ADENOSINE REGULATION OF DANGER SIGNALING

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN
MOLECULAR BIOLOGY AND GENETICS

By

İmran Akdemir

July 2017

ADENOSINE REGULATION OF DANGER SIGNALING

By İmran Akdemir

July 2017

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

_____

Çağlar ÇEKİÇ (Advisor)

_____

Duygu SAĞ

_____

Serkan İsmail GÖKTUNA

Approved for the Graduate School of Engineering and Science:

_____

Ezhan KARAŞAN

Director of the Graduate School

Abstract

ADENOSINE REGULATION OF DANGER SIGNALING

İmran AKDEMİR
M.S. in Molecular Biology and Genetics
Advisor: Çağlar ÇEKİÇ
July, 2017

Metabolic and immune related activities converge as main triggers of adenosine accumulation in extracellular space. Adenosine by engaging adenosine A2A and A2B receptors strongly suppresses innate and adaptive immune responses. Although adenosine receptors are being targeted in preclinical and clinical studies, how different danger signals are regulated by adenosine is poorly understood. Here we showed that adenosine receptor stimulation strongly inhibited inflammatory responses while sparing Type-I interferon responses downstream of different danger signals in dendritic cells and macrophages. Mechanistically, danger signals associated with MyD88-dependent inflammatory pathways such as LPS and CpG but not the danger signals associated with IRF3/Type-I interferon pathways such as pA:U and cGAMP increase the expression of adenosine A2A and A2B receptors. Expression of anti-inflammatory NR4A1 was increased after adenosine receptor stimulation in the presence of TLR ligands known to activate MyD88 pathway but not in the presence of cGAMP and pA:U. Overall these results indicate that there is a differential modulation of danger signaling by adenosine rather than overall suppression. Our results have important implications for developing combinatorial approaches to target adenosine and danger signaling pathways to cure immune-related diseases.

Keywords: Adenosine receptors, danger signaling, dendritic cells, macrophages

Özet

ADENOZİNİN TEHLİKE SİNYALLERİNİ REGÜLASYONU

İmran AKDEMİR
Moleküler Biyoloji ve Genetik, Yüksek Lisans
Tez Yöneticisi: Çağlar ÇEKİÇ
Temmuz, 2017

Metabolik ve bağışıklıkla alakalı aktiviteler hücre dışında adenozin birikmesinin en büyük nedenleridir. Adenozin A2A ve A2B reseptörlerini harekete geçirerek hem doğal bağışıklığı hem de edinilen bağışıklığı güçlü bir şekilde baskılar. Adenozin reseptörleri klinik öncesi ve klinik aşamalarda hedeflenmesine rağmen tehlike sinyallerinin adenozin tarafından nasıl regüle edildiği bilinmemektedir. Biz bu tezde adenozinin dendritik hücre ve makrofajlarda, değişik tehlike sinyallerinin harekete geçirdiği inflamatuvar tepkileri güçlü bir şekilde baskılarken Tip-1 interferon tepkilerini etkilemedeğini gösterdik. Mekanik olarak, LPS ve CpG gibi MyD88'e bağlı sinyaller A2A ve A2B reseptörlerinin ekspresyonunu artırırken, pA:U ve cGAMP gibi IRF-3 ve Tip-1 interferonlar'la alakalı sinyaller bu reseptörlerin ekspresyonunu etkilemedi. Anti-inflamatuvar bir molekül olan NR4A1 ekspresyonu da MyD88'i aktive ettiği bilinen TLR sinyallerin varlığında artarken pA:U ve cGAMP varlığında artmadı. Genel olarak bu sonuçlar adenosinin farklı tehlike sinyalleri üzerinde genel bir baskılayıcı etkiden ziyade farklı türde modülasyonu olduğunu göstermektedir. Çalışmamız ileride bağışıklıkla alakalı hastalıkları tedavi etmek amacıyla tehlike reseptörlerini ve adenozin reseptörlerini hedefleyen birleşimsel yaklaşımlar geliştirilmesi adına önemli çıkarımlar içermektedir.

Anahtar Sözcükler: Adenozin reseptörleri, tehlike sinyali, dendritik hücreler, makrofajlar

Acknowledgement

First, I would like to express my gratitude to my advisor, Asst. Prof. Dr. Çağlar Çekiç for giving me the opportunity to work in his lab and for his guidance, support and patience. Thanks to him I became more qualified both in terms of academics and technical skills.

I want to give my special thanks to Asst. Prof. Dr. Duygu Sağ and Asst. Prof. Dr. Serkan İsmail Göktuna for accepting to become members in my jury and sparing time to improve my thesis. I appreciate their valuable comments and suggestions.

I feel very lucky to be a member of CClab because we were not only labmates but also good friends. I thank, from the bottom of my heart, to my lab mates Altay Koyaş, Ali Can Savaş and Merve Kayhan for their friendship, support and every other thing I can't fit in a sentence. It was a privilege to work with you all!

Other than CClab, I would like to thank all MBG family specially Gürsel Group members for always helping me and answering my questions. I also thank Gamze Aykut for helping me with animal experiments.

The most special thanks goes to my precious family for always supporting my dreams. I know very well that none of these things would come true without them. I'm very grateful to my mother Vahide and my father Mehmet and I love them so much. I also want to thank Sefa Kurak and Hasan Hüseyin Kurak since they are no different than parents to me. Last but not least I would like to thank my beloved boyfriend Anıl Sayal for always being there for me and for his love and support.

I would also like to thank to The Scientific and Technological Research Council of Turkey (TUBITAK), EMBO and TUBA for their financial support. I was financially supported by Bilkent University, Department of Molecular Biology and Genetics and TUBITAK (project no: 115Z170 and 215S729).

Table of contents

List of Figures

List of Tables

Abbreviations

ACK	Ammonium-Chloride-Potassium
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Antigen presenting cell
AR	Adenosine receptor
ATP	Adenosine triphosphate
BCR	B cell receptor
BM	Bone marrow
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
cAMP	cyclic Adenosine monophosphate
CD	Cluster of differentiation
cDC	Conventional DC
CDN	Cyclic dinucleotides
cDNA	Complementary deoxyribonucleic acid
cGAMP	cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cyclic GAMP synthase
CXCL10	C-X-C motif chemokine 10
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
ddH2O	Double-distilled water
dH2O	Distilled water
dsRNA	Double-stranded RNA
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting

FBS	Fetal bovine serum
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G protein coupled receptor
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
IL	Interleukin
ILC	Innate Lymphoid Cell
IRF	IFN-regulatory factor
L	Liter
LPS	Lipopolysaccharide
M	Molar
MAP	Mitogen-activated protein
M-CSF	Macrophage colony-stimulating factor
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
mL	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NECA	5' -*N*-Ethylcarboxamidoadenosine
NF- κB	Nuclear factor- κB
N	Normal
ng	Nano gram
NK	Natural killer
NLR	NOD-leucine rich repeat receptors
NOD	Nucleotide-binding oligomerization domain
NR4A	Nuclear Receptor 4A
ODN	Oligodeoxynucleotide
pA:U	Polyadenylic–polyuridylic acid

PAM	PAM2CSK4
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PRR	Pattern recognition receptor
Q-RT-PCR	Quantitative real time polymerase chain reaction
RLR	RIG-I like receptor
RNA	Ribonucleic acid
Rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
STING	Stimulator of Interferon Genes
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TFH	T follicular helper cell
TH	T helper
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMB	Tetramethylbenzidin
TNFα	Tumor necrosis factor α
TRAF	TNF receptor-associated factor
TRAM	Translocating chain-associating membrane
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
µ	micro

Chapter 1

Introduction

1.1 The Immune System

The immune system is the collection of defense mechanisms of the body, which protects the host from infectious agents, their toxins and the damage they cause. These defense mechanisms are composed of three levels: first anatomic and chemical barriers, second the innate immune system and last the adaptive immune system. The first level of defense – anatomic and chemical barriers – includes the skin, mucosal surfaces, and antimicrobial proteins [1]. The innate and adaptive immune systems include specialized cells called white blood cells or leukocytes, that arise from pluripotent hematopoietic stem cells of the bone marrow. If a pathogen could breach the anatomic and chemical barriers, it will encounter the components of innate immune defense first. This encounter will be sensed by receptors that are able to recognize a broad range of molecular patterns, found on innate immune cells. The response of innate immune defenses is fast and serve to induce inflammation by producing several cytokines and chemokines [2]. Although the innate immune cells can recognize a variety of molecular patterns thanks to their specialized receptors, their response is not antigen-specific, which is a limiting factor to their responses. At that point, the slower response of the adaptive immune system comes to aid, with its cells equipped with antigen-specific receptors and with a unique feature of generating immunological memory [2]. Despite the fact that the innate and adaptive responses are different in nature and how fast they are triggered, their integrated dynamics are crucial for a full and effective clearance of pathogens.

1.1.1 Innate Immunity

As mentioned before, innate immunity is the first line of immunological defense after a pathogen succeeds to evade the anatomical and chemical barriers. The innate responses are rapid as most of the invaders can be detected and cleared by innate immunity within minutes or hours. The innate defenses include antimicrobial enzymes and peptides, complement system and specialized cells such as macrophages, granulocytes, and dendritic cells [3]. These specialized cells are equipped with a variety of pattern recognition receptors (PRRs) that can detect pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and then initiate an inflammatory response [3]. Each innate immune cell reacts to pathogens in a different and specialized way. Macrophages, granulocytes and DCs are all phagocytes, which means they are able to phagocyte a pathogen upon encountering with it. The greatest phagocytic activity is done by macrophages, which are the major phagocyte population in the normal tissues, and neutrophils, which are the rapid responders to inflammation. These cells are the scavengers of innate immunity, as they use phagocytosis with the purpose of killing. DCs, on the other hand, use phagocytosis with a special intend, to process proteins to generate peptide antigens and to present those antigens to T cells [4]. Therefore, dendritic cells are important bridges between innate and adaptive immune responses.

1.1.1.1 Pathogen Recognition Receptors

PRRs are composed of several innate sensor systems that are able to detect PAMPs and DAMPs [5]. They are classified into four groups based on their location and function: free receptors in serum; membrane-bound phagocytic receptors; membrane-bound signaling receptors, and cytoplasmic signaling receptors. Activation of a PRR in immune cells, such as macrophages or neutrophils, leads to secretion of several inflammatory cytokines and chemokines that serve to amplify the immune response [6]. Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-1-like receptors (RLRs), and cytosolic DNA sensors such as STING are among several distinct classes of PRRs [7].

1.1.1.1.1 Toll-Like Receptors

Toll-like receptors (TLRs) are evolved to recognize distinct PAMPs [8]. There are 10 TLR subtypes in humans (TLR 1-10) and 12 (TLR1-9, TLR11-13) in mouse discovered so far [9]. They are well characterized as compared to other PRRs. They can be localized on cellular or endosomal membranes. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 and they mainly recognize the membrane- or cell surface-associated patterns in pathogens. Whereas intracellular TLRs include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13, which recognize nucleic acids derived from bacteria or viruses [5, 9, 10].

TLRs are composed of an ectodomain, which takes role in PAMP recognition, a transmembrane domain, and also a cytoplasmic domain, which contains a TIR (Toll-IL-1 receptor) domain that takes role in the initiation of downstream signaling [11]. The recognition of PAMPs leads to a signaling pathway that results in the activation of NF-KB, IRFs, or MAP kinases to regulate the expression of cytokines, chemokines, and type I IFNs. This is mediated by several adaptor proteins such as MyD88, TRIF, TIRAP/MAL, or TRAM [12-14]. MyD88 is utilized by all TLRs except for TLR3 and activates the transcription factor NF-KB that induces the expression of inflammatory cytokines. TRIF is utilized by TLR3 and TLR4, which leads to activation of IRF3 that induces the expression of type I IFNs [15, 16]. The overall scheme for TLR signaling can be found in figure 1.1.

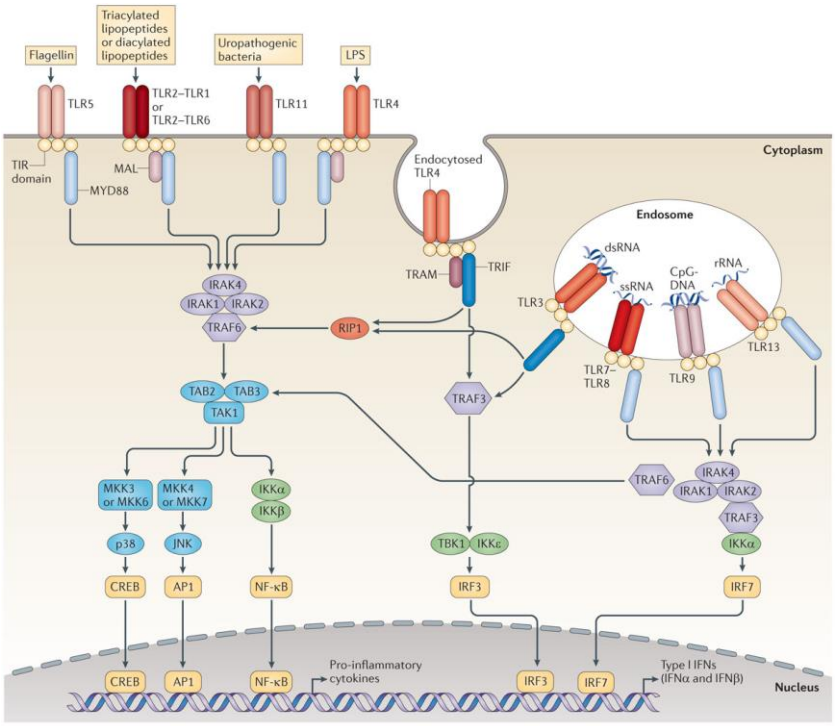

Figure 1.1: Toll like receptor localization and signaling pathways[17].

1.1.1.1.1.1 TLR1, TLR2 and TLR6 signaling

TLR2 is a cell surface receptor, which recognizes a variety of PAMPs including lipoproteins and lipopeptides, peptidoglycan and lipoteichoic acid from mostly Gram-positive and some Gram-negative bacteria [18]. Ligand binding to TLR2 induces heterodimer formation with TLR1 or TLR6. This heterodimer formation is actually the reason for why there is a variety of PAMPs that can be recognized by TLR2 complexes [19]. This receptor is coupled to MyD88 adaptor protein that leads to NF-KB activation [16].

1.1.1.1.1.2 TLR3 signaling

TLR3 is an intracellular TLR expressed on endosomal surfaces and recognizes dsRNA, which is produced by viruses during replication [20]. One example for dsRNA viruses are rotaviruses that causes gastroenteritis in human. TLR3 is coupled to TRIF adaptor protein that leads to activation of IRF3 to induce synthesis of type I IFNs and exert anti-viral activities [21].

1.1.1.1.1.3 TLR4 signaling

TLR4 is a cell surface receptor and recognizes lipopolysaccharide (LPS) of Gram-negative bacteria such as *E.coli* and *Salmonella*. Unlike other TLRs, TLR4 utilizes the accessory proteins MD-2 and CD14 to recognize its ligand [22]. It is coupled to both MyD88 and TRIF leading to induction of both inflammatory cytokines and type I IFNs [23].

1.1.1.1.1.4 TLR9 signaling

TLR9 is an intracellular TLR expressed on endosomal surfaces and recognizes unmethylated CpG dinucleotides [24]. This receptor is primarily expressed on pDCs, which have a great capacity to produce type I IFNs [25]. Normally TLR9 is coupled to MyD88 adaptor protein that leads to NF-KB activation, but it can induce type I IFNs through IRF7 [26]. Presence of this receptors in intracellular compartments allow this interaction and activation of type-I interferon pathway unlike TLR2.

1.1.1.1.2 STING signaling

STING (stimulator of interferon genes) is a cytosolic sensor of infection, which recognizes bacterial cyclic dinucleotides (CDNs) [27]. CDNs are bacterial second messengers, which results in type I interferon production by leading to activation of TBK1 that activates IRF3 upon recognition by STING [28]. Besides, bacterial infection, STING also has a role in antiviral immunity. This antiviral immunity is mediated by the second messenger cGAMP (cyclic guanosine monophosphate-adenosine monophosphate), which is generated by the enzyme cGAS (cyclic GAMP synthase) upon DNA entry into the cells. These events leads to production of type I interferons [29]. The intracellular STING signaling is summarized in figure2.

