MOLECULAR MECHANISM FOR ADENOSINE REGULATION OF DENDRITIC CELLS

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By

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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.

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Abstract

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Cell death, inflammation or other cellular stress factors cause accumulation of adenosine in the extracellular space. Adenosine has immunosuppressive effects on antigen presenting cells. However, molecular mechanisms for adenosine regulation of dendritic cells are poorly understood. Here we showed that adenosine receptor signaling promotes an anti-inflammatory dendritic cell phenotype. While adenosine receptor signaling increased intracellular cAMP levels, phosphoactivation of major inflammatory pathways such as MAPKs, NF-κB and IRF3 were not affected. Adenosine's effects were phenocopied by cAMP. Specific cAMP analogs for EPAC and PKA pathways indicated that adenosine activates both intracellular cAMP receptors to inhibit dendritic cell activation. Anti-inflammatory cFOS and NR4A receptor family expressions were increased by adenosine or EPAC and PKA specific cAMP analogs. Furthermore, T cells incubated with the medium of dendritic cells, which prestimulated with adenosine receptor agonist and PKA-EPAC specific cAMP analogs, produced less IFNγ. Overall our data suggest that dendritic cells are regulated by adenosine through both PKA and EPAC pathways and increased the expression of NR4A nuclear orphan receptors and cFOS. Our findings suggest that for effective targeting of adenosine or other cAMP-inducing receptors both PKA and EPAC are important to modulate immune responses.

Keywords: Adenosine receptors, cAMP signaling, PKA, EPAC, dendritic cells

Özet

ADENOZİNİN DENDRİTİK HÜCRELER ÜZERİNDEKİ ETKİLERİNİN MOLEKÜLER MEKANİZMASI

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Hücre dışı adenozin konsantrasyonu ve adenozin reseptörü ekspresyonları hücre ölümü, yaralanması veya inflamasyon sonucu artar. Antijen sunan hücreler adenozin ile karşılaştığında immun baskılayıcı şekilde hareket ederler. Bu bilgilere rağmen dendritik hücrelerin adenozin tarafından nasıl etkilendiği ve etki mekanizmaları hala bilinmemektedir. Biz bu çalışmada adenozin reseptörü stimülasyonunun dendritik hücre aktivasyonu üzerinde immun baskılayıcı bir etkisi olduğunu gösterdik. Adenozin reseptör sinyali hücre içi cAMP miktarını arttırırken MAPK, NF-κB ve IRF3 gibi önemli immune yolakların fosforilasyonunu etkilemedi. Özellikle beraber kullanıldıklarında EPAC ve PKA spesifik cAMP analogları adenozinin etkilerini fenokopiledi. Adenozin ve PKA-EPAC spesifik cAMP analogları anti-inflamatuar olarak bilinen cFos ve NR4A reseptörlerinin ekspresyonlarını arttırdı. Dahası, aynı şekilde adenozin analoğu ve PKA-EPAC spesifik cAMP analoğu ile işlem görmüş dendritik hücre besiyeri T hücreleri ile kültür edildiğinde T hücrelerinden salınan IFNγ miktarında düşüş gözlendi. Sonuç olarak bu bilgiler doğrultusunda dendritik hücrelerin adenozin tarafından aktive edilen yolaklarında PKA ve EPAC proteinlerinin ikisinin birlikte iş gördüğü ve NR4A ve cFOS moleküllerinin ekspresyonlarında bir artış olduğu gözlemlenmiştir. Bu bulgular bağışıklık sistemi ile alakalı kanser veya alerji gibi hastalıklarda adenozin reseptörlerinin hedeflenerek yeni tedavi edici yaklaşımların geliştirilmesine katkıda bulunabilir.

Anahtar Sözcükler: Adenozin reseptörleri, cAMP sinyali, PKA, EPAC, dendritik hücreler

To my precious family…

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Abbreviations

Ab	Antibody
AC	Adenylyl cyclase
ACK	Ammonium-Chloride-Potassium
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AIM2	Absent in melanoma 2
AKAP	A-kinase anchoring protein
ALR	AIM2 like receptors
AMP	Adenosine monophosphate
APC	Antigen presenting cell
AR	Adenosine receptor
ATF-1	activating transcription factor-1
ATP	Adenosine triphosphate
BCR	B cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
cAMP	cyclic Adenosine monophosphate
CBD	cyclic nucleotide binding domain
CCR	CC chemokinr receptor
CD	Cluster of differentiation
cDC	Classical DC
cDNA	Complementary deoxyribonucleic acid
cFOS	Cellular oncogene c-fos
CLR	C-type lectin receptor
CNGCs	cyclic nucleotide gated ion channels
CRE	cAMP-responsive element
CREB	cAMP responsive element binding prtotein
CREM	cAMP response element modulator
CTL	Cytotoxic T lymphocyte

CTLA-4	Cytotoxic T lymphocyte antigen 4
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
ddH2O	Double-distilled water
DEP	Dishevelled, Egl-10 and Pleckstrin domain
dH2O	Distilled water
DLN	Draining lymph node
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECL	Enhanced Chemiluminescance
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
g	gram
GEF	guanine nucleotide exchange factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G protein coupled receptor
HBSS	Hank's balanced salt solution
HRP	Horse radish peroxidase
IFN	Interferon
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukine
IRF	IFN-regulatory factor
JNK	c-jun N-terminal kinase
KIR	Killer inhibitory receptor
l	Liter
LPS	Lipopolysaccharide

M	Molar
MAP	Mitogen-activated protein
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
mM	Micromolar
MPL-A	Monophosphoryl Lipid A
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NDLN	Non-draining lymph node
NECA	5' -*N*-Ethylcarboxamidoadenosine
NF- κB	Nuclear factor- κB
NFDM	Non-fat dry milk
ng	Nano gram
NK	Natural killer
NLR	NOD-leucine rich repeat receptors
NOD	Nucleotide-binding oligomerization domain
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
pDC	Plasmacytoid DC
PDE	Phospho diesterase
PKA	Protein kinase A
PRR	Pattern recognition receptor
Q-RT-PCR	Quantitative real time polymerase chain reaction
RA	Ras-association domain
REM	Ras exchange domain
RLR	RIG-I like receptor
RNA	Ribonucleic acid
Rpm	Revolution per minute

RPMI	Roswell Park Memorial Institute
RT	Room temperature
TBS	Tris buffered saline
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
Th	T helper
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMB	Tetramethylbenzidin
TNFa	Tumor necrosis factor α
TRAF	TNF receptor-associated factor
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
UTP	Uracil triphosphate
h	hour
UTP	Uracil triphosphate

Chapter 1

Introduction

1.1 The immune system

From bacteria to human, organisms developed protection mechanisms to pathogenic invaders and other foreign molecules over the course of evolution. Our body continually encounter non-self microbes or molecules by the air we breathe, the food we swallow or the objects we touch [1]. Majority of these molecules are not capable to pass the physical barrier of our body; the skin and mucosal surfaces. If they can, specialized cells and molecules take in charge for a complete protection. All of these physical, chemical and biological barriers create the immune system [2].

One of the most important jobs of immune protection is to distinguish self from non-self to avoid attacks to the host organism. By doing so our immune system can recognize, neutralize and destroy the pathogen and create an immunological memory for faster protection of the living organism during future invasions. The immune system provides these by orchestrating a network of cells and molecules. These cells can kill pathogens and infected cells, produce antibodies, mark pathogens, remember enemies in repeated infections and also activate other cells. To accomplish these sophisticated events, immune system is divided into two main arms; innate and adaptive according to their handling mechanisms of pathogens [3]. Disorganizations in the immune system can cause many biological problems such as autoimmune diseases, inflammatory diseases, immunodeficiency or cancer. To avoid these, innate and adaptive immune system should work in harmony and be sure that homeostasis restored.

1.1.1 Innate Immune system

The innate immune system comprises of all rapid and non-specific responses to a foreign molecule in our body. These responses can be physical by the skin, or chemical by mucous, saliva, gastric acid or other secreted liquids. If the pathogen can overcome these barriers, cell based defense come into play. Various players of the innate immunity include complement proteins, antimicrobial peptides, plasma proteins, circulating or tissue resident phagocytes and cells like macrophages, dendritic cells, NK cells and granulocytes [4]. These players can exert their effects by engulfing the pathogen directly, destroying the essential structures of the microbes that are needed for the survival or infectivity of them, opsonization of the foreigners to be marked for phagocytosis, attracting macrophages and neutrophils to the site of inflammation and trapped the infection in the site of inflammation for adaptive immunity set to work [5]. Innate immune system use these mechanisms to cope with an infection quickly without any help of adaptive immune system most of the time.

The innate immune system not only creates the first line of defense but also triggers the adaptive immune system to provide the complete protection for the organism. Therefore, any failure in innate immune mechanisms is supposed to be lethal.

Innate immune system can identify the structures of the microbes called as pathogen associated molecular patterns (PAMPs) or released particles of damaged or dead cells called damage associated molecular patterns (DAMPs). These patterns are evolutionary conserved among microorganisms which makes them easily to be detected by immune system. Recognition of foreign molecules is regulated by pathogen recognition receptors (PRRs) on the host cells. PRRs are encoded in the germline and they can be membrane bound, cytoplasmic or secreted into bloodstream [6]. There are many different types of PRRs known as Toll like receptors (TLRs), nucleotide oligomerization receptors (NLRs), C-type lectin receptors (CLRs), retinoic acid inducible gene like receptors (RLRs), AIM2 like receptors (ALRs) and cytosolic DNA sensors [6]. Immune activation occurs through the secretion of pro-inflammatory cytokines or chemokines when these types of receptors are activated by a danger molecule.

One of the well-known and first identified PRRs are the family of Toll Like receptors. TLRs can response a wide range of patterns like protein and lipid-based compartments of bacteria, viruses, fungi and parasites. First TLR, known as TLR4, is found for the first time on *Drosophila melanogaster*. Since then, 10 human and 13 mice TLRs have been identified [7, 8]. Once recognition of a proper ligand, TLRs activate some intracellular signaling pathways such as NF-K B, IRF3 and several MAP kinase pathways. This activation induces the secretion of inflammatory cytokines, chemokines, type I interferons and costimulatory molecules and thus, fire the initiation of innate immunity to further generate the adaptive immune response [9, 10]. An example of immunological synapse illustrated with Figure 1.1.

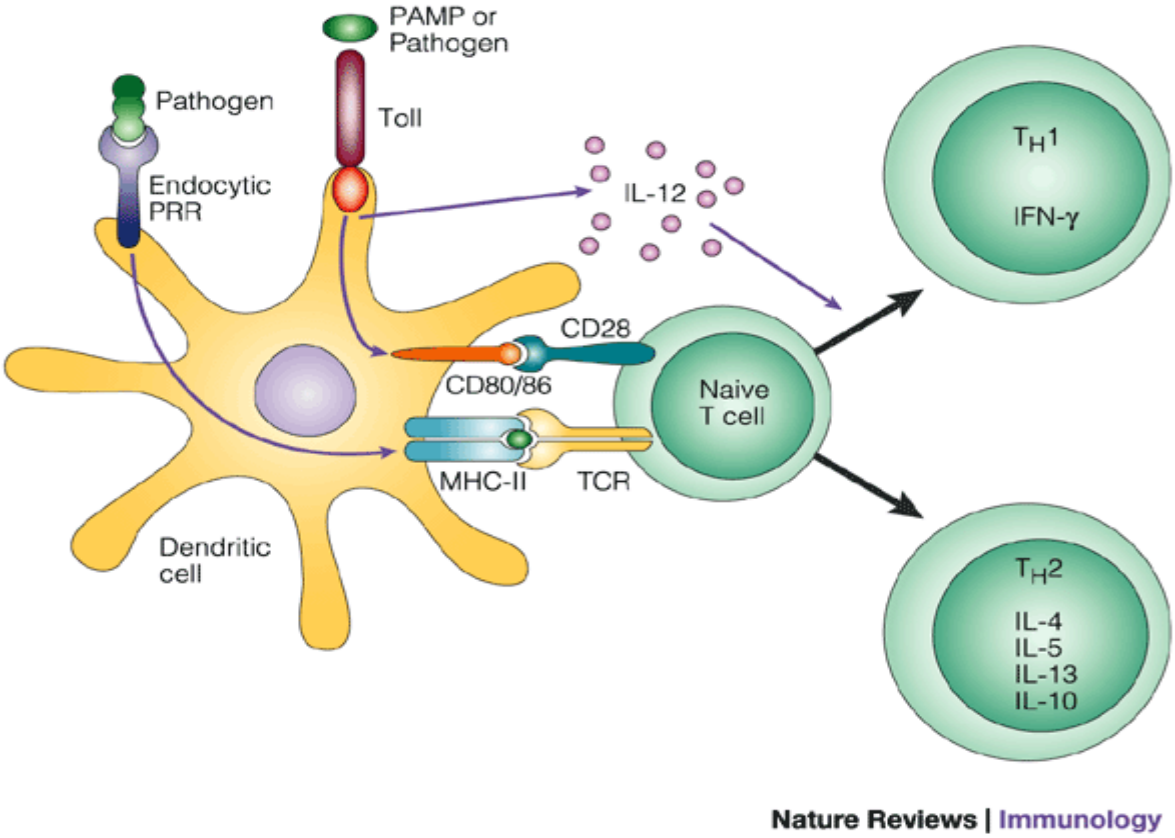


Figure 1.1: Recognition of PAMPs by TLRs on APCs induce T cell response [11].

1.1.2 Adaptive Immune System

Adaptive immune system steps in when the innate immune system is insufficient to clear out the pathogens effectively in the body. While innate immune system is fighting with the pathogenic reproduction, it also awakes the adaptive immune response by antigen procession and presentation. Thus, adaptive immune system known as the second layer of protection and highly specific to foreign antigens unlike the innate immunity which attacks only to general threats. Adaptive immune response is slower that takes several days or even weeks, yet more efficient because it can create an immunological memory by learning about the threat and undergoes a change in its specialized cell types by somatic gene rearrangements.

There are two types of adaptive immune response with its important types of cells; B and T lymphocytes. B lymphocytes create the humoral immunity. When naïve B cells come across to an antigen or Th cells, they differentiate into plasma cells which are capable of producing and secreting antibodies. Those antibodies can recognize circulating pathogens and either activates complement system to phagocyte the pathogen, or neutralize them by blocking pathogen binding sites to the host cells [12].

T progenitor cells are produced in bone marrow and migrate into thymus to mature. While in thymus they developed T cell receptors (TCRs). Unlike B cell receptors (BCRs) that can detect any kind of antigen, T cells can recognize only processed protein structures via their TCRs. This recognition is possible only by peptide - MHC complex and other co-stimulatory molecules on the surface of APCs. If a T cell recognizes a self antigen, it is destroyed before matured to avoid any attack to the host.

According to their CD4 or CD8 co-receptor expression, their roles determined as cytotoxic, helper or regulatory T cells. Upon activation, naïve $CD8^+$ T cells are differentiated into cytotoxic or memory T cells, while naïve $CD4^+$ T cells can differentiate into Th1, Th2, Th9, Th17 or Treg. These T cell subsets are characterized by their cytokine secretion and receptor expression profiles. As a result of this differentiation, they can kill the target cells, activate and improve the function of other immune cells and through Tregs suppress immune response to prevent autoimmunity [3, 13].

1.1.3 APCs as key players mediating innate and adaptive immune responses

The correct and effective translation of the innate signals is the key factor for any communication between innate and adaptive immune system. Antigen presenting cells make this conversation by recognizing the pathogens, processing their peptides and present them to the other cells. Beside presenting antigens in the peptide form, APCs have the capacity to present self or foreign lipids to CD-1 specific T cells or NK cells [14]. There are several factors affect antigen presentation and procession; (1) physical form of the antigen, (2) the delivery method, (3) the property of the APC [15].

Even though, any nucleated cell that express MHC can present antigens, only some can initiate B and T cell functions. Macrophages, dendritic cells and B cells can effectively prime adaptive immunity and known as professional antigen presenting cells (pAPC). Antigen presentation has two major signaling pathways; MHC-I and MHC-II to present epitopes to the TCRs of $CD8^+$ T lymphocytes or $CD4^+$ T lymphocytes, respectively. Although macrophages and B cells play very important roles as pAPCs, only dendritic cells can activate those naïve to an antigen. Thus, they stay in the center of immunology by bridging innate and adaptive immunity [16].

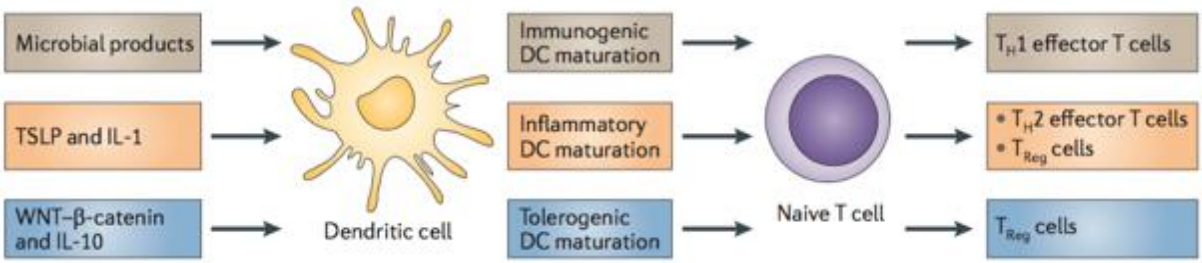


Figure 1.2: Different types of dendritic cell maturation stimulates different types of T cells [17].

Dendritic cells are bone marrow derived cells that can be found in many tissues in our body. Before they encounter a foreign antigen, they remained in immature or steady state in the peripheral tissues such as skin or mucosa. In the immature state they present only self antigens. Because they express very low levels of MHC and co-stimulatory receptors

in the immature state, they can not facilitate T or B cell activation. Instead, they lead to immune tolerance either by T cell deletion, anergy or differentiation of T cells into Tregs [18]. Once they come up with a non-self antigen, they migrate from peripheral tissues into the lymph nodes where they stimulate T and B cells. Figure 1.2 is shown as an example of different dendritic cell-T cell interaction. The factors drive dendritic cell migration includes; the type of the foreign antigen, the molecules that expressed by DCs and surrounding immune cell responses to the microbes. During migration, antigen loaded DCs get matured by upregulating their MHC molecules, co-stimulatory molecules, cytokine secretion ability as well as downregulating antigen capture activity [15].

Even though adaptive immune response is cellular or humoral, the large scale of the immune response is shaped by variety of dendritic cells that are activated and differentiated. Dendritic cells are divided into several subsets upon their phenotypic and functional criteria but commonly known 2 major subsets are myeloid DCs (also known as classical or conventional DCs) and plasmacytoid DCs (pDCs) [19]. Different types of dendritic cells have different cytokine expression profiles upon binding to corresponding antigen through their PRRs. Thus, DCs have the functional flexibility to respond inflammatory changes in their environment. Several number of CLRs, TLRs or intracellular helicases expressed by DCs can control the antigen uptake and selection of the antigen that have the potential to be presented. Because they regulate the control of DC maturation and their cytokine production as well, DC specific TLR signaling is of interest to immunologists while studying antigen procession [20].

Once activated by a proper signal, APCs use two main pathways for antigen processing; MHC-I pathway for endogenous, cytosolic antigens and MHC-II pathway for exogenous antigens. Beside other APCs, dendritic cells primarily cross-present exogenous antigens by using MHC-I pathway to CD8 T cells called as cross-presentation [21]. This mechanism gives DCs the ability to present antigens without infected by the pathogen. Dendritic cells can also efficiently phagocyte small membrane pieces of living cells, a process known as nibbling [22].

After activated and maturated, dendritic cells modulate T cell activation by 4 main signals. First signal is the detection of the presented antigen by MHC molecules on DC surface by

T cell receptors (TCRs). This signal is antigen specific as mentioned. Second signal consists of interaction between costimulatory molecules of B7 proteins CD80, CD86 on DC surface and CD28 or CTLA-4 on T cells [23]. In the absence of second signal T cells can not be activated. Thus, second signal provides whether T cells will respond to activated and maturated DCs or not. Third signal provides the functional polarization of T cells into certain T cell subsets. Varied number of PAMPs and danger signals induce different types of cytokine secretion from DCs and thereby affect T cell differentiation. Upon fourth signal, T cells are stimulated for their migration into the location where DCs first encounter with the pathogen and orchestrate the immune response through their secretion of cytokines such as IL-17, IFN-γ, IL-4, IL-10 and others [24]. Thereby, DCs play essential roles in T cell priming, T cell homeostasis, tolerance induction and interaction with other immune cells.

1.2 Purinergic Signaling

Nucleotides and nucleosides such as ATP and adenosine are considered to be intracellular molecules found in all cells in the past. Burnstock and his colleagues discovered that ATP or a related nucleotide not only work inside the cells but also play important roles in the extracellular space [25]. Since then, extracellular accumulation of purine nucleotides and nucleosides are known to create the purinergic signaling.

