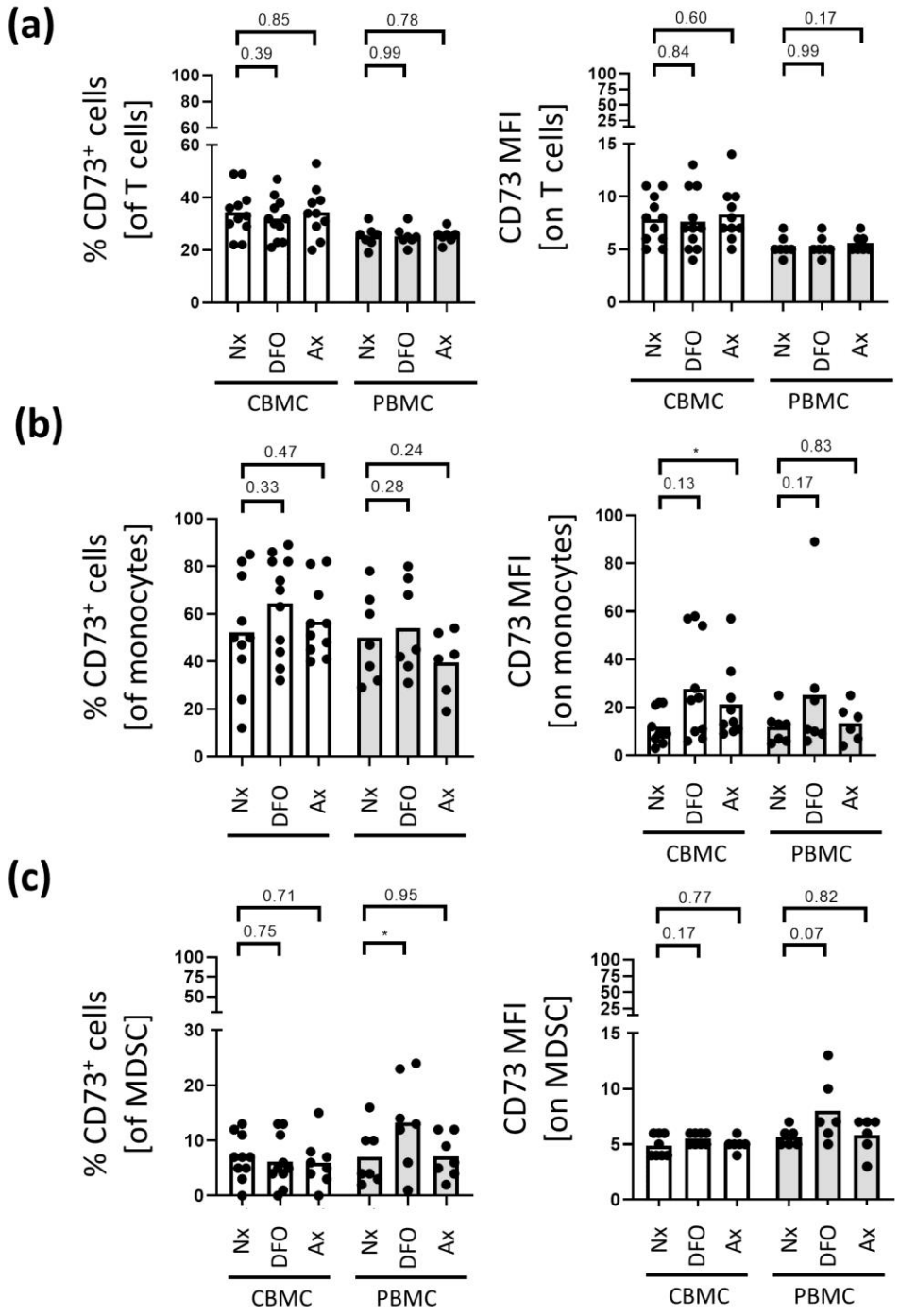


Figure 8.2. CD73 expression on cord blood and adult blood immune effector cells after 48-hour incubation under anoxia, normoxia and in presence of deferoxamine (DFO). *Cord blood (CBMC, left) and peripheral blood mononuclear cells (PBMC, right) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to $2*10^6$ cells per ml and let incubate for 48 hours under following conditions – anoxia (<1% O_2), DFO [10^{-4} M], normoxia. After 48 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Ax – anoxia (<1% O_2), DFO – deferoxamine (Fe^{2+} chelator), Nx – normoxia. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%). CBMC n=6-11, PBMC n=6-7,* = p<0.05*

4.6. CD73 expression by T cells is diminished in presence of PAMPs

Next we determined whether pathogen-associated molecular patterns (PAMPs) are able to induce or enhance the expression of the ectonucleotidases. Interestingly, none of the PAMPs applied in the assay, induced the expression of CD39 by T cells (both cord blood and adult) (Figure 9.2a).

Further, more than 90% of all monocytes (both, cord blood and adult blood) expressed CD39 under sterile conditions as well as in presence of PAMPs. The CD39 density differed depending on the PAMPs and its concentration used in the assay. Treatment with 10 µg/ml Pam3Cys enhanced the CD39 density in cord blood monocytes compared to the untreated control (75 ± 3 MFI vs. 50 ± 15 MFI, n=3, $p<0.05$, Figure 9.2b) as well as in adult blood monocytes (90 ± 10 MFI vs. 50 ± 6 MFI, n=3, $p<0.05$, Figure 9.2b). This effect was dose-depedent.

Enhanced CD39 expression upon treatment with Pam3Cys [10 µg/ml] was also observed on cord blood MDSC (65 ± 5% vs. 55 ± 20%, n=3, $p=0.48$, Figure 9.2c) as well as adult blood MDSC (60 ± 5% vs. 30 ± 10%, n=3, $p<0.05$, Figure 9.2c). Interestingly, LTA [10µg/ml] led to down-regulation of CD39 by cord blood MDSC (35 ± 20% vs. 55 ± 25%, n=3) but not adult MDSC (30 ± 10% vs. 30 ± 7%, n=3). Additionally, CD39 expression pattern did not correspond to dose-dependent manner in contrast to that of monocytes.

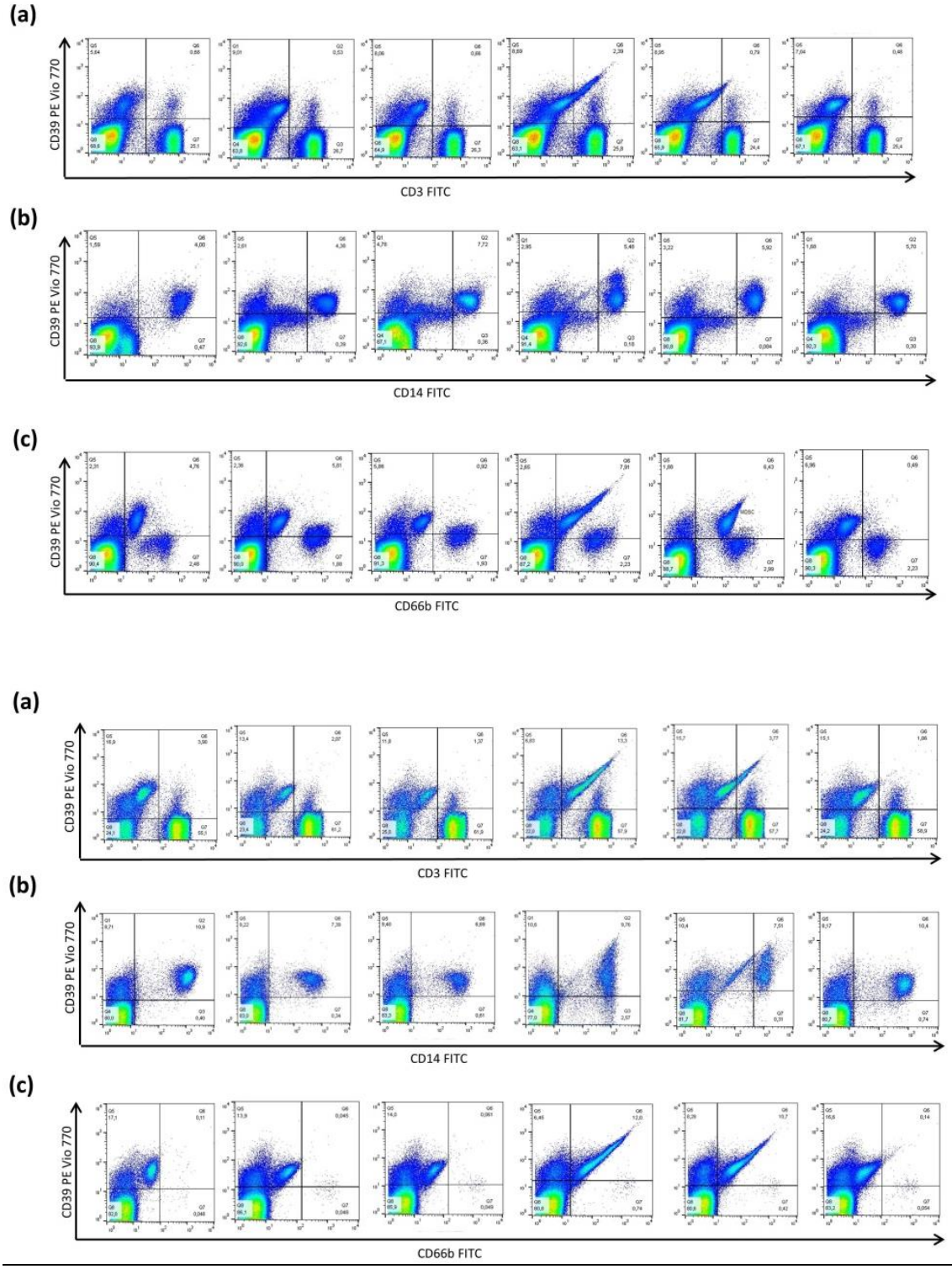


Figure 9.1. CD39 expression on cord blood and adult blood immune effector cells after 24-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC, upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubated for 24 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 24 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Given are representative density plots (upper part: CBMC, lower part: PBMC, from left to the right: untreated, LTA 10 µg/ml, LPS 1 µg/ml, Pam3Cys 10 µg/ml, Pam3Cys 1 µg/ml, Pam3Cys 0.1 µg/ml), n=3-4, * = p<0.05*

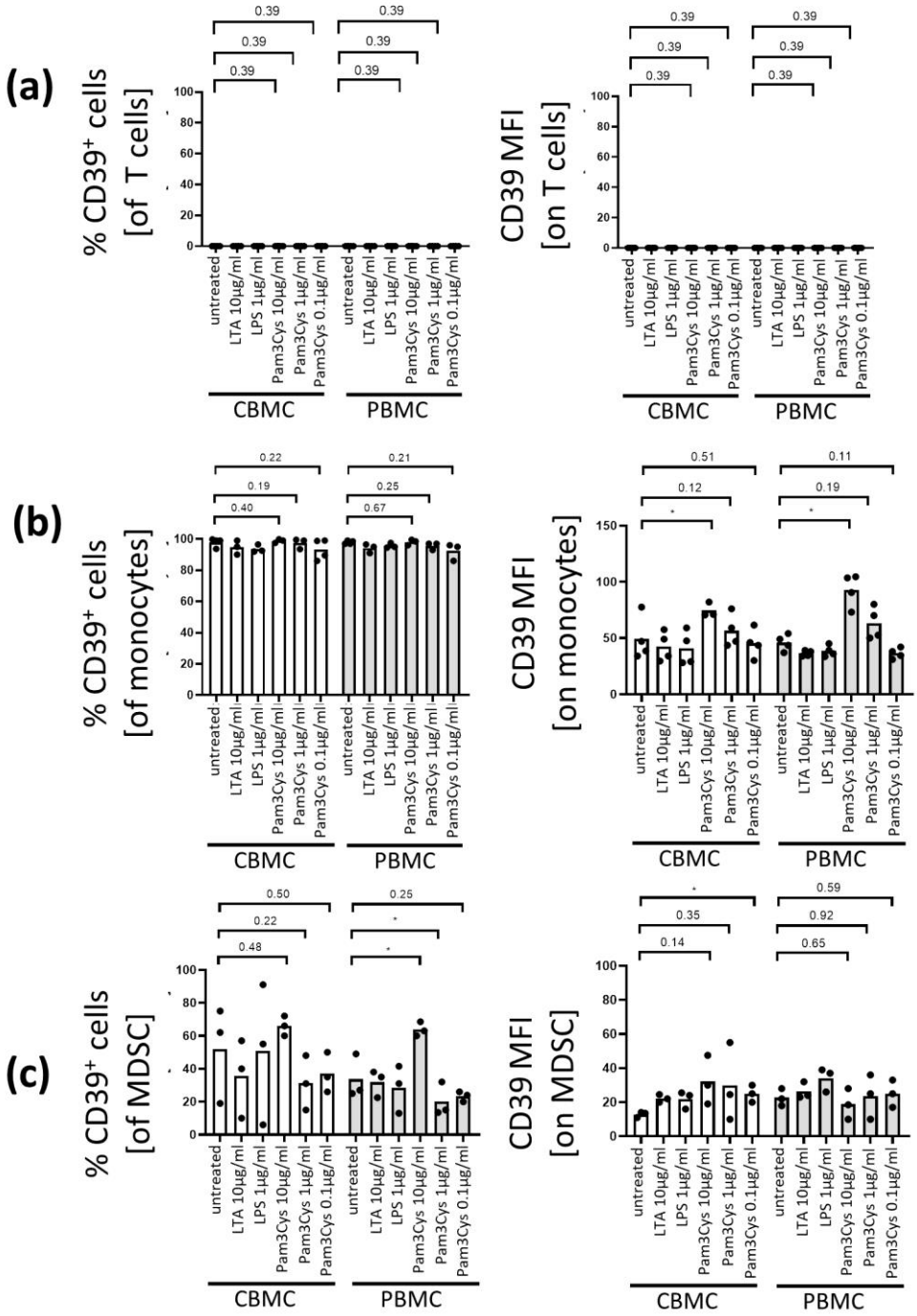


Figure 9.2. CD39 expression on cord blood and adult blood immune effector cells after 24-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC, upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubated for 24 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 24 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%), n=3-4, * = p<0.05*

4.7. Treatment with Pam3Cys induced CD73 expression in monocytes

Only 20% of all cord blood T cells expressed CD73 and the CD73 expression showed dose-dependent expression pattern (Figure 10.2a). Similarly, dose-dependent CD73 expression was observed by adult T cells, but to a lesser extent (Figure 10.2a). In case of cord blood T cells, CD73 densities remained the same independent from stimuli (35 ± 15 MFI, n=4). CD73 densities by adult T cells were lower (30 ± 12 MFI, n=4) in presence of LTA, LPS or let untreated, respectively. Contrary, treatment with 10µg/ml Pam3Cys led to increased CD73 densities by adult T cells in comparison to the untreated sample (42 ± 10 MFI vs 30 ± 12 MFI, n=4, p=0.07, Figure 10.2a). This effect was dose-dependent.

In case of monocytes, an interesting phenomenon was observed. Cord blood monocytes did not respond to treatment with LTA [10µg/ml] and LPS [1µg/ml] by CD73 upregulation and remained single-positive. Contrary, adult monocytes responded to both stimuli LTA [10µg/ml] and LPS [1µg/ml] and became CD73/CD39-double positive. Treatment with Pam3Cys [10µg/ml] led to upregulation of CD73 in both, cord blood monocytes (80 ± 5%,n=3, $p<0.001$, Figure 10b) as well as adult monocytes (90 ± 6%, n=3, $p<0.01$, Figure 10.2b). Similarly, CD73 densities increased – cord blood monocytes (75 ± 15 MFI, n=3, $p<0.05$, Figure 10.2b) and adult monocytes (110 ± 20 MFI, n=3, $p<0.05$, Figure 10.2b). This effect was dose-dependent.

MDSC (both, cord blood and adult) did not upregulated CD73 and remained CD39 single-positive independent from stimuli (Figure 10.2c).

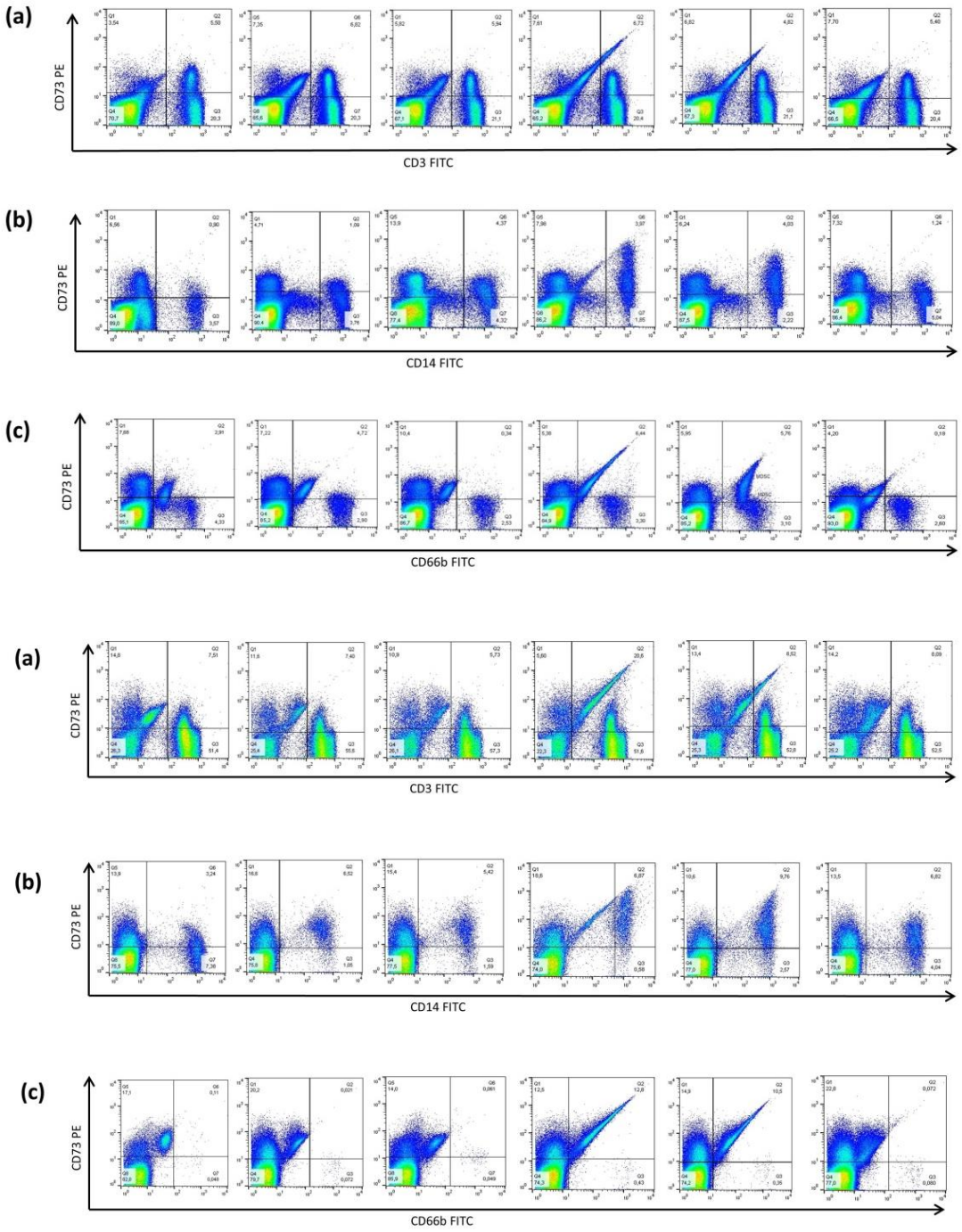


Figure 10.1. CD73 expression on cord blood and adult blood immune effector cells after 24-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC, upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubated for 24 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 24 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Given are representative density plots (upper part: CBMC, lower part: PBMC, from left to the right: untreated, LTA 10 µg/ml, LPS 1 µg/ml, Pam3Cys 10 µg/ml, Pam3Cys 1 µg/ml, Pam3Cys 0.1 µg/ml), n=3-4, * = p<0.05, ** = p<0.01*

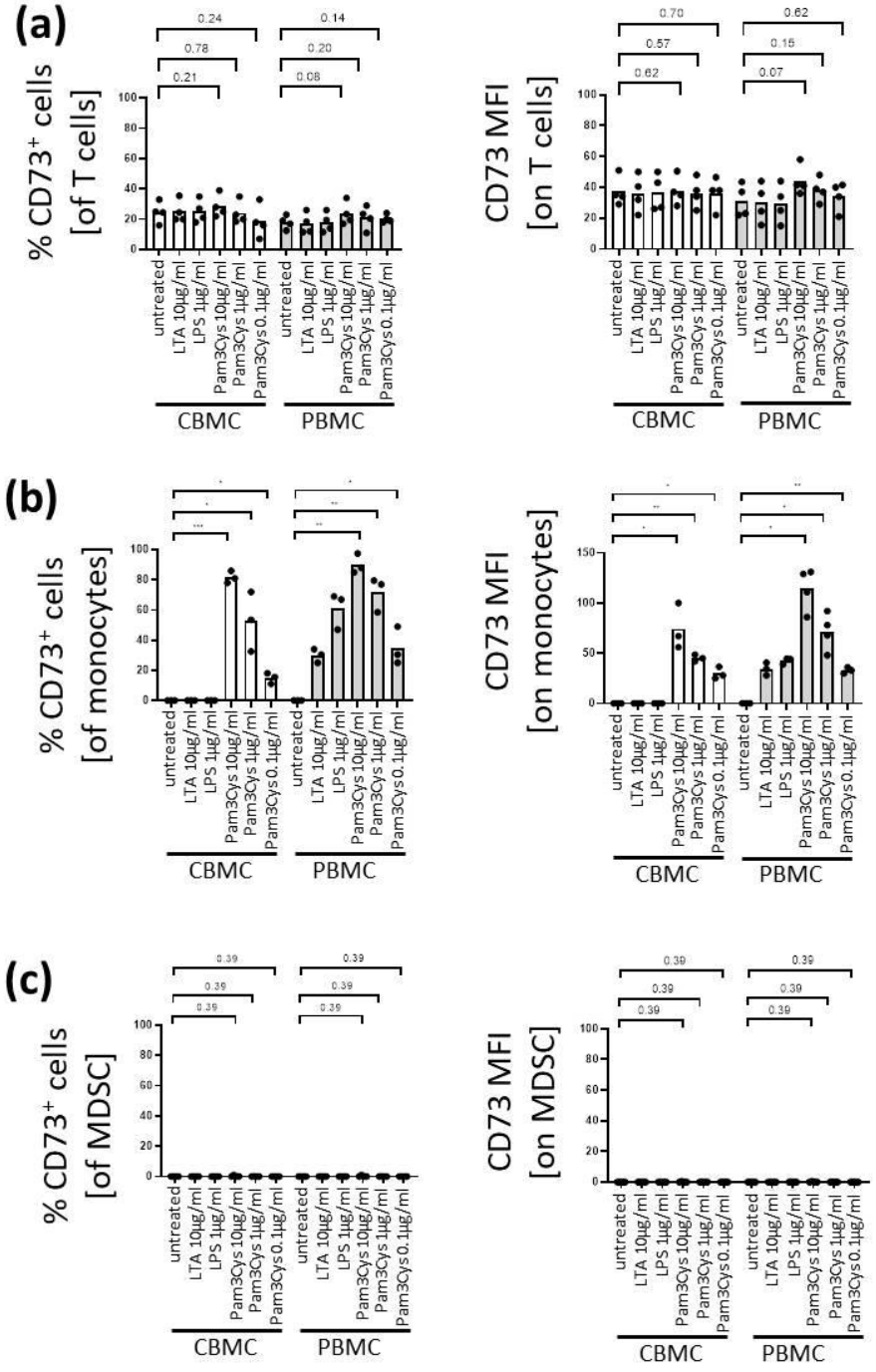


Figure 10.2. CD73 expression on cord blood and adult blood immune effector cells after 24-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC, upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubated for 24 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 24 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%), n=3-4, * = p<0.05, ** = p<0.01*

4.8. Pam3Cys induced strong upregulation of CD39 on cord blood monocytes after 48 hours incubation

PBMC and CBMC were incubated in presence of LPS, LTA and Pam3Cys for 48 hours to determine whether extended exposure to PAMPs has an influence on the expression pattern of bifunctional ectonucleotidase (CD39). T cells did not upregulate CD39 and remained single-positive even after extended exposure (Figure 11.2a).