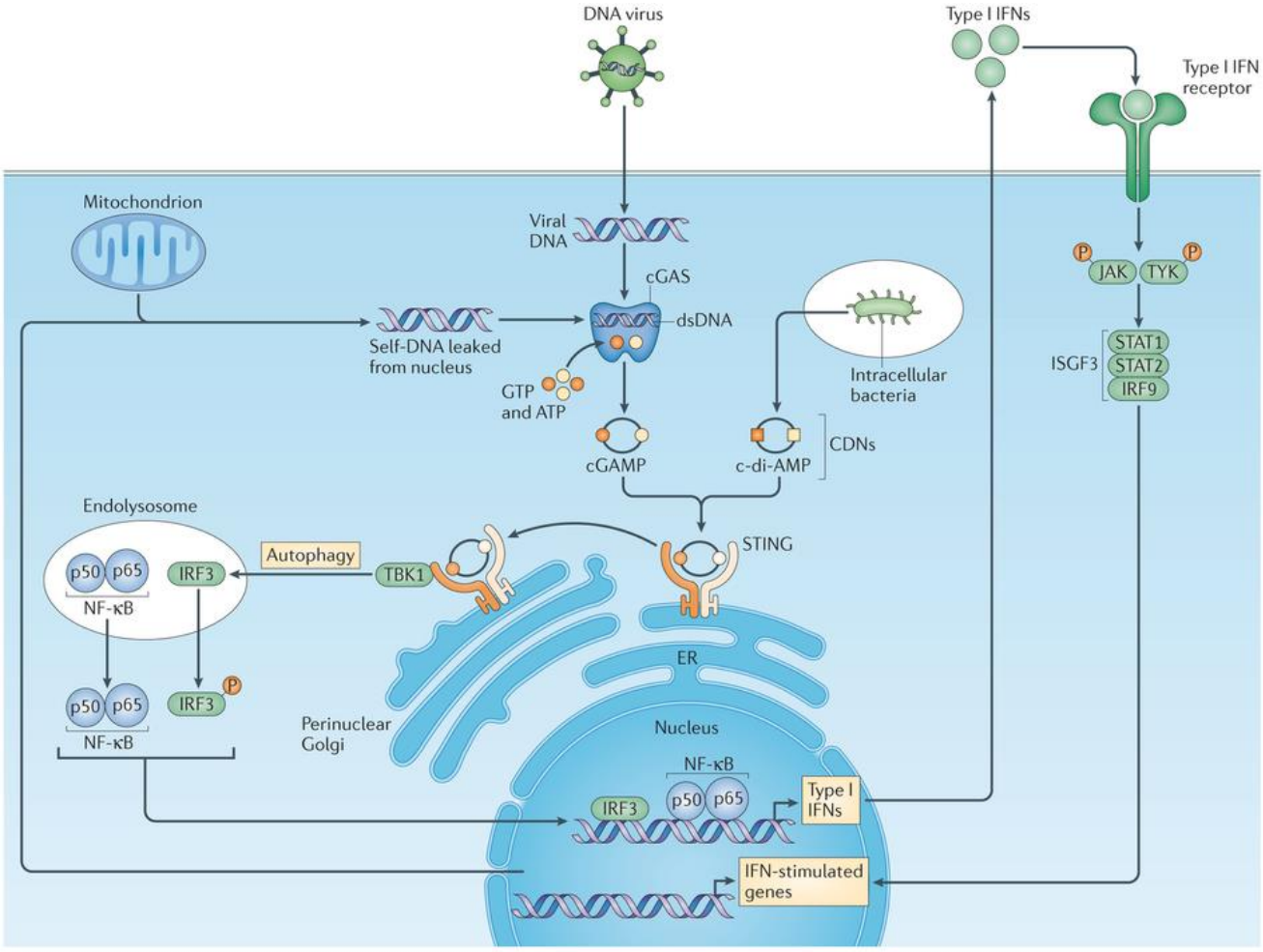

Figure 1.2: STING signaling cascade [30].

1.1.1.2 Innate Immune Responses after Danger Signaling

As mentioned before, recognition of PAMPs and DAMPs via PRRs by innate immune cells leads to inflammation. Inflammatory responses after pattern recognition are further amplified by autocrine-paracrine recognition of secreted inflammatory cytokines and chemokines [2]. These cytokines include pro-inflammatory cytokines such as TNF-a, IL-1beta, and IL-6, and anti-viral cytokines such as type I interferons. The pro-inflammatory cytokines promote inflammation by means of activating lymphocytes, vascular endothelium, increasing vascular permeability and fluid drainage to lymph nodes [31]. Type I interferons on the other hand, help defend against viral infections by inducing a cellular cascade that results in the degradation of viral RNA, and inhibition of viral replication, and proliferation of infected or adjacent cells [32]. Besides the production of cytokines, PRR activation in macrophages and DCs also leads to induction of co-stimulatory molecules, which enables antigen presentation forming a link between innate and adaptive immunity. CD80 and CD86 are two important co-stimulatory molecules, which are recognized by co-stimulatory receptors expressed by adaptive immune cells [33].

1.1.1.3 Macrophages

Macrophages are phagocytic cells that can be found in almost all tissues. Because they are high in number and they show superior phagocytic activity as compared to other immune cells, they provide a first line of innate immune defense against pathogens [34]. Besides their phagocytic activity, they play a crucial role in the initiation of inflammation by releasing cytokines and also take role in the amplification of adaptive responses by promoting antigen presentation to T cells [35].

1.1.1.4 Dendritic Cells

Dendritic cells (DCs) are the professional antigen-presenting cells (APCs) of the innate immunity. There are two types of DCs: conventional DC (cDC), and plasmocytoid DC (pDC) [36]. The main function of cDCs is to process and present the antigens to T cells of adaptive immunity. They acquire these antigens via phagocytosis of pathogens or infected cells [37]. This makes them an important bridge between innate and adaptive immunity. pDCs on the

other hand, are specialized to produce type I interferons [38]. These two types of DCs can be distinguished with different cell-surface markers and transcription factors.
When a cDC encounter pathogen in the peripheral tissues, it is activated by PAMPs. This activation leads to processing of antigens, expression of co-stimulatory molecules and migration to lymphoid tissues, where naïve T cells reside. In the lymphoid tissues, DCs can prime both CD4 and CD8 T cells via their MHC class I, MHC class II, and co-stimulatory molecules. This priming leads to initiation of adaptive immune responses [38, 39].

1.1.2 Adaptive Immunity

While PRR recognition of PAMPs provides limited specificity to innate immune system, the adaptive immune system cells are equipped with a variety of antigen specific receptors, which results in more specificity and sensitivity [40]. There are two major classes of adaptive immune cells: B lymphocytes or B cells, and T lymphocytes or T cells. B cells are the source of antibodies, whereas T cells require the peptides from pathogens to be presented o MHC class I or MHC class II molecules by infected cells or APCs. T cells are further divided into 2 types: CD8 (cytotoxic) T cells, and CD4 (helper T cells). Once activated, CD4 T cells are further divided into subsets based on the signals they receive [2, 40, 41].
Adaptive immune responses are initiated in peripheral lymphoid tissues, where T and B cells can be activated by APCs or helper T cells. After activation they start losing signals for their homing to lymphoid organs, they exit the lymph nodes, and they clear the host of pathogens in an antigen specific manner [40, 42].

1.1.3 Integrated Immune Response

Immune system of the host resists infections in several ways. First step is the innate immune defenses, which is a very rapid process and involves the recognition of the pathogens with limited specificity via PRRs [3]. However, some pathogens are able to evade the innate immune defenses. In this case, the specialized cells of innate immunity should initiate the adaptive responses, which require several days to develop due to their requirement for antigen presentation, selection of right T and B cell clones and proliferation of antigen specific B and T cells [40]. Meanwhile, innate lymphoid cells (ILCs) and NK cells take the stage as they are intermediate between innate and adaptive cells and have unique mechanisms for recognizing

stresses and infected cells and also helping activation of other innate and adaptive immune cells by production of various cytokines [43].

Both subsets of T cells of the adaptive immunity require priming by DCs in lymphoid tissues to get activated. For this, CD4 T cells require the antigens to be presented on MHC class II molecules, whereas CD8 T cells require them to be presented on MHC class I molecules. The peptide antigen:MHC complexes are recognized by TCRs and the co-receptors (CD4 and CD8) of T cells. For naïve T cells to be primed, the interaction between co-stimulatory molecules on DCs and co-stimulatory receptors on T cells is crucial as well. After a T cell is primed however, it does not require interactions with co-stimulatory molecules to recognize the antigen [44-47]. The other signal that is required for activation of T cells is the cytokines. Cytokines leads to differentiation of CD4 T cells into subsets such as: TH1, TH2, TH17, TFH, and Treg cells [46]. All of these subsets require different combinations of cytokines to be generated and take role in defense against different types of pathogens and immune modulation [48]. In contrast to several different subsets of CD4 T cells, CD8 T cell activation results only in effector cytotoxic T cell formation, which is specialized in target specific elimination of infected cells [49].

B cells recognize the antigens via their B cell receptor (BCR), and don't require APCs to present the antigens. They can both recognize the soluble antigens and antigens on antigen-presenting cells or on pathogens. However, after antigen recognition, they receive help from CD4 T cells to be able to expand and to produce higher affinity antibodies [50-52].

An effective and successful immune response, primed by innate immune responses, leads to a state of protective immunity. This state is achieved by the sensor cells, effector cells and molecules produced initially, and also immunological memory. This is enabled by the successful integration of innate and adaptive immune responses.

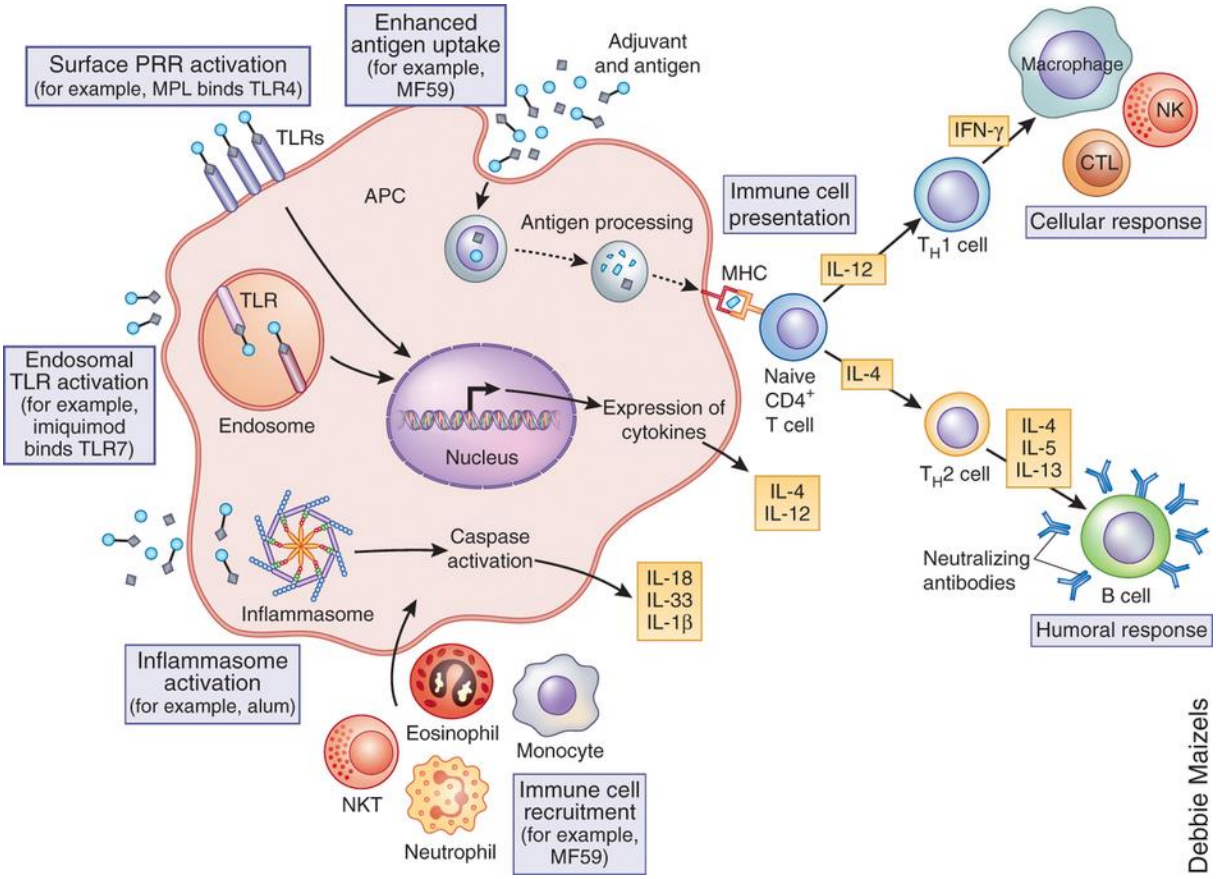

Figure 1.3: Integrated innate and adaptive immune responses [53].

1.2 Purinergic Signaling and Adenosine Receptors

As the name implies, purinergic signaling is a form of extracellular signaling induced by purine nucleotides or nucleosides such as ATP and adenosine. ATP, the energy currency of cells, can be released into extracellular environment from damaged or stressed cells. ATP can be absorbed by immune cells and activate inflammasome complex resulting in release of highly pro-inflammatory cytokine IL-1beta. Therefore, extracellular ATP acts as a danger signal and activates innate immune responses [54]. After its release to extracellular environment, ATP is hydrolyzed to ADP, AMP, and adenosine by ectonucleotidases such as CD39 and CD73. After this, extracellular adenosine is recognized by P1 receptors, which are specialized to sense adenosine [55, 56]. P1 receptors are GPCRs and divided into 4 subtypes: A1, A2A, A2B, and A3 receptors. A2A and A2B are G_s coupled receptors and their activation leads to accumulation of cAMP levels in the cell. A1 and A3 receptors are G_i coupled receptors and their activation decreases intracellular cAMP [56, 57]. The overall scheme for purinergic signaling can be found in figure 1.4.

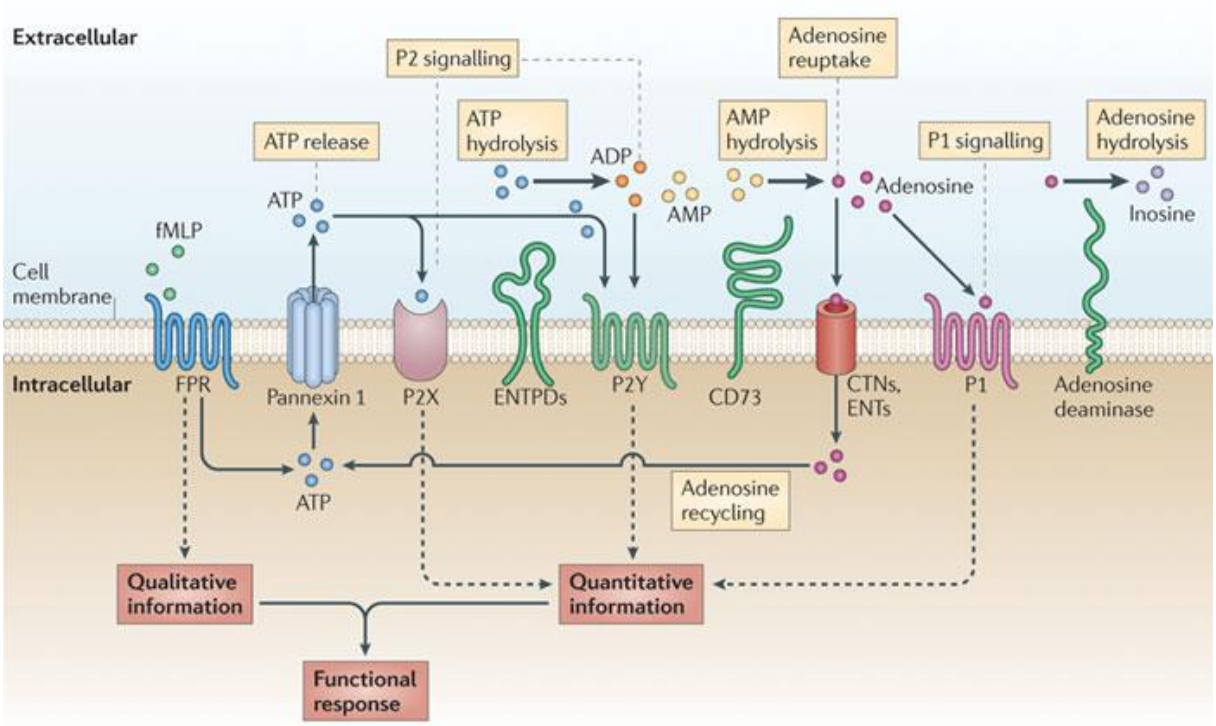

Figure 1.4: Purinergic signaling and its components [56]

1.2.1 Adenosine Signaling and Immune Response

Immune cells express adenosine receptors on their cell surface, which makes them responsive to extracellular adenosine. This effect of adenosine on immune cells has reported to suppress their activation. This suppressive effect is most clearly observed in inflammatory conditions [58]. It has been shown that adenosine affects the cytokine production from macrophages and DCs through mainly A2A and A2B receptors [59]. Adenosine decreases the secretion of TNF-a in macrophages, and shifts the cytokine profile from pro-inflammatory to anti-inflammatory phenotype in DCs [57]. In DCs adenosine suppresses the production of IL-12, while increasing the production of anti-inflammatory IL-10. This shift in cytokine responses results in the reduced capacity to induce TH1 cells, whereas an increased capacity to induce TH2 or Treg cells [57]. Besides macrophages and DCs, effect of adenosine on neutrophils was also observed. Adenosine inhibits the recruitment of neutrophils to the site of inflammation via altering the expression of adhesive molecules on their cell surface [57]. Apart from its ability to affect the lymphocytes through innate cells, adenosine can also directly affect the

lymphocytes. It was shown that adenosine exerts this effect mainly through A2A receptors [60]. Adenosine, by inhibiting IL-2 secretion, reduces the proliferation of activated CD4 T cells. It also suppresses the production of IL-4 and IFN-gamma produced by naïve or polarized CD4 T cells. It also results in upregulation of negative co-stimulatory molecules. Adenosine decreases IL-2 production by CD8 cells as well. It was shown that immunosuppressive Treg cells were dependent on adenosine to exert their immunosuppressive effects, as Tregs isolated from CD39 knock-out mice were not able to suppress proliferation of CD4 T cells [54, 56, 57, 60]. All these observations suggest that adenosine is generally viewed as an immunosuppressive molecule.

