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University of Turku

Faculty of Science and Engineering
Department of Biochemistry
Doctoral Program in Molecular Life Sciences
Turku Centre for Biotechnology

Supervised by

Andrey V. Zavialov, Ph.D.
Turku Centre for Biotechnology
University of Turku

Reviewed by

Hongxia Zhao, Ph.D., Docent
Institute of Biotechnology
University of Helsinki

Gong-Hong Wei, Ph.D., Professor
Faculty of Biochemistry and Molecular Medicine
University of Oulu

Opponent

Li Tian, Ph.D., Professor
Helsinki Institute of Life Science
University of Helsinki
and
Institute of Biomedicine and Translational Medicine
Department of Physiology, Faculty of Medicine
University of Tartu, ESTONIA

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To my family and dear friends

献给我的家人和好友

Chengqian Liu

Interplay between adenosine receptors and adenosine deaminases

Department of Biochemistry, Turku Centre for Biotechnology, Doctoral Program of Molecular Life Sciences, University of Turku, Finland.

ABSTRACT

Adenosine deaminases (ADAs) are key enzymes involved in purine metabolism and immune system. Human have two distinct adenosine deaminase isoforms, ADA1 and ADA2. ADA1 deficiency was reported as the first discovered molecular cause of human severe combined immunodeficiency (SCID) in 1972. In 2014, it was reported ADA2 mutations could cause early-onset stroke and systemic vasculopathy. However, the underlying mechanisms causing the symptoms present in the deficiency of ADA2 (DADA2) patients remain undefined.

This thesis is focused on the function of human ADAs in the immune system and the application of human ADA1 in sandwich enzyme-linked immunosorbent assay (ELISA). The data showed that ADA1 and ADA2 bind to different subsets of immune cells and modulate their response to extracellular adenosine by interplaying with adenosine receptors. Deficiency in human ADA2 may alter cellular responses to extracellular adenosine. In addition, both ADA1 and horseradish peroxidase (HRP) used in sandwich ELISA gave identical results, suggesting that ADA1 could be used as an alternative detection enzyme to measure antigen concentrations in biological fluids.

In summary, this thesis provides new data on the mechanisms causing those vascular inflammatory phenotypes of ADA2 mutations and suggests that ADA1 can be used as a valuable enzyme for amplification in sandwich ELISA.

Keywords: immunodeficiency, ADA1, ADA2, sandwich ELISA, HRP, adenosine receptors，SCID, DADA2

TIIVISTELMÄ

Adenosiini deaminaasit (ADA:t) ovat avainentsyymejä, jotka liittyvät puriinimetabolian ja immuunijärjestelmän toimintaan. Ihmisellä on kaksi erillistä adenosiini deaminaasin isoformia, ADA1 ja ADA2. Niistä ADA1:n havaittiin olevan vaikean perinnöllisen immuunipuutoksen (SCID) molekulaarinen syy vuonna 1972. Vuonna 2014 havaittiin, että ADA2:n mutaatiot saattavat aiheuttaa lapsuusiän halvauksia ja verisuonitulehduksia. Siitä huolimatta se mekanismi, joka aiheuttaa ADA2:n (DADA2) viallisesta toiminnasta johtuvia oireita, on selvittämättä.

Tämä väitös keskittyy ihmisen adenosiini deaminaasien toimintaan immuunijärjestelmässä sekä ihmisen ADA1:n hyödyntämiseen entsyymivälitteisessä immunosorbenttimäärityksessä (ELISA). Tutkimustulosten perusteella osoitettiin, että ADA1 ja ADA2 sitoutuvat immuunijärjestelmän solujen eri alaluokkiin ja muuntavat niiden vastetta solun ulkopuoliselle adenosiinille vuorovaikuttamalla adenosiinireseptorien kanssa. Ihmisen ADA2:n viallinen toiminta muuttaa solun vastetta solun ulkopuoliselle adenosiinille. Lisäksi, sandwich-ELISA määrityksessä havaittiin, että sekä ADA1:llä että piparjuuriperoksidaasilla (HRP) tehdyt määritykset antoivat samanlaiset tulokset. Tämän perusteella näyttää siltä, että ADA1:tä voitaisiin käyttää vaihtoehtoisena määritysentsyyminä antigeenin määrittämiseen erilaisista biologisista nesteistä.

Lyhyesti, tässä väitöksessä esitetään uutta tietoa mekanismeista, joilla ADA2 mutaatiot aiheuttavat verisuonitulehduksia. Lisäksi tulosten perusteella esitetään, että ADA1:tä voitaisiin käyttää tärkeänä entsyyminä sandwich-ELISA määrityksen vahvistamisessa.

Avainsanat: immuunipuutos, ADA1, ADA2, sandwich ELISA, HRP, adenosiinireseptorit, SCID, DADA2

摘要

腺苷脱氨酶（Adenosine deaminase, ADA）参与嘌呤的代谢并具有重要的免疫调节功能。我们人类拥有两种腺苷脱氨酶 ADA1 和 ADA2。1972 年的一篇研究论文报道了 ADA1 缺乏症的病例，是第一个被发现的严重复合型免疫缺乏症（Severe combined immunodeficiency，SCID）的分子机制。我们的课题组以及合作者于 2014 年公开报道了 ADA2 基因缺陷的病例，ADA2 基因突变可能导致早发性中风以及系统性血管疾病等症状，但其内在的分子机制还有待进一步的研究。

我的论文主要研究腺苷脱氨酶 ADA 在免疫系统中的功能，并将 ADA1 用于酶联免疫吸附试验（Enzyme-linked immunosorbent assay，ELISA)。实验结果表明腺苷脱氨酶 ADA1 和 ADA2 能结合到不同的免疫细胞表面并调节这些免疫细胞对腺苷的应答反应。ADA1 和辣根过氧化物酶（horseradish peroxidase，HRP）都能用于酶联免疫吸附试验并获得一致的试验结果，因此 ADA1 可代替 HRP 用于酶联免疫吸附试验测量样品中抗原浓度等。

综上，该论文为 ADA2 缺乏症的疾病机理提供了新的论据，还证明了腺苷脱氨酶 ADA1 可用于酶联免疫吸附试验。

关键词：免疫缺陷，腺苷脱氨酶，ADA1， ADA2， 酶联免疫吸附试验 ELISA，辣根过氧化物酶 HRP，腺苷受体，严重复合型免疫缺乏症 SCID， ADA2 缺乏症 DADA2

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ABBREVIATIONS

ADA	Adenosine Deaminase
hADA1	Human Adenosine Deaminase 1
hADA2	Human Adenosine Deaminase 2
mADA1	Mouse Adenosine Deaminase 1
bADA1	Bovine Adenosine Deaminase 1
ADP	Adenosine Diphosphate
ADGF	Adenosine Deaminase Growth Factor
AMP	Adenosine Monophosphate
dAMP	2'-deoxyadenosine 5'-monophosphate
ATP	Adenosine Triphosphate
dATP	2′-deoxyadenosine 5′-triphosphate
ADORs	Adenosine Receptors
A1R	Adenosine A1 receptor
A2aR	Adenosine A2a receptor
A2bR	Adenosine A2b receptor
A3R	Adenosine A3 receptor
CD8	Cluster of differentiation 8
CD26	Dipeptidyl peptidase-4 (DPP4)
CD39	Ectonucleoside triphosphate diphosphohydrolase-1
CD73	ecto-5'-nucleotidase
CTL	Cytotoxic T Lymphocyte/Cell
DADA2	Deficiency of Adenosine Deaminase 2
DC	Dendritic cell
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
ELISA	Enzyme-Linked Immunosorbent Assay
ENT1	Equilibrative Nucleoside Transporter 1
HRP	Horseradish Peroxidase

IL-6	Interleukin 6
LGLL	Large Granular Lymphocytic Leukemia
LAMs	Lipopolysaccharides Activated Monocytes
LPS	Lipopolysaccharides
MCP-1	Monocyte Chemoattractant Protein 1
MDSC	Myeloid derived suppressor cell
MIP-1α	Macrophage Inflammatory Protein 1-alpha
NK cell	Natural killer cell
PAN	Polyarteriris Nodosa
PBMCs	Peripheral Blood Mononuclear Cells
SCID	Severe Combined Immunodeficiency
TAM	Tumor-associated macrophages
T_{eff}	effector T cell
T-LGL	T cell large granular lymphocytic
TNF-α	Tumor Necrosis Factor alpha
T_{reg}	regulatory T cell

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred in the text by roman numerals (I-IV).

I. ***Liu, C.**, *Skaldin M., Wu, C., Lu, Y., Zavialov, A. 2016 Application of ADA1 as a new marker enzyme in sandwich ELISA to study the effect of adenosine on activated monocytes. Scientific Reports, 6, 31370; doi: 10.1038/srep31370

II. *Kaljas, Y., ***Liu, C.**, *Skaldin, M., *Wu, C., Zhou, Q. Lu, Y., Aksentijevich, I., Zavialov, A. 2017 Human adenosine deaminases ADA1 and ADA2 bind to different subsets of immune cells. Cellular and Molecular Life Sciences 74: 555. doi: 10.1007/s00018-016-2357-0

III. ***Liu, C.**, *Kaljas, Y., Trotta, L., Martelius, T., Saarela J., Seppanen, M., Zavialov, A. Deficiency in human adenosine deaminase 2 alters cellular responses to extracellular adenosine. (manuscript)

IV. ***Liu, C.**, *Kaljas, Y., Trotta, L., Saarela, J., Seppanen, M., Zavialov, A. Interplay between adenosine deaminases and A_2 adenosine receptors regulates immune responses to activating signals in the course of inflammation (manuscript)

*equal contribution

MANUSCRIPTS NOT INCLUDED IN THE THESIS

1. *__Liu, C.__, *Kaljas, Y., Wu, C., Lu, Y., Zavialov, A. V. Cellular response to extracellular adenosine depends on the activating signal.

2. *Skaldin M., *__Liu, C.__, Kaljas, Y., Porcel, J., Zavialov, A.V. Development of anti-ADA2 single chain antibodies coupled to alkaline phosphatase for the diagnosis of pleural tuberculosis.

3. *Kaljas, Y., *__Liu, C.__, Wu, C., Zhou, Q., Aksentijevich, I., Lu, Y, Zavialov, A.V. Quantitative determination of the human adenosine deaminase 2 in plasma for the diagnostics of ADA2 deficiency.

*equal contribution

1. INTRODUTION

Adenosine has been known as a key signaling molecule that plays an important role in the regulation of cellular response of nearby cells. Adenosine can signal through four distinct G-protein coupled receptors: adenosine A1 receptor (A1R), adenosine A2a receptor (A2aR), adenosine A2b receptor (A2bR) and adenosine A3 receptor (A3R) (Burnstock 2008; Fredholm et al. 2011). Adenosine receptors (ADORs) are therapeutic targets in many diseases including Parkinson's disease, asthma and rheumatoid arthritis (Cekic & Linden 2016). For example, adenosine itself is already used for treating certain types of irregular heartbeat (Headrick et al. 2011). Several clinical trials are targeting different ADORs and many 'proof of concept' studies on cellular models or animals related on ADORs hopefully will lead to further clinical trials (Burnstock 2017; Cronstein & Sitkovsky 2017).

It has been shown that adenosine plays an important role in the regulation of immune responses (Fredholm 2007). Adenosine accumulates at sites of inflammation and tumor growth and binds to ADORs that are expressed on the cell surface of the immune cells in response to activation signals. However, the role of adenosine deaminases (ADAs), which can convert adenosine to inosine and reduce the concentration of adenosine, is still unclear. Moreover, why humans possess two different ADAs, ADA1 and ADA2, with adenosine deaminase activity still remains a puzzle (Zavialov & Engstrom 2005). ADA1 can be largely defined as an intracellular enzyme that breaks down adenosine and reduces the concentration of the adenosine derivatives that are toxic to immune cells. It has been found that mutations in ADA1 gene lead to a severe combined immunodeficiency (SCID) characterized by decreased antibody production and lower numbers of T and B cells (Giblett et al. 1972). In contrast to ADA1, ADA2 is a protein secreted mainly by the cells of the myeloid lineage, such as monocytes, macrophages and dendritic cells (Zavialov & Engstrom 2005; Zavialov, Yu, et al. 2010; Zavialov, Gracia, et al. 2010). The deficiency of ADA2 (DADA2) is characterized by an early onset vasculopathy with livedoid skin rash associated with systemic manifestations and mild immunodeficiency (Caorsi et al. 2017; Schepp et

al. 2017; Zhou et al. 2014). Recently, our collaborators in Helsinki expanded phenotype and mutation spectrums of DADA2 and described for the first time DADA2 patients with lymphoproliferation phenotype and T cell large granular lymphocytic (T-LGL) disease (Trotta et al. 2018).

In this thesis work, the function of human ADAs and ADORs was investigated using peripheral blood mononuclear cells (PBMCs) from DADA2 patients and healthy donors. The human ADA1 (hADA1) is also successfully used in sandwich enzyme-linked immunosorbent assay (ELISA).

2. REVIEW OF LITERATURE

2.1. Adenosine as a guardian angel in many human diseases

Adenosine, a ubiquitous purine nucleoside, is available in all human tissues and organs. Nucleosides and nucleotides are well known for their intracellular functions as building blocks for DNA and RNA as well as cellular energy currency. Adenosine is an integral component of adenosine triphosphate (ATP), the major source of energy found in the mitochondria of all plant and animal cells (Antonioli et al. 2013). Adenosine is also a homeostatic regulator and regulates the function of every tissue and organ in human body mainly through the activation of four adenosine receptors (ADORs) that are differentially expressed in almost all human cells and have different affinities for adenosine (Fredholm 2007; Cekic & Linden 2016). Adenosine combats organ dysfunction in multiple pathological states and protects against cell damage in those areas of increased tissue metabolism. Adenosine levels increase in areas of pain, hypoxia, inflammation and cancer, in which adenosine behaves like a guardian angel (Borea et al. 2016).

The concentrations of extracellular adenosine change depending on the tissue types and physiological conditions. For example, the normal physiological concentration of plasma adenosine ranges from 20 to 200 nM (Gessi et al. 2011). At the sites of inflammation and tumor growth, extracellular adenosine accumulates rapidly to the μM range and regulates the immune responses of nearby cells (Figure 1) (Laghi Pasini et al. 2000; Moser et al. 1989; Vijayan et al. 2017). High local concentrations of adenosine may be achieved during specific conditions such as tissue inflammation, tumor microenvironment and injury (Leone et al. 2015; Eltzschig et al. 2012). Interestingly, plasma adenosine levels in healthy lowlanders were found to increase rapidly during initial ascent to high altitude and reach even higher levels upon re-ascent, which is associated with quicker acclimatization. The level of adenosine is regulated by several enzymes including equilibrative nucleoside transporter 1 (ENT1), which plays a major role in extracellular adenosine uptake. Xia and colleagues reported the enzyme ENT1 levels of red blood cells reduced in individuals at high altitude and in mice under hypoxia. Reduced ENT1 levels were maintained upon re-ascent or re-exposure to hypoxia, which is responsible for increased plasma adenosine concentration (Song et al. 2017). Altitude raised blood levels of adenosine,

which dilates blood vessels, boosting blood flow. In humans, the level of extracellular adenosine is also regulated by two adenosine deaminases hADA1 and hADA2. Decrease in ADAs enzyme activity due to genetic defects in the ADA genes leads to serious perturbation in the immune system function while increase in ADAs activity associates with numerous immune diseases and cancers.