ATP can be found almost at millimolar concentrations inside the resting cells of a healthy tissue, whereas it is around nanomolar concentrations in the extracellular space [26]. The concentration of adenosine in the extracellular fluid depends on: (i) the balance between its release from cells, (ii) its generation by ectonucleotidases on the cell surface, and (iii) its re-uptake by adenosine transport processes that is illustrated in Figure 1.3 [27].

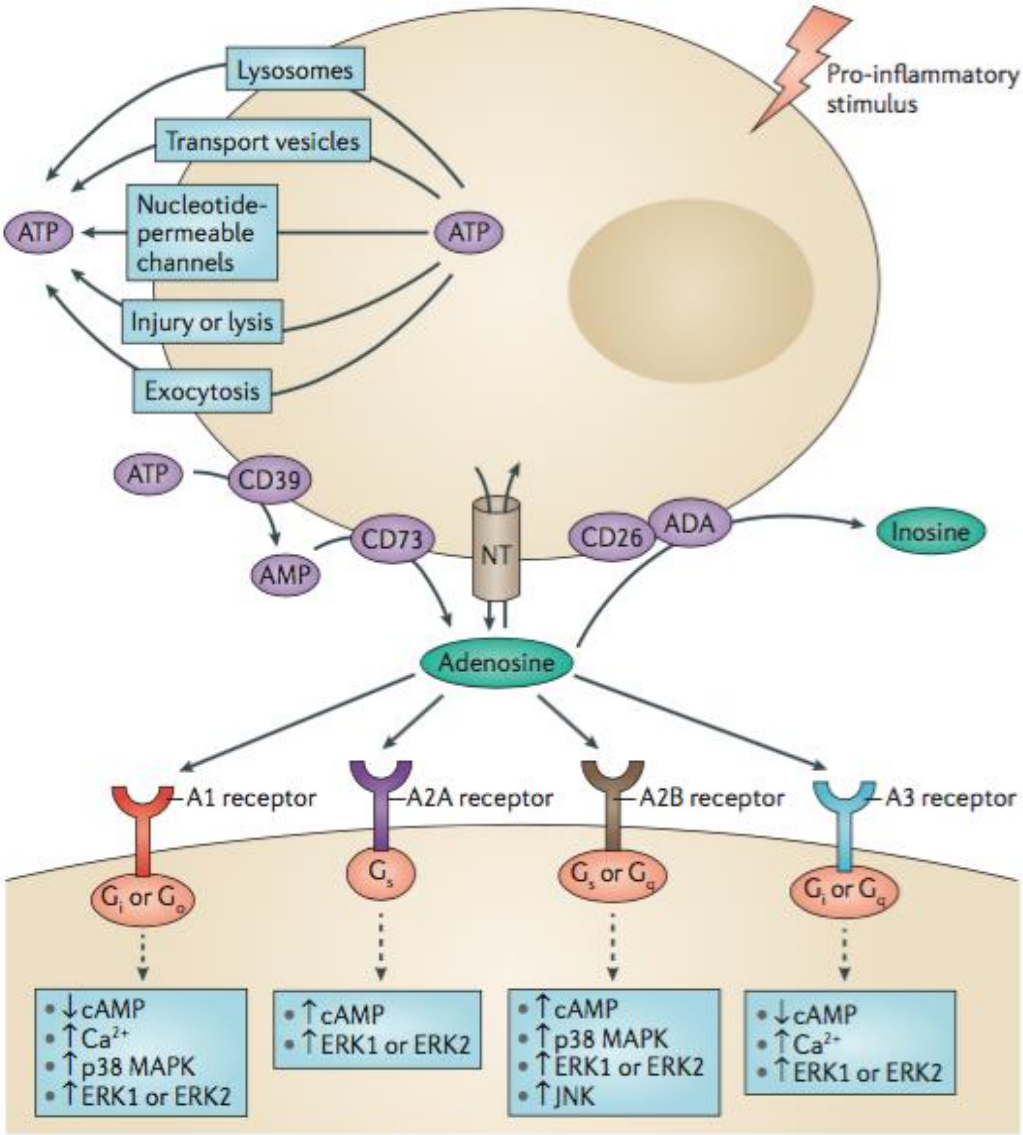


Figure 1.3: An example of adenosine receptor signaling [28].

There are some mechanisms trigger nucleotide release. One of the well known mechanism is cell damage and death. Apoptotic or necrotic cells release ATP and other nucleotides and create danger signals for nearby cells [29-31]. Other mechanisms contain different types of stress factors; hypoxia, pathogen invasion, mechanical stimulation and osmotic swelling [32, 33].

Once nucleotides accumulate in the extracellular fluids, various types of ectonucleotidases rapidly convert them into their degraded products. Ecto-ATPases catabolyze ATP to ADP, ecto-apyrases degrade ATP and ADP to AMP and ecto 5’-nucleotidases degrade AMP to

adenosine. CD39 has been discovered to be the major ecto-apyrase while CD73 is known to be an ecto 5'-nucloetidase [34].

Purinergic signaling have 2 main receptor types; P1 and P2 receptors. P1 receptors are the receptors for adenosine. On the other hand, ATP receptors are referred to as P2 receptors and can be subdivided into 2 groups; P2X for ionotropic ATP gated channels and P2Y for G protein coupled metabotropic receptors [35].

1.2.1 Adenosine receptors

Once produced, extracellular adenosine exerts its effects by four transmembrane G protein coupled receptors; A1, A2A, A2B and A3 [36-38]. These receptors, also known as P1 receptors, have varied affinities for adenosine. While A1, A2A and A3 receptors display high affinity for low amounts of adenosine, A2B receptors exhibit low affinity for higher doses of the nucleotide [39]. Throughout evolution, A1, A2A and A2B receptors are 80-95% homologous and conserved, whereas A3 receptor is variable between species [40].

Depending on cell type, activation status, stage of cell differentiation and inflammatory mediators present, each receptor expression levels can be changed [41]. A1 and A3 receptors are coupled with G_i, G_q and G_o proteins and function by promoting cell activation. By contrast, A2A and A2B receptors are coupled to G_s proteins and exhibit their effects by suppressing cellular responses via activation of adenylyl cyclase causing the production of cAMP [42].

1.2.2 Adenosine signaling of immune cells

Immune cells need danger signals to be activated to perform their duties in immune surveillance. Adenosine has all the pre-requisites for being defined as a danger signal for immune system; (i) it can be quickly and easily generated upon any cell damage or death, (ii) its concentration is very low in the extracellular space under resting conditions, so any excessiveness can be realized immediately, (iii) it is a small molecule to be diffused easily, (iv) it has specific receptors to be recognized by immune cells, and (v) it is easy to be destroyed because every plasma cell has ectonucleotidases [43]. All of these properties

make adenosine an important and interesting small molecule to be studied on immune cells.

Distinct subsets of immune cells are affected by extracellular adenosine in different ways. This can be either stimulatory or inhibitory, depending on the amount and time of the adenosine release. The type of the adenosine receptor that participate in the signaling also affects the immune cell activation. For example, adenosine receptor activity in neutrophils is regulating the production of reactive oxygen species and phagocytosis. Adenosine has an inhibitory effect generated mainly by A2A and A2B receptors, while A1 and A3 receptors promote directed migration (chemotaxis) of neutrophils [44]. Similarly, mast cell degranulation is stimulated through A3 receptors but inhibited by A2A and A2B receptors [45]. Adenosine have powerful inhibitory effects on T cell proliferation. One study showed that adenosine signaling suppressing the IL-2 production and thereby restrict T cell responses to costimulatory signals and T cell proliferation. In addition to that, adenosine signaling leads to expansion of Treg cells. Treg cells through expansion of extracellular ectonucleotidases CD39 and CD73 cause production of more adenosine and inhibit the activity of dendritic cells [46]. A2AR deletion of C57BL/6 mice strongly effects naïve T cell survival and development showed the importance of adenosine regulation in T cell homeostasis [47, 48]. These and other studies indicate that adenosine receptor expressions are changed depending on the activation status of immune cells and effect their functional properties.

1.2.3 Adenosine regulation of APCs

APCs have central roles in modulating immunological processes by their antigen presentation capability and clearance of foreign cells. Thus, adenosine's effect on APC function (especially macrophages and dendritic cells) and their adenosine receptor activity have attracted considerable attention in the past years. Recent studies indicate that PRRs interfere with the intracellular pathways that are activated by adenosine signaling. For example, Figure 1.4 representing the altered amounts of cAMP levels through adenosine signaling of APCs. Extracellular adenosine inhibits LPS induced release of TNF-α, IL-12, MIP-1α and nitric oxide (NO) independent of NF-κB, whereas augments production of anti-inflammatory IL-10 levels of macrophages [49-51]. Several number of studies indicate

that adenosine have A2A and A2B dominant effects on macrophage function. As the phagocytosis of apoptotic cells by macrophages are drived by upregulation of A2A receptors and downregulation of A3 receptors, adenosine gradually shows its suppressive effects on inflammation while apoptotic cells are engulfed by macrophages [52]. Adenosine and its receptors also triggers the release of VEGF through TLR-4, TLR-2, TLR-7 and TLR9 agonists [53]. In addition to that, adenosine signaling play preventive roles on monocyte differentiation into macrophages through A2 receptors by blocking monocyte development at a stage to create dendritic cell like monocytes [54, 55]. Finally, it has been shown that A2A and A2B receptors increase the process of M2 polarization of macrophages and additionally, M1 macrophages have lower expression of ectonucleotidases compared to M2 macrophages [56].

Dendritic cells link innate immunity to adaptive immunity by acting as professional antigen presenting cells and stimulating T cell mediated immune response as mentioned before. For this reason, it is crucial to understand how proliferation, differentiation and activation status of dendritic cells are affected by adenosine signaling.

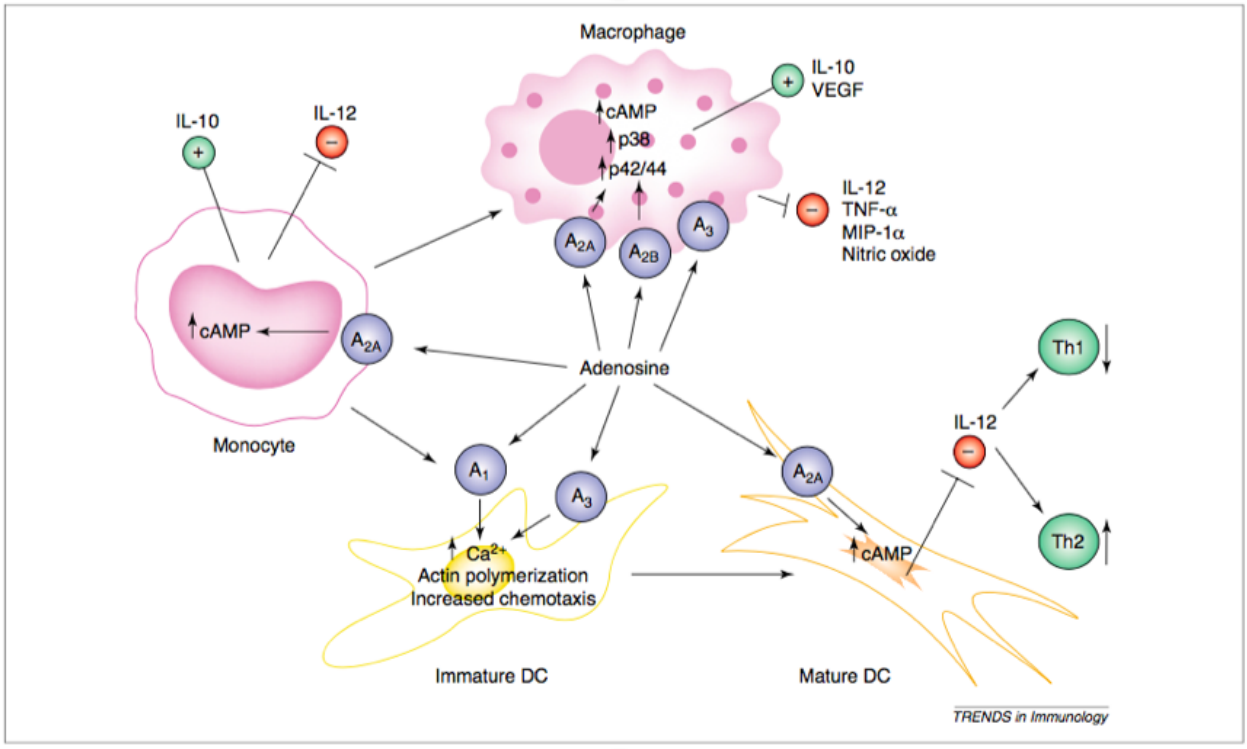


Figure 1.4: Adenosine regulate APC functions through altering intracellular cAMP level [57].

Several data from human and mice studies show the strong modulatory effects of adenosine receptors on dendritic cell functions. The regulation by adenosine receptors depends on whether adenosine is found in chronically low or acutely high concentrations surrounding dendritic cells. [43].

Dendritic cells contain all four types of adenosine receptors but they change expression levels of those upon variety of extracellular signals (pathogens, inflammatory cytokines, neurotransmitters), their maturation status and progenitors where they differentiate from [58]. G_i coupled adenosine receptors (A1, A3 or both) are mostly expressed in both immature myeloid and plasmacytoid dendritic cells. Immature dendritic cells also express A2A receptors at low levels but they are not able to induce accumulation of intracellular cAMP. During their maturation process A1R and A3R expression levels downregulated while A2AR and A2BR upregulated. As a result of A1 receptor activity, immature plasmacytoid dendritic cells are triggered to migrate into the site of inflammation [59, 60]. In the absence of TLR activation, adenosine triggers chemotaxis, mobilization of intracellular calcium and actin cytoskeleton reorganization. [59] Furthermore, adenosine cause an increase in the LPS induced CD80, CD86, CD54, MHC-I and HLA-DR molecules on immature dendritic cells, as well as decreases the ability to stimulate naïve T cells. Adenosine also manipulates the profound secretion of IL-10 while increasing the micropinocytosis ability of immature dendritic cells [58].

When DCs are LPS matured, adenosine inhibits TNF-α and IL-12 secretion but enhanced IL-10 secretion by binding A2ARs. Therefore, helps prevent the tissue injury that occur because of overwhelming immune response mediated by innate immune system [44]. In addition, adenosine increases the secretion of CCL17 and decreases CXCL10 secretion of mature dendritic cells. By this way, adenosine inhibit DCs to induce the chemotaxis of Th1 cells [44]. Another study supports that blockade of A2BR on dendritic cells enhance DC activation and stimulates lymphocyte activation via secreted CXCR3 and CXCL10 to promote anti-tumoral responses [61]. Adenosine can also affect dendritic cells while they differentiate via stimulating production of proinflammatory, angiogenic and tolerogenic effectors. However, they exhibit completely different cell surface markers and showed distinct types of dendritic cell populations [62].

These types of studies indicate that, proliferation, maturation, differentiation status, phagocytosis, cytokine and chemokine production of APCs are highly regulated by adenosine through activation of adenosine receptors.

1.3 cAMP

Extracellular signals that are large and hydrophobic need cell surface receptors to cross the membrane. These signals can be hormones, neurotransmitters, lipid mediators or pharmacological agents called as 'first signal' [63, 64]. Binding of these ligands to their corresponding receptors of the cell membrane is not enough for external signals to be transmitted inside the cell. Second messengers such as phospholipids, Ca^{+2} and cyclic nucleotides are responsible for converting and amplifying these signals and create signal transduction processes as an intermediate molecule inside of the cells.

Cyclic adenosine monophosphate (cAMP) is the first identified and well characterized second messenger as a cyclic nucleotide [65]. Because cAMP signaling pathway involved in many cellular events by affecting transcription and translation of genes, conformational changes and localization of other proteins, its amount in the cytosol is regulated by special enzymes adenylyl cyclase (produce cAMP), phosphodiesterase (degrade cAMP) and at a subcellular level to better coordinate cellular response [66].

Once the ligands are bound G protein coupled receptors (GPCRs), conformational changes occur and GTP binding proteins (heterotrimeric G protein complex) get activated. This activation results in exchange of GDP for GTP and then separation of $G\alpha$ subunit from G protein $\beta\gamma$ dimers via protein domain dynamics as illustrated in Figure 1.5. As a result, those separated subunits further activate other proteins by phosphorylation events and provide continuance of the signal transduction. For cAMP production, adenylyl cyclase (AC) should be activated by free $G\alpha$ subunit. Activated AC then generate cAMP and pyrophosphate by cyclization of ATP. In contrast to AC, phosphodiesterases (PDEs) work by decomposition of cAMP into AMP by hydrolisation. There are 10 differentially expressed AC isoforms and 11 PDE gene families known to be expressed in different cell types and tissues in mammalian cells [67, 68].

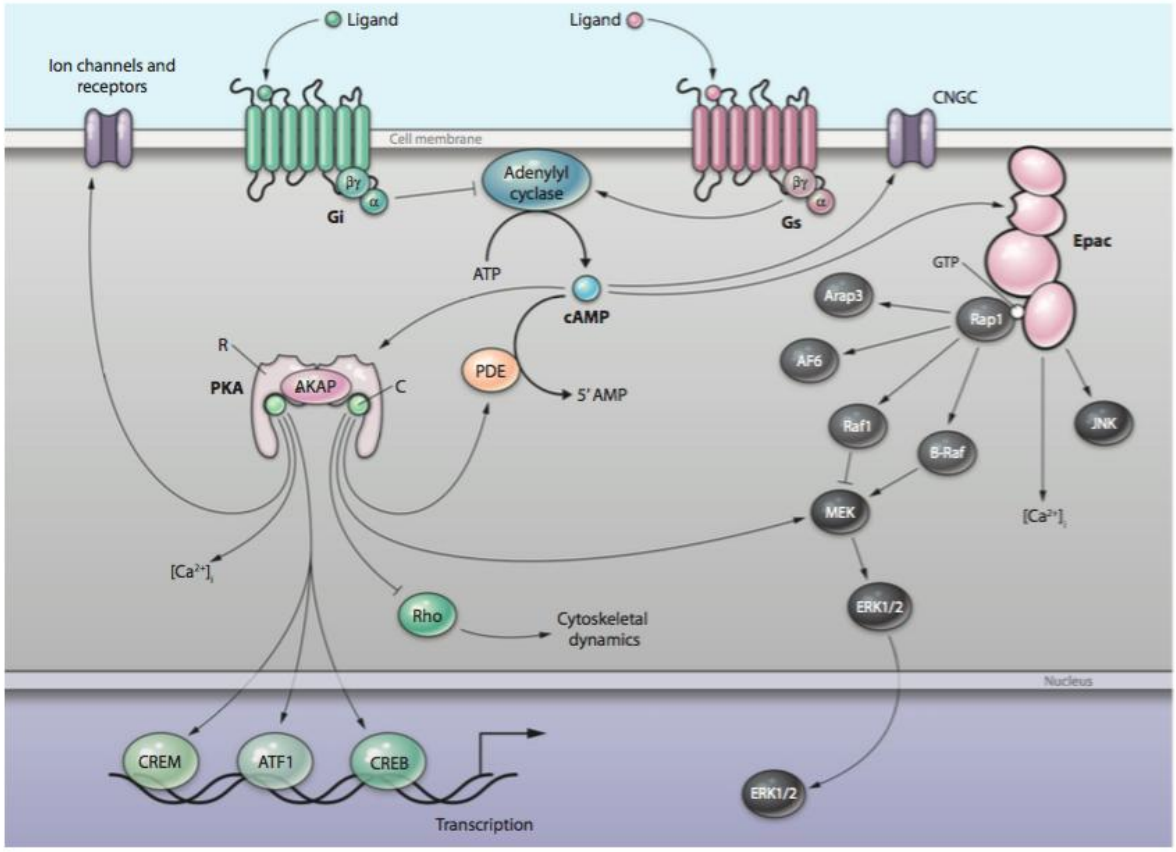


Figure 1.5: Summary of cAMP signaling pathway [69].

After its generation by AC, cAMP function mainly through cAMP-dependent protein kinase A (PKA). Other PKA-independent targets of cAMP are the two isoforms of exchange protein activated by cAMP (EPAC) and cyclic nucleotide gated ion channels (CNGCs) [69].

The PKA is a holoenzyme that is first discovered and best studied among other direct cAMP targets. It has a tetrameric structure composed of two catalytic subunits (C) and two regulatory subunits (R) bound each other non-covalently (R2C2 complex). When the cytosolic cAMP level increased, 4 cAMP molecules bind regulatory subunits of PKA (2 cAMP molecule for each R subunit). This will cause regulatory subunit to dissociate from the complex. Consequently, catalytic subunits will be free to phosphorylate other proteins on their serine or threonine residues both in the cytoplasmic and the nuclear compartments, as well as the regulatory subunit itself [70]. The R subunit on the other hand, bind to catalytic subunit and prevent its activation in the absence of cAMP [71]. In addition to that,

regulatory subunit associate with A-kinase anchoring proteins (AKAPs) for localization of PKA in particular cellular organelles and to the plasma membrane [72].