1.2.2 Therapeutic Targeting of Adenosine Receptors

Adenosine receptors are expressed by many tissues in a subtype-specific manner and play important roles in several different physiological processes. For instance, A1 and A2A receptors have a role in cardiovascular system such as reduction of heart rate or atrial contractility [61]. These receptors also have important effects on nervous system as they regulate the neurotransmitter secretion [62]. The most well-known antagonist for adenosine receptors to take role in the regulation of nervous system is caffeine. Besides these effects on several tissues, as described before, they take role in the regulation of several immune cells. Therefore, adenosine receptors are viewed as important drug targets for immune related conditions. The receptor types and targeting strategies to different types of diseases and conditions can be found in figure 1.5.

Other than the conditions summarized in figure 1.5, there is a great evidence that adenosine accumulates in tumor microenvironment and form an immunosuppressive shield against infiltrating immune cells while promoting angiogenesis [58]. Since adenosine receptors are also expressed on cancer cells, adenosine can directly regulate cancer survival and metastasis. These effects on cancer cells and tumor microenvironment make adenosine receptors a potential target or cancer therapies.

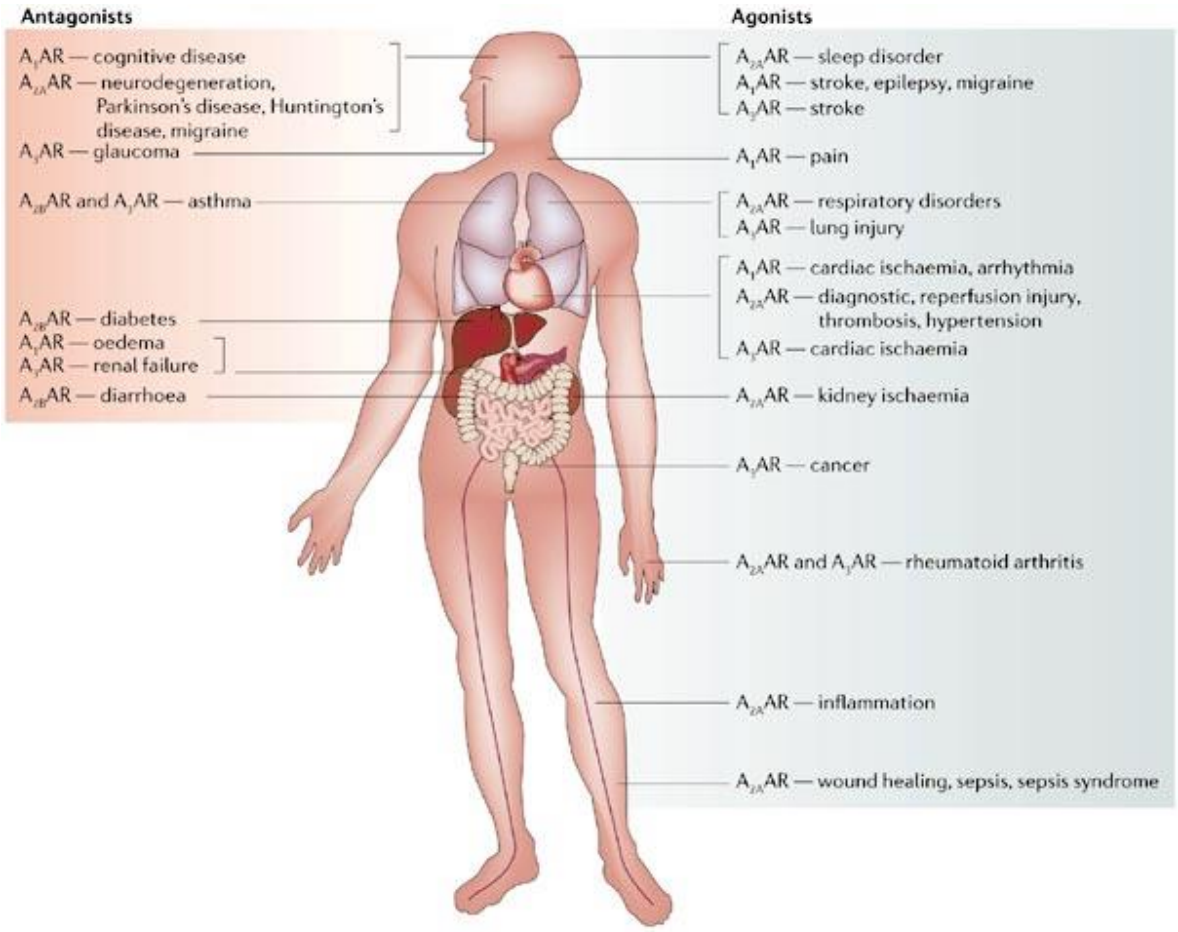

Figure 1.5: Adenosine receptor types related to different conditions [62].

1.3 Aim of the Study

In summary immunological danger signals come in different forms, can be from endogenous or exogenous resources, and recognized by antigen presenting cells (APCs) and phagocytes via their pattern recognition receptors (PRRs). PRRs recognize danger associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) [5,6]. They are found on cytoplasmic membrane, endosomal membranes or in the cytosol. Toll like receptors (TLRs) are a type of PRRs that can recognize a variety of PAMPs and can induce different responses via different adaptor proteins. These are MyD88, which leads to production of inflammatory cytokines, and TRIF that mainly leads to production of type-I interferons [12,15]. Other than TLRs, STING is a recently discovered DNA-sensing pathway that can induce expression of type-I interferons, when activated by the cytosolic second messenger

cGAMP [28]. ATP is released into extracellular environment from cells in stressed conditions, which makes it an indicator of stress and thus stimulates immune response. ATP is hydrolyzed to adenosine in the extracellular environment by CD39 and CD73 enzymes. Adenosine is known to act as an immunosuppressive metabolite as a negative feedback [54-56].

This thesis investigates the role of adenosine on different danger signaling pathways, particularly on TLR-2, TLR-3, TLR-4, TLR-9 and STING pathways. These receptors are chosen based on their locations and adaptor proteins they utilize to cause immunological responses. TLR-2 and TLR-4 are located on cellular membrane, whereas TLR-3 ad TLR-9 are located on endosomal membranes, and STING is a cytosolic sensor. TLR-2 and TLR-9 are MyDD8-dependent pathways, whereas TLR-3 is TRIF-dependent. TLR-4 is dependent on both MyD88 and TRIF. Although adenosine is known to suppress immune responses, basal level of adenosine in extracellular space is sufficient to activate all major adenosine receptors. Yet both innate and adaptive immune responses can take place even in the presence of extracellular adenosine suggesting that adenosine can be permissive to certain aspects of danger signaling. This study investigates if adenosine signaling particularly affects one danger signaling more than the other. Targeting aforementioned TLRs with known signaling patterns and using key cytokines as readouts downstream of these signaling pathways we tried to answer this important question, which also has very important implications in targeting adenosine receptors to develop novel immunotherapies or to formulate new vaccines.

Chapter 2

Materials, Solutions and Buffers

2.1 Materials

2.1.1 General Laboratory and Cell Culture Reagents and Materials

The materials for general usage and cell culture studies can be seen in Table 2.1. All plastic materials for cell culture usage such as flasks, multi-well plates and cryovials were purchased from Greiner Bio-One GmbH, Austria.

Material	**Catalog No**	**Brand**
ADA (Adenosine Deaminase)	10102105001	Roche, USA
HBSS	BE10-547F	Lonza, Switzerland
RPMI 1640 medium	21875-034	Gibco, USA
Sodium Pyruvate (100 mM)	S8636	Sigma, USA
Accutase	SCR005	Merck Millipore, Germany
Penicillin-Streptomycin	17-745E	Lonza, Switzerland
Trypsin	BE17-161E	Lonza, Switzerland
FBS	S181G-500	Biowest, USA
AccuGENE molecular biology water	BE51200	Lonza, Switzerland
DMSO	D12345	Life Technologies, USA
Trypan Blue	BI03-102-1B	Biological Industries, USA

Stericup-GP 0.22um Filter	SCGPU05RE	Merck Millipore, Germany
Freezing container, Nalgene® Mr. Frosty	NG-5100-0001	Nalgene, USA

Table 2.1: General materials used in laboratory and for cell culture.

2.1.2 PRR and Adenosine Receptor Ligands

Ligands used to stimulate PRRs and adenosine receptors can be seen in Tables 2.2 and 2.3. K ODN was a kind gift from Gürsel lab.

Material	**Catalog No**	**Brand**
Ultrapure LPS from Salmonella minnesota R595	tlrl-smlps	Invivogen, USA
Pam2CSK4	tlrl-pm2s-1	Invivogen, USA
Poly(A:U)	tlrl-pau	Invivogen, USA
2'3'-cGAM(PS)2 (Rp/Sp) (Sting ligand)	tlrl-cga2srs	Invivogen, USA
NECA	35920-39-9	TOCRIS Bioscience, Bristol, UK

Table2.2: PRR and adenosine receptor ligands used in experiments

Material	**Sequence**	**Brand**
K23-PS	TCGAGCGTTCTC	Alpha-DNA, Canada

Table2.3: K ODN used in experiments

2.1.3 BMDC and BMDM generation

Chemicals and other reagents used for BMDC and BMDM generation can be found in table 2.5

Chemical	**Catalog No**	**Brand**
Recombinant Mouse GM-CSF	576306	Biolegend, USA
Recombinant Mouse M-CSF	576404	Biolegend, USA
B-Mercaptoethanol		

HEPES solution	H0887-100ML	Sigma Aldrich, USA
L929 cell line		

Table2.4: Chemicals for BMDC and BMDM generation

2.1.4 ELISA

Materials and chemicals used for ELISA experiments can be found in table 2.6.

Chemical	**Catalog No**	**Brand**
Mouse IL-12 (p40) ELISA MAX™ Standard	431602	Biolegend, USA
Mouse TNF-α ELISA MAX™ Standard	430902	Biolegend, USA
Mouse IL-10 ELISA Kit with Pre-Coated Plates	431418	Biolegend, USA
Mouse IFN-β ELISA Kit with Pre-coated Plates	439408	Biolegend, USA
Nunc-Immuno™ MicroWell™ 96 well solid plates	M9410-1CS	Sigma Aldrich, USA
TMB substrate set	421101	Biolegend, USA

Table2.5: Chemicals and materials used for ELISA experiments

2.1.5 RNA Isolation, cDNA Synthesis, and qPCR

For RNA isolations, NucleoSpin® RNA kit (Cat. No: 740955.50) from Macherey-Nagel is used. cDNA from RNA is synthesized by High-Capacity cDNA Reverse Transcription Kit (Cat. No: 4368814) from Applied Biosystems, USA. SUPERase In™ RNase Inhibitor (Cat. No: AM 2694) was used from Life Technologies, USA as RNase inhibitor in cDNA synthesis experiments. Quality control of RNAs and cDNAs is done with the Thermo Scientific™ NanoDrop™. qPCR from cDNAs is performed by using TaqMan® Universal Master Mix II, no UNG (Cat. No: 4440040) from Thermo Scientific, USA. For some genes, SYBRgreen Fast Mix (Cat. No: P/N84067) from Quanta Biosciences, USA is used as well. Probes used for qPCR experiments can be found in table 2.7.

Chemical	**Catalog No**	**Brand**
Mm01308023_m1 (Adora1)	4331182	Life Technologies, USA
Mm00802075_m1 (Adora2a)	4331182	Life Technologies, USA
Mm00839292_m1 (Adora2b)	4331182	Life Technologies, USA

Mm01296602_m1 (Adora 3)	4331182	Life Technologies, USA
Mm00443258_m1 (TNFa)	4331182	Life Technologies, USA
Mm00434174_m1 (IL-12 p40)	4331182	Life Technologies, USA
Mm00439552_s1 (interferon beta 1)	4331182	Life Technologies, USA
Mm00439614_m1 (IL-10)	4331182	Life Technologies, USA
Mm00607939_s1 (beta actin)	4331182	Life Technologies, USA
Mm01300401_m1 (NR4A1)	4331182	Life Technologies, USA
Mm00443060_m1 (NR4A2)	4331182	Life Technologies, USA
Mm00450074_m1 (NR4A3)	4331182	Life Technologies, USA
CXCL10 primer	QT00093436	Qiagen, Germany
GAPDH primer	QT01658692	Qiagen, Germany

Table2.6: Probes used for qPCR experiments

2.1.6 Flow Cytometry

The antibodies used for flow staining can be found in table 2.8

Chemical	**Catalog No**	**Brand**
Mouse Anti-CD86 PE	12-0861-83	Ebiosciences, USA
Mouse Anti-CD80 APC		
Mouse Anti-MHCI AF647	116512	Biolegend, USA
Mouse Anti-MHCII PECy5		
Mouse Anti-CD16/CD32	14-061-85	Ebiosciences, USA
LIVE/DEAD® Fixable Green Dead Cell Stain Kit	L34970	Life Technologies, USA.

Table2.7: Antibodies used for flow cytometry experiments

2.2 Solutions and Buffers

2.2.1 Cell Culture Solutions

All solutions for cell culture are stored at 4 ^{o}C.

RPMI-1640 (with L-Glutamine)

10% FBS (heat inactivated at 55 °C and filtered)

5 ml Penicillin/Streptomycin

5 mL Na Pyruvate

Freezing Medium

10% DMSO in FBS (heat inactivated at 55 °C and filtered)

BMDC Medium

Complete RPMI

50 μM 2-mercaptoethanol

5 ng/ml GM-CSF

BMDM Medium

Complete 5% RPMI

10mM HEPES

10ng/mL M-CSF,

30% L929 conditioned medium.

ACK Lysis Buffer

8.3g NH_4Cl

1g $KHCO_3$

1L double distilled water

pH adjusted to 7,4

2.2.2. ELISA Buffers

Coating Buffer:

8.4g $NaHCO_3$

3.56g Na_2CO_3

1L ddH_2O

pH adjusted to 9.5

Stored at 4 ^oC.

Assay Diluent

10% FBS (Heat inactivated at 55 ^oC and filtered) in PBS

Stored at 4^0C

Wash Buffer

0.05% Tween20 in PBS

Stored at room temperature.

10X PBS (Phosphate Buffered Saline)

80 g NaCl

2 g KCl

15,2 g Na_2HPO_4. H_2O

2,4 g KH_2PO_4

1 L ddH_2O

pH adjusted to 7.4

Sterile filtered

Stored at room temperature.

Stop Solution

2N H_2SO_4 in dH_2O

Sterile filtered

2.2.3 Flow Cytometry Buffers

FACS Buffer

2% FBS and 0.25% Sodium Azide in HBSS

Stored at +4 °C

Chapter 3

Methods

3.1 Cell Culture Procedures

Cell Counting: After cells are collected via centrifugation, they are solved in appropriate volume of medium according to their confluency. Then 10 µl of this solution is mixed with 10 µl of tryphan blue. 10 µl of this mix is placed on hemocytometer and all 4 corners are counted. The cell number is found by dividing the result with 4, multiplying with 2, volume of medium (as mL) and 10^4.

Freezing of Cells: Cells should reach to an optimum confluency. They are washed twice with HBSS. Detached by appropriate method (Accutase/trypsin treatment or scraping). Collected into a 15-mL falcon and centrifuged at 1500 rpm for 5 minutes. Supernatant is aspirated and suspended in 1 mL FBS (heat inactivated, filtered). They are counted with hemocytometer and distributed into cryovials so that each cryovial will contain $5x10^6$ cells. The volume of the FBS in the vials are adjusted to 500 µl. Finally, 500 µl of 20% freezing medium is added into vials, so that the final concentration of DMSO will be 10% in freezing medium. The cryovials are placed in -80^{o}C freezer within the Mr. Frosty container. For long term storage, the vials are transferred to liquid nitrogen.

Thawing of Cells: The vials are placed in 37^{o}C water bath as soon as they are taken from liquid nitrogen tanks. They are thawed rapidly. Partially melted vials are solved with cold medium and transferred into 15-mL falcon containing cold medium. Centrifuged at 1500 rpm

for 5 minutes at 4ºC. Supernatant is aspirated and cells are solved in 37ºC medium and transferred to appropriate vial.

3.2 Stem Cell Isolation from Bone Marrow

Appropriate number of C57/BL6 mice are sacrificed. Femur and tibia are taken, placed into a falcon containing ice cold HBSS. One edge of the bones is cut and placed into 0.6 mL tubes with wholes at the bottom, the cut edge of the bones facing the whole. These 0.6 mL tubes are placed in 1.5 mL tubes. Centrifuged at 7000 rpm for 30 seconds. The cells collected at the bottom of 1.5 mL tube are suspended with 0.5 mL HBSS and transferred into 50 mL falcons. 3 mL of ACK lysis buffer is added and incubated for 3 minutes at room temperature. The rest of the falcon is filled with HBSS and centrifuged at 1200 rpm for 5 minutes. Supernatant is aspirated and cells are solved in appropriate volume of appropriate medium. They are counted and used either for BMDC or BMDM generations.