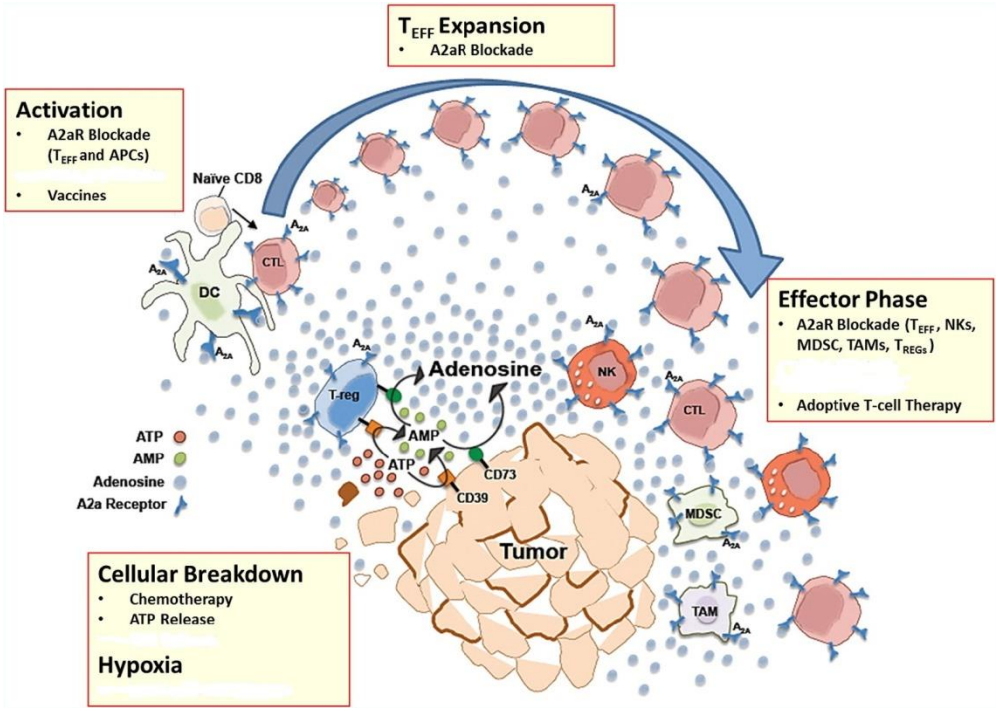


Figure 1 High extracellular adenosine concentration in tumor microenvironment and A2a adenosine receptors expressed by different subsets of immune cells. ATP are released into the tumor microenvironment with increasing tumor cell breakdown in the setting of hypoxia and chemotherapy. ATP is further catabolized to adenosine by CD39 and CD73. Adenosine has profound effects on all phases of immune function. Pharmacologic blockade of A2a receptors on cytotoxic T lymphocytes (CTLs), effector T cells (Teff), regulatory T cells (Tregs), natural killer cells (NKs), dendritic cells (DCs), myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) may counteract the immunosuppressive cloud of adenosine and enhance multiple phases of the immune response, including T cell activation, expansion and effector function. (Modified from Leone, Lo, and Powell 2015)

2.1.1. ADA1 and severe combined immunodeficiency

Dr. Eloise Giblett and colleagues reported two unrelated children with severely impaired cellular immunity whose red cells have no measurable activity of the enzyme hADA1 (Giblett et al. 1972). This discovery led to the development of novel therapies for hADA1-SCID and other immunodeficiency diseases (Giblett et al. 2012; Blackburn & Thompson 2012).

Elevated levels of adenosine were found in the body fluids of hADA1-deficient patients. It was shown that 2′-deoxyadenosine 5′-triphosphate (dATP) levels were elevated for hADA1-deficient patients, which was the toxic metabolite in this disorder. The high rate of cell death following T cell section events results in a source of DNA which is degraded to deoxyadenosine, another substrate of ADA. Deoxyadenosine is converted first to 2'-deoxyadenosine 5'-monophosphate (dAMP) and finally to dATP. The hADA1 deficiency coupled with high levels of deoxynucleoside kinases leads to accumulation of high levels of dATP in the thymi of patients. The dATP-triggered cytochrome c release from mitochondria could activate an apoptotic cascade and lead to failure of T cell development. Those findings led to the eventual development of successful chemotherapeutic approaches for hairy cell leukemia (Blackburn & Thompson 2012).

In the early 1970s, several primary immunodeficiency diseases including SCID and Wiskott-Aldrich syndrome were well recognized by pediatric immunologists. However, the only cure option for severe immunodeficiency diseases was bone marrow transplantation from a histocompatible donor. Nowadays, for those with life-threatening immunodeficiency such as SCID, allogeneic hematopoietic stem cell transplant (HSCT) has been the main curative therapy. For those who lack matched donors, optimal therapy may be achieved with autologous HSCT using gene modified stem cells. The hADA1 deficiency was the first immunodeficiency disorder for which the molecular defect was identified and the first inherited disease to be treated by gene therapy. In 1990, the first clinical trial of gene therapy was attempted in a 4-year-old child with hADA1-SCID (Blaese et al. 1995).

The breakthrough in the treatment of hADA1-SCID patients came with the development of polyethylene glycol (PEG)-modified bovine bADA1 by the company Enzon. PEG-bADA1 (Adagen) was the first FDA-approved PEG-modified protein drug, which was used as a therapy for hADA1-deficent patients (Hershfield et al. 1987). Many patients who do not have suitable bone marrow donors have been able to lead reasonably normal lives as a result of treatment with PEG-bADA1(Kohn & Gaspar 2017).

Gene therapy with non-myeloablative conditioning is another option to be considered for those SCID patients due to hADA1 deficiency who lack an HLA-identical sibling donor (Aiuti et al. 2009). Clinical trials for hADA1-deficiency have demonstrated that gene transfer into hematopoietic stem cells and autologous transplant can provide clinical improvement for many patients (Kuo & Kohn 2016). As of April 2012, 40 patients have been treated for hADA1-SCID in three programs. All have survived and most of them remain disease-free (Scott & DeFrancesco 2016). Recently, Cicalese and colleagues reported excellent medium-term survival (100%, 2.3-13.4 years follow up; median, 6.9 years) and immune reconstitution outcomes for 18 hADA1-SCID patients treated with genetically modified CD34+ cells. Some patients who had previously failed haploidentical HSCT or who had been on PEG-bADA1 were also successfully treated (Cicalese et al. 2016).

2.1.2. Deficiency of adenosine deaminase 2

In 2014, two back-to-back studies reported 33 patients with mutations in the ADA2, encoded by the *CECR1* gene, named as deficiency of ADA2 (DADA2) (Navon Elkan et al. 2014; Zhou et al. 2014). To date, more than 130 DADA2 cases were reported (Hashem, Kelly, et al. 2017). DADA2 patients display multiple health problems including early onset systemic inflammation, recurrent ischemic strokes, and vasculitis/vasculopathy and antibody deficiency (Schepp et al. 2016; Gonzalez Santiago et al. 2015). Strikingly, the patients who are homozygous for a rare mutation, Gly47Arg, have symptoms of polyarteritis nodosa (PAN). Therefore, this loss-of-function mutation in ADA2 is the first identified molecular cause of a monogenic vasculitis. The symptoms of the DADA2 patients are distinct from patients with hADA1-SCID. Several reports suggested that hematopoietic stem cell transplantation (HSCT) was successful in 5

DADA2 patients (Schepp et al. 2017). Hashem and colleagues recently reported HSCT was an effective and definitive treatment for 14 DADA2 patients (Hashem, Kumar, et al. 2017). More recently, our collaborators in Helsinki identified a multi-case family originating from Eastern Finland diagnosed with cutaneous PAN and central nervous system complications. They have expanded the phenotypic and mutation spectrum of DADA2 and reported the first link between DADA2 and early-onset T cell clonal expansion (Trotta et al. 2018).

Studies of hADA2 have been hampered due to its low abundance in human tissues, the absence of a homologous protein in mice and a general assumption that hADA2 might be an isoform of hADA1. *CECR1* was initially identified as a possible candidate gene responsible for a rare genetic disorder called Cat Eye Syndrome (CES), which is associated with several developmental abnormalities linked to a partial triplication of chromosome 22q11 (Riazi et al. 2000). The hADA2 protein was purified as a by-product of commercially available immunoglobulin preparations (Zavialov & Engstrom 2005). It was shown that the gene coding for hADA2, *CECR1*, belongs to a new family of adenosine deaminase growth factors (ADGFs). The founding member of the ADGF family was originally identified as the only growth factor required for the proliferation and survival of embryonic insect cells. *In vivo* experiments with *Drosophila* demonstrated that ADGF-A, an ADA2 homolog, is required for larval development (Mondal et al. 2011). Therefore, ADA2 may also participate in embryonic development.

2.2. Regulation of adenosine level in the immune system

Since Drury and Szent-Gyorgyi described the effects of adenosine upon the mammalian heart beating and intestinal movements by intravenous injection of adenosine into whole animals in 1929, it has become clear that extracellular adenosine has regulatory effects in multiple organs and tissues that have been extensively studied in many species (Drury & Szent-Györgyi 1929). As an extracellular signaling molecule, adenosine plays an important role in many physiological processes. Interestingly, recent study in fruit fly suggested extracellular adenosine signal from immune cells could free up energy for the immune system to more energy at the expense of other tissues (Bajgar et al. 2015). Among the molecules and cytokines produced by immune cells, extracellular adenosine is

responsible for the regulation of the immune response in physiological and pathological conditions mainly through the stimulation of four different G protein–coupled adenosine receptors: A1R, A2aR, A2bR and A3R (Cekic & Linden 2016).

Extracellular adenosine may accumulate by 1) transport from intracellular adenosine via membrane nucleoside transporters and 2) by extracellular metabolism converting ATP or NAD to adenosine. The concentrations of extracellular adenosine are regulated by the enzymes of adenosine metabolism including adenosine deaminases (ADAs), CD38, CD203a, CD39 and CD73 as well as equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs). Extracellular adenosine can be produced from ATP or nicotinamide adenine dinucleotide (NAD^+) by two pathways: the classical CD39/CD73 pathway and the alternative CD38/CD203a/CD73 pathway (Figure 2) (Ferretti et al. 2018). CD38 can generate ADP-ribose from NAD+. CD203a, a key molecule in the alternative adenosine production pathway, may convert ADP-ribose to AMP. CD39 can generate AMP from ATP and ADP and is involved in a classical pathway independent of CD203a. Both pathways converge to CD73 that converts AMP to adenosine.

Adenosine may be produced through the catabolism of ATP by CD39 and CD73 (Bono et al. 2015). Whereas extracellular ATP concentrations are considerably lower, the intracellular levels of ATP are normally in the hundreds micromolar range. For example, the physiological ATP or adenosine concentrations in plasma are at submicromolar level. However, there may be a drastic elevation of extracellular ATP during various physiological and pathophysiological events including inflammation, hypoxia, ischemia, platelet aggregation, sympathetic nerve stimulation or cellular damage. Accordingly, extracellular adenosine concentration can also rise markedly through ATP catabolism. During hypoxia, inflammation or sepsis shock, extracellular adenosine concentration might significantly increase to the micromolar range. Under many conditions including inflammation, multiple cell types release nucleotides such as ATP or ADP, from their intracellular compartments into the extracellular space. CD39 (also known as Ecto-nucleoside triphosphate diphosphohydrolase 1, ENTPD1) can dephosphorylate both ATP and ADP, converting ATP into AMP by sequential hydrolysis (Takenaka et al. 2016).

It was shown that CD39 is expressed by lymphocytes, dendritic cells and a variety of other types of cells. Probably, the lymphoid CD39 is important for coordinate regulation of immune responses via the removal of ATP.

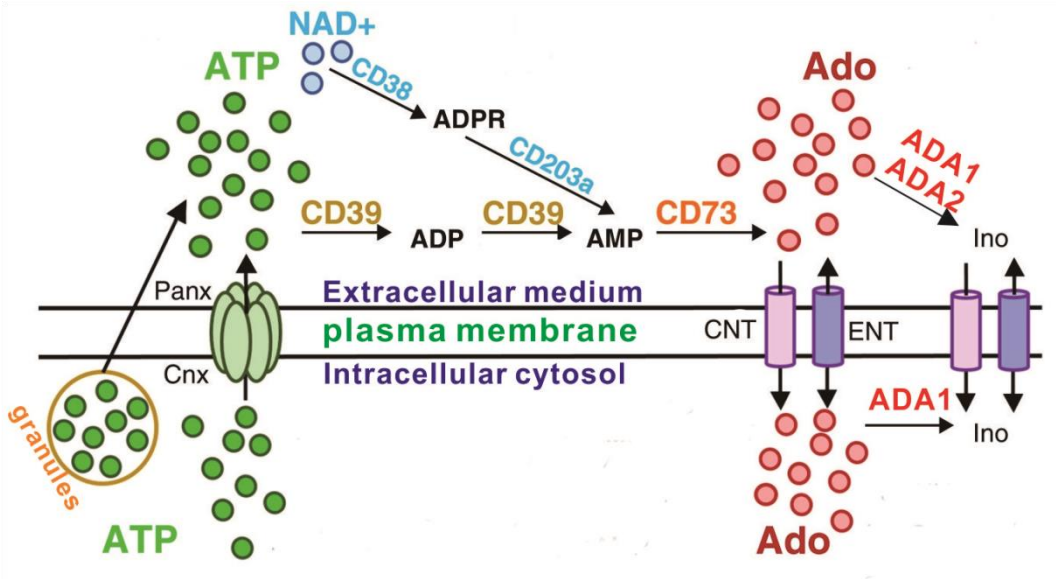


Figure 2 Regulation of extracellular adenosine level. Under inflammatory conditions, metabolic or hypoxic stress, ATP can be released into the extracellular space. ATP is mainly released through the secretion of pre-formed ATP-containing granules and by the intermediate of channels or transporters including pannexin/connexin channels (Panx/Cnx). Extracellular ATP can be converted into adenosine by CD39 and CD73. Extracellular nicotinamide adenine dinucleotide (NAD+) can also be converted into adenosine by CD38 and CD203a. Adenosine can be converted into inosine by ADA1 or ADA2. In addition, adenosine level is regulated by equilibrative or concentrative nucleoside transporters (ENT and CNT) (modified from Allard et al. 2016).

CD73 (also known ecto-5'-nucleotidase) can hydrolyses 5'-AMP into adenosine(Beavis et al. 2012). The CD73 expression is restricted in certain lymphocytes cell types and correlates with cell maturity even though CD73 is expressed in different tissues including liver, kidney and heart. Specifically, CD73 is co-expressed with CD39 on the surface of CD4+CD25+Foxp3+ Treg cells. CD39 and CD73 together comprise

suppressive machinery by converting ATP/ADP via AMP into adenosine, an anti-inflammatory mediator inhibiting T cell proliferation and secretion of cytokines. In addition to CD73, which constitutes the final enzymatic link of the purinergic cascade that leads to the formation of extracellular adenosine, two other enzymes are also important for the regulation of extracellular adenosine levels, i.e., adenosine deaminase (ADA) and equilibrative nucleoside transporters (ENT). Concentrative nucleoside transporters (CNT) actively transport extracellular adenosine to intracellular space (Yegutkin 2014).

2.3. Adenosine receptors

Adenosine receptors (ADORs) are therapeutic targets in many diseases including Parkinson's disease, asthma and rheumatoid arthritis (Chen et al. 2013). Adenosine binds to four ADORs that are differentially expressed on almost all human cells and show different affinities for adenosine. Adenosine itself is already used clinically (in the form of the generic drug Adenocard) to treat certain types of irregular heartbeat such as supraventricular tachycardia. Clinical trials targeting A2aR or A3R are underway to treat Parkinson's disease or psoriasis respectively.

Two out of four ADORs, A1R and A3R, have higher affinities and can be activated by normal adenosine level in the plasma. In contrast to normal physiology, inflammation triggers excess release of ATP from lysed and activated cells. ATP is rapidly converted to adenosine by cell surface ecto-enzymes CD39 and CD73 (Antonioli et al. 2013; Eltzschig et al. 2012). The concentration of adenosine in inflammation and the sites of tumor growth can reach micromolar levels, resulting in activation of the low affinity A2aR and A2bR (Antonioli et al. 2015; Fredholm 2007). In response to cell activation, expression levels of the cell surface ADORs increase. Therefore, the signals conducted by ADORs would be greatly amplified when the expression of an ADOR receptor is increased. ADORs control cellular responses such as proliferation, differentiation and secretion of cytokines by activation of downstream G-proteins. In general, the activation of A2aR and A2bR results in increase of intracellular cyclic AMP, while the activation of A1R and A3R decreases its concentration. Other transduction pathways including MAP kinase, Inositol kinase, NF-kB activation are also regulated by activated adenosine receptors (Gessi et al. 2011).