An interesting phenomenon could be observed in culture of both cod blood and adult monocytes. Slight downregulation of CD39 (85 ± 8%, n=3) was observed if treated with LTA [10µg/ml], in comparison to the other conditions (95 ± 5%, n=3, Figure 11.2b).

Treatment of cord blood monocytes with LTA [10µg/ml] led to decreased CD39 density in comparison to untreated control (40 ± 5 MFI vs. 75 ± 20 MFI, n=3, Figure 11.2b). Similarly, LPS [1µg/ml] led to decreased CD39 density in comparison to untreated control (45 ± 5 MFI vs. 75 ± 20 MFI, n=3, Figure 11.2b). Similarly, adult monocytes expressed lower levels of CD39 upon treatment with LTA (25 ± 5 MFI vs. 55 ± 8 MFI, n=3, Figure 11.2b) as well as with LPS (20 ± 5 vs. 55 ± 8 MFI, n=3, Figure 11.2b) in comparison to untreated control.

Most interesting was the effect of Pam3Cys on both, cord blood and adult monocytes. In comparison to untreated control, Pam3Cys [10µg/ml] led to increased CD39 density in cord blood monocytes (110 ± 20 MFI vs. 75 ± 20 MFI, n=3, $p<0.05$, Figure 11.2b) as well as in adult monocytes (125 ± 10 MFI vs. 55 ± 8 MFI, n=3, $p<0.0001$, Figure 11.2b).

Percentage of CD39-positive cord blood MDSC decreased slightly upon treatment with LTA (5 ± 2% vs. 10 ± 3%, n=3, Figure 11.2c) and LPS (7.5 ± 2% vs. 10 ± 3%, n=3, Figure 11.2c). Contrary, treatment with Pam3Cys [10µg/ml] led to increased percentage of CD39-positive cord blood MDSC in comparison to untreated control (75 ± 10% vs. 10 ± 3%, n=3, $p<0.05$, Figure 11.2c), but dose-dependency of this effect could not be observed.

Adult MDSC responded to treatment with LTA [10µg/ml] by upregulation of CD39 expression (60 ± 8% vs. 5 ± 2%, n=3, Figure 11.2c). Similarly, LPS [1µg/ml] induced upregulation CD39 expression (45 ± 15% vs. 5 ± 2%, n=3, Figure 11.2c).

Treatment with Pam3Cys [10µg/ml] induced upregulation of CD39 expression in comparison to untreated control (25 ± 3% vs. 5 ± 2%, n=3, $p<0.05$, Figure 11.2c). In contrast to cord blood MDSC, treatment with Pam3Cys-upregulated CD39 expression was weaker (25 ± 3% vs. 75 ± 10%, n=3, Figure 11.2c).

In cord blood MDSC, CD39 density remained relatively stable (20 ± 3 MFI, n=3, Figure 11.2c) independent from stimuli with exception of Pam3Cys [10µg/ml] (25 ± 7 MFI, n=3, Figure 11.2c) and Pam3Cys [1µg/ml] (30 ± 10 MFI, n=3, Figure 11.2c). Even in this case dose-dependency of this effect could not be detected.

Contrary, in case of adult MDSC, strong induction of CD39 expression was observed upon treatment with LTA [10µg/ml] (70 ± 25 MFI, n=3) and LPS [1µg/ml] (40 ± 15, n=3, Figure 11.2c). After treatment with Pam3Cys [10µg/ml] density of CD39 expressed by adult MDSC remained low (10 ± 2 MFI, n=3, Figure 11.2c). Additionally, dose-dependent effect was not observed – Pam3Cys [1µg/ml] (0 ± 0, n=3, $p<0.05$, Figure 11.2c), Pam3Cys [0.1µg/ml] (0 ± 0, n=3, $p<0.05$, Figure 11.2c).

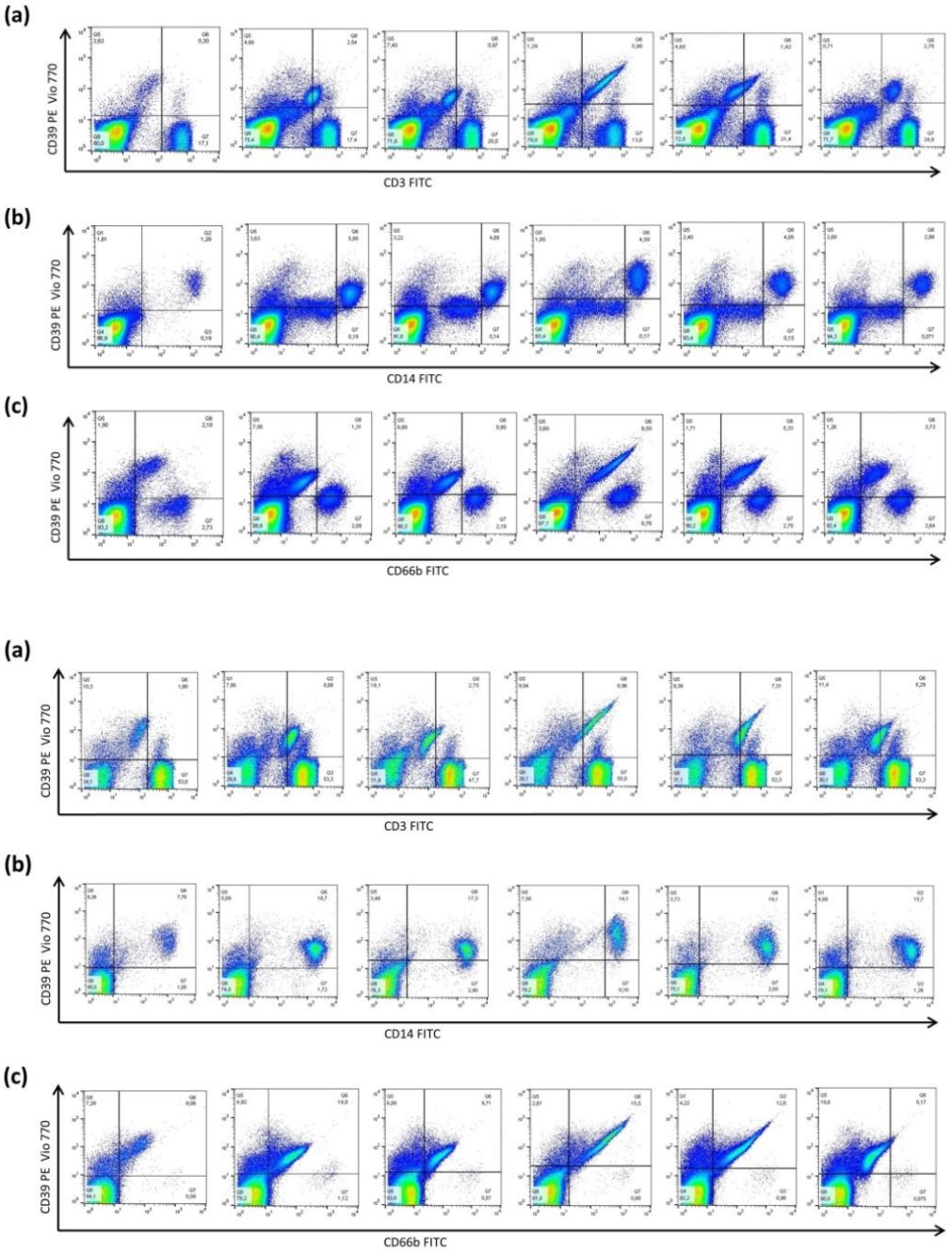


Figure 11.1. CD39 expression on cord blood and adult blood immune effector cells after 48-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC,upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubated for 48 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 48 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Given are representative density plots (upper part: CBMC, lower part: PBMC, from left to the right: untreated, LTA 10 µg/ml, LPS 1 µg/ml, Pam3Cys 10 µg/ml, Pam3Cys 1 µg/ml, Pam3Cys 0.1 µg/ml), n=3, * = p<0.05*

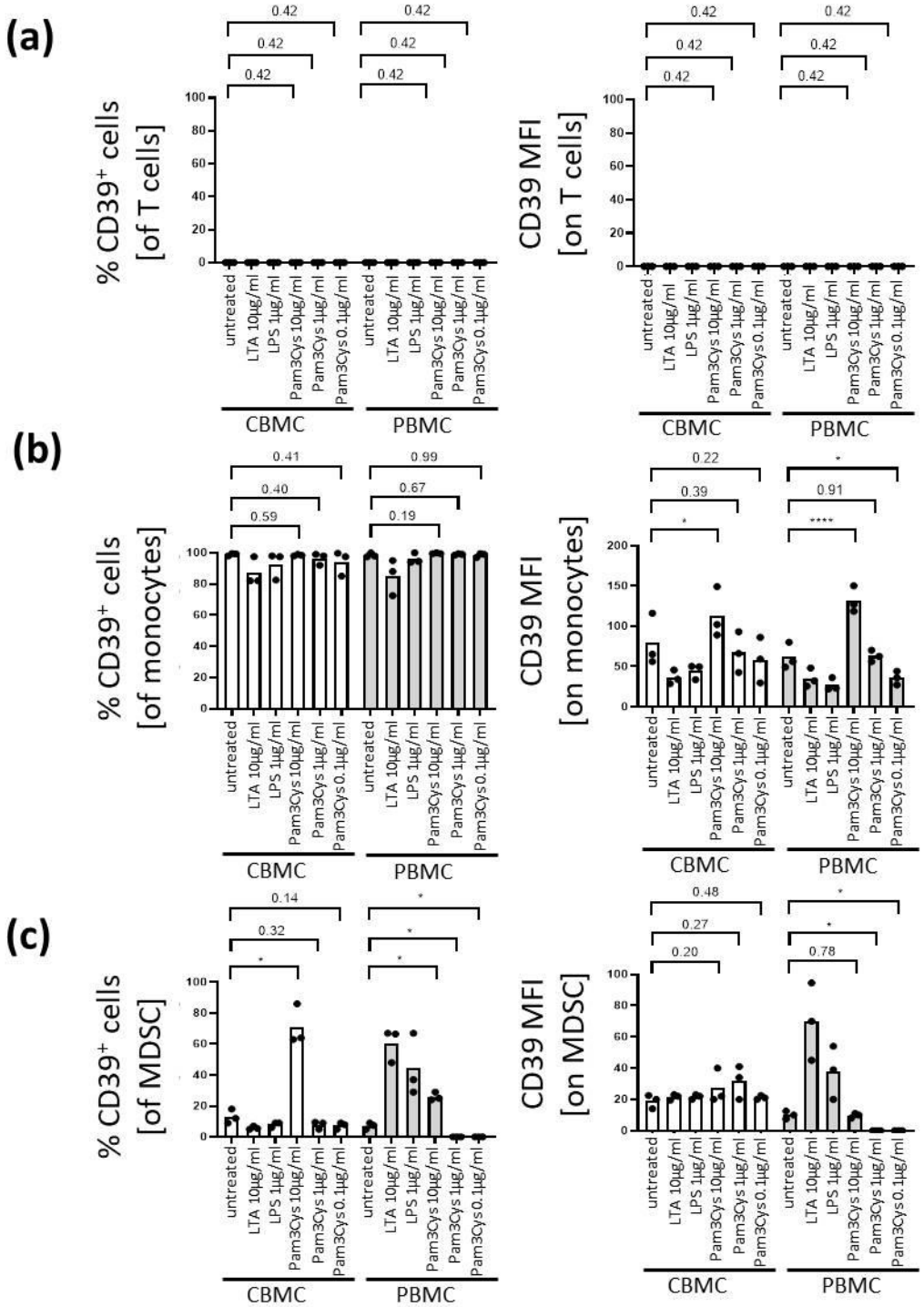


Figure 11.2. CD39 expression on cord blood and adult blood immune effector cells after 48-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC, upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubate for 48 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 48 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD39. Expression was quantified by flow cytometry. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%), n=3, * = p<0.05*

4.9. Pam3Cys does not influence CD73 expression of monocytes

PBMC and CBMC were incubated in presence of LPS, LTA and Pam3Cys for 48 hours to detemine whether extended exposure to PAMPs has an influence on the expression pattern of 5´-Ectonucleotidase (CD73).

Minority (~20%) of cord blood T cells express CD73 after 48 hours incubation independent from stimuli. Treament with Pam3Cys led to dose-dependent manner of CD73 expression (30 ± 7%, n=3, p<0.01, [10µg/ml], Figure 12.2a) vs. (25 ± 4%, n=3, p<0.05, [1µg/ml], Figure 12.2a) vs.(20 ± 5%, n=3, p<0.05, [0.1µg/ml], Figure 12.2a). CD73 density on cord blood T cells remained stable (35 ± 10 MFI, n=3, Figure 12.2a) with the exception of Pam3Cys [10µg/ml] (42 ± 12 MFI, n=3, p=0.58, Figure 12.2a).

T cells express CD73 with moderate density (35 ± 10 MFI, n=3, Figure 12.2a) with the exception of Pam3Cys [10µg/ml] leading to slightly increased CD73 expression density (42 ± 10 MFI, n=3, p=0.58, Figure 12.2a).

CD73 is present on less than 20% of all adult T cells (20 ± 5%, n=3, Figure 12a) independent from the stimuli. The only exception is the treatment with Pam3Cys [10µg/ml] (22 ± 6%, n=3, p=0.11, Figure 12.2a). Treatment with lower concentrations of Pam3Cys [1µg/ml] and [0.1µg/ml] has led to dose-dependent manner of CD73 expression by adult T cells (19 ± 2%, n=3, p<0.01, Figure 12.2a) and (15 ± 4%, n=3, p=0.80, Figure 12.2a).

CD73 density on adult T cells slightly increased upon treatment with LTA [10µg/ml] in comparison to untreated control (25 ± 5 MFI vs. 22 ± 3 MFI, n=3, Figure 12.2a). Contrary, CD73 density upon treatment with LPS [1µg/ml] remained unchanged (22 ± 4 MFI vs. 22 ± 3 MFI, n=3, Figure 12.2a). Treatment with Pam3Cys [10µg/ml] led to slight increased CD73 density (26 ± 5 MFI vs. 22 ± 3 MFI, n=3, p=0.49, Figure 12.2a). Two other concentrations of Pam3Cys [1µg/ml] and [0.1µg/ml] led even to diminished CD73 density in comparison to untreated control (15 ± 2 MFI vs. 22 ± 3 MFI, n=3, p<0.05, Figure 12.2a) and (15 ± 3 MFI vs. 22 ± 3 MFI, n=3, p<0.05, Figure 12.2a).

In cord blood monocytes, CD73 became upregulated upon treatment with PAMPs. Similar effect had LTA (35 ± 5%, n=3, Figure 12.2b) and LPS (40 ± 3%, n=3, Figure 12.2b). Pam3Cys applied in three distinct concentrations [10 – 1 – 0.1µg/ml] led to dose-dependent expression of CD73 in cord blood monocytes (90 ± 5%, n=3, $p<0.01$, Figure 12.2b) vs. (60 ± 20%, n=3, $p<0.05$, Figure 12.2b) vs. (50 ± 8%, n=3, $p<0.01$, Figure 12.2b).

CD73 density on cord blood monocytes treated with LTA and LPS remained low (30 ± 2 MFI, n=3, Figure 12.2b) and (35 ± 4 MFI, n=3, Figure 12.2b). CD73 density on cord blood monocytes showed dose-dependency (110 ± 40 MFI, n=3, $p=0.05$, Figure 12.2b) vs. (50 ± 5 MFI, n=3, $p<0.05$, Figure 12.2b) vs. (25 ± 2 MFI, n=3, $p<0.01$, Figure 12.2b) when Pam3Cys applied in three different concentrations [10 – 1 – 0.1µg/ml].

Adult blood monocytes upregulated CD73 upon treatment with PAMPs. Less than half (45 ± 15%, n=3, Figure 12.2b) of adult monocytes expressed CD73 upon treatment with LTA [10µg/ml]. Similar effect had treatment with LPS [1µg/ml] (42 ± 15%, n=3, Figure 12.2b). Similar to cord blood monocytes, adult monocytes upregulated CD73 in dose-dependent manner (95 ± 5%, n=3, $p<0.001$, Figure 12.2b) vs. (80 ± 10%, n=3, $p<0.01$, Figure 12.2b) vs. (55 ± 15%, n=3, 0.05, Figure 12.2b) upon treatment with Pam3Cys of three distinct concentrations [10 – 1 – 0.1µg/ml].

CD73 density of adult monocytes became moderate upon treatment with LTA [10µg/ml] (50 ± 15 MFI, n=3, Figure 12.2b) and LPS [1µg/ml] (50 ± 1 MFI, n=3, Figure 12.2b). Pam3Cys [10µg/ml] induced strong CD73 expression on adult monocytes (175 ± 25 MFI, n=3, $p<0.05$, Figure 12.2b). Applying two lower concentrations [1µg/ml] and [0.1µg/ml] induced dose-dependent manner of CD73 expression levels.

MDSC (both, cord blood and adult) did not upregulate CD73 and remained CD39 single-positive independent from stimuli (Figure 12.2c).

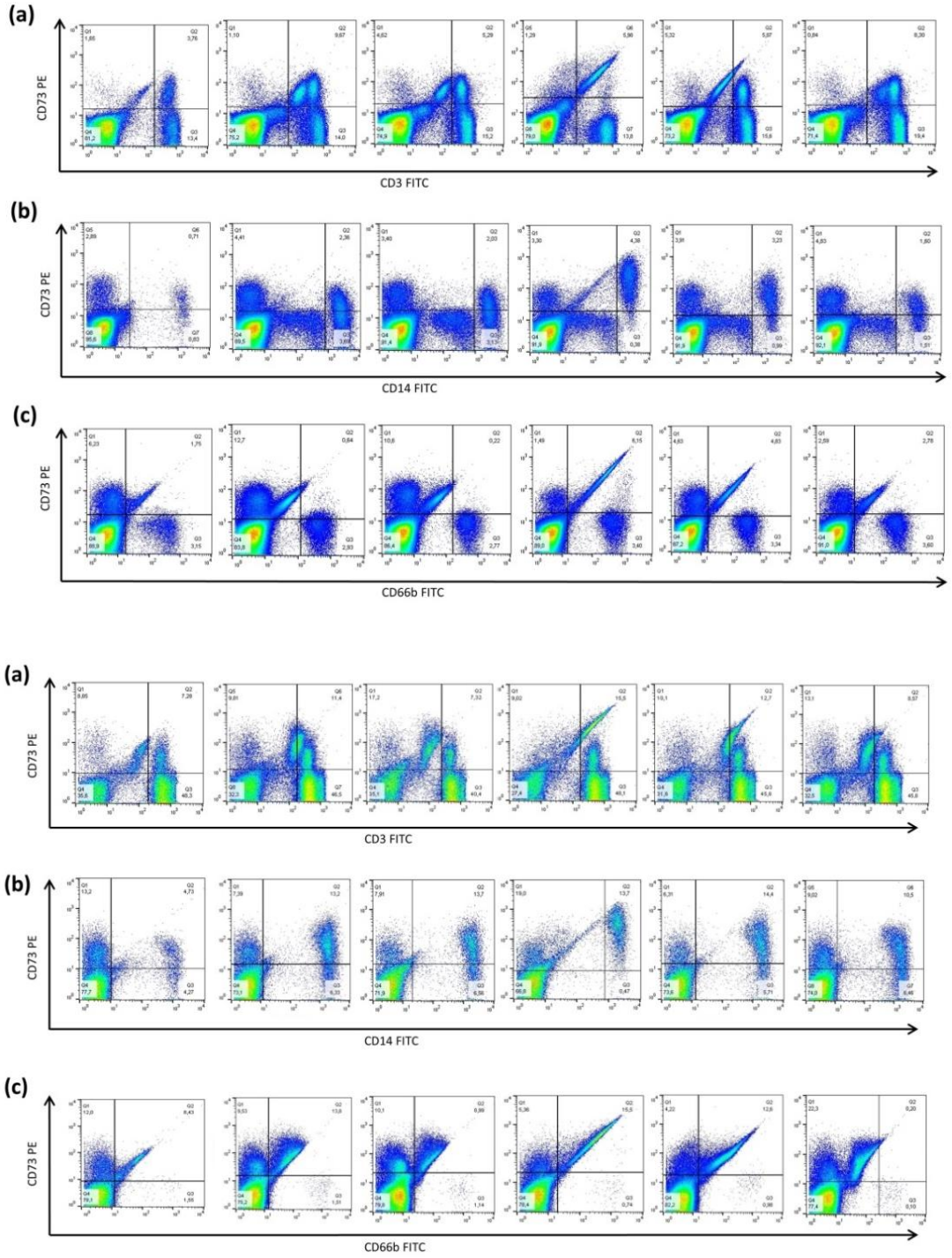


Figure 12.1. CD73 expression on cord blood and adult blood immune effector cells after 48-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC,upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubated for 48 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 48 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Given are representative density plots (from left to the right: untreated, LTA 10µg/ml, LPS 1µg/ml, Pam3Cys 10µg/ml, Pam3Cys 1µg/ml, Pam3Cys 0.1µg/ml), n=3*

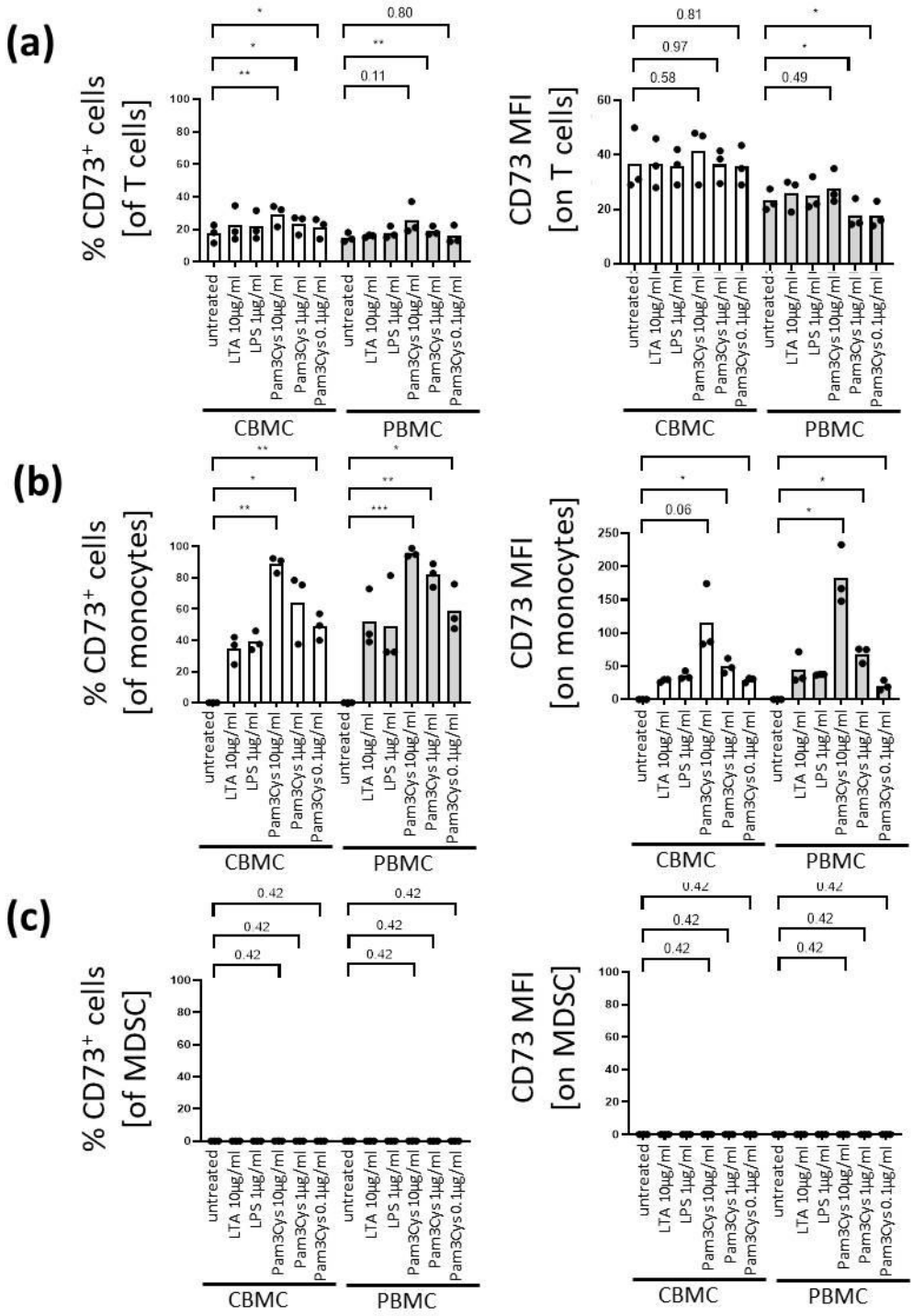


Figure 12.2. CD73 expression on cord blood and adult blood immune effector cells after 48-hour incubation in presence of LTA, LPS, Pam3Cys. *Cord blood (CBMC,upper part) and peripheral blood mononuclear cells (PBMC, lower part) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) to 2*10^6 cells per ml and let incubated for 48 hours under distinct conditions - lipoteichoic acid (LTA, c_{end}=10 µg/ml), lipopolysaccharides (LPS, c_{end}=1 µg/ml), Pam3Cys (c_{end}=10; 1; 0.1 µg/ml). After 48 hours, cells were harvested and stained for lineage markers for (a) T cells (CD3), (b) monocytes (CD14) and (c) MDSC (CD66b) and CD73. Expression was quantified by flow cytometry. Given are mean fluorescence intensities and percentage of positive cells (%), n=3, * = p<0.05, ** = p<0.01, *** = p<0.001*

4.10. Adenosine A_3 receptor expression by cord blood T cells remained unchanged in presence of adenosine but enhanced in presence of Pam3Cys

Fifty percent of freshly isolated cord blood T cells expressed adenosine A_{2A} receptor (50 ± 15%, n=3, Figure 13.2). Percentage of A_{2A}-positive T cells increased during 24 hours incubation independent from stimuli – untreated (70 ± 3%, n=3, p=0.26, Figure 13.2), adenosine [100μM] (65 ± 5%, n=3, p=0.26, Figure 13.2), Pam3Cys [10μg/ml] (68 ± 4%, n=3, p=0.41, Figure 13.2).

