SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Functional analysis of adenosine receptor 2A interacting proteins

by Adrienn Skopál, Pharm. D.

Supervisor: Endre Kókai, PhD.

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2023

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By Adrienn Skopál, Pharm. D., Pharmacist

Supervisor: Endre Kókai, PhD.

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Defense Committee:**	László Csernoch, PhD, DSc
Reviewers:	Mónika Szentandrássyné Gönczi, PhD
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	János András Mótyán, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1 PM, 1st of December, 2023.

1. INTRODUCTION

My work has investigated the functional relationship of adenosine 2A receptor ($A_{2A}R$) interacting proteins in mouse macrophage cell vesicular transport processes. Macrophages play important roles in mammalian physiological processes, constantly monitoring and communicating with their environment. They play a key role in combating inflammatory processes by recognising, engulfing and eliminating pathogens. They activate T and B cells of the immune system and produce cytokines, chemokines, reactive oxygen and nitrogen derivatives and prostaglandins. $A_{2A}R$-dependent signalling in macrophages plays a key role in the regulation of inflammation. Cell surface $A_{2A}Rs$ regulate the production of cytokines and chemokines involved in inflammatory processes, as well as immune cell proliferation, differentiation and migration. The processes that regulate the cell surface expression of $A_{2A}Rs$ and their degradation in macrophages are not fully understood. Therefore, we aimed to gain further insight into the function of the receptor by identifying proteins that interact with $A_{2A}R$ and by functional characterization of the interaction. The molecular and functional interactions suggest that this interaction may play a key role in macrophage viability and inflammatory processes.

2. REVIEW OF LITERATURE

2. 1. The role of adenosine in the living organism

The physiological role of adenosine was discovered as early as 1927, when it was shown in heart muscle cells that the presence of adenosine can slow down heart rate. Then, in the 1980s, the effect of adenosine was demonstrated in the diagnosis and treatment of supraventricular tachycardia. Since then, research on the role of adenosine in the human body has been carried out not only in cardiology but also more widely. Adenosine is an endogenous purine nucleoside with low concentrations in the interstitial space in its basal state and is not found in synaptic vesicles. However, under hypoxia, ischemia, inflammation or tissue damage, intracellular ATP is degraded and adenosine levels increase. Adenosine is produced by ATP dephosphorylation. In the extracellular space, adenosine deaminase controls its concentration, catalysing the conversion of adenosine to inosine. When a critical intracellular concentration is reached, it exits to the extracellular space via nucleoside transporters. The transport works in two directions, via a specific nucleoside carrier. Adenosine exiting into the extracellular space activates adenosine receptors on the surface of the target cells and initiates a signalling pathway to restore homeostasis. It protects the cell from excessive inflammation and signals potential damage to surrounding tissues in an autocrine and paracrine manner. Extracellular adenosine is also produced by the heart, lungs, intestines, brain and immune cells in the human body. Adenosine can also bind to the cell surface receptor and directly affect the function of the cells that produce it or neighbouring cells.

2. 2. The structure of adenosine receptors

$A_{2A}R$ belongs to the largest family of integrated membrane proteins, the G-protein coupled receptor (GPCR) family. G-protein coupled 7-transmembrane receptor. The N-terminal domain is located extracellularly and the C-terminal domain is located in the cytoplasm. The helices are composed of 25-30 amino acids, linked by a total of six loops. Several glycosylation sites are present on the N-terminal, while the C-terminal part can be phosphorylated or palmitoylated. It also plays a role in receptor desensitization and internalization mechanisms. The primary protein structure of human $A_{2A}R$ differs from that of other members of the receptor family. Unlike the other three adenosine receptors, it has a flexible intracellular domain of 122 amino acids, which allows it to function not only as a receptor but also to participate in protein-protein interactions. Since the palmitoylation site is absent in $A_{2A}R$, the C-terminal domain is free to move in the intracellular space. Furthermore, structural studies of the receptor (X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy studies) show that the C-terminal region of the receptor between amino acids 307-412 contains disordered structural units, making it a potential target for functional studies and identification of possible interacting proteins.

2. 3. Distribution of A_{2A}Rs in mammalian organisms

A_{2A}Rs are present in many tissues of the mammalian body and have a very diverse distribution. Their effects include the central nervous system, heart, lungs, kidneys and the immune system. The amount and activation or inhibition of $A_{2A}R$ is also involved in the regulation of other signalling pathways. $A_{2A}R$ plays a role in synaptic plasticity, promotes glutamate release and

enhances NMDA receptor actions. $A_{2A}R$ inhibits the glutamate uptake transporter GLT-1 and stimulates glutamate release in astrocytes. Adenosine has complex effects on the heart involving A1 and A2 receptors. $A_{2A}R$ exerts a presynaptic anticholinergic effect, which is manifested by inhibition of AV and sinus node activity. The antiarrhythmic effect is used in supraventricular tachycardia. $A_{2A}Rs$ are located in the smooth muscle and endothelium of the cardiac vascular wall and are involved in the regulation of the coronary vasculature. The cardioprotective effect of $A_{2A}Rs$ is due to their potent anti-inflammatory activity. $A_{2A}Rs$ are most abundant on macrophage cells. In inflammatory processes, adenosine regulates cytokine production by monocytes/macrophages, as macrophage-derived mediators are important participants in inflammatory responses. Adenosine reduces the production of inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 and 12 (IL-6, IL-12) and increases the release of anti-inflammatory cytokines such as interleukin-10 (IL-10), which is necessary to prevent excessive tissue damage.

2. 4. The $A_{2A}R$ mediated signalling mechanism

$A_{2A}R$ has the highest affinity for adenosine. Receptor activation can occur through three signalling pathways. The classical pathway is based on the direct regulation of cAMP. The pathway involving β-arrestins is known to play an important role in receptor endocytosis and signal transduction. They recruit proteins and different signalling molecules involved in endocytosis to receptors and link G-protein coupled receptors to different signalling pathways. During agonist-stimulated receptor endocytosis, β-arrestins function as endocytic adaptors, also known as scaffold proteins, and interact with proteins. Signal transduction may also occur through a third mechanism, where $A_{2A}R$ forms heterodimers with other proteins. It is now known that G-protein coupled receptors can also activate signalling pathways through mechanisms independent of G-protein. G protein-coupled receptors are the largest family of membrane proteins in the human genome and mediate various biological processes through signal transduction across the cell membrane. Upon activation, $A_{2A}R$ is coupled to the stimulating G-protein of the Gsαβγ heterotrimer, resulting in nucleotide exchange and dissociation of α and βγ subunits.

2. 5. $A_{2A}R$ desensitisation

Continued or repeated application of a receptor agonist results in a receptor-mediated response in the plateau phase. In receptor kinetics, this is called desensitization. Mechanisms underlying rapid desensitization involve phosphorylation of the receptor by members of G protein-coupled receptor kinases (GRKs) and, as a result, binding to β-arrestin molecules. This aids desensitization by uncoupling the receptor from its G-protein, which alters the function of the receptor. Following desensitization, GPCRs are internalized in an arrestin-dependent manner through clathrin-coated vesicles, leading to eventual intracellular dephosphorylation of the receptor. It can then be reinserted into the cell membrane in order to re-enter the resensitized state. Prolonged agonist activation shuttles the internalized receptor to a lysosomal vesicle, after which the receptor activity is reduced. $A_{2A}R$ rapidly decreases activity, usually over an hour. Understanding the process and regulation of receptor desensitisation is important for drug development. $A_{2A}R$ desensitisation is mediated by several agonist-dependent processes that are separated in time. Short-duration agonist application triggers rapid desensitization of $A_{2A}R$-

stimulated adenylate cyclase activity, which entails a decrease in the extent of receptor G_s-protein coupling and agonist-stimulated phosphorylation of $A_{2A}R$. However, prolonged agonist treatment results in a decrease in total receptor abundance. The structural changes that occur during this process take place at the C-terminal.

2. 6. $A_{2A}R$ interacting partners

$A_{2A}R$ has a long C-terminal domain that can be phosphorylated or palmitoylated. It also plays a role in receptor desensitization and internalization mechanisms. Since the palmitoylation site of $A_{2A}R$ lacks the C-terminal domain, it is free to move in the intracellular space. As mentioned earlier, the C-terminal region of the receptor between amino acids 307-412 contains disordered structural units, making it a potential target for the identification of interacting proteins. The identification of interacting proteins is important because, in addition to their role in signal transduction, it may provide insights into additional functions. The following proteins have already been identified as $A_{2A}R$ interacting proteins: synapse associated protein of 102 kDa (SAP102), α-actinin, calmodulin, neuronal calcium binding protein 2 (Necab2), translin-associated protein-X (TRAX), ARNO. In addition, Gas-2 like 2 (G2L2), HSP90, D2R, USP4 and NCS-1 have also been identified as $A_{2A}R$ partners. The interactors identified by our group are CtsD protease and NPC1. A further 22 interactors were identified as a result of computer analysis using the University of Reading bioinformatics web server (http://www.reading.ac.uk/bioinf/PINOT/PINOT_form.html). The analyses were based on yeast two-hybrid methods, co-immunoprecipitation and fluorescence methods.

2. 7. The role of $A_{2A}R$ in macrophages

Macrophages have many functions. These include: phagocytosis of pathogens, infected and dead cells and cell debris, antigen presentation by displaying processed antigens, and production of different types of cytokines (IL-1, IL-6, TNF-α). They also play an important role in the progression of inflammatory diseases and in tumours. Macrophages, as part of the innate immune system, are essential to effectively fight infections, remove debris and dead cells. They also promote tissue repair and wound healing. Macrophages can enhance antimicrobial defences or blunt harmful inflammation. The local cytokine environment can control the polarisation of macrophages. Two distinct macrophage subpopulations exist. M1 is the classically activated or pro-inflammatory subset and M2 is the alternatively activated or anti-inflammatory macrophage subset. M1 macrophages regulate tissue damage and responses to inflammation. M2 macrophages, however, are key in the resolution of inflammation. M2 macrophages are responsible for clearing dead cells, cell debris. Following phagocytosis, macrophages kill most microorganisms. Macrophages form a heterogeneous cell population and show functional plasticity in response to dynamic microenvironmental cues. M1 or classical activation of macrophages is induced by Toll-like receptor (TLR) agonists. M1 macrophages possess antiproliferative and cytotoxic activity, which is carried out by reactive oxygen and nitrogen radicals such as hydrogen peroxide, superoxide, nitric oxide (NO), as well as peroxynitrite and anti-inflammatory cytokines. The regulatory effects of adenosine on M1 macrophages are mainly mediated by $A_{2A}Rs$. By combining $A_{2A}R$-deficient mice with pharmacological agents, adenosine has been shown to inhibit the release of TNF-α, IL-6 and IL-12, and to enhance the production of IL-10 by lipopolysaccharide (LPS)- or bacteria-activated macrophages, mostly via $A_{2A}Rs$. It reduces the levels of pro-inflammatory cytokines

(IL-1β, IL-6, TNF-α) and enhances the production of anti-inflammatory cytokines (IL-4, IL-10).