2.4. Adenosine deaminases

Adenosine deaminase (ADA, EC 3.5.4.4), a key enzyme of the purine metabolism, can catalyze the deamination of adenosine or deoxyadenosine to inosine or deoxyinosine, respectively. ADA has been found in bacteria, fungi, plants, invertebrates, vertebrates and mammals including humans (Maier et al. 2005). In humans, there are two distinct ADAs, hADA1 and hADA2 (Zavialov & Engstrom 2005). Both of them are found in the human plasma with ADA2 being a more abundant enzyme in normal physiology. ADA2 is a secreted protein and myeloid cells such as monocytes, macrophages and dendritic cells, are the main source for ADA2 in humans (Iwaki-Egawa et al. 2006). On contrary, ADA1 is mainly an intracellular enzyme that converts adenosine to inosine thus preventing accumulation of toxic adenosine derivatives and protecting the cells from death. The later may explain why the absence of functional ADA1 results in B and T-cells death and leads to severe combined immunodeficiency (SCID) (Shaw et al. 2017).

Though ADA1 has no signal sequence, the enzyme was found outside the cells where it binds to dipeptidyl peptidase IV (or CD26) expressed on the effector T cells or associates with ADORs and other unknown receptors expressed on monocytes and DCs. Although ADA1 is not secreted and its primary role is to regulate the intracellular adenosine concentration, it has been found that human ADA1 can bind dipeptidyl peptidase IV (CD26) and associate with A1R, A2aR and A2bR adenosine receptors expressed on the surface of immune cells (Franco et al. 2007; Ciruela et al. 1996). It was further proposed that ADA1 simultaneously binds to dendritic cells (DCs) and T cells via adenosine receptors and CD26, respectively, bridging the cells by forming an "immunological synapse" (Franco et al. 2007). This could explain why ADA1-dependent stimulation of T cell proliferation in the presence of DCs does not require ADA1 to be enzymatically active. However, according to other reports, the enzymatic activity of ADA1 is required to counteract regulatory T cell-mediated T cell suppression. According to the proposed scenario, ADA1 binds to CD26 on the surface of effector T cells and protects the effector T cells from the extracellular adenosine produced from ATP degradation by the ecto-enzymes expressed on the regulatory T cells. It was suggested that in the absence of ADA1, adenosine reduces T cell proliferation and cytokine release by activating the A2aR (Franco et al. 2007).

Although ADA1 is present in virtually all human tissues, the highest expression levels are found in the lymphoid system such as lymph nodes, spleen and thymus, where it prevents the buildup of ADA substrates. The high rate of cell death in the thymus following T cell section events results in a source of DNA which is degraded to deoxyadenosine, a substrate of ADA. ADA1 deficiency coupled with high levels of deoxynucleoside kinases leads to accumulation of high levels of dATP in the thymi of patients (Franco et al. 2007; Van De Wiele et al. 2002).

ADA2 is a secreted protein, and myeloid cells, such as monocytes, macrophages, and dendritic cells, are the main source of ADA2 in humans (Zavialov, Gracia, et al. 2010). Recently, autosomal recessive germline mutations of CECR1 were found to result in ADA2 deficiency (DADA2) leading to cytopenias, pure red cell aplasia, lacunar strokes, polyarthritis nodosa (PAN phenotype), and large granulocyte lymphocyte leukemia (LGLL phenotype) (Trotta et al. 2018; Zhou et al. 2014). Allogeneic hematopoietic stem cell transplantation (HSCT) and anti-TNF-α inhibitors are currently the main strategies for the treatment of DADA2 (Hashem, Kumar, et al. 2017; Montfrans et al. 2014).

3. AIMS OF STUDY

The aim of my thesis study was to investigate the role of adenosine receptors and adenosine deaminases in human immune system. Specifically, their roles on monocytes are mainly discussed in this thesis. In addition, the possibility of application of human adenosine deaminase hADA1 in standard ELISA was also evaluated.

The specific aims were:

1. To investigate the binding capacity of PBMCs with human and mouse ADAs
2. To explore the effect of adenosine and ADAs on the cytokine secretion of LPS stimulated monocytes
3. To evaluate the possibility of human ADA1 as amplification enzymes in standard ELISA

4. MATERIALS AND METHODS

Table 1. Summary of all the materials and methods

Materials and methods	publications
Expression construct of human and mouse ADAs	I, II, IV
Expression, purification and modification of recombinant ADAs	I, II, IV
Analysis of blood cells from healthy donors and DADA2 patients	II, III
Monocytes isolation and culture	I, II, III, IV
ELISA assay using human ADA1	I
Western blot analysis of ADA1 binding to THP1 and HuT78 cells	II

4.1. Expression construct of human and mouse ADAs

The open reading frames (ORFs) of human and mouse ADA genes were amplified with corresponding forward and reverse primers in Table 2 and sub-cloned into the pCR2.1-TOPO vector. The ADA ORFs were excised using *Xho* I and *BamH* I and ligated into a *Xho* I/*BamH* I digested self-inactivating transfer plasmid (pHR-cPPT-hB7-SIN) for expression of wild type ADAs (Wu & Lu 2010; Wu & Lu 2007). To express ADA2 with the His-tag and biotinylation site, the wild type ADA2 construct were digested with *Xho* I and *Sac* I, and ligated with the *Sac* I/*Sal* I excised fragment with His-tag and biotinylation site of the insect cell expression plasmid (Zavialov, Yu, et al. 2010). Subsequently, the ADAs with His-tag and biotinylation site were amplified with the primers ADA2-F and ADA2-biotin in Table 2 and ligated into the transfer plasmid (pHR-cPPT-hB7-SIN).

Table 2 Primers for plasmid constructs of adenosine deaminase

Species	Names			Used in
Human	ADA1-F	AGCTCGAGACCGGTCCACCATGGCC CAGACGCCCGCCT	F	I, II, IV
	ADA1-R	ATGGATCCGCTAGCTCAGAGGTTCT GCCCTGCAG	R	I, II, IV
	ADA2-F	ATCTCGAGCCACCATGTTGGTGGAT GGCCCATCTG	F	II, IV
	ADA2-R	TAGGATCCTCACTTTGTAGCCACAT CTGC	R	II, IV
	ADA2-Biotin	GCAGATCTGCTAGCGTCGACTTAGT GATGATGATG	R	II, IV
	ADA2-H86G-1	GTCACGATGCCAATGTCACCGAGGT GCAAGGCAGCC	R	II, IV
	ADA2-H86G-2	GGCTGCCTTGCACCTCGGTGACATT GGCATCGTGA	F	II, IV
	ADA2-H86G-3	GCTCTCCACTGAGCTCATACACCGG CAGCAG-	R	II, IV
Mouse	mADA1-F	AGCTCGAGACCGGTCCACCATGGCC CAGACACCCGCAT	F	II
	mADA1-R	TCAGATCTATTGGTATTCTCTGTAG AGC	R	II

To introduce the point mutation of H88G in ADA2, three additional primers including two primers ADA2-H86G-1 and ADA2-H86G-2 containing the desired mutation were used for fusing PCR. The fusing PCR product containing H88G mutation of ADA2 using the primers ADA2-F and ADA2-H86G-3 was then ligated with His-tag and biotinylation site, and inserting into the transfer plasmid (pHR-cPPT-hB7-SIN). All the constructs were sequenced for confirmation.

4.2. Expression, purification and modification of recombinant ADAs

HEK 293T cells were transfected with lentiviral transfer plasmids expressing wild-type or mutant ADA, pCMV-VSV-G envelope and pCMV ΔR8.2 packaging plasmids using the calcium phosphate method (Wu & Lu 2007). The lentiviral vectors were concentrated from the conditioned

medium of transfected HEK 293T cells, and were used to infect new HEK 293T cells. The 293T cells were grown in DMEM medium as described in Table 3.

Table 3 Cell lines and culture conditions used

HEK293	DMEM	DMEM medium (Sigma), supplemented with 5% FBS, 100 U/ml penicillin, 2 mM L-glutamine, and 100 μg/ml streptomycin
THP1, HuT78	RPMI	RPMI 1640 medium, supplemented with 10% FBS, 100 U/ml penicillin, 2 mM L-glutamine, 100 μg/ml streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 0.1% 2-mercaptoethanol

The recombinant human and mouse ADA1 was purified from the transfected 293T cell lysates. The 293T cell pellet was lysed through three freeze-thaw cycles using liquid nitrogen and a water bath at 42 °C. Then the cell lysate was re-suspended in ice-cold Buffer A (50 mM Tris-HCl buffer pH 6.8, 50 mM NaCl, 10 μM $Zn(OAc)_2$, 0.02% NaN_3). The supernatant containing ADA1 was collected after 4000 g centrifugation for 20 min, filtered using 0.45 μm filters, and applied onto a DEAE-Sepharose column equilibrated with the Buffer A. The flow through was collected and then adjusted to pH 8.4 with Tris base buffer. The ADA1 was applied to the DEAE-Sepharose column equilibrated with Buffer B (50 mM Tris-HCl buffer pH 8.5, 10 μM $Zn(OAc)_2$, 0.02% NaN_3). The ADA1 bound to the DEAE-Sepharose column was eluted using 0-500 mM NaCl gradient. The fractions which have ADA activity were pooled, concentrated using 10-kDa centrifuge ultraconcentrators, and further purified on a Superdex 200 column equilibrated with PBS buffer containing 10 μM $Zn(OAc)_2$ and 0.02% NaN_3. Purified ADA1 was chemically biotinylated using a G-Biosciences kit.

Recombinant ADA2 was purified from conditioned medium of the transfected 293T cells as described previously (Zavialov & Engstrom 2005; Zavialov, Yu, et al. 2010). For cell staining experiment, ADA2 was enzymatically biotinylated and conjugated with streptavidin labeled with FITC from eBioscience as described (Zavialov, Gracia, et al. 2010).

4.3. Analysis of blood cells from healthy donors and DADA2 patients

The study was undertaken in compliance with the principles of the Helsinki Declaration and was approved by the ethics committees of Helsinki and Oulu University Central Hospitals, Finland. The clinical evaluations of the patients in the manuscript III were carried out at the Helsinki University Hospital. All the patients in publication II were evaluated at the NIH Clinical Center. The patients enrolled in the study had been approved by institute review board, and the patients or their parents provided written informed consent. Blood cells were obtained fresh blood after the red blood cells lysis using RBC buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats of healthy donors (Red cross, Helsinki, Finland) or fresh blood of DADA2 patients using a Ficoll density gradient and stored frozen at -80 °C before the analysis. The PBMCs or blood cells were stained with cell surface markers from BD Biosciences.
For the binding of ADA2, the cells were first incubated with 30 µM ADA2 in FACS buffer (2% FCS in PBS) for 10 min at 4 °C, then washed with FACS buffer before being stained with 3 µg/mL anti-ADA2 rabbit polyclonal antibodies. Anti- ADA2 antibodies were purified using protein G Sepharose from the plasma of rabbits immunized with ADA2 and then using ADA2-streptavidin column (Zhou et al. 2014). The anti-ADA2 antibodies labeled with either FITC or CF633 dye (Sigma). A FoxP3 staining kit from eBioscience was used for Intracellular staining with anti-FoxP3 antibodies, which involves multiple washes. So a complex containing two ADA2 molecules linked by streptavidin was created to stabilize the binding of ADA2 to the cells.

4.4. Monocytes isolation and culture

Human CD14+ monocytes were purified from fresh blood of health donors or the buffy coats obtained from the Red Cross, Helsinki, Finland. Peripheral blood mononuclear cell (PBMCs) was first isolated by a Ficoll density gradient and then positive selection of CD14+ monocytes were done using anti-CD14-conjugated magnetic microbeads (Miltenyi), as described previously (Zavialov, Gracia, et al. 2010). The purity of recovered cells was 95–99% CD14+, as determined by flow cytometry using the FITC conjugated mouse anti-human CD14 mAb (BD

Biosciences). Complete RPMI 1640 medium was used in the cell cultures of monocytes. Monocytes were cultured in 5 ml polypropylene tubes (Falcon) in suspension at 0.5x106 cells/ml in 0.5 ml complete RPMI 1640 medium. Monocytes were activated using 10 ng/mL LPS in the presence of (I) agonist NECA and four different kinds of antagonists in Table 4, (II) adenosine, 15 μg/ml ADA2 or the mutant ADA2 (H88G) as indicated. The tubes with monocytes were incubated for 20 hours at 37 °C and 5% CO2. Then the cells were separated by centrifugation (300g, 5 min) and the cytokines TNF-α or MCP-1 concentrations in the supernatants were determined by ELISA, with HRP or human ADA1 as amplification enzymes. ADA2 concentration in the plasma of healthy donors or DADA2 patients was determined using the assay described in previous study and the TNF-α concentration was determined by ELISA (Zhou et al. 2014).

Table 4 Agonist and antagonists of ADORs used in this study

Name in this study	Common code	Specificity for ADORs
ANT	CGS 15943	A1 A2a A2b A3
AGO	NECA	A1 A2a A3
A1 ANT	PSB 36	A1
A2a ANT	SCH 442416	A2a
A3 ANT	CAY10498	A3

4.5. Western blot analysis of ADA1 binding to THP1 and HuT78 cells

The THP1 or HuT78 cells were cultured in the complete RPMI 1640 medium (Table 3). The cells were collected and stained with 100 μg/ml of biotinylated mADA1 or hADA1 in 60 μl FACS buffer for 10 min at 4 °C. For the competition experiment, THP1 cells were incubated with 100 μg/ml wild type hADA1 in 400 μl of FACS buffer for 10 min. The THP1 cells were centrifuged for 5 min at 300 g to remove the supernatant with unbound hADA1 before staining with biotinylated mADA1 as described above. After the staining with biotinylated ADAs, the cells were washed three times with 1 ml cold FACS buffer and lysed on ice using 50 μl buffer with 20 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM PMSF. Then the cell lysates were sonicated using a Bioruptor bath sonicator (Diagenode). And 25 μl cell lysates were applied

into the 4-20% Mini-PROTEAN RGX Gel (Bio-Rad). The proteins were transferred from the gel to a nitrocellulose membrane and biotinylated ADAs were visualized using avidin-HRP (eBioscience) and chemiluminescent western blot detection reagent (ThermoFischer).

4.6. ELISA assay using human ADA1

The concentrations of selected cytokines were determined using BioLegend ELISA kits according to the manufacturer's instructions. For the experiments with hADA1-based ELISA, avidin-HRP was substituted with hADA1-streptavidin as the following procedures:

1. Mix hADA1 with streptavidin in the molar ratio 1:2 to prepare 100 µg/ml hADA1-streptavidin (based on the streptavidin concentration)
2. Dilute 100 µg/ml hADA1-Streptavidin to 500 ng/ml using a buffer with 0.5% BSA, PBS and 0.02% NaN_3.
3. Add 100 µl of 500 ng/ml hADA1-Streptavidin to each well of a 96-well plate, and incubate at room temperature on a plate shaker for 30 min.
4. Wash the plate 3 times with PBS buffer including 0.05% Tween-20.
5. Wash the plate 1 time with PBS buffer.
6. Add 100 µl adenosine in PBS and 5 µM Zn^{2+}.
7. Incubate the plate at 37 °C for several hours.
8. Transfer the reaction mix to a UV-transparent plate (Costar) containing water, to make up 200 µl of 0.1 mM adenosine. Read the plate on a Synergy H1 hybrid Reader (Biotek) at 245 and 265 nm to get the 265/245 ratio, and determine the concentrations according to the standard curve. The UV-transparent plate can be reused multiple times.

Alternatively, 40 µl of the reaction mix were transferred onto a 96-well cell culture plate (Greiner) containing 60 µl of the reaction, containing 0.0625 U/ml purine nucleoside phosphorylase, 0.025 xanthine oxidase, 0.01 U/ml horse radish peroxidase, 1.25 mM 4-aminoantipyrine, and 0.75mM N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (all from Sigma-Aldrich) in 0.1 M Tris-HCl pH 6.8. The plate was incubated at 37 ^{o}C for 30 min, and the absorbance at 550 nm was determined using a Synergy H1 hybrid Reader (Biotek). For the detection using a fluorescent

reagent Ampliflu Red (Sigma-Aldrich), 1.25 mM 4-aminoantipyrine and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline were substituted with 0.1 mM Ampliflu Red, and the fluorescent intensity (excitation 530 nm and emission at 590 nm) was determined using a Synergy H1 hybrid Reader (Biotek).