In contrast to cord blood T cells, percentage of freshly isolated adenosine A_{2A} receptor-positive adult T cells is higher (65 ± 5%, n=3, Figure 13.2) and the expression pattern did not change during incubation for 24 hours independent from stimuli – untreated (66 ± 3%, n=3, Figure 13.2), adenosine [100μM] (64 ± 2, n=3, p=0.12, Figure 13.2), Pam3Cys [10μg/ml] (65 ± 4%, n=3, p=0.31, Figure 13.2). A_{2A} receptor density on freshly isolated T cells (both, cord blood and adult) is low - cord blood monocytes: (11.5 ± 1.5 MFI, n=3, Figure 13.2) and adult monocytes (11.5 ± 2 MFI, n=3, Figure 13.2).

Density of A_{2A} receptor on cord blood T cells decreased independently from stimuli – untreated (7.5 ± 1 MFI, n=3, Figure 13.2), adenosine [100μM] (9 ± 2 MFI, n=3, p=0.06, Figure 13.2) and Pam3Cys [10μg/ml] (10 ± 4 MFI, n=3, p=0.26, Figure 13.2). Similarly, adult T cells slightly downregulated A_{2A} receptor during 24 hours incubation independent from stimuli – untreated (8 ± 1 MFI, n=3, Figure 13.2), adenosine [100μM] (8.5 ± 2 MFI, n=3, p=0.28, Figure 13.2), Pam3Cys [10μg/ml] (9 ± 2 MFI, n=3, p=24, Figure 13.2).

Majority of freshly isolated T cells expressed A_3 receptor - cord blood T cells (75 ± 12%, n=3, Figure 13.2), adult T cells (80 ± 2%, n=3, Figure 13.2). During 24 hours incubation, A_3 receptor expression was downregulated if untreated (65 ± 5%, n=3, Figure 13.2), remained unchanged in presence of adenosine [100μM] (75 ± 7%, n=3, p=0.06, Figure 13.2), or was upregulated in presence of Pam3Cys [10μg/ml] (80 ± 2%, n=3, p=0.06, Figure 13.2).

Contrary, adult T cells seemed not to be sensitive against these stimuli and their A_3 receptor expression remained unchanged (80 ± 3%, n=3, Figure 13.2).

Freshly isolated T cells (both, cord blood and adult) expressed moderate levels of A_3 receptor (15 ± 5 MFI, n=3, Figure 13.2). If the cord blood T cells remained untreated, A_3 expression level did not change over 24 hours (15 ± 2 MFI, n=3, Figure 13.2). Higher A_3 receptor expression levelsl were detected upon treatment with adenosine [100µM] (16 ± 2 MFI, n=3, p=0.23, Figure 13.2) as well as with Pam3Cys [10µg/ml] (17 ± 5 MFI, n=3, p=0.19, Figure 13.2). Adenosine A_3 receptor expression levels in adult T cells remained the same if cells remained untreated for 24 hours (15 ± 1 MFI, n=3, Figure 13.2). Slight decrease in A_3 receptor expression levels was observed upon treatment with adenosine [100µM] (14 ± 2 MFI, n=3, p=0.35, Figure 13.2). Contrary, treatment with Pam3Cys [10µg/ml] induced slight increase (16 ± 2 MFI, n=3, p=0.16, Figure 13.2).

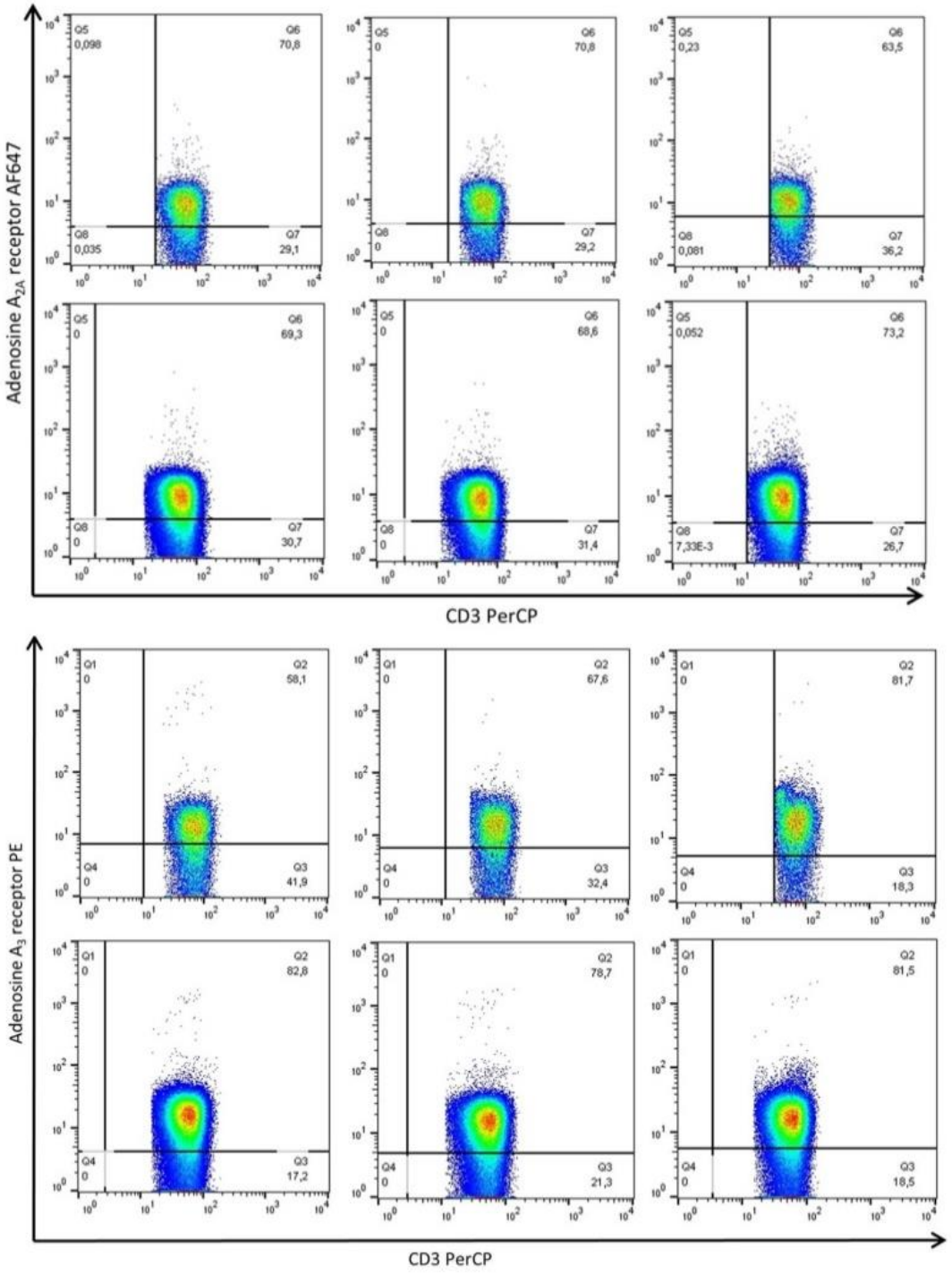


Figure 13.1. Adenosine A_{2A}, A_3 receptors expression on T cells after 24-hour incubation under distinct conditions. *Cord blood (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine), diluted up to $2*10^6$ cells per ml and let incubated for 24 hours in presence of adenosine [100 µM], Pam3Cys [10 µg/ml], or left untreated. After 24 hours cells were harvested, washed by spinning down and stained for lineage marker CD3 as well as for A_{2A} and A_3 receptor. Expression was quantified by flow cytometry. Given are representative density plots (1st and 3rd row: cord blood T cells, 2nd and 4th row: peripheral blood T cells, from left to right:untreated, adenosine [100 µM], Pam3Cys [10 µg/ml]), n=3.*

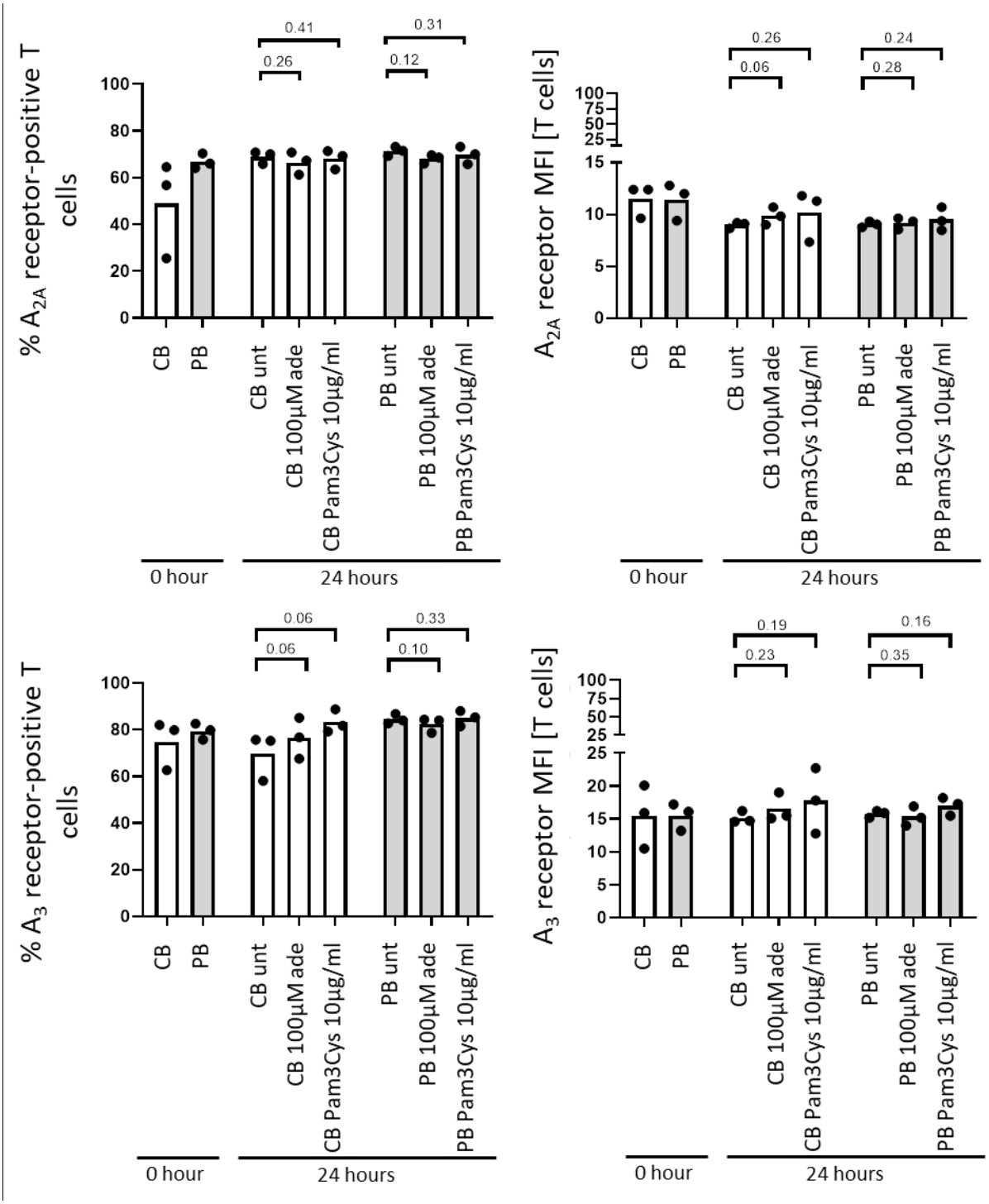


Figure 13.2. Adenosine A_{2A}, A_3 receptors expression on freshly isolated T cells and after 24-hour incubation under distinct conditions. *Cord blood (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine), diluted up to $2*10^6$ cells per ml and let incubated for 24 hours in presence of adenosine [100 µM], Pam3Cys [10 µg/ml], or left untreated. After 24 hours cells were harvested, washed by spinning down and stained for lineage marker CD3 as well as for A_{2A} and A_3 receptor. Expression was quantified by flow cytometry. Given are mean fluorescence intensities and percentage of positive cells (%), n=3.*

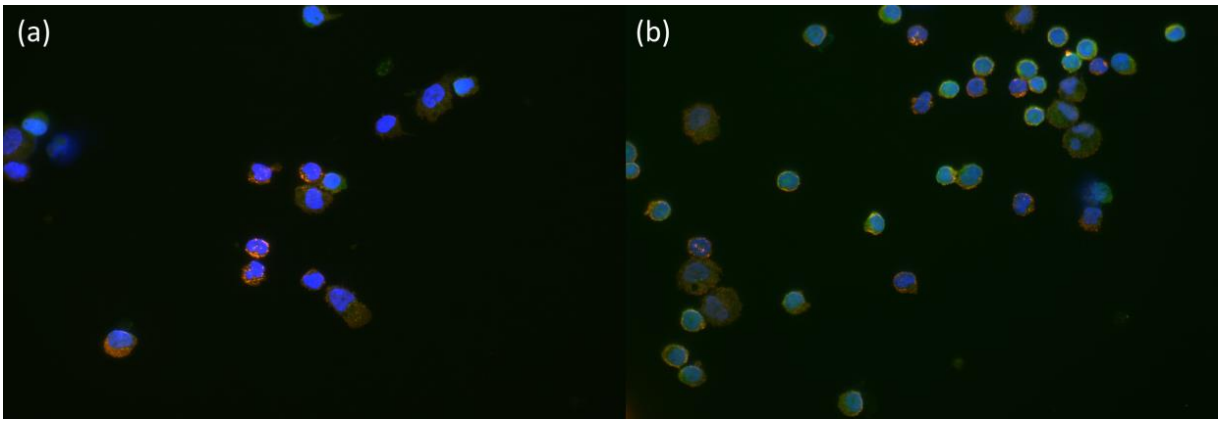


Figure 13.3. Immunofluorescence images of adenosine A_{2A} receptor on cord blood T cells. *Purified CBMC were incubated for 24 hours in (a) presence or (b) absence of adenosine [100 μM]. On next day, cells were harvested and the immunofluorescence staining was done as written in Materials and Methods. Antibodies – A_{2A} receptor AlexaFluor 647 dilution 1:50, CD3 FITC dilution 1:50, DAPI for nucleus staining.*

4.11. Expression of adenosine A_3 receptor was enhanced by monocytes in presence of adenosine and Pam3Cys

Up to 90% of freshly isolated monocytes (cord blood and adult) are positive for A_{2A} receptor. Incubation for 24 hours induced slight increase in A_{2A} expression independent from conditions (95 ± 3%, n=3, Figure 14.2). Similar situation was in case of adult monocytes, when A_{2A} receptor expression was slightly upregulated if treated with adenosine [100 µM] (95 ± 1, n=3, p=0.15, Figure 14.2), or left untreated (95 ± 1, n=3, Figure 14.2), respectively. Treatment with Pam3Cys Pam3Cys [10µg/ml] led to decreased A_{2A} expression on adult monocytes (90 ± 2%, n=3, p=0.05, Figure 14.2).

Expression levels of A_{2A} receptor on freshly isolated cord blood monocytes were moderate (17 ± 4 MFI, n=3, Figure 14.2) and remained unchanged over 24 hours incubation if the cells remained untreated. Treatment with adenosine [100 µM] induced slight increase in A_{2A} expression level (19 ± 6 MFI, n=3, p=0.26, Figure 14.2). Incubation for 24 hours in presence of Pam3Cys [10µg/ml] induced upregulation of A_{2A} receptors on cord blood monocytes (22 ± 4 MFI, n=3, $p<0.05$, Figure 14.2).

Expression levels of A_{2A} receptor on freshly isolated adult monocytes were slightly higher (19 ± 3 MFI, n=3, Figure 14.2) than those of freshly isolated cord blood monocytes. Slight increase in A_{2A} receptor expression level was observed upon 24 hours incubation on untreated monocytes (20 ± 1 MFI, n=3, Figure 14.2). Contrary, treatment with adenosine [100µM] led to decreased A_{2A} receptor expression levels (18 ± 3, n=3, p=0.26, Figure 14.2). Pam3Cys [10µg/ml] increased the A_{2A} receptor expression levels (21 ± 2 MFI, n=3, p=0.22, Figure 14.2).

Up to 80% of all freshly isolated cord blood monocytes expressed A_3 receptor. Interestingly, population of freshly isolated A_3-positive adult monocytes is greater (90 ± 2%, n=3, Figure 14.2). Incubation for 24hours induced slight increase in A_3-positive cord blood monocytes

dependening on the stimuli – untreated (90 ± 3%, n=3, Figure 14.2), adenosine [100 µM] (87 ± 7%, n=3, p=0.35, Figure 14.2) and Pam3Cys [10µg/ml] (85 ± 3, n=3, p=0.08, Figure 14.2).

In case of adult monocytes, A_3 receptor expression became enhanced during 24 hours incubation (95 ± 3, n=3, Figure 14.2) and the expression remained unchanged independent from conditions.

A_3 receptor expression levels on freshly isolated monocytes (cord blood and adult) seemed to be comparable (25 ± 8 MFI vs. 27 ± 5 MFI, n=3, Figure 14.2). In cord blood monocytes, A_3 expression level were increased after 24 hours incubation with a variable strength depending on the conditions – untreated (42 ± 8 MFI, n=3, Figure 14.2), adenosine [100 µM] (50 ± 8 MFI, n=3, $p<0.05$, Figure 14.2) and Pam3Cys [10µg/ml] (65 ± 15 MFI, n=3, p=0.12, Figure 14.2).

A_3 receptor expression levels on adult monocytes also increased during 24 hours incubation. Similar to cord blood monocytes, the strength varied according to the conditions – untreated (45 ± 5 MFI, n=3, Figure 14.2), adenosine [100 µM] (50 ± 5 MFI, n=3, p=0.24, Figure 14.2) and Pam3Cys [10µg/ml] (70 ± 6 MFI, n=3, $p<0.05$, Figure 14.2).