2. 8. Macrophages as specialised enforcers of the immune system

Macrophages are a major type of phagocytic cell of the innate immune system. Their important function is to recognise, engulf, remove and destroy bacteria and viruses that can be dangerous to the body. Pattern recognition receptors expressed on macrophages are involved in the pathogen recognition process and ensure that foreign substances can be distinguished. Following recognition of the bacteria, the target cell encapsulates the material in the phagosome and fused with the lysosome to form a phagolysosome. This compartment contains an acidic pH environment that is not favourable for the bacterial cell. These processes eventually lead to the death of the bacteria and allow the processing of peptide antigens, which later appear on MHC class II molecules. This process leads to the activation of helper T cells and stimulates the acquired immune response. Therefore, macrophages, as antigen-presenting cells, form a bridge between non-specific (innate) and specific (adaptive) immunity. Macrophages, as efficient phagocytes and antigen-presenting cells, are able to perform their functions due to the presence of cathepsins, which play a role in the regulation of innate (PRR signalling, pathogen killing, apoptosis) and adaptive (antigen processing and presentation) immune responses. Cathepsins belong structurally to a heterogeneous group of proteases, which were first described as intracellular enzymes of protein-degrading activity in mildly acidic environments. Currently, the name cathepsin refers to two serine proteases (cathepsins A and G), two aspartyl proteases (cathepsins D and E) and eleven lysosomal cysteine proteases (cathepsins B, C, F, H, K, L, O, S, V, X and W). In most cases, despite their lysosomal localization, under certain conditions cathepsin can be released from lysosomes into the cytoplasm, where they perform proapoptotic functions by activating caspases and promoting the release of mitochondrial proapoptotic molecules. In macrophages, cathepsin D expression is highly differentiation-dependent and increases as monocytes mature into macrophages. Cathepsin proteases can be differentiated during the differentiation of macrophages into two main functional phenotypic forms: the classical activation phenotype M1 and the alternative activation phenotype M2. The direction of polarization of M1 and M2 macrophages depends on the nature of the immune response they elicit. The expression and activity of cathepsin B, L and S mRNAs, which regulate macrophage function, are significantly increased in M2 macrophages. From this, we conclude that cathepsins play a key role as regulators of macrophage differentiation and function and may influence disease outcome.

2. 9. The role of lysosomes in macrophages

Lysosomes are mostly found in the cytoplasm of cells. Their interiors are acidic, mostly composed of hydrolytic enzymes. It follows that lysosomes are key players in intracellular degradation processes such as intracellular digestion and autophagy. Substances taken up by endocytosis are transported into membrane-confined vesicles. During autophagosome digestion, lysosomes fuse with vesicles to allow enzymes to access their contents. The main function of the lysosome is to break down macromolecules transported by endocytosis or autophagy. If it is unable to degrade these macromolecules or transport their degradation products to the cytosol, the material accumulates in the lysosomes and causes abnormal lysosomal dysfunction. There are currently about fifty known lysosomal storage disorders, many of which are characterised by neurodegeneration, severe organ failure or premature death. Lysosomal abnormalities may be involved in the development of cancer, atherosclerosis, and

may also be associated with adverse outcomes in Alzheimer's and Huntington's diseases. Cells undergo a number of intracellular transport processes that deliver newly synthesised lysosomal membranes or soluble proteins to the intracellular space. Early endosomes constitute the first transport stage after uptake for most pathways, although this is most closely related to the clathrin-mediated pathway. The pH of this compartment is ~6.5, which favours ligand-receptor dissociation. Early endosomal antigen 1 (EEA1) is a widely used early endosomal marker, and its role has also been described in "recycling" pathways. EEA1 is an essential protein for endosome docking and fusion mechanisms. EEA1 is related to small GTP-binding proteins of the Rab family. Once the endosomal membrane associates with EEA1, it triggers endosomes for subsequent fusion. Most vesicle contents are transported to late endosomes and then to lysosomes. Both compartments contain acidic and degrading enzymes. In these compartments, the pH is reduced to ~ 5.5 and ~ 4.5, respectively, allowing catalytic activity of these degradative enzymes. We have also utilized lysosome-associated membrane protein-2 (LAMP2) as a lysosomal membrane protein in our experiments as a lysosomal marker. The molecular mechanisms and regulation of cellular processes involving vesicular transport are highly complex. For this reason, vesicular transport is implicated in many diseases. For example, neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, autoimmune diseases and cancers have been associated with alterations in endocytic transport, lysosomal processing, autophagy. In most cases, these dysfunctions are associated with increased accumulation of undigested substances. This typically leads to nutrient deficiencies, altered signal transduction or metabolism, apoptosis and inflammatory processes, which ultimately contribute to disease progression. Lysosomes contain acidic hydrolases that perform important degradative functions, such as the breakdown of various substrates.

2. 10. Characterization of cathepsin D protease

Our work qroup performed yeast two-hybrid screening with the A_{2A}R C-terminal domain using the mouse intraperitoneal macrophage (IPMΦ) cDNA library and identified cathepsin D (CtsD) protease as an interacting protein in 5 independent clones. The putative A_{2A}R-CtsD interaction was confirmed by several independent methods (immunoprecipitation, pull-down method). We confirmed the colocalization of the C-terminal domain of A_{2A}R and CtsD protease in RAW 264.7 macrophage and IPMΦ cells. CtsD is a lysosomal aspartyl endopeptidase. It is generated in the rough endoplasmic reticulum as procathepsin D. After removal of the signal peptide, it is transported to the lysosome via an M6P receptor-dependent or independent pathway. When it enters the acidic medium endosomal and lysosomal compartments, cleavage of the N-terminal propeptide of 44 amino acids results in the single-stranded, intermediate, active enzyme form with a molecular mass of 48 kDa. Further cleavage yields the mature form of CtsD with globular structure. The mature form is composed of light (14 kDa) and heavy chains (34 kDa), which are non-covalently linked. The maturation of CtsD is due to the autocatalytic activity of cysteine proteases and CtsD. Under normal physiological conditions, proCtsD is located in the lysosome. CtsD is the focus of research in several diseases, such as Alzheimer's disease and atherosclerosis. Protease functions of CtsD include metabolic degradation of intracellular proteins, activation and degradation of polypeptide hormones, and activation of enzymatic precursors. In addition to these functions, it also regulates the process of programmed cell death. CtsD degrades partially degraded or abnormally structured proteins. Proteolytic cleavage leads to the inactivation of these proteins. Activators of the CtsD enzyme are e.g. glycine ethyl ester, phospholipids, polyphosphates. Inhibitors are α-2-microglobulin and pepstatins. Damage to the

cell-forming membrane of the lysosome releases CtsD into the cytoplasm where it can interact with other proteins. This process is known as lysosomal membrane permeabilisation (LMP) and triggers a signalling cascade that can lead to apoptosis. The LMP mechanism can release catepins and protons from the lysosome simultaneously. The proteolysis of CtsD is pH-dependent. Cathepsins are very active at acidic pH, but become inactive at the neutral pH of cytoplasm. Nevertheless, they can stabilize when a substrate is bound to them. They are able to retain their proteolytic activity for minutes after release into the cytoplasm, allowing transient activity. The stability of the lysosome and the occurrence of programmed cell death are influenced by the cholesterol content of the lysosome, damage to the membrane component, minor modifications to the membrane structure and changes in membrane fluidity. The following proteins have previously been identified as substrates of human CtsD in the cytoplasm and their role in the induction of apoptosis has been demonstrated: sphingosine kinase-1, X-linked apoptosis inhibitory protein (XIAP), B-cell lymphoma-2 (Bcl-2), caspases. In macrophage cells, CtsD is most abundantly expressed. Cathepsin E is another member of the enzyme family, but this protease is expressed at low levels. In view of this, our experiments have used pepstatin A penetratin as an aspartyl protease inhibitor to specifically inhibit CtsD. It has been previously observed that CtsD aspartyl protease plays a role in the clathrin-mediated internalization and degradation of the TNF-α receptor, a mechanism by which the receptor is spliced into a so-called multivesicular body.

2. 11. Characterisation of the Niemann-Pick C-1 protein

NPC1 is found in all tissues, but is expressed to the greatest extent in the liver. It plays an important role in the regulation of intracellular cholesterol homeostasis and is therefore a widely studied protein. The Niemann-Pick C-1 (NPC1) protein is composed of 1278 amino acids. It contains 13 transmembrane helices. NPC1 is synthesized in the endoplasmic reticulum and then continues to mature in the Golgi and is eventually transported to late endosomes/lysosomes. Mutations in the NPC1 protein can lead to a severe disorder, called Niemann - Pick type C disease, which leads to accumulation of cholesterol and glycosphingolipids in lysosomes, particularly in the liver, spleen and brain, and eventually to premature death. The analysis of the NPC1 protein is challenging for a number of reasons, including the fact that the cholesterol substrate is hydrophobic and partitioned in membranes containing NPC1. The function of NPC1 proteins is to transport cholesterol within lysosomes and between late endosomes/lysosomes and other cellular components such as the endoplasmic reticulum and plasma membrane. Bernardo and colleagues have studied conditions in the brain in which NPC1 receptors fail to function normally, the main consequences of which are defects in cerebellar neurodegeneration, neuroinflammation and myelination. The elevation of adenosine levels and stimulation of $A_{2A}R$ may represent a therapeutic perspective in NPC diseases, considering their beneficial effects on dysmyelination. Adenosine also plays a key role in modulating the myelination process, as evidenced by its ability to influence oligodendroglial cell migration, proliferation and maturation. Coppi et al. characterized the function of $A_{2A}R$ in oligodendrocyte progenitor cells and showed that *in vitro* stimulation of the receptors with a selective $A_{2A}R$ agonist (CGS21680) delayed their differentiation into oligodendrocytes without affecting cell viability.