5. RESULTS

5.1. Recombinant human ADA1 expressed by HEK 293T cells are active (I, II, IV)

Coleman and colleagues reported the production of recombinant human ADA1 in baculovirus-infected insect larvae (Medin et al. 1990). In this study, human ADA1 was expressed in the cytoplasm of HEK-293T cells transfected with lentiviral particles. The hADA1 was purified from the cell lysate using two-step chromatography (I, Figure 1D). The high purity of purified hADA1 was shown by SDS-PAGE gel (I, Figure 1B). It was shown that this hADA1 had similar catalytic parameters to the recombinant hADA1 expressed in bacteria (K_{cat}=190 s^{-1} and Km=26 μM) (I, Figure 1C) (Gracia et al. 2008). The purified ADA1 was biotinylated chemically without any loss of its enzymatic activity, indicating that the biotinylated ADA1 might be used to amplify the detection signals in commercial ELISA kits.

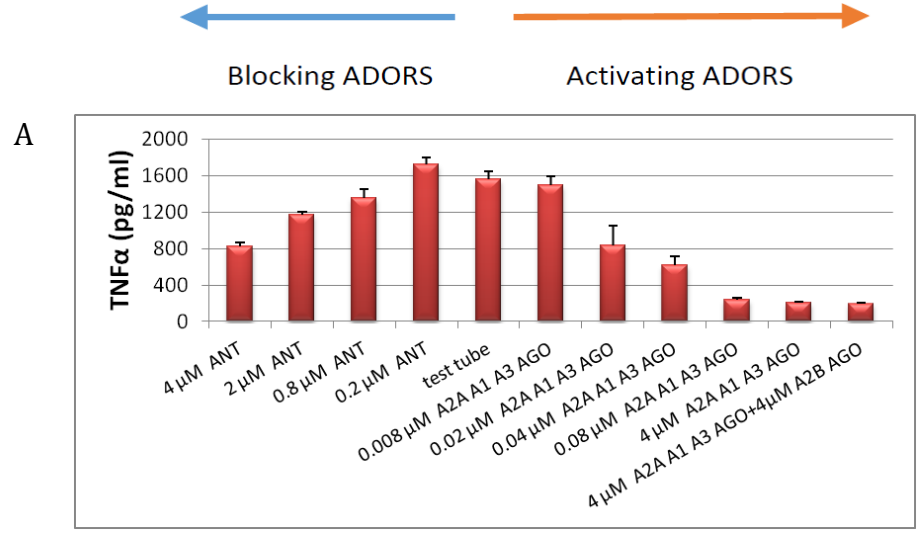
5.2. Human and mouse ADA1 bind to CD16- monocytes and THP1 cells (II)

Human ADA1 is best known as the first discovered molecular cause of rare immunodeficiency SCID (Giblett et al. 1972; Giblett et al. 2012). It was shown that human ADA1 present on the cell surface as ecto-ADA1 which is localized on the external side of membrane of monocytes and co-expressed with CD26 on the surface of T cells or CD26-tranfected Jurkat cells (Kameoka et al. 1993). In this study, biotinylated recombinant ADA1 of human or mouse was used to investigate the binding affinity of ADA1 to human immune cells and THP1 cells. The binding of biotinylated ADA1 to cells was analyzed using streptavidin conjugated to a fluorochrome and flow cytometry. In accordance with previous study (Kameoka et al. 1993), human ADA1 binds to the CD26 expressing fraction of lymphocyte subset (II, Figure 4C). In addition, the binding of biotinylated hADA1 can be completely abolished by adding unmodified hADA1, suggesting that biotinylated hADA1 binds specifically to the lymphocytes (II, Figure 4D). Among three subsets of CD14+ monocytes, CD14++CD16- classical monocytes had the highest capacity to bind biotinylated hADA1 (II, Figure 4A-B). Interestingly, mouse ADA1 was also shown to bind CD14++CD16- monocytes and human THP1 cells, a monocyte-like cell line (II, Figure 4E).

However, mouse ADA1 did not bind the HuT78 T-cell line expressing CD26 (II, Figure 4E). This is in agreement with previous report suggesting that mADA1 does not bind to CD26 directly (Dong et al. 1997).

5.3. Effect of non-specific ADORs agonists and antagonists on monocytes (I, IV)

It was shown that the levels of A1R and A2aR receptors increases in the LPS-activated monocytes (LAMs) (Chavez-Valdez et al. 2009), and non-selective ADORs agonists bound to A2aR decrease the secretion of TNF-α by LAMs (Zhang et al. 2005). We compared the effect of a non-selective ADORs agonist and a non-selective ADORs antagonist on the cytokine secretion by LAMs. As demonstrated in Figure 4, the release of cytokines from LAMs drops with the increase of either ADORs agonist or antagonist concentrations (I, Figure 6A). Interestingly, the secretion of TNF-α and MCP-1 by LAMs was more sensitive to the presence of ADOR agonist and antagonist compared to IL-6 (Figure 4A-C). Accordingly, the level of MCP-1 secretion decreased 5 times and the level of TNF-α decreased 8 times in the presence of both 4 μM nonselective $A_1A_3A_{2A}$ agonist and 4 μM A_{2B} agonist. At the same time, it was noted that the number of live monocytes increased with the increase in the ADOR agonist concentration (Figure 4D).



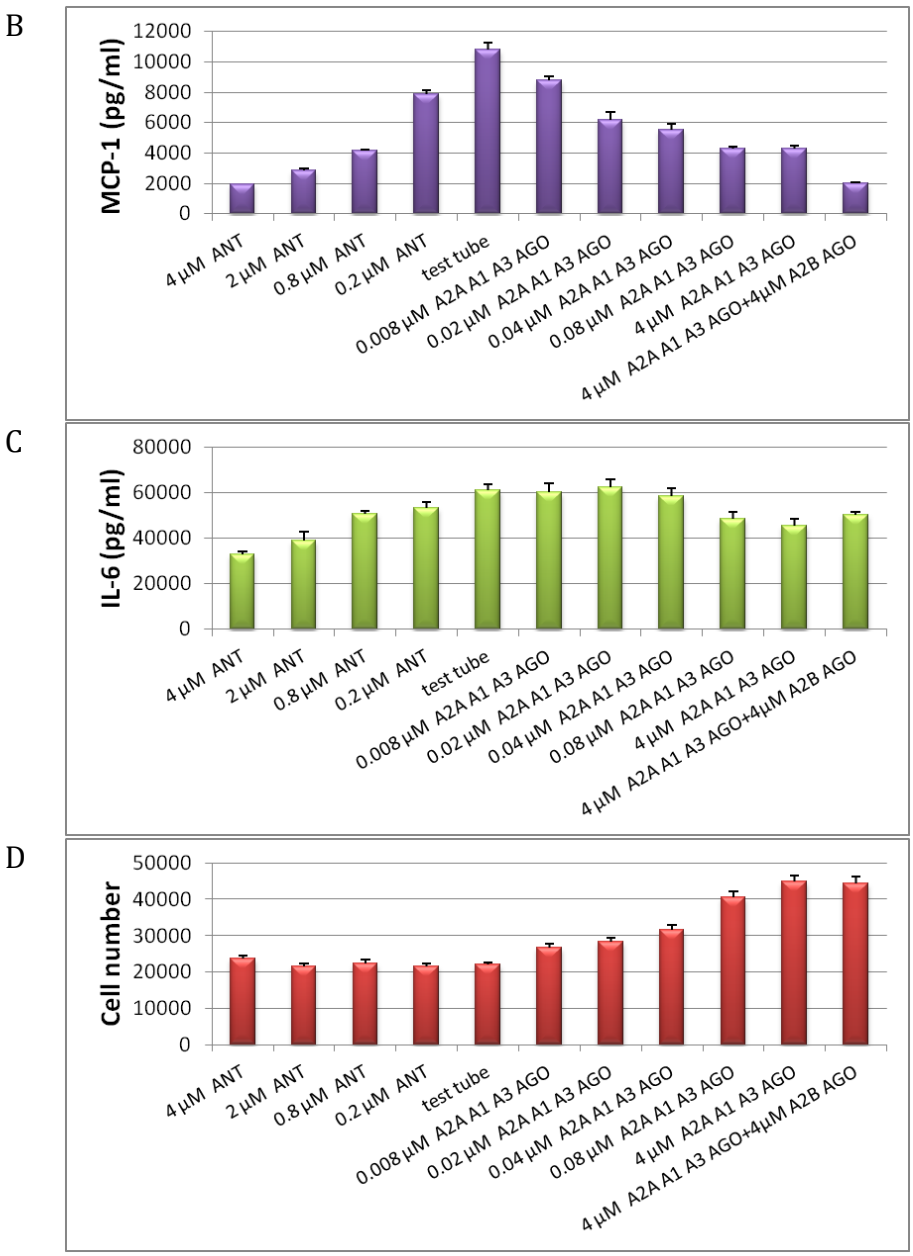
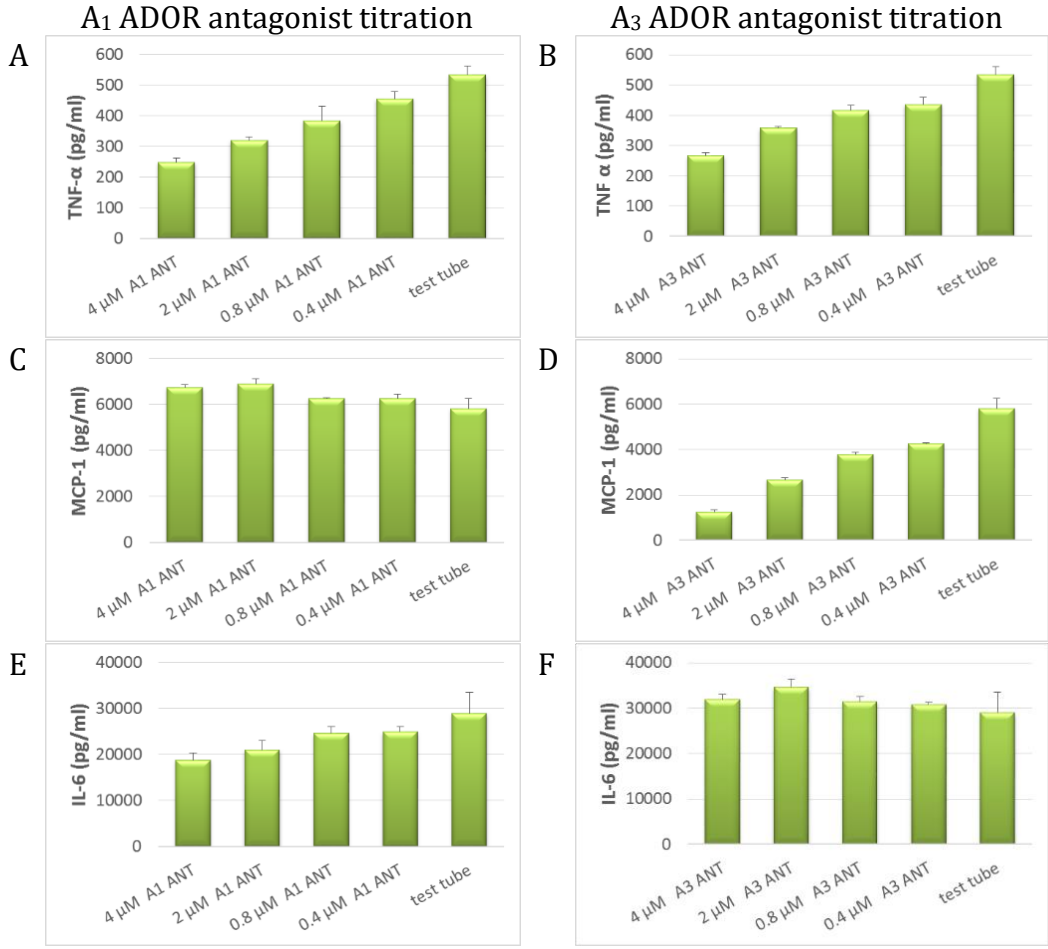


Figure 3 The effect of non-specific ADORs agonists and antagonists on the cytokine secretion by LAMs. (A-C) The concentration of TNF-α, MCP-1, IL-6 in the culture medium of monocytes after a 20-hour incubation period with 10 ng/ml LPS in the presence or absence of increasing concentrations of non-selective A2aA2bA1A3 antagonist (ANT, CGS 15943) or A2aA1A3 agonist (A2A A1 A3 AGO, NECA) with or without the addition of A2b agonist (A2B AGO). (D) The relative number of live monocytes remaining in the cell medium after 20 hours of incubation.

5.4. Effect of A1 and A3 ADORs antagonists on LAMs (I, IV)

It was shown A_1 and A_3 receptors are already activated by endogenous adenosine present in the test tube (I, Figure 6A). A competitive blocking of these receptors with ADOR antagonist results in a decrease in TNF-α and MCP-1 production by LAMs (Figure 4A-D). We also studied a dose-dependent effect of selective A_1 and A_3 antagonists on the cytokines released by LAMs. It was shown that MIP-1α and MIP-1β secretion is controlled by both A_1 and A_3 receptors (Figure 4G-J), while MCP-1 secretion is regulated by A_3 receptor (Figure 4C-D). On the contrary, the decrease in IL-6 secretion at low adenosine concentration was mainly due to A_1 receptor activation (Figure 4E-F).



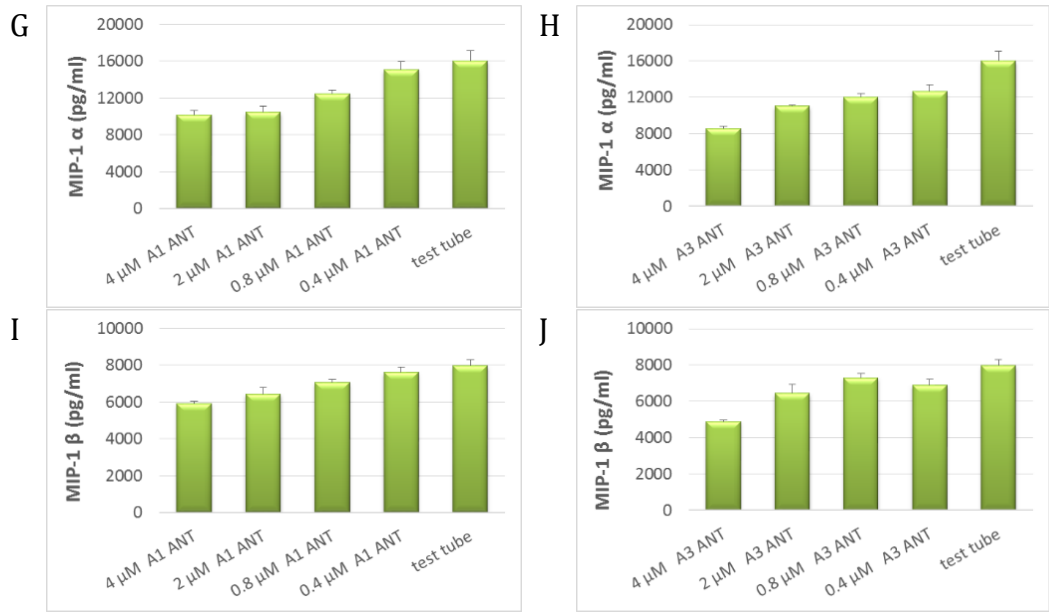


Figure 4 The effect of A1 and A3 ADOR antagonists on the secretion of the cytokines by LAMs. The concentration of TNF-α, MCP-1, IL-6, MIP-1α and MIP-1β in the culture medium of LAMs after a 20-hour-long incubation with 10 ng/ml LPS in the presence of increasing concentrations of selective A1R antagonist or A3R antagonist. Each point represents a mean of three replicates.