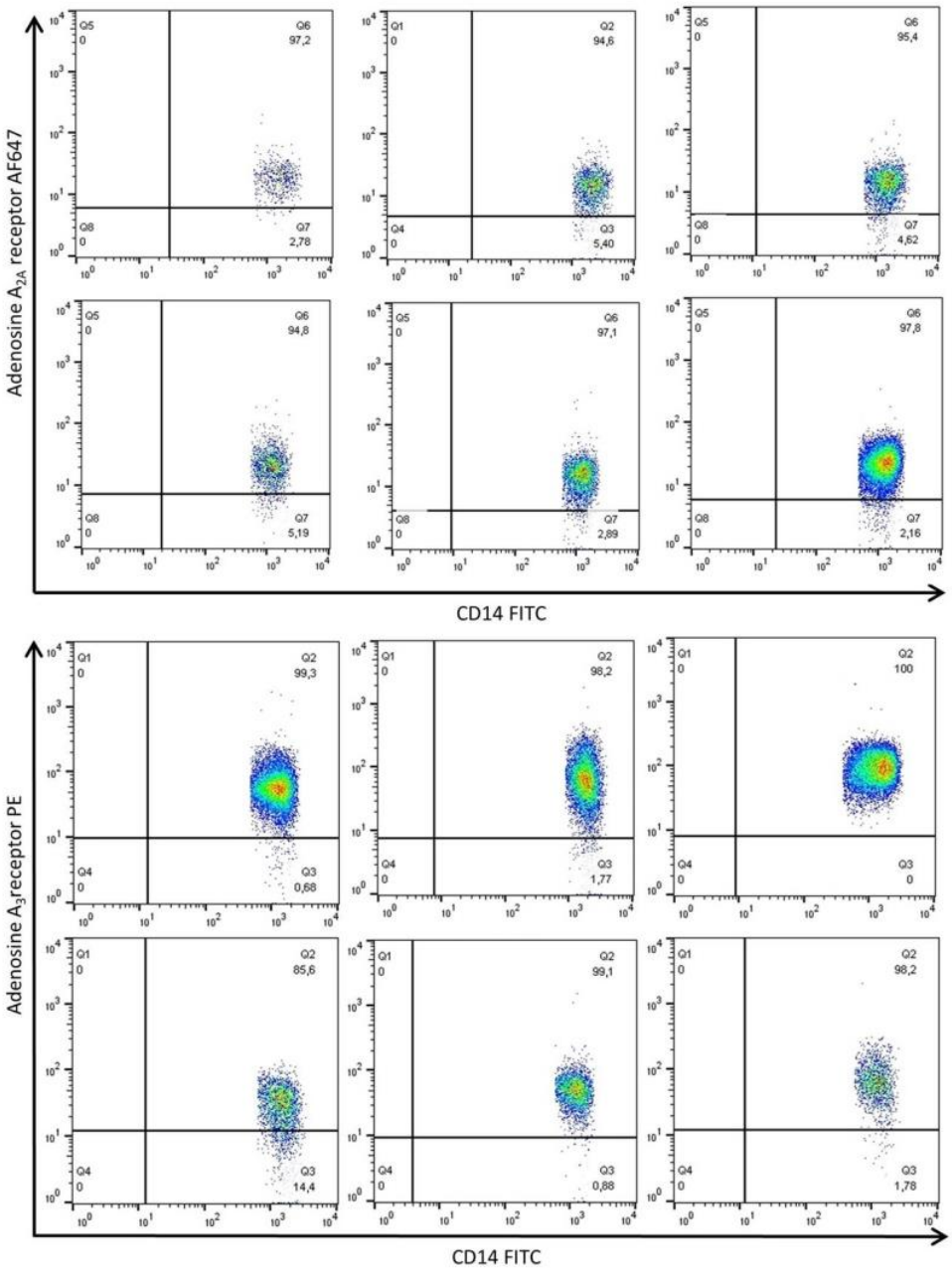


Figure 14.1. Adenosine A_{2A}, A_3 receptors expression on monocytes and after 24-hour incubation under distinct conditions. *Cord blood (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) and diluted up to $2*10^6$ cells per ml and let incubated for 24 hours in presence of adenosine [100 µM], Pam3Cys [10 µg/ml], or left untreated. After 24 hours cells were harvested, washed by spinning down and stained for lineage marker CD14 as well as for A_{2A} and A_3 receptor. Expression was quantified by flow cytometry. Given are representative density plots (1st and 3rd row: cord blood monocytes, 2nd and 4th row: peripheral blood monocytes, from left to right:untreated, adenosine [100 µM], Pam3Cys [10 µg/ml]) (Ďurčo et al. submitted)*

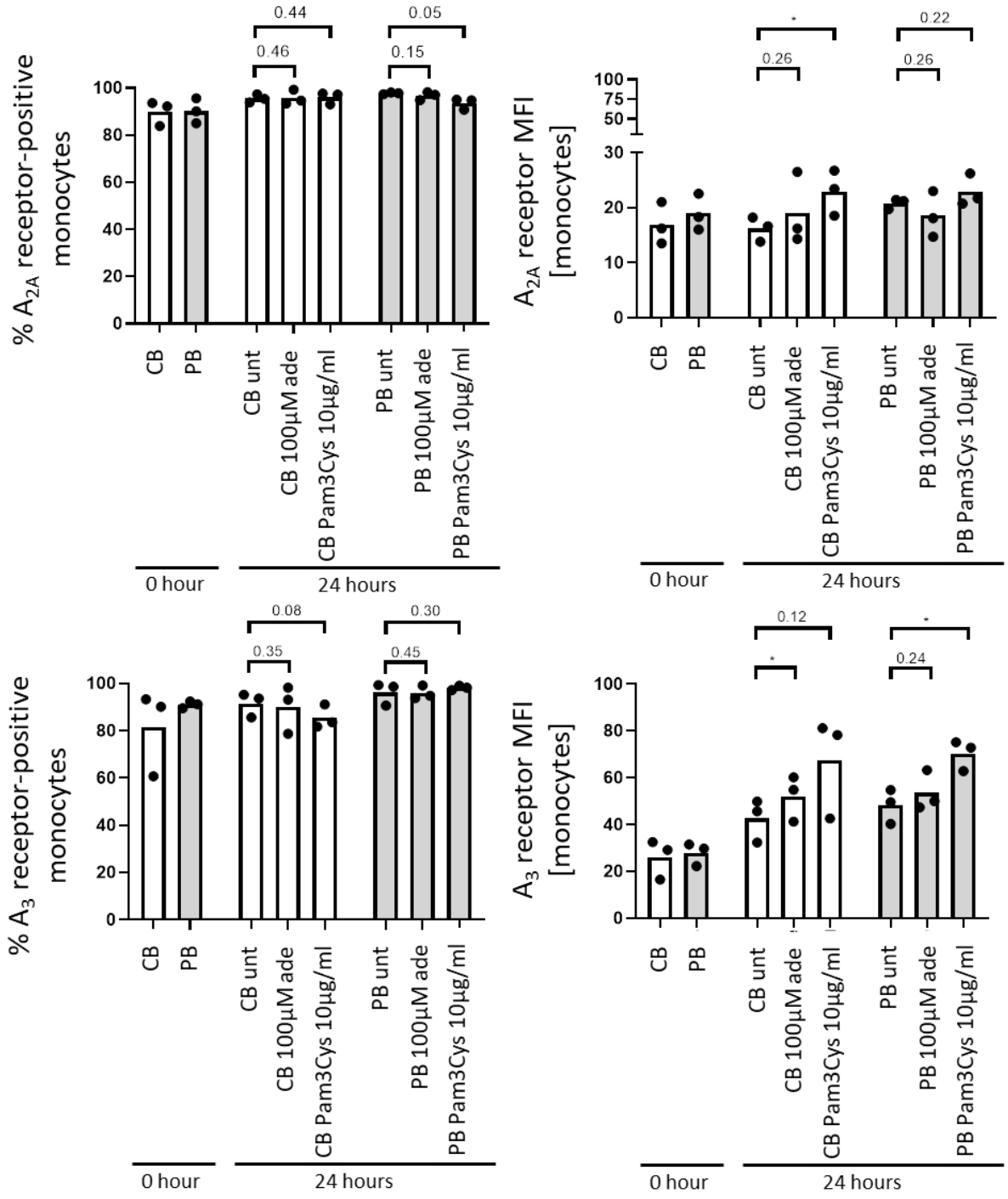


Figure 14.2. Adenosine A_{2A}, A_3 receptors expression on freshly isolated monocytes and after 24-hour incubation under distinct conditions. *Cord blood (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) and diluted up to $2*10^6$ cells per ml and let incubated for 24 hours in presence of adenosine [100 µM], Pam3Cys [10 µg/ml], or left untreated. After 24 hours cells were harvested, washed by spinning down and stained for lineage marker CD14 as well as for A_{2A} and A_3 receptor. Expression was quantified by flow cytometry. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%, n=3, * = p<0.05) (Ďurčo et al. submitted)*

4.12. The population of A_3-positive cord blood MDSC became smaller after treatment with Pam3Cys but the expression rate per cell increased.

Independent from origin, majority (80 ± 5%) of freshly isolated MDSC expressed A_{2A} receptor. The incubation for 24 hours increased the population of A_{2A}-positive cord blood MDSC, whereas the increase was dependent on the conditions – untreated MDSC (85 ± 3%, n=3, Figure 15), MDSC treated with adenosine [100 µM] (90 ± 5, n=3, p=0.11, Figure 15.2) and MDSC treated with Pam3Cys [10µg/ml] (87 ± 3, n=3, p=0.34, Figure 15.2).

Adult MDSC also upregulated A_{2A} receptor during the 24 hours incubation (85 ± 3%, n=3, Figure 15.2). Moreover, treatment with Pam3Cys [10µg/ml] led to strongest upregulation (90 ± 5%, n=3, $p<0.05$, Figure 15.2) of A_{2A} receptor expression from the given conditions.

Expression levels of A_{2A} receptor on freshly isolated MDSC (cord blood and adult) have been shown to be moderate – cord blood MDSC (18 ± 6, n=3, Figure 15.2), adult MDSC (20 ± 7, n=3, Figure 15.2). Incubation of cord blood MDSC for 24 hours led to slight downregulation of A_{2A} receptor under all three given conditions – untreated (13 ± 4 MFI, n=3, Figure 15.2), adenosine [100 µM] (16 ± 4 MFI, n=3, $p<0.05$, Figure 15.2) and Pam3Cys [10µg/ml] (18 ± 3, n=3, p=0.12, Figure 15.2).

In comparison to freshly isolated adult MDSC, adult MDSC downregulated A_{2A} receptor during 24 hours incubation if untreated (11 ± 1 MFI, n=3, Figure 15.2), or in presence of adenosine [100 µM] (14 ± 1 MFI, n=3, $p<0.05$, Figure 15.2). Intriguingly, A_{2A} receptor expression levels became higher (22 ± 10 MFI, n=3, p=0.17, Figure 15.2) after 24 hours incubation in presence of Pam3Cys [10µg/ml].

Adenosine A_3 receptor is commonly present on MDSC (cord blood and adult) – 85 ± 15% cord blood MDSC and 85 ± 5% adult MDSC are positive for adenosine A_3 receptor. After incubation for 24 hours percentage of A_3-positive cord blood MDSC increased – untreated (87 ± 1%, n=3, Figure 15.2), adenosine [100 µM] (90 ± 5%, n=3, p=0.25, Figure 15.2).

Contrary, number of A_3-positive cord blood MDSC decreased (78 ± 6%, n=3, p=0.11, Figure 15.2) in presence of Pam3Cys [10µg/ml].

During the 24 hours incubation population of A_3-positive adult MDSC became slightly greater (90 ± 3%, n=3, Figure 15.2) in comparison to freshly isolated adult MDSC. Treatment with Pam3Cys [10µg/ml] and adenosine [100 µM] for 24 hours led to comparable increase of A_3-positive MDSC (95 ± 3%, n=3, Figure 15.2).

Adenosine A_3 receptor expression levels have been shown to be moderate in both, freshly isolated cord blood MDSC (35 ± 5 MFI, n=3, Figure 15.2) and adult MDSC (41 ± 25 MFI, n=3, Figure 15.2). The incubation for 24 hours without treatment and in presence of adenosine [100 µM] led to diminished expression levels of A_3 receptor in comparison to freshly isolated cord blood MDSC – untreated (30 ± 8 MFI vs. 35 ± 5 MFI, n=3, Figure 15.2) and with adenosine [100µM] (30 ± 3 MFI vs. 35 ± 5 MFI, n=3, Figure 15.2). Contrary, incubation with Pam3Cys [10µg/ml] increased the A_3 receptor expression levels in comparison to the freshly isolated cord blood MDSC (40 ± 10 vs. 35 ± 5 MFI, n=3, Figure 15.2).

Adult MDSC have been shown to express another pattern. If untreated, A_3 receptor was downregulated in comparison to freshly isolated adult MDSC (30 ± 5 MFI, n=3, Figure 15.2). If incubated with adenosine [100 µM], A_3 receptor expression levels remained unchanged (40 ± 10 MFI, n=3, Figure 15.2) in comparison to the freshly isolated adult MDSC. Incubation with Pam3Cys [10µg/ml] led to diminished A_3 receptor expression levels (33 ± 6 MFI, n=2, Figure 15.2) in comparison to the freshly isolated adult MDSC.

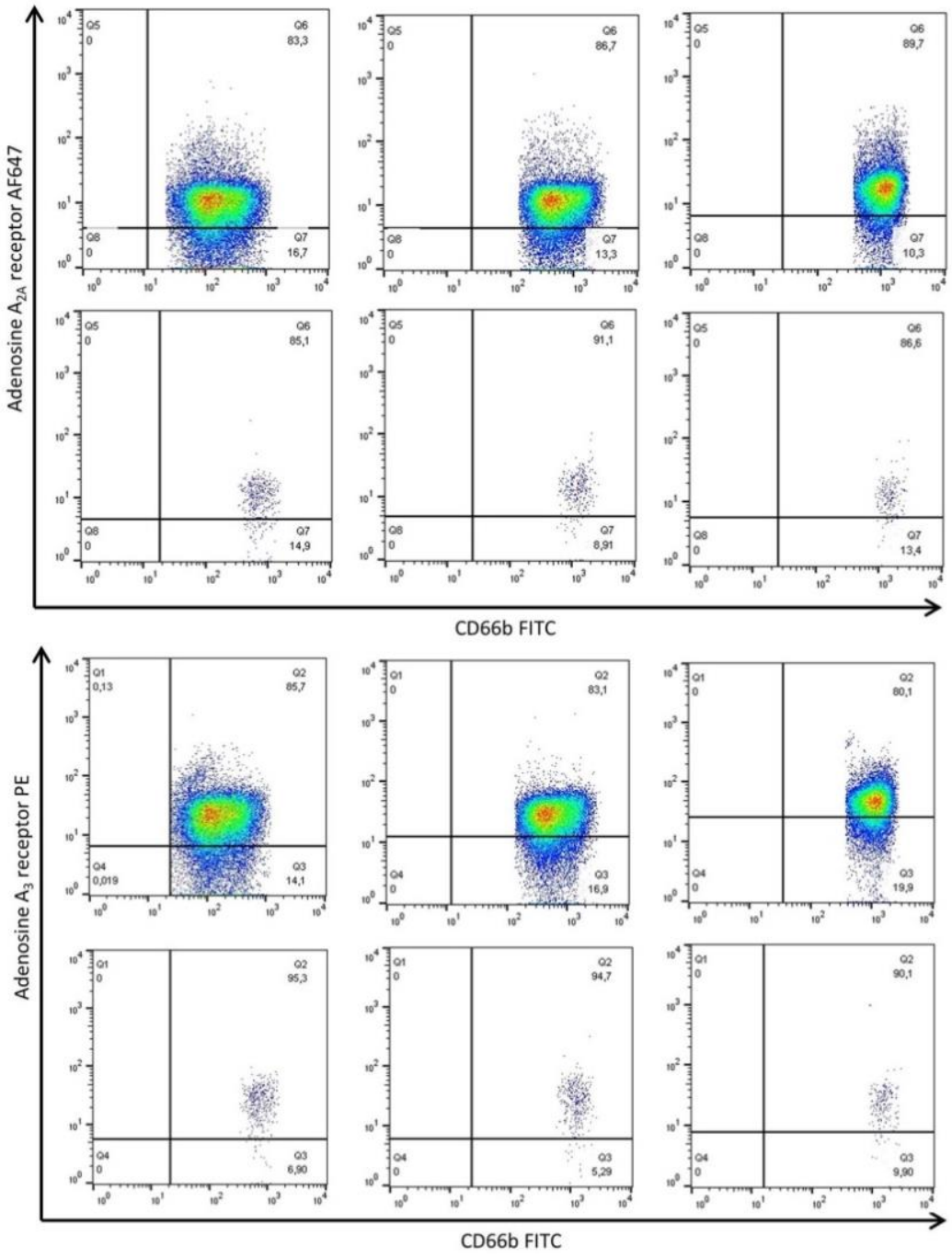


Figure 15.1. Adenosine A_{2A}, A_3 receptors expression on MDSC and after 24-hour incubation under distinct conditions. *Cord blood (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) and diluted up to $2*10^6$ cells per ml and let incubated for 24 hours in presence of adenosine [100 µM], Pam3Cys [10 µg/ml], or left untreated. After 24 hours cells were harvested, washed by spinning down and stained for lineage marker CD66b as well as for A_{2A} and A_3 receptor. Expression was quantified by flow cytometry. Given are representative density plots (1^{st} and 3^{rd} row: cord blood MDSC, 2^{nd} and 4^{th} row: peripheral blood MDSC, from left to right:untreated, adenosine [100 µM], Pam3Cys [10 µg/ml]).*

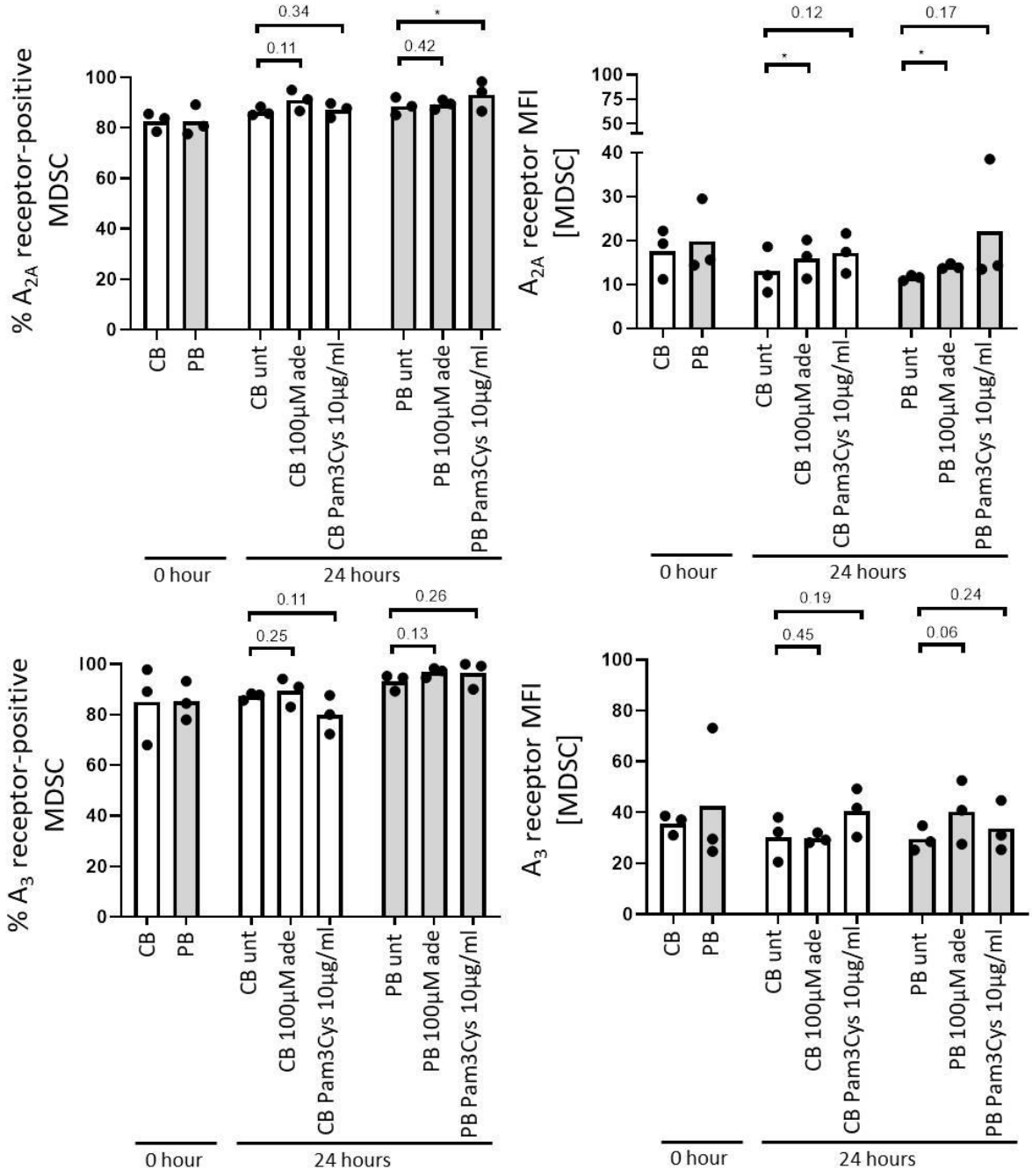


Figure 15.2. Adenosine A_{2A}, A_3 receptors expression on freshly isolated MDSC and after 24-hour incubation under distinct conditions. *Cord blood (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) and diluted up to $2*10^6$ cells per ml and let incubated for 24 hours in presence of adenosine [100 µM], Pam3Cys [10 µg/ml], or left untreated. After 24 hours cells were harvested, washed by spinning down and stained for lineage marker CD66b as well as for A_{2A} and A_3 receptor. Expression was quantified by flow cytometry. Given are mean fluorescence intensities (MFI) and percentage of positive cells (%), n=3, * = p<0.05*

4.13. Adenosine has a direct negative effect on T cell proliferation

OKT3/IL-2-induced T cell proliferation (both, cord and adult blood) is dose-dependently subdued with increasing adenosine concentration. Adenosine [1µM] diminished proliferation of cord blood T cell by 10% in comparison to OKT3/IL-2-stimulated cord blood T cells without adenosine. Adult T cells seemed to be more resistant against adenosine – their proliferation was diminished by 6.25% in comparison to OKT3/IL-2-stimulated adult T cells. Ten-times higher adenosine concentartion [10µM] seemed not to have a stronger inhibition effect on cord blood T cell proliferation than 1µM adenosine. Contrary, proliferation of adult T cells in presence adenosine [10µM] was downregulated by 14.75%. Increasing the adenosine concentration to 100µM, proliferation was downregulated by 17.67% (cord blood T cells), 35.875% (adult T cells).The highest adenosine concentration [1000µM] led to diminishing T cell proliferation by 47% (cord blood), or 68.4% (adult blood), respectively.

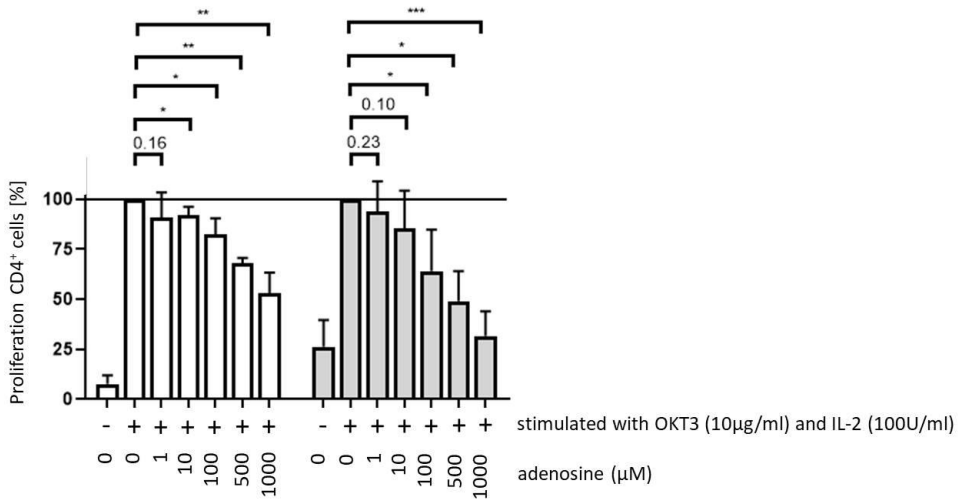
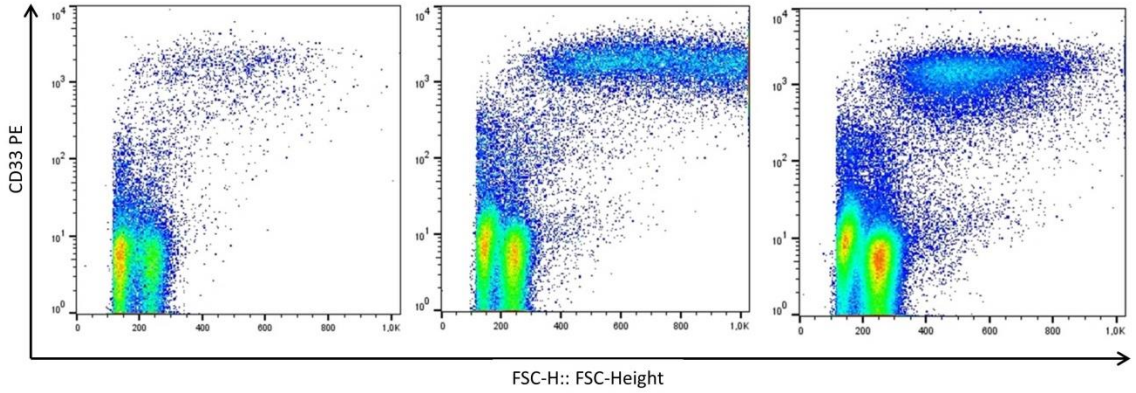


Figure 16. T cell proliferation inhibition in presence of ascending adenosine concentration. *Cord blood (white bars) and peripheral blood mononuclear cells (PBMC, light grey bars) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) and diluted up to 5*10^5 cells per ml and let incubate in presence of adenosine [100 µM] for 4 days at 37°C, 5% CO_2. On day 4 cells were harvested, stained for CD4 and the proliferation rate was examined by flow cytometry. Given is the proliferation rate in percentage (%) ± SD, n=4, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.*

4.14. The potency of adenosine to induce MDSC is comparable with that of GM-CSF

Applying adenosine to suspension of PBMC led to a strong induction of adult $CD33^+$ MDSC in comparison to the untreated control (14.6 ± 5% vs. 8.7 ± 5%, $p<0.001$, n=15, Figure 17). Interestingly, the yield was comparable to that of GM-CSF (14.6 ± 5% vs. 14.7 ± 5%, n=7, Figure 17) taken as a benchmark. Relative accumulation of induced MDSC was not due to decrease of other cell types (see Figure 21). Thus my results show for the first time that adenosine is capable to induced MDSC in healthy individuals (Ďurčo et al. submitted).