2. 12. The tools of cell membrane repair

Eukaryotic cells are separated from the extracellular environment by a cell membrane consisting of a phospholipid bilayer, which contains proteins that regulate the entry and exit of molecules into and out of the cells. Loss of function can lead to impaired cell homeostasis and

cell death. Most cells are exposed to mechanical or chemical stresses that can affect the integrity of the cell membrane. Small membrane damage can be repaired by the cell, but it interacts with the cytoskeleton and extracellular matrix through the integral membrane proteins in the cell membrane. These interactions cause mechanical tension on the cell membrane, which when damaged forms pores in the membrane. Membrane damage allows intracellular components to leave the cell. From the other direction, Ca^{2+} and other components of the extracellular milieu enter the cell and can reach toxic levels. So, if these abnormalities are not addressed quickly, they can lead to cell death. To address this, cells have developed active methods to repair cell membrane disorders. Plasma membrane damage and repair occurs *in vivo*. Membrane repair may involve fusion of vesicles at the site of injury or into the cell membrane. Endocytotic mechanisms may be involved in repair of larger membrane defects, while smaller 100 nm breaks are repaired by reclosure and exocytosis. Repair by entrapment involves finding the membrane damage and excising the damaged membrane. Endocytosis contributes to membrane repair by internalising the damaged membrane.

2. 13. The role of $A_{2A}R$ in human diseases

Adenosine receptors play an important role in immune function and defence against pathogens. It follows that $A_{2A}Rs$ regulate a number of anti-inflammatory signalling pathways in the periphery. They play a role in rheumatoid arthritis (RA), acute lung inflammation, chronic obstructive pulmonary disease (COPD), asthma, irritable bowel syndrome, wound healing processes. Methotrexate, used in rheumatoid arthritis, increases adenosine production. $A_{2A}R$ activation delays the progression of arthritis by preventing oxidative and nitrosative damage, vlamin reduces TNF-α, IL-1β and IL-6 levels. Methotrexate reduces bone breakdown in RA patients and regulates anti-inflammatory effects via $A_{2A}Rs$. $A_{2A}Rs$ in the brain are mainly located in the striatum, where colocalisation with D_2R has been demonstrated. Since 2006, istradefillin as an $A_{2A}R$ antagonist in combination with levodopa has been successfully used in Japan for the treatment of Parkinson's disease. It has also been observed to be able to form heterodimers with D_2R. $A_{2A}R$ antagonists given at high concentrations reduce the affinity and function of D_2R agonists. In both transgenic young and old mice, $A_{2A}R$ deficiency in astrocytes was shown to increase long-term memory. By inhibiting glutamate uptake, $A_{2A}R$ can cause synaptic dysfunction and excitotoxic cell death, which underlies many neurodegenerative diseases. As an $A_{2A}R$ antagonist, caffeine has a number of prophylactic effects that have been observed in Parkinson's and Alzheimer's diseases, attention deficit hyperactivity disorder, brain injury, depression and stroke. In $A_{2A}R$-deficient mouse models, $A_{2A}R$ has been observed to play a key role in the regulation of adenosine-based motor activity. $A_{2A}R$ antagonists (e.g., caffeine) enhance the motor effect on post-synaptic neurons.

3. AIMS

I. Our objective was first to demonstrate the functional interaction between $A_{2A}R$ and CtsD protease. We first sought to characterize how the interaction between the two proteins is affected by the activation of $A_{2A}R$ and the inhibition of CtsD protease. Second, we wanted to ascertain whether $A_{2A}R$ could be a substrate for CtsD.

- We first aimed to investigate the colocalization of the $A_{2A}R$ C-terminal domain and CtsD protease in IPMΦ cells. To achieve this, $A_{2A}R$ and CtsD specific labelling was used and colocalisation was detected by confocal microscopy.
- We planned to investigate the putative interaction between the C-terminal domain of $A_{2A}R$ and CtsD protease in RAW 264.7 macrophage cells by immunoprecipitation and western blotting.
- To identify potential CtsD cleavage sites, we planned to use three different *in silico* computer programs to analyse the primary amino acid sequence of the C-terminal of $A_{2A}R$.
- We planned to investigate how inhibition of the CtsD enzyme affects the cell surface expression of $A_{2A}R$. For this purpose, we planned to use $A_{2A}R$-specific labelling, to detect the quantitative changes in $A_{2A}R$ by confocal microscopy and to evaluate them with the associated software.
- In further functional studies, we planned to test the effect of CtsD enzyme inhibition on $A_{2A}R$-mediated cytokine production.

After demonstrating the functional interaction between $A_{2A}R$ and CtsD protease, we aimed to identify additional $A_{2A}R$ interacting partners.

II. We further investigated how $A_{2A}R$ activation affects the transport of early and late endosomes and lysosomes in macrophage cells.

- To follow this up, we first wanted to investigate changes in LAMP2 protein localization using laser scanning cytometry (LSC). We then planned to monitor LAMP2 and early endosomal antigen 1 (EEA1) protein expression using a microscopy method that combines imaging with confocal microscopy and quantitative data analysis.
- Our objectives were to investigate the effect of $A_{2A}R$ activation on the cell surface expression of LAMP2 and EEA1 protein in LPS-activated macrophages.
- We investigated how $A_{2A}R$ activation affects NPC1 mRNA and protein expression.
- In order to verify the fucional interaction of NPC1 protein and $A_{2A}R$, we planned to detect changes in NPC1 protein localization following receptor-specific activation.

4. MATERIALS AND METHODS

4. 1. Cell culturing

RAW 264.7 mouse macrophages, primary mouse IPMΦ and human HEK-293 cells, which persistently express human $A_{2A}R$ labeled with Flag and SNAP peptide at its N-terminal (e.g., HEK-293-Flag-$A_{2A}R^{SNAP}$- cells /provided by Francesco Ciruela, Department of Pathology and Experimental Therapeutics, University of Barcelona, Spain/) were cultured in DMEM with high glucose content. Cultivation was performed in T75 cell culture flasks, Falcon Multiwell cell culture dishes, 96-well cell culture dishes and 8-well cell culture dishes. The cell culture fluid contained 10% fetal bovine serum (FBS), 50 IU/ml penicillin, 50 µg/ml streptomycin and 2 mM L-Glutamine (hereafter complete DMEM). Cells were cultured in a humidified (80%) incubator at constant temperature (37°C) and CO2 concentration (5 v/v%).

4. 2. Animal models

Experiments were performed with 8-12 weeks old male C57BL6/J wild-type mice (The Jackson Laboratory, Farmington, CT, USA). All mice were kept under specific, pathogen-free conditions in the Laboratory Animal Core Facility and all animal experiments were performed in accordance with the Declaration of Helsinki and approved by the University of Debrecen Working Animal Experiment Committee (DEMÁB 2016/15).

4. 3. Isolation of mouse peritoneal macrophages

C57BL6/J wild-type male mice were injected intraperitoneally with 2 ml of sterile Brewer's thioglycolate (TG) solution (4% m/v). Four days later, cervical dislocation was performed on the mice and peritoneal macrophage cells (IPMΦ) were washed from the abdominal cavity of the mice with 10 ml DMEM. Cells were collected by centrifugation (10 min, 300 g, 4°C) and resuspended in 2 ml red blood cell lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA). Incubated on ice for a few minutes, then the volume was made up to 10 ml with DMEM solution. Macrophages were centrifuged again for 10 min at 300 g at 4°C and resuspended in complete DMEM. Cells were divided into Falcon Multiwell cell culture dishes, 96-well cell culture dishes, and 8-well cell culture dishes. The cell culture dishes were then incubated at 37°C in a humidified incubator for five hours to allow the cells to adhere to the plate surface. Non-adherent cells were removed by washing with serum-free DMEM and the cells were resuspended in complete DMEM. Eighteen hours after distribution into tissue culture dishes, macrophages were treated with the various pharmacological compounds.

4. 4. Pharmacological treatment of macrophages

Pharmacologically active substances were dissolved in water. Pepstatin A penetratin 3 or 9 µM, CGS21680 100 nM and LPS 100 ng/ml were used as final concentrations. Macrophages were pre-incubated for 20 min in the presence of Pepstatin A penetratin or CGS21680 before activation of macrophages with LPS for 4 h. After treatment, total RNA was isolated with TRI reagent, reverse transcribed and used as template for RT-qPCR to analyse relative expression changes. After protein isolation, protein concentration was determined from the cell lysate and used for IP and pull-down methods. The immunostained samples were imaged by confocal microscopy and analyzed using appropriate software. Cytokine levels were measured from the medium and aspartyl protease activity from the cell lysate.

4. 5. RNA isolation, reverse transcription and quantitative real-time PCR

RNA was purified with TRI reagent according to the manufacturer's instructions. 2 µg cDNA was added to 8 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X). RT-qPCR was performed under the following conditions: at 95°C for 10 min, followed by 50 cycles of 94°C for 10 s, 60°C for 10 s and 72°C for 10 s. Reactions were performed in triplicates and data were normalized to the measured hand values of the housekeeping genes, beta-2-microglobulin (β2M) and glycerylaldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative real-time PCR was performed using a Roche LightCycler 480 II (Roche, Basel, Switzerland).