5.5. ADAs alone has no significant effect on TNF-α secretion from LAMs (II, IV)

An experiment similar to the one shown in Figure 4 was performed with monocytes from three different healthy donors and the cells from a PAN DADA2 patient. As shown in Figure 5, the inhibition of TNF-α released from the cells from a PAN DADA2 patient requires much higher ADOR agonist concentrations, suggesting the lower impact of A_{2A} receptors in the regulation of the secretion of cytokines in LAMs from a DADA2 patient. The disturbance in ADOR signaling explains why the remaining concentration of TNF-α in the cell culture of the LAMs from a PAN DADA2 patient (778±30 pg/ml) in the presence of 4 μM ADOR agonist was still significantly much higher compared to the cytokine concentration in the cell culture of LAMs from healthy donors (116±75 pg/ml). In the experiment described in Figure 5, we also added either ADA1 or ADA2 to

monocytes before the activation of the cells with LPS to demonstrate that the presence of ADAs does not result in a decrease in TNF-α secretion from LAMs, which was opposite to the effect of ADOR antagonist addition (Figure 4A-C and Figure *5*). This suggests that ADAs cannot compete with A_1 and A_3 receptors for the binding to adenosine that is already present in the test tube at a low concentration. The monocytes from the PAN DADA2 patient responded to the ADOR antagonists similar to the cells of the healthy donors.

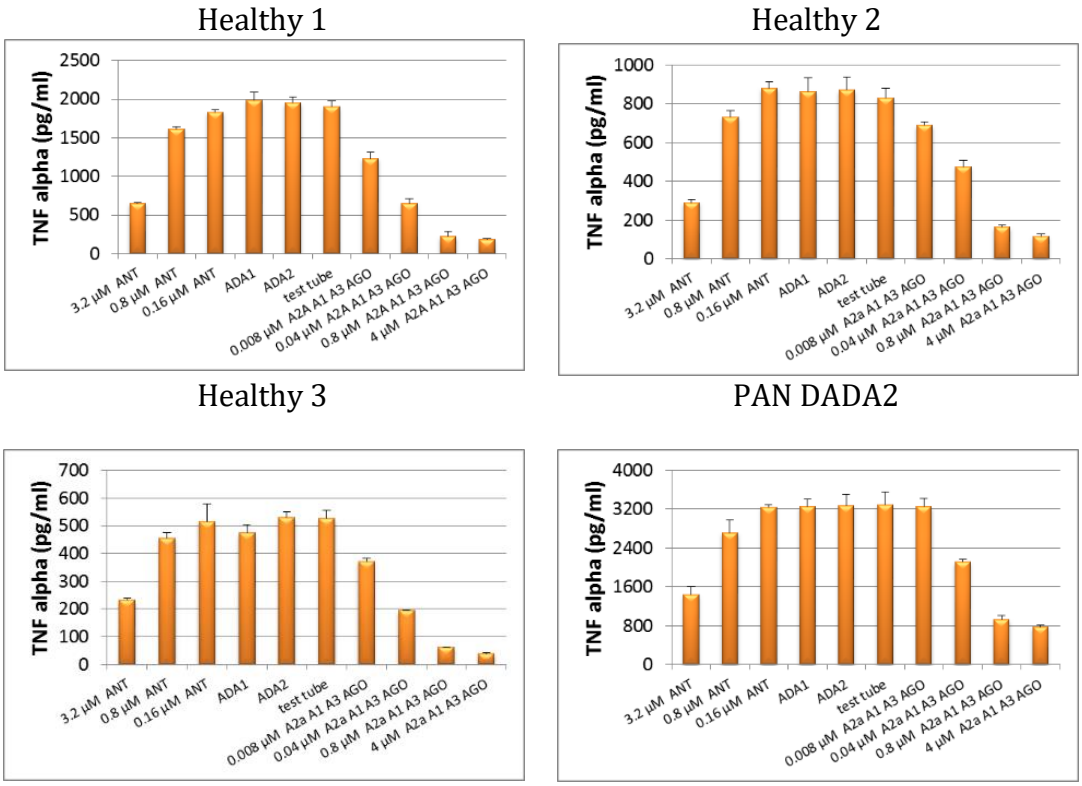
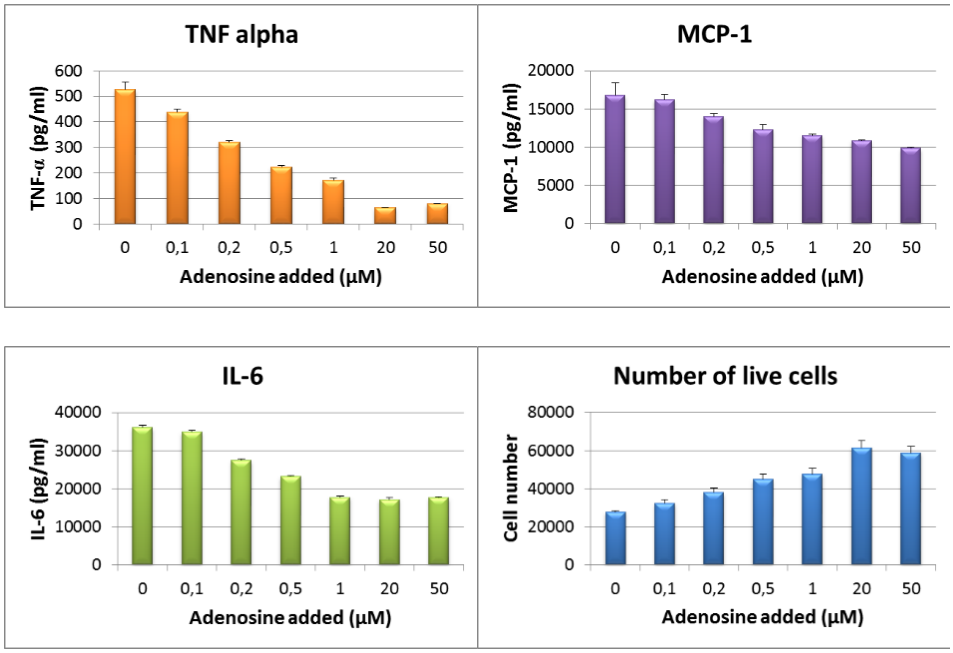


Figure 5 The effect of non-specific ADORs agonists or antagonists on the activation of the monocytes with LPS in the presence of ADAs. The concentration of TNF-α in the culture medium of monocytes after a 20-hour-long incubation with 10 ng/ml LPS in the presence or absence of increasing concentrations of non-selective $A_{2a}A_{2b}A_1A_3$ antagonist (ANT, CGS 15943) or $A_{2a}A_1A_3$ agonist ($A_{2A}A_1A_3$ AGO, NECA), 6.2 µg/ml hADA1 and 5.8 µg/ml hADA2. Each point represents a mean of three replicates.

5.6. Adenosine inhibits cytokine secretion from LAMs (II, IV)

The decrease in TNF- α released from the cells was seen at the concentrations of adenosine as low as 0.1 µM (II, Figure 6A). As shown in Figure 6A, adenosine at a high concentration almost completely stopped the secretion of TNF-α from LAMs. However, the effect of adenosine on MCP-1 and IL-6 secretion from the cells was not as dramatic compared to TNF-α, suggesting that either the secretion of TNF-α from LAMs is more sensitive to the presence of high concentrations of adenosine or the release of cytokines is regulated by adenosine at different time points after the activation with LPS (see below 5.8). Similar to Figure 3D, the number of cells remaining in the cell culture increased with the elevated adenosine concentration, indicating that the presence of adenosine at high concentration increases the lifespan of the cells.

A



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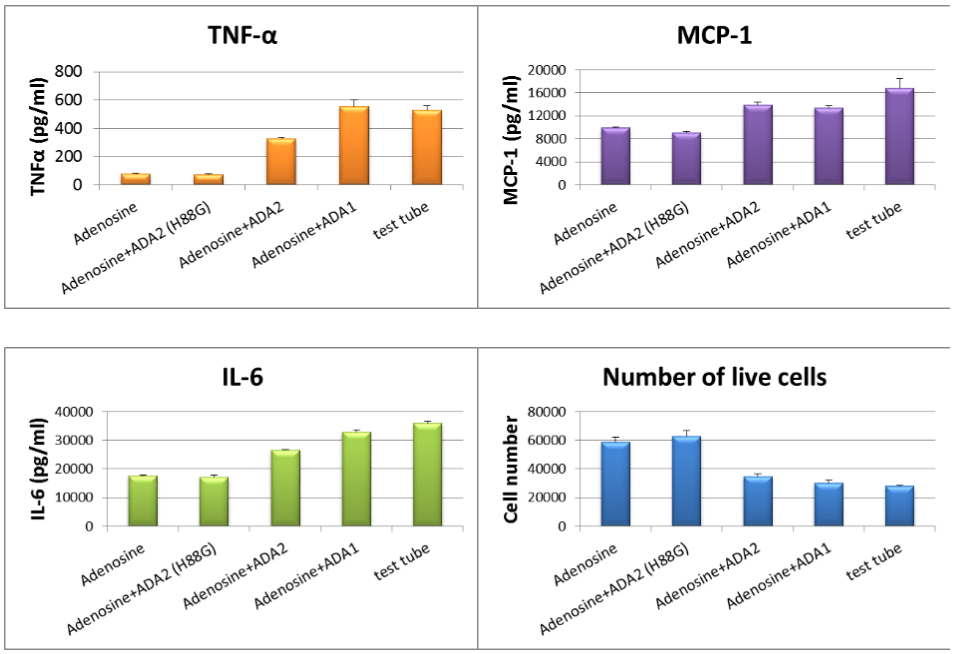

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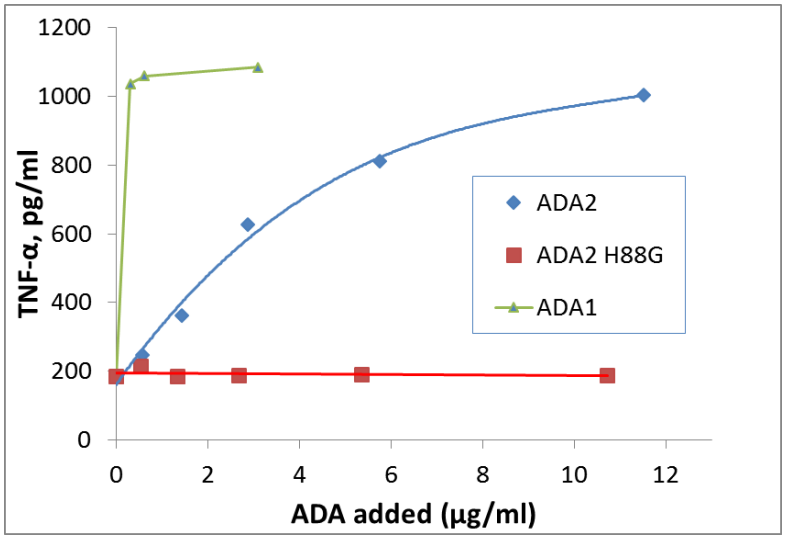

Figure 6 ADAs restore the activity of monocytes at a high adenosine concentration

(A) The concentration of TNF-α, MCP-1, IL-6 in the culture medium of monocytes after a 20-hour incubation period with 10 ng/ml LPS with or without the addition of adenosine. The last panel on the figure shows the relative number of live monocytes remaining in the cell medium after 20

hours in the same experiment. Each point represents a mean of three replicates.

(B) The concentration of TNF-α, MCP-1, IL-6 in the culture medium of monocytes after a 20-hour-long incubation with 10 ng/ml LPS in the presence of increasing concentrations of adenosine with and without the addition of 6.2 μg/ml ADA1, 5.8 μg/ml ADA2 or 5.4 μg/ml ADA2 H88G. The last panel on the figure shows the relative number of live monocytes remaining for 20 hours in the cell medium in the same experiment.

(C) The concentration of TNF-α in the culture medium of monocytes after a 20-hour incubation period with 10 ng/ml LPS in the presence of increasing concentrations of ADAs (ADA1, ADA2 and ADA2 H88G) in the presence of 50 μM adenosine. Each point represents a mean of three replicates.

5.7. ADAs restore the activity of LAMs suppressed by adenosine at high concentrations (II, IV)

Human ADA1, ADA2, or ADA2 H88G mutant, which lacks ADA activity, were added together with 50 μM adenosine. As we see in Figure 6A, addition of 50 μM adenosine to the test tube inhibits the release of TNF-α and IL-6 from LAMs. However, addition of hADA1 or hADA2 restores the level of cytokines released from LAMs (Figure 6B). This was due to the ADA activity resulting in a decreased adenosine concentration. Hence, the addition of ADA2 H88G did not have any effect on the activity of the LAMs. Moreover, the inclusion of ADA1 almost completely restored the activity of the monocytes because of the higher enzymatic activity of ADA1 compared to ADA2 at 50 μM adenosine concentrations (Figure 7). On the contrary, addition of the ADAs in the presence of 50 μM adenosine decreased the number of live cells in the culture medium, which is in line with the observation shown in Figure 6A. Our results demonstrate that ADAs hydrolyze extracellular adenosine and efficiently restore the activity of the cells. As shown in Figure 6C, the level of cytokines secretion by LAMs in the presence of 50 μM adenosine was directly proportional to the activity and concentration of ADA1 or ADA2 and it did not change in the presence of ADA2 H88G.

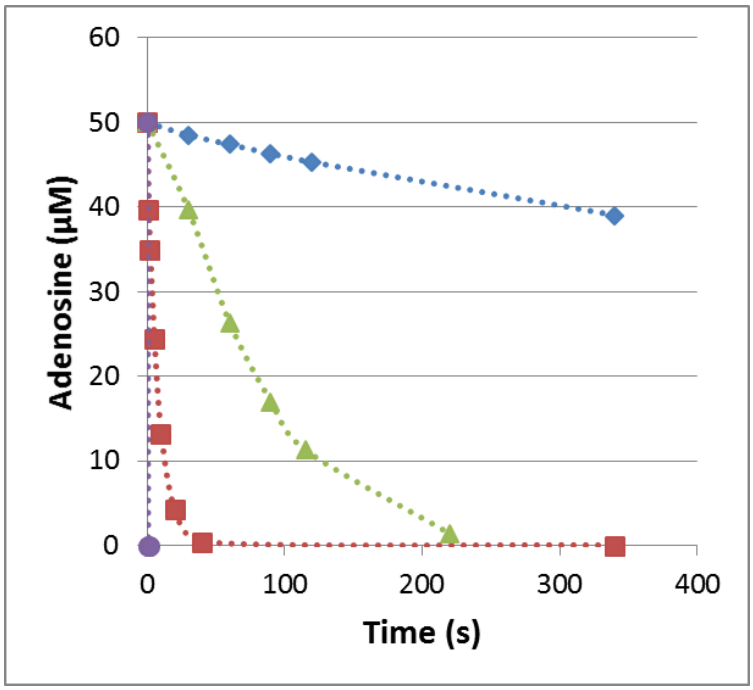

Figure 7 Kinetics of adenosine hydrolysis by bovine ADA1 present in the cell culture medium, recombinant ADA1, ADA2 and ADA2 H88G. Adenosine (50 µM) was incubated together with either ADA1 (circles), ADA2 (squares), cell culture medium (triangles), or ADA2 H88G (diamonds). The level of adenosine was estimated at different incubation times.

5.8. Exposure of LAMs to adenosine at different time points regulates secretion of different cytokines (IV)

We studied the effect of adenosine addition to monocytes at different times following LPS activation. As it is shown in Figure 8A, the addition of adenosine together with the activation with LPS or 20 minutes after the addition of LPS results in almost complete inhibition of TNF-α secretion from the cells. However, the addition of adenosine at later points (at 60 and at 175 min) had a much lesser effect on TNF-α concentration in the cell culture medium of LAMs. This result suggests that a major amount of TNF-α was already secreted 175 minutes after the activation of the cells, and thus, the effect of adenosine addition after this time point was negligible. Indeed, as it is shown in Figure 8C, TNF-α was readily secreted

by monocytes starting from the activation of the cells with LPS and reaching its maximum amount at 200 minutes. Consistently, adenosine, added together with LPS, inhibited TNF-α secretion (Figure 8C) However, the addition of adenosine at 140 and 200 minutes did not have much effect on the release of TNF-α in the cell culture medium by LAMs. Similar results were obtained for MIP-1α and MIP-1β, albeit the rate of the secretion of chemokines by LAMs was slower compared to TNF-α (Figure 8D-E). As it is shown in Figure 8F, the low rate of MCP-1 secretion by LAMs can explain the lack of inhibition with adenosine added together with LPS.