Because PKA is the main controller for majority of the cAMP signaling pathway, it regulates a vast number of cellular processes including proliferation, differentiation, growth, metabolism, gene regulation or apoptosis. Specificity of these responses depend on the participation of R and C isoforms that are expressed and activated on a given cell. The regulatory subunit of PKA consists of two isoforms RI and RII and they further divided into four: RIα, RIβ, RIIα and RIIβ at the genetic level and show different functions [73, 74]. By contrast, the catalytic subunits are divided into 3; Cα, Cβ and Cγ with identical kinetics and functions [75]. These and additional isotypes arise from splice variants display different functional and biochemical properties and implies specificity of PKA functions.

Transcriptional regulation of cAMP on the nucleus is mediated through activation of cAMP response element binding protein (CREB), cAMP response element modulator (CREM) and activating transcription factor-1 (ATF-1) [76]. Each of these targets of PKA has a conserved kinase binding domain which PKA can specifically bind and activate when it is translocated in the nucleus. Once phosphorylated by PKA, these transcription factors bind and regulate the DNA sequence called cAMP-responsive element (CRE). The genes contain CRE consensus sequences in their promoters respond to cAMP signaling as a result of these series of reversible phosphorylation events. In summary, the catalysis of PKA can be direct; by protein phosphorylation to increase or decrease the protein activity, or indirect; by first activating CREB and other transcription factors for further regulating other genes and synthesis of other proteins.

Exchange protein activated by cAMP (Epac) is a cAMP dependent protein which has guanine nucleotide exchange factor (GEF) activity. When cAMP binds, Epac catalyzes the exchange of GDP for GTP by GEF activity and further activates Ras superfamily small GTPase homologous Rap1 and Rap2 [77]. Therefore, many cellular processes, that Ras family proteins are included, also affected by cAMP activation of Epac.

Two different isoforms of Epac protein have been described in mammalians: Epac1 and Epac2. They have broad tissue distribution, described in a variety of cell types with different physiological functions, but have similar domain structures and working

principles. Both isoforms include N-terminal regulatory and C-terminal catalytic regions. N terminal region consist of a DEP domain and one (Epac1) or two (Epac2) cyclic nucleotide binding domains (CBD). While DEP domain is responsible for anchoring to the membrane, CBD domain, which is a homologous to R subunit of PKA, binds to the cAMP [78]. On the other hand, catalytic region contains GEF domain called CDC25-homology domain (CDC25-HD), a regulatory domain named Ras exchange domain (REM) and an additional Ras-association domain (RA) for again anchoring to the plasma membrane. When cAMP is low, regulatory domain work as auto-inhibitor by preventing Rap association to the CDC25-HD domain [79, 80]. Upon cAMP activation, CBD domain unfolds the Epac and let catalytic region to bind Rap GTPase [81].

All signaling events that have been previously attributed to PKA signalling, may actually involve Epac since their binding affinities to cAMP have been found to be very similar [77]. Thus, without any kinase activity but being a direct target of cAMP, Epac acts either alone or with PKA and creates an important alternative cAMP messenger system.

1.3.1 PKA vs. EPAC mediated effects of cAMP on immune cells

Since its discovery, cAMP has been shown to regulate diverse cellular functions, such as proliferation, growth, adhesion, migration, growth arrest, gene transcription and apoptosis in mammalian cells through its targets PKA, EPAC and Ca^{+2} [82]. Studies about the role of cAMP signaling pathways on immune cells revealed cAMP as an important immunomodulatory illustrated in Figure 1.6. Even though elevation of cAMP is known to be immunosuppressive, recent reports demonstrate cAMP as a positive player in immune activation [83, 84]. The cell type involved, the amount of cAMP, localization of cAMP and cAMP effectors involved determine the regulation of immune system by cAMP [85].

The regulators of cAMP signaling A-kinase anchoring proteins (AKAPs), Phosphodiesterases (PDEs), and Adenylyl Cylase (ACs) change the amount and localization of intracellular cAMP and affect the immune cell responses by targeting PKA and EPAC [85]. Adenylyl cylases, which hydrolize ATP to cAMP, have nine membrane bound and one soluble isoforms found in mammalian cells [86]. Out of several number of

studies, AC7 isozyme is found to be greatly expressed in macrophages and dendritic cells as well as T lymphocytes and play important roles in immune regulation for example participating in DC-T cell interaction via activating the PKA pathway [87, 88]. In addition to that activators of AC such as PGE2 were found to induce production of IL-23 in DCs, which leads stimulation of Th17 cells [89, 90].

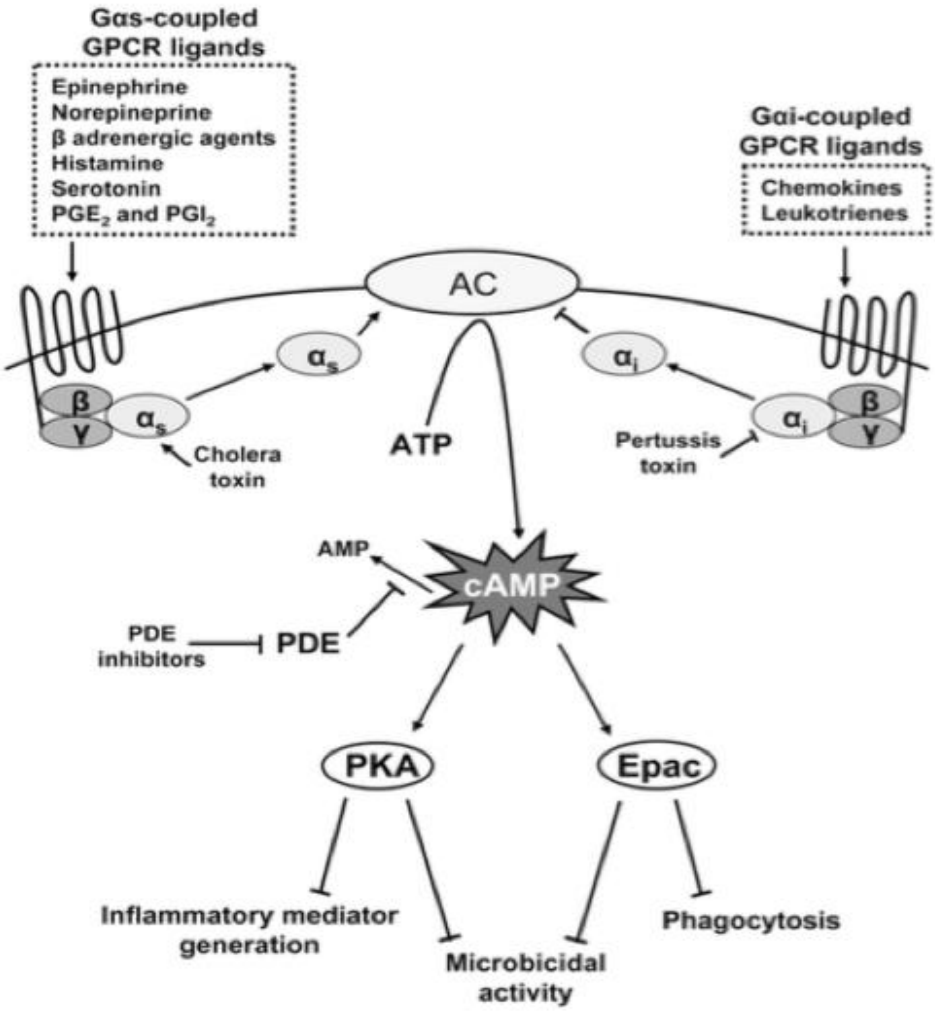


Figure 1.6: Antimicrobial functions differentially regulated by particular cAMP effectors [91].

Phosphodiesterases (PDEs) are the enzymes convert cAMP into AMP and have 11 family of proteins differentially expressed among immune cells. Some pharmacological agents inhibit PDE activity and thereby increase cAMP levels is of interest a lot because of anti-inflammatory effects of cAMP [29]. Out of other isotypes, PDE4 is the predominant

enzyme that metabolite cAMP in order to regulate immune response. Both exogenous cAMP analogs and agents increase the intracellular cAMP levels have anti-inflammatory effects upon secretion of pro-inflammatory cytokine and growth arrest of stimulated lymphocytes. Because of their tissue specific distribution, drugs developed to specifically inhibit PDE4 in APCs in order to decrease the production of pro-inflammatory cytokines to reduce allergic response. PDE4 inhibition also increases the production of IL-10 in DCs through PKA pathway which will further inhibit stimulated T cell proliferation and their cytokine production again for asthma like diseases [92].

Interactions of R subunit of PKA with AKAP proteins target them to specific subcellular localization. Because AKAPs have almost 50 members, their interaction gives specificity to PKA-derived actions [93, 94]. Through PKA activation by elevated cAMP levels, antigen presentation and DC maturation are increased. AKAPs are expressed differentially during the maturation process of DC and they are necessary for PKA binding to lipid rafts of dendritic cells to regulate antigen presentation, CD4 T cell activation, TNF-α and IL-10 production, expression of costimulatory molecules and chemokine receptor CCR7 [95].

These regulators of cAMP generally studied as PKA-dependent pathways. It is now widely accepted that EPAC have many important contributions on cAMP directed immune cell responses. Epac1 is the major isoform expressed in immune cells and it has either suppressive or stimulatory functions [96]. Although principal effects of cAMP are mediated by PKA signaling in T cells, recent studies provide evidence that Epac is very important for T cell activation and proliferation [97]. The first study of Epac1 function in macrophages revealed that Epac1 inhibits Fc receptor phagocytosis in alveolar macrophages while PKA specific cAMP analog has no effect on this process [98]. On the other hand, Epac1-Rap1 mediated signaling pathway leads to an increase in proliferation of macrophages in coordination with PKA pathway. This cooperation is also true for attenuated phagocytosis of microglia and peritoneal macrophages. Other studies showed that while LPS induced TNF-α production is suppressed by a PKA dependent manner, phagocytosis is inhibited by Epac1 dependent pathway in macrophages [99, 100]. For the case of dendritic cells, Epac1-Rap1 signaling pathway is still under investigation. However, some studies showed that Epac regulate chemokine release and cell motility of DCs, which is very important because constant circulation and motion of immune cells are

critical for the immune system [101]. The dendritic cell migration enhanced by Tregs, which promote cAMP production upon sensing extracellular adenosine produced by these cells [102]. Beyond its suppressive effects on cytokine release in DCs, the immune synapses between APC-T cells also abrogated by Epac1 signaling [103]. As a result of these studies, APC functions including; integrin-dependent cell adhesion, chemotaxis, polarization, and phagocytosis are regulated by Epac pathway [104].

Although initial studies indicate PKA as the main regulator of cAMP directed immune responses, Epac1 and Epac2 have been found to act as novel cAMP mediators that use mechanisms independent of PKA in both innate and adaptive immune system [105]. Thus, both mediators (either alone/or together) have crucial functions in distinct immune responses. Depending on the strength and duration of the signaling, and also their relative abundance, distribution and localization, PKA and EPAC may have redundant, independent or even opposing effects in regulating a specific cellular function [106].

1.4 Aim of the study

As an extracellular purine nucleoside adenosine have immunosuppressive effects on antigen presenting cells. In macrophages, several number of studies indicate that adenosine suppresses LPS induced proinflammatory cytokine secretion and increases IL-10 production. Changes in surface molecule expression of macrophages also reported by the effects of adenosine signaling. Adenosine has immunosuppressive effects on APCs through the elevation of cAMP levels as mentioned earlier. One of the intracellular receptors of cAMP, PKA, contributes to this process. Even though the effects of adenosine signaling have been studied on macrophages, the downstream modulators of the pathway haven't been shown yet. In addition to that, the detailed mechanism of adenosine stimulation on dendritic cells remained a mystery when we compare our knowledge of DC studies with macrophage studies.

As we have lack of information about the downstream modulators of adenosine mediated cAMP signaling pathway, we aim to investigate the details of cAMP signaling pathway when adenosine shape the dendritic cell functions. Our major aim was to understand which intracellular cAMP receptor is responsible for adenosine and/or cAMP-mediated polarization of DCs into an anti-inflammatory phenotype. To address this question after validating previous studies indicating the anti-inflammatory effects of adenosine signaling and cAMP, we employed PKA and EPAC specific cAMP analogs and tested DC responses in inflammatory conditions. Along with pro- and anti-inflammatory cytokine expression, and cell surface activation molecules we have also tested DC conditioned medium for its effects on T cell activation. We have also used cFOS expression and NR4A expression as known anti-inflammatory mediators of adenosine/cAMP signaling as our readouts to test the respective contributions of PKA and EPAC pathways [107, 108]. Results of this study has important implications for the regulation of immune system by targeting adenosine receptors or cAMP pathway.

Chapter 2

Materials

2.1 General Laboratory & Cell Culture Reagents and Materials

Filter tips, cell culture flasks, cell culture plates were purchased from Greiner Bio One, Austria. Cell scrapers, serological pipettes, cell strainers, culture dishes and Falcon round bottom polystyrene tubes were purchased from Corning Life Sciences Inc., USA. Mr. Frosty container and Nunc 96 well polypropylene plates for ELISA were purchased from Thermo Scientific, USA. Pipettes, electronic pipettes and multichannel pipettes were purchased from Gilson, USA and Rainin United Kingdom. X-Ray films were purchased from Kodak, Rochester, NY, USA. q-PCR plates were purchased from Roche Applied Science, Mannheim, Germany. V-shaped reagent vessels were supplied from Biohit, Finland. Levoplus motorized pipette was purchased from Scilogex, USA. 1.1 mL singles polypropylene tubes were purchased from VWR, Radnor, PA, USA. 1⁄2 Area 96 well white plates for cAMP assay were purchased from Perkin Elmer Waltham, Massachusetts, USA. Working racks were purchased from StarLab, UK. PVDF membrane roll was purchased from Biorad, USA. All plastic and metallic materials used in cell culture such as scissors, forceps, erlenmayer flasks, graduated cylinders, 2 mL cryovials, glass bottles, tube racks, magnetic stirring rods were purchased from Isolab, Wertheim, Germany.

2.1.1 Machines and Equipments

Cell culture hoods, incubators and waterbath were supplied from Nüve, Ankara, Turkey. Axiovision 4.3 microscopy was purchased from Carl Zeiss, Munich, Germany. -20 Fridge was purchased from Arçelik, İstanbul, Turkey. -80 freezer was purchased from Hettich, Geldermansen, Holland. Nanodrop and PCR machine was purchased from Thermo Fisher Scientific, Waltham, MA, USA. All western blot equipments and power supplies were purchased from Biorad, Hercules, CA, USA. X-ray cassette and X-ray hyper processor were purchased from Amersham Pharmacia Biotech, Amersham, UK. LightCycler for q-PCR was purchased from Roche Applied Science, Mannheim, Germany. CytoFLEX Flow Cytometer was purchased from Beckman Coulter, USA. ELISA reader was supplied by Biotek, USA. Vortex, shakers, mini centrifuge, rotators, plate mixer, timers were purchased from Isolab, Wertheim, Germany. List of chemicals and reagents, taqman probes, western blot antibodies, flow antibodies and kits used in the experiments can be found in Table 2.1-2.5 respectively.

2.1.2 Chemicals and Reagents

Table 2.1: List of chemicals and reagents

Name	**Catalog No**	**Brand**
2.5 mM dNTP mix	4367381	Invitrogen, USA
5x first strand buffer	18064022	Invitrogen, USA
6-Bnz-cAMP sodium salt	5255	Tocris, UK
8-pCPT-2-O-Me-cAMP-AM	4853	Tocris, UK
AccuGENE molecular biology water	51200	Lonza, Switzerland
Acrylamide/Bis-acrylamide	BA004	Introbio

ADA (Adenosine Deaminase)	10102105001	Roche, USA
BSA	SC-2323	Chem Cruz
cAMPS-Sp, triethylammonium salt	1333	Tocris, UK
cDNA Supermix	P/N84034	Quantabio, USA
Clarity western ECL substrate	1705060	Biorad, USA
ESI 09	4773	Tocris, UK
FBS	S181G-100	Biowest, France
Goat IGG fraction to hamster IGG	55397	MPbio, USA
HBSS	BE10-547F	Lonza, Switzerland
HEPES	H0887-100ML	Sigma, USA
Incuwater-clean	A5219	Applichem, USA
Ionomycin	I3909	Sigma, USA
KT 5720	1288	Tocris, UK
Monensin Solution (1,000X)	420701	Biolegend, USA
NECA	35920-39-9	Tocris, UK
Normal rat serum	13551	StemCell, Canada
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	26620	Thermo Scientific, USA
Penicillin/Streptomycin	17-745E	Lonza, Switzerland
Perchloric acid	311421	Sigma, USA
Phosphatase inhibitor cocktail	04 906 837 001	Roche, USA

Pierce™ 16% Formaldehyde (w/v), Methanol-free	28908	Life Technologies, USA
Potassium carbonate	P5833-500G	Sigma, USA
Protease inhibitor cocktail tablet	04 693 124 001	Roche, USA
Random primer (10X)	4319979	Invitrogen, USA
Recombinant mouse GM-CSF	576306	Biolegend, USA
Reverse transcriptase	4308228	Invitrogen, USA
RNaseZap	R2020-250ML	Sigma, USA
Rolipram	0905	Tocris, UK
RPMI-1640	21875-034	Gibco, USA
Sodium azide	71289-5G	Sigma, USA
Sodium pyruvate	S8636	Invitrogen, USA
StemPro® Accutase® Cell Dissociation Reagent	SCR005	Millipore, USA
SUPERase• In™ RNase Inhibitor	AM2696	Life Technologies, USA
SYBR green fastmix	P/N84067	Quantabio, USA
Tagman Universal Master Mix	4440040	Thermo Scientific, USA
TEMED	161-0801	Biorad, USA
TMB substrate set	421101	Biolegend, USA
Trypan blue	03-102-1B	Biological industries, USA

Ultrapure LPS from Salmonella minnesota R595	tlrl-smlps	Invivogen

Table 2.2: List of taqman probes

Name	**Product**	**Species**	**Brand**
B-actin	Mm00607939_s1 (beta actin probe/primer)	Mouse	Life Technologies, USA
cFOS	Mm00487425_m1 (c-Fos taqman probe/ primer)	Mouse	Life Technologies, USA
IFN-B	Mm00439552_s1 (interferon beta 1, fibroblast)	Mouse	Life Technologies, USA
IL-10	Mm00439614_m1 (IL-10)	Mouse	Life Technologies, USA
IL-12	Mm00434174_m1 (IL-12 p40 taqman probe)	Mouse	Life Technologies, USA
NR4A1	Mm01300401_m1 (NR4A1)	Mouse	Life Technologies, USA
NR4A2	Mm00443060_m1 (NR4A2 taqman probe/ primer)	Mouse	Life Technologies, USA
NR4A3	Mm00450074_m1 (NR4A3)	Mouse	Life Technologies, USA
Ptsg2	Mm00478374_m1 (Ptgs2)	Mouse	Life Technologies, USA
TNF-α	Mm00443258_m1 (TNF-α taqman probe)	Mouse	Life Technologies, USA

Table 2.3: Western Blot antibodies

Antibody Name	**Catalog number**	**Brand**
cFOS	2250S	Cell Signalling, USA
CREB	4820S	Cell Signalling, USA
ERK 1/2	9102	Cell Signalling, USA
goat a-rabbit ıgG HRP conjugate	1706515	Biorad, USA
HRP Goat anti-mouse IgG	405306	Biolegend, USA
IKKα/β	sc-7607	Santa Cruz, USA
IRF-3	sc-9082	Santa Cruz, USA
SAPK/JNK	9252	Cell Signalling, USA
p-cFOS	5348S	Cell Signalling, USA
p-ERK 1/2	9461	Cell Signalling, USA
p-IKK	3033s	Cell Signalling, USA
p-IRF3	29047s	Cell Signalling, USA
p-JSAPK/NK	9251	Cell Signalling, USA
p-p38	9913S	Cell Signalling, USA
p-CREB	9198S	Cell Signalling, USA
β-Actin	sc-47778	Santa Cruz, USA

Table 2.4: Flow Cytometry antibodies

Antibody Name	Species	Catalog number	Brand
CD11b Percpcy5.5	Mouse	557397	Biolegend, USA
CD11c PE	Mouse	60-0114-U100	Tonbobio, USA
CD16/32 (Fc block)	Mouse	14-061-85	eBiosicences
CD25 BV421	Mouse	102034	Biolegend, USA
CD4 Percp Cy5.5	Mouse	65-0041-U100	Tonbobio, USA
CD44 Biotin	Mouse	10232	StemCell, USA
CD45 Af700	Mouse	103128	Biolegend, USA
CD69 Percp Cy5.5	Mouse	45-0691-82	eBiosciences
CD8 APC cy7	Mouse	25-0081-U100	Tonbobio, USA
CD80 APC	Mouse		Biolegend, USA
CD86 PE	Mouse	12-0861-83	eBiosciences
Green fluorescent reactive dye, LDG	Mouse	L349780	Life Technologies, USA
IFN-γ PE-CY7	Mouse	60-7311-U100	Tonbobio, USA
IL-17 APC	Mouse	506904	Biolegend, USA
IL-4 BV421	Mouse	504127	Biolegend, USA
IL-9 PE	Mouse	514104	Biolegend, USA
LyG C FITC	Mouse	128033	Biolegend, USA
LyG G Af647	Mouse	25-1276-U100	Tonbobio, USA