4. 6. Protein isolation

After treatment with different reagents, RAW 264.7 and IPMΦ cells were washed with phosphate buffered saline (PBS). Then, ice-cold modified radioimmunoprecipitation essay buffer (RIPA) (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1x protease inhibitor cocktail and 1 mM phenylmethyl methylsulfonyl fluoride (PMSF) were added. The lysate was collected by centrifugation (10 min, 10000 g, 4°C) and the supernatants were collected. Protein concentrations were determined using a Direct Detect spectrophotometer (Merck-Millipore, Darmstadt, Germany) and a Bicinchoninic acid (BCA) Protein Assay Kit using a Tecan Spark instrument (Tecan, Mannedorf, Switzerland). The standard protein calibration curve was prepared by plotting the OD value at 562 nm corrected by the blank sample value for each BSA standard. The standard curve was then used to determine the protein concentration of each sample.

4. 7. Immunoblot

Protein lysates were denatured for 10 min at 95°C in the presence of SDS sample buffer, and samples containing 10 µg proteins were separated by SDS-PAGE (4-12% gradient or 10% gel, 100 V, 60 min). Separated proteins were transferred to nitrocellulose membranes (400 mA for 90 min). After blocking with 3% BSA in 1x Tris buffered saline Tween 20 (TBST) buffer, membranes were incubated with cMyc-, GST-, CtsD-, or Flag-specific antibodies overnight at 4°C. The next day, membranes were incubated with anti-goat-rat peroxidase (HRP), anti-mouse HRP and β-actin antibodies for 1 h at room temperature. Bands were detected using ECL Western Blotting Detection reagent. The signal was detected using the Chemidoc Touch Imaging System (Bio-Rad Laboratories, Hercules, California, USA) and the bands were evaluated by densitometry using Image Lab (Bio-Rad Laboratories, Hercules, California, USA) and Image J software.

4. 8. Enzyme-linked immunosorbent assay (ELISA)

Interleukin-6 (IL-6) and interleukin-10 (IL-10) cytokine concentrations were determined from cell culture media using commercially available ELISA DuoSet kits according to the manufacturer's instructions.

4. 9. *in silico* CtsD cleavage site analysis by prediction method

The primary structure of $A_{2A}R$ (mouse, UniProt ID: Q60613) was analyzed by two different in silico CtsD cleavage site prediction methods. The arrows indicate potential CtsD cleavage sites and the color codes refer to the prediction methods. Red: Prosper Protease Specificity Prediction Server PROSPER: Protease substrate specificity webserver (monash.edu.au); Green: Site Prediction https://www.dmbr.ugent.be/prx/bioit2-public/SitePrediction/.

4. 10. Preparation of pCMV - cMyc-$A_{2A}R^{284-410}$ vector

The C-terminal sequence encoding amino acids 284-410 of $A_{2A}R$ was inserted into a pCMV mammalian expression vector (Clontech) by PCR to generate restriction enzyme (Nde I, Xho I) specific recognition sites at both ends of the sequence. Following restriction cleavage of the DNA fragments, the C-terminal sequence encoding amino acid $A_{2A}R^{284-410}$ was inserted into the Nde I and Xho I restriction cleavage sites of the pCMV vector. The C-terminal part of $A_{2A}R$ was expressed as a fusion protein with a cMyc epitope tag in HEK-293 mammalian cells.

4. 11. Immunoprecipitation of cMyc-$A_{2A}R^{284-410}$, full-length $A_{2A}R$, CtsD and NPC1 proteins

RAW 264.7, HEK-293 or HEK-293-Flag-$A_{2A}R^{SNAP}$ cell lysates containing 500 µg of total protein were supplemented with PBS to 500 µl in RIPA buffer and 100 mg/ml of protease inhibitor cocktail (PIC) and 100 mg/ml of PMSF were added. To the complex, 10 µg anti-CtsD antibody or control IgG of the appropriate isotype; 1 µg anti-cMyc antibody and control IgG1 of the appropriate isotype or 8,5 µg anti-$A_{2A}R$ antibody were added and incubated overnight at 4°C with rotation. The next day, equilibrated Protein G-specific Dynabead magnetic bead suspension was added to the lysates and incubated for 1 hour at 4°C with rotation. Beads were washed three times with RIPA-PBS buffer (1:2 ratio) supplemented with 100 mg/ml PIC and 100 mg/ml PMSF. Bound proteins were eluted from the immunocomplexes with 48 µl of ice-cold RIPA buffer, followed by the addition of 12 µl of 5 x SDS sample buffer. The eluted samples were denatured at 95°C for 10 min, separated by SDS-PAGE (10 % gel, 100Vm 60 min) and then assayed by wesrten blot using anti-cMyc antibody, anti-CtsD and anti-NPC1 antibodies, respectively. The immunocomplex formed in RAW 264.7 cell lysate expressing cMyc-$A_{2A}R^{284-410}$ with cMyc-specific antibody was also examined by MS analysis.

4. 12. Treatment of HEK-293-Flag-$A_{2A}R^{SNAP}$ cell lysates and mouse GST-$A_{2A}R^{284-410}$ with CtsD protease, GST recombinant proteins

After washing the HEK-293-Flag-$A_{2A}R^{SNAP}$ cells once with ice-cold PBS, the cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl pH=7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF). Cells were then harvested by centrifugation (10 min, 10000 g, 4°C). Supernatants were collected and protein concentrations were determined using a Direct Detect spectrophotometer (Merck-Millipore, Darmstadt, Germany). The cell lysates (50 µg protein) were incubated with recombinant mouse CtsD at a concentration of 1.5 µg/ml for 1 and 2 h at 37°C. Mouse GST-$A_{2A}R^{284-410}$ and GST recombinant proteins (200 ng) were incubated with recombinant mouse CtsD at a concentration of 10 µg/ml for 5, 10 and 30 min at 37°C. Prior to the procedure, CtsD was preincubated for 10 min at room temperature. The mixture was supplemented with Assay buffer (0.1 M sodium acetate, 0.2 M NaCl, pH=3.5). After incubation with mammalian cell lysate and CtsD, 10 times the sample volume was added of cold (-20°C) acetone, and the samples were vortexed and incubated for 30 min at -20°C. The mixture was centrifuged at 10000 g for 10 min at 4°C. The supernatant was removed and the pellets were taken up in 72 µl of RIPA buffer and supplemented with 18 µl of 5 X SDS sample buffer. After incubation of mouse GST-$A_{2A}R^{284-410}$, GST proteins and CtsD in 48 µl Assay Buffer, enzyme reactions were stopped with 12 µl 5 X SDS sample buffer. All samples were then denatured at 95°C for 10 min and separated by SDS-PAGE (10 % gel, 100V, 60 min). While HEK-293-Flag-$A_{2A}R^{SNAP}$ cell samples were analyzed by WB assay using anti-Flag

antibody, mouse GST-$A_{2A}R^{284-410}$ and GST protein samples were visualized by silver staining in the gel.

4. 13. Pull-down study of the interaction between mouse GST-$A_{2A}R^{284-410}$ and endogenous CtsD

The recombinant mouse GST and GST-$A_{2A}R^{284-410}$ proteins were expressed in *E. coli* strain BLR and bound from the bacterial cell supernatants using the GST-specific MagneGST protein purification system (V8600, Promega, Madison, WI, USA). Cells from 10 ml O/N bacterial cultures were plated in 1 ml lysis buffer (10 mM Tris buffer pH: 8, 150 mM NaCl, 1 mM EDTA) supplemented with 10 µl PIC (100 mg/ml), 10 µl PMSF (100 mg/ml) and 10 µl lysozyme (100 µg/ml), resuspended and vortexed vigorously. The mixtures were incubated on ice for 15 minutes, then supplemented with 50 µl dithiothreitol (DTT) (100 mM) and 75 µl sarcosil (20 v/v%), vortexed again and sonicated three times for 30 seconds with 30 second breaks (50 cycles, 5 Micro Tip Limit) (Branson Sonifer 250). The supernatant fraction of the lysates was separated by centrifugation (10 000 g for 5 min at 4 °C), to which 11,5 µl of Triton-X solution (10 v/v%) and 30 µl of equilibrated magnetic beads were added. The mixtures were incubated overnight at 4°C with continuous rotation, and the next day the GST-specific complexes were washed twice with 250 µl wash buffer and twice with 250 µl PBS. Then, 1/5 of the washed mouse GST and GST-$A_{2A}R^{284-410}$ bound beads were eluted in 33 µl elution buffer (50 mM reduced glutathione, 50 mM Tris buffer pH 8.1) for 15 min at 4°C with continuous rotation. The mixture was denatured in the presence of SDS sample buffer (10 min, 95°C) and separated by SDS-PAGE (10 % gel, 100 V, 60 min). Separated proteins were visualized by Coomassie Brillant Blue G250 staining and the signal was detected using a Fluorchem FC2 Imaging System (Alpha Innotech, #22424, NIBE Industriar AB, Markaryd, Sweden). For CtsD pull-down (PD) experiments, 145 µg of total protein IPMΦ cell lysate was incubated with 2/5 of washed mouse GST or GST-$A_{2A}R^{284-410}$ bound beads in 500 µl final volume of RIPA buffer supplemented with 1x PIC for 2 h at 4°C. PD complexes were then washed once with 600 µl RIPA buffer and twice with PBS supplemented with 1x PIC. The bound proteins were eluted from the PD complexes with 100 µl ice-cold RIPA buffer containing 33 µl 5x SDS sample buffer. The eluted samples were denatured at 95°C for 10 min, separated by SDS-PAGE (4-12% gradient gel, 100V, 60 min), analysed by WB with CtsD-specific antibody and sent for mass spectrometric analysis.