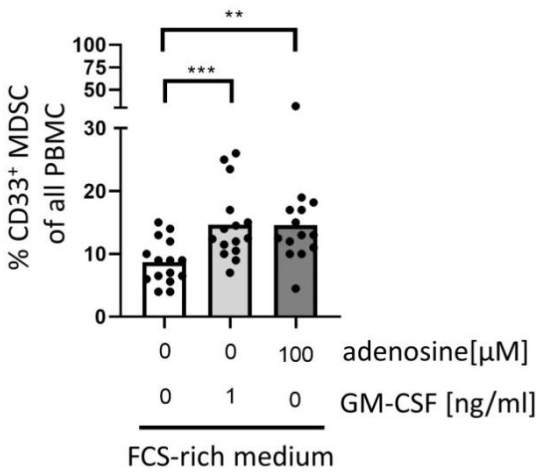


Figure 17. MDSC induction in presence of GM-CSF or adenosine in FCS-rich medium. *Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in complete medium (RPMI 1640 + FCS + P/S + L-glutamine) and diluted up to $5*10^5$ cells per ml and let incubate in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] or left untreated for 7 days at 37°C, 5% CO_2, with medium change on day 4. On day 7, cells were harvested and examined for CD33-positive cells by flow cytometry, Given are representative density plots (from left to right: negative control, GM-CSF, adenosine) as well as percentage (%) of CD33-positive cells n=14-15.* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (Ďurčo et al. submitted) (2nd paper in preparation)*

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4.15. Adenosine-based MDSC induction is diminished in cord blood plasma-rich medium

Our group tries to elucidate the basic mechanisms of immune responses in fetus and in neonate. That´s why I decided to replace the fetal calf serum in complete medium by freshly isolated cord blood plasma to evaluate the influence of cord blood plasma on the MDSC induction. An interesting phenomenon could be observed when adenosine-based MDSC induction yielded smaller amount of $CD33^+$ MDSC in comparison to adenosine-based induction made in FCS-rich complete medium (6.3 ± 6% vs. 13.1 ± 6%, $p<0.01$, Figure 18). Contrary, GM-CSF-based induction in presence of freshly isolated cord blood plasma yielded higher amount of $CD33^+$ MDSC than GM-CSF-based induction done in FCS-rich medium (16.47 ± 10% vs. 13.48 ± 6%, $p<0.05$, Figure 18) (2nd paper in preparation).

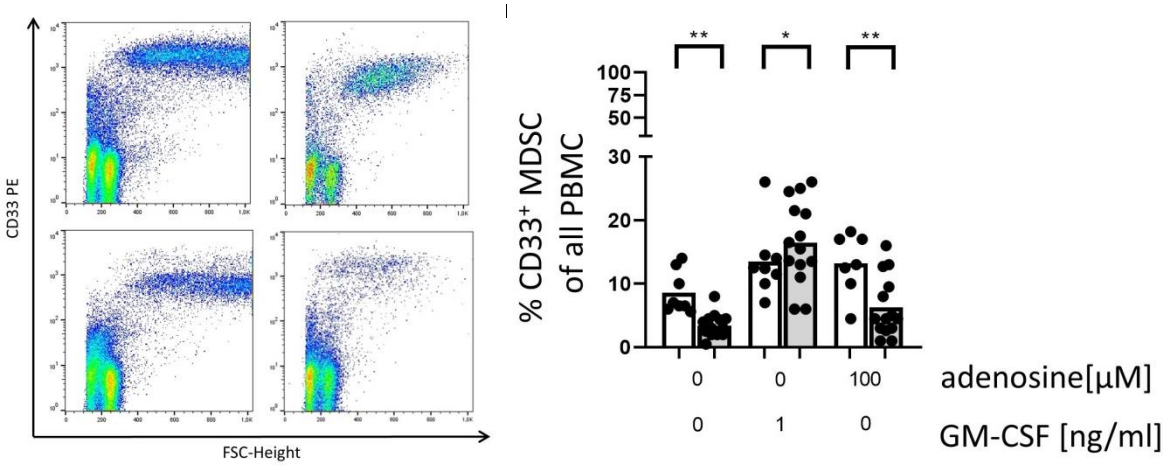


Figure 18. MDSC induction in presence of GM-CSF or adenosine in cord blood plasma-rich medium and FCS-rich medium. *Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in medium containing either FCS (RPMI 1640 + FCS + P/S + L-glutamine) (white bars) or cord blood plasma (RPMI 1640 + cord blood plasma + P/S + L-glutamine) (gray bars) and diluted up to $5*10^5$ cells per ml and let incubate in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] or left untreated for 7 days at 37°C, 5% CO_2, with medium change on day 4. On day 7, cells were harvested and examined for CD33-positive cells by flow cytometry. Given are representative density plots (left: GM-CSF, right: adenosine, upper row: FCS-rich medium, lower row: cord blood plasma-rich medium) as well as percentage (%) of CD33-positive cells, FCS-rich medium n=7-8, cord blood plasma-rich medium n=14,* = p < 0.05, ** = p < 0.01 (Ďurčo et al. submitted) (2nd paper in preparation).*

4.16. Diminished adenosine-based MDSC induction was also observed during incubation in autologous adult blood plasma-rich medium

To get a broader overview over effect of human blood plasma on the MDSC induction, I replaced freshly isolated cord blood plasma by freshly isolated autologous adult blood plasma. Modified medium was applied to induce $CD33^+$ MDSC. In comparison to the FCS-rich complete medium, adenosine-based MDSC induction was reduced (5.8 ± 4% vs. 13.1 ±7%, $p<0.01$, FCS-rich medium n=7, autologous adult blood plasma-rich medium n=12, Figure 19). Interestingly, GM-CSF-based MDSC induction in presence of autologous adult blood plasma-rich medium did not differ strongly from that made in the FCS-rich medium (14.6 ± 10% vs. 13.5 ± 8%, p=0.45, FCS-rich medium n=8, autologous adult blood plasma-rich medium n=12, Figure 19).

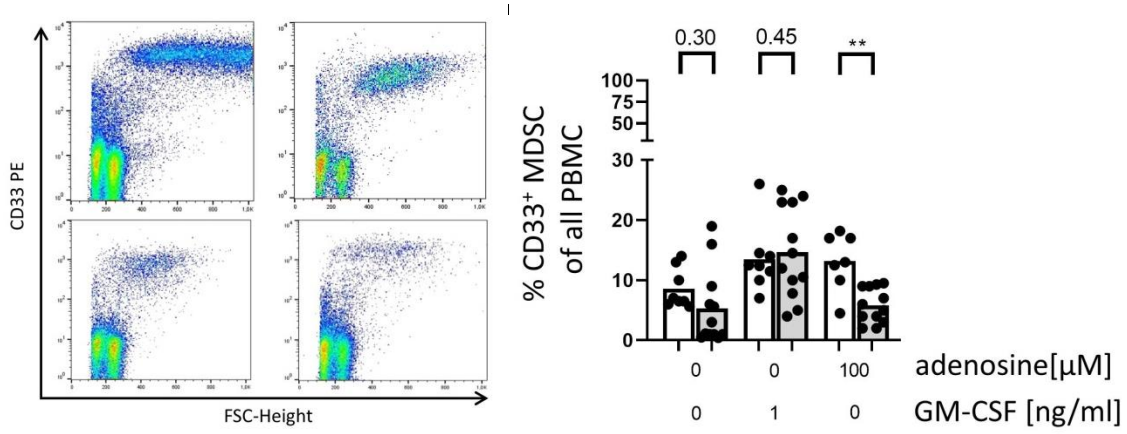


Figure 19. MDSC induction in presence of GM-CSF or adenosine in medium containing autologous adult blood plasma. *Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in medium containing either FCS (RPMI 1640 + FCS + P/S + L-glutamine) (white bars) or autologous adult blood plasma (RPMI 1640 + autologous adult blood plasma + P/S + L-glutamine) (gray bars) and diluted up to $5*10^5$ cells per ml and let incubate in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] or left untreated for 7 days at 37°C, 5% CO_2, with medium change on day 4. On day 7, cells were harvested and examined for CD33-positive cells by flow cytometry. Given are representative density plots (left: GM-CSF, right: adenosine, upper row: FCS-rich medium, lower row: autologous adult blood plasma-rich medium) as well as percentage (%) of CD33-positive cells, FCS-rich medium n=7-8, autologous adult blood plasma-rich medium n=12, * = p < 0.05, ** = p < 0.01.*

4.17. Applying of heterologous adult blood plasma resulted in diminished adenosine-based MDSC induction

To get a complete overview of the effect of human blood plasma on the MDSC induction, I also applied freshly isolated heterologous adult blood plasma instead of FCS. Similarly to previous modifications, MDSC induction done in this modified medium yielded smaller amount of adenosine-induced MDSC in comparison to induction done in FCS-rich complete medium (8.6 ± 8% vs. 13.2 ± 7%, n<0.05, FCS-rich medium n=7, heterologous adult blood plasma-rich medium n=6, Figure 20). Further, GM-CSF-based MDSC induction (9.1 ± 4% vs. 13.5 ± 7%, n=0.08, FCS-rich medium n=8, heterologous adult blood plasma n=6, Figure 20).

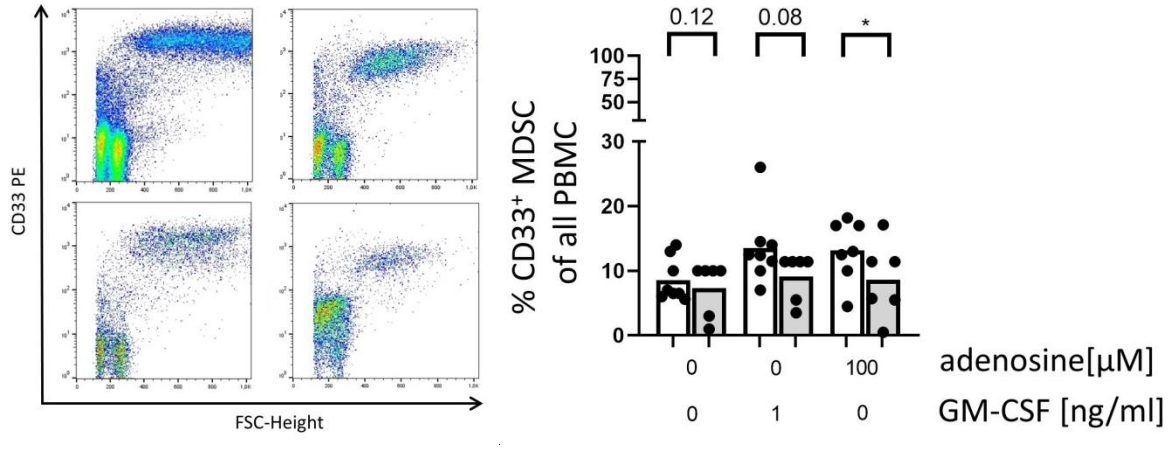


Figure 20. MDSC induction in presence of GM-CSF or adenosine in medium containing heterologous adult blood plasma. *Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended in medium containing either FCS (RPMI 1640 + FCS + P/S + L-glutamine) (white bars) or heterologous adult blood plasma (RPMI 1640 + heterologous adult blood plasma + P/S + L-glutamine) (gray bars) and diluted up to 5*10^5 cells per ml and incubated in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] or left untreated for 7 days at 37°C, 5% CO_2, with medium change on day 4. On day 7, cells were harvested and examined for CD33-positive cells by flow cytometry. Given are representative density plots (left: GM-CSF, right: adenosine, upper row: FCS-rich medium, lower row: heterologous adult blood plasma-rich medium) as well as percentage (%) of CD33-positive cells, FCS-rich medium n=7-8, heterologous adult blood plasma-rich medium n=6, * = p < 0.05, ** = p < 0.01.*

4.18. The highest survival rate of seeded PBMC was achieved in FCS-rich medium

Another aspect worth to mention was the survival rate of the seeded PBMC after 7 days incubation. As shown in the Figure 21, cells incubated in FCS-rich medium showed the best fitness. Within this group, survival rates varies dependent on the conditions – if cells remained untreated, one-fourth (24.7 ± 20%) of seeded cells survived, addition of GM-CSF [1ng/ml] to the cell culture increased the cells survival of seeded cells in comparison to the untreated cell culture (33.2 ± 30% vs. 24.7 ± 20%, n=16, $p<0.05$, Figure 21). Slightly increased survival rate of seeded cells was also observed after addition of adenosine [100µM] (28 ± 25% vs. 24.7 ± 20%, n=16, $p=0.11$, Figure 21).

Replacing FCS by freshly isolated cord blood plasma led to diminished survival rate, in general. If untreated, only 8.6 ± 8% survived in modified medium. Treatment with GM-CSF [1ng/ml] promoted cell survival (20.7 ± 13% vs. 8.6 ± 8%, n=14, $p<0.01$, Figure 21). Addition of adenosine [100µM] resulted in slightly increased cell survival in comparison to untreated cells (12.4 ± 12% vs. 8.6 ± 8%, n=14, $p<0.05$, Figure 21).

Similarly, applying autologous adult blood plasma instead of FCS resulted in reduced yield in comparison to the induction in FCS-rich medium. Incubation of untreated sample resulted in survival of 17.9 ± 12% of seeded cells. Addition of GM-CSF [1ng/ml] slightly increased the survival rate in comparison to the untreated cells (20.8 ± 12% vs. 17.9 ± 12%, n=13, $p=0.12$, Figure 21). Treatment with adenosine [100µM] resulted in slightly reduced survival rate of seeded cells in comparison to the untreated cells (16.1 ± 12% vs. 17.9 ± 12%, n=13, $p=0.24$, Figure 21).

Heterologous adult blood plasma-rich medium also led to diminished yield of survived cells after 7 days incubation. Minority of PBMC (6.8 ± 5%) survived if untreated. Presence of GM-CSF [1ng/ml] led to slight increase in cell survival in comparison to the untreated cells (8.5 ± 4% vs. 6.8 ± 5%, n=6, p<0.05, Figure 21). Interestingly, yield of adenosine-treated culture was comparable to that of GM-CSF (8.6 ± 8%).

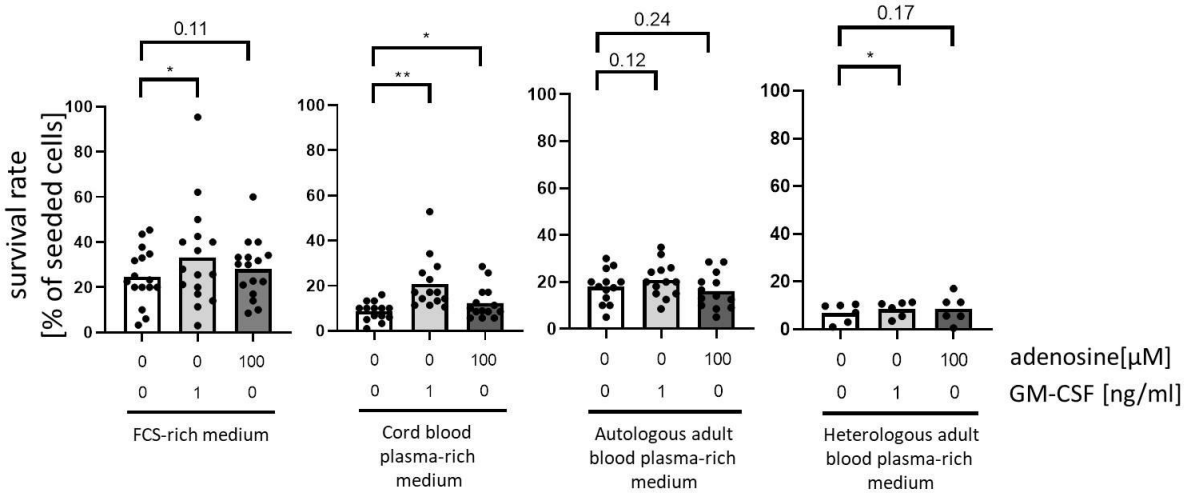


Figure 21. Survival rate of seeded cells during MDSC induction. *Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended and diluted up to 5*10^5 cells per ml in medium (RPMI + 1% P/S + 1% L-Glutamine) containing (from left to right) either fetal calf serum or cord blood plasma or autologous adult blood plasma or heterologous adult blood plasma, respectively. Cell suspensions were incubated in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] for 7 days at 37°C, 5% CO_2, with medium change on day 4. On day 7, cells were harvested, an aliquot was stained with Trypan Blue and cells were counted using a binocular. Given are percentages of survived cells (%) as well as mean. FCS-rich medium n=16, cord blood plasma-rich medium n=14, autologous adult blood plasma n=13, heterologous adult blood plasma n=6, * = p<0.05, ** = p<0.01.*

4.19. MDSC induction done in cord blood plasma-rich medium resulted in the highest yield of separated cells

Next aspect to be investigated was the percentage of cells separated by AutoMACS using CD33-positive magnetic beads after the process of harvesting. An interesting phenomenon was observed in cell cultures cultivated in FCS-rich medium. On one hand, incubation in FCS-rich media resulted in highest survival rate (see Figure 21), on the other hand one-week long incubation in FCS-rich medium resulted in a small amount of cells (3 ± 3%, n=14, p=0.48, Figure 22) yielded by AutoMACS separation using CD33-positive magnetic beads.

Contrary, when cord blood plasma-rich medium was applied, survival rate of seeded cells was lower than those incubated in FCS-rich medium (see Figure 21) but the percentage of cells separated by AutoAMCS using CD33-positive magnetic beads was higher - GM-CSF (6.5 ± 4%, n=4) and adenosine (5.4 ± 3.5%, n=6, p=0.48, Figure 22).

Applying adult blood plasma (autologous and heterologous) led to reduced survival rate of seeded cells (see Figure 21) as well as to small yield of cells separated by AutoMACS using CD33-positive magnetic beads (5 ± 4.5%, n=5, p=0.38, Figure 22).

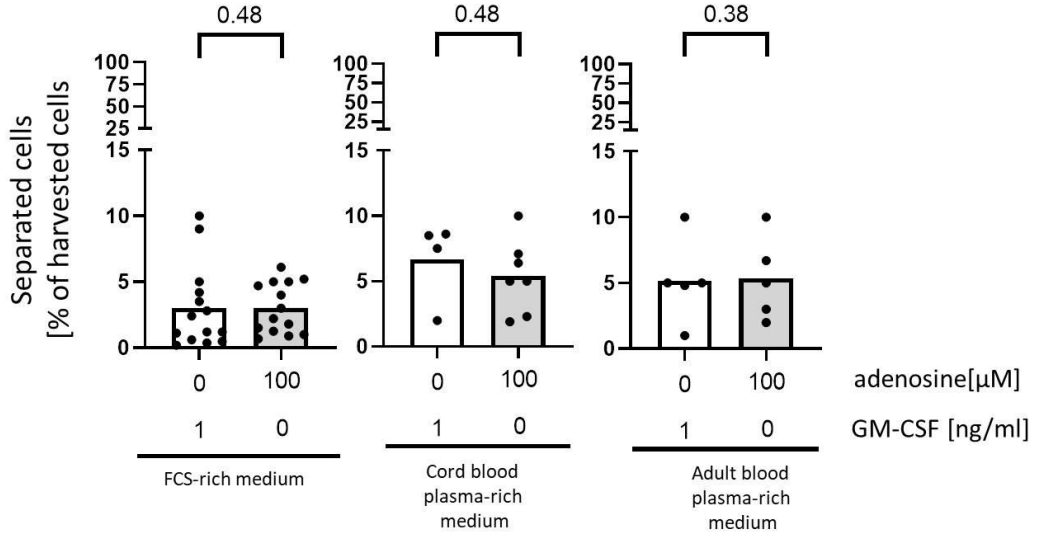


Figure 22. Rate of separated cells upon harvesting on day 7. *Peripheral blood mononuclear cells (PBMC) were isolated by Biocoll gradient centrifugation, resuspended and diluted up to 5*10^5 cells per ml in medium (RPMI + 1% P/S + 1% L-Glutamine) containing (from left to right) either fetal calf serum or cord blood plasma or adult blood plasma, respectively. Cell suspensions were incubated in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] for 7 days at 37°C, 5% CO_2, with medium change on day 4. On day 7, cells were harvested, isolated by AutoMACS using CD33-positive magnetic beads and counted using a binocular. Given are percentages of cells separated by AutoMACS (%) as well as mean, FCS-rich medium n=14, cord blood plasma-rich medium n=4-7, adult blood plasma-rich medium n=5.*

4.20. Adenosin-induced MDSC effectively suppressed T cell proliferation

For functional analysis of adenosine-induced MDSC we next tested their capability to inhibit T cell proliferation, a pivotal effect of MDSC. Co-cultivation of adenosine-induced MDSC with freshly isolated autologous PBMC in different ratios dose-dependently inhibited T cell proliferation. The adenosine-induced MDSC exerted inhibitory capacity comparable to that of GM-CSF-induced MDSC used as a control (31.6 ± 8% vs. 27.7 ± 8%, p=0.21, n=5-9, Figure 23). Thus adenosine may exert its immunomodulatory properties via induction of activated MDSC (Ďurčo et al. submitted).

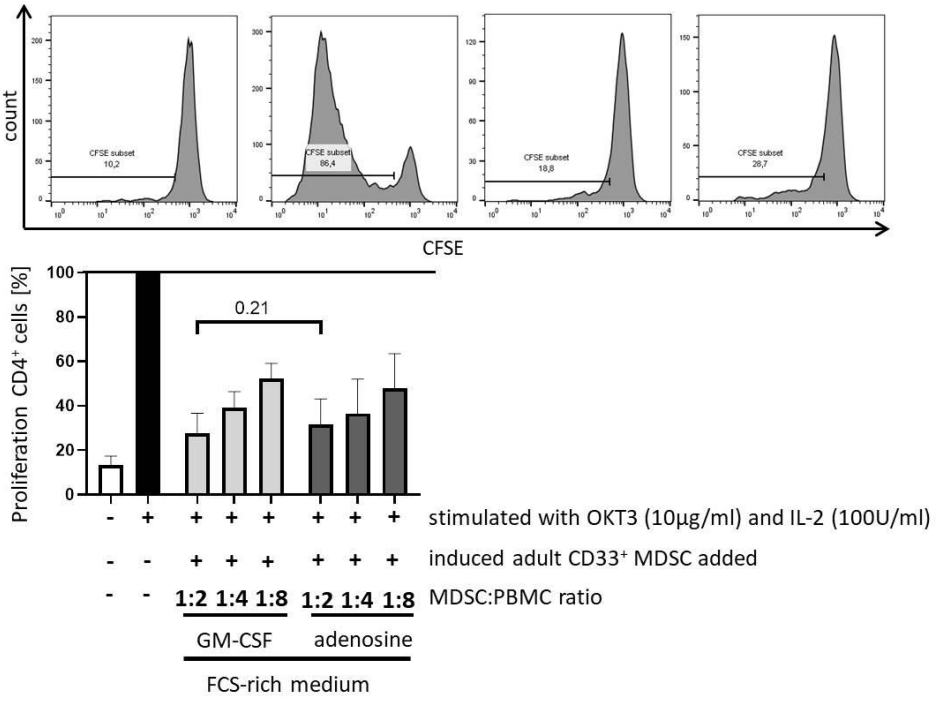


Figure 23. T cell proliferation inhibition by MDSC induced in FCS-rich medium. *Peripheral blood mononuclear cells (PBMC) were isolated, resuspended in RPMI 1640 medium + 10% fetal calf serum + 1% penicillin/streptomycin + 1% L-glutamine and let incubate in presence of Granulocyte-macrophage colony-stimulating factor (GM-CSF) [1 ng/ml] or adenosine [100 µM] for 7 days at 37°C, 5% CO_2 with medium change on day 4. On day 7, cells were harvested applying Detachin™ and using cell scraper to detach the cells from the plastic dish bottom and investigated on CD33 expression by flow cytometry. The CD33-positive cells were isolated by AutoMACS Separator using anti-CD33 microbeads, counted under the binocular, resuspended in a sterile 1x phosphate buffered saline and diluted to $1*10^6$ cells per ml. In the mean time autologous PBMC were freshly isolated using Biocoll density gradient centrifugation, diluted with a sterile 1x phosphate buffered saline to $1*10^7$ cells per ml and stained with CFSE. Stained cells were counted on the Sysmex, resuspended and diluted to $2*10^6$ cells per ml in complete medium containing autologous blood plasma (10%), RPMI 1640, penicillin / streptomycin (1%) and L-glutamine (1%) and split into two aliquots. One aliquot remained unstimulated as a negative control, the second aliquot was stimulated with IL-2 (100U/ml) and OKT3 (c_{end}=10µg/ml, stock 1mg/ml). The stimulated cells were put in a co-culture with CD33-positive cells in different ratios and let incubate for 4 days at 37°C, 5% CO_2. On day 4 cells were harvested, stained for CD4 and the proliferation rate was examined by flow cytometry. Given is the proliferation rate in percentage (%) ± SD and histograms (from left to right: negative control, positive control, 2:1 PBMC : GM-CSF-induced MDSC, 2:1 PBMC : adenosine-induced MDSC). n=5-9 (Ďurčo et al. submitted).*

4.21. Potency of induced MDSC to inhibit the T cell proliferation increased in presence of cord blood plasma

MDSC induced in presence of adenosine [100µM] either in FCS-rich medium or in cord blood plasma-rich medium were isolated and co-incubated with autologous OKT3/IL-2-stimulated PBMC to assess their inhibitory capacity. Surprisingly, MDSC induced in cord blood plasma-rich medium exerted much stronger inhibitory activity against proliferating T cells in comparison to that induced in FCS-rich medium (17.9 ± 8% vs. 31.6 ± 10%, $p<0.01$, n=5-9, Figure 24). Additionally, dose-dependency of the T cell proliferation inhibition was remained independently from the medium in which the MDSC were induced.