MHC-I Af647	Mouse	116512	Biolegend, USA
MHC-II PE Cy5	Mouse	11-5321-81	eBiosicences
Streptavidin PE	Mouse	S866	Invitrogen, USA
Streptavidin PE	Mouse	S866	Invitrogen, USA

Table 2.5: List of kits

Name	**Brand**
EasySep Mouse CD4+ T cell enrichment kit	StemCell, Canada
High-Capacity cDNA Reverse Transcription Kit	Life Technologies, USA
LANCE® Ultra cAMP kit	Perkin Elmer, USA
Mouse IL-10 ELISA MAX™ Standard	Biolegend, USA
Mouse IL-10 pre-coated plates	Biolegend, USA
Mouse IL-12 (p40) ELISA MAX™ Standard	Biolegend, USA
Mouse TNF-α ELISA MAX™ Standard	Biolegend, USA
Nucleospin RNA Isolation kit	Macherey-Nagel, Germany

2.2 Solutions, Buffers and Culture Media

2.2.1 Cell Culture Media

RPMI-1640 Medium (10%)

- 10% filtered FBS
- 1% Penicillin/Streptomycin
- 1% 1X Na-Pyruvate
- All ingredients dissolved in 500 mL sterile RPMI medium
- Storage condition: +4°C

Freezing Medium

- 10% DMSO dissolved in FBS
- Storage condition: +4°C

BMDC Medium

- 50 μM 2-mercaptoethanol (prepared stocks with complete RPMI and stored at -20°C)
- 5 ng/mL GM-CSF (stored at -20°C)
- All ingredients dissolved in complete 10% RPMI medium
- Medium was freshly made

ACK Lysis Buffer

- 8.3g NH_4Cl
- 1g $KHCO_3$
- All ingredients dissolved in 800 mL ddH_2O
- pH adjusted to 7.2-7.4 and the volume finalized 1L with ddH_2O

- Storage condition: -20°C

2.2.2 Flow Cytometry Buffers

FACS Buffer

- 2% FBS
- 0.05% NaN_3
- All ingredients dissolved in 500 mL sterile HBSS
- Storage condition: +4°C

Permeabilization Buffer

- 1% FBS
- 0.1% Sodium Azide
- 0.1% Saponin
- All ingredients dissolved in sterile 500 mL HBSS
- pH adjusted to 7.4-7.6
- Storage condition: +4°C

4% Fixation Buffer

- 16% formaldehyde dissolved in sterile HBSS
- Storage condition: -20°C

2.2.3 ELISA Buffers

Coating Buffer

- 8.4g $NaHCO_3$
- 3.56g Na_2CO_3
- All ingredients dissolved in 800 mL ddH_2O

- pH adjusted to 9.5 and the volume finalized 1L with ddH_2O
- The buffer sterilized by filtering or autoclaving
- Storage condition: +4^oC

Assay Diluent

- 10% FBS dissolved in 1X PBS
- The buffer sterilized by filtering
- Storage condition: +4^oC

Wash Buffer

- 5 mL Tween 20
- 1L 10X PBS
- All ingredients dissolved in 9L ddH_2O
- Storage condition: Room Temperature

Stop Solution (2N H_2SO_4)

- 98,08 mL H_2SO_4 dissolved in 1 L ddH_2O
- Storage condition: Room Temperature

2.2.4 Western Blotting Buffers

10X PBS

- 80g NaCl
- 2g KCl
- 15.2g $Na_2HPO_4.H_2O$
- 2.4g KH_2PO_4
- All ingredients dissolved in 800 mL ddH_2O
- pH adjusted to 7.4 and the volume finalized 1L with ddH_2O

- The buffer sterilized by filtering or autoclaving
- Storage condition: Room Temperature

10X TBS

- 80g NaCl
- 2g KCl
- 30g Tris base
- All ingredients dissolved in 800 mL ddH_2O
- pH adjusted to 8 and the volume finalized 1L with ddH_2O
- The buffer sterilized by filtering or autoclaving
- Storage condition: Room Temperature

Stripping Buffer (Mild)

- 15 g glycine
- 1 g SDS
- 10 ml Tween20
- All ingredients dissolved in 800 mL dH_2O
- pH adjusted to 2.2 and volume bring up to 1L with dH_2O
- Storage Condition: Room Temperature

RIPA Buffer

- 750 µl 2M NaCl
- 1 mL 10% NP-40
- 500 µl 10% Sodium DOC
- 100 µl 10% SDS
- 250 µl 2M Tris HCL
- 500 µl 1M NaF
- 100 µl 100mM $NaVO_4$

- All ingredients dissolved in 6.5 mL dH_2O
- 2 tablets of protease inhibitor and 2 tablets of phosphatase inhibitor added
- The volume finalized 10 mL with dH_2O
- Storage condition: -20^oC

5X Sample (loading) buffer

- 10 g SDS
- 25 mL 2-mercaptoethanol
- 50 mL Glycerol
- 0.25 g Bromophenol blue
- 3.94 g Tris-HCL
- All ingredients dissolved in 25 mL dH_2O
- Storage condition: -20^oC

10X Running Buffer

- 10.08 g SDS
- 30.3 g Tris
- 144 g Glycine
- All ingredients dissolved in 1L dH_2O
- Storage condition: Room Temperature

Transfer Buffer

- 6.06 g Tris Base
- 28.8 g Glycine
- All ingredients dissolved in 1.2L dH_2O
- 400 mL MeOH added
- The volume finalized 2L with dH_2O

- Storage condition: +4^{0}C

TBS-T

- %0.1 Tween dissolved in 1X TBS
- Storage condition: Room Temperature

5% NFDM

- 2.5 g NFDM dissolved in 50 mL TBS-T
- Buffer was freshly made

10% APS

- 5 g APS dissolved in 50 mL dH_2O
- Storage condition: -20°C

10% SDS

- 5 g SDS dissolved in 50 mL dH_2O
- Storage condition: Room Temperature

1 M Tris pH 6.8

- 6.057 g Trizma base dissolved in 20 mL dH_2O
- pH adjusted to 6.8 and volume finalized to 50 mL with dH_2O
- Storage condition: Room Temperature

1M Tris pH 8.8

- 6.057 g Trizma base dissolved in 20 mL dH_2O
- pH adjusted to 8.8 and volume finalized to 50 mL with dH_2O

- Storage condition: Room Temperature

2.2.5 cAMP Assay Buffers

Stimulation Buffer

- 75 µl of 1M HEPES
- 200 µl of 7.5% BSA Stabilizer (included in the kit)
- All ingredients dissolved in 14 mL sterile HBSS
- pH adjusted to 7.4 and volume finalized to 15 mL with HBSS
- Buffer was freshly made

Chapter 3

Methods

3.1 Maintenance of Cells

3.1.1 Bone Marrow Derived Dendritic Cell Generation

Isolating cells from bone marrow (day 0)

Femur and tibia were collected from C57/BL6 mice and put in a bacterial plate contains ice cold RPMI medium. Bones were cut on the edge and placed in a 0.5 mL tubes with wholes at the bottom. 0.5 mL tube then placed into a 2 mL Eppendorf tube to be centrifuged at 7000 rpm for 15 sec. The pellet dissolved in 0.5 mL HBSS by making single cell suspensions and transferred into 50 mL falcon. To lyse the RBCs, 3 mL of ice cold ACK lysis buffer was added for ~3min. Rest of the falcon was filled with HBSS and spun down at 1200 rpm for 5 min. Supernatant removed and the pellet was resuspended in fresh BMDC medium to be counted and placed in bacterial petri dish. Each petri dish contained 10 mL aliquots of BMDC medium at cell concentration of $2x10^6$ cells. Petri dishes placed into the cell culture incubator, which is set for 37 °C with 5% CO_2.

Cell Growth

Day 3: Cells were fed with 10 mL new BMDC medium in every dish

Day 6: 10 mL old medium was removed from each dish, centrifuged at 1200 rpm for 5min

and cell pellet was resuspended in 10 mL fresh BMDC medium. Then it was transferred back in to the original dish.

Day 8: Cells were fed with 10 mL new BMDC medium in every dish

Harvest (Day 10)

Half of the medium was removed to 50 mL falcon tube. Remained medium was pipetting up and down to collect the low attached cells. Falcons then centrifuged at 1200 rpm for 5 min. Cell pellet was resuspended with RPMI medium to be counted and used for the following experiments or frozen with freezing medium as $5x10^6$ cells/cryovial. The quality control of dendritic cells checked with flow cytometry.

3.1.2 RAW macrophages

RAW macrophages are strongly adherent murine macrophage cell line. They were detached by using either scraper or accutase depending on the experiment type. They were grown in complete RPMI medium in 37^0C with 5% CO_2 incubator. Once they reached around 60-80% confluency, they were passaged. General thawing and cryopreservation techniques were applied for RAW macrophages.

3.2 General Cell Culture Techniques

3.2.1 Cell Freezing

Cells were collected with suitable method and centrifuged. Supernatant was aspirated and cell pellet resuspended in freezing medium to be placed in cryovials immediately. Cryovials then transferred into Mr. Frosty Freezing Container and placed to -80 ^{o}C freezer. Cryopreservation can be placed at -80 ^{o}C up to several months or in liquid nitrogen for a longer time.

3.2.2 Cell Thawing

Cryovials taken from -80 ºC or liquid nitrogen were immediately thawed at 37 ºC water bath. When ice melted, cell suspension transferred into 15 mL falcon which contains 10 mL 10% RPMI medium to reduce the amount of DMSO in the freezing medium. Falcon tubes centrifuged with appropriate rpm for 5min and then supernatant was aspirated immediately. Cell pellet was resuspended with fresh RPMI medium to be used for following experiments.

3.2.3 Cell Counting

Hematocytometer was used to count single cell suspensions. 10 µl of cell suspension mixed with 10 µl of Trypan Blue to selectively color dead cells. From that mix, 10 µl was taken and only live cells were counted under the light microscope with 10x objective. Cell number was calculated with the estimated formula by considering the dilution factors.

3.3 ELISA

One day before running the ELISA, capture antibody supplied by the kit were diluted 1:200 in coating buffer. The wells were coated with 50 µl of this diluted capture antibody and placed at +4 ºC fridge overnight. Next day, the plate was washed with wash buffer 4 times and dried out. 200 µl assay diluent were used to block the wells for 1 hr at RT by slowly shaking. Then ELISA plate washed with wash buffer 4 times again. 96 well plate was taken from 37 ºC incubator and centrifuged at 1200 rpm for 5 min. The supernatant was collected carefully to another 96 well plate and either used freshly or stored at -20 ºC. 50 µl supernatant was used per well either directly or by diluted with assay diluent. The plate incubated with supernatant for 2h at RT by slowly shaking. ELISA plate then washed again the same way and 50 µl 1:200 diluted detection antibody supplied by the kit were put per well for 1 hr at RT by slowly shaking. Plate was washed 4 times and each well was incubated with 50 µl 1:1000 diluted Avidin-HRP supplied by the kit for 30 min at RT by slowly shaking. The plate was washed 5 times for a longer time and dried out. TMB solution mixed in 1:1 ratio was put for 50 µl per well and incubated for 15-30 min at RT at dark. Once the blue color was visible enough, 50 µl stop solution was put per well. The ELISA plate was ready to be read at 450nM with ELISA reader.

3.4 Flow Cytometry

3.4.1 Surface Staining

Cell culture plates were centrifuged and the medium was removed. Cells were washed with ice cold HBSS and placed into FACS plate. Remaining and strongly attached cells were incubated with ice cold accutase for 5-10 min at 37°C. Detached cells were washed with HBSS twice to get rid of any accutase. Antibody mix (1:400) and Fc block (1:200) were prepared in 50µl/well FACS buffer and cells were incubated in that ab mix for 30 min on ice in a dark place. Then samples washed once with FACS buffer and the procedure continue with either fixation protocol or running with Cytoflex machine.

3.4.2 Fixation

Before intracellular staining, cells need to be fixated in order to permeabilize them for abs to enter the cell. Cell pellets were resuspended in 50µl ice cold HBSS and then 50 µl of 4% formaldehyde solution. Samples were incubated in that mix for 30 min on ice, in a dark place.

3.4.3 Intracellular Staining

Cells were incubated with PMA, Ionomycin and Monensin for 6 h before starting FACS stainings. Fixated cells were washed with FACS buffer by centrifugation. Cell pellet then resuspended with Fc block (1:200) and ab mix (1:100) in perm buffer and incubated for 40min at RT in a dark place. Cells then washed with perm buffer twice and finally resuspended in FACS buffer to be run with Cytoflex machine. Samples were kept on ice until run by avoiding light.

3.5 RNA Isolation

RNA Isolation was done according to the manufacturer's protocol in a sterilized place by RNaseZap. After treatment was done, FACS tubes were immediately put on ice to stop the reaction. Then they were washed twice with 2 mL ice cold HBSS at 1200 rpm for 5 min. Supernatant was removed and 350 µl Lysis buffer was added and mixed well. Then the

lysate was transferred into eppendorf tubes and 3.5 µl 2-mercaptoethanol was added. In this step, the lysate can be kept at -80°C for several days. Next step was to filtrate lysate by using Nucleospin Filter components supplied by the kit. After that, adjustment of RNA binding conditions, binding RNA, desalting silica membrane and digesting DNA steps were followed respectively. After that, silica membrane was washed and dried by kit's solutions. Final step was to elute the RNA by centrifugation at 11000 g for 1 min. The amount and purity of RNA was measured by using nanodrop and isolated RNAs were kept at -80 ^{o}C until used for cDNA experiment.

3.6 cDNA preparation

cDNA protocol applied after RNA isolation was done. The protocol was performed as described in the kit. Master mix was prepared in respect to Table 3.1.

Table 3.1: cDNA master mix

Components	**Volume (ul) 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

10 µl of master mix and 10 µl of RNA sample was mixed in 0.2 mL PCR tube. Samples were run with PCR thermocycler using the program given at Table 3.2

Table 3.2: cDNA program

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time	10 min	15 sec	1 min	∞

Samples were diluted 1:10 to be used for qRT-PCR and kept at -20°C.

3.7 QPCR

Even though the kit protocol is for 20 µl, our reactions were prepared for 10 µl. Reaction mix was prepared based on the Table 3.3;

Table 3.3: PCR reaction mix

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

10 µl reaction mix was placed into 96 well q-PCR plate. Then plate was sealed carefully and centrifuged at 1000 rpm for 1 min at 4 ^{o}C. Running protocol was based on Table 3.4. Samples were kept on ice until run.

Table 3.4: QPCR program

	Step 1	**Step 2**	**Step 3**
Temperature (°C)	95	95	60
Time	10 min	15 sec	1 min
Cycle	1 cycle	50 cycle	

3.8 Western Blot

3.8.1 Protein Isolation

Treatments for western blot were performed with FACS tubes. Protein amount was equalized by using BCA Protein Assay Kit as instructed. Cells were taken from 37 ^{o}C incubator and immediately placed on ice to stop the reaction after the treatment was done. Then 2mL ice cold HBSS added and FACS tubes centrifuged with proper rpm for 5 min. Washing with HBSS performed twice. Supernatant was removed and cell pellet was dissolved in 50 µl of RIPA buffer. Cells were incubated with RIPA buffer for 30 min on ice by vortexing the tubes every 10 min. Then eppendorf tubes were centrifuged at 13000 rpm for 13 min at 4 ^{o}C. Supernatant was carefully seperated from the pellet to another eppendorf tube. Sample buffer was added for the suitable amount and samples were kept either at -20 ^{o}C or -80 ^{o}C until run.

3.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Before pouring gels, glass plates were cleaned with dH_2O and 70% ethanol. First, 10% resolving gel was prepared based on the followings;

For 10 mL Resolving Gel;

- 4 ml dH20
- 3.3 ml acrylamide mix
- 2.5 ml 1 M Tris pH 8.8
- 100 µl 10 % SDS
- 100 µl 10 % ammonium persulfate
- 6 µl TEMED

Water saturated isopropanol was put as a layer on the top of resolving gel. Once the gel was polymerized, isopropanol was removed and stacking gel was poured with 10 or 15 well comb in it. It was prepared based on the followings;

For 5 mL Stacking Gel;

- 3.4 ml dH20
- 830 ml acrylamide mix
- 630 ml 1 M Tris pH 6.8
- 50 µl 10% SDS
- 50 µl 10% ammonium persulfate
- 5 µl TEMED

While the gel was polymerized, protein samples were melted and heated at 95 ºC for 5 min. Then samples were prepared with 5x sample buffer and loaded on to gels for between 5-20 µg/well. Empty wells were loaded with diluted sample buffer only. Running procedure was done with 100 Volt for about 90 min.

3.8.3 Western Blotting

4 whatman paper were cut in 7x9 cm^2 and moistened with transfer buffer. PVDF membrane same size with whatman papers was first dipped in 100% methanol to be activated for about 10sec then placed on the top of the gel. The gel and PVDF membrane was surrounded by 4 layers of whatman papers and 1 layer of sponge was covered them.

Then that sandwich was placed in transfer apparatus containing Transfer Buffer and run at 120 Volts for 1 hour, at +4 ºC. After run, PVDF membrane was cut in particular kDa and incubated with 5% of NFDM for 1 hour at room temperature on a shaker slowly.

After blocking, the membrane was quickly washed to remove any excessive NFDM Incubation with antibodies was performed with 50 mL falcons. The membrane was incubated with primary antibody either for overnight at +4 ºC or 1 hour at room temperature on a shaker slowly.

After primary antibody incubation, membranes were washed with 1X TBST for 25 min by changing the TBST in every 5 min at room temperature. Membranes were then incubated in secondary antibody for 1 h at room temperature on a shaker slowly. After secondary antibody incubation, membranes were again washed with 1X TBST for 25-30 min by

changing the TBST in every 5 min at room temperature. After washing, membranes were placed on X-ray cassette and incubated with ECL reagents, mixed in 1:1 ratio, for 5 min at dark. X-ray films were exposed to membranes at full dark place for different time periods ranging from 1 second to 30 minutes and then developed. In addition to that, the membranes were developed also with Amersham Western blotting imaging machine with different time points.

3.8.4 Stripping

PVDF membranes were washed with TBST for 5 min to get rid of ECL solution after developed. The membrane then washed twice with stripping buffer for 5-10 min, depending on the brightness of the signal, on a slow shaker. Then the membranes were washed twice with 1X PBS for 5 min on shaker. After washing with PBS, the membranes washed twice with 1X TBST for 5 min on shaker. The membrane was again developed to be sure that there were not any residue of an antibody on the membrane. After stripping was done, the PVDF membrane blocked with 5% NFDM again for 40 min on shaker slowly. After blocking, the membrane was ready to detect any other proteins in the same way.

3.9 CD4+ T Cell Isolation

Spleen or lymph nodes were taken from C57/BL6 mice in a 15 mL falcon contains 5-6 mL cold 10% FBS in HBSS. They were passed through a sterile 40 um cell strainer to get single cell suspension on 50 mL falcon. The falcon was then centrifuged at 1200 rpm for 5 min. Supernatant was removed and the pellet was dissolved with proper amount of 10% FBS in HBSS solution depending on the desired number of cells with the isolation.

3.9.1 Mouse CD4+ T cell enrichment

EasySep Mouse CD4+ T cell enrichment kit was used based on the protocol of the kit. Cell suspension dissolved in 50 µl/mL normal rat serum and the solution was transferred into FACS tube. 50 µl/mL EasySep CD4+ T cell enrichment cocktail was added and incubated

in refrigerator for 15 min. 100 µl/mL EasySep Biotin Selection Coctail 2 was added, mixed and incubated in refrigerator for 15 min. EasySep D2 Magnetic Particles were vortexed for 30 sec and 100 µl/mL of that was added and incubated in refrigerator for 5min. Cell suspension total volume was brang up to 2.5 mL with 10% FBS in HBSS and the FACS tube was placed into the magnet without the cap, incubated for 5 min. In one move, the supernatant was poured to another FACS tube and enriched cells were counted and used for the following experiment.

3.10 cAMP Assay Kit

cAMP assay was done according to the manufacturer's protocol. BMDCs were isolated and seeded as $1x10^5$ cells per well in 96 well plate. After treatment was done, supernatant was aspirated and 100 µl 0.6M Perchloric acid was added. Then 15 µl 2.5M potassium carbonate was added for neutralization. The lysate was transferred into eppendorf tube and kept on ice for at least 30min. Eppendorf tubes were quickly spun down to remove perchlorate salt and centrifuged at 10000 rpm for 10 min. The clear lysate was collected and 20 µl of the lysate were moved in to half area flat bottom well white plate. 10 µl 4X Eu cAMP tracer and 10 µl 4X ULight-anti-cAMP solution was added and waited for 1 h in RT. The half are plate was run on the SpectraMax plate reader and could be kept to be run until the next 24 h.