4. 14. Immunostaining of $A_{2A}R$, LAMP2, EEA1 and NPC1 proteins

IPMΦ cells ($3x10^5$) and RAW 264.7 ($5x10^4$) were cultured in 300 µl complete DMEM in 8-well cell culture dishes and IPMΦ (10^5) and RAW 264.7 ($2x10^4$) cells in 100 µl complete DMEM in 96-well Cell Carrier Ultra cell culture dishes. Cells were treated with Pepstatin A penetratin (1, 3 and 9 µM) and $A_{2A}R$ agonist (CGS21680, 100 nM) for 20 min prior to immunostaining before LPS (100 ng/ml) was added to cells for 4 h. After treatment, the cell culture medium was replaced with fresh complete DMEM. $A_{2A}R$ and LAMP2 protein were labeled in the cell culture medium for 30 min at 37°C with anti-$A_{2A}R$ antibody (2 µg/ml) and anti-LAMP2-Alexa-488 antibody (2 µg/ml), respectively. Cells were then fixed with 4% m/v PFA solution for 20 min and incubated in blocking buffer (BSA dissolved in 2 m/v% PBS) for 30 min at room temperature. Cells were washed three times with 300 µl PBS and Alexa-488-conjugated anti-rabbit labeling was used for the primary anti-$A_{2A}R$ antibody. For EEA1- and NPC1-specific immunostaining, cells were fixed in 4 m/v% PFA solution for 20 min and

incubated in blocking buffer (2 m/v% BSA dissolved in PBS) for 30 min at room temperature. Anti-EEA1 (1 µg/ml) and anti-NPC1 (5 µg/ml) antibodies were added to the samples and the cells were incubated overnight at 4 °C. Cells were washed three times with 300 µl PBS, and for staining of EEA1 and NPC1, Alexa-488 conjugated anti-rabbit secondary antibody (5 µg/ml) was added to blocking buffer and incubated for 1 hour at room temperature. Nuclei were stained with DAPI (20 µg/ml) for 1 hour at room temperature in blocking buffer. After staining and chamber removal, slides were coverslipped in the presence of 5 µl Mowiol-Dabco coverslip medium. Photographs were taken with a Leica SP8 confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA) with a 63× oil immersion objective (NA: 1.4). In a 96-well Cell Carrier Ultra plate, blocking buffer was replaced with 50 µl PBS and images were acquired with an Opera Phenix High Content Confocal System (Perkin Elmer, Waltham, MA, USA). To visualize the localization of $A_{2A}R$, 50-210 fields of view and 370-6100 cells per well were recorded, and laser-based autofocus was applied at each imaging position. Images of the DAPI and Alexa-488 channels were acquired at 2 µm from the Z image plane using a 63× aqueous objective (NA: 1.15). Primary data were analyzed using Harmony 4.8 software (Perkin Elmer, Waltham, MA, USA) according to the "Spot Analyses Ready to Made Solution" with additional custom modifications. Cells were identified by the DAPI signal and cell phenotypes were characterized by the Alexa-488 signal. Parameters such as the number of Alexa-488 positive spots, total area of spots, relative intensity of spots in the membrane and cytoplasmic regions were determined. Statistical analyses of the parallel data set were performed using GraphPad Prism 8 software. Data evaluation was based on individual analysis of 550-5450 different cells and presented as mean SEM. *p <0.05, **p <0.01 and ***p <0.001 vs control #p <0.05, ##p <0.01 and ###p <0.001 vs LPS treated cells.

4. 15. Laser scanning cytometry

RAW 264.7 (10^5) or IPMΦ (20^5) cells were labeled with anti-LAMP-2-Alexa-488 antibody (0.5 mg/ml) and the nucleus was stained with DAPI (20 µg/ml) for 1 h at room temperature in blocking buffer. Samples were measured using a laser scanning iCys Research Imaging cytometer (Thorlabs Imaging Systems, Sterling, VA, USA). The resulting fluorescence signals (405 nm and 488 nm) were collected with a 40× (NA 0.75) objective into 2 detection channels (blue and green channels). The blue channel PMT was set to 22 V, gain to 100%, offset to -0.14 V, and the green channel PMT was set to 40 V, gain to 100%, offset to -0.06 V. The X-step size was 0.25 µm. The field size was 250*192 µm. The resolution was 1024*768 and the pixel size was 0.25 µm*0.25 µm.

4. 16. Mass spectrometry

Tryptic fragments of cMyc-$A_{2A}R^{284-410}$ and isotype-control specific immunocomplexes from RAW 264.7 cell lysate and mouse GST and GST-$A_{2A}R^{284-410}$ specific pull-down complexes from mouse IPMΦ were analyzed by matrix-assisted laser desorption ionization "time-of-flight" mass spectrometry as described previously.

4. 17. Statistical analysis

Data were presented as the mean ± SEM of three to six independent experiments. The D'Agostino and Pearson test was used to analyse normality. In cases where the data showed a normal distribution, one-way ANOVA with Sidak's post hoc test was performed. In the other case, if the data did not show a normal distribution, the data were transformed and then one-way ANOVA was performed, also with Sidak's post hoc test. P-values <0.05 were considered

statistically significant ($*p<0.05$; $**p<0.01$; $***p<0.001$). Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA).

5. RESULTS

5. 1. Identification of proteins that interact with the C-terminal domain of $A_{2A}R$

$A_{2A}R$, unlike other members of the receptor family, has a long, C-terminal, intracellular domain (human: 122 amino acids, mouse: 120 amino acids). This domain is responsible for interacting with other proteins. However, no such protein-protein interaction has been identified in macrophage cells to date. Therefore, we aimed to identify $A_{2A}R$-interacting proteins in mouse macrophage cells. To achieve this, Dr. Endre Kókai first performed yeast two-hybrid library screening of the C-terminal domain of $A_{2A}R$ using a mouse peritoneal macrophage library and identified the CtsD interacting protein. We then used immunoprecipitation and MS analysis following a pull-down method to demonstrate the association of the $A_{2A}R$ C-terminal with the NPC1 protein in macrophages. The latter methods also confirmed the interaction of $A_{2A}R$ and CtsD. Yeast two-hybrid screening identified the CtsD coding sequence in 15 independent clones. Three different sets of positive clones with overlapping sequences indicated that the hit was genuine and not the result of multiple repetitions of the CtsD coding sequence fragment in the cDNA library. In order to confirm the putative interaction between the C-terminal domain of $A_{2A}R$ and the CtsD protease, our team used several independent methods to confirm this (e.g. immunoprecipitation, pull-down method). My task was to perform further experiments to confirm the functional relationship between the two proteins.

In a mass spectrometric study of immuno- and pull-down complexes with the C-terminal domain of $A_{2A}R$, Dr. Endre Kókai identified 27 interacting proteins, including the NPC1 protein, using two independent methods. These methods confirmed the presence of CtsD protease in the complexes, thus confirming the yeast two-hybrid screening result. The further task of my PhD thesis was to characterize in detail the functional relationship between $A_{2A}R$ and NPC1 in mouse macrophage cells among the 27 proteins. Regarding the role of $A_{2A}R$ in vesicular transport processes, Visentin et al. have previously shown that activation of $A_{2A}R$ restores mitochondrial membrane potential and cholesterol accumulation in fibroblasts from Niemann-Pick C1 patients and in human neuronal and oligodendroglial NPC1 cell lines. With these in mind, we chose NPC1 protein as one of eight other potential interacting partners involved in vesicular transport and continued our experiments by investigating the functional interaction of NPC1 protein and $A_{2A}R$ in macrophages.

5. 2. Confirmation of the interaction between $A_{2A}R$ and CtsD protease in macrophages

Immunocytochemistry was used to investigate whether endogenous $A_{2A}R$ and CtsD share a common localization in IPMΦ cells. The result of the experiment showed that endogenous $A_{2A}R$ and CtsD showed a high degree of colocalization. In this case, the Pearson coefficient was 0.81. Next, we investigated whether either LPS-mediated inflammatory stimulation, $A_{2A}R$ activation or inhibition of aspartyl protease affected the $A_{2A}R$-CtsD interaction, which was verified by co-IP experiments. Based on the results of our experiments, we found that treatment of RAW 264.7 cells with LPS and aspartyl protease inhibitor significantly reduced the receptor interaction in both the intermediate and mature forms of CtsD. Incubation of cells with $A_{2A}R$ specific agonist only affected the interaction of the receptor with the CtsD HC form. This treatment, therefore, affects the regulation of protease maturation. LPS treatment is used to model inflammatory processes. The effect of the treatment affected the interaction between $A_{2A}R$ and CtsD, so this physiological process also affects the interaction. LPS treatment reduced the expression of CtsD. Aspartyl protease inhibition suggests that the inhibited form of the CtsD enzyme is less able to interact with $A_{2A}R$.

5. 3. $A_{2A}R$ is a putative substrate of the CtsD protease

Yeast two-hybrid method, IP, PD and immuno-localization experiments were used to demonstrate the interaction of $A_{2A}R$ and CtsD. In order to determine whether $A_{2A}R$ directly interacts with CtsD and is a substrate for the enzyme, the primary structure of the receptor was analyzed by two different protease cleavage site prediction methods. Based on the results of the computational analysis, the algorithms identified several putative CtsD cleavage sites in $A_{2A}R$. We also found four peptide sequences that were identified by two independent methods (AA40-41, AA45-46, AA84-85, AA375-376) in the primary structure of the receptor. We observed that the AA375-376 peptide sequence is located in the C-terminal region of the receptor used as a binding partner in yeast two-hybrid screening, co-IP and PD methods. We further aimed to experimentally investigate whether CtsD protease directly cleaves $A_{2A}R$. For our assays, we used a HEK-293 cell line that persistently expresses Flag-$A_{2A}R^{SNAP}$ (i.e. HEK-293-Flag-$A_{2A}R^{SNAP}$ cells /provided by Francesco Ciruela, Department of Pathology and Experimental Therapeutics, University of Barcelona, Spain/). From these human HEK-293 cells, $A_{2A}R$ was immunoprecipitated and protein extracts were incubated with recombinant mouse CtsD enzyme (1.5 μg/ml) for 1 or 2 hours. Immunoblotting of HEK-293-Flag-$A_{2A}R^{SNAP}$ cells revealed a protein band of ~67 kDa, corresponding to the SNAP-conjugated $A_{2A}R$ protein (~48 kDa + ~19 kDa). In addition, protein bands of higher molecular weight were observed, probably corresponding to glycosylated forms of Flag-$A_{2A}R^{SNAP}$, as previously reported by Burgueno et al. These data suggest that CtsD treatment significantly reduced the amount of full-length Flag-$A_{2A}R^{SNAP}$ R (~67 kDa) after 1 h of incubation. In addition to the full-length Flag-$A_{2A}R^{SNAP}$ band, two smaller molecular size bands (~63 kDa and ~52 kDa) were also detected with the anti-Flag antibody. The ~63 kDa A2AR protein band corresponds to the putative CtsD cleavage of the receptor at the predicted site of computer prediction (AA375-376), thus generating an $A_{2A}R$ lacking the last AA33-39. However, the appearance of the ~52 kDa $A_{2A}R$ protein band is not expected as it does not correspond to any of the predicted CtsD cleavage sites. This result suggests the existence of additional, as yet unpredicted CtsD-sensitive proteolytic sites in the C-terminal domain of $A_{2A}R$. Finally, to confirm whether CtsD is able to cleave the C-terminal domain of $A_{2A}R$, recombinant mouse GST-$A_{2A}R^{284-410}$ fragments were incubated with the CtsD enzyme (10 μg/ml) for 5, 10 and 30 min. The results of the experiment showed that CtsD decreased the expression of mouse GST-$A_{2A}R^{284-410}$ protein in a time-dependent manner, and concomitantly produced a shorter form of mouse GST-$A_{2A}R^{284-410}$, probably lacking the last 33-39 AA. In addition, CtsD degraded the 14 kDa degradation product produced during purification of mouse GST-$A_{2A}R^{284-410}$. The results of immunoprecipitation experiments indicated that $A_{2A}R$ could be a potential substrate for the CtsD enzyme.