3.3 Generation of Bone Marrow Derived Dendritic Cells

On day 0, After stem cells isolated from bone marrows are counted, they are placed in bacterial petri dishes in 10 mL of BMDC medium containing $2x10^6$ cells. They are placed in 37ºC cell culture incubators. On days 3 and 8, the dishes are supplemented with further 10 mL of BMDC medium. On day 6, 10 mL from the dishes are taken into 50 mL falcons and centrifuged at 1200 rpm for 5 minutes. Supernatant is aspirated, cells are solved in new 10 mL BMDC medium and placed into dishes again. On day 10, the suspension cells and poorly adherent cells are collected by pipetting. They are taken into 50 mL falcons and centrifuged at 1200 rpm for 5 minutes. After that cells can be used in an experiment or can be frozen. Some of the cells are taken for quality control and stained with CD11b, CD11c, Gr1, Live-dead antibodies according to flow cytometry protocol.

3.4 Generation of Bone Marrow Derived Macrophages

On day -1, after the stem cells isolated from a mouse, they are placed in a cell culture dish in 10 mL of BMDM medium without L929 medium addition. The dish is placed in a 37ºC cell

culture incubator. On day 0, 15 mL BMDM medium that contains 50% L929 medium is mixed with the medium that contains non-adherent cells from day -1. This mixture is distributed into 6-well low attachment plate as 4 mL per well. On days 3 and 5, 1.5 mL of BMDM medium is added to wells. On day 7, the cells are collected into 50 mL falcons from the wells by pipetting. They are washed twice with HBSS by centrifugation at 1200 rpm for 5 minutes. They are either frozen or used in an experiment after they are pre-incubated overnight.

3.5 Cell treatments for ELISA and Flow Cytometry

Fresh or thawed cells are seeded into 96-well plates in 100 µl RPMI medium. For DCs, 300.000 cells per well is used, whereas for macrophages 100.000 cells per well is used. Cells are pre-incubated for 2 hours and the medium is discarded. 100 µl of fresh medium with ADA (1 unit) is added into wells. Cells are incubated for 1 hour. Cells are stimulated with 50 µl medium that contains PRR ligands and 50 µl medium that contains NECA. For wells that do not contain NECA, medium containing same volume of DMSO is added. Cells are incubated overnight. After that, the plate is centrifuged at 1000 rpm for 5 minutes. Supernatant is taken into new plate and it is either used in ELISA experiment or stored at -20^{o}C. The cells are detached by cold HBSS and Accutase treatment and used in flow staining.

3.6 Cell treatments for qPCR

Fresh or thawed cells are placed into 50 mL falcon in 20 mL RPMI medium. They are pre-incubated for 2 hours while the lid of the falcon is partially closed and placed inclined. They are centrifuged at 1200 rpm for 5 minutes. The medium is aspirated and cells are solved in fresh medium that contains 1 unit ADA. They are distributed into round bottom polystyrene tubes in 200 µl medium so that each tube contains $1x10^6$ cells. They are incubated for 1 hour. Cells are stimulated with 100 µl medium that contains PRR ligands and 100 µl medium that contains NECA. For tubes that do not contain NECA, medium containing same volume of DMSO is added. They are incubated for appropriate times. After that the tubes are placed on ice and washed twice with HBSS by centrifugation at 1400 rpm for 5 minutes. They are lysed with 350 µl of RNA isolation kit component RA1 buffer. 3.5 µl of B-mercaptoethanol is

added into tubes. After that either RNA isolation is performed or the samples are stored at -80°C.

3.7 RNA Isolation with NucleoSpin® RNA kit

Isolation is done according to manufacturer's guide. The only exception is the last step, which is done in 2 steps by using 20 µl of nuclease free water in each step. Thus the final volume in which the RNA eluted is 40 µl. The isolated RNAs are kept at -80°C until the cDNA synthesis is done.

3.8 cDNA Synthesis

cDNA synthesis is done with the High-Capacity cDNA Reverse Transcription Kit. Master mix ingredients and volumes can be seen in table 3.1.

2X Master mix (for 1 reaction)	
Components	**Volume (µl) per Reaction**
10X RT Buffer	2
25X dNTP Mix (100mM)	0,8
10X RT Random Primers	2
Reverse Transcriptase	1
Rnase Inhibitor	1
Nuclease-free Water	3,2
Total per Reaction	10

Table 3.1: Ingredients and volumes of 2X master mix for cDNA synthesis per reaction.

After the master mix is prepared, 10 µl of master mix is distributed into PCR tubes. 10 µl of RNA sample is added into corresponding tube. The tubes are spun down. Then they are placed in PCR machine and the program seen in table 3.2 is applied. After the program is

completed, quality assessment is done and they are diluted 1:5 with nuclease free water. For long term storage, they are placed at -20ºC.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25	37	85	4
Time	10 min	120 min	5 min	∞

Table 3.2: Program for cDNA synthesis

3.9 Quantitative-Real Time-PCR

To measure the relative mRNA expressions, Q-RT-PCR is used. For most of the genes Taqman probes are used. The ingredients for master mix prepared for taqman probes can be seen in table 3.3. For SYBR green mixture, see table 3.4

Components	**Volume per 10 μl Reaction (μl)**
20X Taqman Gene Expression Assay	0.5
2X Taqman Gene expression Master Mix	5
cDNA Template	2
RNase free water	2.5

Table 3.3: Ingredients and volumes of master mix for taqman probes

Components	**Volume per 10 μl Reaction (μl)**
20X Taqman Gene Expression Assay	1
2X PerfeCTa SYBR Green FastMix	5
cDNA Template	2
RNase free water	2

Table 3.4: Ingredients and volumes of master mix for SYBR green protocol

The mix is prepared without the cDNA template and 8 µl of it is distributed into the wells of PCR plate. cDNA is added into corresponding wells. The plate is sealed and centrifuged at 1000 rpm for a minute. Then it is placed in PCR machine. For taqman probes, the program seen in table 3.5 is applied. For SYBR green, the program seen in table 3.6 is applied.

Step	**Temperature (°C)**	**Duration**	**Cycle**
Initial Denaturation	95	5 min	1x
Denaturation	95	10 secs	50x
Annealing	60	10 secs	
Elongation	72	15 secs	
Final Extension	72	5 min	1x
Hold	4	∞	

Table 3.5: Program for Q-RT-PCR with taqman probes

Step	**Temperature (°C)**	**Duration**	**Cycle**
Initial Denaturation	95	3 min	1x
Denaturation	95	15 secs	40x
Annealing	60	60 secs	
Final Extension	72	5 min	1x
Hold	4	∞	

Table 3.6: Program for Q-RT-PCR with SYBR green

3.10 ELISA

IFN-b and IL-10 detections: ELISA protocols are done according to manufacturer's guide.

TNF-a and IL-12 detections: ELISA protocols are done according to manufacturer's guide with few exceptions. Coating and detection antibodies, TMB substrate and stop solution is

used 50 µl per well instead of 100 µl. The highest standard concentration is 1000 pg/mL instead of 500 pg/mL, which is followed by 6 two-fold serial dilutions.

3.11 Flow Cytometry

All steps of flow cytometry protocol are performed on ice. Cells are taken into v-bottom plates. They are washed twice with 200 µl FACS buffer by centrifugation at 1400 rpm for 5 minutes. Samples are resuspended in 50 µl FACS buffer containing 1:50 diluted Fc block (Anti-CD16/CD32 antibody). They are incubated at dark for 5 minutes. 50 µl FACS buffer containing 1:200 diluted antibody cocktail is added. Samples are incubated for 30 minutes at dark. They are washed with 200 µl FACS buffer by centrifugation at 1400 rpm for 5 minutes. Samples are suspended in 150 µl FACS buffer and transferred into flow tubes. 120 µl of each sample is read.

Chapter 4

Results

4.1 Adenosine Receptors Expressions with or without the Danger Signals in BMDCs

Previous studies including ours have demonstrated the importance of adenosine receptor signaling in antigen presenting cells. In this study we aimed to test if the effect of adenosine on antigen presenting cells changes based on the nature of danger signaling. For this aim we used bone marrow-derived dendritic cells (BMDCs) as a primary professional antigen presenting cells type because: 1. These cells can represent inflammatory dendritic cells while having antigen cross-presentation capability, 2. Very high numbers of these cells can be obtained to do biochemical and cellular analysis [63, 64]. To mimic adenosine receptor signaling we used NECA, a cell impermeable and stable adenosine analog [65]. This way we were sure the effects we observed is not through adenosine's potential effects through other intracellular pathways than receptor signaling. We pretreated the cells with the enzyme ADA (Adenosine deaminase), which breaks done the adenosine molecules, to eliminate the effect of endogenous adenosine. As danger signals we used 1. ultrapure *S. Minnesotta* LPS, which activates only TLR4 [66], which occurs especially during gram negative bacterial infections or during tumor release of HMGB1, an endogenous signal for TLR4 [67], 2. PAM_2CSK_4 (PAM) a synthetic peptide that mimics lipoproteins on both gram positive and gram negative bacteria and activates TLR2 pathway [68], 3. Stable cGAMP which activates STING/IRF3/type I interferon pathway during auto-inflammatory or sterile inflammatory conditions as a danger signal [69], 4. CpG DNA, which is prevalent among bacterial DNA and activates TLR9 [70]. In some experiments we used pA:U, which is a TLR3 ligand and activates only the TRIF pathway [71]. For the experiments in which pA:U was used, we had

to switch to bone marrow-derived macrophage (BMDM) system because BMDCs were not responsive to pA:U. Before testing the effect of adenosine signaling on these danger signals we optimized the concentrations of these chemicals. We performed dose response experiments to see the optimum dose in which the danger signals can lead to highest secretion of their corresponding cytokines. We saw that the following concentrations were optimum for these chemicals in BMDCs: LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, pA:U: 30 μg/ml, and NECA: 3 μM (Data not shown). Next we tested the expression of all adenosine receptor types in response to danger signals in the presence or absence of NECA in BMDCs to test what adenosine receptor subtypes are affected by danger signaling and if the presence of adenosine influences the expression of adenosine receptor subtypes. Our results indicated that A2A, A2B, A3 but not A1 adenosine receptors are expressed in BM-DCs (Figure 4.1). All the danger signals were able to increase expression of both A2A and A2B receptor. Among all the danger signals the ones activating the cell surface TLRs, TLR4 and TLR2, were the most potent in increasing adenosine receptor expression (Figure 4A. and B.). A3 receptor expression did not change after BMDC stimulation. Addition of NECA slightly increased especially A2B receptor expression after LPS, PAM and CpG but not the other adenosine receptors. NECA alone did not have any significant effect on adenosine receptor expression (Figure 4.1).

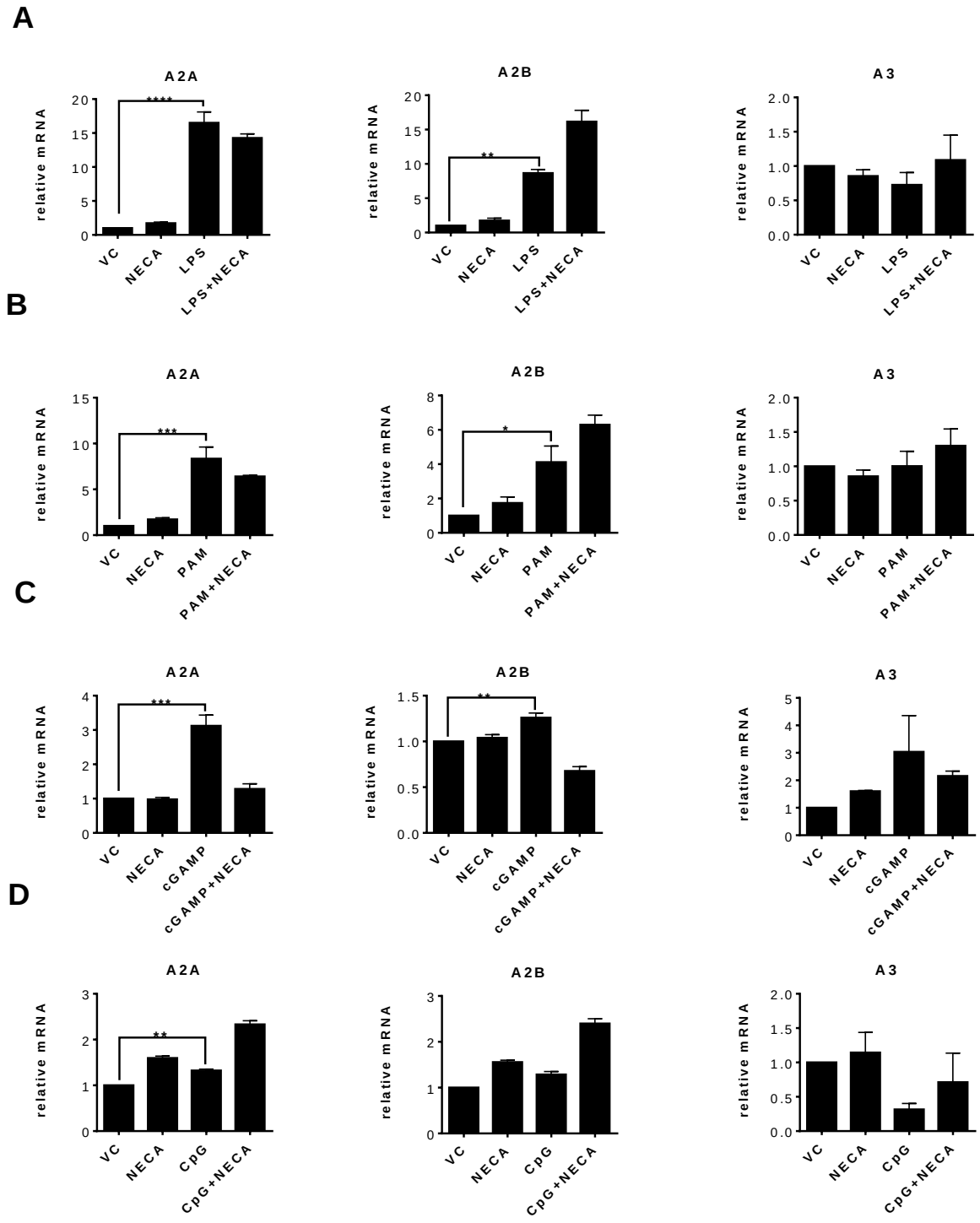

Figure 4.1: Danger signals increase the expression of A2A and A2B receptors. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for 3 hours (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). mRNA quantification is done with taqman probes. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.2 Immunomodulatory Effects of Adenosine on BMDCs

After the investigation of the effect of danger signaling on adenosine receptor expression in BMDCs, we tested the effect of adenosine on cytokine secretion in BMDCs. We used TNF-alpha and IL-12p40 as immuno-modulatory and pro-inflammatory cytokines as readouts. We also tested IFN-beta, and IP-10 as an indication for activation of Type-I interferon pathway downstream of LPS, cGAMP and CpG signaling but not PAM signaling since TLR2 activation weakly stimulates type-I interferon production and TLR2-induced IP-10 expression does not rely on TRIF or IRF3 signaling [72]. IL-10 was tested to show the effect of adenosine receptor singling on generation of anti-inflammatory phenotype.

4.2.1 Effect of Adenosine on Pro-Inflammatory Cytokine Secretion

We observed a significant suppression by adenosine of both TNF-alpha and IL-12p40 secretion with the danger signals associated with MyD88. Interestingly, no significant modulation of these cytokines by adenosine is observed with cGAMP signaling, which primarily stimulates IRF3 signaling suggesting that adenosine receptor signaling may more strongly affect MyD88-dependent outcomes. (Figure 4.2).