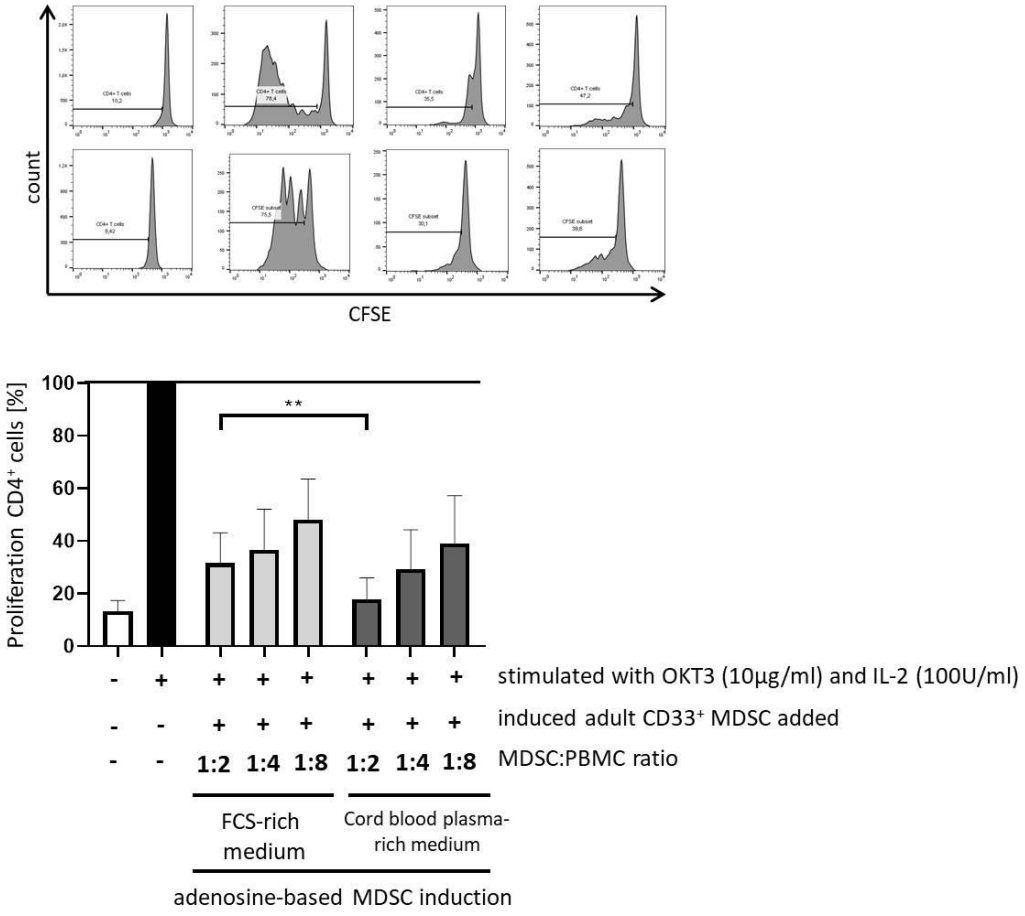


Figure 24. T cell proliferation inhibition by MDSC induced in FCS-rich and cord blood plasma-rich medium. *Peripheral blood mononuclear cells (PBMC) were isolated, resuspended either in FCS-rich medium (RPMI 1640 medium + 10% FCS + 1% P/S + 1% L-glutamine) (light gray bars) or cord blood plasma-rich medium (RPMI 1640 medium + 10% cord blood plasma + 1% P/S + 1% L-glutamine) (dark gray bars) and let incubate in presence of adenosine [100 µM] for 7 days at 37°C, 5% CO_2 with medium change on day 4. On day 7, cells were harvested applying Detachin™ and using cell scraper to detach the cells from the plastic dish bottom and investigated on CD33 expression by flow cytometry. The CD33-positive cells were isolated by AutoMACS Separator using anti-CD33 microbeads, counted under the binocular, resuspended in a sterile 1x phosphate*

*buffered saline (PBS) and diluted to 1*10^6 cells per ml. In the mean time autologous PBMC were freshly isolated using Biocoll density gradient centrifugation, diluted with a sterile 1x phosphate buffered saline to 1*10^7 cells per ml and stained with CFSE according to manufacturer´s recommendation. Stained cells were counted on the Sysmex, resuspended and diluted to 2*10^6 cells per ml in complete medium containing autologous blood plasma (10%), RPMI 1640, P/S (1%) and L-glutamine (1%) and split into two aliquots. One aliquot remained unstimulated as a negative control, the second aliquot was stimulated with IL-2 (100U/ml) and OKT3 (c_{end}=10ug/ml, stock 1mg/ml). The stimulated cells were put in a co-culture with CD33-positive cells in different ratios and let incubate for 4 days at 37°C, 5% CO_2. On day 4 cells were harvested, stained for CD4 and the proliferation rate was examined by flow cytometry. Given is the proliferation rate in percentage (%) ± SD and histograms (upper row: in FCS-rich medium induced MDSC, lower row: in cord blood plasma-rich medium induced MDSC, from left to right: negative control, positive control, 2:1 PBMC : adenosine-induced MDSC, 4:1 PBMC : adenosine-induced MDSC), n=5-9, ** = p < 0.01.*

4.22. Both, GM-CSF- and adenosine-based MDSC induction in presence of cord blood plasma resulted in MDSC exerting greater inhibitory capacity

Comparing the inhibitory capacity of MDSC induced either by GM-CSF [1ng/ml] or adenosine [100µM] incubated in two distinct media (FCS-rich and cord blood plasma-rich medium) revealed another interesting phenomenon - MDSC induced in cord blood plasma-rich medium exerted much stronger inhibitory capacity than those induced in FCS-rich medium - GM-CSF-induced MDSC (33.0 ± 5% vs. 17.6 ± 8%, n=6-7, p=0.24, Figure 25) and adenosine (45 ± 5% vs. 17.9 ± 8%, n=6-7, p<0.05, Figure 25).

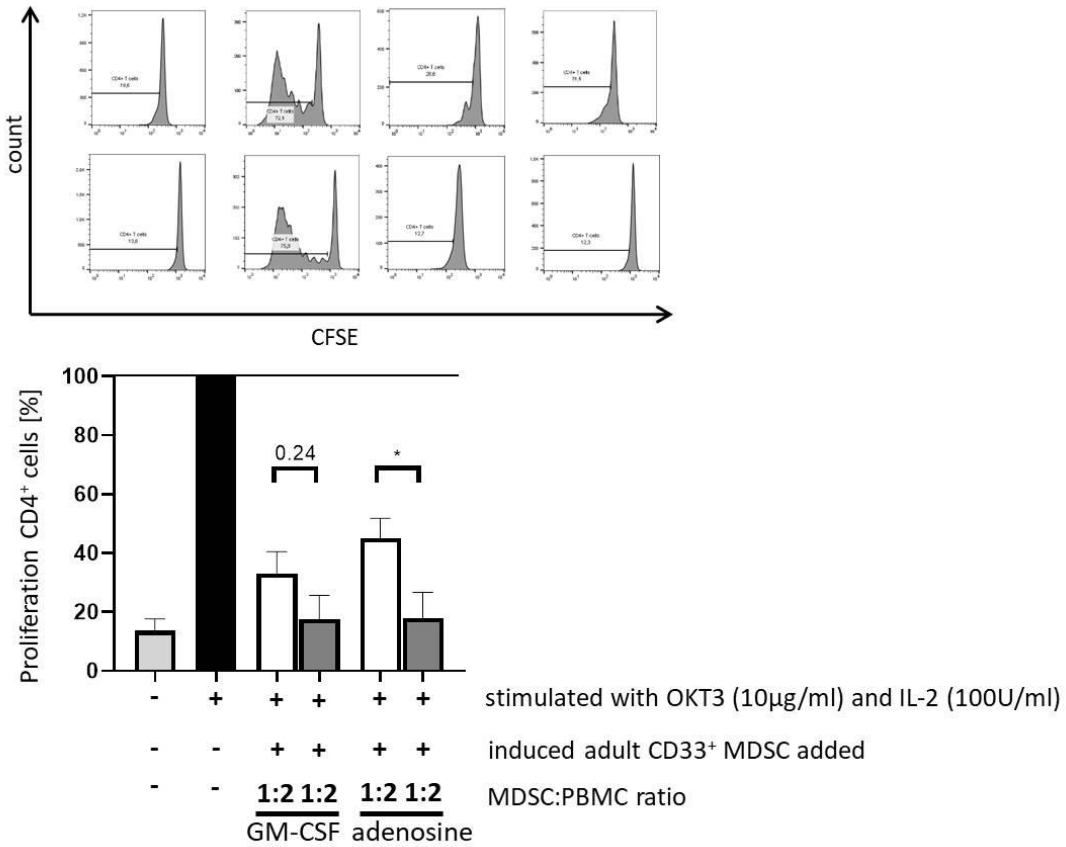


Figure 25. T cell proliferation inhibition by MDSC induced in FCS-rich and cord blood plasma-rich medium. *Peripheral blood mononuclear cells (PBMC) were isolated, resuspended either in FCS-rich medium (RPMI 1640 medium + 10% FCS + 1% P/S * 1% L-glutamine)(white bars) or in cord blood plasma-rich medium (RPMI 1640 medium + 10% cord blood plasma + 1% P/S * 1% L-glutamine) (dark grey bars) and let incubate in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] for 7 days at 37°C, 5% CO_2 with medium change on day 4. On day 7, cells were harvested applying Detachin™ and using cell scraper to detach the cells from the plastic dish bottom and investigated on CD33 expression by flow cytometry. The CD33-positive cells were isolated by AutoMACS Separator using anti-CD33 microbeads, counted under the binocular, resuspended in a sterile 1x phosphate buffered saline (PBS) and diluted to $1*10^6$ cells per ml. In the mean time autologous PBMC were freshly isolated using Biocoll density gradient centrifugation, diluted with a sterile 1x phosphate buffered saline to $1*10^7$ cells per ml and stained with CFSE according to manufacturer´s recommendation. Stained cells were counted on the Sysmex, resuspended and diluted to $2*10^6$ cells per ml in complete medium containing autologous blood plasma (10%), RPMI 1640, P/S (1%) and L-glutamine (1%) and split into two aliquots. One aliquot*

*remained unstimulated as a negative control, the second aliquot was stimulated with IL-2 (100 U/ml) and OKT3 (c_{end}=10 ug/ml, stock 1 mg/ml). The stimulated cells were put in a co-culture with CD33-positive cells in ratio 2:1 (PBMC:MDSC) and let incubate for 4 days at 37°C, 5% CO_2. On day 4 cells were harvested, stained for CD4 and the proliferation rate was examined by flow cytometry. Given is the proliferation rate in percentage (%) ± SD and histograms (upper row: FCS-rich medium induced MDSC, lower row: cord blood plasma-rich medium induced MDSC, from left to right: negative control, positive control, 2:1 PBMC : GM-CSF-induced MDSC, 2:1 PBMC : adenosine-induced MDSC,. n=6-7, * = p < 0.05.*

4.23. Applying autologous adult blood plasma induced MDSC of greater inhibitory capacity

Similar situation was observed when MDSC induced either in FCS-rich or autologous adult blood plasma-rich medium were co-incubated with autologous OKT3/IL-2-stimulated PBMC. MDSC induced in autologous adult blood plasma-rich medium exerted stronger inhibitory capacity against proliferating T cell than those induced in FCS-rich medium – GM-CSF (20.7 ± 2% vs. 36.4 ± 14%,p=0.05) and adenosine (17.4 ± 2% vs. 40.2 ± 10%, n=4-7, p<0.05, Figure 26).

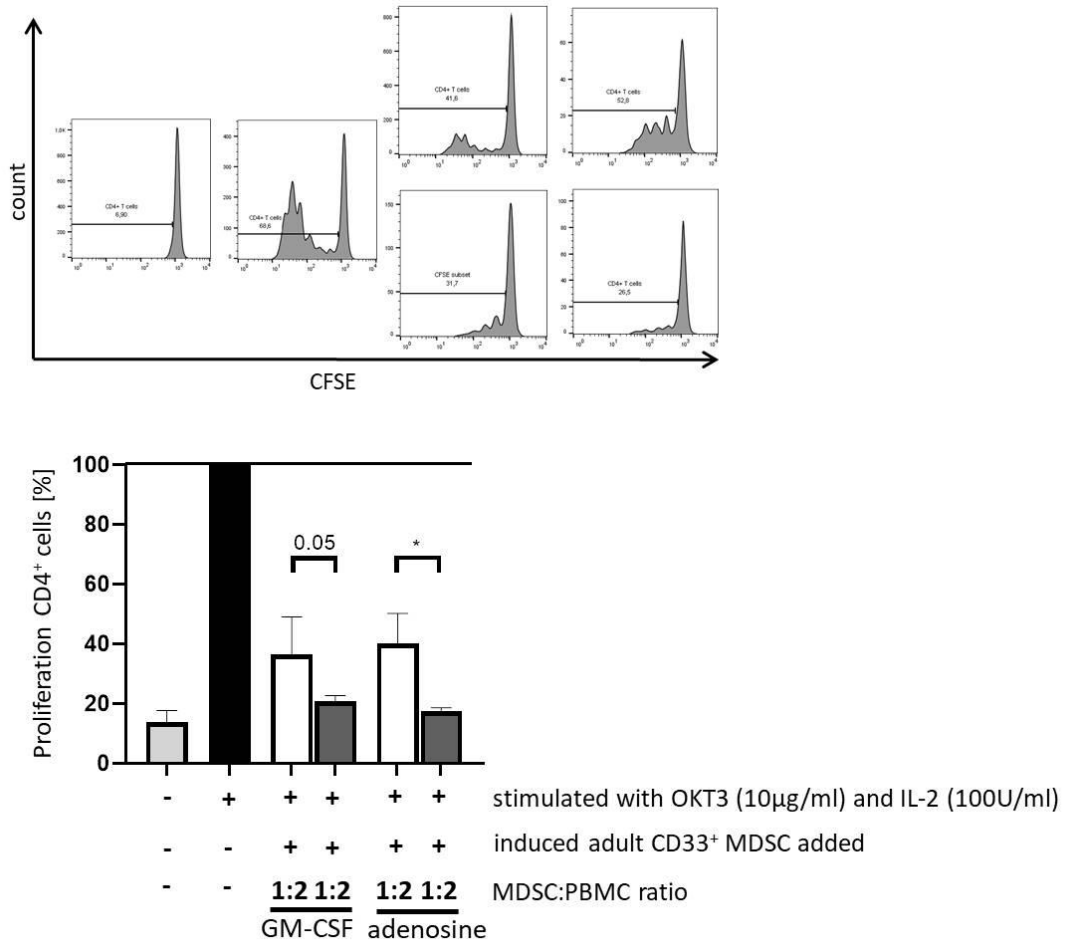


Figure 26. T cell proliferation inhibition by MDSC induced in FCS-rich and autologous adult blood plasma-rich medium. *Peripheral blood mononuclear cells (PBMC) were isolated, resuspended either in FCS-rich medium (RPMI 1640 medium + 10% FCS + 1% P/S * 1% L-glutamine)(white bars) or in autologous adult blood plasma-rich medium (RPMI 1640 medium + 10% autologous adult blood plasma + 1% P/S * 1% L-glutamine) (dark gray bars) and let incubate in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] for 7 days at 37°C, 5% CO_2 with medium change on day 4. On day 7, cells were harvested applying Detachin™ and using cell scraper to detach the cells from the plastic dish bottom and investigated on CD33 expression by flow cytometry. The CD33-positive cells were isolated by AutoMACS Separator using anti-CD33 microbeads, counted under the binocular, resuspended in a sterile 1x phosphate buffered saline (PBS) and diluted to 1*10^6 cells per ml. In the mean time*

*autologous PBMC were freshly isolated using Biocoll density gradient centrifugation, diluted with a sterile 1x phosphate buffered saline to $1*10^7$ cells per ml and stained with CFSE according to manufacturer´s recommendation. Stained cells were counted on the Sysmex, resuspended and diluted to $2*10^6$ cells per ml in modified medium containing autologous blood plasma (10%), RPMI 1640, P/S (1%) and L-glutamine (1%) and split into two aliquots. One aliquot remained unstimulated as a negative control, the second aliquot was stimulated with IL-2 (100 U/ml) and OKT3 (c_{end}=10 ug/ml, stock 1 mg/ml). The stimulated cells were put in a co-culture with CD33-positive cells in ratio 2:1 (PBMC:MDSC) and let incubate for 4 days at 37°C, 5% CO_2. On day 4 cells were harvested, stained for CD4 and the proliferation rate was examined by flow cytometry. Given is the proliferation rate in percentage (%) ± SD and histograms (from left to right: negative control, positive control, 2:1 PBMC : GM-CSF-induced MDSC, 2:1 PBMC : adenosine-induced MDSC). n=4-7, * = p < 0.05*

4.24. Diminished inhibitory capacity of MDSC induced in heterologous adult blood plasma-rich medium

The same pattern of T cell proliferation inhibition was observed when MDSC induction was conducted in the medium containing heterologous adult blood plasma – MDSC induced in modified medium exerted stronger inhibitory capacity against proliferating T cell than those induced in FCS-rich complete medium - GM-CSF-induced MDSC (36.4 ± 10% vs. 17 ± 2%, n=4-6, p=0.07, Figure 27) and adenosine-induced MDSC (45 ± 5% vs. 31.5 ± 1%, n=4-6, p=0.06, Figure 27).

Intriguingly, inhibitory capacity of adenosine-induced MDSC was much lower (31.5 ± 1%, n=4-6, p=0.06, Figure 27) in comparison to the inhibitory capacities of adenosine-induced MDSC incubated in cord blood plasma-rich medium (17.9 ± 8%, n=6-7, Figure 25) and autologous adult blood plasma-rich medium (17.4 ± 2%, n=4-7, Figure 26).

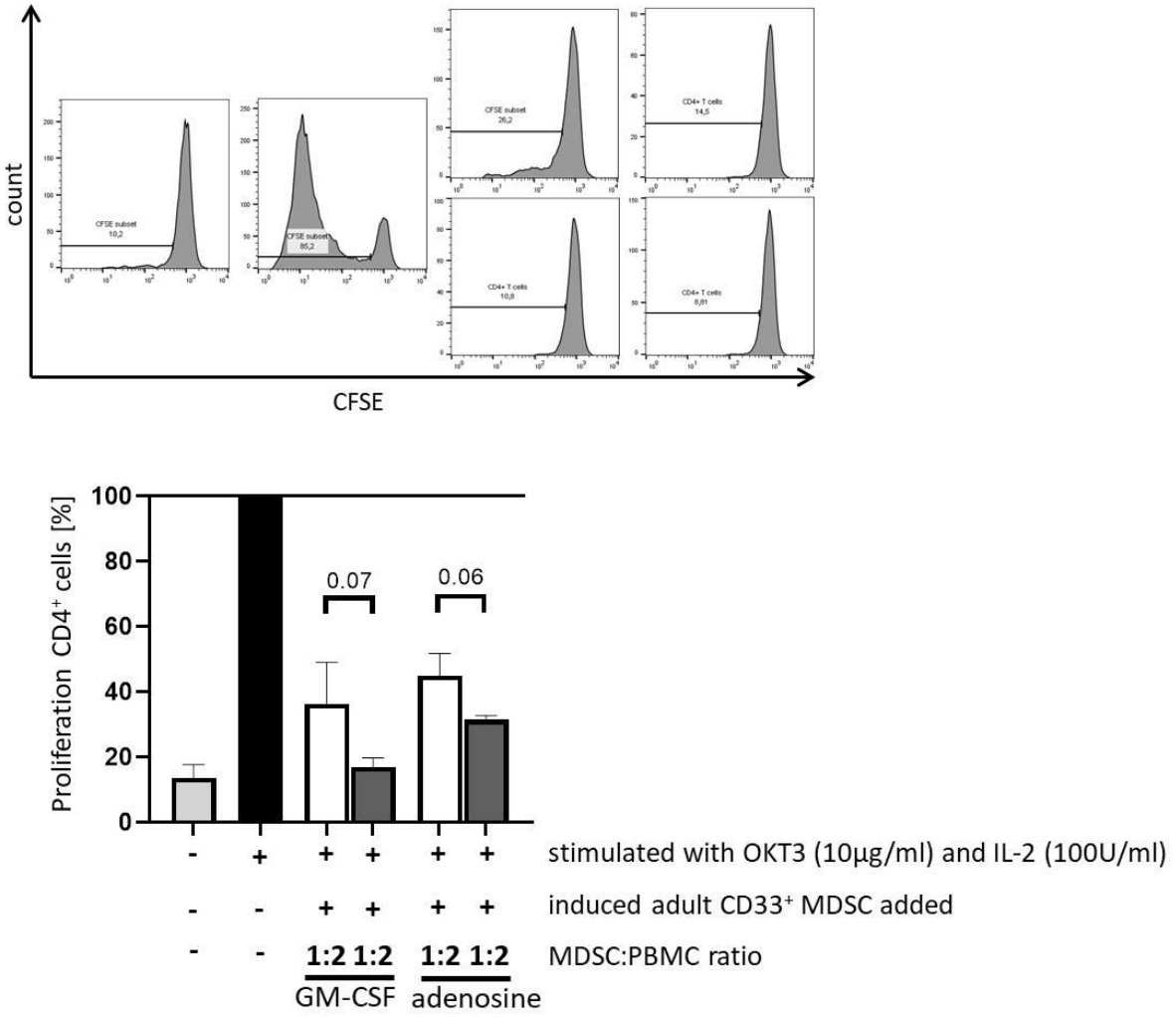


Figure 27. T cell proliferation inhibition by MDSC induced in FCS-rich and heterologous adult blood plasma-rich medium. *Peripheral blood mononuclear cells (PBMC) were isolated, resuspended either in FCS-rich medium (RPMI 1640 medium + 10% FCS + 1% P/S * 1% L-glutamine)(white bars) or in heterologous adult blood plasma-rich medium (RPMI 1640 medium + 10% heterologous adult blood plasma + 1% P/S * 1% L-glutamine) (dark gray bars) and let incubate in presence of adenosine [100 µM] or GM-CSF [1 ng/ml] for 7 days at 37°C, 5% CO_2 with medium change on day 4. On day 7, cells were harvested applying Detachin™ and using cell scraper to detach the cells from the plastic dish bottom and investigated on CD33 expression by flow cytometry. The CD33-positive cells were isolated by AutoMACS Separator using anti-CD33 microbeads, counted under the binocular, resuspended in a sterile 1x phosphate buffered saline (PBS) and diluted to $1*10^6$ cells per ml. In the mean time autologous PBMC were freshly isolated using Biocoll density gradient centrifugation, diluted with a sterile 1x phosphate buffered saline to $1*10^7$ cells per ml and stained with CFSE according to manufacturer´s recommendation. Stained cells were counted on the Sysmex, resuspended and diluted to $2*10^6$ cells per ml in complete medium containing autologous blood plasma (10%), RPMI 1640, P/S (1%) and L-glutamine (1%) and split into two aliquots. One aliquot remained unstimulated as a negative control, the second aliquot was stimulated with IL-2 (100 U/ml) and OKT3 (c_{end}=10 µg/ml, stock 1 mg/ml). The stimulated cells were put in a co-culture with CD33-positive cells in ratio 2:1 (PBMC:MDSC) and let incubate for 4 days at 37°C, 5% CO_2. On day 4 cells were harvested, stained for CD4 and the proliferation rate was examined by flow cytometry. Given is the proliferation rate in percentage (%) ± SD and histograms (upper row: in FCS-rich medium induced MDSC, lower row: in heterologous adult blood plasma-rich medium, from left to right: negative control, positive control, 2:1 PBMC : GM-CSF-induced MDSC, 2:1 PBMC : adenosine-induced MDSC). n=4-6.*

4.25. Adenosine upregulates MDSC effector enzyme IDO

To investigate the mechanism of MDSC-mediated inhibition of T cell proliferation, we next quantified expresssion of typical MDSC effector enzymes IDO, ArgI, iNOS. Freshly isolated MDSC from cord blood and adult blood expressed IDO at basal level within the range of 45 – 50 MFI (Figure 28). Cultivation for 24 hours did not change basal expression, while in presence of adenosine IDO expression was upregulated in cord blood MDSC (76.1 ± 25 MFI vs. 53.6 ± 23 MFI, $p<0.01$, n=7, Figure 28). Incubation of adult MDSC for 24 hours in presence of adenosine led to increased IDO expression in comparison to the untreated cells (79.3 ± 20 MFI vs. 55.4 ± 30 MFI, $p<0.01$, n=7, Figure 28).