5. 4. Aspartyl protease enzyme inhibition increases $A_{2A}R$ expression in mouse macrophages

We then examined the effect of inhibition of CtsD activity on $A_{2A}R$ expression in mouse IPMΦ cells. To achieve this, cells were incubated with oligopeptide-conjugated pepstatin A penetratin [RQIKIWFQNRRMKWKK (pAntp(43-58)], a potent inhibitor of aspartyl proteases, while $A_{2A}R$ expression and subcellular localization were monitored by immunostaining followed by confocal microscopy analysis. Albee and colleagues found that pepstatin A can inhibit additional aspartyl proteases, including cathepsin E, pepsin or renin. However, the inhibitory effect of pepstatin A on penetratin is primarily due to inhibition of the enzyme CtsD, because it is the most highly expressed intracellular aspartyl protease. In immune cells, CtsD is the

predominant aspartyl protease produced, while cathepsin E mRNA and protein expression is also very low (https://www.genecards.org/cgi-bin/carddisp.pl?gene=CTSE#expression). IPMΦ Cells were cultured in the presence or absence of LPS, modeling the inflammatory environment of macrophages. In the presence of LPS, $A_{2A}R$ expression was increased in macrophages as previously described by Ramanathan and Köröskényi et al. Our results showed that $A_{2A}R$ localized in the plasma membrane and cytoplasm. Subsequently, our confocal microscopy images confirmed that pepstatin A penetratin treatment increased the number, size and intensity of $A_{2A}R$-specific fluorescent spots in a concentration-dependent manner. This was observed in both LPS-activated and control IPMΦ cells, as well as in the plasma membrane and cytoplasm. These observations are in agreement with our previous results that $A_{2A}R$ may be a substrate for CtsD and that inhibition of proteolytic cleavage maintains higher levels of the receptor. In addition, we have also demonstrated colocalization of both proteins in RAW 264.7 macrophage and IPMΦ cells.

5. 5. Pepstatin A penetratin treatment modulates cytokine production by LPS-activated IPMΦ cells

Macrophages play a prominent role in inflammatory processes. This physiological process is characterised by the regulation of inflammatory and anti-inflammatory cytokine production. Since $A_{2A}R$ stimulation has previously been shown to decrease IL-6 secretion and increase IL-10 cytokine release by macrophages, we investigated whether inhibition of aspartyl proteases, which increases $A_{2A}R$ expression in IPMΦ cells, could also induce $A_{2A}R$ stimulation. To achive this, we measured changes in IL-6 and IL-10 levels in IPMΦ cells by ELISA. In the previous experiment, it was demonstrated that treatment with the aspartyl protease inhibitor pepstatin A penetratin affects $A_{2A}R$ expression, and therefore we investigated how it affects cytokine production in macrophages. Pepstatin A penetratin treatment decreased IL-6 and increased IL-10 cytokine levels in LPS-activated IPMΦ cells. This implied that aspartyl protease inhibition could induce similar effects on the production of pro-inflammatory and anti-inflammatory cytokines as $A_{2A}R$ stimulation.

5. 6. Detection of the interaction between $A_{2A}R$ and NPC1 protein

Among the $A_{2A}R$ interacting proteins identified by IP and PD follow-up MS analysis with the C-terminal domain of $A_{2A}R$, we focused on the NPC1 protein. This was because the functional relationship between the two proteins has been described previously. The results indicated a potential therapeutic role for $A_{2A}R$ in the treatment of this rare genetic disorder. Therefore, we first investigated the relationship between $A_{2A}R$ and NPC1 produced in transgenic HEK cells and then the interaction of endogenous $A_{2A}R$ and NPC1 in RAW 264.7 cells. For this purpose, HEK-293-Flag-$A_{2A}R^{SNAP}$ cells, previously used in experiments demonstrating the interaction of CtsD and $A_{2A}R$, were used as embryos. The interaction between $A_{2A}R$ and NPC1 was verified by IP using an $A_{2A}R$ specific antibody. The specificity of the interaction between the two proteins was confirmed by the absence of NPC1 protein in the immunocomplex when rabbit control serum was used. We then examined whether the interaction between endogenous $A_{2A}R$ and NPC1 also occurs in macrophages. To achieve this, co-IP experiments were performed in RAW 264.7 cells. We were also able to immunoprecipitate NPC1 protein in RAW 264.7 cell lysate using the anti-$A_{2A}R$ antibody previously used, similar to HEK-293-Flag-$A_{2A}R^{SNAP}$ cells. Importantly, the NPC1-specific protein band was not observed in the absence of anti-$A_{2A}R$

antibody in the co-IP experiment. Taken together, these results showed that full-length $A_{2A}R$ and NPC1 interact not only in HEK-293-Flag-$A_{2A}R^{SNAP}$ cells but also in macrophages.

5. 7. $A_{2A}R$ activation reduces NPC1 mRNA expression and protein expression in LPS-activated macrophages

We then investigated the possibility of a functional interaction between NPC1 and $A_{2A}R$. To achieve this, we first studied the effect of $A_{2A}R$ activation on NPC1 mRNA expression and protein expression in LPS-activated macrophages. When LPS-activated IPMΦ cells were treated with $A_{2A}R$ agonist, a decrease in NPC1 mRNA expression was observed. The relative amount of NPC1 mRNA decreased upon LPS treatment and $A_{2A}R$ stimulation enhanced this decrease in LPS-induced macrophages. Furthermore, when we examined NPC1 protein expression, we found that it was significantly decreased by combined LPS-$A_{2A}R$ agonist treatment, similar to mRNA levels. These results suggest that $A_{2A}R$ signaling regulates NPC1 expression in macrophages.

5. 8. $A_{2A}R$ activation regulates NPC1 cell surface expression in mouse macrophages

We then aimed to determine whether $A_{2A}R$ activation affects the localization and intracellular abundance of NPC1. To achieve this, we specifically labelled the NPC1 protein and then used confocal microscopy to follow the intracellular distribution of NPC1 in $A_{2A}R$ agonist-treated macrophages (i.e. RAW 264.7, IPMΦ) in the absence and presence of LPS. Upon activation of RAW 264.7 and IPMΦ cells with LPS, there was an increasing trend in the number of protein-specific spots of NPC1, the relative intensity of spots, the total area and number of spots, and the number of NPC1-specific spots per area in both the plasma membrane and the cytoplasmic region of cells compared to untreated cells. $A_{2A}R$ agonist pretreatment reduced the number of NPC1-specific spots and the total spot area in the membrane region in LPS-stimulated RAW264.7 cells. In the IPMΦ, $A_{2A}R$ agonist treatment reduced the number and total area of NPC1-specific spots found in the cytoplasm and the intensity of relative NPC1-specific spots identified in the plasma membrane region. This suggests that $A_{2A}R$ stimulation reduces NPC1 protein expression in the cell membrane environment not only in LPS-activated macrophage cell lines but also in primary peritoneal macrophages.

5. 9. $A_{2A}R$ stimulation reduces lysosome-associated membrane protein 2 (LAMP2) expression in mouse macrophages

The NPC1 protein defect is a genetic disorder that affects the transport of LDL-derived cholesterol from the lysosome lumen to the membrane. Treatment with an $A_{2A}R$ agonist reduces the cell surface expression of LAMP2 protein as a lysosomal marker in both healthy fibroblasts and fibroblasts from NPC1-deficient patients. By monitoring LAMP2, we can infer changes in the localization of these cell organelles. Therefore, our aim was to investigate the effect of $A_{2A}R$ activation on the intracellular distribution of LAMP2 in quiescent and activated macrophages. To achieve this, RAW 264.7 and IPMΦ cells were pretreated with $A_{2A}R$ agonist, incubated in the absence or presence of LPS, and LAMP2 cell surface abundance was monitored by confocal microscopy after specific immunostaining. We then determined the number and fluorescence intensity of LAMP2-specific spots in the plasma membrane and cytoplasmic regions of RAW 264.7 cells and IPMΦ. LPS activation significantly increased the number of LAMP2-specific spots, the total area of spots and the number of spots per area in both plasma membrane and cytoplasmic regions in IPMΦ cells. In addition, similar changes were observed in the

cytoplasmic regions of IPMΦ macrophage cells when pretreated with $A_{2A}R$ agonist. These results indicate that activation of $A_{2A}R$ reduces the amount of LAMP2 protein in LPS-activated macrophages, thus affecting the cell surface expression of LAMP2 protein. Since both LAMP2 and NPC1 proteins are present in the lysosome, this observation suggests that $A_{2A}R$ activation may also affect NPC1 protein expression.