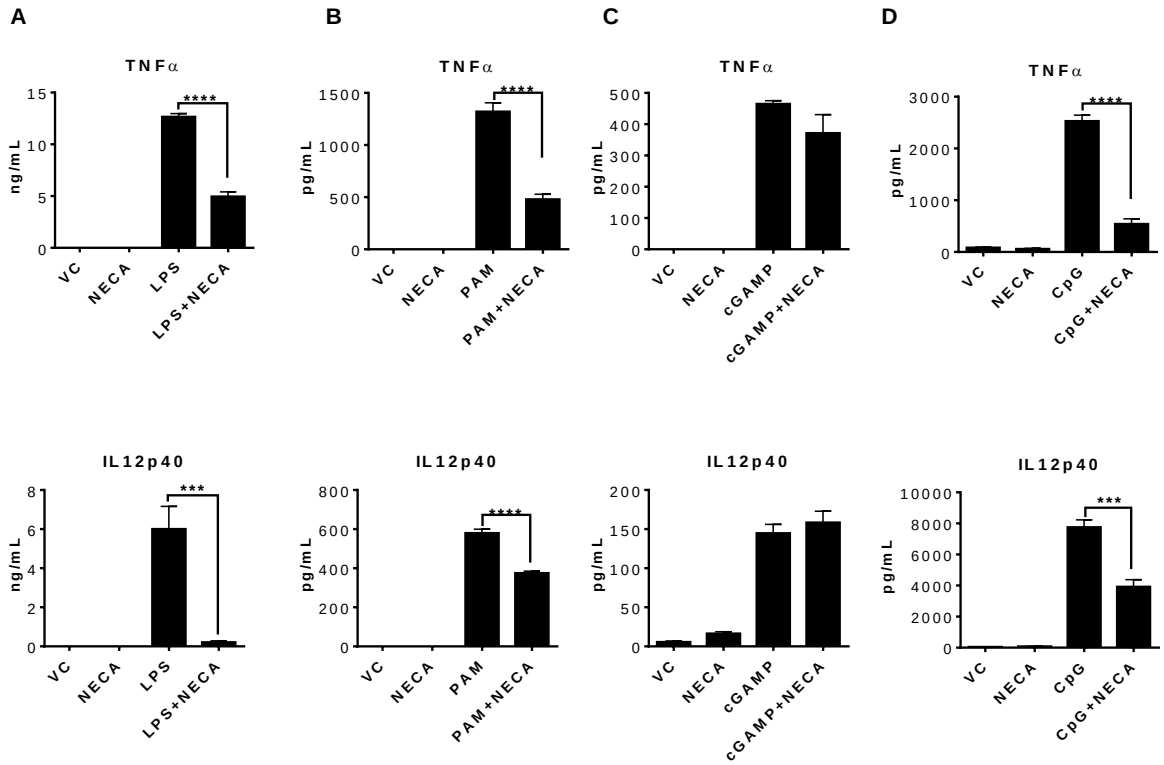

Figure 4.2: Adenosine receptor signaling decreases the secretion of pro-inflammatory cytokines in the presence of danger signals associated with MyD88 adaptor proteins. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for overnight (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). Cytokine secretion is detected from media. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.2.2 Effect of Adenosine on Type-I Interferon Cytokine Secretion

After pro-inflammatory cytokines, we next tested the modulation of Type-I interferon pathways using IFN-beta and IP-10 as a readout because secretion of these cytokines are mediated mostly by MyD88-independent fashion except for IP-10 downstream of TLR2 as indicated earlier. CpG DNA also use MyD88 as an adaptor but it can activate IRF7 signaling and downstream type I interferon responses as being an endosomal danger sensor [26]. Our results indicated that adenosine receptor signaling decreased IFN-beta and IP-10 secretion, but it was not as strong as that of pro-inflammatory cytokines (Figure 4.3A). TLR2 activation by PAM activates only the MyD88-dependent pathway on the cell surface of dendritic cells; therefore, fails activating type I interferon pathway [73]. Accordingly, Figure 4.3B shows no increase in IFN-beta secretion after TLR2 activation. However, TLR2 strongly increased IP-10 secretion and this was suppressed by adenosine receptor signaling. cGAMP increased both IFN-beta and IP-10 and this increase is not affected by adenosine receptor stimulation (Figure 4.3C). Similarly, CpG DNA increased both IFN-beta and IP-10 secretion and this was not influenced by adenosine receptor stimulation (Figure 4.3D). Overall these results suggest that the effect of adenosine receptor signaling on pathways causing type I interferon activation is not as prominent as that of pro-inflammatory cytokines.

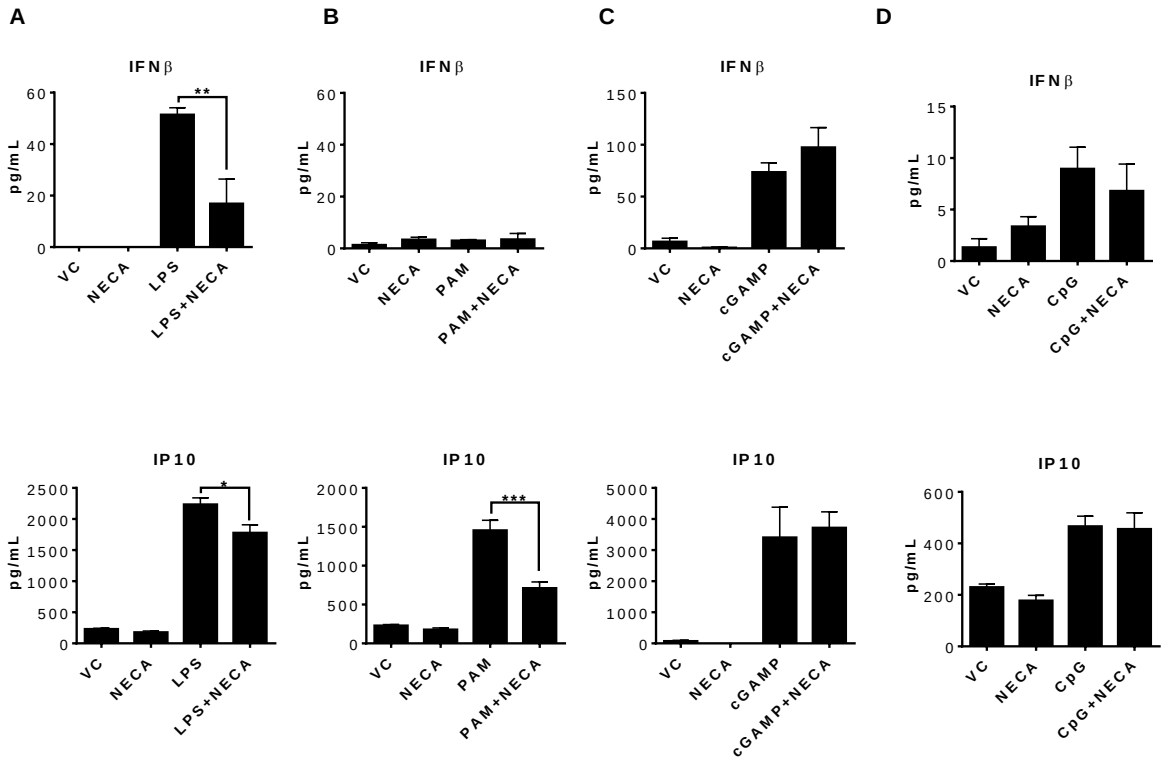

Figure 4.3: Adenosine receptor signaling decreases the secretion of Type-I Interferons in the presence of danger signals associated with MyD88 adaptor proteins. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for overnight (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). Cytokine secretion is detected from media. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.2.3 Effect of Adenosine on Anti-Inflammatory Cytokine Secretion

After testing the modulation of pro-inflammatory cytokines and Type-I interferons by adenosine, we next tested the effect adenosine signaling on secretion of anti-inflammatory cytokine IL-10. Addition of NECA with all MyD88-coupled danger signals significantly increased IL-10 secretion (Figure 4.4A, 4.4B, 4.4D). However, adenosine receptor signaling significantly decreased IL-10 secretion by cGAMP. This data again suggests that adenosine signaling polarize DCs into an anti-inflammatory phenotype when there is MyD88-coupled danger signals, whereas it may not have such an effect on IRF3 related pathways such as cGAMP signaling (Figure 4.4C).

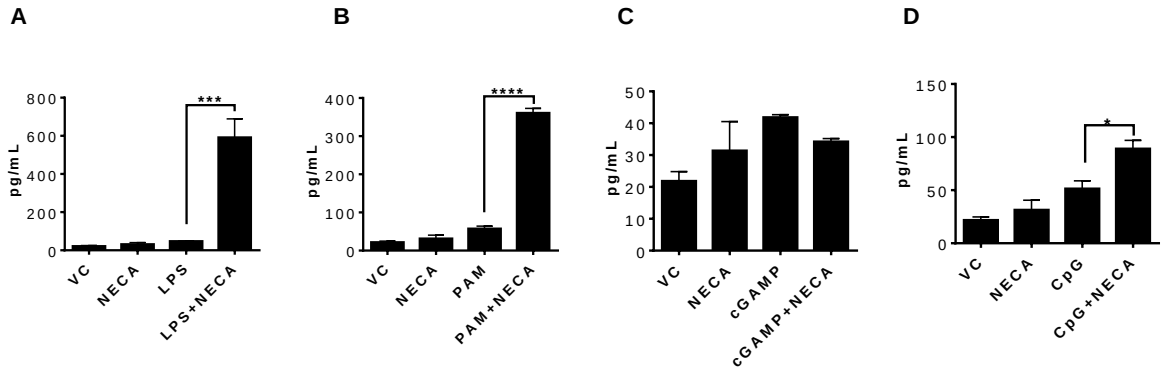

Figure 4.4: Adenosine receptor signaling increases IL-10 secretion in BMDCs stimulated with danger signals that can activate MyD88-dependent signaling events. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for 6 hr. (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). Cytokine secretion is detected from media. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.2.4 Effect of Adenosine on cell surface activation markers on DCs

Since DCs are professional APCs, their cell surface co-stimulatory molecule expression profile should be observed to assess their antigen presenting capacity. We tested the changes in both CD80 and CD86 expression after the activation of DCs with different danger signals in the presence or absence of NECA by flow cytometry. A significant decrease in CD86 is observed in the presence of NECA when MyD88-coupled danger signals were present. Interestingly, an increase in CD80 expression is observed in same conditions. In contrast, an increase in CD86 levels is observed after addition of NECA and cGAMP. (Figure 4.5).

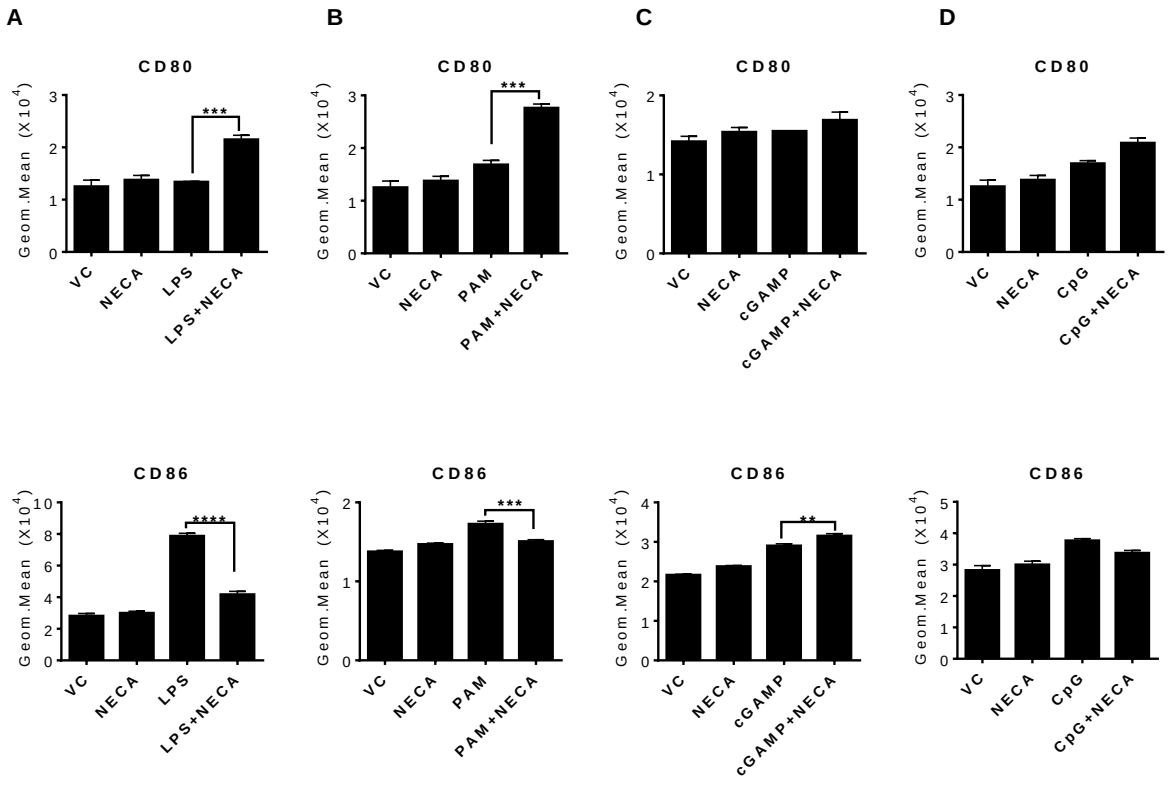

Figure 4.5: Adenosine receptor signaling modulates the expression of co-stimulatory molecules in the presence of danger signals. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for overnight (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). Expression of co-stimulatory molecules is detected by flow cytometry. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

Besides the co-stimulatory molecules, MHC-I and MHC-II molecule expressions are crucial for antigen presentation. They are the molecules onto which antigens are loaded by intracellular machinery and presented on the cell surface. Any effect on their expression severely impacts their antigen presentation capacity. Therefore, we tested the expression of these molecules in the presence or absence of NECA and danger signals by flow cytometry. Addition of NECA almost completely reversed the increased expression of MHCI and MHCII after danger signals associated with MyD88. No decrease or increase is observed in the presence of NECA and cGAMP as compared to cGAMP-only group. (Figure 4.6). These results suggest that presence of adenosine decreases the ability of BMDCs to present antigen

when they are primed by MyD88-dependent danger signals but the presence of adenosine has no effect on the antigen presentation ability of BMDCs if they were primed with cGAMP.

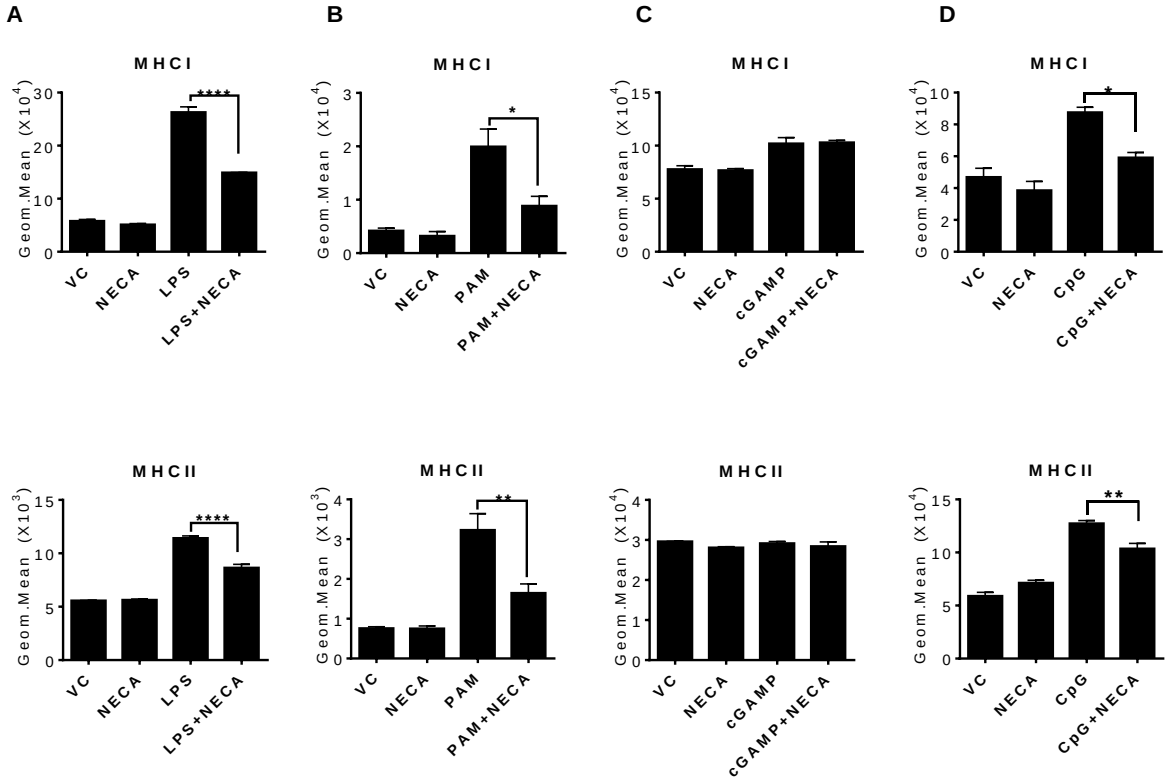

Figure 4.6: Adenosine receptor signaling decreases the expression of activation markers in the presence of danger signals associated with MyD88 adaptor proteins. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for overnight (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). Expression of co-stimulatory molecules is detected by flow cytometry. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.3 Effects of Adenosine on BMDCs at Transcriptional Level

Since we tested the modulation of cytokine secretion by adenosine, we also wanted to test the impact of adenosine signaling on expression of these cytokines at mRNA level. This is because adenosine has been shown to regulate expression post translationally. We tested TNF-alpha and IL-12p40 as pro-inflammatory cytokines, IFN-beta and IP-10 as a Type-I interferon readout, and IL-10 as an anti-inflammatory cytokine profiling. Other than cytokines, we tested the expression of NR4A1 because it is suggested as one of the

mechanisms through which adenosine regulates immune cells [74]. Therefore, it is important to know if changes in adenosine regulation depending on the danger signaling are also correlated with NR4A1 expression.

4.3.1 Effect of Adenosine on mRNA Expression of Pro-Inflammatory Cytokines

All danger signals strongly increased TNF-alpha transcript levels. Adenosine signaling could significantly reduce TNF-alpha mRNA after LPS and PAM stimulation (Figure 4.7 A and B). Adenosine signaling could also significantly reduce the expression of IL-12p40 after LPS and CpG treatment. IL12p40 mRNA expression did not significantly change after addition of NECA and cGAMP or PAM as compared to cGAMP or PAM only group, respectively. These results suggest that Adenosine receptor signaling may regulate cytokine secretion at both transcriptional and post-transcriptional level.