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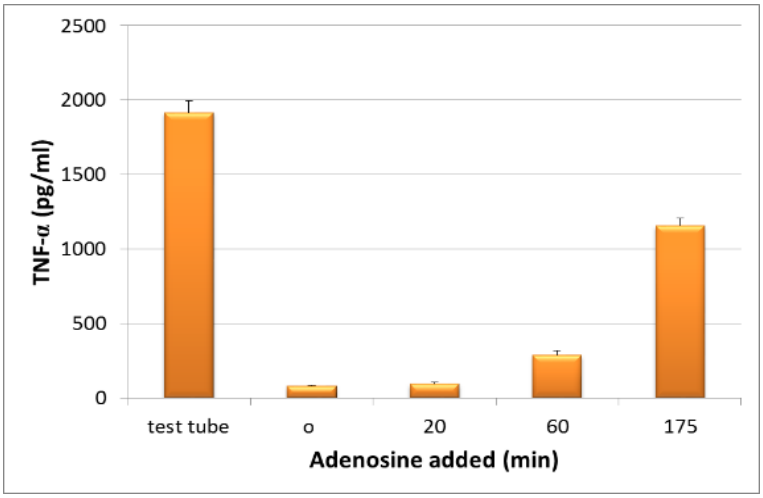

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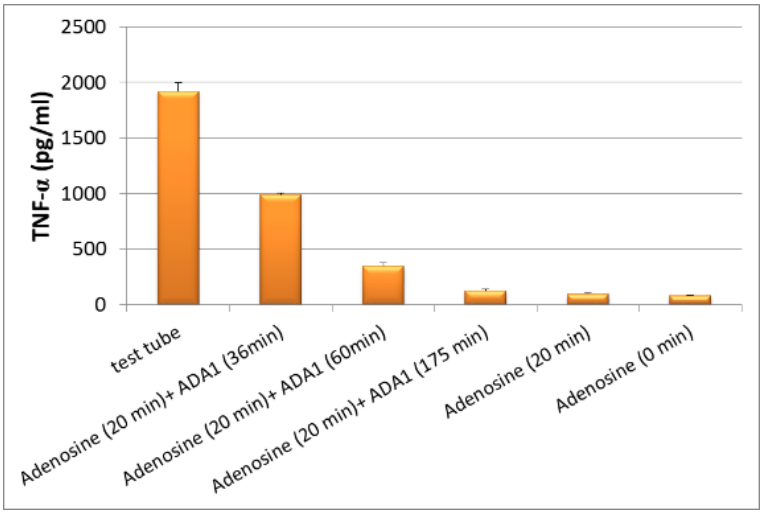

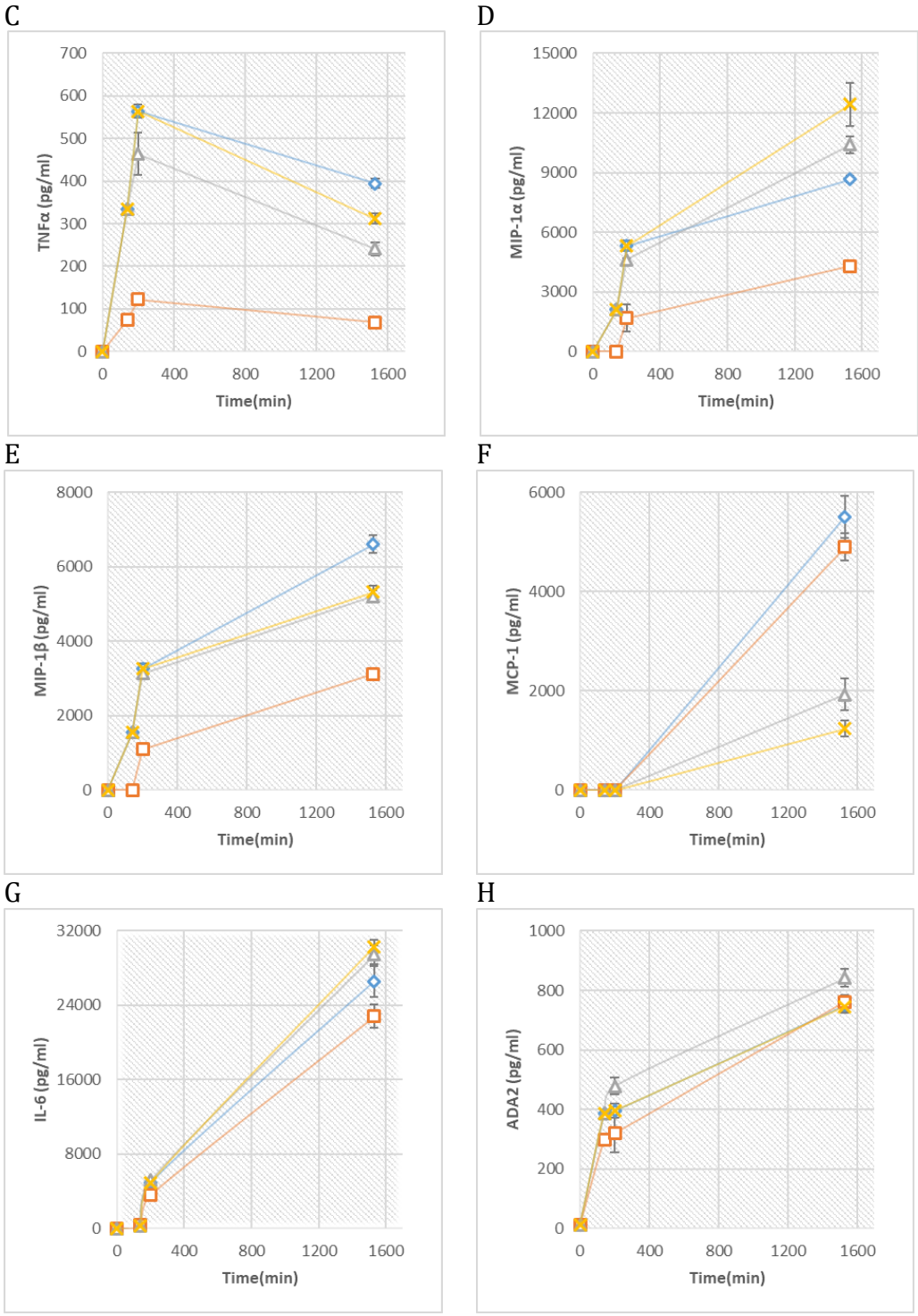

Figure 8 The effect of adenosine addition to LAMs at different time points on the cytokines released from the cells. (A) The concentration of TNF-α

in the culture medium of monocytes after a 20-hour-long incubation with 10 ng/ml LPS in the presence of 50 μM adenosine added at different points of time after LPS. (B) Exposure of LAMs to 50 μM adenosine added after incubation with LPS at different times and removed by addition of 6.2μg/ml ADA1 after the incubation time indicated in the graphs. Each point represents a mean of three replicates (C-H). The concentration of TN F-α, MCP-1, IL-6, MIP-1α, MIP-1β, and ADA2 in the culture medium of monocytes at different time points after the addition of 10 ng/ml LPS in the absence (diamonds) or presence of 50 μM adenosine added at 0 (squares), 140 (triangles) and 200 (crosses) min after LPS.

We have to note that in contrast to ADOR agonist, adenosine gets degraded by bovine ADA1, which is present in the cell culture (Figure 7), and thus, the effect of adenosine addition to LAMs differs from the non-hydrolysable ADOR agonist (Figure 3A-C). Therefore, addition of adenosine together with LPS did not inhibit MCP-1 secretion from the cells (Figure 6A), because adenosine was readily hydrolyzed by bovine ADA1 before MCP-1 started to release from LAMs (Figure 8F). Interestingly, minimal effect of adenosine addition was seen on IL-6 and ADA2 secretion by LAMs at any point in time after the activation of the cells with LPS (Figure 8G-H). Similar to TNF-α, ADA2 was secreted as soon as the cells were exposed to LPS, indicating that the enzyme participates in the regulation of the immune cells activity from the start of inflammation. In contrast, there was a 140–200-minute delay in the secretion of IL-6 and MCP-1 from LAMs compared to the other cytokines, suggesting that these two cytokines are required at the later stages of inflammation.

In the next experiment, LAMs were pulsed with adenosine that could be completely removed by the addition of ADA1 at different times after the activation of the cells with LPS (Figure 8B). It was demonstrated that an exposure of LAMs with adenosine for 16 minutes was sufficient to inhibit TNF-α production by half. Further incubation with adenosine for 40 minutes resulted in an almost complete inhibition of TNF-α release from the cells. This suggests that adenosine binds to the pre-existing adenosine A_{2A} receptors expressed on the monocytes and blocks the secretion of TNF-α from the cells.

5.9. Monocytes from PAN DADA2 do not respond to high concentrations of adenosine (II, III)

Many DADA2 patients have high concentrations of TNF-α in their blood samples (II, Figure 6C). However, some of the patients had an undetectable level of TNF-α as the healthy donors. This observation could be explained by the differences in the DADA2 patients' phenotypes (Caorsi et al. 2016). For instance, DADA2 patients with PAN phenotype may have higher levels of TNF-α, and thus, they respond well to the treatment with TNF-α inhibitors (Gonzalez Santiago et al. 2015). DADA2 patients without PAN phenotype may not have elevated concentrations of TNF-α in their blood. Here, we have analyzed the blood sample of a DADA2 patient with PAN phenotype and compared to that of a DADA2 patient with a recently discovered LGLL phenotype (Trotta et al., 2018). Both patients carried CECR1 transcripts with an identical c.G506A (p.Arg169Gln) mutation. As shown in Table 5, the patient with PAN had normal distribution of the cell subsets compared to the healthy donors. However, the DADA2 patient with LGLL-like phenotype showed a dramatic reduction in the NK, CD25+ B cells and an increase in the CD8+ T cells, a characteristic feature of the LGLL-phenotype. Both DADA2 patients had undetectable levels of ADA2 in plasma (Table 5).

Table 5 Lymphocyte subsets and ADA2 concentrations in DADA2 patients. The cell subsets were gated and expressed in per cents, as it was in (II, Table 1).

DADA2 patient phenotype		**PAN** DADA2	**LGLL** DADA2	**Controls**
Mutation		Arg169Gln	Arg169Gln	
ADA2, ng/ml		<0.25	<0.25	120±22
TNF-α, pg/ml		164±10	<8	<8
Cell subset	**Gated cells**	**%**	**%**	**%**
NK cells	3-/56+	7.48	0.47	12.2±5.6
CD8+ T cells	3+/8+	9.75	54.0	21.7±5.0
CD19+ B cells	19+	15.8	0.29	10.0±2.7
CD25+CD19+ B cells	19+/CD25+	15.1	6.36	25.2±7.2

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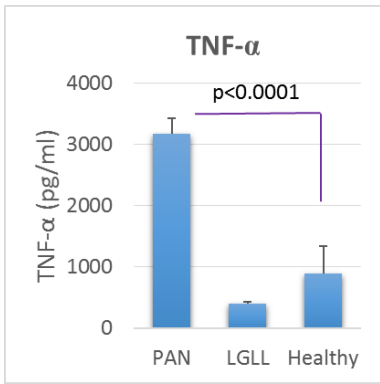

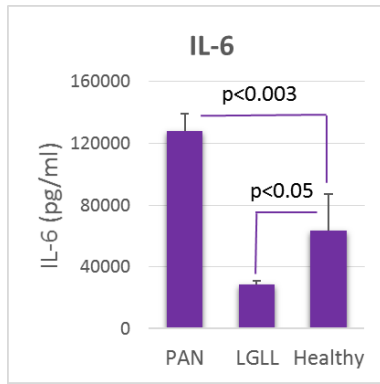

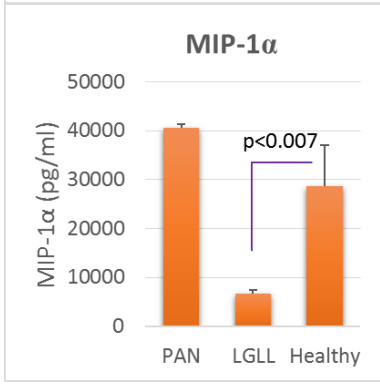

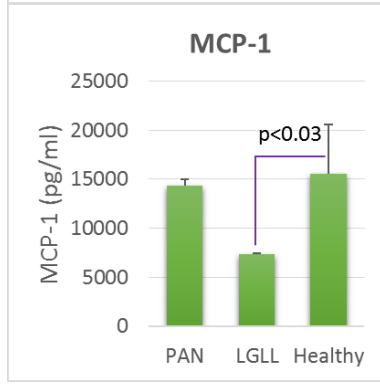

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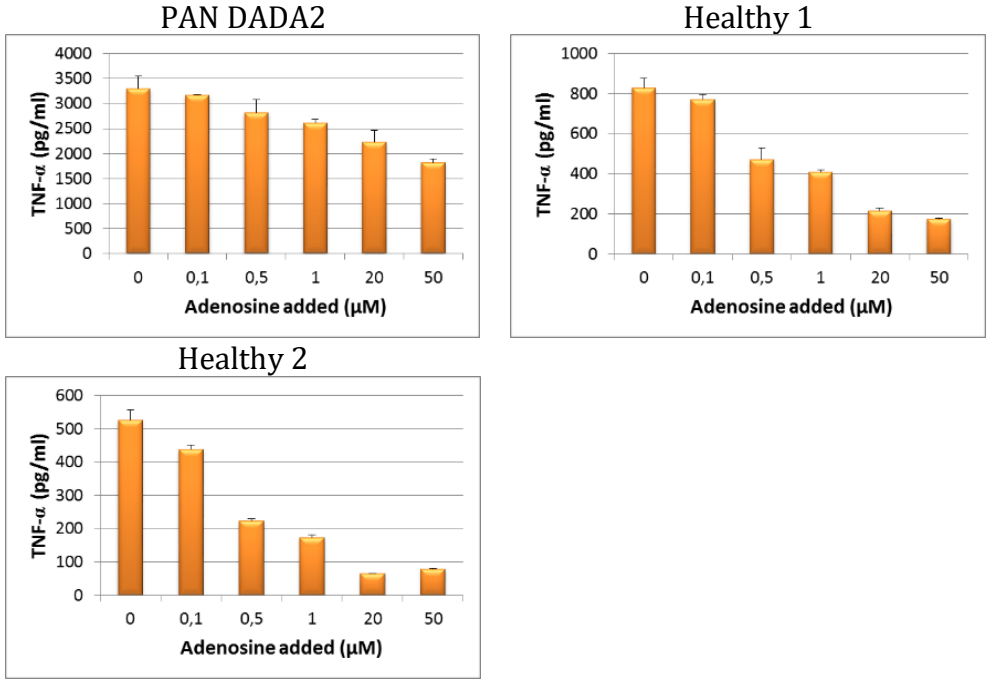
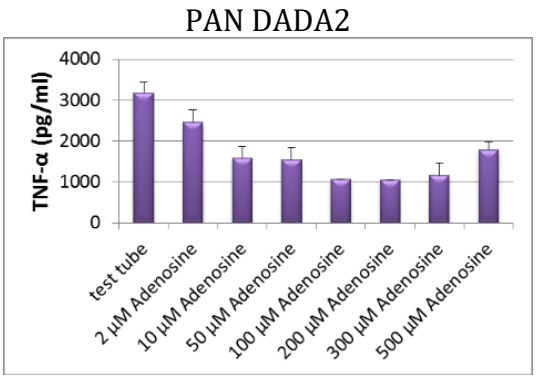
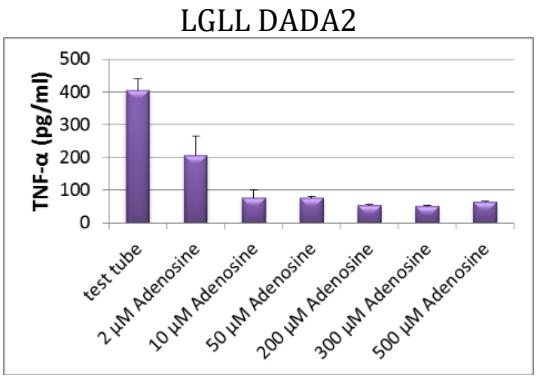


Figure 9 Monocytes from the PAN DADA2 patient overproduce TNF-α and IL-6 and do not respond to adenosine. (A) The concentrations of TNF-α, IL-6, MIP-1α, and MCP-1 in the culture medium of monocytes from

DADA2 patients with PAN phenotype (PAN), LGLL phenotype (LGLL), and 6 healthy donors after 20-hour incubation with 10 ng/ml LPS. (B) The concentrations of TNF-α in the culture medium of monocytes from PAN DADA2 patient and 2 healthy donors after 20-hour incubation with 10 ng/ml LPS in the presence of adenosine. Each column represents a mean of three replicates.