The IDO expression in freshly isolated cord blood and adult blood monocytes, both was within the range of 18 - 20 MFI (Figure 28). Likewise MDSC, cultivation of cord blood monocytes for 24 hours did not change basal IDO expression (23.7 ± 10 MFI), while IDO expression was upregulated in the presence of adenosine (33.4 ± 5 MFI vs. 23.7 ± 8 MFI, $p<0.01$, n=7, Figure 28). Incubation of adult monocytes for 24 hours in presence of adenosine led to increased IDO expression in comparison to untreated cells (34.3 vs. 28.3 MFI, $p<0.05$, n=7, Figure 28) (Ďurčo et al. submitted).

Remaining two effector enzymes (ArgI and iNOS) did not show any changes upon treatment with adenosine (data not shown).

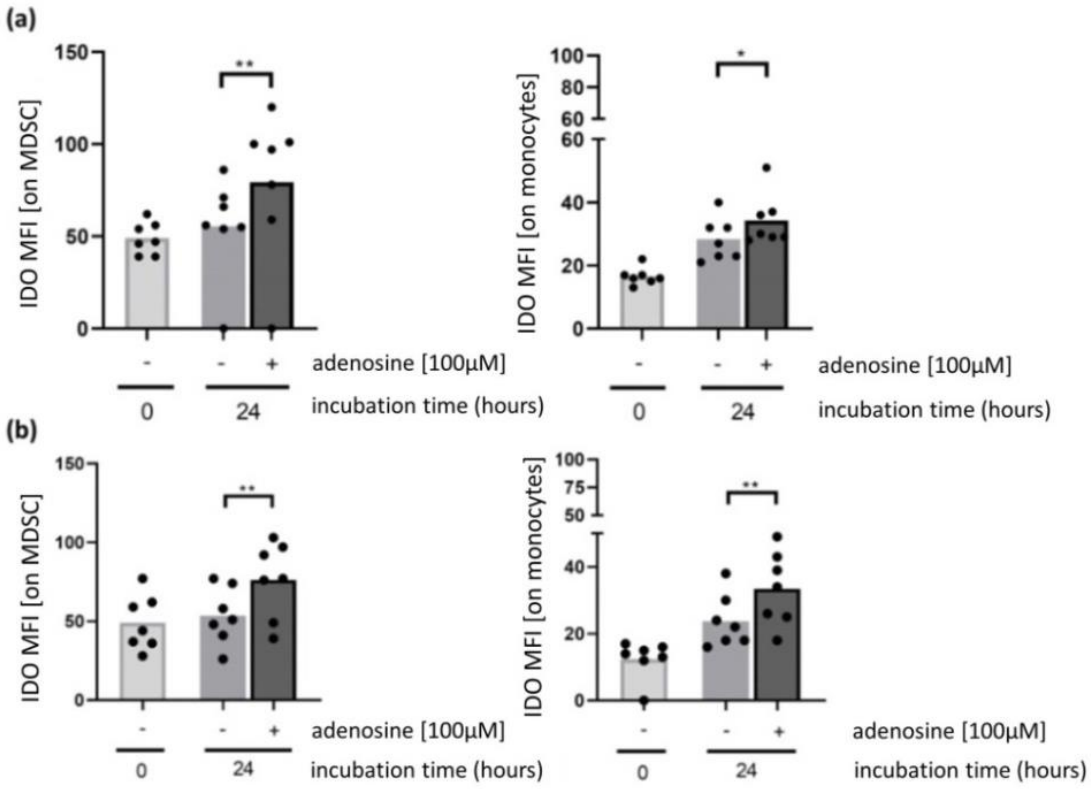


Figure 28. Expression of IDO (indoleamine-2,3-dioxygenase) in presence or absence of adenosine. *(a) Peripheral blood mononuclear cells (PBMC) and (b) cord blood mononuclear cells (CBMC) were isolated, resuspended in complete medium (RPMI 1640 medium + 10% fetal calf serum + 1% penicillin / streptomycin + 1% L-glutamine) and stained for immediate detection (t=0h) as well as incubated in presence (+) or absence (-) of adenosine [100 µM] for 24 hours. After incubation, aliquots were stained for MDSC lineage marker (CD66b), monocyte lineage marker (CD14) and for IDO and examined by flow cytometry. Mean fluorescence intensities (MFI) as well as representative density plots (after 24 hours) are shown – from left to right: MDSC w/o adenosine, MDSC + adenosine [100 µM], monocytes w/o adenosine, monocytes with adenosine [100 µM], n=7, * = p<0.05, ** = p <0.01 (Ďurčo et al. submitted).*

5. Discussion

The neonatal sepsis is one of the major risc factors for neonatal morbidity and mortality [1]. Pathophysiologically, the presence of several mechanisms acting immunosuppressively as a remnant of fetal development, contributes to the susceptibility to infections in neonates. Adenosine is an immunomodulatory metabolite of increased concentration in neonatal blood plasma [60]. Main question of the doctoral thesis was how adenosine exerts immunomodulatory activity in neonatal immune effector cells.

I found that (i) CD39 is dominantly expressed by monocytes and CD73 by T cells, (ii) ectonucleotidase expression pattern can be modified by external factors, e.g. PAMPs, oxygen shortage or iron ion chelator, (iii) neonatal immune effector cells do express adenosine receptors (A_{2A}, A_3), adenosine receptor expression pattern can be influenced by external factors, e.g. adenosine, PAMPs, (iv) adenosine is potent to induce MDSC and the potency is comparable with that of GM-CSF, (v) yield of adenosine-induced MDSC depends on medium, (vi) adenosine-induced MDSC effectively suppressed T cell proliferation, (vii) potency of induced MDSC to inhibit the T cell proliferation increased in presence of cord blood plasma, (viii) adenosine upregulates effector enzyme IDO in monocytes and MDSC.

5.1. Neonatal effector immune cells are capable of adenosine production

Several studies have already shown, that in peripheral adult blood, CD39 is constitutively expressed on more than 90% of monocytes, 20%-30% of $CD4^+$ T cells and 5% of $CD8^+$ T cells. CD73 is expressed on approximately 50% of $CD8^+$ T cells, 10% of $CD4^+$ T cells, and MDSC can co-express CD39 and CD73 [84], [112] and (Longhi, unpublished). Thus, our results on the expression pattern of CD39 and CD73 on neonatal immune effector cells resemble those from adults. Similar to adults, at least two neonatal immune cell types, i.e.monocytes and T cells (Figure 3 and 4), may take part in neonatal adenosine production, occurring as a two-step degradation of extracellular ATP by CD39 and CD73 (Ďurčo et al. submitted).

According to several studies CD39 cleaving ATP and ADP to AMP contributes to the protective effect under hypoxic conditions [129], [130]. My results showed that population of adult CD39-positive monocytes under anoxia became smaller in comparison to the adult monocytes incubated under normoxia (90 ± 10% vs. 96 ± 3%, n=11-18, p=0.05, Figure 5a+b). In parallel, comparing the expression rate of CD39 per cell became weaker under anoxic conditions (30 ± 8 MFI vs. 42 ± 10 MFI, n=11-18, p<0.001, Figure 5a+b).

Ectonucleotidase CD73 expression rate can be influenced by different stimuli. According to studies done by Tóth et al. CD73 expression can be increased dose-dependently by endogenous TNFα and thus the percentage of CD73-positive T_H cells in the periphery become higher [113]. Experiments with murine models have shown that CD73 expression can be strongly up-regulated by TGFβ on conventional $CD4^+$ and $CD8^+$ T cells [131]. The CD73 expression can be also influenced by substances present in the body under physiologic conditions, e.g. retinoic acid [132] or the active form of vitamin D [133].

Iron bioavailability is another factor which may play a crucial role in the correct function of ectonucleotidases. Iron ions can be depleted by binding to siderophores – e.g. deferoxamine (DFO) [134]. Applying DFO (10^{-4}M) to a cell culture CD73 expression pattern did not change over 24 hours (Figure 7a+b) and the two-step ATP cleavage on a co-operative way between CD39-positive monocytes and CD73-positive T cells remained unchanged.

Adenosine seems to be a critical mediator during ischemia and hypoxia [82]. Studies done by Synnestvedt et al. revealed that CD73 expression in the intestinal eptihelial cells may be regulated by hypoxia whereas one of the induction pathway involves Hypoxia-inducible Factor 1 (HIF-1) [77]. Further, Saito et al. have shown that plasma adenosine concentration increased (21nM to 51nM) in human volunteers subjected to hypoxia (SpO_2 = 80% over 20 minutes) and pre-treated with dipyridamole (an adenosine re-uptake inhibitor) [135]. Based on these results I want to examine the CD73 expression under anoxic conditions resembling the hypoxic conditions in uterus during prenatal development.

Experiments with cord and adult blood cells showed no changes in the CD73 expression during 24 hours incubation (Figure 7a + b).

Phelan et al. have shown that macrophages infected with *Mycobacterium tuberculosis* showed stronger expression of genes involved in the glycolysis upon simultaneous treatment with DFO [136]. Further, Phelan et al. discovered that DFO boosted production of pro-inflammatory cytokines, e.g. IL1β and TNFα in macrophages infected with live attenuated *Mycobacterium tuberculosis* [136]. This is in accordance with findings done by Serafín-Lopéz pointing out reduced TNFα and IL-1β production and enhanced mycobacterial growth in presence of iron ions [137]. Moreover, DFO stabilizes transcription factor HIF1α [138], [139] which in turn induces the IL-1β secretion [140].

Based on these results I decided to investigate the effect of DFO on the ectonucleotidase expression pattern. An increase in CD73-positive monocytes has been observed during the 48 hours incubation in presence of DFO in comparison to the untreated cells (62 ± 25% vs. 50 ± 30%, n=11, p=0.33, Figure 8a + b). Additionally, expression rate per cell increased in presence of DFO (25 ± 20 MFI, n=10, p=0.13, Figure 8a + b). in comparison to untreated (10 ± 10 MFI) or anoxic conditions (20 ± 10 MFI, n=10, $p<0.05$, Figure 8a + b), respectively.

Adenosine produced by tandem action of CD39/73 negatively regulates the immune response. Efficient clearance of infection requires efficient innate immune responses, thus adenosine production needs to be subdued. Investigations done by Costales et al. discovered that *Salmonella* infection inhibits the CD73 expression and thus contributes to the enhanced antimicrobial activity. Moreover, Costales postulated that even *Salmonella* whole cell lysate contributes to down-regulation of CD73 and enhances the pro-inflammatory response simultaneously. Production of nitric oxide and pro-inflammatory cytokines (IL-1β, TNFα) as well as clearance of internalized bacteria were more efficiently in absence of CD73 [141].

I have discovered that minority of cord blood T cells (20 ± 5%, Figure 10a + b) expressed CD73 in presence of PAMPs. Compared to the cord blood T cells incubated under sterile normoxic conditions (35 ± 15%, Figure 10a + b), reduction of CD73-positive T cell population could be observed. These findings are in accordance with those done by Costales et al. CD73 is the rate-limiting factor in adenosine production [142], thus its expression regulation contributes to the adenosine production. Further, as Costales et al. postulated, in case of infection with *Salmonella*, or with its whole cell lysate, immune system has to be able to induce inflammation. That´s why production of metabolites with negative effect on the immune system needs to be subdued [141].

Pam3Cys and LPS as pathogen-associated molecular patterns may induce changes in the cell physiology of immune cells. As shown by Eichin et al., isolated human monocytes were stimulated either with lipopolysaccharides (LPS) and tumor necrosis factor α (TNFα) or with interleukin 4 (IL-4) and monocytes colony-stimulating factor (M-CSF). After 3 days stimulation, LPS/TNFα-treated pro-inflammatory polarized M1 macrophages expressed CD73 at high rate. Contrary, IL-4/M-CSF-stimulated immunosuppressive-polarized M2 macrophages did not express CD73 [143].

Applying Pam3Cys led to upregulation of CD73 in both, cord blood monocytes (80 ± 5%,n=3, $p<0.001$, Figure 10a + b) as well as adult monocytes (90 ± 6%, n=3, $p<0.01$, Figure 10a + b). Furthermore, applying Pam3Cys in 3 different concentration [10 – 1 – 0.1 µg/ml] led to dose-dependent CD73 expression by monocytes. The CD73 upregulation in presence of Pam3Cys seems to be a physiologic brake preventing the immune system from exhaustion. As already mentioned, CD73 as rate-limiting factor [142] cleaves AMP to adenosine acting immunosuppressively by binding to appropriate adenosine receptors. Stronger CD73 expression may result in enhanced adenosine production and thus to down-regulation of immune response. Levy et al. showed that TNFα production in neonates upon Pam3Cys treatment is attenuated in comparison to the adults [60].

Results of my study pointed out dose-dependently increased CD39 density per cell upon treatment with Pam3Cys in cord blood monocytes (110 ± 20 MFI vs. 75 ± 20 MFI, n=3, $p<0.05$, Figure 11a + b) as well as in adult monocytes (125 ± 10 MFI vs. 55 ± 8 MFI, n=3, $p<0.0001$, Figure 11a + b). Together with the CD73 expression induction by monocytes, it may play as a physiologic brake, when monocytes start to produce large quantities of adenosine to prevent an excessive immune reaction.

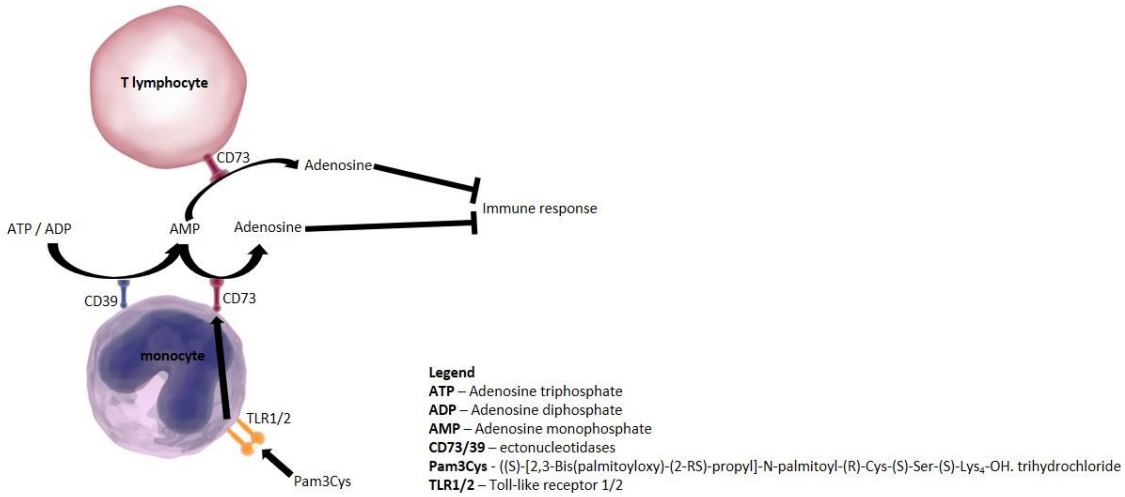


Figure 29. Upregulation of CD73 by monocytes after treatment with Pam3Cys.

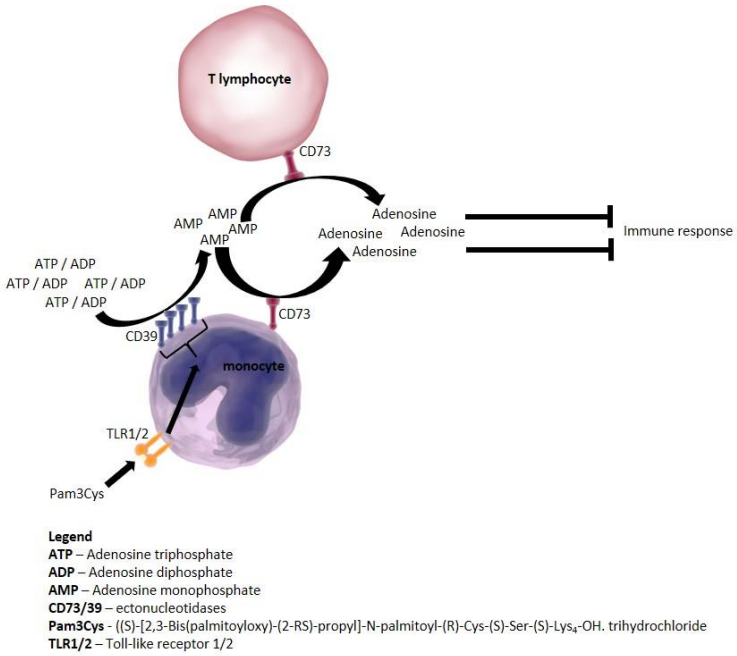


Figure 30. Enhanced upregulation of CD39 expression by monocytes after treatment with Pam3Cys.

5.2. Neonatal effector immune cells are perceivable to adenosine

Zarek et al. demostrated that the interaction between tissue-derived adenosine and adenosine A_{2A} receptor may be an important negative regulator of T cell function. First, development of effector T cells is inhibited. Second, T cell development is skewed towards regulatory T cell development. In case of A_{2A} receptor engagement, even the antigen recognition by T cell is impaired and thus, it may lead to anergy [144]. Further, adenosine as A_{2A} receptor agonist binds to the A_{2A} receptor activating Gs protein, which in turn leads to cAMP production and protein kinase A (PKA) activation. Active protein kinase A results in downregulation of type 1 cytokine production [145], [146].

Adenosine-rich microenvironment inhibits the inflammation and prevents tissue destruction [147], [148]. Experiments with A_{2A}R null mice done by Ohta and Sitkovsky have shown the indispensable role of adenosine A_{2A} receptor. Immune cells of these mice were not capable to control the inflammation process resulting in massive tissue destruction [149].

Adenosine A_3 receptor is present on majority of the immune cell types mediating the role of adenosine in process fo inflammation [150], [151].

Majority of freshly isolated T cells expressed A_3 receptor - cord blood T cells (75 ± 12%, n=3, Figure 13a + b), adult T cells (80 ± 2%, n=3, Figure 13a + b). During 24 hours incubation, A_3 receptor expression by cord blood T cells was downregulated if untreated (65 ± 5%, n=3, Figure 13a + b), remained unchanged in presence of adenosine [100µM] (75 ± 7%, n=3, p=0.06, Figure 13a + b), or was upregulated in presence of Pam3Cys [10µg/ml] (80 ± 2%, n=3, p=0.06, Figure 13a + b). Contrary, adult T cells seemed not to be sensitive against these stimuli and their A_3 receptor expression remained unchanged (80 ± 3%, n=3, Figure 12).

Freshly isolated T cells (both, cord blood and adult) expressed moderate levels of A_3 receptor (15 ± 5 MFI, n=3, Figure 13b). If the cord blood T cells remained untreated, A_3 expression level did not change over 24 hours (15 ± 2 MFI, n=3, Figure 13b). Higher A_3 receptor expression levels were detected upon treatment with adenosine [100µM] (16 ± 2

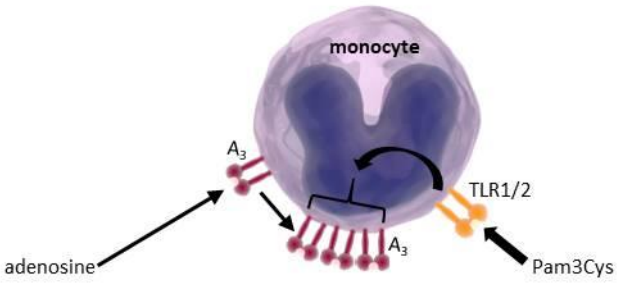
MFI, n=3, p=0.23, Figure 13b) as well as with Pam3Cys [10µg/ml] (17 ± 5 MFI, n=3, p=0.19, Figure 13b).

Adenosine A_3 receptor expression levels in adult T cells remained the same if cells remained untreated for 24 hours (15 ± 1 MFI, n=3, Figure 13b). Slight decrease in A_3 receptor expression levels was observed upon treatment with adenosine [100µM] (14 ± 2 MFI, n=3, p=0.35, Figure 13b). Contrary, treatment with Pam3Cys [10µg/ml] induced slight increase (16 ± 2 MFI, n=3, p=0.16, Figure 13b).

Binding to the A_3 receptors, adenosine modulates the monocyte-macrophages functions. It inhibits the respiratory burst and IL1β, TNFα, chemokine macrophage inflammatory protein 1 α and iNOS gene expression [152], [153]. Further, IL-18-dependent up-regulation of ICAM-1 expression on monocytes is reduced and production of IL-12, IFNγ and TNFα is abolished [154].

A_3 receptor expression levels on freshly isolated monocytes (cord blood and adult) seemed to be comparable (25 ± 8 MFI vs. 27 ± 5 MFI, n=3, Figure 14b). In cord blood monocytes, A_3 expression level were increased after 24 incubation with a variable strength depending on the conditions – untreated (42 ± 8 MFI, n=3, Figure 14b), adenosine [100 µM] (50 ± 8 MFI, n=3, $p<0.05$, Figure 14b) and Pam3Cys [10µg/ml] (65 ± 15 MFI, n=3, p=0.12, Figure 14b).