3.11 Statistical Analysis

All graph drawings and data analysis were performed by using GraphPad Prism 6.0 software. One way ANOVA followed by Tukey's multiple comparison was used to determine statistical differences.

Chapter 4

Results

4.1 AR Stimulation Promotes DC Polarization into an Anti-Inflammatory Phenotype

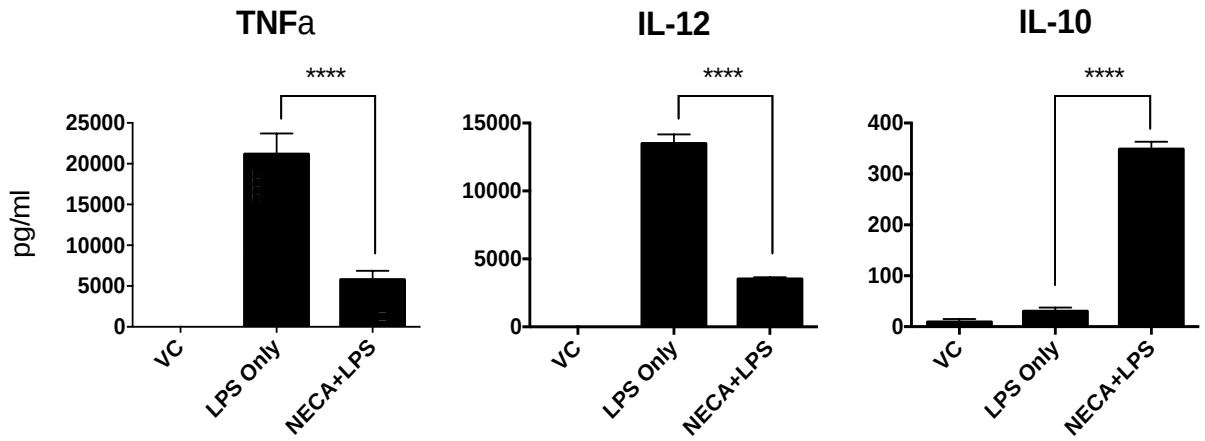
4.1.1 Adenosine Signaling affect cytokine secretion profile of Dendritic cells

Lipopolysaccharide (LPS) is the main component of Gram (-) bacteria cell's outer membrane that provides the structural integrity and security against an attack to the bacteria. It causes a strong pro-inflammatory response in many cells such as macrophages, monocytes or dendritic cells by binding their CD14/TLR4/MD2 receptor complex [109]. Several number of studies indicate that LPS strongly induces cytokine production of many cells by activating both Myd88 and TRIF dependent signaling pathways, as the ideal immune stimulator. Even though it is commonly used to trigger innate immune system, commercial LPS is generally contaminated by other bacterial components and may activate TLR2 pathway as well as TLR4 pathway. For this reason, ultrapure LPS which only activate TLR4 signaling pathway was used in our experiments as an inflammatory stimuli.

To investigate effects of adenosine signaling induced dendritic cells after LPS stimulation, we used 5' -*N*-Ethylcarboxamidoadenosine (NECA). NECA is a cell impermeable non-selective adenosine receptor agonist. It was first discovered to be a specific A2AR selective compound but turned out to be a non-selective agonist with high affinity to other adenosine receptor subtypes as well [110, 111]. Because it is not cell permeable, its effects are going to be only through adenosine receptors on the cell surface. For this reason, NECA is known to be one of the most prominent adenosine analog when studying adenosine receptor signaling.

Dendritic cells were generated from bone marrow of C57/BL6 mice as explained in Chapter 2. Quality control of our BMDCs is illustrated in Appendix Figure 1. Dendritic cells were pre-treated with adenosine deaminase (ADA) in all experiments by which NECA treatment was involved. ADA is the enzyme promotes irreversible conversion of adenosine to inosine and thereby play essential roles in regulating purine metabolism [112]. The reason why we used ADA pre-treatment is because we wanted to eliminate the basal levels of adenosine, caused by several environmental factors, in order to see the real effects of NECA in our cells. For this reason, all experiments involve NECA actually pre-treated with ADA (final:1U) for 1 h unless indicated otherwise. In addition to that, cell number experiments, dose response and time course experiments were done with BMDCs and RAW.264.7 macrophages to pick the correct and optimal dose and time period for all of our experiments (data not shown here).

A)



B)

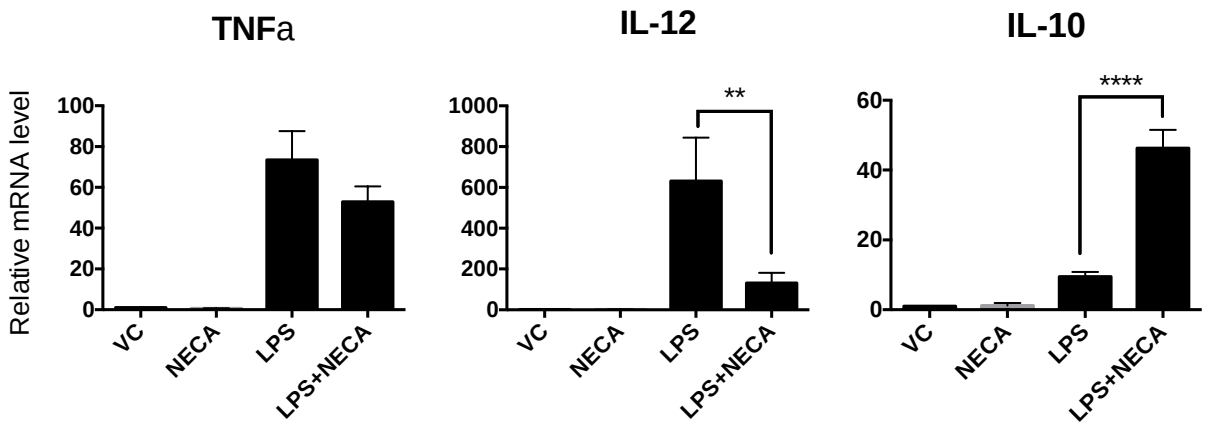


Figure 4.1: Adenosine receptor signaling decreases LPS-induced effector cytokine secretion such as TNF-α and IL-12, whereas promotes secretion of anti-inflammatory IL-10 in dendritic cells. *A) BMDCs ($3x10^5$/well) were treated with 1μg/ml LPS and 3μM NECA for overnight. TNF-α, IL-12 and IL-10 cytokine levels were measured by ELISA. B) BMDCs ($1x10^6$/tube) were treated with 1μg/ml LPS and 3μM NECA for 1 h. RNA Isolation and cDNA preparation was performed as mentioned in Chapter 2. Relative mRNA levels were measured by Q-PCR. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

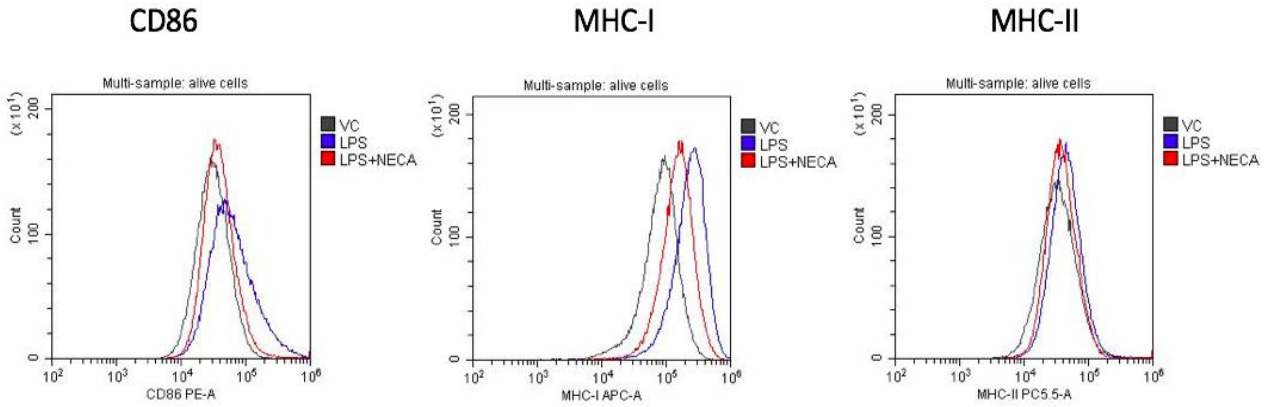
There are many studies indicating that adenosine signaling decreases LPS-induced pro-inflammatory cytokine secretion such as IL-6, IL-1β or TNF-α in murine macrophages [60, 113]. Similarly, NECA has been shown to suppress LPS-induced TNF-α and IL-6 in PBMCs or THP-1 cells [108]. LPS-induced IL-10 secretion of macrophages was increased by adenosine signaling [114]. Consistent with these previous studies, we showed that adenosine receptor agonist NECA suppresses the pro-inflammatory TNF-α and IL-12 secretion, while elevating IL-10 secretion in dendritic cells in both mRNA and protein levels (Figure 4.1). Besides, NECA alone had no effect on expression of cytokines tested here (data not shown). These results confirm other studies showing adenosine signaling promotes dendritic cells into an anti-inflammatory phenotype.

4.1.2 AR stimulation inhibits upregulation of cell surface activation markers in DCs

Dendritic cells are key players by bridging innate immunity to adaptive immunity. As mentioned in Chapter 1, T cells need to be educated by antigen presenting cells to get activated and polarized into different functional subsets. Once DCs encounter with a non-self antigen and danger molecules, they get matured and further initiate T cell activation by upregulating their surface molecule expressions such as MHCs and costimulatory molecules. In this experiment, we checked how NECA affect MHC-I and MHC II molecule and CD86 co-stimulatory molecule expressions of dendritic cells by using flow cytometry. As can be seen in Figure 4.2, addition of NECA reduced LPS induced MHC-I

and MHC-II expressions as well as CD86 expressions. These results indicate that adenosine receptor signaling not only promotes anti-inflammatory phenotype but also suppresses important mediators of APC function of dendritic cells.

A)



B)

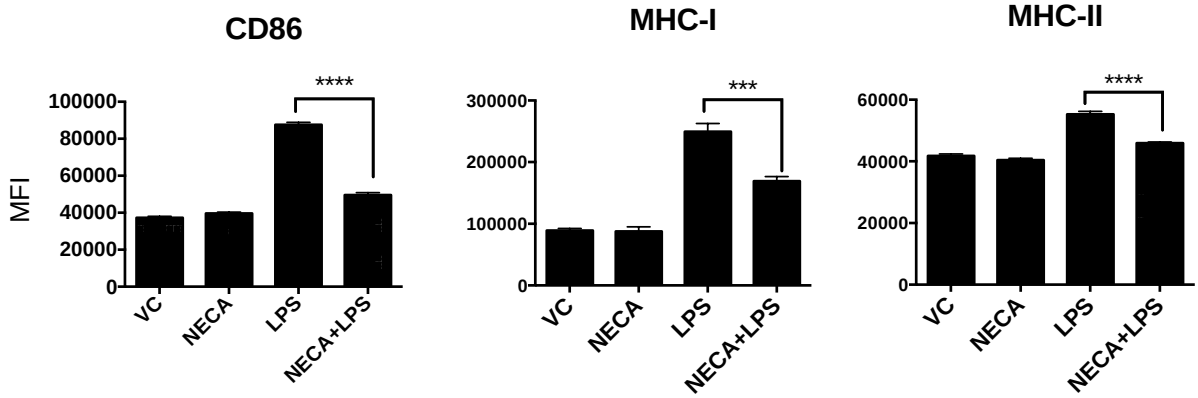


Figure 4.2: Adenosine receptor stimulation decreases co-stimulatory molecule and MHC molecule expression levels on dendritic cells. *BMDCs ($3x10^5$/well) were treated with 1μg/ml LPS and 3μM NECA for overnight. MFI rates were analyzed by using flow cytometry. A) The histogram overlay represented. B) The quantitative results represented. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

4.2 Adenosine receptor signaling promotes downstream cAMP signaling without affecting phosphoactivation of major inflammatory pathways

4.2.1 Adenosine did not decrease the phosphoactivation of inflammatory signaling pathways

When LPS used as a ligand, it binds to TLR4 receptor on the cell surface and activates the TLR4 signaling pathway through Myd88 dependent or independent pathways. As a result, several number of phosphorylation events regulate TLR-mediated signaling pathways. When Myd88 (Myeloid Differentiation Primary Response Gene 88) and its adaptor associated protein TRIP (TIR Domain-Containing Adaptor Protein) are activated by a proper ligand, they mediate pro-inflammatory cytokine production and further activation of IKK and MAPK cascades. The TRIF dependent pathway on the other hand, involves in activation of Interferon Regulatory Factor-3 (IRF3) and further promotes type-1 interferon production [11, 115, 116].

LPS stimulated wild type macrophages showed increased levels of MAPK pathway activation and further lead to an increased phosphorylation of p38, ERK and JNK proteins [117]. In order to understand the effects of adenosine signaling in LPS induced inflammatory pathways in dendritic cells, we performed western blot analysis for several targets of Myd88 and TRIF dependent pathways; IKKαβ, JNK, ERK, IRF3 and as a loading control β-actin. As can be seen in Figure 4.3 NECA didn't change the LPS-induced phosphoprotein levels of IKKαβ, JNK, ERK and IRF3 in 10 min or 30 min. In addition to that we also checked the effects of 2 min, 5 min, 2 h and 3 h after NECA and LPS treatment but again adenosine signaling didn't affect the phosphorylation levels of these proteins (data not shown). Therefore, these data suggest that adenosine receptor signaling does not influence the phosphoactivation of these major pathways to regulate DC responses.

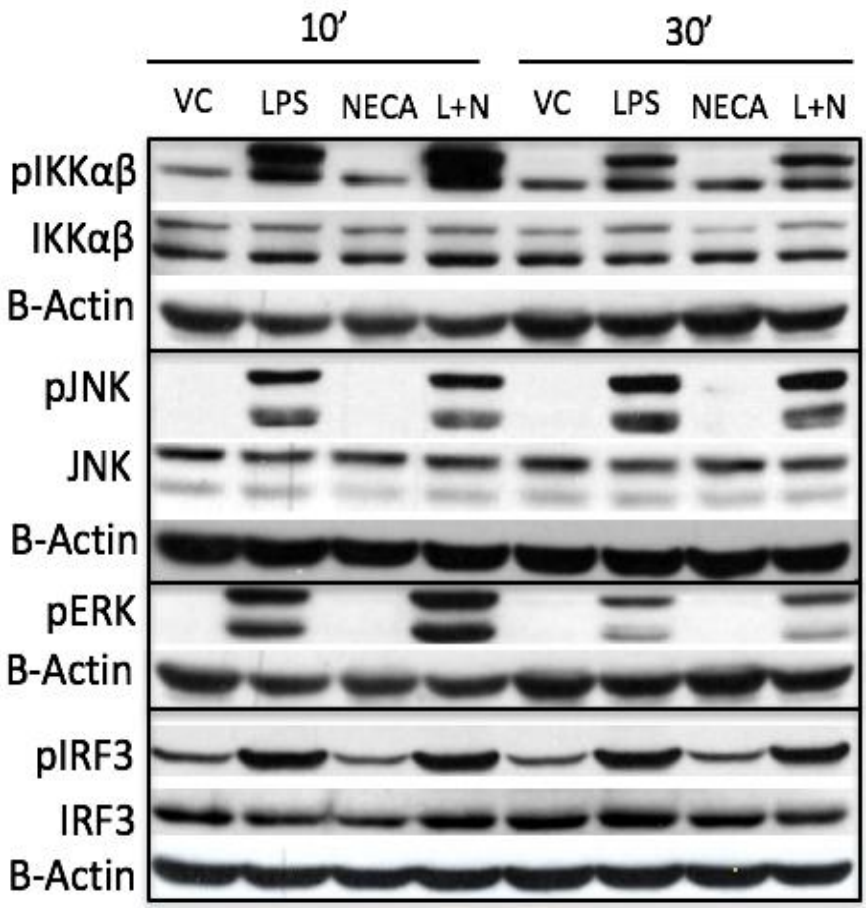


Figure 4.3: Effects of NECA on some key inflammatory pathways of DCs. *BMDCs ($1x10^6$/tube) were treated with 1μg/ml LPS and 3μM NECA for 10 min and 30 min. Phospho and total protein levels were analyzed by western blot. Representative of three experiments.*

4.2.2 Adenosine promotes elevation of cAMP levels and downstream cAMP signaling protein CREB as well as cFOS.

Adenosine receptors belong to family of GPCRs called P1 purinergic receptors and regulate the intracellular cAMP levels to affect the cellular responses. Even though it is known that A1 and A3 receptors are G_i coupled and decrease the cAMP levels, G_s coupled A2ARs and A2BRs are known to rise the intracellular cAMP levels. In order to see the effect of NECA in the accumulation of intracellular cAMP in dendritic cells, we treated our BMDCs in different doses with NECA for 10 min after LPS prestimulation. As can be

seen in Figure 4.4, NECA treatment significantly elevates intracellular cAMP level in a dose dependent manner. Even if NECA is known to be a general AR agonist, this result suggest that its effects are mainly generated through A2ARs and A2BRs and elevating the cAMP levels.

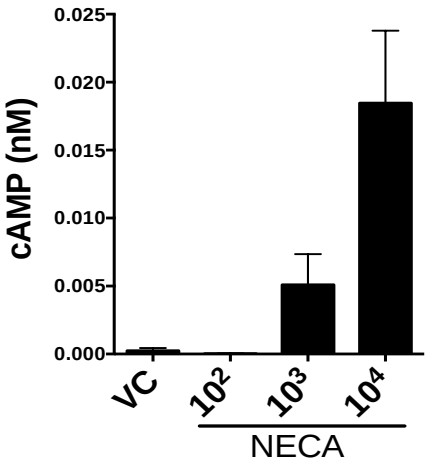


Figure 4.4: NECA elevates cAMP levels in BMDCs. *BMDCs ($3x10^5$/well) were treated with 1μg/ml LPS for 6 h. After that they were washed with warm HBSS and treated with different doses of NECA and 10μM rolipram for 10min. Then perchloric acid and potassium carbonate was added in order to collect cell lysate. After 30 min incubation on ice, cells were centrifuged, clear lysate was collected and cAMP assay kit was performed as the manufacturer's protocol. n=4 Representative of three experiments.*

After we showed NECA increases the intracellular cAMP levels in dendritic cells, we wanted to check one of the main targets of cAMP signaling pathway - cAMP responsive element protein (CREB) phosphorylation to further see how NECA activates cAMP signaling. CREB induce CRE-driven gene expressions after phosphorylated by cAMP mediators such as PKA or Ca^{+2} and translocated in the nucleus. As can be seen in Figure 4.5 NECA strongly increase phospho CREB levels confirming adenosine receptor signaling in dendritic cells activates cAMP pathway.

Recently it was shown that cAMP pathway regulates inflammation through increasing cFos expression [118]. Inflammatory NF-kB activation was shown to be directly inhibited by cFos. Therefore, we tested whether cAMP stimulating NECA can also increase anti-

inflammatory cFos expression rather than changing phosphoactivation of NFkB, MAPK and IRF3 pathways. As compared to VC, NECA increased cFos protein levels and mRNA levels in the absence or presence of LPS, suggesting increased cFos can be another mechanism for adenosine regulation of dendritic cells (Figure 4.5).