The TNF-α level in the blood of the PAN DADA2 patient was elevated compared to that of the DADA2 patient with LGLL phenotype and of healthy donors. We conducted a similar experiment, as described above, but with monocytes from DADA2 patients activated with LPS (Figure 9). It was found that monocytes from PAN DADA2 patient released significantly more TNF-α and IL-6 compared to those from healthy donors (Figure 9A). In contrast, monocytes from LGLL-like DADA2 patient produced significantly less IL-6, MIP-1α, and MCP-1 than those from healthy controls. This result suggests that the secretion of cytokines by monocytes from DADA2 patients is dysregulated. Indeed, the release of TNF-α from monocytes of the PAN DADA2 patient was marginally inhibited by a high concentration of adenosine as compared to monocytes from the LGLL-like DADA2 patient and healthy donors (Figure 9B).





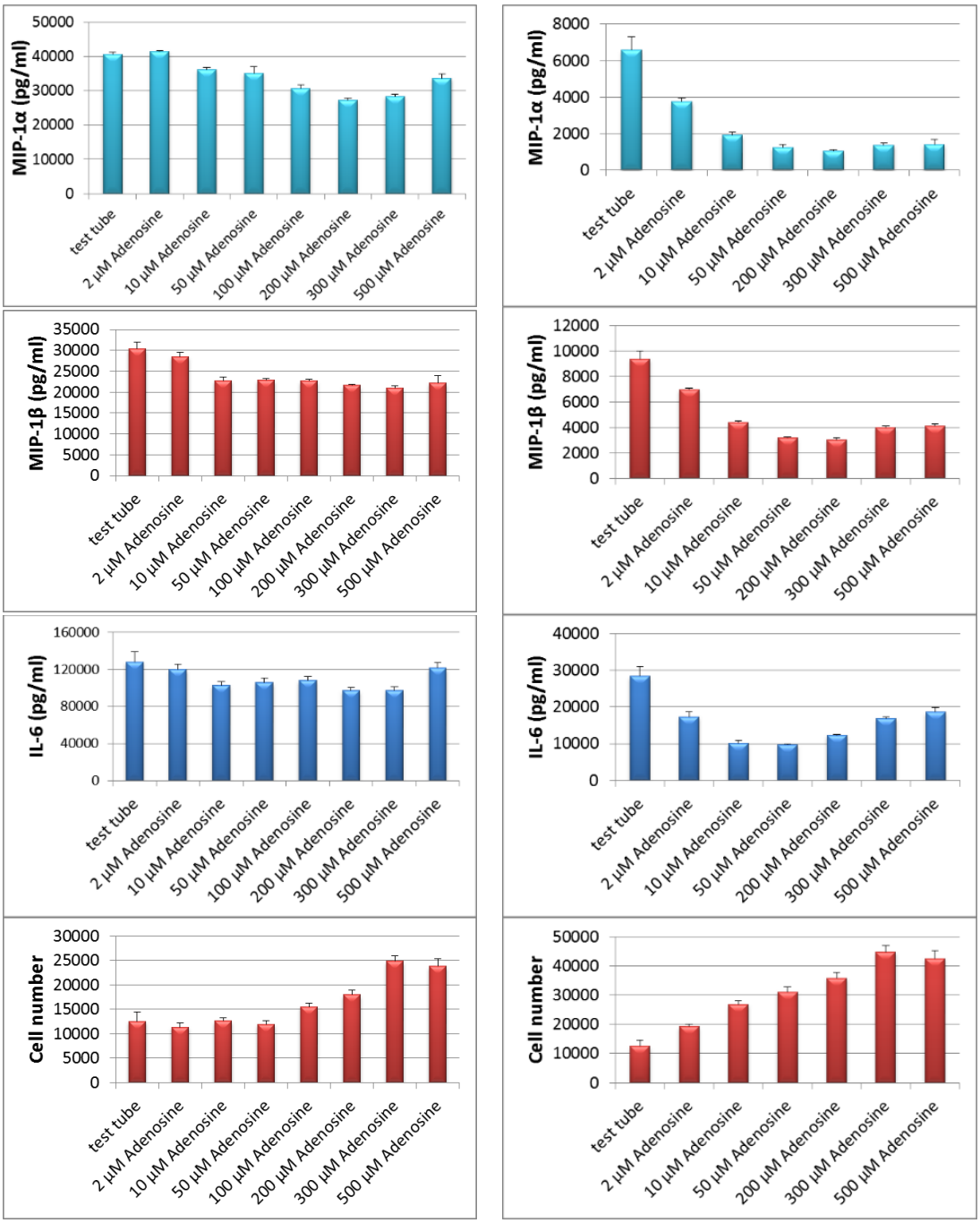


Figure 10 Monocytes from DADA2 patients respond differently to extracellular adenosine. The concentration of TNF-α, MIP- 1α, MIP-1β, IL-6 and the number of live cells remaining in the culture medium of monocytes from DADA2 patients with the PAN phenotype (PAN DADA2)

or LGLL phenotype (LGLL DADA2) after a 20-hour incubation with 10 ng/ml LPS and adenosine.

Even at an adenosine concentration of 500 μM, the level of TNF-α in the cell culture of monocytes from the PAN DADA2 patient remained higher than the concentration of TNF-α produced by LAMs from healthy controls without the addition of adenosine (Figure 10). Therefore, the fact that the LAMs from the PAN DADA2 patient are not regulated by a high concentration of adenosine may explain the high concentration of TNF-α in the blood of this patient. The lack of regulation could be due to a diminished expression of the A2aR on the LAMs from the PAN DADA2 patient. Indeed, an addition of A2aR antagonist to LAMs from the PAN DADA2 patient did not have any effect on the level of cytokines secreted by the cells. In contrast, an addition of A2aR antagonist to LAMs of the LGLL-like DADA2 patient and that of the healthy controls resulted in a significant increase in the release of TNF-alpha, MIP-1α, MIP-1β, and IL-6 from the cells (Figure 11). This indicates that in contrast to LAMs from the PAN DADA2 patient, the LAMs from the LGLL-like patient and healthy controls express A2aR and a fraction of these receptors is bound to adenosine produced by LAMs.

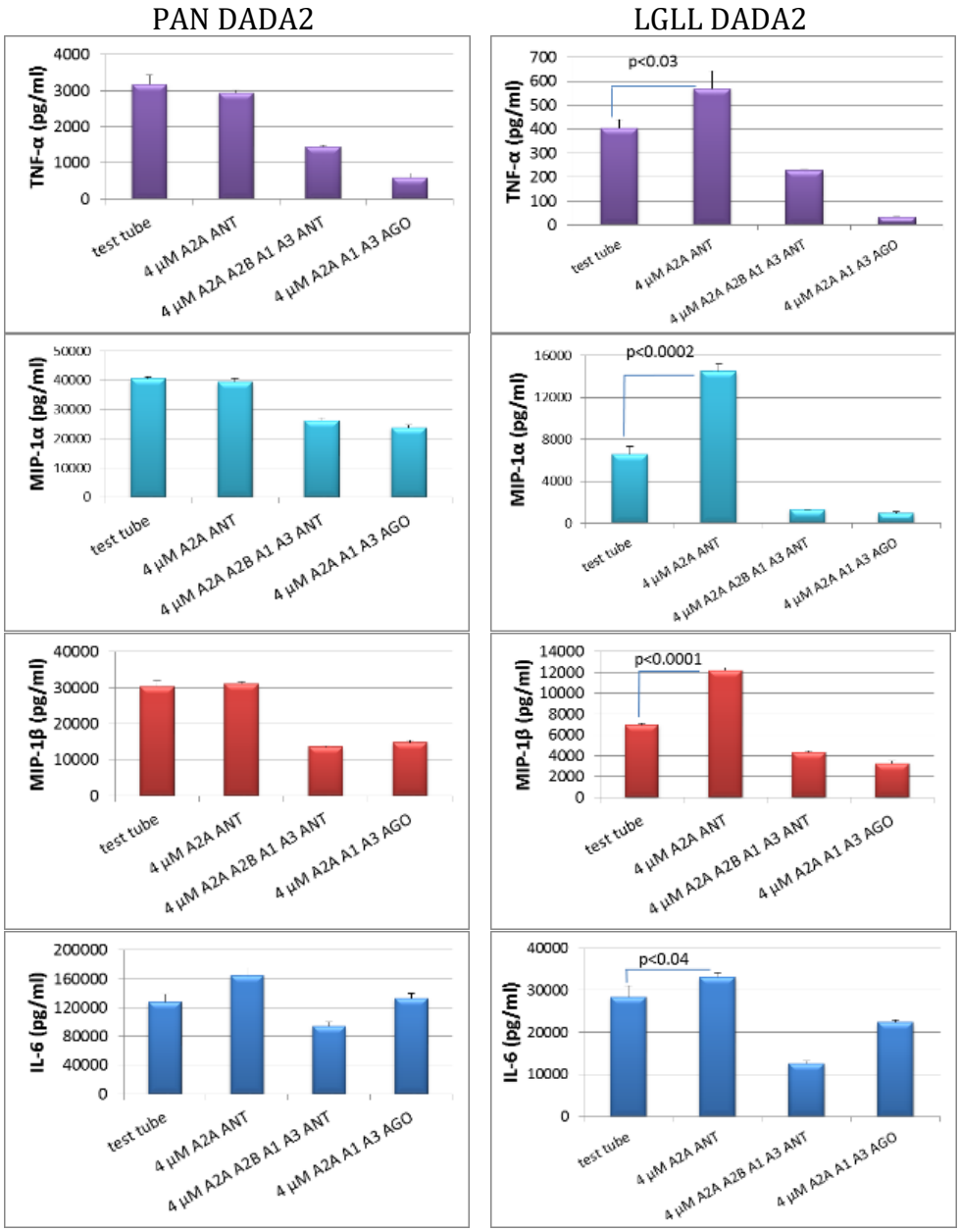


Figure 11 Monocytes from DADA2 patients do not respond to a specific A2aR antagonist. The concentration of TNF-α, MIP- 1α, MIP-1β, IL-6 in the culture medium of monocytes from DADA2 patients with the PAN phenotype and LGLL phenotype after 20-hour incubation with 10 ng/ml LPS and A2aR antagonist (A2A ANT, SCH 442416), A2aA2bA1A3 antagonist (A2AA2BA1A3 ANT, CGS 15943) or A2aA1A3 agonist (A2AA1A3 AGO, NECA). Each column represents a mean of three replicates.

5.10. hADA1-based ELSIA can be detected using three different assays (I)

To prove that hADA1 can be used as an amplification enzyme for detection, hADA1-streptavidin complex was used to substitute the HRP-avidin in commercial ELISA kits from BioLegend. First, the analyte of interest was sandwiched between the capture antibodies and biotinylated detection antibodies following standard procedures of ELISA kits (I, Figure 2A). Then hADA1-streptavidin was added to each well of the ELISA plate. Adenosine was used as the substrate of hADA1 to determine the analyte concentrations according to the amount of inosine produced by hADA1. Three different assays were used to determine the inosine concentration in the reaction mix of adenosine and inosine.

First, the UV assay was successfully used to measure the concentration of ADA2 in human plasma (Zhou et al. 2014). The reaction mix was transferred to a UV transparent 96-well plate containing water to dilute the nucleosides to 0.1 mM concentration. Then the UV-transparent plate was read at 245 nm and 265 nm using a Synergy H1 hybrid Reader (Biotek). The reaction of deamination catalyzed by hADA1 was followed by an increase in the ratio between the absorbances at 245 nm and 265 nm. The ratio was proportional to the concentration of hADA1 and analyte (I, Figure 2B). This UV assay does not require any additional reagents, and the reaction mix in the ELISA plate can be analyzed several times.

Second, the color assay produces quinone dye for detection in the visible spectrum. The inosine, product of adenosine catalyzed by hADA1, is converted by purine nucleoside phosphorylase (PNP) and xanthine oxidase (XOD) to uric acid and hydrogen peroxide, which is further reacted with reacted with N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to generate quinone dye (Delacour et al. 2010). Quinone dye was detected at 550 nm in the visible spectrum, which allows using standard 96-well plates and a simple plate reader (I, Figure 2C).

Third, the sensitivity of inosine detection could be significantly increased by substituting N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline and 4-aminoantipyrine with a fluorescent reagent, Ampliflu Red.

Accordingly, Ampliflu red is converted into a highly fluorescent compound, resorufin, and the reaction of adenosine deamination is monitored by the increase in fluorescent intensity (I, Figure 4B).

The sensitivity of the hADA1 assay for a given ELISA kit or antigen concentration can be adjusted either by changing adenosine concentrations or incubation times (I, Figure 3). In general, the increase in the incubation time and the use of lower adenosine concentrations allow reaching the maximum sensitivity of the assay. Therefore, hADA1 ELISA could be used for a wide range of antigen concentrations, ranging from nanograms to picograms per milliliter (I, Figure 4A). The use of fluorescent reagents adds even higher sensitivity to the assay (I, Figure 4B). Biotinylated hADA1 is very stable and could be stored at +4 °C in a PBS buffer with NaN_3. The hADA1 remains fully active for an extended period of time providing reliable and reproducible results (I, Figure 5).

6. DISCUSSION AND GENERAL PERSPECTIVES

Adenosine has been shown to be an important immunomodulator (Cekic & Linden 2016). Stimulation of monocytes with antigens such as LPS induces the expression of adenosine receptors on the surface of the cells, thereby increasing the sensitivity of the cells to extracellular adenosine (Chavez-Valdez et al. 2009). Although it is generally assumed that adenosine bound to adenosine receptors suppresses the function of immune cells, our results showed the requirement of adenosine for the activation of monocytes (I, Figure 6). This suggested the activation or suppression of the cells is dependent on the concentration of adenosine. In this study, we analyzed the level of cytokines secreted by monocytes in response to different concentrations of ADOR agonist, antagonist, and adenosine in the presence of ADAs to demonstrate that the release of the cytokines is controlled by adenosine and ADAs in the extracellular environment. We also concluded that monocytes can display distinct functions in response to low and high adenosine concentrations (Figure 12). The finding that LAMs from PAN DADA2 patients release a high concentration of TNF-α and decrease their response to extracellular adenosine in the absence of ADA2 suggests that ADA2 plays an important role in the regulation of immune responses by controlling the level of TNF-α and other pro-inflammatory cytokines.

It has been shown that specific A2aR agonists inhibit TNF-α secretion from the activated monocytes, while A1R and A3R agonists potentiate secretion of the cytokines (Hamano, Takahashi et al. 2008). Our results also suggest that the activation of high affinity adenosine receptors A1R and A3R at a low concentration of adenosine is required for the activation of monocytes (I, Figure 6). In this study, we thoroughly analyzed the impact of ADOR agonists and antagonists as well as adenosine and ADAs on the regulation of the secretion of cytokines and the survival of the LAMs. We confirmed that the addition of both ADOR agonists and adenosine results in the inhibition of the release of TNF-α, MCP-1, MIP-1α, MIP-1β, and IL-6 from LAMs (Zhang et al. 2005). Interestingly, it was found that the number of live cells remaining in the cell culture increases upon increasing ADOR agonist and adenosine

concentrations. This could either be due to the reduced activity of the cells or the reduced secretion of pro-apoptotic factors, both of which can potentially induce the death of LAMs. In contrast to the cytokines, the expression of ADA2 was not sensitive to extracellular adenosine and it can thus be said that the enzyme can be secreted by LAMs even at high concentrations of adenosine. Strikingly, the blocking of adenosine receptors with a non-selective ADOR antagonist decreased the secretion of TNF-α and MCP-1 from LAMs 3 times and 7 times respectively. This suggests that the release of adenosine from the cells and its binding to A1R and A3R high affinity receptors results in the autocrine activation of the LAMs (I, Figure 6). Indeed, the experiments with selective A1R and A3R antagonists show that blocking of A1R or A3R inhibits the release of cytokines from LAMs. While the secretion of TNF-α, MIP-1α, and MIP-1β was controlled by both A1R and A3R, the secretion of MCP-1 and IL-6 was regulated by A3R and A1R respectively. It has been shown that monocytes express A1R immediately after LPS activation, suggesting that this receptor could be important for the activation of the cells in the initial stage of inflammation (Chavez-Valdez et al. 2009).