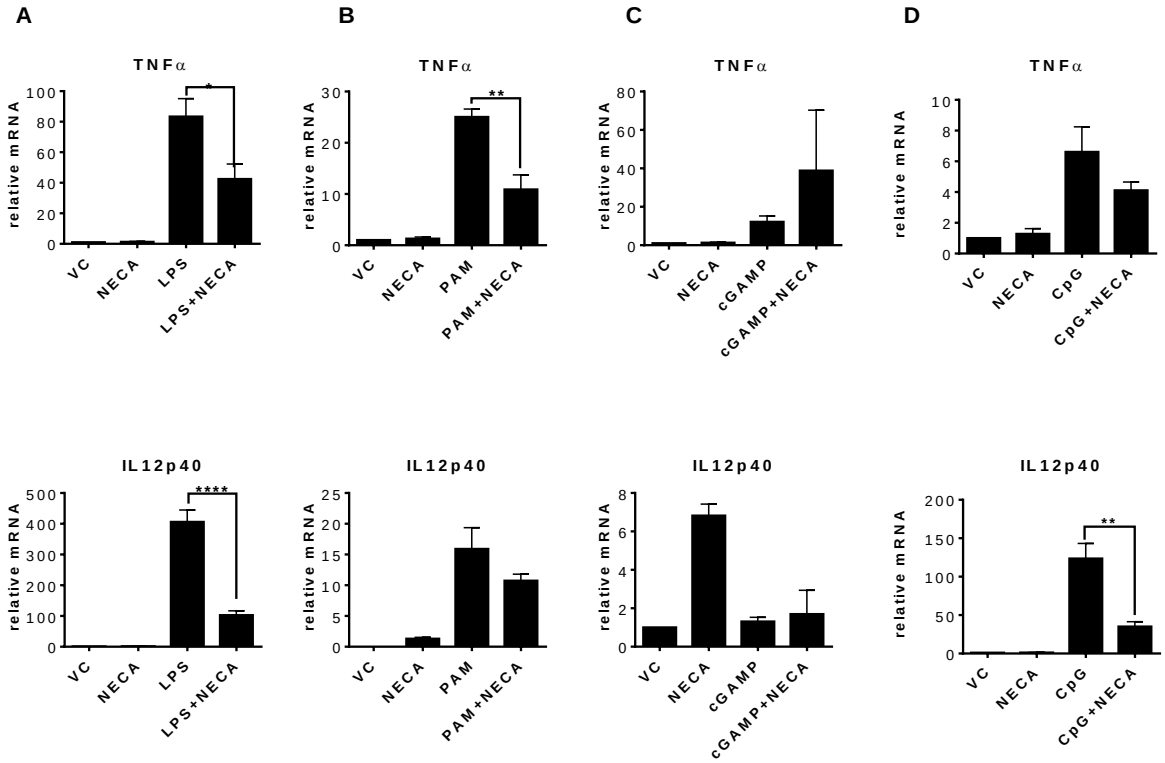

Figure 4.7: Adenosine receptor signaling decreases the mRNA expression of pro-inflammatory cytokines in the presence of danger signals associated with MyD88 adaptor proteins. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for 3 hours (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). mRNA quantification is done with taqman probes. For statistical analysis, one-way ANOVA was used. **

*p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.3.2 Effect of Adenosine on mRNA Expression of Type-I Interferons

When we have tested cytokine secretion profile we have observed that only after LPS stimulation NECA addition significantly decreased IFN-beta secretion. Secretion of the other Type-I interferon-related cytokine IP-10 was not affected by the addition of NECA in the presence of all the danger signals except PAM (Figure 4.3). At mRNA level we observed no change in IFN-beta expression after NECA addition in the presence of danger signals as compared to danger signal only groups. (Figure 4.8, upper panel). Similarly, addition of NECA along with any danger signals tested did not change IP10 mRNA level as compared to danger signal alone (Figure 4.8, lower panel). These results confirm the observation that Type-I interferon pathway may not be strongly regulated by adenosine receptor signaling.

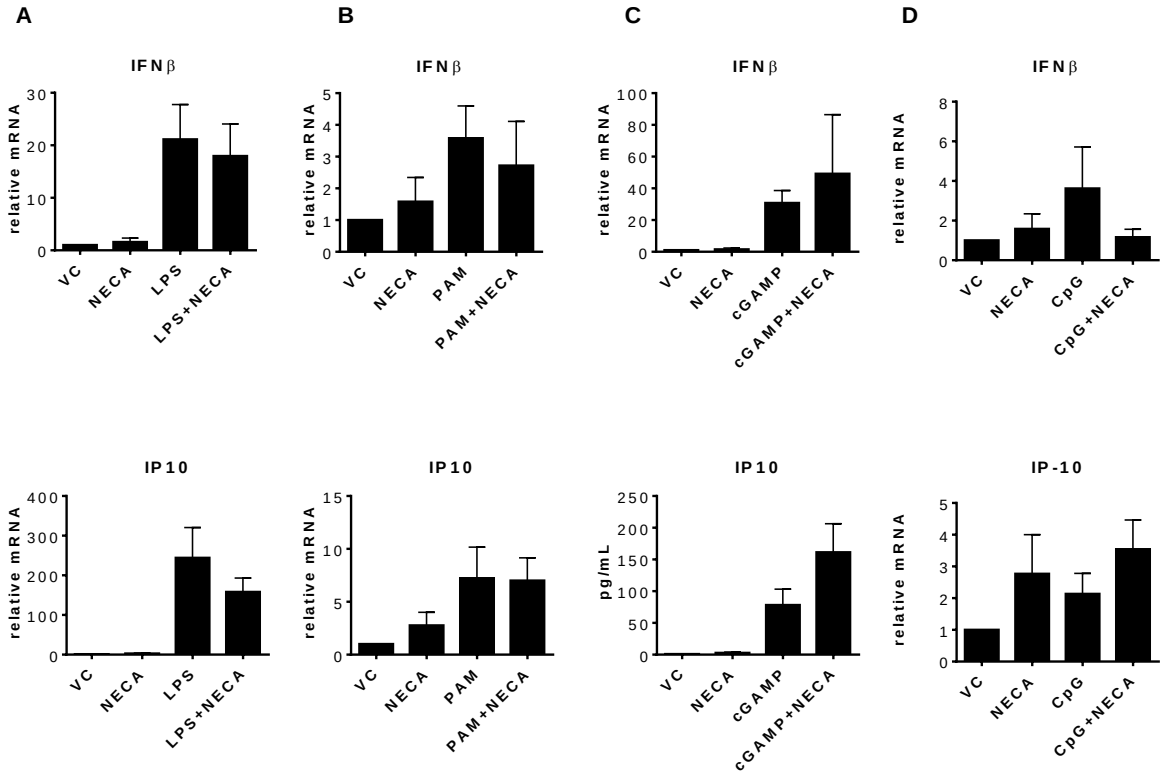

Figure 4.8: Adenosine receptor signaling has no effect on the mRNA expression of type-I Interferons in the presence of danger signals. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for 3 hours (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). mRNA quantification is done with taqman probes. For*

*statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.3.3 Effect of Adenosine on mRNA Expression of Anti-Inflammatory Cytokines

Consistent with ELISA results, we observed an increase in IL-10 mRNA expression in the presence of NECA when only MyD88-coupled receptors are activated (Figure 4.9 A,B,D). The difference was particularly significant when NECA added with LPS or CpG oligonucleotides.

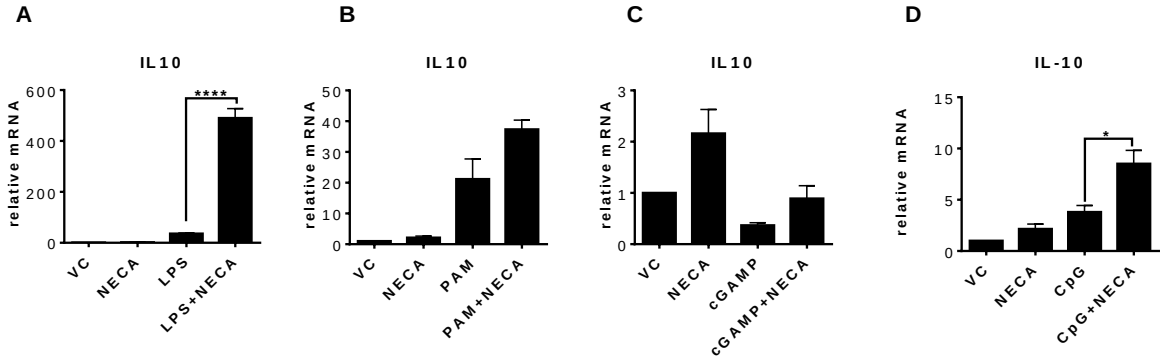

Figure 4.9: Adenosine receptor signaling increases the mRNA expression of IL-10 in the presence of danger signals associated with MyD88 adaptor proteins. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for 3 hours (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). mRNA quantification is done with taqman probes. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.3.4 Effect of Adenosine on mRNA Expression of NR4A

After we observed the modulation of cytokines, co-stimulatory molecules, and activation markers by adenosine, we wanted to test the expression of an anti-inflammatory transcription factor regulated by adenosine [74]. We observed a significant increase in the mRNA levels of NR4A1 in the presence of NECA only in the presence of MyD88-coupled danger signals. No modulation by NECA is observed after cGAMP signaling (Figure 4.10). These data suggest

that adenosine may modulate MyD88-dependent pathways through modulation of NR4A1 molecule, since it is an anti-inflammatory transcription factor.

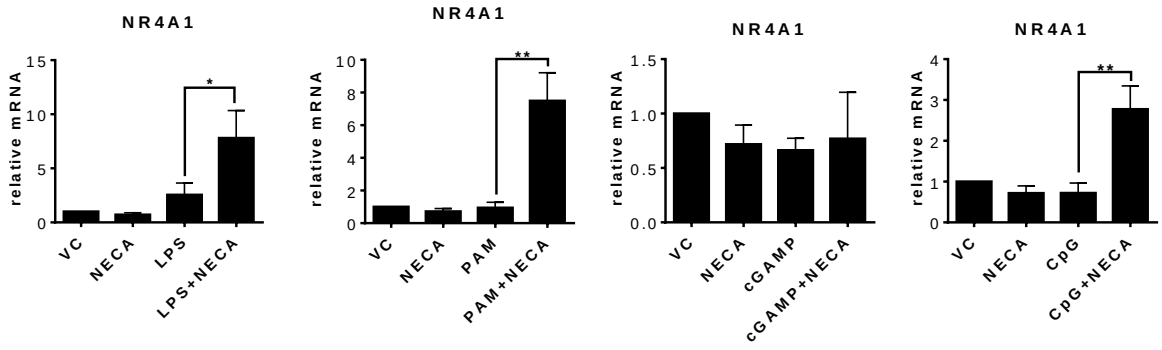

Figure 4.10: Adenosine receptor signaling increases the mRNA expression of NR4A in the presence of danger signals associated with MyD88 adaptor proteins. *ADA pretreated BMDCs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for 3 hours (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). mRNA quantification is done with taqman probes. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.4 Adenosine Receptors Expressions with or without the Danger Signals in BMDMs

After we observed the modulation by adenosine on BMDCs, we wanted to test the response of another important regulator of inflammation and antigen presentation to validate the MyD88-biased regulation cytokine responses in another primary immune cell culture system. For this, we chose BMDMs, and tested the expression of adenosine receptor types on these cells as well. As in BMDCs, we saw that out of four adenosine receptor types, A1 receptor is not expressed both in the presence or absence of danger signals, whereas A2A, A2B, and A3 receptors are expressed. We observed a significant upregulation of A2A and A2B receptors in the presence of danger signals associated with MyD88 adaptor protein, whereas there was no such upregulation after cGAMP signaling. (Figure 4.11). Interestingly, in BMDMs addition of NECA along with danger signals further increased the A2AR or A2BR expression suggesting a positive feedback loop of auto regulation of inflammatory responses.

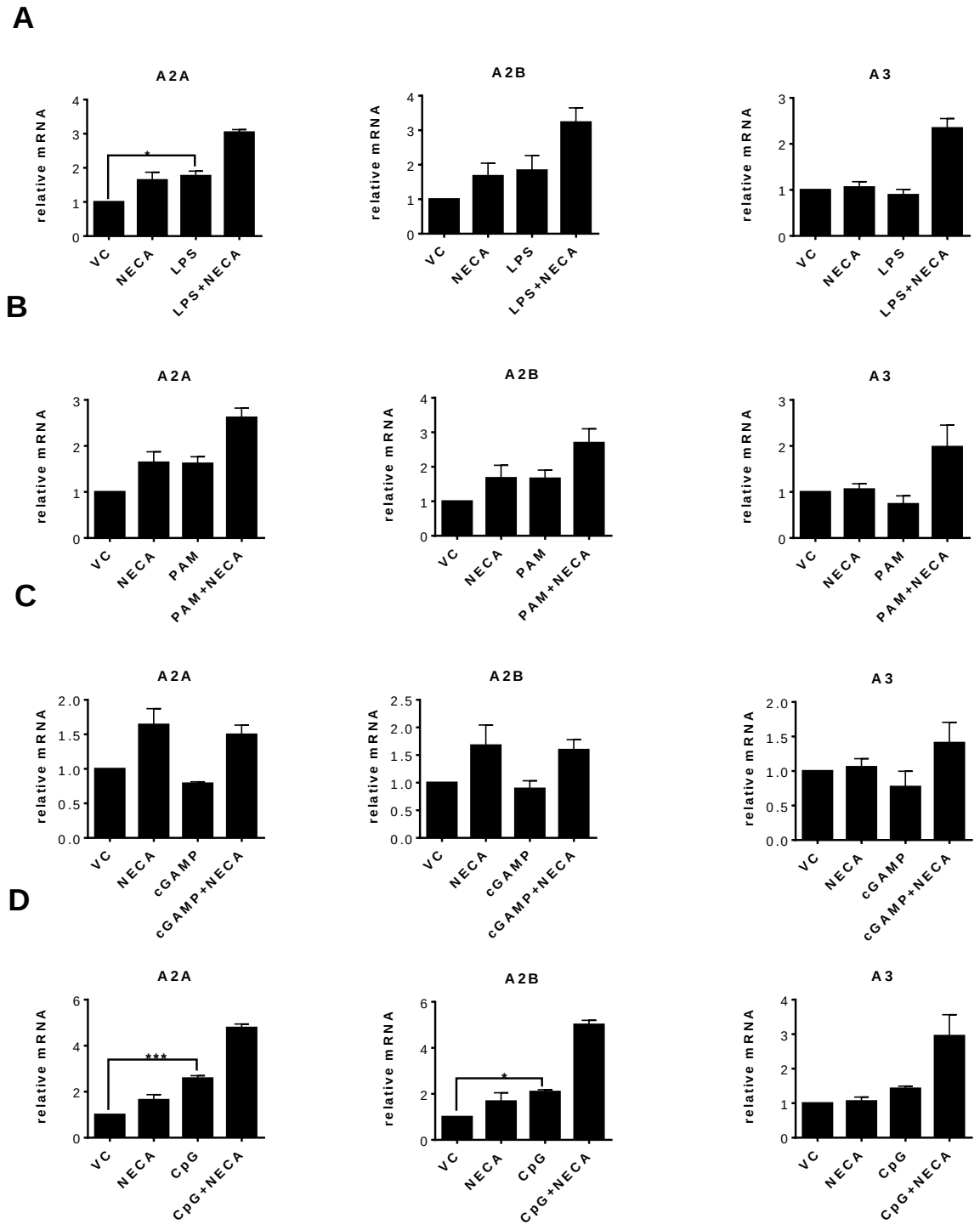

Figure 4.11: Danger signals associated with MyD88 adaptor proteins increase the expression of A2A and A2B receptors. *ADA pretreated BMDMs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for 3 hours (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). mRNA quantification is done with taqman probes. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.5 Immunomodulatory Effects of Adenosine on BMDMs

To assess the immunomodulatory effects of adenosine on BMDMs, we tested TNF-alpha and IL-12p40 as pro-inflammatory cytokines. We tested IFN-beta, as an indication of activation Type-I interferon pathway and IL-10 was tested to show the effect of adenosine receptor singling on generation of anti-inflammatory phenotype.

4.5.1 Effect of Adenosine on Pro-Inflammatory Cytokine Secretion

Consistent with what we observed in BMDCs, presence of NECA with the MyD88-coupled danger signals leads to a decrease in pro-inflammatory cytokine secretion in the BMDMs as well. TNF-alpha secretion with cGAMP signaling is not affected in the presence of NECA. No IL-12 secretion is observed in the presence of cGAMP from BMDMs (Figure 4.12).