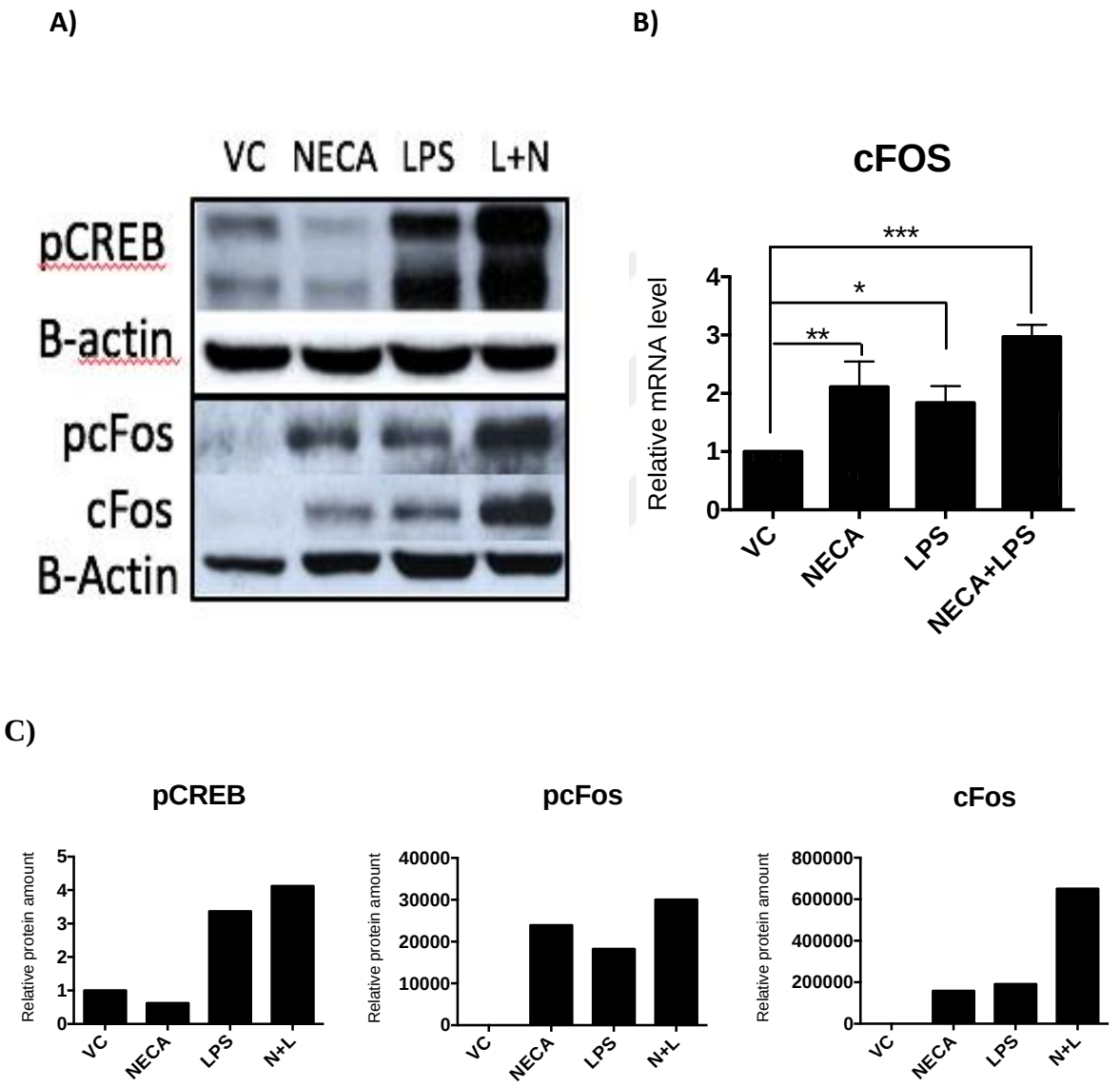


Figure 4.5: NECA increase the phosphorylation status of CREB and cFOS. *A) BMDCs (1x10^6/tube) were treated with 1μg/ml LPS and 3μM NECA for 30 min. Phospho and total protein levels were analyzed by western blot. B) BMDCs (1x10^6/tube) were treated with 1μg/ml LPS and 3μM NECA for 1 h. RNA Isolation and cDNA preparation was performed as mentioned in Chapter 2. Relative mRNA levels were measured by Q-*

*PCR for cFOS. C) Quantitative results of western blots (normalized to β-actin). Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

4.3 Adenosine receptor signaling increases expression of anti-inflammatory NR4A nuclear orphan receptors.

NR4A orphan nuclear receptors (NR4A1-3) are nuclear receptor family of intracellular transcription factors that are characterized as key transcriptional regulators. They function as ligand-independent transcription factors and known to be immediate/early response genes. They can be induced rapidly just like cFOS. Several number of studies showed the contribution of NR4A genes in cell viability, proliferation, transcriptional control of metabolism and inflammation [119]. For example, NR4A1 have important functions in regulating monocyte differentiation. Deletion of NR4A family members result in hyperinflammatory responses that are shaped by enhanced TLR signaling [119-121]. Moreover, NECA has been shown to modulate NR4A family member expressions to elicit anti-inflammatory effects in mast cells, RAW macrophage 264.7 and in other cells of myeloid origin [122]. However, if adenosine receptor stimulation causes changes in NR4A family of orphan nuclear receptors is not known in dendritic cells.

Thus, we set out an experiment to investigate how NR4A1-3 gene expressions are affected by adenosine signaling in our cells. As can be seen in Figure 4.6, NECA significantly increases the expression of all NR4A genes at mRNA level in dendritic cells. These results suggest that adenosine receptor signaling may regulate DC cell activation and inflammatory responses by regulating cFos or NR4A expression rather than interfering the phosho-activation of major inflammatory signaling molecules.

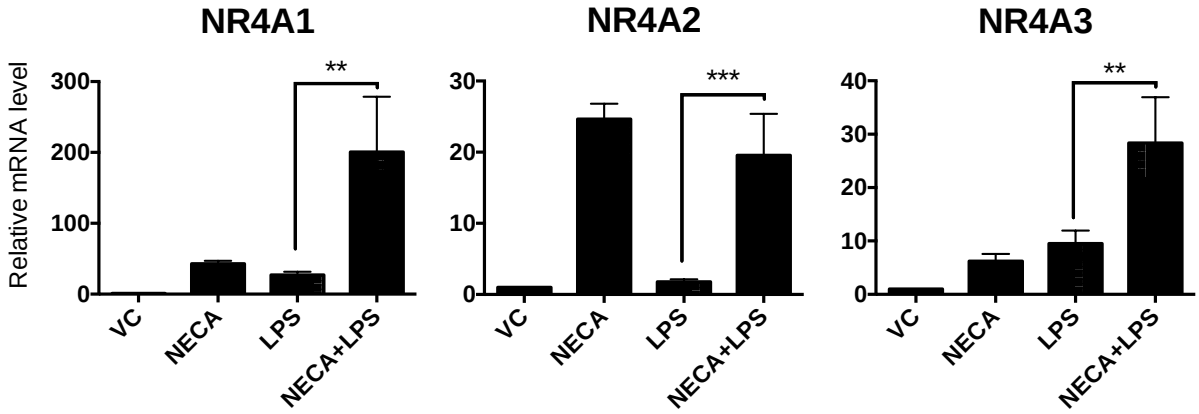


Figure 4.6: NECA increases mRNA expression levels of NR4A nuclear orphan receptors. *BMDCs (1x10^6/tube) were treated with 1μg/ml LPS and 3μM NECA for 1 h. RNA Isolation and cDNA preparation was performed as mentioned in Chapter 2. Relative mRNA levels were measured by Q-PCR. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

4.4 cAMP mimics the effects of adenosine receptor signaling in DCs

Our previous studies so far indicated that NECA modulates dendritic cells by elevating the intracellular cAMP levels and creates an immunosuppressive environment by reducing pro-inflammatory cytokine secretion and co-stimulatory molecule expressions. Next step was to support our findings by using the same readouts as we used previously but this time with a cAMP analog. For this reason, we used cAMPS Sp. cAMPS Sp is a cell permeable cAMP analog that can activate cAMP mediators such as PKA and EPAC. It is a triethylammonium-salt that is used to show the involvement of cAMP-dependent protein kinases in several number of studies [74, 123]. For this reason, we used this general cAMP analog-cAMPS Sp in order to see whether cAMP analog phenocopies polarization of dendritic cells into an anti-inflammatory phenotype by adenosine receptor signaling. Consistent with the previous data, Figure 4.7 showed that similar to NECA, cAMP Sp reduces the pro-inflammatory TNF-α and IL-12 whereas strongly increases anti-inflammatory IL-10 levels of dendritic cells.

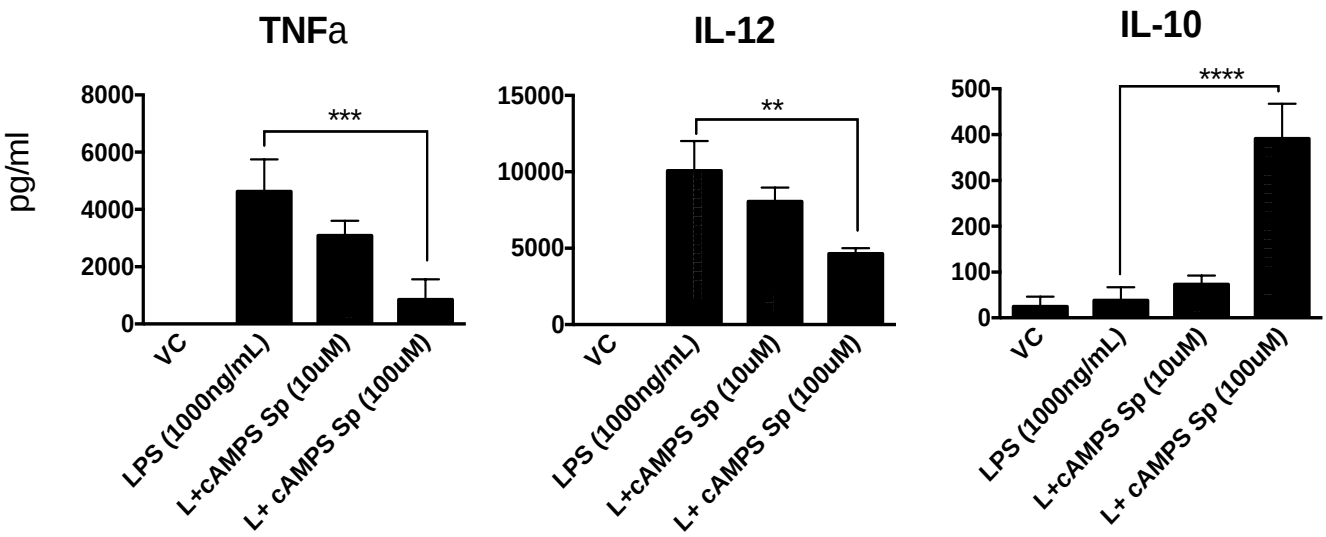


Figure 4.7: General cAMP analog cAMPS Sp stimulation promotes an anti-inflammatory phenotype after LPS stimulation in dendritic cells. *BMDCs ($3x10^5$/well) were treated with 1μg/ml LPS and 10μM or 100μM cAMPS Sp for overnight. TNF-α, IL-12 and IL-10 cytokine levels were measured by ELISA. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of two experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

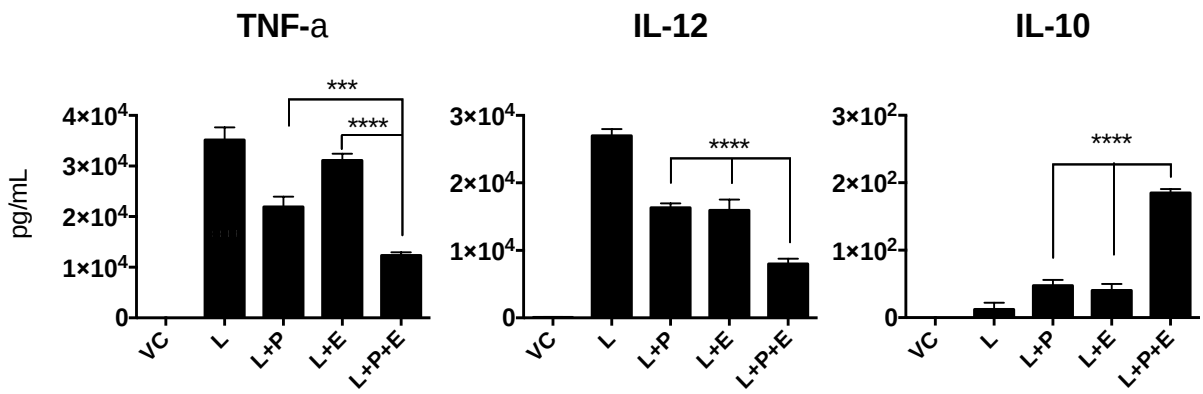
4.5 Additive effect of combinatorial stimulation of both EPAC and PKA pathway on inhibition of DC activation

As explained in Chapter 1, cAMP modulates the cellular functions mainly binding to its intracellular receptors, PKA and EPAC. Because their relative contribution is important when cAMP shapes the immune response, we wanted to use PKA and EPAC specific cAMP analogs and aim to show which cAMP specific pathway that adenosine work through to regulate dendritic cells. For these experiments, we used highly selective cell permeable cAMP analogs; 6-Bnz cAMP sodium salt that selectively activates PKA but not EPAC and 8-pCPT-2-O-Me cAMP-AM that selectively activates EPAC.

So far, we showed the effects of adenosine signaling on intracellular cAMP level and how this regulation affect the innate immunity by using several number of readouts. That's why

we wanted to investigate the details of cAMP specific signaling activation by performing similar experiments that we used in NECA treatments using PKA and EPAC specific analogs alone or in combination rather than NECA. These experiments; therefore, would be important to show not only how Adenosine/cAMP pathway regulates DC responses, but also how in general cAMP regulating other agents would regulate DC responses in different immunological microenvironments/responses. First, we attempted to see the changes in TNF-α, IL-12 and IL-10 cytokine levels. As illustrated in Figure 4.8A, PKA and EPAC specific cAMP analogs alone partially regulated secretion of these cytokines. However, when both analogs used together, we saw drastic changes. PKA and EPAC together suppress LPS-induced TNF-α and IL-12 levels whereas strongly stimulates IL-10 secretion. This result suggests that PKA and EPAC have additive effects while working on adenosine stimulated dendritic cells. The additive effect was particularly obvious for IL-10 in both protein and mRNA level whereas TNFalpha levels were strongly regulated by addition of these analogs particularly in protein level suggesting these pathways can post-transcriptionally regulate expression of these cytokines. Please note that when used in the absence of LPS these analogs did not significantly change the expression of these cytokines except for a tendency of an increased IL-10. (Appendix Figure 2).

A)



B)

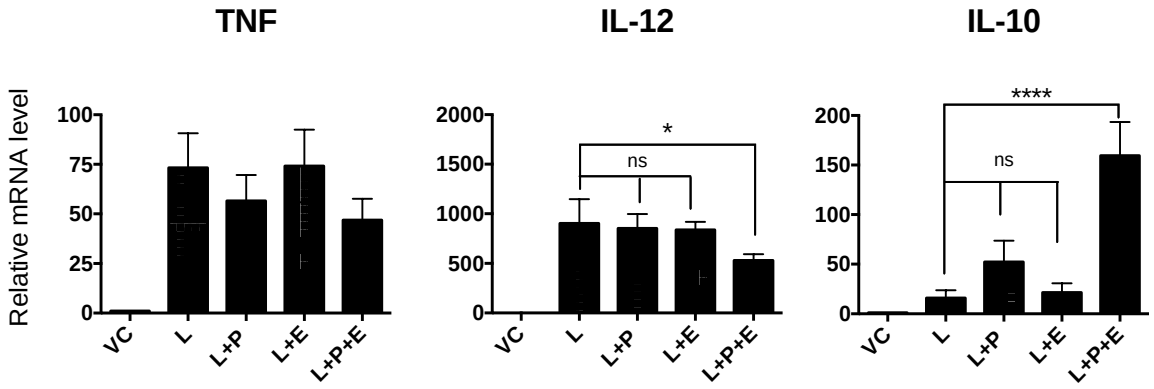
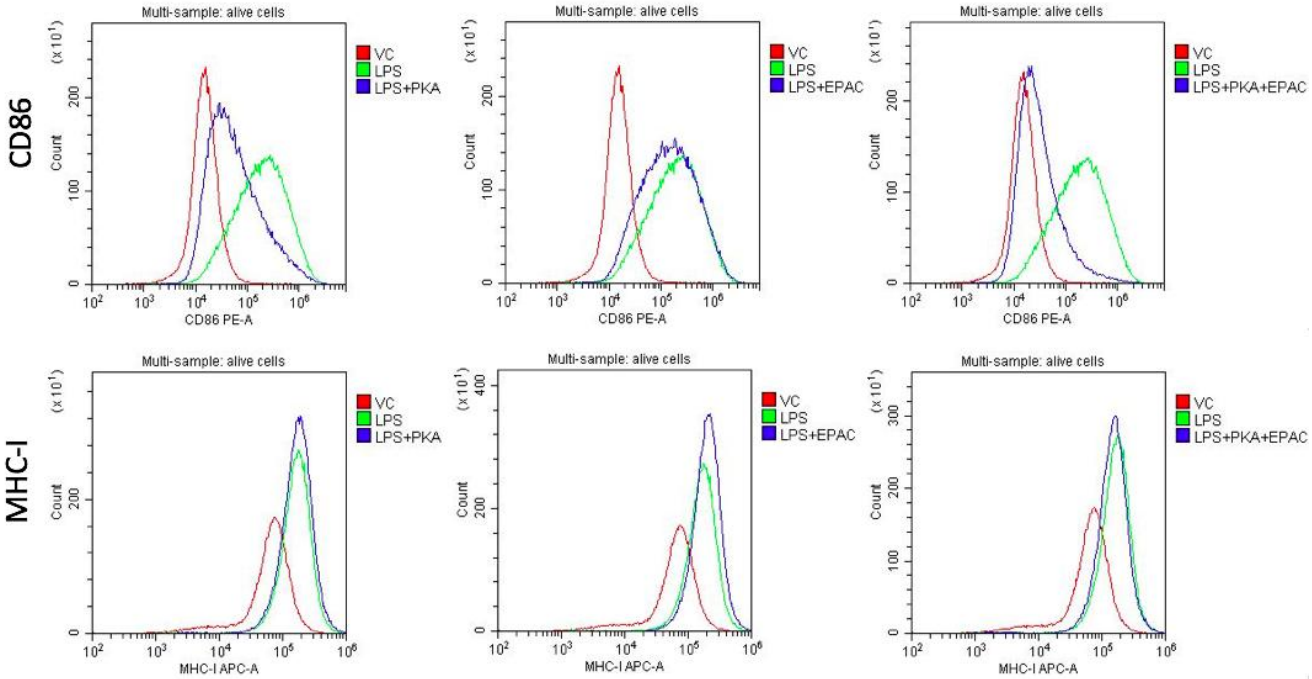


Figure 4.8: PKA and EPAC specific cAMP analogs stimulate suppression of pro-inflammatory cytokine levels while elevate the anti-inflammatory IL-10 secretion. *A) BMDCs (3x10^5/well) were treated with 1μg/ml LPS, 100μM PKA or 3μM EPAC specific cAMP analog for overnight. TNF-α, IL-12 and IL-10 cytokine levels were measured by ELISA. B) BMDCs (1x10^6/tube) were treated with 1μg/ml LPS, 100μM PKA and 3μM EPAC specific cAMP analogs for 1 h. RNA Isolation and cDNA preparation was performed as mentioned in Chapter 2. Relative mRNA levels were measured by Q-PCR. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

Next step was to determine the effects of specific cAMP analogs on dendritic cell surface molecule expression markers by flow cytometry. Consistent with the previous results of NECA, CD86 co-stimulatory molecule and MHC-I expressions decreased upon our treatment with PKA or EPAC specific analogs. Moreover, the expression levels significantly decreased, almost returned back to the basal level, when we use both cAMP analogs together, suggesting the additive effect of these analogs when they are used together.

A)



B)

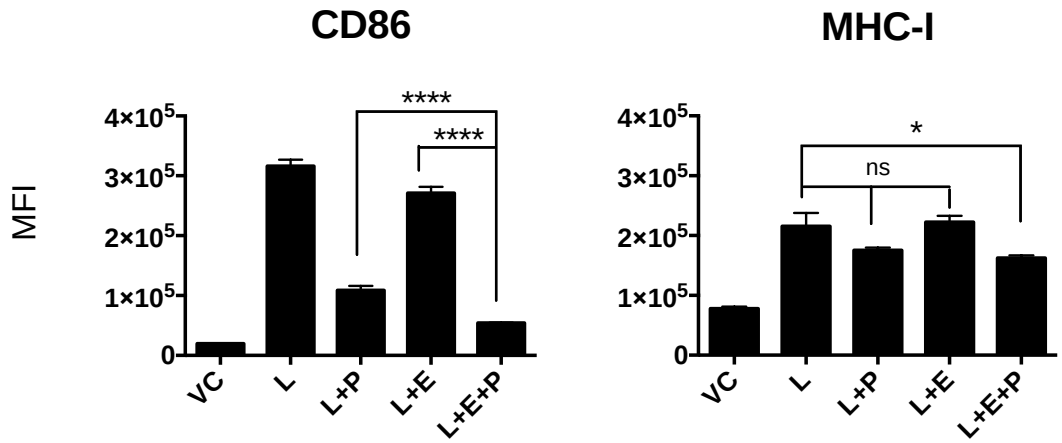


Figure 4.9: PKA and EPAC specific cAMP analogs stimulate suppression of CD86 and MJC-I levels of dendritic cells. *BMDCs (3x10^5/well) were treated with 1μg/ml LPS, 100μM PKA or 3μM EPAC specific cAMP analog for overnight. MFI rate was analyzed by using flow cytometry. A) Histogram overlay B) Quantitative results for CD86 and MHC-I. Statistics were calculated using one-way ANOVA followed by Tukey's multiple*

*comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

4.6 Stimulation of PKA and EPAC pathway together strongly elevates NR4As and cFOS

According to our observation, when we use NECA as adenosine receptor agonist, the amount of NR4A orphan nuclear receptor and cFOS mRNA expression levels are upregulated. Moreover, we also observed increased amounts of cFOS phosphorylation upon NECA treatment in dendritic cells. Thereby in the next step, we wanted to check the effects of cAMP specific analogs on cFOS and NR4As. First, we attempted to see the change in mRNA level. As can be seen in Figure 4.10, PKA and EPAC analogs can increase cFOS, NR4A2 and NR4A3 mRNA only when used together. Although PKA analog alone could significantly increase NR4A1 alone, combination of both PKA and EPAC specific analog more strongly increased NRA41 expression.