ADAs are natural antagonists of ADORs since ADAs can reduce the concentration of adenosine. The efficiency of ADAs depends on their catalytic parameters, such as turnover rate (k_{cat}) and Km value. ADA1, which possesses a Km that is 40 times lower than ADA2, is more active at a low adenosine concentration and thus, the rate of adenosine hydrolysis is much higher with ADA1 in our experimental settings. However, in contrast to ADOR antagonist, the addition of ADAs to LAMs did not result in a decrease of the secretion of the cytokines from the cells. This suggests that ADAs reduce the concentration of adenosine at the level at which A1R, A3R, and a fraction of A2aR still bind adenosine. Therefore, A1R and A3R receptor-dependent autocrine activation of LAMs at a low concentration of adenosine is independent of ADAs (Figure 12). In contrast, when the concentration of adenosine is high, ADAs decrease the level of adenosine below the level of concentration required to bind and activate A2aR. Therefore, the addition of ADAs to LAMs at a high adenosine concentration increases secretion of cytokines. The effect of ADAs on the increase of TNF-α secretion from LAMs in the presence of 50 μM

adenosine is dependent on the concentration of ADAs and their catalytic activity.

It was also demonstrated that the inhibitory effect of adenosine correlates with the rate of the cytokine secretion by LAMs. The addition of adenosine to monocytes together with LPS had a strong inhibitory effect on TNF-α from the cells, while the release of MCP-1 from the cells was only slightly inhibited. The secretion of MCP-1 by LAMs is delayed by 3 hours following the addition of LPS compared to TNF-α, MIP-1α, and MIP-1β. At this point, adenosine added with LPS is already hydrolyzed by bADA1 present in the culture medium. However, a strong inhibitory effect of adenosine on the release of MCP-1 was seen when adenosine was added to LAMs 140 minutes after the activation of the cells with LPS. Therefore, adenosine can inhibit the release of cytokine from LAMs when the cells actively secrete the cytokines. There was either no or less inhibitory effect of adenosine on the secretion of TNF-α by LAMs when adenosine was removed by ADAs before the release of the cytokines from the cells. However, the full inhibitory effect of adenosine on TNF-α secretion by LAMs and the number of live cells remaining in the cell culture could already be seen after the incubation of the cells with adenosine for just 40 minutes. This means that adenosine binds to the pre-existing A_{2A} receptors and can express its inhibitory activity on TNF-α release soon after the activation of the cells. In contrast, the regulation of MCP-1 secretion from LAM may require the expression of additional A2aR adenosine receptors.

Our results suggest that both the regulation of adenosine on the secretion of cytokine and the timing of the secretion after LPS activation of the monocytes is crucial for controlling the activity of the cells in the inflamed tissue and the recruitment of the cells at the site of inflammation. TNF-α is secreted immediately after the activation of monocytes with LPS and can induce local inflammation (Scott & Kingsley 2006). At the same time, the release of TNF- α can be stopped by a slight increase in the concentration of extracellular adenosine. This could be important for the protection of tissues from excessive damage, i.e., in sepsis. Intriguingly, we found that ADA2 is also secreted from LAMs following the activation

of the cells with LPS. This suggests that ADA2 contributes to the regulation of the immune system in response to the inflammatory signals. As it was suggested before, ADA2 bound to the cell surface of myeloid cells, neutrophils, B cells, NK cells, and Treg cells can regulate their response to the extracellular adenosine by decreasing the local adenosine concentration (II, Figure 1).

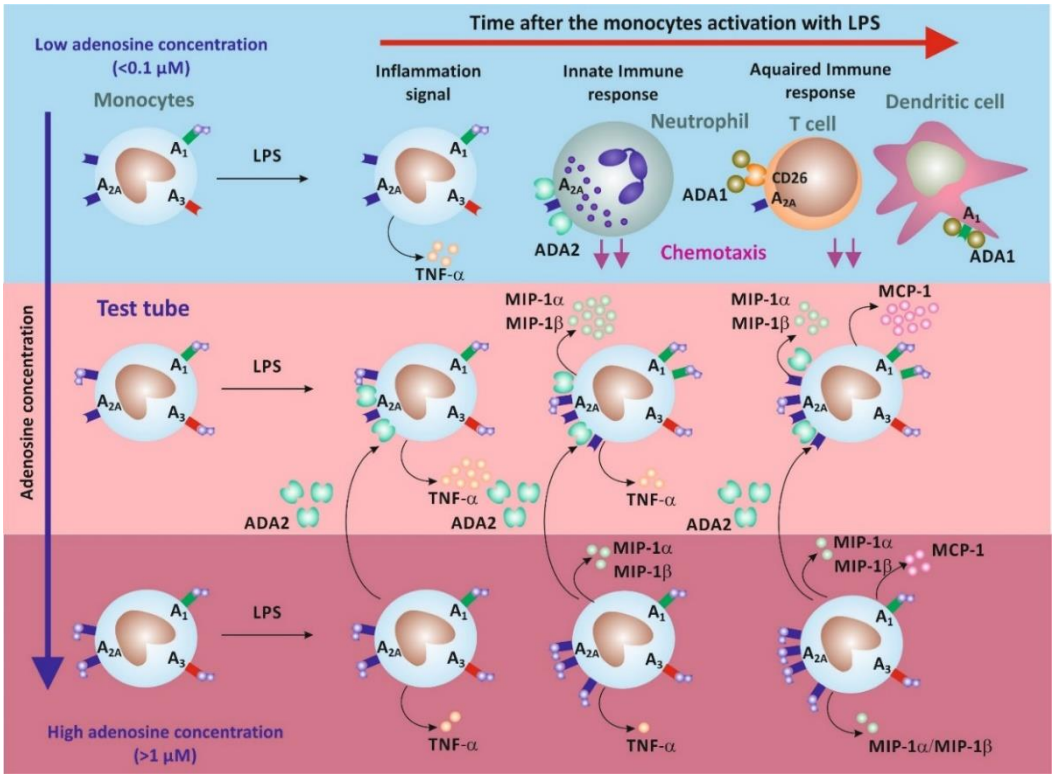


Figure 12. A scheme showing the proposed events following the activation of the monocytes with the LPS and the role of extracellular adenosine and ADA2 in the regulation of inflammation. Inflammation triggers the release of ATP, which is further converted to adenosine. At low concentration (<0.1 μM), adenosine binds mainly to A1R and A3R and stimulates the secretion of cytokine from LAMs. At high concentration (>1 μM), adenosine saturates A2aR and blocks TNF-α, MIP-1α, MIP-1β, and MCP-1 secretion from LAMs. The expression level of both A2aR and A1R on LAMs is increased with time. ADA2 bound to the surface of the cells lowers the local concentration of adenosine by hydrolyzing adenosine to inosine and thus potentiating the secretion of cytokines by LAMs (Test

tube). TNF-α is secreted immediately after the activation of the monocytes with LPS and induces the local inflammation. Chemokines MIP-1α, MIP-1β and MCP-1 reach the maximum expression level by LAMs at different times points following the release of TNF-α and recruit the cells of innate (neutrophils) and acquired (Effector T cells) immune system to the site of inflammation. ADA1 and ADA2 bind to the corresponding cell subsets and hydrolyze extracellular adenosine and regulate the activity of the immune cells.

In this study, we analyzed the response of the monocytes from DADA2 patients with identical mutations (Arg169Gln) but with two different phenotypes, PAN DADA2 and LGLL DADA2 (Table 5). The absence of functional ADA2 is often associated with chronic or recurrent systemic inflammation resulting in endothelium damage and severe/ lethal disease that may affect multiple organs of the body (Caorsi et al. 2016). Systemic polyarthritis nodosa (PAN), immunodeficiency, ischemic, or hemorrhagic strokes are common symptoms found in DADA2 patients (Zhou et al. 2014; Schepp et al. 2016; Schepp et al. 2017). However, a new phenotype characterized by T-LGL like leukemia was also recently found, further reiterating the important role of ADA2 in the regulation of both inflammation and cancer (Trotta et al. 2018). Our data shows that LAMs from PAN DADA2 patients secrete more TNF-α and IL-6 than the cells from the healthy donors. As it was demonstrated, adenosine bound to A1R and A3R at a low adenosine concentration increases the level of the secretion of cytokines from LAMs. In contrast, at a high concentration of adenosine, binding of adenosine to A2aR inhibits the release of cytokine from the cells. Therefore, the excessive expression of A1R and A3R over A2aR may result in an overproduction of TNF-α in PAN DADA2 patients.

It was shown that the majority of PAN DADA2 patients have an increased level of TNF-α in plasma (II, Figure 6C). However, the level of TNF-α in some of the patients was found to be nearly equal to the TNF-α level in the healthy donors. The increase of TNF-α in the plasma of the majority of PAN DADA2 patients could be explained by the release of cytokine from the activated monocytes and neutrophils due to the chronic inflammation. Moreover, the activated monocytes from the PAN DADA2

patients overproduce TNF-α due to an unbalanced regulation of the cell responses by extracellular adenosine. As we suggested, the cells from PAN DADA2 patients may express more A1R and A3R and less A2aR as compared to the healthy donors, resulting in excessive TNF-α production at a low adenosine concentration. Interestingly, LAMs from LGLL DADA2 patients express significantly less IL-6, MIP-1α, and MCP-1 compared to the PAN DADA2 patients and healthy donors. Contrary to that of PAN DADA2 patients, the cells from the LGLL DADA2 patients may express more A2aR over A1R and A3R because of a different compensatory mechanism as a result of ADA2 deficiency. The imbalance in the expression of ADORs in the cell surface of the immune cells from DADA2 patients may explain the diversity of DADA2 patients phenotypes and prove that ADORs and ADAs play an important role in the regulation of inflammation and cancer (Hashem, Kelly, et al. 2017).

The number of live monocytes in PAN DADA2 patients that have remained in the cell culture after 20 hours of incubation with the LPS was found to be much lower compared to LGLL DADA2 patients. Therefore, it could be suggested that an excessive production of TNF-α or other pro-apoptotic factors by PAN DADA2 LAMs may result in the death of the cells. This can explain different types of cytopenia observed in DADA2 patients (Zhou et al. 2014; Hashem, Kelly, et al. 2017). Interestingly, the level of MCP-1 secretion by PAN DADA2 LAMs was similar to LAMs from the healthy donors. This may suggest that the excessive TNF-α production by PAN DADA2 LAMs is due to the overexpression of A1R that do not regulate the level of MCP-1 secretion. On the contrary, at a high concentration of adenosine, the cells from PAN DADA2 patients have a decreased sensitivity to adenosine that could be explained by the down-regulation of the expression of A2aR. This low sensitivity to adenosine could be beneficial to keep the level of TNF-α high such that it will combat bacterial infections, but it may also result in an aggravated inflammation associated with the symptoms found in the DADA2 patients with PAN phenotype. On the contrary, in DADA2 patients with LGLL phenotype, the response to high concentrations of adenosine is similar to that of the healthy subjects. However, the concentration of TNF-α in plasma and the amount of cytokine released by LAMs from LGLL DADA2 patient is much lower compared to that of PAN DADA2 patient, suggesting that

extracellular adenosine inhibits the secretion of TNF-α in this patient. The difference between PAN and LGLL DADA2 patients could be due to the different compensatory mechanisms developed in the absence of ADA2, resulting in dysregulation of the expression of ADORs on the immune cells and adenosine signaling.

Following the initial burst in the release of TNF-α, chemokines such as MIP-1α and MIP-1β are also actively secreted by LAMs after a short time of activation with the LPS. This could result in recruitment and activation of the cells of the innate immune system, such as neutrophils, eosinophils, and basophils. The release of these chemokines is strictly controlled by extracellular adenosine. As we have shown in (II, Figure 1), neutrophils are the main target for ADA2, and thus, ADA2 might be crucial for the regulation of the activity of the neutrophils at the sites of inflammation. This may also explain why the absence of ADA2 in DADA2 patients is associated with an up-regulation of neutrophils-expressed genes leading to excessive inflammation associated with elevated levels of TNF-α and PAN (Belot et al. 2014). In contrast to the release of TNF-α, ADA2, MIP-1α, and MIP-1β, the release of IL-6 and MCP-1 from LAMs is delayed. This could indicate that MCP-1 dependent recruitment of the monocytes, dendritic cells, and memory T cells follows the bacterial clearance by leukocytes. Therefore, the recruitment of the cells of the acquired immune system by MCP-1 could be shut down by adenosine 3 hours post infection to limit the immune response by the cells of innate immune system. The delayed production of IL-6 and a low sensitivity of the cytokine released by LAMs to a high concentration of adenosine suggests that IL-6, at a later stage, may have the anti-inflammatory function and induce tissue remodeling, followed by inflammation (Hunter & Jones 2015). The resolution of inflammation may also be associated with an increased concentration of adenosine due to the decreased level of the secretion of ADA2 from the monocytes/macrophages, which could be downregulated by IFN-γ released from the activated cells (Zavialov, Gracia, et al. 2010).

The interplay between ADA2 and TNF-α may also explain high levels of ADA2 in patients with tuberculosis and HIV, where high levels of TNF-α

and low levels of adenosine could be required to induce pro-inflammatory responses to the viral and bacterial pathogens. Similarly, it can also explain why breast, gastric, colon cancers, and lymphomas are associated with high levels of ADA2. In accordance with our results, ADA2 might be required to control the release of TNF-α from the tumor-associated macrophages (TAMs). Preliminary results obtained by Halozyme Therapeutics show that pegylated ADA2 could be used as an anti-cancer drug to combat solid tumors by reducing adenosine concentration at the site of the tumor growth and recruiting T cells (Wang et al. 2016). It has also been shown that the gene therapy aimed to treat ADA1 deficiency does not result in insertional oncogenesis, as compared to other SCID therapies (Kohn & Gaspar 2017). The latter suggests that ADA1 expression in the cells may suppress the oncogenesis, making ADAs promising anti-cancer drugs.

Here, we present a new mechanism of monocyte regulation with adenosine and ADAs. We show that adenosine concentration and the timing of the exposure of monocytes to extracellular adenosine is crucial for the regulation of the inflammatory responses to the pathogens. Our data suggests that ADORs play an important role in DADA2 and the use of recombinant pegylated ADAs, A1R and A3R antagonists and A2aR agonist could be an alternative strategy for DADA2 treatment. The understanding of an interplay between ADAs and ADORs will provide valuable information on the development of the new therapies to combat cancers, treat autoimmune disorders, and manage inflammatory responses.

7. CONCLUSIONS

The publications in this thesis work highlight the critical role of human adenosine deaminases ADA1 and ADA2 in the regulation of cytokine secretion from cells. Furthermore, this study demonstrates that ADA2 binds neutrophils, monocytes, NK cells, B cells and regulatory T cells. It was suggested that different adenosine receptors are involved in the monocytes function at low and high concentrations of adenosine. It was shown that human adenosine deaminase hADA1 binds to CD26. However, the unknown receptor of hADA2 remains to be investigated in the future.

In this thesis, we have developed a new type of sandwich ELISA using hADA1 to measure the cytokine levels. With this method, identical results were obtained comparing to standard ELISA with HRP, suggesting that hADA1 could substitute HRP in commercial ELISA kits. The hADA1 or other ADA can be used in more ELISA assays in the near future.

Our results demonstrated that hADA1 and hADA2 bind to different cell subsets of immune system and modulate their response to extracellular adenosine by interplaying with adenosine receptors. Understanding of the interplay between adenosine deaminases and adenosine receptors will allow important insights on new approaches in the management of deficiency in ADA2 or ADA1.

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Turun yliopisto
University of Turku

INTERPLAY BETWEEN ADENOSINE RECEPTORS AND ADENOSINE DEAMINASES

Chengqian Liu

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