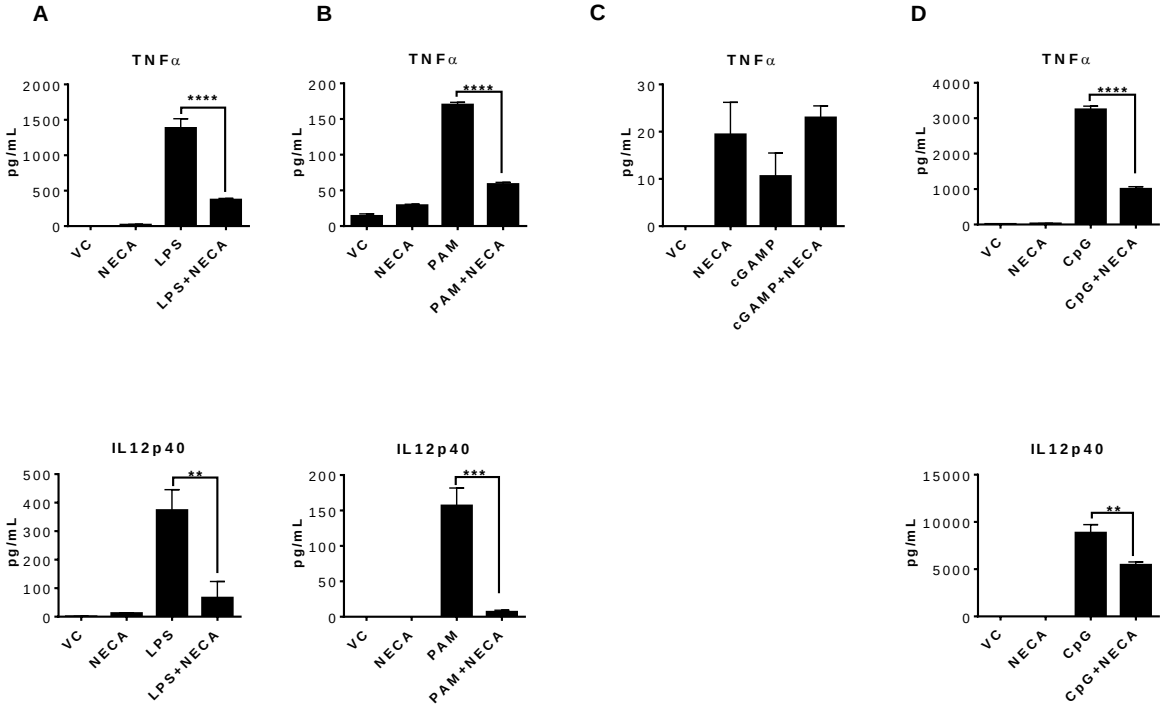

Figure 4.12: Adenosine receptor signaling decreases the secretion of pro-inflammatory cytokines in the presence of danger signals associated with MyD88 adaptor proteins. *ADA pretreated BMDMs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for overnight (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). No IL-12 secretion is detected with cGAMP treatment. Cytokine secretion is detected from media. For statistical*

*analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.5.2 Effect of Adenosine on Type-I Interferon Secretion

Type-I Interferon secretion from BMDMs appears to be not modulated by adenosine signaling. As can be seen in Figure 4.13. danger signals known to be coupled with IRF/Type-I interferon signaling significantly increased IFN-beta secretion, whereas PAM failed to do so, which is not a strong stimulator of Type-I interferons. Addition of NECA did not decrease the secretion of IFN-beta when added with any of the danger signals tested. Surprisingly, we observed an increase in the secretion of IFN-beta in the presence of adenosine with the CpG. These results suggest that adenosine receptor signaling may particularly regulate MyD88-dependent inflammatory pathways.

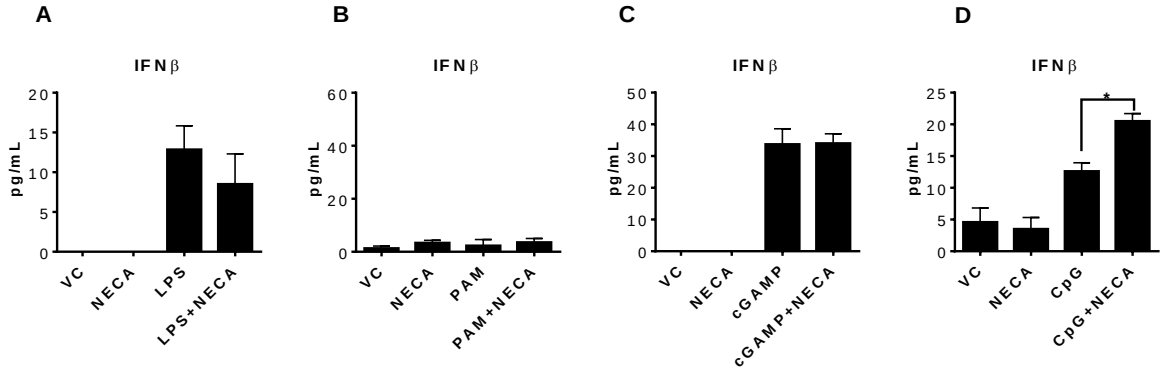

Figure 4.13: Adenosine receptor signaling does not have an effect on the secretion of type-I Interferons in the presence of danger signals. *ADA pretreated BMDMs stimulated with LPS (A), Pam2CSK4 (B), cGAMP (C), and CpG ODN (D) for overnight (LPS: 300 ng/ml, Pam2CSK4: 10 ng/mL, cGAMP: 1μg/ml, CpG: 3 μM, NECA: 3 μM). No IL-12 secretion is detected with cGAMP treatment. Cytokine secretion is detected from media. For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

4.5.3 Effect of Adenosine on TLR3 pathway in BMDMs

Although throughout the study we emphasized the responses depending on MyD88-dependent inflammatory pathways, we only showed cGAMP signaling as being MyD88-independent. Another important tool to test the hypothesis that adenosine receptor signaling mainly targets MyD88-dependent but not independent pathways is using pA:U as a danger signal. pA:U activates TLR3/TRIF/Type-I interferon pathway but not MyD88/inflammatory pathways. Our initial observations indicated a very weak to no responses to pA:U when we use BMDCs as target cell populations. Contrary to BMDCs, we observed a response to pA:U stimulation in BMDMs. We observed no expression of A1 receptor and no significant upregulation of remaining adenosine receptors types in the presence of pA:U (Figure 4.14A). Then we tested the secretion of TNF-alpha, IL12p40, and IFN-beta cytokines in the presence and absence of NECA. Figure 4.14B and C shows that pA:U is not a strong inducer of inflammatory cytokines. However, we observed a significant increase in IFN-beta secretion in the presence after pA:U stimulation. Addition of NECA did not change the accumulation of IFN-beta after pA:U treatment. Overall along with data related to cGAMP signaling the results of our study suggests adenosine receptor signaling causes an MyD88-biased regulation of antigen presenting cells. Results of this study has important implications for the modalities targeting adenosine receptors for cancer therapy or other inflammatory diseases.

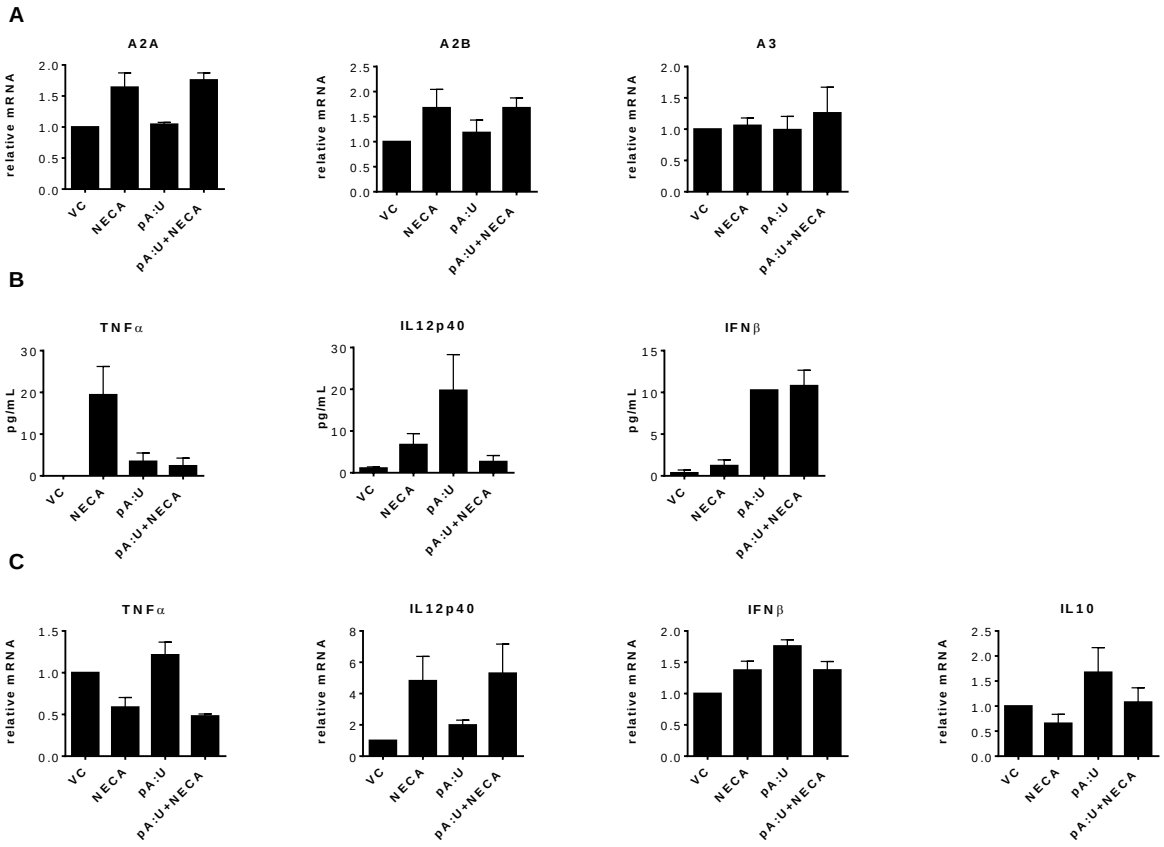

Figure 4.14: Effect of Adenosine receptor signaling on TLR3 pathway in BMDMs. *ADA pretreated BMDMs stimulated with pA:U for 3 hours (A) for overnight (B), for 3 hours (C). mRNA quantification is done with taqman probes. Cytokine secretion is detected from media (pA:U: 30μg/ml, NECA: 3 μM). For statistical analysis, one-way ANOVA was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results are from 2 or more independent experiments with similar results. (n=3)*

Chapter 5

Discussion

Our immune system has specialized to recognize a wide range of danger signals and respond to them. By doing this, it enables protection for several pathogens and infectious agents and for the damage they cause [2]. It is crucial to understand the mechanisms by which immune system is regulated to develop novel strategies to tackle many diseases and conditions. Increased efforts in research focusing on understanding immune cell regulation lead to new strategies against the deadliest conditions such as infectious diseases and cancer. Vaccines are one of the most intriguing examples for that. Besides vaccines, immune regulatory agents have been also studied to overcome several conditions. Immune system is affected not only by the pathogen or non-self molecules but also from metabolites produced by the host and these metabolites may have several modulatory effects [75]. Thus understanding the regulatory mechanisms of metabolites on immune system is also crucial to be able to regulate the immune system in many conditions. Since many of the regulatory agents used in auto-immune or infectious diseases have serious adverse effects, understanding the immune regulatory mechanisms of metabolites will also enable us to develop treatments without any adverse effects. In this study, our aim was to understand the adenosine regulation of several immunological danger signals, which can be used to develop combinatorial therapies for several conditions.

As it can be seen in Figure 1.5, agonists and antagonists of different adenosine receptor types are being tested to tackle different disease conditions. Apart from that, the role of adenosine receptors in the regulation of the immune system is more appreciated in recent years. The

research done in this area to this day suggests that adenosine has an immunosuppressive effect on immune cells [58]. The insight from these research activities had led to target adenosine receptors in preclinical and clinical studies. However, the concept of "how different danger signals are regulated by adenosine" is poorly understood. The most studied and understood effect is on TLR4 signaling and several studies showed that adenosine has a strong immunosuppressive effect on this pathway [76]. Nonetheless effects of adenosine on other danger signaling pathways is not clear. This situation led us to investigate these effects to see whether the immunosuppressive modulation is a universal effect on danger signals or if there is a differential modulation.

To investigate this, we chose five different danger signaling pathways based on their location and adaptor proteins: TLR2, TLR3, TLR4, TLR9 and STING pathways. TLR-2 and TLR-4 are located on cellular membrane, whereas TLR-3 ad TLR-9 are located on endosomal membranes, and STING is a cytosolic sensor. TLR-2 and TLR-9 are MyD88-dependent pathways, whereas TLR-3 is TRIF-dependent. TLR-4 is dependent on both MyD88 and TRIF [18,20,22,24,27]. We chose BMDCs to study this effect since they are the specialized APCs and a link between innate and adaptive immune responses [63]. BMDCs are also primary cell cultures, giving a more similar response to in vivo conditions compared to cell lines. To study the effect of adenosine, we used the stable and cell impermeable adenosine analog NECA [65]. As danger signals we used 1. ultrapure *S. Minnesotta* LPS, which activates only TLR4 [66], 2. PAM_3CSK_4 (PAM) a synthetic peptide that mimics lipoproteins on both gram positive and gram negative bacteria and activates TLR2 pathway [68], 3. Stable cGAMP which activates STING/IRF3/type I interferon pathway [69], 4. CpG DNA, which is prevalent among bacterial DNA and activates TLR9 [70], 5. pA:U, which is a TLR3 ligand and activates only the TRIF pathway [71]. For the experiments in which pA:U was used, we had to switch to BMDM system because BMDCs were not responsive to pA:U.

First, we tested the expression of adenosine receptor subtypes in the absence and presence of danger signals to see if there is an upregulation. We observed in BMDCs, that in the presence of all the danger signals tested, there was an upregulation of A2A and A2B receptors, suggesting that there may be an overall immune suppression by adenosine on the danger signal pathways. Also, the presence of NECA had no significant effect on the expression of adenosine receptors. To investigate the effect of adenosine on danger signaling pathways, we tested the secretion levels of cytokines with the danger signals and in the presence or absence

of NECA. We observed that presence of NECA lead to a significant decrease in the levels of pro-inflammatory cytokines released after stimulation of MyD88-coupled pathways, but not cGAMP. Other than pro-inflammatory cytokines, we tested secretion of type-I interferons, since cGAMP mostly leads to secretion of type-I interferons rather than pro-inflammatory cytokines. We observed a similar effect: adenosine did not influence the secretion of type-I interferons after activation of STING pathway. The suppressive effect of adenosine signaling on type I interferon responses after LPS stimulation was much less prominent as compared to that on pro-inflammatory cytokines. In addition to pro-inflammatory cytokines and type-I interferons, we tested the modulation of anti-inflammatory cytokine IL-10. As expected from an immunosuppressive molecule, adenosine signaling increased the secretion of IL-10 after activation of MyD88-coupled receptors. Surprisingly, it led to a decreased secretion of IL-10 after cGAMP treatment. To investigate the immunomodulatory effects further, we tested the expression of co-stimulatory molecules and activation markers by flow cytometry. We observed a significant decrease in the expression of CD86, MHCI, and MHCII molecules in the presence of NECA in LPS, PAM, and CpG treated groups, but not in cGAMP treated groups, suggesting that adenosine decreases the antigen presentation ability of BMDCs with the dangers signals that can activate MyD88 pathway. Surprisingly, we observed an increase in CD80 expression levels after NECA treatment, which may be part of a compensatory loop permitting antigen presentation even in the presence of high adenosine concentrations. All of these data suggests that adenosine signaling causes a biased suppression towards MyD88-coupled pathways.

To understand if adenosine receptor signaling primarily modulates cytokine responses at mRNA level or through post-translational mechanisms we have also tested expression of effecter cytokines at mRNA level by qPCR. We observed a similar pattern with mRNA expression; however, for certain danger signals, such as TLR2, the difference was somewhat less pronounced. Adenosine signaling could still suppress the mRNA expression of both TNF-alpha and IL-12p40 after LPS stimulation, suggesting that adenosine acts as a strong immunosuppressive agent for TLR-4 signaling. Interestingly although adenosine receptor stimulation significantly suppresses IFN-beta secretion after LPS stimulation, it had no effect on IFN-beta expression at mRNA level suggesting adenosine can modulate certain cytokine using posttranslational mechanisms. We also tested the expression of anti-inflammatory molecule NR4A1, to be able to suggest a molecular mechanism to suppression of MyD88-coupled danger signals. Only in the presence of MyD88-coupled signals, the expression of

NR4A1 was increased by NECA, suggesting adenosine receptor signaling requires activation of MyD88-dependent pathways to increase the expression of this anti-inflammatory molecule.

We observed an immunosuppressive effect by adenosine receptor signaling on MyD88-coupled pathways, but not with TRIF coupled or IRF3 related pathways in BMDCs. To validate these findings further we wanted to test this hypothesis in another immune cell type. For this, we chose BMDMs, since they are also primary cells with important functions in inflammatory responses and they are responsive to most of the danger signal receptors. We again tested the expression of adenosine receptor types first. In the BMDMs, LPS, PAM, and CpG treated groups upregulated the expression of A2A, and A2B receptors but not pA:U and cGAMP. Then we tested the secretion of pro-inflammatory cytokines and type-I interferons from BMDMs. Consistent with BMDC results, we observed a decrease in the secretion of pro-inflammatory cytokines in the presence of adenosine with the danger signals associated with MyD88, but not cGAMP, and pA:U. However, in BMDMs, no decrease in IFN-beta levels is observed with any of the danger signals tested. Surprisingly, an increase in IFN-beta levels was observed in the presence of adenosine with CpG.