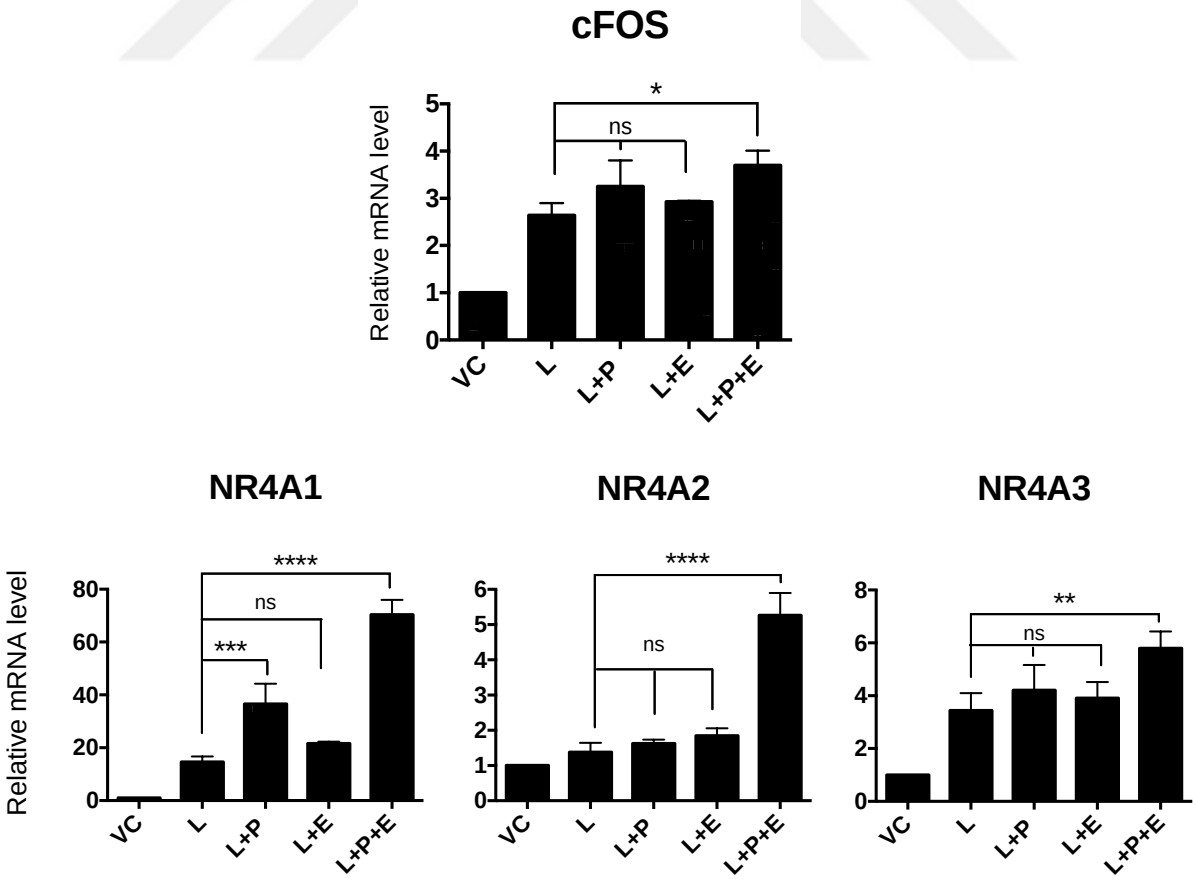
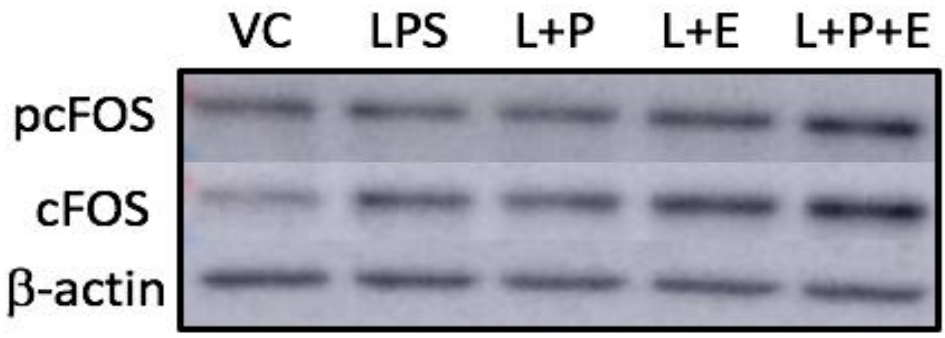


Figure 4.10: PKA and EPAC specific cAMP analogs increase cFOS and NR4A orphan nuclear receptor mRNA expressions. *BMDCs (1x10^6/tube) were treated with 1μg/ml LPS, 100μM PKA and 3μM EPAC specific cAMP analogs for 1 h. RNA Isolation and cDNA preparation was performed as mentioned in Chapter 2. Relative mRNA levels were measured by Q-PCR. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

cFOS protein phosphorylation level didn't change much when we use PKA and EPAC specific cAMP analogs alone. However, addition of EPAC together with PKA drastically change one of our target proteins phosphorylation, cFOS, suggesting the additive effect of PKA and EPAC specific cAMP analogs in our LPS stimulated dendritic cells (Figure 4.11). These results suggest that NECA can potentially stimulate both PKA and EPAC specific pathways to regulate dendritic cell responses.

A)



B)

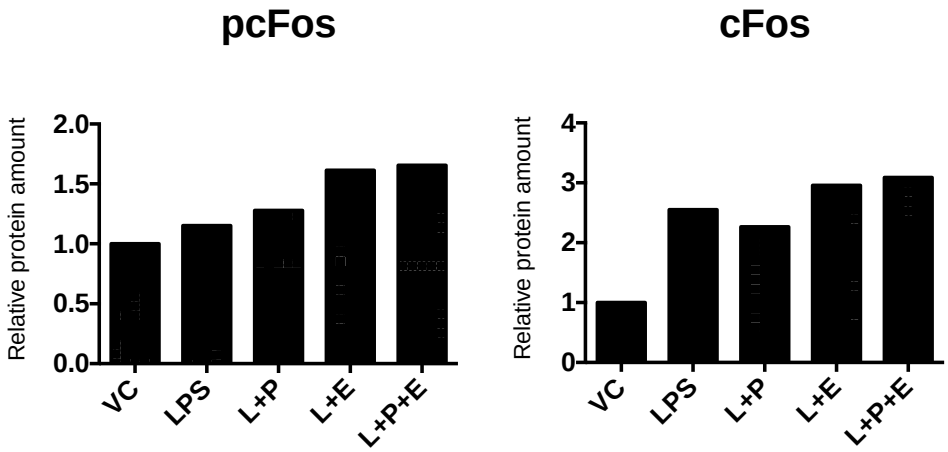
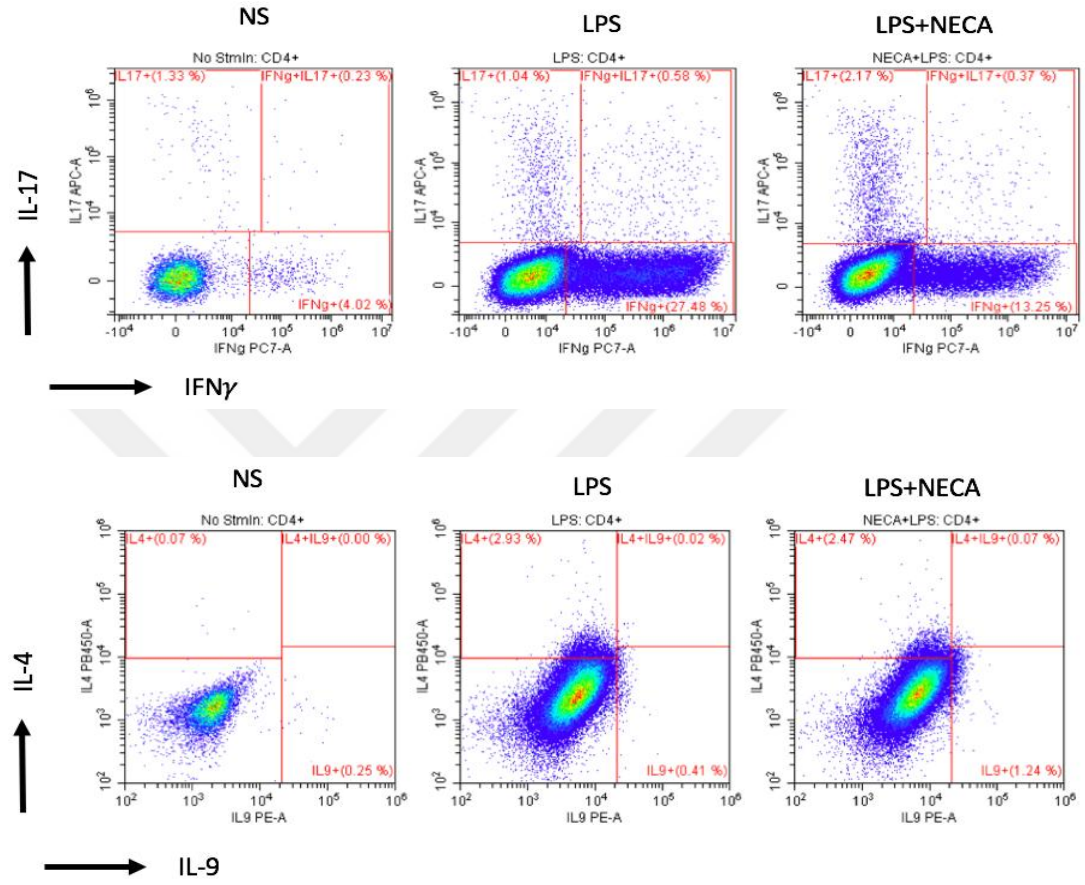


Figure 4.11: PKA and EPAC specific cAMP analogs increase the phospho and total protein levels of cFOS. *A) BMDCs (1x10^6/tube) were treated with 1μg/ml LPS, 100μM PKA and 3μM EPAC specific cAMP analogs for 1 h. Phospho and total protein levels were analyzed by western blot. B) Quantitative results of western blots (normalized to β-actin). Representative of three experiments.*

4.7 Adenosine receptor agonist and cAMP specific PKA and EPAC analogs stimulate a similar cytokine secretion profile in dendritic cells causes suppression of Th1 deviation

In previous experiments, we showed how dendritic cell activation and maturation status are affected by adenosine signaling as well as cAMP signaling. Next step was to confirm our results by showing how these changes are functionally important. Cytokines and chemokines that are secreted from dendritic cells can regulate the activation of T cells. For example, IL-10 is an immunosuppressive cytokine that can suppress T cell responses. To see the effects of our treatments in DCs ability to regulate T cell activation, dendritic cells were prestimulated for 3 hours with NECA, PKA or EPAC specific analogs alone or in combination in the presence or absence of LPS. And then we washed the cells and added fresh medium. After overnight culture supernatants were collected and added onto T cells activated by anti-CD3 and anti-CD28. Of note, CD4+ T cells were isolated as mentioned in Chapter 2 (Appendix Figure 3) and then cultured with anti-CD3/anti-CD28 in the IgG coated wells. As can be seen Figure 4.12 conditioned medium from DC cultures treated

with NECA+LPS caused decreased proportion of IFNg+ T cells. Although NECA treatment increased proportion of IL-9+ and IL-17+ cells, proportion of these cells were around only 1% and could be influenced by the decrease in proportion of IFNg+ T cells.



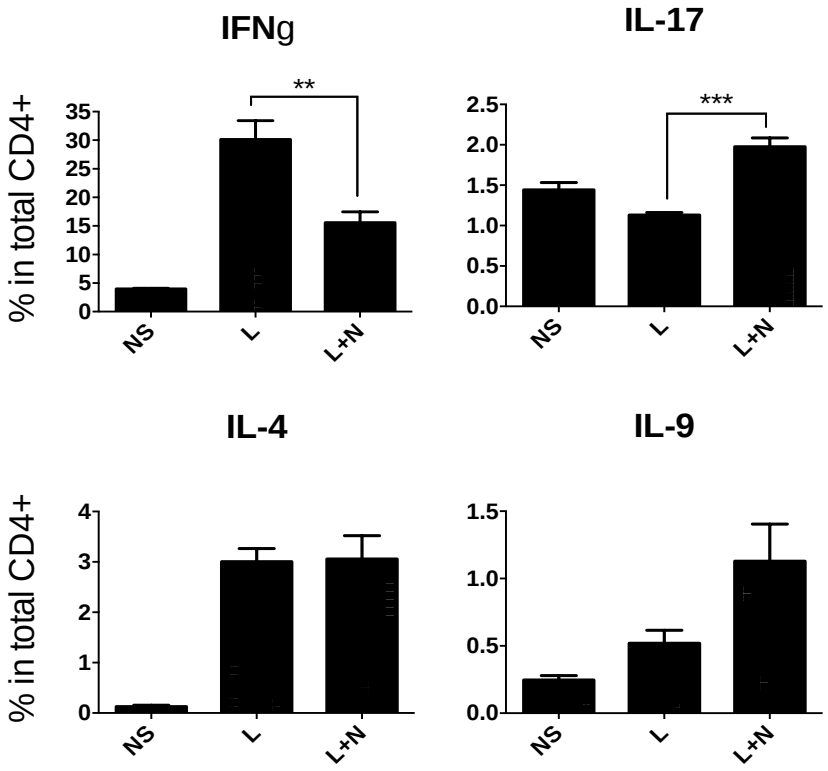
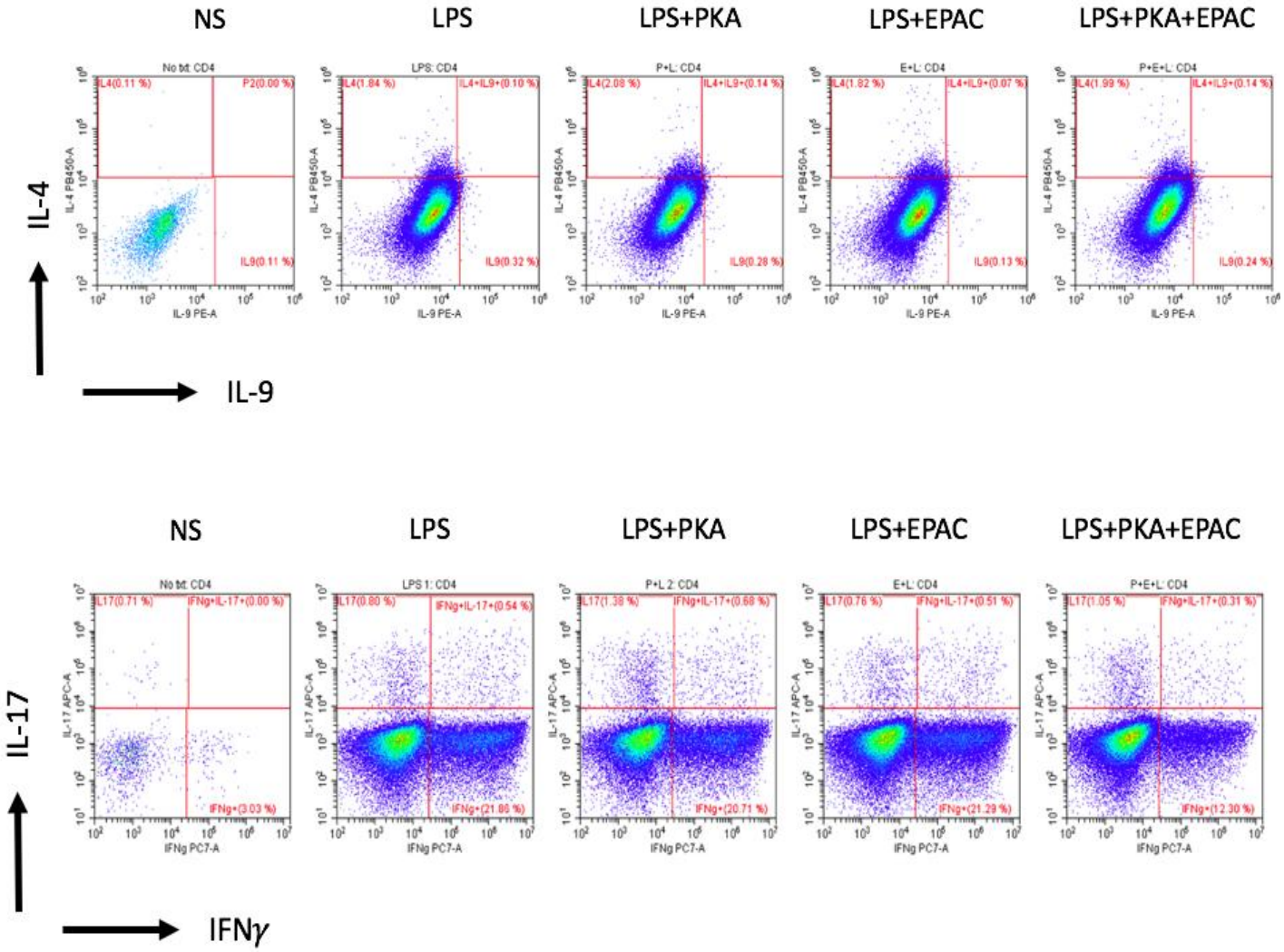


Figure 4.12: Dendritic cells secrete mediators that affect the polarization of naïve CD4+ T cells upon NECA treatment. *BMDCs (3x10^5/well) were treated with 1μg/ml LPS and 3 μM NECA for overnight. The day after naïve CD4+ T cell were cultured with those preconditioned DC mediums for 4 days. At day 4 T cells were washed and restimulated with 1mg/ml ionomycin and 0,1mg/ml PMA for 6h. The last 4 h of restimulation was done with 1X monensin in addition to PMA and ionomycin. Intracellular cytokine levels were measured by flow cytometry with intracellular staining protocol as mentioned at Chapter 2. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=4 Representative of two experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

Similarly**,** conditioned medium from DC cultures treated with PKA and EPAC specific analogs in combination+LPS caused decreased proportion of IFNg+ T cells (Figure 4.13). Although these analogs increased the proportion of IL-9+ and IL-17+ cells, proportion of these cells were around only 1% and could be influenced by the decrease in proportion of IFNg+ T cells as observed after NECA stimulation.

It is important to note that even in the absence of LPS treatment of DCs with NECA or combination of PKA and EPAC specific analogs tend to regulate DC responses because DC conditioned medium for these conditions also tend to decrease T cell activation suggesting that in BM-DCs there is basal level of activation of immunological pathways which can be regulated by Adenosine/cAMP signaling (Appendix Figure 4).



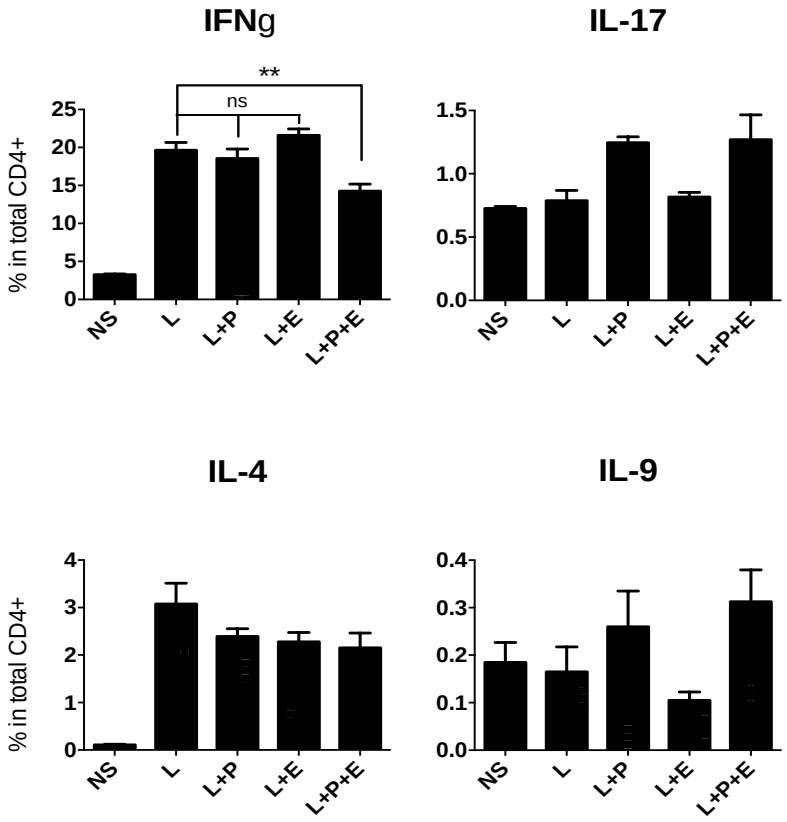


Figure 4.13: Dendritic cells secrete mediators that affect the polarization of naïve CD4+ T cells upon PKA and EPAC specific cAMP analog treatment. *BMDCs ($3x10^5$/well) were treated with 1μg/ml LPS, 100μM PKA and 3μM EPAC specific cAMP analogs for overnight. The day after naïve CD4+ T cell were cultured with those preconditioned DC mediums for 4 days. At day 4 T cells were washed and they were restimulated with 1mg/ml ionomycin and 0,1mg/ml PMA for 6h. The last 4 h of restimulation was done with 1X monensin in addition to PMA and ionomycin. Intracellular cytokine levels were measured by flow cytometry with intracellular staining protocol as mentioned at Chapter 2. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=4 Representative of two experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

Chapter 5

Discussion

The immune system creates defense mechanisms in response to pathogenic invaders or altered-self molecules by using specialized biological structures and processes. In humans, the immune system is divided into 2 groups as innate or adaptive; by the duration of response, specificity and components that are used. Out of many others, a special cell type 'dendritic cells' connect innate immunity to adaptive immunity by working as professional antigen presenting cells. Any signal from the environment is important for dendritic cell activation especially because they are standing at the center of two main immune system.