5. 10. $A_{2A}R$ activation modulates expression of early endosome antigen 1 (EEA1) in mouse macrophages

EEA1, a protein involved in recycling of early endosomes, has been used as a marker of early endosomes during macrophage endocytosis. In CHO cells carrying the NPC1-null mutation, an increase in endocytotic vesicle size was observed when EEA1 was studied as an endosome marker. This suggests that the NPC1-null mutation may lead to alterations in the endocytic pathway and the formation of larger endocytic vesicles, which may contribute to NPC1-related cellular dysfunction. In these enlarged peripheral vesicles, proteins accumulated and endolysosome fusion was inhibited. We therefore examined the effect of $A_{2A}R$ stimulation on EEA1 protein expression in mouse IPMΦ cells. EEA1 expression and specific immunofluorescence labeling were detected by high-throughput confocal microscopy and evaluated using preprocessing software. Our results showed that LPS treatment of cells increased the total area and number of EEA1 spots in the plasma membrane environment, but did not result in a similarly significant change in the cytoplasm. Stimulation with the $A_{2A}R$ agonist reduced the number of EEA1-specific spots and the total area of spots in the plasma membrane environment, as well as the total area of spots in the cytoplasm in LPS-treated cells.

6. DISCUSSION

Adenosine receptors are found in many tissues of the mammalian body. Their distribution is diverse. Their effects include the central nervous system, cardiovascular and immune systems, and their role in cancer and wound healing is of major importance. The physiological relevance of $A_{2A}R$ is observed in the nervous system, where the amount and activation or inhibition of $A_{2A}R$ is involved in the regulation of other signalling pathways. $A_{2A}R$, unlike other members of the receptor family, has a long, motile intracellular C-terminal domain of 122 amino acids in humans and 120 amino acids in mice. It lacks a palmitoylation site. Due to its protein structure and flexibility, this domain is suitable for protein-protein interactions. This assumption is supported by the identification of several interacting proteins with the C-terminal domain of the receptor. Also, this domain has been shown to regulate receptor expression and function in non-immune cells. However, no such interaction has been described in immune cells. In addition to the classical signalling pathway mediated by $A_{2A}R$, it has been shown that regulatory events such as anchoring of the receptor to the actin cytoskeleton; regulation of receptor recycling; regulation of the mitogen-activated protein kinase signalling pathway, "cross talk" with other transmembrane receptors, modulation of receptor activation and exit from the ER occur through proteins that interact with the C-terminal domain. Our study provided new insights into the mechanism of $A_{2A}R$ desensitization in mouse macrophages. $A_{2A}R$ numbers, as for all GPCRs, are regulated by receptor synthesis, recycling and elimination processes. Optimal levels of $A_{2A}R$ expression and activation on the surface of macrophages led to an anti-inflammatory phenotype and protected the body from tissue damage. Previously, it has been shown that activation of $A_{2A}R$ on macrophages prevents ischemia-reperfusion injury in liver, lung and kidney. The molecular mechanism of $A_{2A}R$ internalization, which may be followed by rapid or slow recycling to the plasma membrane or complete degradation in the lysosome, has been partially characterized. The topic of this PhD thesis is the functional characterization of the relationship between $A_{2A}R$ and its intracellular C-terminal domain interacting proteins in macrophage cells. Previously, our group identified CtsD protease as a protein associated with $A_{2A}R$ using a yeast two-hybrid method. The interaction of CtsD with G-protein-coupled receptor 54 (GPR54), which plays a fundamental role in the development and maintenance of mammalian reproductive functions, has also been shown. In the present work, we focused on the identification of proteins that interact with $A_{2A}R$. This will allow us to gather additional information on receptor-mediated regulatory pathways and desensitization through protein-protein interactions in mouse macrophages. The aim was therefore to verify the functional relationship between $A_{2A}R$ and CtsD in a mammalian cell model and in mouse macrophages. As a first step, we investigated whether the interaction between the C-terminal A2AR and CtsD could be detected in higher eukaryotic cells. To answer this question, IP experiments were designed in a Flag-tagged $A_{2A}R$ overexpressing cell line HEK-293 (HEK-293-Flag-$A_{2A}R^{SNAP}$). To confirm the association between endogenous proteins, RAW 264.7 and mouse peritoneal macrophages were used for IP and immunostaining. Our confocal microscopy studies in macrophage cells confirmed that $A_{2A}R$ and CtsD expressed within the cell show high colocalization. The result of our IP experiments in RAW 264.7 macrophage cells confirmed that the interaction between the two endogenous proteins also occurs in macrophage cells. We then tested whether the $A_{2A}R$ sequence contains potential CtsD protease cleavage sites. We performed the assay with two independent *in silico* prediction programs and found that several potential aspartyl protease cleavage sites were present in the amino acid sequence of $A_{2A}R$. The algorithms used identified multiple CtsD cleavage sites within $A_{2A}R$. Four peptide sequences

were identified by two independent methods (AA40-41, AA45-46, AA84-85, AA375-376). It was observed that the AA375-376 peptide sequence is located in the C-terminal region of the yeast two-hybrid screening, co-IP and PD methods bait receptor. The results of our studies showed that it specifically interacts with CtsD protein via the C-terminal domain of $A_{2A}R$ in HEK-293-Flag-$A_{2A}R^{SNAP}$ cells. We then experimentally tested whether the recombinant mouse CtsD enzyme can cleave Flag-tagged $A_{2A}R$ overexpressed by HEK293 cells. The result of our experiment showed that CtsD significantly reduced the amount of Flag-tagged $A_{2A}R$ in the cell lysate. Thus, we found that the *in silico* prediction is valid in reality and that CtsD is able to cleave $A_{2A}R$. $A_{2A}R$ immunostaining was performed by treating IPMΦ cells in the presence or absence of LPS, modeling the inflammatory environment of macrophages. As a result of this experiment, we found that $A_{2A}R$ expression in macrophages increased in the presence of LPS. Furthermore, our confocal microscopy images confirmed that pepstatin A penetratin treatment increased the number, size and intensity of $A_{2A}R$-specific fluorescent spots in a concentration-dependent manner. This was observed in both LPS-activated and control IPMΦ cells, as well as in the plasma membrane and cytoplasmic regions. Treatment of macrophages with the cell permeable CtsD inhibitor pepstatin A increased the cell surface expression of $A_{2A}R$, confirming that CtsD regulates $A_{2A}R$ expression *in vivo* and plays a role in the regulation of receptor abundance. Furthermore, this regulatory mechanism may also affect $A_{2A}R$-mediated immunomodulatory mechanisms. Based on our results, we have extended the list of interacting partners of $A_{2A}Rs$ and described this protein-protein interaction for the first time in macrophage cells. These results suggest that $A_{2A}R$-mediated activation of CtsD, which in turn leads to cleavage of the C-terminal of $A_{2A}R$. We also examined the effect of CtsD enzyme inhibition on $A_{2A}R$-regulated signalling through changes in the levels of different cytokines (inflammation inducing and inflammation inhibiting). The $A_{2A}R$-mediated Gs-protein coupled pathway enhances cAMP production upon adenylate cyclase activation. In our study, the aspartyl protease inhibitor produced an effect similar to $A_{2A}R$ activation. Treatment with pepstatin A penetratin significantly decreased the production of inflammation-inducing IL-6 and increased the production of anti-inflammatory IL-10 in the same cell type. Thus, the cytokine level regulation effect obtained through inhibition of aspartyl proteases is equivalent to the effect of $A_{2A}R$ activation, both enhancing the anti-inflammatory effect of macrophages. In the second part of this thesis, I will describe the interaction between $A_{2A}R$ and NPC1 proteins. The background of this observation is that our group identified the NPC1 protein as an interacting partner of $A_{2A}R$ by two independent methods. Furthermore, a functional relationship between $A_{2A}R$ and NPC1 in the nervous system has been previously demonstrated. The observation suggests that NPC1 protein is functionally linked to the $A_{2A}R$-mediated signaling pathway. Visentin et al. demonstrated that activation of $A_{2A}R$ restores mitochondrial membrane potential and cholesterol accumulation in fibroblasts from NPC1 patients, as well as in human neuronal and oligodendroglial cell lines. They demonstrated that receptor activation significantly reduces the defect in intracellular transport of endocytosed cholesterol in NPC1-deficient fibroblasts and oligodendrocytes. The role of the NPC1 protein in the immune system was demonstrated by Grinstein and colleagues in RAW 264.7 macrophage cells and fibroblasts, where they observed that the NPC1 protein plays a role in the intracellular accumulation of cholesterol. And macrophages are known to be involved in vesicular transport to fight infections and in inflammatory responses. Taken together, these findings suggest that the interaction between $A_{2A}R$ and NPC1 protein is biologically plausible and that $A_{2A}R$ may play a role in regulating NPC1 protein function in macrophages. Bernardo and colleagues investigated myelin defects