Overall, our results suggest that, adenosine acts as an immunomodulatory agents rather than being an exclusively immunosuppressive agent and it selectively suppresses the danger signals associated with MyD88-dependent inflammatory pathways such as LPS and CpG but not the danger signals associated with IRF3/Type-I interferon pathways such as pA:U and cGAMP. This may be an evolutionary adaptation to survive the viral infections, because viral infections result in cell lysis and the presence of adenosine in the extracellular environment and thus adenosine signaling suppressing the inflammatory response would be negatively selected to be able to survive. Also adenosine may exert the immunosuppressive effect on MyD88-dependent pathways through anti-inflammatory NR4A1 molecule, since its expression was increased after adenosine receptor stimulation in the presence of TLR ligands known to activate MyD88 pathway but not in the presence of cGAMP and pA:U. Overall these results indicate that there is a differential modulation of danger signaling by adenosine rather than overall suppression. Our results have important implications for developing combinatorial approaches to target adenosine and danger signaling pathways to cure immune-related diseases.

As a future perspective, we want to test and observe this effect in vivo, to see whether there is a differential modulation in vivo. We also want to investigate the role of adenosine on STING signaling in detail, since it is a recently discovered pathway, and currently there is no studies investigating this effect. Other danger signaling pathways, besides the ones studied in this thesis, may also be studied to see the effect of adenosine on these danger signals. Lastly, infectious and autoimmune disease models related to MyD88-coupled pathways can be studied for potential treatments, since we saw an immunosuppressive effect by adenosine on these pathways.

Bibliography

1. Parkin, J. and B. Cohen, *An overview of the immune system.* Lancet, 2001. **357**(9270): p. 1777-89.
2. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
3. Hoffmann, J. and S. Akira, *Innate immunity.* Curr Opin Immunol, 2013. **25**(1): p. 1-3.
4. Nowarski, R., et al., *Innate immune cells in inflammation and cancer.* Cancer Immunol Res, 2013. **1**(2): p. 77-84.
5. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* Int Rev Immunol, 2011. **30**(1): p. 16-34.
6. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clin Microbiol Rev, 2009. **22**(2): p. 240-73, Table of Contents.
7. Kumagai, Y., O. Takeuchi, and S. Akira, *Pathogen recognition by innate receptors.* J Infect Chemother, 2008. **14**(2): p. 86-92.
8. Janssens, S. and R. Beyaert, *Role of Toll-like receptors in pathogen recognition.* Clin Microbiol Rev, 2003. **16**(4): p. 637-46.
9. Sasai, M. and M. Yamamoto, *Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors.* Int Rev Immunol, 2013. **32**(2): p. 116-33.
10. Lester, S.N. and K. Li, *Toll-like receptors in antiviral innate immunity.* J Mol Biol, 2014. **426**(6): p. 1246-64.
11. Qian, C. and X. Cao, *Regulation of Toll-like receptor signaling pathways in innate immune responses.* Ann N Y Acad Sci, 2013. **1283**: p. 67-74.
12. Brown, J., et al., *TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk.* J Dent Res, 2011. **90**(4): p. 417-27.
13. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.
14. McGettrick, A.F. and L.A. O'Neill, *Localisation and trafficking of Toll-like receptors: an important mode of regulation.* Curr Opin Immunol, 2010. **22**(1): p. 20-7.
15. Akira, S., *TLR signaling.* Curr Top Microbiol Immunol, 2006. **311**: p. 1-16.
16. Takeda, K. and S. Akira, *TLR signaling pathways.* Semin Immunol, 2004. **16**(1): p. 3-9.

17. O'Neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors - redefining innate immunity.* Nat Rev Immunol, 2013. **13**(6): p. 453-60.
18. Liu, Y., et al., *TLR2 and TLR4 in autoimmune diseases: a comprehensive review.* Clin Rev Allergy Immunol, 2014. **47**(2): p. 136-47.
19. van Bergenhenegouwen, J., et al., *TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors.* J Leukoc Biol, 2013. **94**(5): p. 885-902.
20. Chattopadhyay, S. and G.C. Sen, *dsRNA-activation of TLR3 and RLR signaling: gene induction-dependent and independent effects.* J Interferon Cytokine Res, 2014. **34**(6): p. 427-36.
21. Ullah, M.O., et al., *TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target.* J Leukoc Biol, 2016. **100**(1): p. 27-45.
22. Plociennikowska, A., et al., *Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling.* Cell Mol Life Sci, 2015. **72**(3): p. 557-81.
23. Lu, Y.C., W.C. Yeh, and P.S. Ohashi, *LPS/TLR4 signal transduction pathway.* Cytokine, 2008. **42**(2): p. 145-51.
24. Kumagai, Y., O. Takeuchi, and S. Akira, *TLR9 as a key receptor for the recognition of DNA.* Adv Drug Deliv Rev, 2008. **60**(7): p. 795-804.
25. Bao, M. and Y.J. Liu, *Regulation of TLR7/9 signaling in plasmacytoid dendritic cells.* Protein Cell, 2013. **4**(1): p. 40-52.
26. Blasius, A.L. and B. Beutler, *Intracellular toll-like receptors.* Immunity, 2010. **32**(3): p. 305-15.
27. Barber, G.N., *STING-dependent cytosolic DNA sensing pathways.* Trends Immunol, 2014. **35**(2): p. 88-93.
28. Tao, J., X. Zhou, and Z. Jiang, *cGAS-cGAMP-STING: The three musketeers of cytosolic DNA sensing and signaling.* IUBMB Life, 2016. **68**(11): p. 858-870.
29. Cai, X., Y.H. Chiu, and Z.J. Chen, *The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling.* Mol Cell, 2014. **54**(2): p. 289-96.
30. Barber, G.N., *STING: infection, inflammation and cancer.* Nat Rev Immunol, 2015. **15**(12): p. 760-70.
31. Riera Romo, M., D. Perez-Martinez, and C. Castillo Ferrer, *Innate immunity in vertebrates: an overview.* Immunology, 2016. **148**(2): p. 125-39.
32. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses.* Nat Rev Immunol, 2014. **14**(1): p. 36-49.

33. Sharpe, A.H., *Mechanisms of costimulation.* Immunol Rev, 2009. **229**(1): p. 5-11.
34. Locati, M., A. Mantovani, and A. Sica, *Macrophage activation and polarization as an adaptive component of innate immunity.* Adv Immunol, 2013. **120**: p. 163-84.
35. Luster, A.D., *The role of chemokines in linking innate and adaptive immunity.* Current Opinion in Immunology, 2002. **14**(1): p. 129-135.
36. Schraml, B.U. and C. Reis e Sousa, *Defining dendritic cells.* Curr Opin Immunol, 2015. **32**: p. 13-20.
37. Joffre, O.P., et al., *Cross-presentation by dendritic cells.* Nat Rev Immunol, 2012. **12**(8): p. 557-69.
38. Colonna, M., G. Trinchieri, and Y.J. Liu, *Plasmacytoid dendritic cells in immunity.* Nat Immunol, 2004. **5**(12): p. 1219-26.
39. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nat Immunol, 2015. **16**(4): p. 343-53.
40. Yatim, K.M. and F.G. Lakkis, *A brief journey through the immune system.* Clin J Am Soc Nephrol, 2015. **10**(7): p. 1274-81.
41. den Haan, J.M.M., R. Arens, and M.C. van Zelm, *The activation of the adaptive immune system: Cross-talk between antigen-presenting cells, T cells and B cells.* Immunology Letters, 2014. **162**(2): p. 103-112.
42. Moser, M. and O. Leo, *Key concepts in immunology.* Vaccine, 2010. **28**: p. C2-C13.
43. Artis, D. and H. Spits, *The biology of innate lymphoid cells.* Nature, 2015. **517**(7534): p. 293-301.
44. Caroll, M.C. and A.P. Prodeus, *Linkages of innate and adaptive immunity.* Current Opinion in Immunology, 1998. **10**(1): p. 36-40.
45. Iwasaki, A. and R. Medzhitov, *Regulation of Adaptive Immunity by the Innate Immune System.* Science, 2010. **327**(5963): p. 291.
46. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nature immunology, 2015. **16**(4): p. 343-353.
47. Medzhitov, R. and C.A. Janeway, *Innate immunity: impact on the adaptive immune response.* Current Opinion in Immunology, 1997. **9**(1): p. 4-9.
48. Schmitt, N. and H. Ueno, *Regulation of Human Helper T Cell Subset Differentiation by Cytokines.* Current opinion in immunology, 2015. **34**: p. 130-136.
49. Andersen, M.H., et al., *Cytotoxic T cells.* J Invest Dermatol, 2006. **126**(1): p. 32-41.
50. Gowthaman, U., S.B. Chodisetti, and J.N. Agrewala, *T cell help to B cells in germinal centers: putting the jigsaw together.* Int Rev Immunol, 2010. **29**(4): p. 403-20.

51. Pieper, K., B. Grimbacher, and H. Eibel, *B-cell biology and development.* J Allergy Clin Immunol, 2013. **131**(4): p. 959-71.
52. Vazquez, M.I., J. Catalan-Dibene, and A. Zlotnik, *B cells responses and cytokine production are regulated by their immune microenvironment.* Cytokine, 2015. **74**(2): p. 318-26.
53. Reed, S.G., M.T. Orr, and C.B. Fox, *Key roles of adjuvants in modern vaccines.* Nat Med, 2013. **19**(12): p. 1597-608.
54. Burnstock, G. and J.-M. Boeynaems, *Purinergic signalling and immune cells.* Purinergic Signalling, 2014. **10**(4): p. 529-564.
55. Burnstock, G., *Purinergic signalling: past, present and future.* Braz J Med Biol Res, 2009. **42**(1): p. 3-8.
56. Junger, W.G., *Immune cell regulation by autocrine purinergic signalling.* Nat Rev Immunol, 2011. **11**(3): p. 201-212.
57. Hasko, G., et al., *Adenosine receptors: therapeutic aspects for inflammatory and immune diseases.* Nat Rev Drug Discov, 2008. **7**(9): p. 759-70.
58. Antonioli, L., et al., *Immunity, inflammation and cancer: a leading role for adenosine.* Nat Rev Cancer, 2013. **13**(12): p. 842-57.
59. Linden, J., *Regulation of leukocyte function by adenosine receptors.* Adv Pharmacol, 2011. **61**: p. 95-114.
60. Linden, J. and C. Cekic, *Regulation of lymphocyte function by adenosine.* Arterioscler Thromb Vasc Biol, 2012. **32**(9): p. 2097-103.
61. Jacobson, K.A., *Introduction to adenosine receptors as therapeutic targets.* Handb Exp Pharmacol, 2009(193): p. 1-24.
62. Jacobson, K.A. and Z.G. Gao, *Adenosine receptors as therapeutic targets.* Nat Rev Drug Discov, 2006. **5**(3): p. 247-64.
63. Galati, D., et al., *Dendritic cells in hematological malignancies.* Crit Rev Oncol Hematol, 2016. **108**: p. 86-96.
64. Mayordomo, J.I., et al., *Bone marrow-derived dendritic cells serve as potent adjuvants for peptide-based antitumor vaccines.* Stem Cells, 1997. **15**(2): p. 94-103.
65. Cusack, N.J. and S.M. Hourani, *5'-N-ethylcarboxamidoadenosine: a potent inhibitor of human platelet aggregation.* Br J Pharmacol, 1981. **72**(3): p. 443-7.
66. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.

67. Tang, D., et al., *High-mobility Group Box 1 [HMGB1] and Cancer.* Biochimica et biophysica acta, 2010. **1799**(1-2): p. 131.
68. Ozinsky, A., et al., *The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13766-71.
69. Wu, J., et al., *Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA.* Science, 2013. **339**(6121): p. 826-30.
70. Ashkar, A.A. and K.L. Rosenthal, *Toll-like receptor 9, CpG DNA and innate immunity.* Curr Mol Med, 2002. **2**(6): p. 545-56.
71. Perrot, I., et al., *TLR3 and Rig-Like Receptor on Myeloid Dendritic Cells and Rig-Like Receptor on Human NK Cells Are Both Mandatory for Production of IFN-γ in Response to Double-Stranded RNA.* Journal of immunology (Baltimore, Md. : 1950), 2010. **185**(4): p. 2080-2088.
72. Re, F. and J.L. Strominger, *Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells.* J Biol Chem, 2001. **276**(40): p. 37692-9.
73. Hoshino, K., et al., *Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation.* Int Immunol, 2002. **14**(10): p. 1225-31.
74. Crean, D., et al., *Adenosine Modulates NR4A Orphan Nuclear Receptors To Attenuate Hyperinflammatory Responses in Monocytic Cells.* J Immunol, 2015. **195**(4): p. 1436-48.
75. Levy, M., C.A. Thaiss, and E. Elinav, *Metabolites: messengers between the microbiota and the immune system.* Genes Dev, 2016. **30**(14): p. 1589-97.
76. Coombs, M.R.P., et al., *The adenosine system modulates Toll-like receptor function: basic mechanisms, clinical correlates and translational opportunities.* Expert review of anti-infective therapy, 2011. **9**(2): p. 261-269.

Copyright Permissions

Copyright Permission for Figure 1.1

Thank you for your order.

This Agreement between Ms. İmran Akdemir ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

Printable details.

License Number	4140850964453
License date	Jul 02, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Immunology
Licensed Content Title	The history of Toll-like receptors [mdash] redefining innate immunity
Licensed Content Author	Luke A. J. O'Neill, Douglas Golenbock, Andrew G. Bowie
Licensed Content Date	May 17, 2013
Licensed Content Volume	13
Licensed Content Issue	6
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Toll like receptor localization and signaling pathways.
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Adenosine Regulation of Danger Signaling
Expected completion date	Jul 2017
Estimated size (number of pages)	80
Requestor Location	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, 06800 Turkey Attn: Ms. İmran Akdemir
Billing Type	Invoice
Billing address	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, Turkey 06800 Attn: Ms. İmran Akdemir
Total	0.00 USD

Copyright Permission for Figure 1.2

Thank you for your order.

This Agreement between Ms. İmran Akdemir ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

Printable details.

License Number	4140851287445
License date	Jul 02, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Immunology
Licensed Content Title	STING: infection, inflammation and cancer
Licensed Content Author	Glen N. Barber
Licensed Content Date	Nov 25, 2015
Licensed Content Volume	15
Licensed Content Issue	12
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	STING signaling cascade
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Adenosine Regulation of Danger Signaling
Expected completion date	Jul 2017
Estimated size (number of pages)	80
Requestor Location	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, 06800 Turkey Attn: Ms. İmran Akdemir
Billing Type	Invoice
Billing address	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, Turkey 06800 Attn: Ms. İmran Akdemir
Total	0.00 USD

Copyright Permission for Figure 1.3

Thank you for your order.

This Agreement between Ms. İmran Akdemir ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

Printable details.

License Number	4140851440594
License date	Jul 02, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Medicine
Licensed Content Title	Key roles of adjuvants in modern vaccines
Licensed Content Author	Steven G Reed, Mark T Orr, Christopher B Fox
Licensed Content Date	Dec 5, 2013
Licensed Content Volume	19
Licensed Content Issue	12
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Integrated innate and adaptive immune responses
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Adenosine Regulation of Danger Signaling
Expected completion date	Jul 2017
Estimated size (number of pages)	80
Requestor Location	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, 06800 Turkey Attn: Ms. İmran Akdemir
Billing Type	Invoice
Billing address	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, Turkey 06800 Attn: Ms. İmran Akdemir
Total	0.00 USD

Copyright Permission for Figure 1.4

Thank you for your order.

This Agreement between Ms. İmran Akdemir ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

Printable details.

License Number	4140860075040
License date	Jul 02, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Immunology
Licensed Content Title	Immune cell regulation by autocrine purinergic signalling
Licensed Content Author	Wolfgang G. Junger
Licensed Content Date	Feb 18, 2011
Licensed Content Volume	11
Licensed Content Issue	3
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Purinergic signaling and its components
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Adenosine Regulation of Danger Signaling
Expected completion date	Jul 2017
Estimated size (number of pages)	80
Requestor Location	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, 06800 Turkey Attn: Ms. İmran Akdemir
Billing Type	Invoice
Billing address	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, Turkey 06800 Attn: Ms. İmran Akdemir
Total	0.00 USD

Copyright Permission for Figure 1.5

Thank you for your order.

This Agreement between Ms. İmran Akdemir ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

Printable details.

License Number	4140860262094
License date	Jul 02, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Drug Discovery
Licensed Content Title	Adenosine receptors as therapeutic targets
Licensed Content Author	Kenneth A. Jacobson and Zhan-Guo Gao
Licensed Content Date	Mar 1, 2006
Licensed Content Volume	5
Licensed Content Issue	3
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Adenosine receptor types related to different conditions
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Adenosine Regulation of Danger Signaling
Expected completion date	Jul 2017
Estimated size (number of pages)	80
Requestor Location	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, 06800 Turkey Attn: Ms. İmran Akdemir
Billing Type	Invoice
Billing address	Ms. İmran Akdemir Bilkent University, Main Campus SB building 2. floor, MBG department Çankaya/Ankara Ankara, Turkey 06800 Attn: Ms. İmran Akdemir
Total	0.00 USD