Purinergic signaling affect both innate and adaptive immunity. It is known that adenosine receptors, a class of purinergic receptors, have immunosuppressive effects on immune cell activation. This is also true for dendritic cells. Adenosine has been reported to be an immunosuppressive agent in DCs by decreasing pro-inflammatory cytokine secretion from DCs and monocytes, preventing antigen presentation, affecting their T cell polarization ability and DC maturation [103].

In this study, we investigated the effects of NECA- the stable adenosine receptor agonist on DC functions including; cytokine production, surface marker and co-stimulatory molecule expression, its ability to affect CD4+ T cell activation. We also tested what components of cAMP signaling can be important for DC regulation through adenosine receptors. To accomplish this, we used bone marrow derived dendritic cells because they

can mimic responses of several different DC types such as production of inflammatory cytokines, antigen cross-presentation and type I interferon production. Also, using BM-DCs provided the advantage of generation of massive quantities of primary cells for the tests we employed.

Our main biological question was: 'How does adenosine signaling modulate DC responses?' Studies showed the immunosuppressive effects of NECA on LPS-induced dendritic cells. Once again to confirm this information, we used cytokine secretion profile including TNF-α, IL-12, IL-10 and surface molecule expressions such as CD86, MHC-I and MHC-II as our read outs to show the immunosuppressive effects of NECA on dendritic cells. After that, because adenosine receptor signaling is known to affect cAMP signaling, we measured intracellular cAMP levels and cAMP response element binding protein (CREB) levels. As a result, we showed that NECA elevates intracellular cAMP levels and CREB phosphorylation of BMDCs. In addition to that, we also investigated the effects of NECA treatment on phosphoactivation of LPS induced inflammatory signaling pathways and observed no significant changes during NECA treatment of our BMDCs.

Our second question was whether adenosine receptor signaling increase the expression of known anti-inflammatory nuclear proteins, cFos and NR4A family of orphan receptors or not. The reason why we asked this because it was shown that downstream messenger of adenosine receptor signaling, cAMP, was shown to increase cFos levels to suppress inflammation in macrophages by negatively regulating NF-kB transcriptional activity [118]. Similarly, NR4A1 was recently shown to act as anti-inflammatory mediator modulated by adenosine or prostaglandins in macrophages [108, 125]. Also, other components of NR4A receptors were induced by adenosine/cAMP signaling in different non-immune cells [126, 127]. We for the first time showed that the expression of these negative regulators of NF-kB is increased after adenosine receptor stimulation in dendritic cells.

After confirming cAMP induced similar polarization of DCs into anti-inflammatory phenotype we next tested the effects of cAMP specific pathways. When intracellular cAMP levels increased in the cytoplasm, it activates mainly PKA and EPAC, two proteins that are ubiquitously expressed in every tissue. They can have opposing, synergistic or

even independent effects depending on the cell type and where they are localized. Mechanism of cAMP mediated activation of APCs have been studied but those studies were emphasizing only the PKA mediated effects on this process. Thus, we asked our third and last question: 'What is the involvement of PKA and EPAC mediated pathways on adenosine regulation of DCs?'. To answer this question, we used highly specific PKA and EPAC analogs. Again, we checked the cytokine secretion, surface molecule expressions, NR4A and cFOS expressions as we did previously with NECA and observed the similar results. Surprisingly, we also realized their additive effects; meaning that even they don't do much when they are alone, combination of PKA and EPAC specific cAMP analogs result in the strongest and most similar to NECA treatment. That was the most important finding of our study, highlighting the additive effect of PKA and EPAC specific cAMP analogs for the first time in dendritic cells. Lastly, we wanted to investigate those findings in a functional test; how those stimulated dendritic cells affect T cell polarization upon their secreted factors. The results of this experiment showed again similar outcomes both for NECA and combination of PKA-EPAC specific cAMP analogs. We conclude our data from experiments with stable adenosine receptor agonist NECA and selective cAMP analogs as adenosine regulation of dendritic cells are mediated by both PKA and EPAC pathways.

Diseases in which extracellular adenosine levels are extremely high in the microenvironment, such as cancer, accumulation of adenosine can be problematic because of its immunosuppressive effects on immune cells [95]. Thereby, studies such as targeting and blocking adenosine receptors of immune cells in tumor microenvironment gave promising results by slowing down the tumor progression [61, 128]. Because we shed light on adenosine mediated signaling of cAMP pathway on dendritic cells, targeting both PKA and EPAC pathways in dendritic cells in such diseases may be an effective therapeutic approach. Excessive amounts of adenosine also found in the asthmatic lung [129, 130]. DCs have been shown to play important roles in allergic sensitization as well as triggering Th2 responses and thereby augmenting the disease [131]. Thus, in such diseases like asthma or chronic allergy, which excessive amount of immune activation occurs, our findings may reveal a new target to keep down the immune response under control.

The importance of this study is arising because we revealed the function of regulators of the most common signaling pathways on immune regulation. Not only adenosine, but also many other extracellular agents such as prostaglandins, cholera toxins, forskolin, other GPCR agonists and adenylyl cyclase inducing agents have been shown to induce PKA mediated cAMP signaling pathway and give similar immunosuppressive response in APCs [95]. Thus, the diseases in which cAMP elevating agents included, may be targeted through PKA and EPAC pathways upon our findings.

As future perspective, one approach can be discovering direct effects of dendritic cell-T cell immunological synapses in addition to conditioned medium experiments that we showed previously. Co-culture experiment of NECA or PKA-EPAC treated and antigen loaded dendritic cells with OT-1 transfected T cells would reveal how our treatments actually change the antigen presentation and T cell stimulation capability of dendritic cells.

As another approach, we aim to use PKA and EPAC specific inhibitors in order to prove the involvement of PKA and EPAC pathways in adenosine mediated signaling of dendritic cells. There have been studies on PKA and EPAC specific inhibitors for macrophages and dendritic cells but either because of their cellular system or the mechanism they are focused on didn't give convincing results. Out of many controversial try of those inhibitors, it has been reviewed that because PKA and EPAC have many catalytic and regulatory subunits, the inhibitors are targeting only one subunit thought to be unsuccessful to inhibit the protein function completely [69]. However, we showed PKA and EPAC have additive effects while shaping the dendritic cell function when they are used together. Thereby we are suggesting and planning to use inhibitors again in combination and expect to reverse the effects of NECA on dendritic cells. EPAC and PKA are highly coordinated cAMP effectors. Targeting only one pathway wouldn't enlighten the exact function of cAMP mediated effects. Thus, either by using pharmacological inhibitors of PKA and EPAC pathways in combination, or silencing their regulatory or catalytic subunits at the same time would be extremely useful while examining the roles of two cAMP mediators PKA and EPAC on adenosine mediated signaling of dendritic cells.

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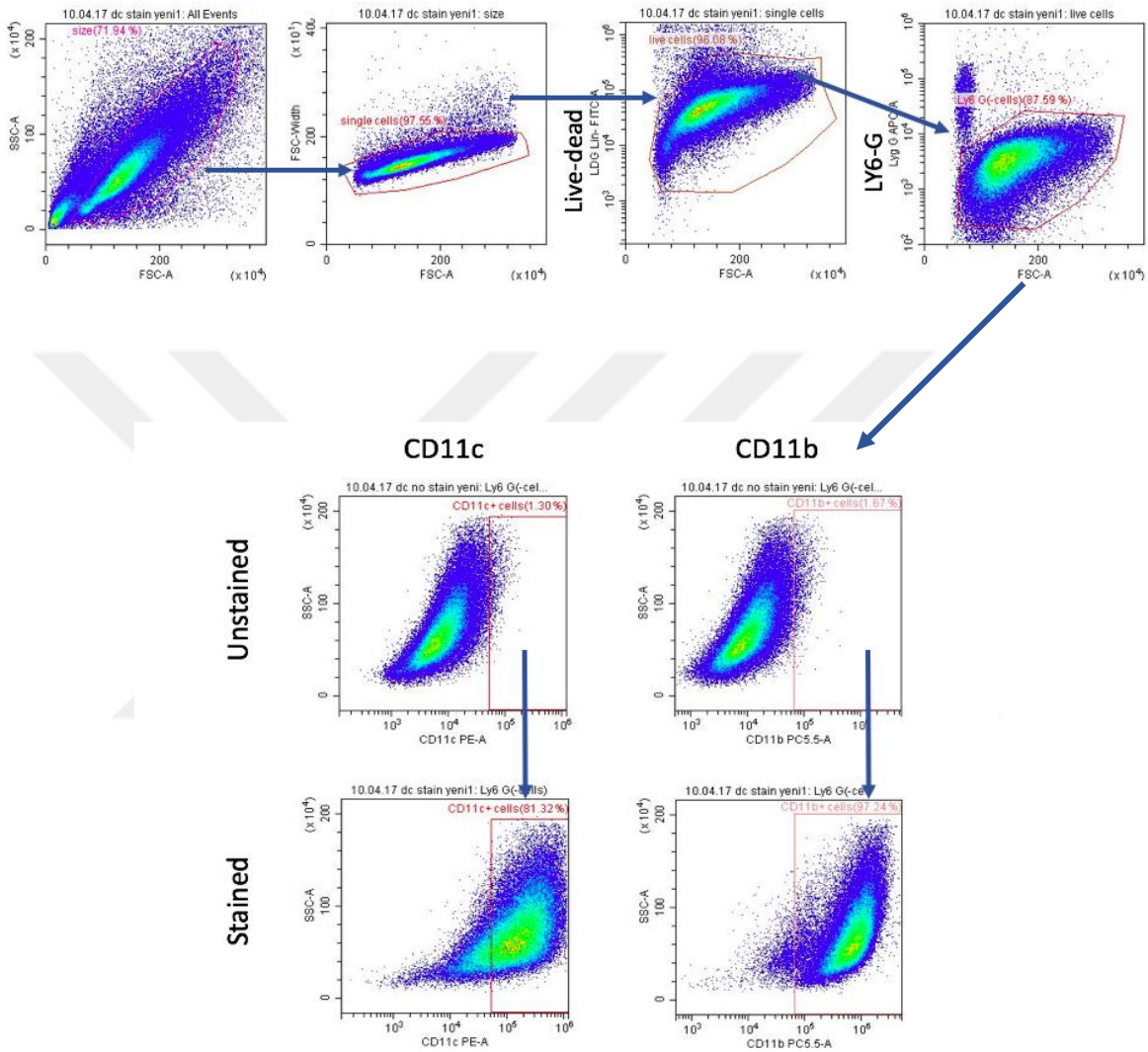
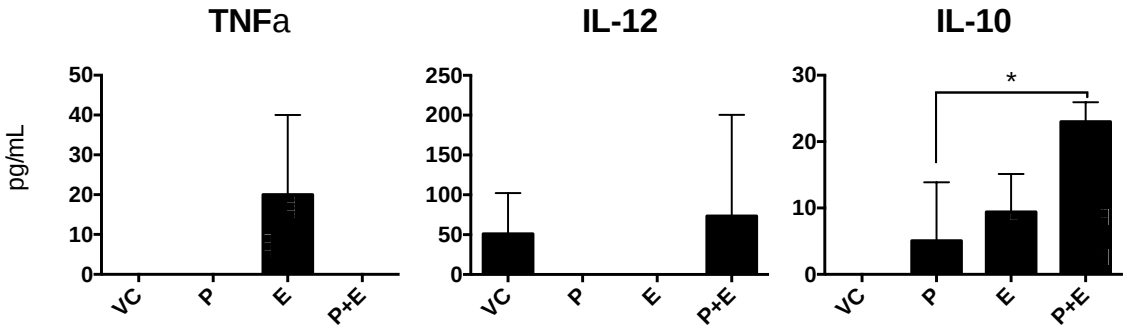
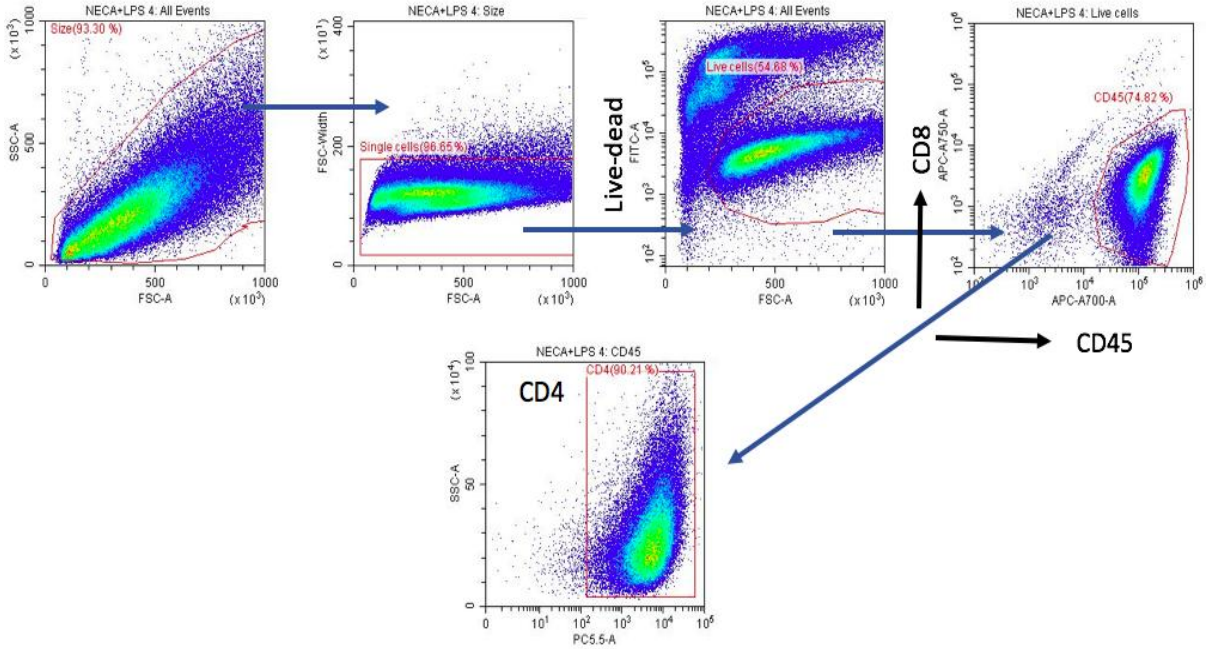
Appendix



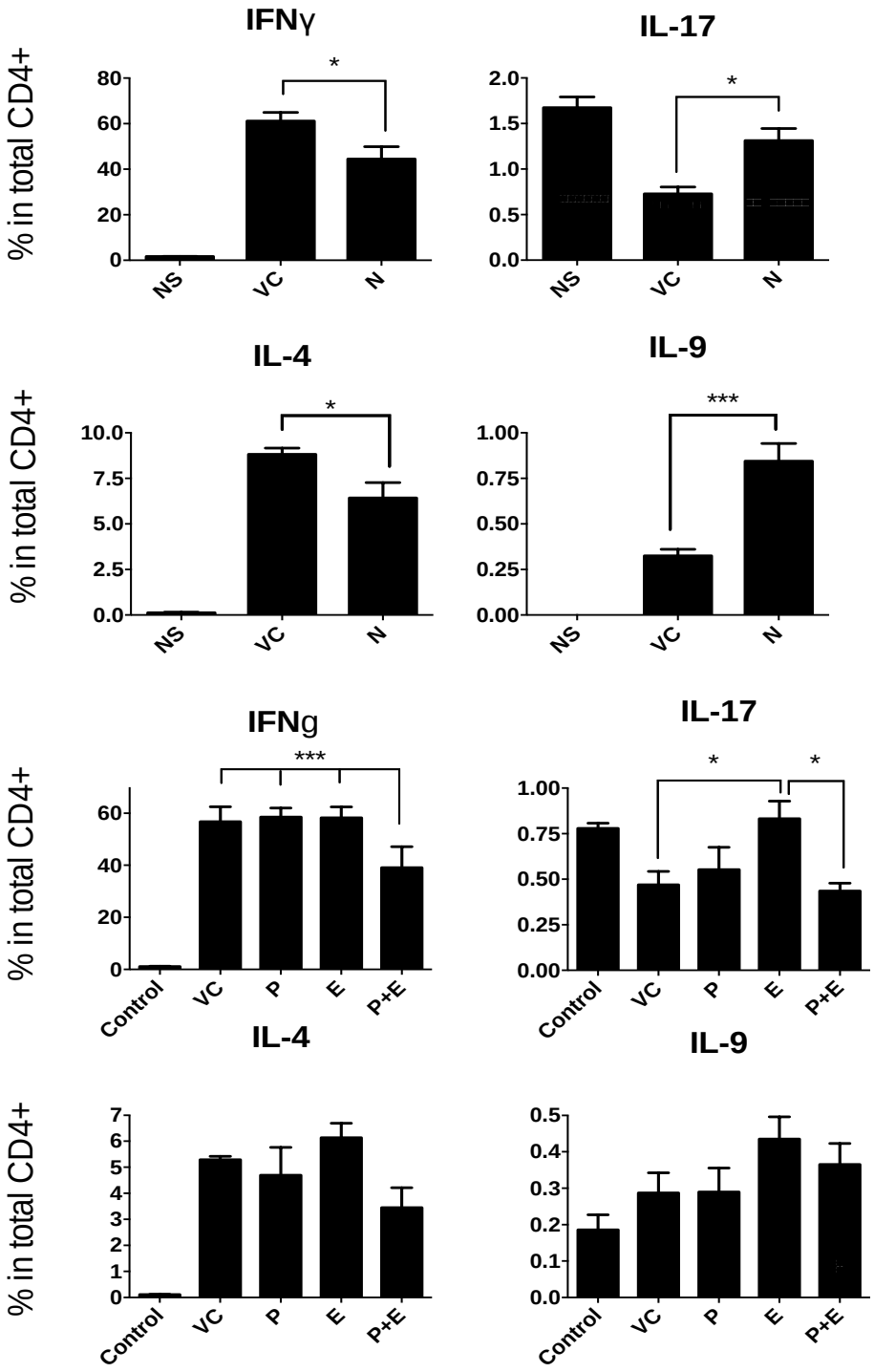
Appendix Figure 1: Gating strategy for quality control of bone marrow drived dendritic cells. *Dendritic cells were generated as mentioned in Chapter 2. The desired cell population was chosen by FSC/SSC graph on the far left. The duplets and triplets were eliminated in FSC-Width/FSC-A graph. Next, the dead cells and lineage negative cells were separated from alive cells by using Live-dead/FITC graph. After that, Ly6-G – cells were chosen and gated for Cd11b+ and Cd11c+ cells of unstained and stained samples.*



Appendix Figure 2: The effects of cAMP specific PKA and EPAC analogs without LPS on cytokine secretion of dendritic cells. *BMDCs ($3x10^5$/well) were treated with 1μg/ml LPS, 100μM PKA or 3μM EPAC specific cAMP analog for overnight. TNF-α, IL-12 and IL-10 cytokine levels were measured by ELISA. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=3 Representative of three experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*



Appendix Figure 3: Gating strategy and quality control of CD4+ T cells. *The desired cell population was chosen by FSC/SSC graph on the far left. The duplets and triplets were eliminated in FSC-Width/FSC-A graph. Next, the dead cells were separated from alive cells by using Live-dead/FITC graph. After that, CD45+ T cells were chosen and gated further to pick the CD4+ T cell population.*



Appendix Figure 4: Dendritic cells secrete mediators that affect the polarization of naïve CD4+ T cells upon NECA/PKA and EPAC specific cAMP analog treatment without LPS stimulation. *BMDCs (3x10^5/well) were treated with either 3 μM NECA or 100μM PKA and 3μM EPAC specific cAMP analogs for overnight. The day after naïve CD4+ T cell were cultured with those preconditioned DC mediums for 4 days. At day 4 T*

*cells were washed and restimulated with 1mg/ml ionomycin and 0,1mg/ml PMA for 6h. The last 4 h of restimulation was done with 1X monensin in addition to PMA and ionomycin. Intracellular cytokine levels were measured by flow cytometry with intracellular staining protocol as mentioned at Chapter 2. Statistics were calculated using one-way ANOVA followed by Tukey's multiple comparison test. n=4 Representative of two experiments. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001.*

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