A_3 receptor expression levels on adult monocytes also increased during 24 hours incubation. Similar to cord blood monocytes, the strength varied according to the conditions – untreated (45 ± 5 MFI, n=3, Figure 14b), adenosine [100 µM] (50 ± 5 MFI, n=3, p=0.24, Figure 14b) and Pam3Cys [10µg/ml] (70 ± 6 MFI, n=3, $p<0.05$, Figure 14b).



Legend
A_3 – Adenosine triphosphate
Pam3Cys - ((S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH. trihydrochloride
TLR1/2 – Toll-like receptor 1/2

Figure 31. Upregulation of A_3 receptor expression in presence of adenosine and Pam3Cys.

Adenosine A_3 receptor is less widely distributed than the other adenosine receptor subtypes. It is considered as a high affinity receptor for adenosine together with A_1 and A_{2A} [155], [86]. Under physiologic conditions, somatic cells express A_3 receptor at very low rate [156], [157], [158]. Elevated adenosine and cytokines levels as factors characteristic of tumor microenvironment (TME) and inflammatory cells can contribute to the adenosine A_3 receptor upregulation [159].

Adenosine A_3 receptor is commonly present on MDSC (cord blood and adult) – 85 ± 15% cord blood MDSC and 85 ± 5% adult MDSC are positive for adenosine A_3 receptor. After incubation for 24 hours in presence of Pam3Cys [10µg/ml] the number of A_3-positive cord blood MDSC decreased (78 ± 6%, n=3, p=0.11, Figure 15a + b).

Contrary, incubation with Pam3Cys [10µg/ml] increased the A_3 receptor expression levels in comparison to the freshly isolated cord blood MDSC (40 ± 10 vs. 35 ± 5 MFI, n=3, Figure 15 a + b).

5.3. Physiology of neonatal effector immune cells may be modulated by adenosine receptors

Upon adenosine binding to A_{2A} receptor, adenylyl cyclase becomes active and produced cAMP accumulates in the region of lipid rafts containing TCR-CD3-ζ chain-complex. Cyclic

AMP activates PKA-1, the most abundant isoform of protein kinase, which in turn phosphorylate and activate C-terminal Src kinase (Csk). Activated Csk inhibits lymphocyte-specific protein kinase (Lck) by phosphorylation of tyrosine residue (Y_{505}). Due to inactivation of Lck, phosphorylation of ITAMs (zeta chain and CD3) does not occur. As consequence, zeta-chain-associated protein kinase of 70kDa (ZAP70) cannot bind and the downstream protein – Linker for activation of T cell (LAT) – remains unphosphorylated and inactive. Additionally, transcription nuclear factor of activated T cells (NF-AT) becomes phosphorylated and inactivated by PKA-1. Orchestrated inhibition of early stage activation and NF-AT inactivation lead to downregulation of T cell activation [145], [160].

In my series of experiments (Figure 16), a downregulation of OKT3/IL-2-induced T cell proliferation was (both, cord and adult blood) observed. This is in accordance with findings done by Linden and Cekic 2012 and Jimenez, Punzo et al. 2001. Moreover, I could observe that T cell proliferation inhibition was dose-dependent. Adenosine [1µM] diminished proliferation of cord blood T cell by 10% in comparison to OKT3/IL-2-stimulated cord blood T cells without adenosine. Adult T cells seemed to be more resistant against adenosine – their proliferation was diminished by 6.25% in comparison to OKT3/IL-2-stimulated adult T cells. Ten-times higher adenosine concentartion [10µM] seemed not to have a stronger inhibition effect on cord blood T cell proliferation than 1µM adenosine. Contrary, proliferation of adult T cells in presence adenosine [10µM] was downregulated by 14.75%. Increasing the adenosine concentration to 100µM, proliferation was downregulated by 17.67% (cord blood T cells), 35.875% (adult T cells), respectively.The highest adenosine concentration [1000µM] led to diminishing T cell proliferation by 47% (cord blood), or 68.4% (adult blood), respectively.

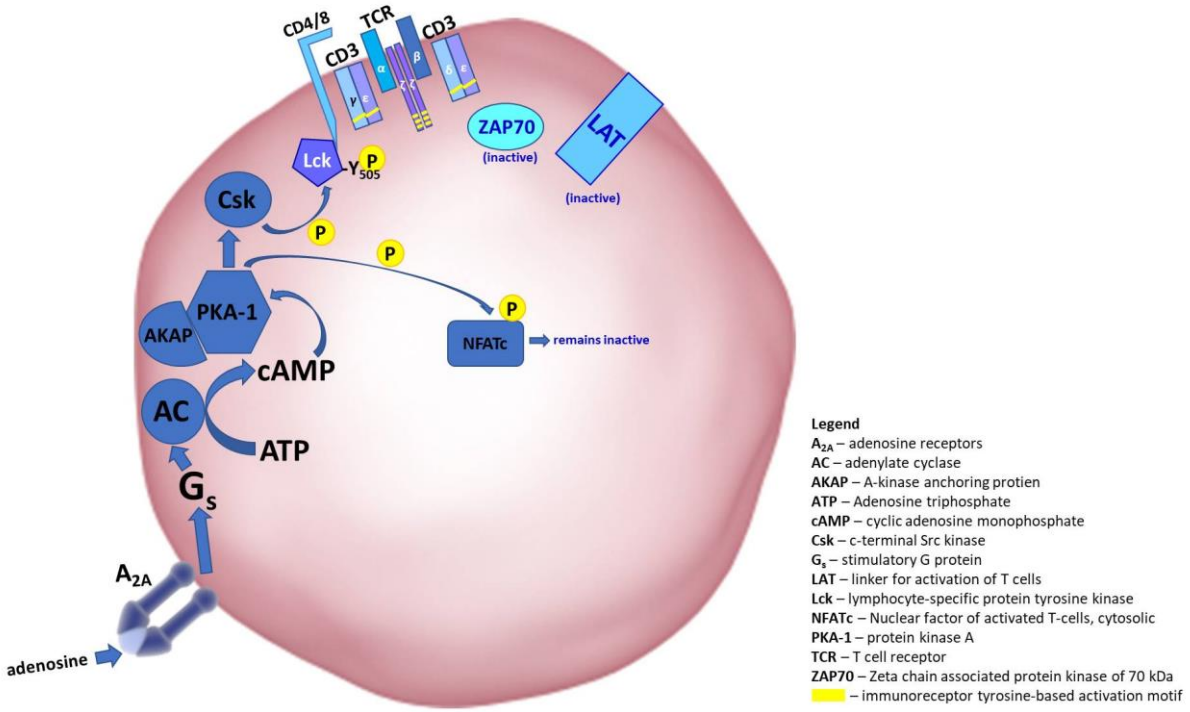


Figure 32. Simplified overview of inhibitory activity of adenosine on T cell proliferation upon binding to A_{2A} receptor. *According to [145], [161], [162], [160]*

As already shown in tumor biology, adenosine produced within tumor microenvironment (TME) suppresses the T cell-mediated antitumor immune response by binding to adenosine A_{2A} receptor expressed on effector T cells. Further, adenosine can affect the accumulation of MDSC within tumor lesions and skew the differentiation of monocytes to tumor-associated macrophages (TAMs) and induce the shift of dendritic cells towards tolerogenic phenotype [163]. According to Virgilio and Adonolfi, tumor microenvironment contains unusually high concentration of ATP and adenosine. The latter one seems to play a key role in the formation of the immunosuppressive tumor millieu [164]. Ex vivo studies done by Ohta et al. showed that adenosine concentration within the tumor core si approximately 30% higher than in the pepriphery. Moreover, the adenosine concentration within tumor seems to be twice higher in comparison to the healthy tissue [165].

In series of experiments, it was shown that adenosine applied to the suspension of PBMC isolated from healthy adult person led to strong induction of $CD33^+$ MDSC in comparison to the untreated control (14.6 ± 5% vs. 8.7 ± 5%, $p<0.001$, n=15, Figure 17) and that the yield was comparable to that of GM-CSF (14.6 ± 5% vs. 14.7 ± 5%, n=7, Figure 17) taken as a benchmark. Additionally, I would like to emphasize that the relative accumulation of induced MDSC was not due to decrease of other cell types. Thus these results show for the first time that adenosine is capable to induce MDSC in healthy individuals. Moreover, the potency of adenosine to induce MDSC is comparable with that of GM-CSF (Ďurčo et al. submitted).

5.4. The ability of adenosine to induce MDSC varies depending on culture media

In our lab, we try to unravel the molecular mechanisms of the feto-maternal tolerance. That´s why it was interesting to find out what happens if the fetal calf serum commonly used in complete medium will be replaced by freshly isolated cord blood plasma. In presence of this modified medium an interesting phenomenon was observed, adenosine-based MDSC induction yielded smaller amount of $CD33^+$ MDSC in comparison to adenosine-based induction made in FCS-rich complete medium (6.3 ± 6% vs. 13.1 ± 6%, $p<0.01$, Figure 18). It is shown for the first time that in-vitro MDSC induction in presence of cord blood plasma results in a smaller yield.

Interestingly, diminished adenosine-based MDSC induction was also observed when the FCS in complete medium was replaced by freshly isolated autologous adult blood plasma (5.8 ± 4% vs. 13.1 ±7%, $p<0.01$, FCS-rich medium n=7, autologous adult blood plasma-rich medium n=12, Figure 19) as well as freshly isolated heterologous adult blood plasma (8.6 ± 8% vs. 13.2 ± 7%, $n<0.05$, FCS-rich medium n=7, heterologous adult blood plasma-rich medium n=6, Figure 20).

Based on these results, it may be assumed that human (cord and adult) blood plasma contains distinct, at the moment unknown, factor having negative effect on the adenosine-

based MDSC induction. These discoveries led to at least two questions - what is this factor like? Is this one distinct substance, or several factors acting in concert? For these purposes it would be good to do a detailed analysis of the cord blood plasma.

Further, I want to find out whether viability and survival rate of seeded PBMC change when fetal calf serum is replaced by human blood plasma (cord, adult). As shown in the Figure 21, cells incubated in FCS-rich medium showed the best fitness. Within this group, survival rates varies dependent on the conditions – if cells remained untreated, one-fourth (24.7 ± 20%) of seeded cells survived, addition of GM-CSF [1ng/ml] to the cell culture increased the cells survival of seeded cells in comparison to the untreated cell culture (33.2 ± 30% vs. 24.7 ± 20%, n=16, $p<0.05$, Figure 21). Slightly increased survival rate of seeded cells was also observed after addition of adenosine [100µM] (28 ± 25% vs. 24.7 ± 20%, n=16, p=0.11, Figure 21).

Contrary, in presence of human blood plasma (cord and adult), survival rate varied within the range of 6 to 20% (Figure 21). Possible explanation is that the fetal calf serum is produced by a company a its production is standardized, thus content variability is minimized. In contrast, isolated human blood plasma is unique and its composition depends on the physiological status of the person at the time point of blood drawing.

Strikingly, highest yield of $CD33^+$ separated cells was achieved by incubation in cord blood plasma-rich medium (Figure 22). Possible explanation may be that cord blood plasma contains distinct factor promoting the differentiation. Discoveries done by Laver et al. underpin this assumption. According to their experiments cord blood plasma contains 19.9 ± 5.2 U/ml GM-CSF. Contrary, GM-CSF was not detectable in adults. Additionally, G-CSF level in cord blood are approximately 16-fold higher (40.8 ± 2.8) than in adult blood plasma (2.5 ± 1.5) [166].

5.5. Adenosin-induced MDSC effectively suppressed T cell proliferation

Katoh and Watanabe showed that cancer cells use several tricks to fool the immune system, e.g. imbalance in T_H1/T_H2 immune response, development of tumor-associated macrophages as well as enhanced presence of immunosuppressive cells – MDSC and regulatory T cells [167]. Further, Gabrilovic and Nagaraj pointed out that under cancer settings, adenosine may skew the differentiation of immature myeloid progenitor cells into MDSCs rather than to mature dendritic cells [5].

In my experiments, I have shown that adenosine is capable not only to induce the re-differentiation of already differentiated PBMC into MDSC (see Figure 16), but those cells exert immunosuppressive activity towards T cells. The adenosine-induced MDSC exerted inhibitory capacity comparable with that of GM-CSF-induced MDSC used as a control (31.6 ± 8% vs. 27.7 ± 8%, p=0.21, n=5-9, Figure 23) (Ďurčo et al. submitted).

As already mentioned above, Laver et al. discovered that GM-CSF concentration in cord blood plasma reaches level of 19.9 ± 5.2 U/ml, but it is not detectable in adult human blood plasma. Also G-CSF levels in cord blood are strongly elevated [166].

In my experiments, I could show that MDSC induced in cord blood plasma-rich medium exerted much stronger inhibitory activity against proliferating T cells in comparison to that induced in FCS-rich medium (17.9 ± 8% vs. 31.6 ± 10%, p<0.01, n=5-9, Figure 24). Elevated GM-CSF and G-CSF levels, as mentioned by Laver et al. [166] may be the possible explanation why MDSC induced in cord blood plasma-rich medium exert stronger immunosuppressive potency in comparison to MDSC induced in FCS-rich medium.

Lechner et al. doing their in vitro experiments, have shown that GM-CSF alone is sufficient to induce immunosuppressive phenotype in PBMC of healthy adults [168] [169]. Moreover, MDSC inhibit the function and proliferation of effector T cells, which might otherwise be able to eliminate tumor cells [170], [171]. According to Laver et al. GM-CSF levels in cord blood plasma reach 19.9 ± 5.2 U/ml [166].

In my series of experiments, an interesting phenomenon was observed - MDSC induced in cord blood plasma-rich medium exerted much stronger inhibitory capacity than those induced in FCS-rich medium - GM-CSF-induced MDSC (33.0 ± 5% vs. 17.6 ± 8%, n=6-7, p=0.24, Figure 25) and adenosine (45 ± 5% vs. 17.9 ± 8%, n=6-7, p<0.05, Figure 25). These findings are in accordance with those mentioned above and elucidate one of the reasons of elevated MDSC count in the cord blood.

Based on the discoveries done by Laver et al. that GM-CSF is not detectable in adult human blood plasma, it was striking to observe that MDSC were induced in autologous adult blood plasma-rich medium. Moreover, these cells exerted stronger inhibitory capacity towards proliferating T cell than those induced in FCS-rich medium – GM-CSF-induced MDSC (20.7 ± 2% vs. 36.4 ± 14%,p=0.05) and adenosine-induced MDSC (17.4 ± 2% vs. 40.2 ± 10%, n=4-7, p<0.05, Figure 26). These results raise the question whether there is another pathway inducing MDSC.

Observation that MDSC induced in heterologous adult blood plasma-rich medium (Figure 27) exert diminished inhibitory capacity seems to be more in accordance than the other mentioned above. This may be explained by undetectable amount of GM-CSF in adult blood plasma [166]. But the question how the MDSC arise in GM-CSF-poor environment remains to be answered.

5.6. The way how adenosine-induced MDSC may inhibit the T cell proliferation

IDO cleaving tryptophan to kynurenine, hydroxykynurenine and anthranilic acid seems to play an important role in T cell proliferation inhibition [172], [173], [174].Tryptophan is an amino acid essential for T cell proliferation. Tryptophan depletion in concert with accumulation of toxic metabolites (kynurenine, hydroxykynurenine, anthranilic acid) leads to arrest of T cells in mid-G1 phase [174] and proliferation inhibition.

Series of experiments revealed an enhanced up-regulation of IDO expression after 24 hours incubation in presence of adenosine [100μM]. Basal IDO expression in freshly isolated

MDSC (both, cord blood and adult) was within the range 45 - 50 MFI (Figure 28). Cultivation for 24 hours in presence of adenosine [100μM] enhanced the IDO expression in cord blood MDSC (76.1 ± 25 MFI vs. 53.6 ± 23 MFI, $p<0.01$, n=7, Figure 28). Similarly, adult MDSC reacted by IDO expression upregulation (79.3 ± 20 MFI vs. 55.4 ± 30 MFI, $p<0.01$, n=7, Figure 28).(Ďurčo et al.submitted).

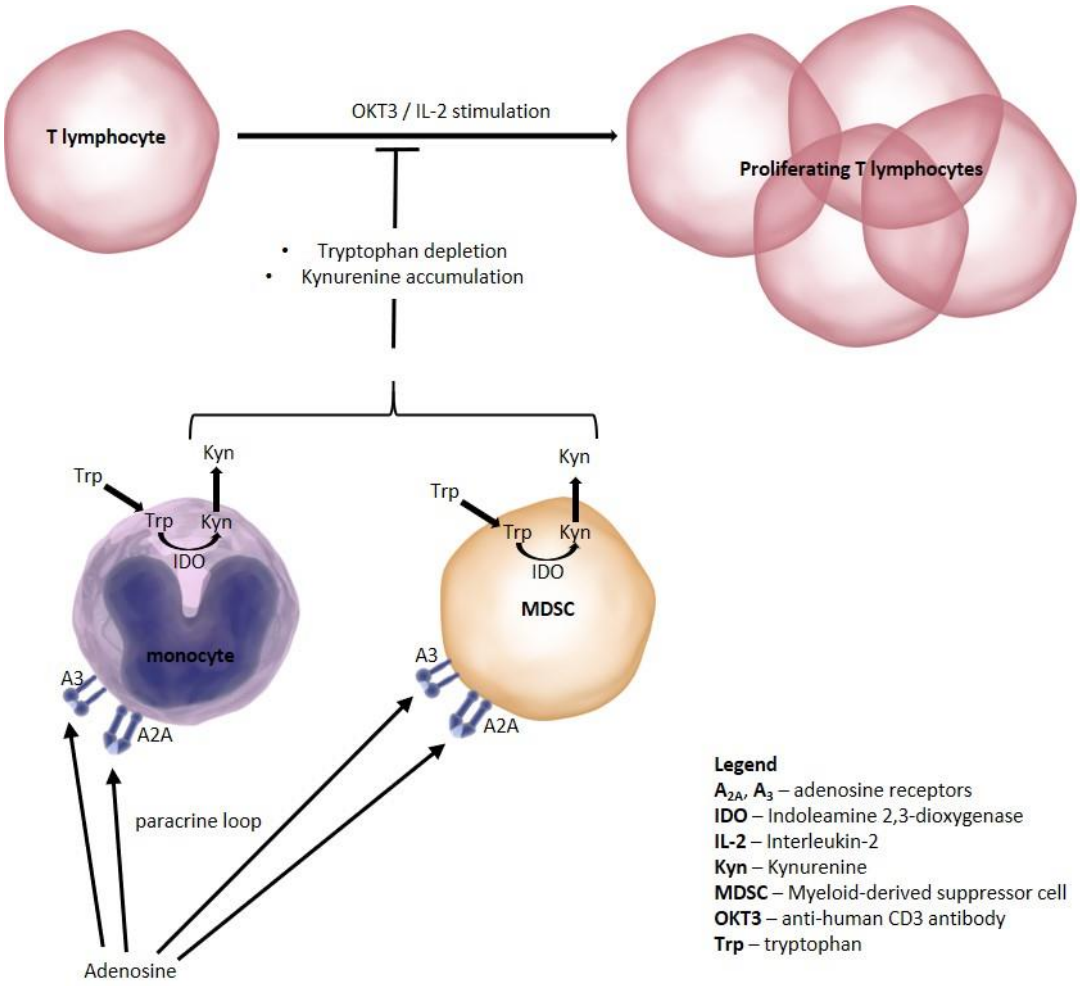


Figure 33. IDO expression upregulation and T cell proliferation inhibition.

5.7. Summary

Summarizing, immunosuppressive activity of adenosine in cord blood of healthy newborns and adults is comparable to that in cancer patients. Adenosine produced from extracellular ATP by ectonucleotidases CD39 and CD73 may influence the physiology and the behaviour of immune cells in autocrine as well as in paracrine fashion by binding to appropriate receptors, e.g. A_{2A} and A_3. Adenosine may exert its immunomodulatory function at several levels (Ďurčo et al. submitted), e.g. (a) downregulation of T cell proliferation via direct binding to A_{2A} receptor, (b) enhanced expression of inhibitory A_3 receptor by monocytes, (c) induction of immunosuppressive MDSC, (d) MDSC-mediated T cell proliferation inhibition, (e) enhanced IDO production by monocytes and MDSC (Ďurčo et al. submitted).

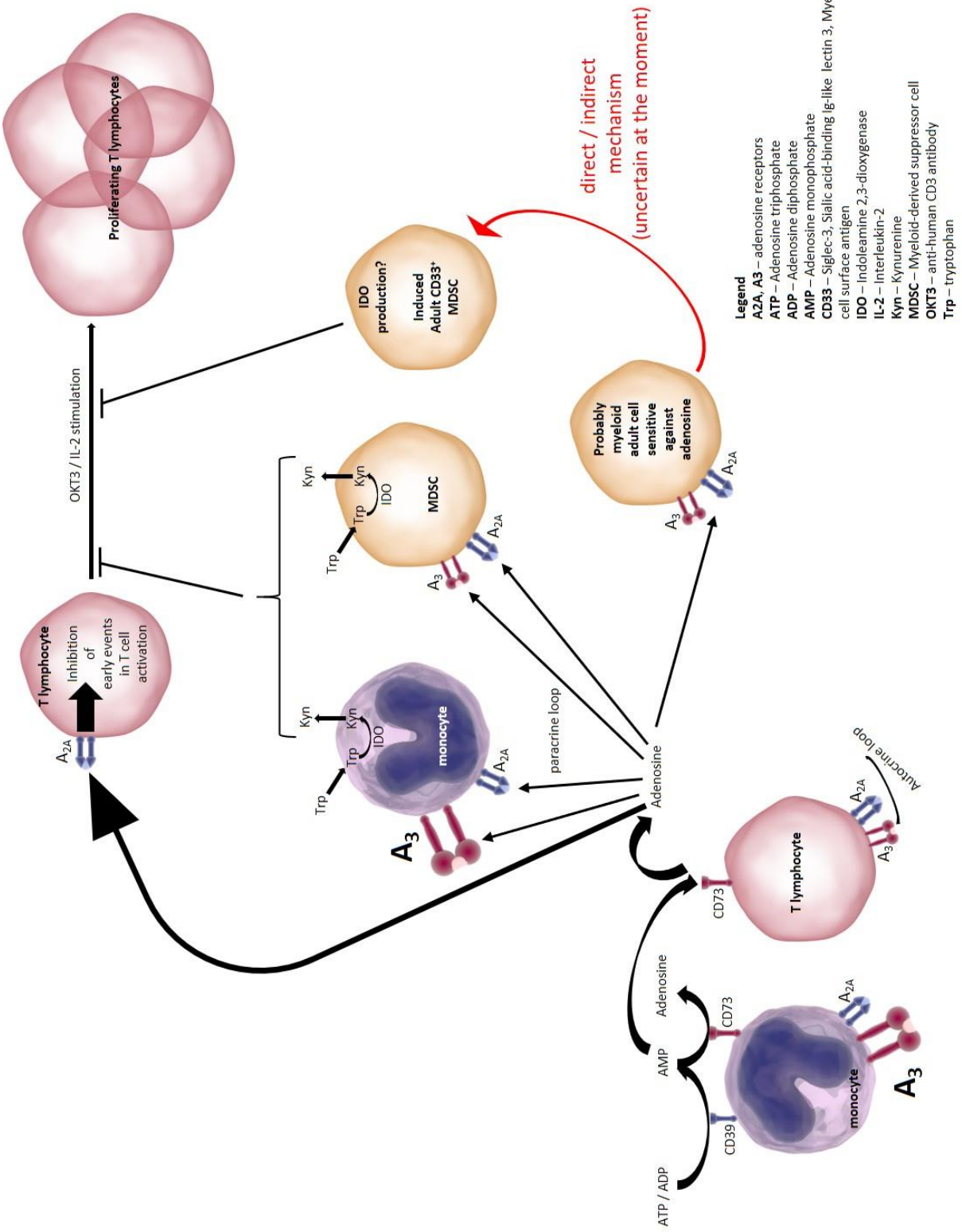


Figure 34. Complex mode of action of adenosine in neonatal immune response regulation.

6. References

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