and the role of cholesterol in myelination. Stimulation of $A_{2A}R$ may offer a therapeutic perspective in the disease, as it has a beneficial effect on dysmyelination. Previously, Npc1 gene expression was observed to be affected by cycloheximide and progesterone. Cycloheximide increases Npc1 mRNA levels. Granulosa cells may be subjected to a transient progesterone-induced Npc1 blockade, resulting in a compensatory increase in Npc1 mRNA and NPC1 protein. In order to investigate how $A_{2A}R$ activation affects NPC1 mRNA and protein expression in IPMΦ cells, we used $A_{2A}R$ agonist treatment, which in both cases reduced NPC1 expression in LPS-activated macrophages. To determine whether $A_{2A}R$ activation directly affects NPC1 protein abundance, changes in protein expression and localization, we used immunostaining and confocal microscopy to examine changes in NPC1 abundance and localization. The results showed that $A_{2A}R$ agonist treatment reduced both the number and area of NPC1-specific spots in the plasma membrane and cytoplasmic regions of macrophages compared to LPS-activated samples. Previously, $A_{2A}R$ signaling has been shown to play a role in the regulation of vesicular transport. Isidoro et al. found that pretreatment with an $A_{2A}R$ agonist induced the movement of endosomes and lysosomes towards the plasma membrane, followed by fusion of cellular components with the plasma membrane. This was evidenced by the appearance of lysosome-associated membrane protein 1 (LAMP1) on the cell surface and the release of lysosomal soluble enzymes in hepatocytes. Since NPC1 is a protein located in the inner membrane of lysosomes and its function is to transport LDL-derived cholesterol from the lumen of the lysosome to the membrane, we investigated changes in the expression and localization of another lysosomal marker, LAMP2 protein, following treatment with the $A_{2A}R$ agonist in macrophage cells. We showed that activation of $A_{2A}R$ significantly reduced the amount of LAMP2 protein in the membrane and cytoplasmic regions of IPMΦ cells compared to the change upon LPS activation. Our results were consistent with the observation that $A_{2A}R$ activation leads to reduced LAMP2 expression in fibroblasts from both healthy and NPC1 patients. Tahirovic and Hecimovic and colleagues observed enlarged early endosomes and recycling endocytic cellular components in CHO cells from NPC1 KO mice. This suggested that the lack of NPC1 protein affects endocytic organelle function through defects in the endolysosomal pathway. Using the EEA1 marker, which has been successfully used previously in macrophages, we investigated how $A_{2A}R$ activation alters EEA1 protein abundance and intracellular localization. Similar to LAMP2, EEA1 protein levels were significantly reduced following $A_{2A}R$ agonist treatment in membrane and cytoplasmic regions of IPMΦ cells, in contrast to increased levels following LPS activation. The intracellular vesicular transport process requires the highly coordinated function of several proteins; therefore, mutations in the genes encoding these proteins often result in inherited disorders that are based on defective function of the vesicular transport pathway. The export of cholesterol requires NPC1 and NPC2, genetic mutations of which can cause Niemann-Pick type C (NPC) disease, a disease characterised by lysosomal accumulation of cholesterol and glycosphingolipids. NPC disease affects many organ systems in the human body, including the liver, spleen and brain, and can lead to the development of severe symptoms. These may include hepatosplenomegaly, progressive neurological deterioration and cognitive decline. NPC1 is located in the membrane of endosomes and lysosomes and is involved in intracellular cholesterol transport. It plays a role in the transport of cholesterol and other types of fats across cell membranes. When this system malfunctions, cholesterol is deposited and can lead to lysosomal storage diseases, which are mainly associated with neurological symptoms. Our results contribute to a more detailed understanding of the molecular mechanism of NPC1 disease by functional analysis of the interaction between NPC1 and $A_{2A}R$.

7. SUMMARY

Adenosine plays an important role in modulating the function of immune cells, especially T-cells and myeloid cells such as macrophages and dendritic cells. A_{2A}R-dependent signalling in macrophages plays a key role in the regulation of inflammation. However, the processes that regulate the cell surface appearance and degradation of A_{2A}R in macrophages are not fully understood. A_{2A}Rs on cell surface regulate the production of pro-inflammatory cytokines and chemokines as well as the proliferation, differentiation and migration of immune cell. It is currently known that the C-terminal domain of A_{2A}R and its interacting proteins regulate receptor recycling, but the role of these proteins in macrophages is not fully determined. Therefore, our aim was to identify A_{2A}R interacting proteins in macrophages that may affect A_{2A}R expression and activity. Our group previously performed yeast two-hybrid screening using the C-terminal domain of A_{2A}R and a macrophage expression library. As a result of this screening, we found that CtsD is a potential interacting partner for A_{2A}R. Subsequently, in my work I investigated the interaction of A_{2A}R and CtsD protease *in vitro* and in cell models, such as RAW 264.7 and mouse peritoneal macrophage cells. Our results demonstrate that A_{2A}R is a substrate of CtsD and that inhibition of CtsD activity increases A_{2A}R density and cell surface expression in macrophages. In conclusion, our results suggest that CtsD is a novel A_{2A}R interacting partner and thus we describe a molecular and functional interaction that may be key in the regulation of A_{2A}R-mediated inflammatory processes. In the second part of my work, we demonstrated the interaction between A_{2A}R and NPC1 protein in macrophages. Previously, our group has used two independent methods to identify the NPC1 protein interacting with the C-terminal domain of A_{2A}R in RAW 264.7 and IPMΦ cells. The interaction between the NPC1 protein and the full-length A_{2A}R was further investigated in HEK-293 cells that persistently express the receptor, and in RAW 264.7 cells that endogenously express A_{2A}R. The results of our experiments show that activation of A_{2A}R reduces NPC1 mRNA and protein expression in LPS-activated mouse IPMΦ cells. In addition, A_{2A}R stimulation negatively regulates plasma membrane transport of NPC1 in LPS-stimulated macrophages. Furthermore, we also show that A_{2A}R activation altered the expression and localization of endosomal markers LAMP2 and EEA1 in macrophages. Taken together, these results suggest a putative A_{2A}R-mediated regulation of NPC1 protein function in macrophages, which is of great importance in Niemann-Pick type C disease, where mutations in NPC1 protein result in accumulation of cholesterol and other lipids in lysosomes.

Main Scientific results:

Detection of functional interaction between $A_{2A}R$ and CtsD protease

- We have demonstrated colocalization of the C-terminal domain of $A_{2A}R$ and CtsD protease in IPMΦ cells.
- We have demonstrated the interaction between the $A_{2A}R$ C-terminal domain and CtsD protease in RAW 264.7 macrophage cells.
- We identified potential aspartyl protease cleavage sites in the primary protein sequence of the $A_{2A}R$ C-terminal domain using three different *in silico* computer programs.
- We experimentally demonstrated that $A_{2A}R$ is a substrate for CtsD protease. Furthermore, inhibition of CtsD activity increases the amount and cell surface expression of $A_{2A}R$ in macrophages.
- We have shown that inhibition of CtsD activity decreases the production of pro-inflammatory IL-6 and increases the production of the pro-inflammatory cytokine IL-10 in LPS-activated IPMΦ cells.

Detection of functional interaction of $A_{2A}R$ and NPC1 protein

- We found that $A_{2A}R$ activation decreases NPC1 mRNA and protein expression in LPS-activated mouse IPMΦ cells.
- We confirmed that A2AR stimulation negatively regulates plasma membrane-directed transport of NPC1 in LPS-activated macrophages.
- Based on our results, we found that activation of $A_{2A}R$ significantly reduces the amount of LAMP2 protein in the membrane and cytoplasmic regions of IPMΦ cells in LPS-activated cells.
- We demonstrated that EEA1 protein levels were significantly decreased following $A_{2A}R$ agonist treatment in the membrane and cytoplasmic regions of IPMΦ cells, in LPS activated cells.

8. ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor, Dr Endre Kókai, who introduced me to the basics of molecular biology and taught me the approach and way of thinking that is essential for the design and execution of successful experiments. I thank him for the advices in my experimental work and in the preparation of my publications and dissertation.

I would like to thank Professor László Virág for allowing me to carry out the experiments on which this thesis is based at the Institute of Medical Chemistry, and also for giving me the opportunity to try my hand at teaching as a member of the Institute. I would also like to thank you for your advice and support.

I would like to thank my colleagues Ildikó Szabó, Dr Zsolt Regdon, Dr Zsuzsanna Polgár, Dr. Katalin Kovács, Dr Csaba Hegedűs, Dr Edina Bakondi, Dr Máté Ágoston Demény, Dr Alexandra Kiss, Dr Ádám Sipos, Máté Nagy-Pénzes, Zoltán Hajnády, Tamás Kéki, Dr Nikolett Király, Eliza Guti, Katalin Berta, Isotta Sturniolo and all members of the department for their professional support, useful advices, encouragement and last but not least their friendship. A special thanks to Dr Béla Tóth, who was instrumental in getting me started as a research student at the Institute of Medical Chemistry.

I would like to thank our laboratory assistants Mihályné Herbály, Erika Olajosné Gulyás, Andrea Tankáné Farkas and Dávid Varga for their help in carrying out the experiments and for teaching me the basics of laboratory work. Thank you also to the staff at the Laboratory Animal Core Facility.

I would like to thank all my co-authors, without whom my publications on the subject of my doctoral thesis would not have been possible. Special thanks to Dr György Haskó and Francisco Ciruela Alfarez for their ideas and for their helpful advice in both my publications. I thank Dr Péter Áron Tóth, Attila Tibor Gerencsér and Gyula Ujlaki for their help in the evaluation and analysis of the results.

I would like to thank Dr Zsolt Bacsó and Dr Csaba Bankó, colleagues and co-authors at the Institute of Biophysics and Cell Biology, for their cooperation and help in carrying out many of my experiments.

I would like to thank Dr Eszter Janka for introducing me to the basics of statistics and for her help and conscientious work in mathematical quantification of the experiments.

Last but not least, the greatest thanks go to my loving parents, without whom this work and this thesis would not have been possible. Thank you for all your patience and endless love. I would like to thank my family, friends and colleagues for all the encouragement and for standing by me.

Applications to support research:

OTKA (MB08A 84685); OTKA-K132193; GINOP-2.3.2-15-2016-00020-TUMORDNS, GINOP-2.3.2-15-2016-00048-STAYALIVE. National Institutes of Health grants: R01GM066189, R01DK113790, R01HL158519, and 1 R35GM145245. PID2020-118511RB-I00; MCIN/AEI/10.13039/501100011033 “ERDF. A way of making Europe”.

List of publications

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Subject: PhD Publication List

Candidate: Adrienn Skopál
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10076420

List of publications related to the dissertation

1. **Skopál, A.**, Ujlaki, G., Gerencsér, A., Bankó, C., Bacsó, Z., Ciruela, F., Virág, L., Haskó, G., Kókai, E.: Adenosine A2A Receptor Activation Regulates Niemann-Pick C1 Expression and Localization in Macrophages.
   Curr. Issues Mol. Biol. 45 (6), 4948-4969, 2023.
   DOI: http://dx.doi.org/10.3390/cimb45060315
   IF: 2.976 (2021)
2. **Skopál, A.**, Kéki, T., Tóth, P. Á., Csóka, B., Koscsó, B., Németh, Z. H., Antonioli, L., Ivessa, A., Ciruela, F., Virág, L., Haskó, G., Kókai, E.: Cathepsin D interacts with adenosine A2A receptors in mouse macrophages to modulate cell surface localization and inflammatory signaling.
   J. Biol. Chem. 298 (5), 1-18, 2022.
   DOI: http://dx.doi.org/10.1016/j.jbc.2022.101888
   IF: 5.486 (2021)

Total IF of journals (all publications): 8,462
Total IF of journals (publications related to the dissertation): 8,462

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

21 June, 2023

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár