MOLECULAR MECHANISMS OF ADENOSINE REGULATION OF HELPER T CELL RESPONSES

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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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Polarizations of helper T cells into different functional subsets is important in order to influence the progression of immune-related diseases. One of the major outcomes in immune cell activation and generation of immunogenic response followed by TCR stimulation, is elevation of extracellular adenosine and upregulation of adenosine A_{2A} receptors. Adenosine A_{2A} receptor stimulation elevates intracellular cAMP to regulate helper T cell responses., Intracellular receptors of cAMP, PKA and EPAC proteins regulate cellular responses downstream of cAMP. In this study, we showed that adenosine differentially suppresses Th1 polarization rather than polarization of other functional T cell subsets. Adenosine signaling strongly decreased T cell accumulation in all the polarizing conditions except for Th2 condition. Adenosine-mediated decrease in T cell accumulation is associated with decreased proliferation and survival. PI3K-AKT pathway by targeting the Akt phospho-activation is one of the essential factors for regulation of immune

response. One of the targets for Akt is Foxo1, which is inhibited by Akt phosphorylation. Foxo1 is known to suppress T cell proliferation and important in T cell trafficking and survival. Mechanistic studies have shown that adenosine signaling decreases the phosphorylation of Akt and Foxo1 molecules downstream of TCR. A Foxo1 inhibitor, AS1842856, reverses the reduction in T cell accumulation after adenosine receptor stimulation in particularly Th1 and Th17 conditions by increasing T cell survival in these conditions rather than T cell proliferation. Further studies using PKA and EPAC specific analogs indicated that both pathways may be required for adenosine mediated suppression of Th1 polarization; however, PKA pathway alone is largely responsible from inhibition of T cell proliferation. Results of this study have major implications to understand potential cell-intrinsic effects of one of the major immunoregulatory pathways.

Keywords: Adenosine, Adenosine Receptors, Foxo1, Helper T cells.

Özet

ADENOZİNİN YARDIMCI T HÜCRESİ YANITLARININ REGÜLASYONUNDAKİ MOLEKÜLER MEKANİZMALARI

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Yardımcı T hücrelerinin, farklı fonksiyonel alt gruplara polarizasyonu, immün kökenli hastalıkların ilerlemesine olan etkileri nedeniyle önem teşkil etmektedir. T hücresi reseptör uyarımını takiben oluşan hücre aktivasyonu ve immünojenik yanıtın ortaya çıkardığı en büyük etkilerden biri, ekstrasellüler adenozin miktarının artışı ve adenozin reseptörü A_{2A} nın up-regülasyonudur. Adenozin A_{2A} reseptör uyarımı, yardımcı T hücre cevaplarının düzenlenmesi için hücre içi cAMP miktarını arttırır. cAMP nin hücre içi reseptörleri, PKA ve EPAC proteinleri, cAMP nin alt yolağı ile oluşan hücresel cevapları düzenler. Bu çalışmada, adenozinin, Th1 polarizasyonunu, diğer fonksiyonel T hücresi altgruplarından farklı olarak baskıladığını göstermekteyiz. Adenozin sinyali, Th2 dışındaki bütün polarizasyon koşullarında T hücresi birikimini kuvvetli bir biçimde azaltmaktadır. Adenozin aracılı T hücresi birikimindeki azalış, azalmış proliferasyon ve sağkalım ile ilişkilidir. PI3K-AKT yolağı, Akt fosfo-aktivasyonunu hedefleyerek, immün yanıtın düzenlenmesindeki en temel faktörlerden biridir. Akt nin hedeflerinden biri olan Foxo1, Akt fosforilasyonuyla inhibe edilmektedir. Foxo1'in T hücresi proliferasyonunu baskıladığı ve T hücresi göçü ve sağkalımında önemli olduğu bilinmektedir. Mekanistik çalışmalar göstermektedir ki, adenozin sinyali, TCR alt yolağı olan Akt ve Foxo1

fosforilasyonunu azaltmaktadır. Foxo1 inhibitörü olan AS1842856, adenozin reseptör uyarımı sonrası azalan T hücresi birikimini, özellikle Th1 ve Th17 koşullarında, proliferasyonu etkilemektense T hücresi sağkalımını arttırarak geri çevirmektedir. PKA ve EPAC spesifik analoglar ile yapılan daha ileri çalışmalar, her iki yolağın da Th1 polarizasyonunun baskılanması için gerekli olabileceğini göstermektedir; bununla beraber, T hücresi proliferasyonunun inhibisyonundan ağırlıklı olarak PKA yolağı sorumludur. Bu çalışmanın sonuçları, başlıca immünoregülatör yolaklarının potansiyel hücre içi etkilerinin anlaşılmasında önemli çıkarımlara sahiptir.

Anahtar kelimeler: Adenozin, Adenozin Reseptörleri, Foxo1, Yardımcı T hücreleri.

En değerlilerim; canım aileme..

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Abbreviations

Ab	Antibody
ABB	Annexin Binding Buffer
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Antigen presenting cell
AR	Adenosine receptor
ATP	Adenosine triphosphate
BCR	B cell receptor
BCL2	B cell lymphoma 2
cAMP	cyclic Adenosine monophosphate
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CREB	cAMP response element binding protein
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
ddH2O	Double-distilled water
dH2O	Distilled water
FOX	Forkhead box
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
HBSS	Hank's balanced salt solution
IBD	Inflammatory bowel disease
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E

IgG	Immunoglobulin G
IL	Interleukin
iNKT	Invariant natural killer T cell
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK	Natural killer
PAMP	Pathogen associated molecular pattern
PD-1	Programmed death 1
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B (also known as Akt)
RPMI	Roswell Park Memorial Institute
SA	Streptavidin
SCID	Severe combined immunodeficiency
Tfh	Follicular helper T cells
TGF	Transforming growth factor
Th	T helper cell
Treg	Regulatory T cell

Chapter 1

Introduction

1.1 THE IMMUNE SYSTEM

Immune system is responsible for protecting the host from pathogens and from cancer formation. In general, during this process, by discriminating self from non-self or modified, misplaced self (pathogens, mutated endogenous proteins or intracellular components); a unique, complex network between the members of immunity and other physiological systems takes role to maintain the homeostasis for healthy lifespan. Although there are various types of microorganisms and molecules that might be harmful for the host, the immunogenic response can be divided into two parts depending on their specificity and speed [1].

1.1.1 INNATE IMMUNE SYSTEM

Innate immunity forms the first line of defense mechanism of the host. When the physical/chemical barriers of the host (e.g. epithelia, mucosal surfaces) are breached by the pathogen; a quick, vigorous immunogenic response occurs encoded by germline, without showing any-specificity and without providing long-lasting protection. One of the key aspects of innate immunity, it is developed earlier during evolution and the response is not changed depending on the source of infection, but it is effective against wide-range of pathogens at the same level even after many encounters with the same type of them, which is caused by the absence of memory against the infectious agent. This branch of immune system consists of phagocytes (monocytes, macrophages, neutrophils, dendritic cells), cells responsible for secretion of inflammatory mediators (basophils, eosinophils and mast cells) together with other elements (complement system, acute-phase proteins, cytokines) and NK cells.

In this system, all cells are originated from pluripotent hematopoietic stem cells and a differentiation process occurred through myeloid lineage predominantly. The discrimination between the self and non-self molecules, which is essential for appropriate immunogenic response, relies on the recognition of PAMPs (Pathogen-associated molecular patterns) of pathogens through their PRRs (Pattern Recognition Receptors). PAMPs are not found on the vertebrates but shared commonly by many pathogens and toxins. Any problem in that detection process can cause the initiation of immunogenic response against host's self-antigens which might result in pathological conditions such as allergy, auto-immune disorders [1-3]. One of functions of the innate immunity is activate cellular adaptive immunity, which is activated as a second line of more specific defense mechanism that is capable of forming immunological memory.

1.1.2 ADAPTIVE IMMUNE SYSTEM

The most characteristic feature of the adaptive immunity is the generation of antigen specific response occurred by the members in that branch of the immune system. Tightly regulated selection mechanisms allow to create lymphocytes that have no immunogenic response against self-molecules of the host but effective to foreign antigens by their specific receptors. In contrast to innate immunity, generation of effector response takes

days rather than hours to develop and provides a long-lasting immunity against subsequent exposures with the same antigen by initiating more rapid and vigorous immune response provided by memory cells. In general, adaptive immunity relies on two fundamental steps; it starts with the recognition/presentation of the antigen, which gives rise to cell activation, differentiation and proliferation of lymphocytes. This step provides the selection of lymphocytes bearing appropriate receptor and multiplying of them under the control of "clonal selection" process, followed by the effector responses either by B lymphocytes via release of antibodies into body fluids and tissues, or by T lymphocytes via orchestrating the immune response and initiating the cytotoxic activity.

All lymphocytes are originated from hematopoietic stem cells within the bone marrow. The maturation process of B cells also occurs within bone marrow, whereas T cells travel to the thymus for this step. After the maturation, they migrate to secondary lymphoid organs of the host and maintain as "naïve" cells. Maturated lymphocytes bear the receptors specific to antigen after the process named as "gene-rearrangement" early in the development which allows for generation of more than 10^8 antigen receptors and antibody types [4]. Four types of genetic fractions, *V* (Variable), *D* (Diversity), *J* (Joining), and *C* (Constant), play the key roles for this process. A unique recombination [5] of genes of each within these segments responsible for unique antigen receptor production together with the inaccuracy in splicing and an enzyme "deoxyribonucleotidyltransferase" capable of inserting nucleotides around segments, further mount up the receptor generation [6]. Whole procedure is conducted under control of enzymes produced by *RAG* (Recombination Activating Genes)1-2. Defects in mechanism of *RAG* genes lead to severe immunodeficiency caused by the absence of lymphocytes bearing appropriate receptors [7].

1.1.2.1 B LYMPHOCYTES

They are responsible for producing antibodies which are important in toxin-neutralizing, blocking pathogen-adhesion, and opsonizing bacteria for favoring phagocytosis [1]. There are two main sites on antibody molecule structure; constant region determines the function of antibody whereas the variable region is responsible for antigen binding. During ontogenesis, B cells formed as "B1" cells; most of them express CD5 functioning in

adhesion and signaling system. At slightly later stages on ontogenesis, they undergo a process ended up with the lack of expression of CD5 molecule and form as “B2” cells. IgM and IgD are co-expressed on mature B2 cells before they encounter with an antigen. Within germinal centers, where B cell response occurs, modification processes can be initiated; *V* gene segment rearrangement allows to “receptor editing” [8] and “class switching” in immunoglobulins, defining as the VDJ unit of antibody amend to different constant region [9] together with the “somatic hypermutation” for adjusting the binding strength; ensures for production of fine-tuned IgA, IgE, or IgD. Before the final response arises, they leave the germinal center as “plasma cells” and initiate antibody secretion within secondary lymphoid tissues [3]. General diagram about B cell development is shown in Figure 1.1

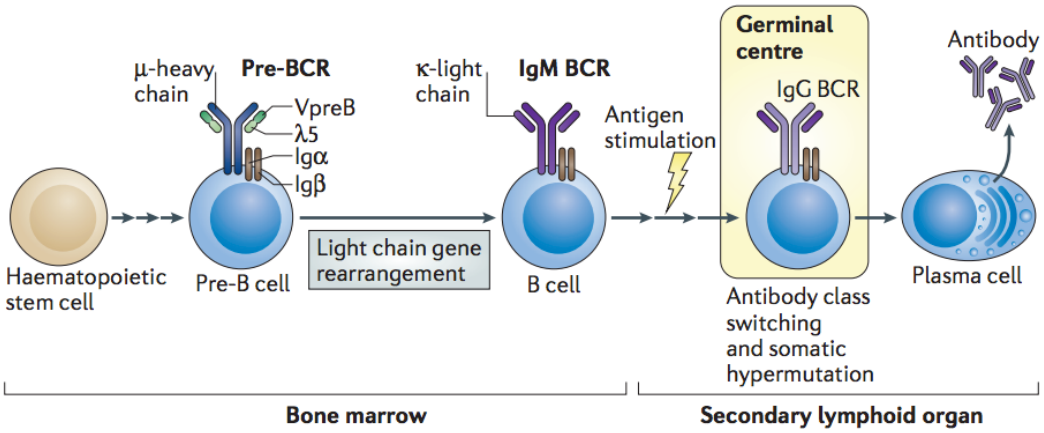


Figure 1.1: The generation and maturation of B cells. [10]

B-cell responses can be initiated by either T-cell dependent or independent manner. In B and T cell interaction, processed antigen is presented to CD4+ T cell, complexed with MHC-II molecule leading to the secretion of cytokines by T cells, necessary for B cell that ended up with B cell division & maturation [1]. On the other hand, repetitive parts of the antigen where the antigen is recognized and bound to, can initiate the short-lived, less specific B cell response without T cells, leading to direct secretion of IgM without class-switching and formation of memory [11].

1.1.2.2 T LYMPHOCYTES

Unlike B cells, T lymphocytes are not capable of recognizing antigen directly, but instead, they are activated after the antigen is presented to them by MHC complex. Antigen is loaded onto MHC complex in two different ways; It might be generated as a result of infected/dysfunctional cells and expressed on the cell surface by MHC I or taken up from the extracellular microenvironment via endocytosis by APCs. All nucleated cells express MHC I complex and capable of presenting the antigen for elimination by CD8+ T cells via cytotoxic effect. Antigen presenting cells are responsible for taking up the antigens, processing into short peptides and transferring them to the secondary lymph nodes where they encounter with naïve T cells and antigen presentation process occurs [1]. Migration of immature T cells from bone marrow to thymus occurs throughout lifespan of the individual [12].

The structure of T cell receptors based on the constant and variable regions similar to B cell receptors, however, they are found as membrane-anchored form. The α and β chains are mostly found among TCR types which recognize the MHC-antigen complex [13, 14] whereas under the 10% of T cells [1] bear γ and δ chains, which are thought to have functions for mucosal defense [3]. T cells bearing α/β TCRs remain in the thymus and undergo various selection process [15] for appropriate immunogenic response to prevent the self-destruction. The cells which are capable of interacting with MHC complex, but not showing any affinity against self-antigens and MHC complex are specifically selected during this process [16, 17]. Furthermore, in thymic regulation process, many various expressed molecules on the surface of T cells are switched depending on their selection. Two major classes of T cells occur after this procedure; T cells that express CD4+ molecule which can interact with MHC II molecule and respond to orchestrate immunogenic functions by secreting cytokines and taking role in B cell activation and the ones that express CD8+ molecule, which are able to recognize MHC I complexed peptides. CD8+ T cells are effective in cytotoxic activity against intracellular pathogens. They also get help from CD4 T cells for better expansion and effector differentiation after activation. After the activation, some T cells still remain in the lymph nodes as “memory cells”, ready to initiate faster secondary responses against the same pathogen exposures. Discrimination between the naïve and memory T cells is based on the CD45 expression on their surfaces.

Naïve T cells express CD45RA whereas CD45RO is found on memory T cells [1, 3]. In activation process, recognition of MHC-antigen complex is not enough for appropriate immune response. The interaction between MHC-antigen complex and CD4 or CD8 molecules together with CD3 only, results in the formation of unresponsive T cells (anergic). Co-stimulatory molecules are also essential for T cell response. Communication between co-stimulatory molecules, e.g. CD80 (B7-1), CD86 (B7-2), CD40 expressed on the APCs and CD28 expressed on the T cell is also essential for activation [1]. A general diagram explaining T cell activation by APCs is shown in Figure 1.2.

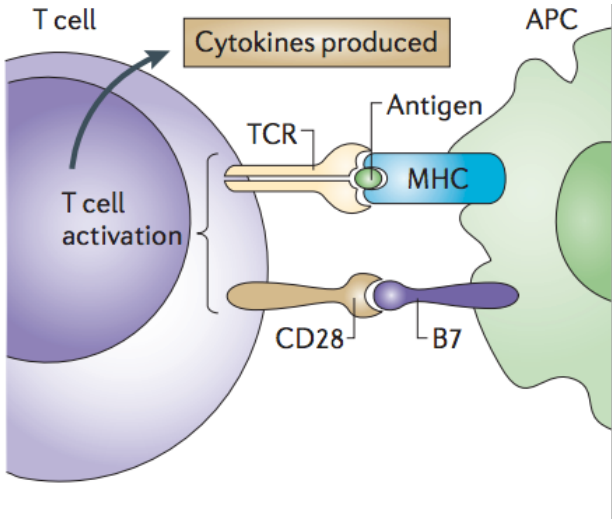


Figure 1.2: The interaction between T cell and APC during T cell activation. [18]

CD8+ T cells and their cytotoxic innate counterpart, NK cells use similar mechanism to kill the target cells. They bind to cells, creating pores on the targeted cell membrane by insertion of perforins followed by the transmission of granzyme-containing cytoplasmic granules to targeted cell's cytoplasm through the pores and manipulate the cell's metabolism by activating caspase enzymes, thereby inducing cell apoptosis for elimination of infection. To activate apoptosis, cytotoxic T cells can also bind to "Fas" molecules on the surface of targeted cells via their Fas ligand "FasL" [1].

CD4+ T cells can be sub-grouped based on cytokine production profile they produce [19]. In early stages of T cell activation, helper (CD4+) T cells defined as "Th0" which mainly responsible for IL-2 production participate in T cell proliferation and stimulation of CD8+ T cells [1]. When the activation signals still continue, they further start to respond these

signals and begin to polarize into different subsets (Th1, Th2, Th17, Th9 and Tfh) relying on the condition at infected zone, responsible for secretion of various cytokines for appropriate immune response [20, 21]. General information about CD4+ T cell subsets is shown in Figure 1.3.

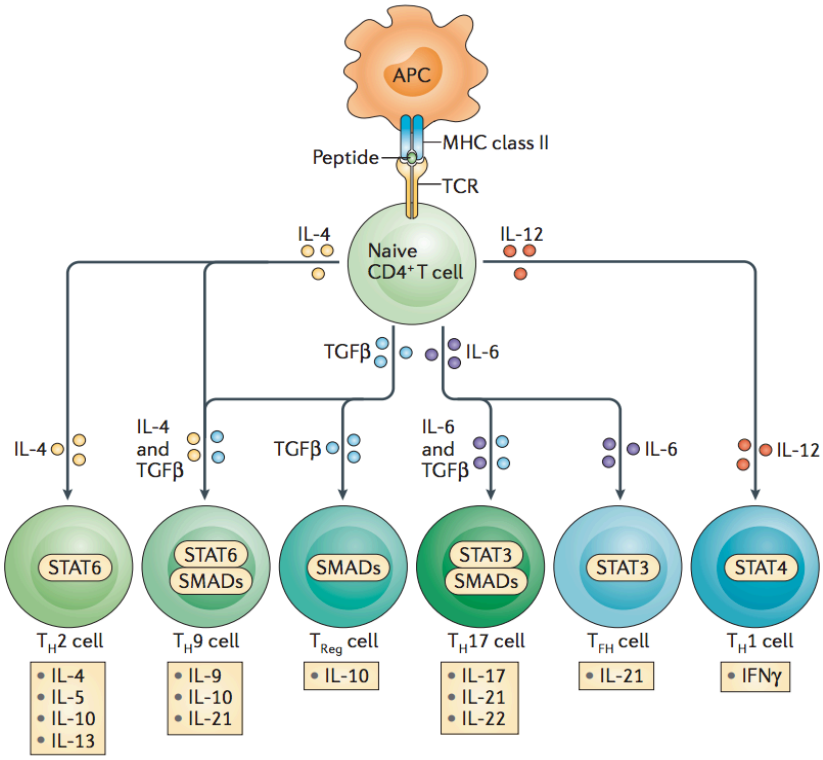


Figure 1.3: CD4+ T cell subsets and their functions in adaptive immunity.[22]

Specific cytokines are responsible for certain differentiation processes; IL-12, as a result of production by NK cells or macrophages enhances Th1 differentiation whereas IL-4 is the key factor in Th2 differentiation process which is secreted by basophils and mast cells as well as Th2 cells themselves. Also, IL-6 and TGF-β together induce the polarization of Th17 whereas TGF-β and IL-4 are responsible in Th9 differentiation. After the polarization process, polarized Th cells begin to express their characteristic proteins to secrete their main cytokines. IL-2 and IFNγ production is a result of Th1 polarization leading to contribution to cell-mediated immune response. Th2 cells are capable of secreting various cytokines; IL-4, IL-5, IL-10 and IL-13, effective in humoral response as well as allergic responses. IL-17, IL-21 and IL-22 production is generated by Th17 cells

that take part in elimination of extracellular pathogens. The Th9 cells are responsible for production of IL-9 predominantly [23-25].

Some part of CD4+ cells have a functional role in downregulating immune responses. These cells are called as “Regulatory T cells” (Treg) which show their characteristic forms by expressing forkhead transcription factor (FoxP3) together with CD25. The regulatory activity comes from the secretion of IL-10 and TGF-β [26]. Some of these cells are generated in thymus known as “natural Treg” (nTreg) whereas some Tregs are transformed from naïve CD4+ T cells at the early stage of activation in the presence of IL-10 and TGF-β in periphery, which are called as “induced Tregs” (iTreg). However, CD25 or FoxP3 expression pattern can be slightly different in this phenotype [27].

A subgroup of T lymphocytes named as “invariant NK T cells (iNKT)” provide rapid response against infection in innate immunity. These cells express, invariant αβ T cell receptor together with natural killer cell markers (e.g. NK1.1) and responsible in recognizing the lipids instead of peptides coming from either the pathogen or damaged cell itself which is presented by APCs through their MHC-class I related molecule CD1d [28, 29].

1.2 PURINERGIC SIGNALING

Among various bio-molecules, ATP is the fundamental energy currency for living cells. It is a member of purine family and consists of an “adenine” base and “ribose” bound to three ionized phosphate groups [30]. It can be generated as a result of different biochemical processes e.g., glycolysis, TCA or Krebs cycle as well as oxidative phosphorylation [31]. Beside the major function of ATP in energy supply, it can also takes part of regulation in the various physiological processes such as cardiovascular system [32], visceral organs, e.g. gut, bladder, vagina [33, 34]. Together with its degradation products, AMP or adenosine, ATP is also vital for activation of inflammasome, key molecule for the secretion of pro-inflammatory interleukin-1β (IL-1β). Therefore, ATP is considered to be one of the endogenous immunostimulatory damage-associated molecular patterns (DAMPs), which will be discussed later [35]. In general, purinergic receptors can

be branched into three groups based on their pharmacological activities and their structural properties [36].

P2X class has a function as ATP-gated ion channels. They specifically respond to ATP with various ligand affinities [37]. After ligand binding, the response is seen as the influx of extracellular cations, including calcium [38]. Among these types of receptors, P2X7, reveals its ion channel function under the condition of micromolar ATP concentrations whereas at higher ATP levels (milimolar range), P2X7 form pores and cause apoptosis [37].
P2Y subclass of purinergic receptors belong to G-protein coupled receptor group shows affinity to ATP, ADP, UTP, UDP, UDP-Glucose whereas P1 subclass recognizes adenosine [38].

Another important function of ATP after its secretion from damaged tissues, stressed or dying cells as "danger signal" is to initiate the appropriate immunogenic response through its recognition by purinergic receptors [39-41]. Extracellular ATP accumulation is predominantly occurrs through Pannexin 1 channels which are members of purinergic receptors. That hemi-channel type, together with connexins are believed to allow intercellular communications [38]. It is also known that TCR stimulation, as well as osmotic stress is responsible for accumulation of ATP through Pannexin 1 [42-44]. Main feature of ATP in immune response can be detected as dual effects. High concentrations of ATP enhance the activation of immune cells and pro-inflammatory response when secreted acutely by the activated cells and when acting in autocrine/paracrine fashion. Nevertheless, when the generation of extracellular ATP is chronically available at lower levels, it may cause an anti-inflammatory response [41, 45, 46]. These effects which are regulated by P1 and P2 receptors, are confirmed in CD4+ T lymphocytes as well as monocytes, macrophages, DCs and neutrophils. In Figure 1.4. general mechanisms of purinergic signaling in T cell types is shown.

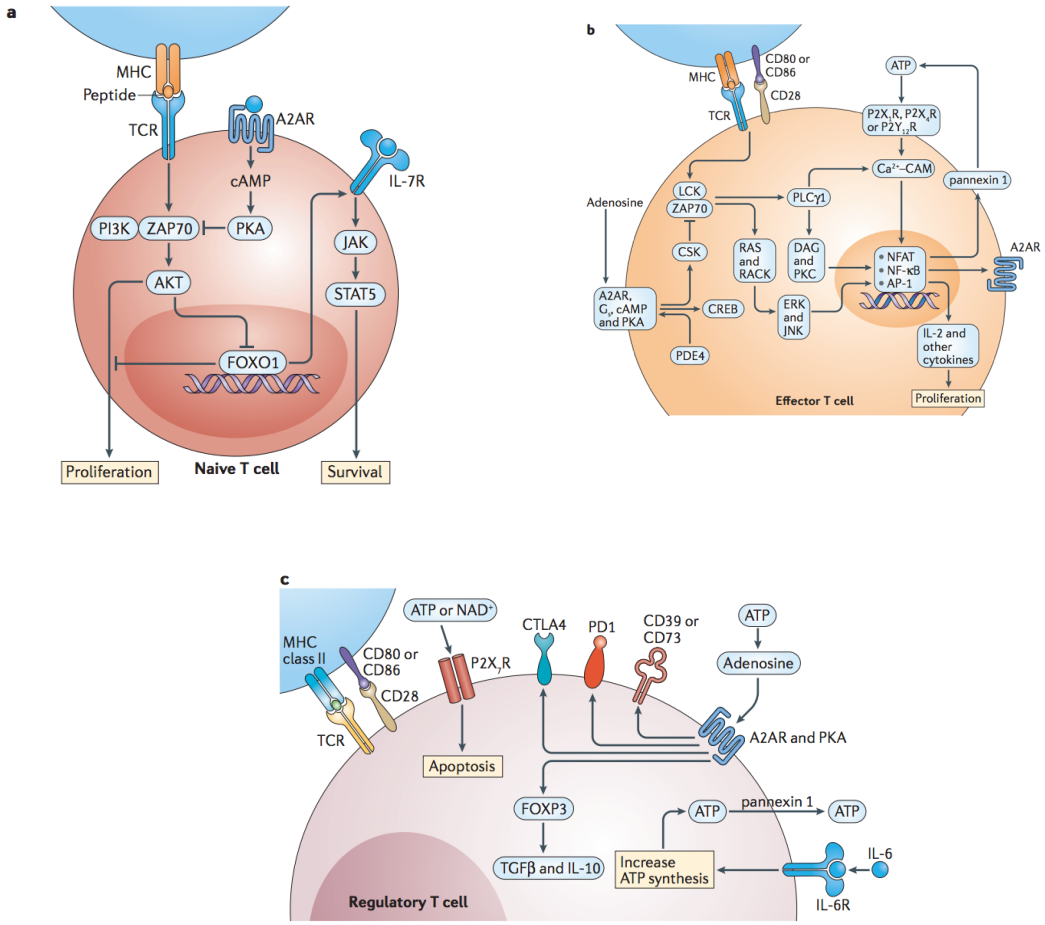


Figure 1.4: Purinergic signaling mechanism in **a)** Naïve T cells, **b)** Effector T cells **c)** Regulatory T cells [47]

1.2.1 ADENOSINE and ADENOSINE RECEPTORS

Adenosine, a nucleoside, member of purine family which is a ligand for P1 receptors has many different roles in physiological systems. It shows various effects in nervous system (such as dementia, sleep, pain, Parkinson's disease), renal system, cardiovascular diseases, pulmonary disorders, cancer, and inflammation [48]. It is responsible in modulating the responses against tissue-disturbing events to provide tissue protection. After stressful conditions occur, such as hypoxia, ischemia, trauma, inflammation; adenosine functions as a tissue protective molecule [49] by increasing the oxygen supply to the tissue, inducing anti-inflammatory responses and stimulating angiogenesis [50]. The concentration of adenosine is low at extracellular area when there is no stressful environment, however, it can be rapidly elevated to regain homeostasis after pathophysiological conditions [29]. ATP level elevation in the extracellular space as "danger signal" causes initiation of a

hydrolyzing process by ectonucleotidases that converts ATP/ADP to AMP via CD39 (also known as; ectonucleoside triphosphate diphosphohydrolase-1) , and from AMP to adenosine via CD73 (also known as ecto-5'-nucleotidase) [51] to terminate P2 receptor activation and preventing from pro-inflammatory effects of ATP. In addition to that, extracellular adenosine can also be found followed by transportation from intracellular area through bidirectional nucleoside transporters [31].

For termination of adenosine signaling, equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs) remove extracellular adenosine from the extracellular space [52, 53]. Furthermore, adenosine deaminase (ADA), an enzyme responsible for converting adenosine into inosine, is also effective for terminating adenosine signaling process [54] whereas adenosine kinase is responsible for conversion of adenosine into AMP [55]. Two different isoforms of ADA; ADA and CECR1 (also known as ADA2) which are responsible for enhancing the co-stimulatory signaling during immune response followed by T-cell proliferation and type 1 cytokine production [56, 57] are found on various cell types including lymphocytes [58].

Several studies point out the importance of ADA, CD39 and CD73 in physiological regulation mechanism by adenosine signaling. Polymorphisms in CD39 is responsible for the elevation of ATP/ADP and lowering of Adenosine levels, cause the predisposition to inflammatory bowel disease (IBD) as well as multiple sclerosis (MS), whereas loss-of-function of CD73 in humans causes arterial calcifications [59-62].

Adenosine exerts its effects by binding to four types of receptors; A_1, A_{2A}, A_{2B} and A_3; which are members of G-protein coupled receptor family (GPCRs). Adenosine receptor signaling regulates cellular events mainly by changing the intracellular level of cAMP by activating or inhibiting adenylyl cyclase activity [29]. A_1, A_{2A}, and A_{2B} are highly homologous whereas A_3 varies among species [63]. A_1 and A_3 are coupled with G_i, G_o, G_q, and decrease intracellular cAMP level by inhibiting adenylyl cyclase, while A_{2A} and A_{2B} are coupled with G_s, G_q, and G_{olf} increase intracellular cAMP level following by activating adenylyl cyclase [64, 65]. The effect of adenosine signaling is determined by the repertoire of receptors expressed on tissue. Adenosine signaling is described briefly in Figure 1.5.

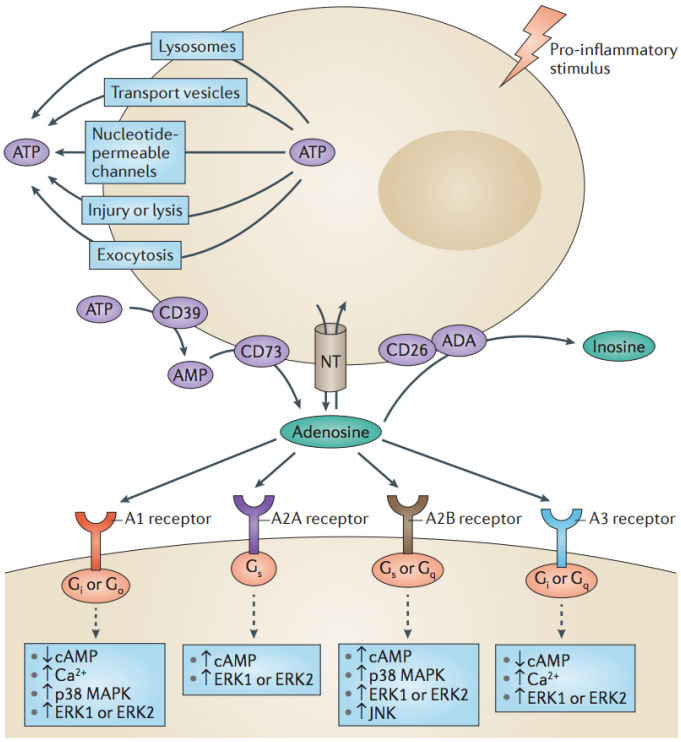


Figure 1.5: General mechanism of adenosine signaling [65]

A_1 activation via pertussis-toxin sensitive G protein, leading to adenylyl cyclase inhibition [66]. Furthermore, release of Ca^{2+} ions from intracellular stores by phospholipase C (PLC) activation is another effect of A_1 receptor activation. Elevated intracellular Ca^{2+} level is capable of activating various enzymes; such as phospholipase A_2 (PLA_2), phospholipase D (PLD), protein kinase C (PKC) to generate an appropriate physiological response [36, 48]. Briefly, activation of A_1 does not create a consensus response; as a matter of fact, both pro- and anti-inflammatory responses can be seen following by the activation. These dual effects of A_1 are seen depending on the tissue/organ and injury stage/progression together with the cell type and other signaling mechanisms for optimum physiological response [31]. It enhances pro-inflammatory response in human neutrophils by fomenting chemotaxis and adherence of these cells to epithelium under the condition when adenosine levels at sub-micromolar concentrations [67, 68]. Moreover, A_1 antagonism is shown to have protective effects in lungs [69] while agonists of A_1 may provide a protection in renal effects which come up with upregulation of cyto-protective genes, together with increased heat-shock protein 27 (HSP27) phosphorylation, which has a role in preventing the denaturation and aggregation of cellular proteins [70] [71]. Absence of A_1 may causes pro-

inflammatory effect in the lungs, demyelination in central nervous system (CNS), induced level of pro-inflammatory cytokine secretion in sepsis-induced mice [72-74]. According to one study, A_1 and A_3 are elevating G-CSF production which is important in stimulation of bone marrow cells, therefore can be used against chemotherapy to reverse its effect by inducing the number of leukocytes after the treatment [75].

A_{2A} and A_{2B} are responsible for elevating the intracellular cAMP level after stimulation of adenylyl cyclase via G_S and G_{olf} and specifically, A_{2B} is capable of activating PLC through G_q [64]. In A_{2A} signaling, elevated cAMP levels lead to regulation of various pathways including Ca^{2+}, K^+ channels, CREB, PLC, MAPK activation [76, 77]. The structure of these two receptors seem quite related, however, A_{2B} consists of the longest extracellular loop 2 (ECL2) among all adenosine receptors which has a major role in ligand binding, together with the highest number of cysteine residues found among all GPCRs. The length of ECL2 in $A_{2B,}$ may partially contribute the decreased affinity of that receptor to ligands compared to A_{2A} [78, 79]. Correspondingly, there are few numbers of A_{2B} agonists which are useful, e.g NECA (Non-selective adenosine receptor agonist) [80], and highly A_{2B} selective agonist, BAY60-6583 [81]. In A_{2A}-dependent signaling by agonists pointed out the several protective effects against myocardial infarction, lung as well as spinal cord [82-84] whereas A_{2B} agonists show their effects in the lungs during hypoxia in relation with HIF-1 transcriptional induction [85]. Furthermore A_{2B} is essential for IL-4 production in human mast cells [29]. More importantly, A_{2A} receptor have also major role in the regulation of immune response together with A_{2B}.

A_3 is shown as the most variable receptor type of adenosine among species. It causes inhibition of adenylyl cyclase and decreasing the intracellular cAMP level, as well as stimulation of PLC and calcium mobilization [48, 86, 87]. Besides its cardioprotective effects through K_{ATP} channels, it is also effective in regulation of various cellular functions, such as cell growth, survival and differentiation [87]. Moreover, an agonist of A_3, IB-MECA is shown to inhibit tumor growth [48, 87] and A3 is shown to mediate the allergic response in mice; e.g histamine release in mast cells [88]. Molecular interactions within adenosine receptors is shown in Figure 1.6.

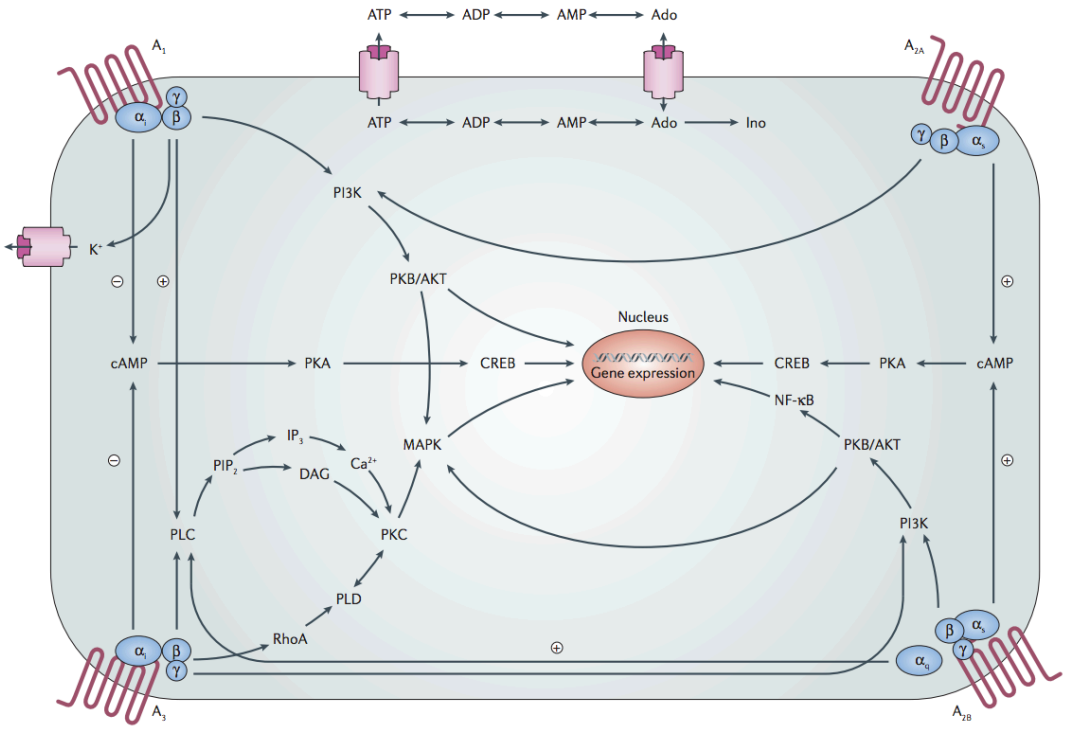


Figure 1.6: The interaction between adenosine receptors in cellular mechanism [48]

1.2.2 ADENOSINE RECEPTOR SIGNALING in IMMUNE SYSTEM

In general, adenosine dependent regulation of immune system based on the repertoire of adenosine receptor and bio-availability of adenosine. During inflammation, neutrophils are the first recruited class of immune cells. In neutrophil response, adenosine decreases neutrophil activation as well as neutrophil migration and release of pro-inflammatory cytokines. Moreover, expression of adhesion molecules is reduced mainly by A_{2A} signaling followed by elevated cAMP which leads to PKA activation [89]. On the other hand A_3 takes part as well in the affecting neutrophil migration and inhibition of superoxide anion generation [90, 91].

In macrophages, A_{2A}, together with A_{2B} reduce the secretion of pro-inflammatory cytokines and provide the accumulation of anti-inflammatory cytokine IL-10 [92, 93]. More importantly, adenosine, together with inosine are shown to inhibit M1-macrophage activation, which has pro-inflammatory response, and direct the M2-like phenotype, which favors angiogenesis and tissue protection [94, 95].

Dendritic cells are also essential for immune system. A_{2A} activation on mature DCs, changes their profiles into anti-inflammatory by reducing TNFα, IL-6, IL-12 production, and inducing the augmentation of IL-10. Moreover, in the presence of adenosine, DCs have limited ability to induce Th1 polarization from naïve CD4+ T cells [96-98]. Stimulation of A_{2B} results in the development of incorrect DCs, functioning as promoting the tumor growth; [99] that are characterized by both expression of low level DC marker, "CD1a" and CD14, a monocyte marker which is not normally expressed on the surface of DC.

1.2.2.1 ADENOSINE SIGNALING in T LYMPHOCYTES

Among all four types of adenosine receptors, A_{2A} is the predominantly expressed subtype in T lymphocytes and is induced after TCR stimulation; [100, 101] thereby primary effects of adenosine occur through A_{2A} receptors. In general, adenosine can inhibit T cell activation, proliferation, secretion of pro-inflammatory cytokines, and cytotoxic activity in an A_{2A} dependent manner [29].

Two downstream molecules of cAMP signaling, PKA and EPAC are responsible for mediating the adenosine signaling response on T cells and most of the effects by cAMP are mediated by PKA [102]. After adenosine binding to A_{2A}, elevation of cAMP level through G_s followed by the protein kinase A (PKA) activation restricts TCR activation [102]. cAMP mediated signaling is directly targets the cAMP response element binding protein (CREB) and phosphorylates nuclear factor of activated T cells (NF-AT) as well as inhibiting the NF-kB [103].

The adenosine dependent, restricted TCR activity, decreases the generation of various processes essential for appropriate immune response; e.g., inhibition of TCR-mediated pro-inflammatory cytokine secretion, such as IFNγ, IL-2, IL-12 and TNFα; CD25 and CD69 expression, and proliferation together with cytotoxic activity by regulating granular exocytosis from both CD4+ and CD8+ T lymphocytes; whereas favor the immunosuppressive molecules, such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), IL-10, TGF-β [101, 104, 105]. In

addition to reduce of Th1 and to lesser extent, Th2 polarization, A_{2A} has also ability to limit IL-17 generation by blocking the IL-6 production; which has essential role in Th17 differentiation and IL-17 secretion, together with TGF-β. Moreover, this process also induces the FoxP3 expression, resulted in favoring the "inducible" Treg differentiation [106]. CD39 and CD73 are co-expressed on Tregs, and different studies show that, adenosine-mediated immunosuppression can also be generated via CD39/CD73 expressed on Treg cells [107, 108].

Extracellular adenosine also affects the maintenance and survival of naïve T cells. Specifically, downregulation of IL-7Rα (CD127) expression after the TCR stimulation is prevented by AKT phosphorylation as a downstream of PI3K signaling, as a result of PKA-dependent adenosine signaling mechanism [109].

1.3 FOXO SIGNALING

Foxo is a subclass of Fox (Forkhead box) transcription factor family which is named after studying of *fkh* (Fork head) gene in *D. melanogaster* by monitoring a spiked-head in adults after the mutation on this gene occurs [110]. They are responsible in regulating the cellular responses and regulated by post-transcriptional mechanisms resulted in the controlling of their cellular localization [111]. Its genetic characterization has studied more specifically via its homologue DAF-16 in *C. elegans*. Activation of DAF-16 resulted in longer lifespan compared to normal nematodes [112]. Together with other transcriptional factors, they provide appropriate responses under the effect of environmental cues. The "O" subclass of Fox family has 4 members: Foxo1, Foxo3, Foxo4 and Foxo6. One of the most enquired kinase that is essential for regulating the Foxo response is Akt, and its phosphorylation function on Foxo is controlled by PI3K in response to various cell stimuli [111]. Nevertheless, according to studies, Akt phosphorylation of Foxo proteins has no direct effects on their functions, yet, it regulates binding for Foxo proteins onto 14-3-3 proteins [113].

14-3-3 proteins are modulators on various cellular processes. After binding to their targets, 14-3-3 proteins can regulate their stabilization, cellular localization, interaction with other

complexes and their enzymatic activity [114-116]. Phosphorylation of Foxo proteins by Akt direct them to accumulate in the cytoplasm after their inactivation [117].

Foxo proteins are responsible in the regulation of cell cycle progress and apoptosis [118]. It has been shown that, in breast cancer, overexpression of Foxo proteins reduce the tumor progression *in vitro* and and tumor size *in vivo* [119, 120]. Cellular localization of them is essential in tumoral response; cytoplasmic localization cause lower survival in breast cancer patients where nuclear localization, under the effect of its response, elevating apoptosis while reducing angiogenesis [119-122]. After finding the importance of this transcription factor family in cancer progression, it was studied among other cancer types, e.g. glioblastoma [123], leukemia [124]; which brings the field of investigation the effect of foxo proteins in immunity.

1.3.1 FOXO1 SIGNALING in T LYMPHOCYTES

Foxo1 proteins are important in maintaining the T cell homeostasis. Among other Foxo members, Foxo1 is upregulated specifically during the T cell maturation [125]. When there is no activation, T cell survival and homeostatic proliferation is predominantly dependent in IL-7 signaling [126]. The physiological effect of IL-7 in T cells generate after binding its receptor which consists of α-chain (IL-7Rα, also known as CD127) and common-γ-chain following by the activation of STAT5 transcriptional factor [127]. IL-7R expression is strongly down-regulated after TCR-activation, however it is re-expressed in memory T cells [128].

Akt-mediated phosphorylation as a result of TCR-activation, influence of CD28 co-stimulatory signaling and cytokine influence, initiates the nuclear export Foxo1 for inactivation in T cells whereas in resting conditions, Foxo1 proteins stay in the nucleus [129, 130]. It has been proposed that Foxo1 inactivation is essential for T cells to enter cell proliferation process based on a study in which the Akt-insensitive Foxo1 mutant expression resulted in the suppression of proliferation [122, 131]. Foxo1-deficiency causes the generation of T cells with activated phenotype and increases the differentiation to effector T cells. In addition to importance of Foxo1 in maintaining the naïve T cells, Th1 and Th17 polarization is also reduced under the influence of Foxo1 [128]. Furthermore, the

genes responsible for stimulatory molecules are upregulated in the absence of Foxo1 [132-134]. General information about Foxo1 signaling in T cells is shown in Figure 1.7.

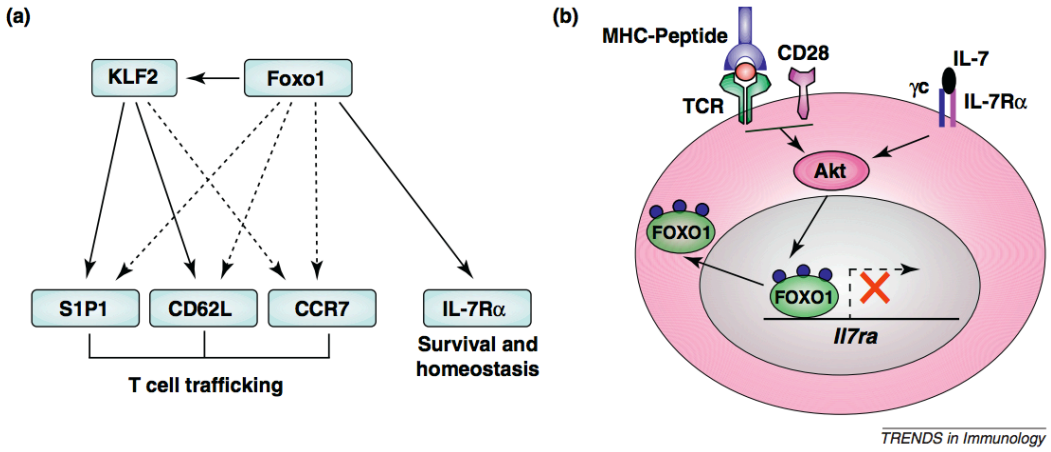


Figure 1.7: General effect of Foxo1 signaling in T cells [111]

There is a balanced mechanism between IL-7R expression and Foxo1 signaling to provide the optimal maintenance and trafficking of T cells. In the absence of Foxo1 or inactivation caused by the TCR triggered AKT phosphorylation following by PI3K signaling, there is a diminished expression of IL-7R; which is also effective in screening the activated phenotype [128, 135]. Thymic exiting and migration of peripheral lymphoid organs, depend on various trafficking molecules; e.g. sphingosine-1-phosphate receptor 1 (S1P1, *Edg1*), CCR7, and CD62L [136]. Migration of T cells into secondary lymphoid organs is reduced due to the low levels of expression of these molecules caused by Krupple-like 2 (KLF2) deficiency, which is a transcription factor selectively expressed on mature T lymphocytes, leading to thymic accumulation of T cells [137]. Foxo1 overexpression resulted in the induced levels of KLF2, whereas absence of Foxo1 leads to reduction of it, correlated with S1P1, CCR7 and CD62L expressions [135, 138]. In addition to the importance of maintenance and tolerance, Foxo1 is also important in Treg differentiation process. There are some binding docks identified for Foxo proteins in *FoxP3* promoter region [139], and it has been pointed out that TGF-β responsiveness in CD4+ T cells is under the influence of Foxo1 [140].

1.4 AIM OF THE STUDY

CD4+ T cells have been studied to understand their effects in different pathological conditions because they not only help CD8+ T cells and B cells but also shape the course of inflammatory responses. CD4+ T cells can polarize into different functional subsets to regulate the immunogenic response. Therefore, these cells can be targeted to cure immune related disorders. Before doing so, it is important to identify how these cells are regulated in different microenvironments. It is also important to know by what molecular mechanisms their responses are regulated. Adenosine is one of the important components of different inflammatory conditions. Also by being a GPCR, it functions similarly by other extracellular regulators of inflammation targeting their corresponding GPCRs. One example for such molecules is prostaglandins. Therefore, the main aim of the study was a thorough analysis of the effect of adenosine receptor signaling in helper T cells by using highly enriched naïve CD4+ T cells instead of testing cytokines from supernatants without doing intracellular cytokine staining and measuring true polarization of T cells. [101, 141, 142].

Our second aim was to identify potential molecular mechanisms for adenosine regulation of helper T cells. Because we observed an anti-proliferative and apoptotic effect of adenosine signaling in certain Th polarizing conditions we focused our attention on Foxo1 transcription factors. This is because Foxo1 transcription factors can inhibit survival and proliferation of T cells. TCR activation causes Akt phospho-activation and Foxo1 is inhibited after being phosphorylated by Akt. Our previous studies and others have shown that adenosine can inhibit Akt activation downstream of TCR. To address the effect of Foxo1 inhibition on T cell accumulation in the presence or absence of adenosine receptor stimulation we used a highly selective Foxo1 inhibitor, AS1842856.

Finally, our last aim was to understand which arm of cAMP pathway, namely PKA or EPAC pathway, is more responsible from suppressive effects of adenosine receptor signaling on T cells. To address this question, we used PKA vs. EPAC specific cAMP analogs

To our knowledge this is the first study testing the effects of adenosine receptor signaling in all major polarizing conditions, testing not only cytokine responses but also T cell accumulation. We also first time identified sustained activation of Foxo1 by reduced phosphorylation of Akt by adenosine may particularly suppress T cell survival rather than proliferation. Another novel finding was the combinatorial targeting of both EPAC and PKA pathways was required to phenocopy the effects of adenosine on IFNγ production/Th1 differentiation. Results of this study has important implications for mechanistic understanding of how helper T cell responses can be shaped in different cytokine and metabokine milieu.

Chapter 2

Materials

2.1 MATERIALS

2.1.1 General Laboratory & Cell Culture Reagents and Materials

All the 96-well cell culture plates were bought from Greiner Bio One, Austria. All pipettes were bought from Gilson, USA and Rainin, United Kingdom. BD Falcon™ 5 ml polystyrene round-bottom tubes were used in cell enrichment procedure. CD4+ enrichment procedure was performed via EasySep Mouse™ Cd4+ T cell Enrichment Kit (Cat. No: 19752) and for magnetic sorting of CD4+ cells, EasySep™ Immunomagnetic Cell Separation equipment (Cat. No: 18000) was used. CFSE, a dye for monitoring the cell proliferation was kindly gifted from Thor Lab (Bilkent, Ankara). For flow cytometry, CytoFlex was used from Beckman Coulter. Raw data were analyzed by GraphPad Prism 6. All equipment that used in western blot experiments were bought from Biorad, Hercules, CA, USA. PVDF membrane was developed by Amersham Imager 600. For cAMP assay,

LANCE® Ultra cAMP kit (Cat. No: TRF0262, Perkin Elmer) was used. ½ Area 96-well plates for cAMP assay, were bought from Perkin Elmer. The list of general chemicals and buffers during the cell culture experiments can be seen in table 1.1

Table 1.1: The list of the cell culture media components and buffers

Product	**Cat No.**	**Brand**
RPMI 1640 Medium	21875-034	Gibco
Fetal Bovine Serum (FBS)	S181G-500	Biowest
Sodium Pyruvate (100 mM)	S8636	Sigma-Aldrich
Penicillin/Streptomycin	17-745E	Lonza
HBSS	BE10-547F	Lonza
HEPES Solution	H0887-100ML	Sigma-Aldrich
β - mercaptoethanol		AppliChem

2.1.2 Cytokines & Antibodies & Reagents

The list of the antibodies, cytokines, enzyme/ligands, and other reagents that had been used during CD4+ cell subset differentiation experiments can be seen in Table 2.1, 2.2, 2.3 and 2.4 respectively.

Table 2.1: The list of the antibodies used to generate CD4+ T cell subsets

Product	**Cat No.**	**Brand**
Goat IgG Fraction to Hamster IgG	55397	MP
LEAF™ Purified Anti-mouse CD3ε	100314	BioLegend
LEAF™ Purified Anti-mouse CD28	102112	BioLegend
LEAF™ Purified Anti-mouse IFN-γ	505812	BioLegend
In Vivo Ready™ Anti-mouse IL-4	40-7041-U500	Tonbobio

Table 2.2: The list of the cytokines used to generate CD4+ T cell subsets

Product	**Cat No.**	**Brand**
Recombinant Mouse IL-2 (carrier-free)	21-8021-U005	Tonbobio
Recombinant Mouse IL-4 (carrier-free)	21-8041-U005	Tonbobio
Recombinant Mouse IL-6 (carrier-free)	575704	BioLegend
Recombinant mouse IL-7	577802	BioLegend
Recombinant Mouse IL-12 (p70) (carrier-free)	21-8121-U010	Tonbobio
Recombinant Mouse IL-23 (carrier-free)	589002	BioLegend
Recombinant Human TGF-β1 (carrier-free)	580702	BioLegend

Table 2.3: The list of the enzyme & ligands used during signaling experiments

Product	**Cat No.**	**Brand**
Adenosine Deaminase (ADA)	10102105001	Roche
NECA	35920-39-9	Tocris
SCH 58261 (A2A Antagonist)	160098-96-4	Tocris
8-pCPT-2-O-Me-cAMP-AM (cAMP analog-Epac specific)	4853	Tocris
6-Bnz-cAMP sodium salt (cAMP analog-PKA specific)	5255	Tocris
Foxo1 Inhibitor, AS1842856	344355-10MG	Merck Milipore

Table 2.4: The list of other reagents used to generate CD4+ T cell subsets

Product	**Cat No.**	**Brand**
Monensin Solution (1000x)	420701	BioLegend
Brefeldin A Solution (1000x)	420601	BioLegend
PMA	P8139-1MG	Sigma
Ionomycin	IO634-1MG	Sigma
Normal Rat Serum	13551	StemCell
Mouse CD4+ T Cell Enrichment Coctail	19752C.3	Easy Sep (StemCell)
Biotin Selection Coctail 2	19653	Easy Sep (StemCell)
D2 Magnetic Particles	19650	Easy Sep (StemCell)
EasySep Mouse CD4+ T Cell Enrichment Kit	19752	Easy Sep (StemCell)

All chemicals and antibodies for flow cytometry and western blot experiments can be seen in table 2.5 and 2.6 respectively.

Table 2.5: The list of flow cytometry chemicals & antibodies

Product	**Cat No.**	**Brand**
FoxP3 Transcription Factor Staining Buffer Set	TNB-0607-KIT	Tonbobio
16% Formaldehyde Stock Solution (Methanol-free)	28908	Thermo Scientific
LIVE/DEAD® Fixable Green Dead Cell Stain Kit (For 488 nm Excitation)	L349780	Life Technologies
LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (For 405 nm Excitation)	L34957	Life Technologies
Sodium Azide (NaN_3)	71289-5G	Sigma

Saponin	84510	Fluka
CD16/32 (Fc Block)	14-061-85	eBiosicences
BCL-2 PE	633508	BioLegend
CD4 PE	12-0041-83	eBiosciences
CD4 PerCpCy5.5	65-0041-U100	Tonbobio
CD4 PE-Cyanine 7	60-0042-U100	Tonbobio
CD44 Biotin – Rat Monoclonal Atb	10232	StemCell
CD45 Alexa Fluor® 700	103128	BioLegend
CD8a PerCpCy5.5	65-1886-U100	Tonbobio
CD8a APC-Cyanine7	25-0081-U100	Tonbobio
FoxP3 AF647	126407	BioLegend
IFNɣ PE-Cyanine7	60-7311-U100	Tonbobio
IL - 17 APC	506916	BioLegend
IL-4 PE	50-7041-U100	TonboBio
IL - 4 Brilliant Violet 421™	504127	BioLegend
IL - 9 PE	514104	BioLegend
Streptavidin - PE	S866	Invitrogen
Streptavidin - APC	17-4317-82	eBiosciences
Streptavidin – BV510	563261	BD

Table 2.6: The List of Western Blot Antibodies & Chemicals

Product	**Cat No.**	**Brand**
p-Akt (s473) rabbit mAb	9271	Cell Signalling
p-FOXO1 (s256) rabbit Ab	9466	Cell Signalling
p-CREB (Ser133)	9198S	Cell Signalling
p-ERK ½	9461	Cell Signalling
AKT	9272	Cell Signalling
FOXO1	9454	Cell Signalling
CREB	4820S	Cell Signalling
β-Actin Antibody (C4)	sc-47778	Santa Cruz
Phosphatase Inhibitor	04 906 837 001	Roche
Protease Inhibitor	04 693 124 001	Roche

Goat α-rabbit IgG HRP Conjugate	1706515	Biorad
HRP Goat α-mouse IgG	405306	BioLegend
Acrylamide / Bisacrylamide	BA004	InroBio
Clarity Western ECL Substrate	1705060	BioRad
TEMED	161-0801	BioRad
BCA Kit	23227	Thermo Fisher
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	26620	Thermo Scientific

2.1.3 Mice

All mice that have been used for experiments were generated at Animal Research Facility in Bilkent University, Dept. of Molecular Biology and Genetics. During the experiments, wild-type C57BL/6 (For Th2 differentiation, Balb/c mice was used as well.) 10-15 weeks old mice were used. All ethic permissions were taken and all procedures are done by following the Experimental Animals Regulations.

2.2 Buffers and Solutions

2.2.1 Flow Cytometry Buffers

- FACS Buffer: 2% FBS and 0,05% NaN_3 in HBSS
- Fixation Buffer: 4% Formaldehyde in HBSS
- Permeabilization Buffer: 1% FBS + 0.1% NaN_3 and 0.1% Saponin in HBSS

2.2.2 Western Blot Buffers

<u>5x Sample Buffer</u>

- 10 g SDS
- 25 mL β - mercaptoethanol
- 50 mL Glycerol
- 0.25 g Bromophenol blue
- 3.94 g Tris – HCl

in 25 ml dH_2O (store at -20^0C)

10x Running Buffer

- 10.08 g SDS
- 30.3 g Tris
- 144 g Glycine

in 1L dH_2O

Transfer Buffer

- 6.06 g Tris Base
- 28.8 g Glycine

in 1.2L dH_2O

- 400 ml MetOH

Adjust to 2L with dH_2O

RIPA Buffer (for 10 ml)

- 750 ul 2M NaCl (2M NaCl: 1.16 g NaCl in 10 ml ddH_2O)
- 1 ml 10% NP-40 (10% NP-40: 1 ml NP-40 in 9 ml ddH_2O)
- 500 ul 10% Sodium DOC (10% Sodium DOC : 1 g Sodium DOC in 10 ml ddH_2O)
- 100 ul 10% SDS (10% SDS: 1 g in 10 ml ddH_2O)
- 250 ul 2M Tris HCl (2M Tris HCl: 3.14 g in 10 ml ddH_2O, ph:8)
- 500 ul 1M NaF
- 100 ul 100mM $NaVO_4$

in 6.5 ml ddH_2O.

Stripping Buffer

- 15 g Glycine
- 1 g SDS
- 10 ml Tween 20

in 800 ml dH_2O (Adjust pH to 2.2)

Adjust to 1L with dH_2O

TBS-T: %0.1 Tween in TBS (1 mL tween in TBS)

5% NFDM: 2.5 g NFDM in 50 mL TBS-T

10% APS: 5g APS in 50 mL dH2O

10% SDS: 5 g SDS in 50 mL dH2O

1 M Tris pH 6.8: 6.057 g Trizma base in 20 mL dH2O, adjust pH to 6.8 then adjust volume to 50 mL with dH2O

1M Tris pH 8.8: 6.057 g Trizma base in 20 mL dH2O, adjust pH to 8.8 then adjust volume to 50 mL with dH2O

2.2.3 Chemicals for cAMP Assay

- 0.6M Perchloric acid (2.14 ml 70% aqueous solution into 48 ml dH_2O)
- 2.5M Potassium Carbonate (17.275 g into 50 ml dH_2O)

Chapter 3

Methods

3.1 Cell Culture Experiments

3.1.1 CD4+ T Cell Isolation & Enrichment

Spleens were isolated from C57BL/6 and Balb/c mice and transferred into HBSS including 10% FBS (v/v) on ice. The organ was mashed and strained through Falcon™ 40 μM Cell Strainer. (Fisher Scientific) (Cat. No: 08-771-1). Centrifugation was performed at 1200 rpm/min for 5 minutes. After the removal of supernatant, cells were resuspended in HBSS with 10% FBS at the appropriate volume according to the number of desired cell numbers for experiments. After the resuspension, cells were transferred into the 5 ml polystyrene round-bottom tubes for CD4+ T cells enrichment, which is necessary for fitting into the magnetic sorter. After the transfer; "Normal Rat Serum" was added to the suspension at 50 ul/ml of the cells (e.g. for 2 ml of cells, 100 μl of cocktail is added) together with the 3 ul of CD44 Biotin antibody; then, "CD4+ T cell Enrichment Cocktail" was added to mix at 50 μl/ml. Suspension was mixed well and incubated in the refrigerator (2-8^{0}C) for 15 minutes. "Biotin Selection Cocktail 2" was added after the 15 min of incubation at the level of 100 ul/ml. Cells were mixed well by pipetting again and incubated in the refrigerator (2-8^{0}C) for another 15 minutes. For binding of the magnetic particles to non-CD4+ T cells, "D2 Magnetic Particles" was added into the mix at 100 ul/ml. It is important that the magnetic particles have to be vortexed vigorously for at least 30 seconds to

dissolve any aggregates and to optimize the binding affinity. After the addition of magnetic particles, and mixing, cells were incubated in refrigerator (2-8^{0}C) for 5 minutes. For optimum magnetic separation, total volume of cells was brought to 2.5 ml by adding HBSS with 10% FBS and gently mixed by pipetting slowly 2-3 times. Tube was placed into the magnet without the cap and waited for 5 minutes. The magnet and the tube was inverted in one continuous motion into the new 5 ml polystyrene tube to collect only CD4+ cells, and get rid of the particles, other materials in the suspension. Shaking the tube and collecting any remaining drops should not be done. CD4+ Enriched cells were counted by hemocytometer and after the calculation the exact number of cells need for the experiment, the suspension was centrifuged, then resuspended in the condition medium (RPMI 1640, with 10% FBS, 10 mM HEPES and 50 μM β-mercaptoethanol). ADA (Adenosine Deaminase) was added to the media at 1U as final concentration, and cells were preincubated for 30 minutes at room temperature to get rid of the effects of endogenous adenosine and creating a new starting point for the experiments.

3.1.2 Carboxyfluorescein Succinimidyl Ester (CFSE) Labeling

In CFSE Labeling, after the counting of the cells, centrifugation was performed at 1200 rpm for 5 minutes than they were resuspended in 1ml HBSS. On the other hand, CFSE Stock Solution was prepared with HBSS in another falcon tube in 2x concentrations (10 uM) at 1 ml. Two pipettes were used for mixing process, cell suspension and CFSE solution were taken through different p1000 pipettes and put them into another 15 ml falcon tube simultaneously. Falcon was gently inverted 1-2 times and waited for 7 minutes at room temperature. It is important that CFSE is a fluorescent reactive dye, so the environment should be as dark as possible while working with it. At the end of the 7 minutes, rest of the falcon was filled with (10% FBS) HBSS quickly, centrifuged at 1200 rpm for 5 minutes. After resuspension of the cells with condition medium include ADA and preincubating for 30 minutes at room temperature in dark, cells were ready for the differentiation processes. During CFSE experiments, 6 x10^4 cells/well were used for CFSE experiments and they were not restimulated with PMA and Ionomycin at the end of the differentiation process. After this period, cells were collected and flow cytometry was performed directly.

3.1.3 T cell activation for Western Blotting

Lymph nodes were harvested from Black/6 mice and mashed, followed by filtering through Falcon™ 40 μM cell strainer. Cells were counted and after centrifugation at 1200 rpm for 5 min, they were resuspended with RPMI 1640, containing 10% FBS, 10 mM HEPES and 50 μM β-mercaptoethanol together with 1x ADA at desired volume according to experiments. For western blotting, $3x10^6$ lymphocytes/90 ul were used. Cells were splitted to 1.5 ml Eppendorf tubes in 90 ul and incubated in $+4^0$C for 2 hours. After that, NECA (1μM), SCH 58261 (1μM), EPAC (3μM), PKA (30μM) analogs together with 1μg/ml α-CD3ε and α-CD28 were added as final concentrations in 10 μl/well from 10x stock solutions. Antagonists were added 10 mins before than agonists and analogs. After 30 min incubation at $+4^0$C, hamster IgG was added to tubes as 50 μg/ml final concentration. Later, tubes were incubated at 37^0C in thermoshaker. After 10 min, cells were moved onto ice to stop the reaction and protein isolation protocol was performed followed by western blot protocol.

3.1.4 CD4+ Th Cell Differentiation

3.1.4.1 Day:0

The 96-well plate was coated with goat IgG fraction to hamster IgG. 50 μM of the IgG was prepared with HBSS and added to wells 50μl at 50μg/ml concentration. Plates were coated with IgG on shaker for 2 hours, at the low-level shaking (≈100-200 rpm/min). At the end of the coating stage, plate was washed with HBSS once, and blocked with RPMI 1640 media for 10-15 min. Later, the media was removed, and the treatments were added to coated wells. Waiting for another 10 – 15 minutes is necessary for the strengthening the interaction between the ligands and antibodies. The cells which preincubated in the medium with ADA were later added to the wells. Before the start of the incubation, plates were centrifuged at 1000 rpm for 5 minutes on low brake. In general, for Th differentiation experiments, $3x10^4$ CD4+ T cells/well were used during the experiments at the 150 μl medium/well. In "Uncoated Groups" cells were plated on well without IgG-coating. Depending on the type of the experiment, medium of uncoated group was included IL-7 (5ng/ml) as well as HEPES (10mM) and β-mercaptoethanol (50 μM). During the experiments, cells stimulated with 1μg/ml α-CD3ε and α-CD28 only were used as control groups.

Table 3.1 Reagent cocktails used to generate Th cell subsets differentiation (Day:0)

Th POLARIZATIONS (Final Concentrations)				
Th1	**Th9**	**Th17**	**Th2**	**Treg**
HEPES (10mM) & β-mercaptoethanol (50 μM)				
α-CD3ε/α-CD28 (1μg/ml)				
IL-12 (10ng/ml)	TGF-β1 (10ng/ml)	TGF-β1 (1ng/ml)	IL-2 (20ng/ml)	TGF-β1 (5ng/ml)
α-IL4 (10μg/ml)	IL-4 (10ng/ml)	IL-6 (50ng/ml)	IL-4 (100ng/ml)	IL-2 (5ng/ml)
	α-IFNγ (10μg/ml)	IL-23 (5ng/ml)	α-IFNγ (10ng/ml)	
		α-IL-4 (10μg/ml)		
		α-IFNγ (5μg/ml)		

3.1.4.2 Day: 2

Plates were centrifuged at 1000 rpm for 5 minutes on low brake, removed 120 ul/well of old medium slowly and added 140 ul/well of freshly prepared medium on top of the existing medium included ligands. It is important that the new medium should include HEPES (10mM), β-mercaptoethanol (50 μM) and 1U of ADA. The materials in medium for feeding procedures are listed below.

Table 3.2 The feeding condition material list (Day:2)

Th1	**Th9**	**Th17**	**Treg**
IL-2 (20ng/ml)	TGF-β1 (10ng/ml)	TGF-β1 (1ng/ml)	TGF-β1 (5ng/ml)
	IL-2 (20ng/ml)	IL-6 (50ng/ml)	IL-2 (5ng/ml)
	IL-4 (10ng/ml)	IL-23 (5ng/ml)	
	α-IFNγ (10μg/ml)	α-IL-4 (10μg/ml)	
		α-IFNγ (5μg/ml)	

For Th2 differentiation conditions, cells are transferred into another uncoated cell culture plate without changing the media and without adding any cytokine or antibody as well as any ligands.

3.1.4.3 Day: 4

At the beginning of day: 4, cells were washed with HBSS once following by complete removal of treatment medium, then restimulation media was administered onto cells. This media includes PMA, Ionomycin and Monensin (For Th17 differentiation, Brefeldin A is used instead of Monensin) together with HEPES (10mM), β-mercaptoethanol (50 μM). Monensin or Brefeldin A was added to restimulation media 1 hour later than administration of PMA and Ionomycin.

Table 3.3 Restimulation process of Th cell subsets

	Restimulation Process			
	Th1	**Th9**	**Th17**	**Treg**
PMA	50 ng/ml	500 ng/ml	500 ng/ml	No Restimulation
Ionomycin	1 µg/ml	500 ng/ml	500 ng/ml	
Golgi Plug	1x Monensin	No Monensin	1x Brefeldin A	
Time	5.5 hours	5.5 hours	5.5 hours	

Th2 restimulation process was performed at Day:5 with 50ng/ml PMA and 1ug/ml Ionomycin together with 1x Monensin for 6 hours. Monensin was added to restimulation media 1 hour later than administration of PMA and Ionomycin.

In Th experiments, all ligands, NECA (1µM), SCH 58261 (300nM), EPAC (3µM), PKA (30µM) and AS1842856 (50nM) were added on both Day:0 and Day:2, except for Th2 which was mentioned above. We have performed Th2 polarizing experiment in both C57BL/6 (Figure 4.1) and Balb/c (Figure 4.2,3 – Figure 4.7,9)

At the end of incubation time, polarized cells were ready to be stained.

3.2 FLOW CYTOMETRY

After the incubation process is done, cells were transferred to Nunc™ 96-Well Polypropylene MicroWell™ Plates (Cat. No: 249944) for staining procedure. After washing the cells twice at 300g for 5 min, with ice cold HBSS and FACS Buffer respectively, cells were incubated with an antibody cocktail containing 1:400 flow antibodies together with 1:200 Fc block in FACS buffer on ice for 30 min at dark for surface staining. After 30 mins, cells were washed once with ice cold HBSS, then dissolved in 50µl/well ice cold HBSS followed by adding 50µl/well 4% Formaldehyde. For fixation, cells were incubated for 30 mins at dark on ice. After washing step for ice cold HBSS to get rid of formaldehyde, another incubation was performed with intracellular staining cocktail including 1:100 antibodies together with 1:200 Fc block dissolved in the permeabilization buffer. This time cells were incubated for 40 min in room temperature at dark. After intracellular staining was done, cells were washed with ice cold FACS buffer

twice to completely remove the permeabilization buffer, and resuspended in FACS buffer. After dissolving in an appropriate volume of FACS buffer, cells were monitored through CytoFlex to analyze the differentiation procedures.

3.3 WESTERN BLOTTING

3.3.1 Protein Isolation

In order to make the protein lysate for western blotting, cells were dissolved in RIPA buffer and incubate for 30 mins on ice by vortexing each 10 mins vigorously. After 30 mins, cells were centrifuged at 13000 rpm for 13 min at $+4^0$C and supernatant is collected. Protein equilibration was done according to BCA Kit's protocol.

3.3.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

At the beginning, resolving and stacking gels were prepared according to the list below:

For 10 ml Resolving Gel

- 4 ml dH_20
- 3.3 ml acrylamide mix
- 2.5 ml 1 M Tris pH 8.8
- 100 ul 10 % SDS
- 100 ul 10 % ammonium persulfate
- 6 ul TEMED

For 5 ml Stacking Gel

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

After pouring of stacking gel on top of resolving gel, waited for polymerizing process. Samples were put at 90^0C for 3 min before loading into wells at appropriate volumes together with sample buffer. Later, gel was ran at 90V until the dye had reached the bottom of the surface.

3.3.3 Western Blotting

For transferring proteins to the PVDF membrane, 4 layers of whatman paper together with 1 layer of sponge were soaked into transfer buffer are prepared. PVDF membrane was sunk into 100% methanol for activation and placed on top of gel following by putting another whatman papers and sponge soaked into transfer buffer as well. After setting up

the transfer apparatus, the procedure was initiated for 1 hour at $+4^0$C. Later, the membrane was cut in size particular size where the protein of interest was localized, and incubate for 1 hour at 5% NFDM on room temperature with shaking in low rpm for blocking.

Following by blocking, the membrane was washed quickly to remove remaining NFDM particles. Incubation with primary antibody was done either at $+4^0$C for overnight or 1 hour at room temperature with slowly rotating in 50 ml falcon at appropriate concentrations of antibody. Membrane was washed after incubation with 1x TBST at room temperature on shaker, for 25 mins via changing the TBST in every 5 min. Then membrane was incubated with secondary antibody for 1 hour at room temperature while slowly rotating. Membrane was washed again with TBST for 25-30 min by changing TBST in every 5 min as well and last incubation was performed with 1:1 mixed ECL solution in full dark place at various time depending on the time for optimum protein developing on the device.

3.4 cAMP ASSAY

Isolated lymphocytes were activated for 1 day in IgG-coated 96 cell culture plate with 1 μg/ml α-CD3/CD28 together with 20ng/ml IL-2. After activation, cells were washed with HBSS and transferred into new cell culture plate after dissolving in the freshly made stimulation buffer. After adding NECA or vehicle control at the level of 1μM as final concentrations, in the presence of 50 μM rolipram, cells were incubated for 10 min at 37^0C. Later, ice cold 0.6M perchloric acid was added to cells at 100 μl/well and following by pipetting, 15 μl/well of 2.5M potassium carbonate was added to cells for neutralizing. Cells were incubated on ice at least 30 min and spun at 10.000 rpm for 10 min after transferring 1.5ml Eppendorf tubes. Clear lysates were collected and cAMP assay was performed according to manufacturer's protocol.

Chapter 4

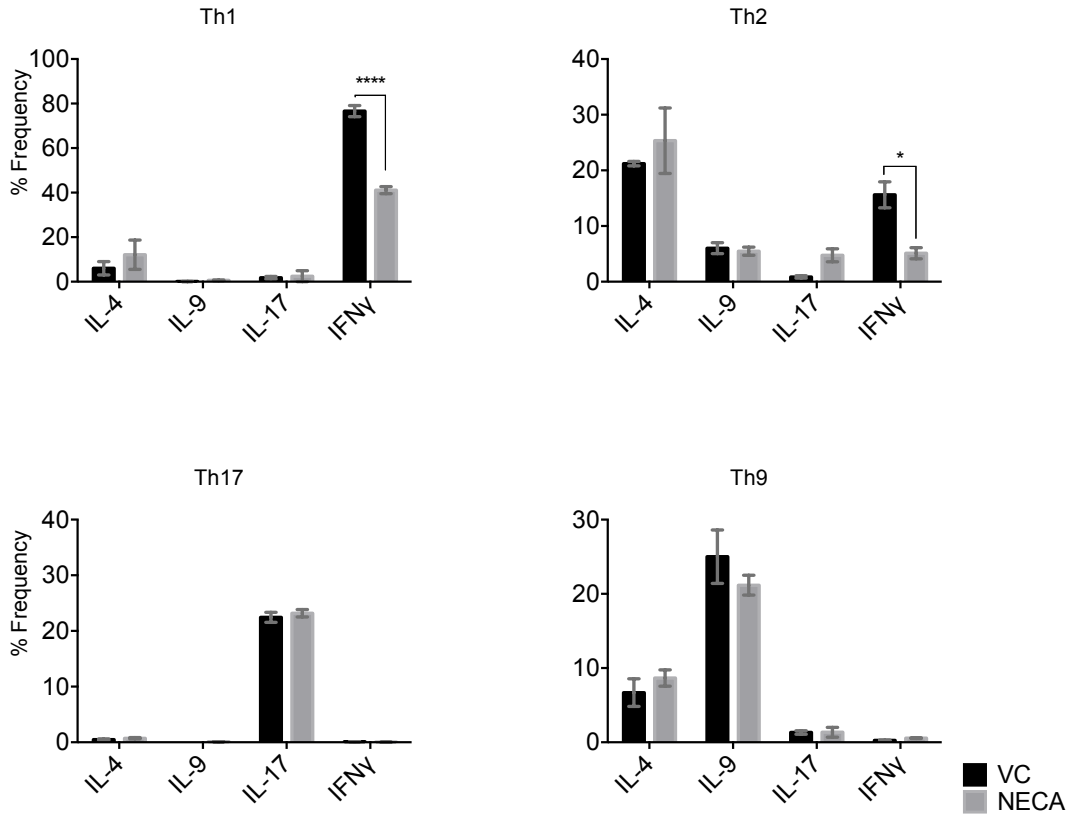
Results

4.1 Adenosine Receptor Signaling Preferentially Inhibits Th1 Polarization

Adenosine is an important regulator in orchestrating the immune response. Although it is known, the adenosine receptor type A_{2A} is predominantly expressed on T lymphocytes and regulates T cell activation [102], a thorough analysis of adenosine regulation of different Th subsets using highly enriched T cells is missing. To prevent the effect of endogenous adenosine during the experiment we pretreated the cells with adenosine deaminase (ADA), which converts adenosine into inosine. And then we treated cells with 5' –*N*-Ethylcarboxamidoadenosine (NECA) at 1μM , a stable adenosine analog which has affinity to all receptors, cannot pass through the cell membrane and cannot be degraded by ADA to observe the adenosine receptor-dependent effects on Th cell responses. CD4+ cells were cultured with in the presence or absence of NECA in different polarizing

conditions as indicated in the methods and they were stained for flow cytometry after restimulation with PMA and Ionomycin. Figure 4.1A indicates that NECA treatment particularly inhibited Th1 polarization *in vitro,* as indicated by decreased proportion of cells producing IFNγ in Th1 conditions. Even in Th2 polarizing conditions, proportion of cells producing IFNγ decreased by NECA suggesting cells resistant to get polarized to other Th subtypes but to Th1 subtype is strongly inhibited by NECA. In contrast, there was no significant difference in proportion of cells positive for corresponding cytokines indicating their polarization pattern. Adenosine receptor stimulation has been known to promote regulatory T cell (Treg) differentiation [143]. Figure 4.1B shows that NECA treatment has no effect differentiation of CD4+ cells in Tregs. Overall these results suggest that adenosine receptor stimulation differentially affects Th1 polarization of helper T cells. Of note, main gating strategy to target CD4+ T cells and their quality control is shown in appendix figure 1. Moreover, gating strategies for each Th polarization conditions are shown in appendix figure 2-6.

A.



B.

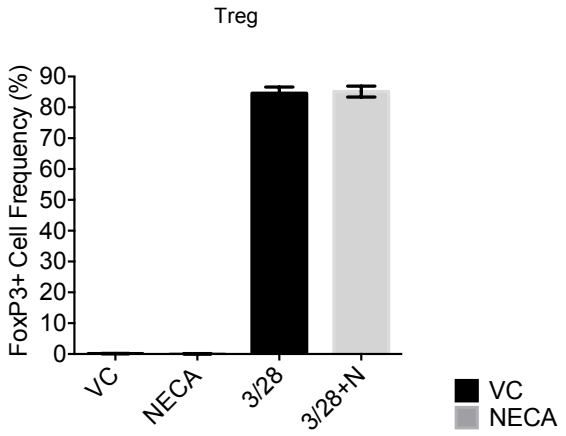


Figure 4.1: Adenosine receptor stimulation reduces the IFNγ production by inhibiting Th1 polarization. *ADA pretreated CD4+ T cells, isolated from C57BL/6 mice, were cultured in differentiating media in the presence or absence of NECA (1 μM). Intracellular staining was performed to detect IFNγ$^+$, IL-4$^+$, IL-9$^+$, IL-17$^+$(Figure 4A) and FoxP3$^+$ (Figure 4B) CD4$^+$ T cells. (3/28: CD4+ T cells were differentiated in Treg condition only, 3/28+N: 1 μM NECA was present in Treg polarizing condition) For statistical analysis, two-way ANOVA was performed. (Experiment was performed as quadruplicate and validated with four independent experiments. Bonferroni's multiple comparison test was used, **** p≤0.0001, * p≤0.05)*

4.2 Adenosine Receptor Signaling inhibits accumulation of Th1, Th17 and Th9 cells but not Th2 cells

Although our study has shown a differential effect on Th1 polarization, there are other studies suggesting adenosine receptors decrease or promote other polarizing conditions. This may be particularly evident if ELISA based assays are employed for several days of cell culture. Because accumulation of cells can also be directly proportional to extracellular cytokine accumulation we, next tested the exact T cell numbers in the presence or absence of NECA, in different polarizing conditions. Figure 4.2 indicated that NECA strongly suppresses accumulation of T cells in all the polarizing conditions except for Th2. We even observed a slight increase in T cell accumulation in Th2 polarizing conditions, suggesting NECA may influence either proliferation or apoptosis of T cells in conditions other than Th2 to influence T cell accumulation.

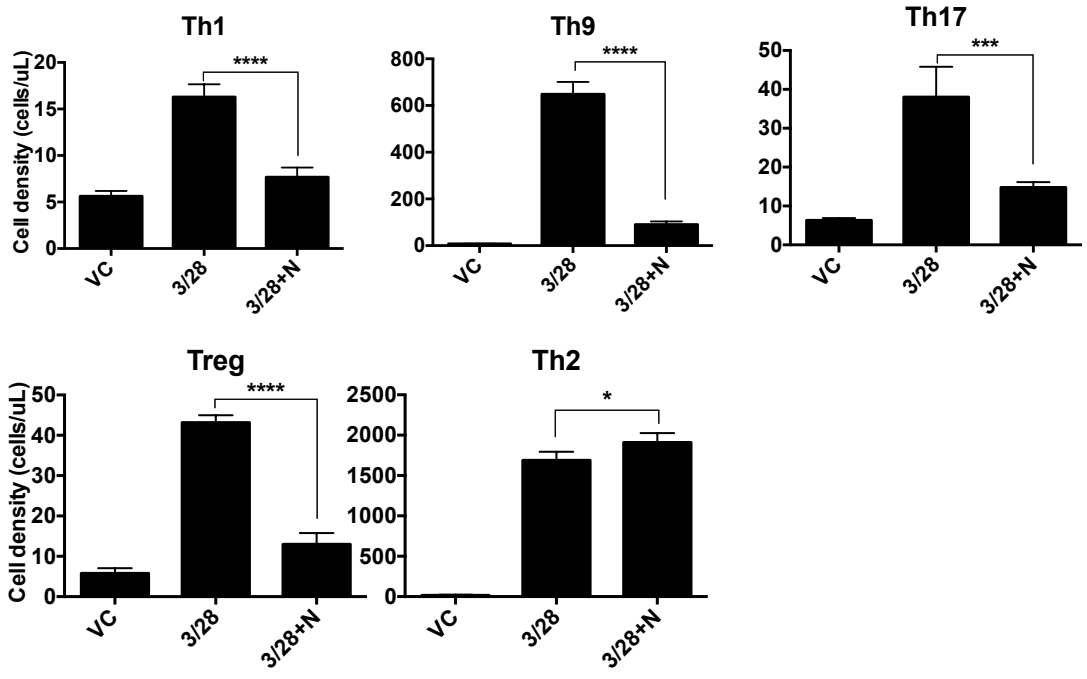


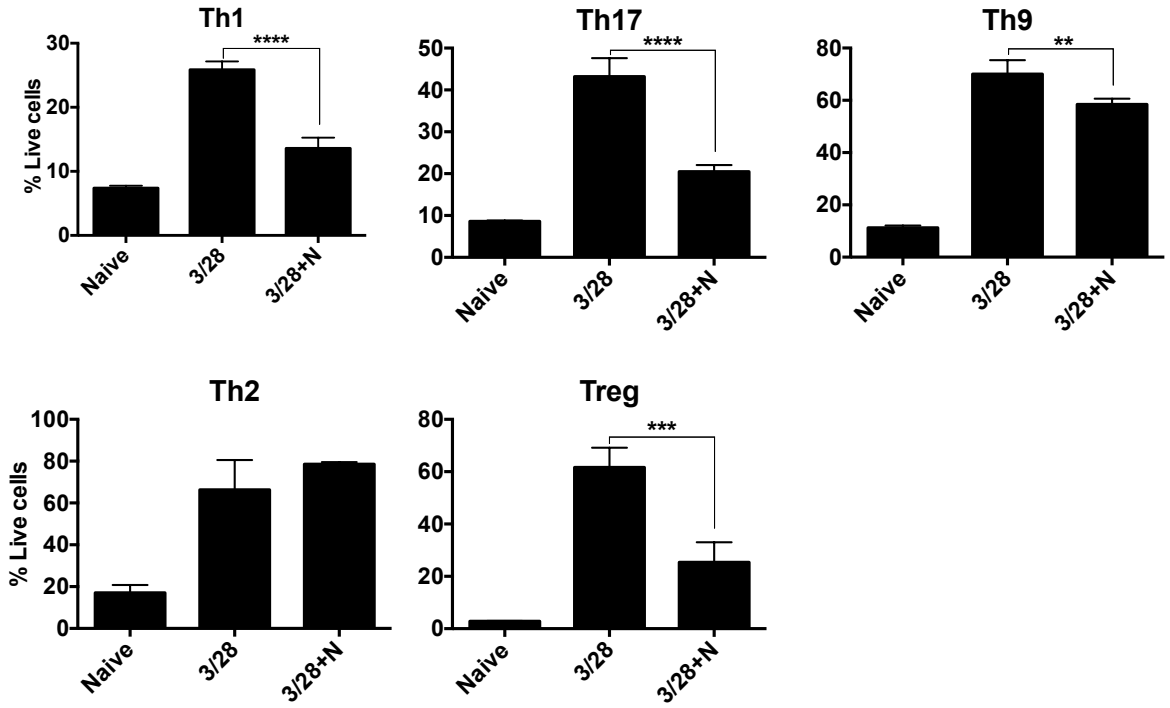
Figure 4.2 Absolute CD4+ T cell numbers diminished by adenosine receptor stimulation in CD4+ Th conditions except for Th2. *ADA pretreated CD4+ T cells were cultured in differentiating media in the presence or absence of NECA (1 μM). Flow staining was performed to show the absolute CD4+ T cell numbers. 3/28: CD4+ T cell group which cultured in polarizing conditions without NECA, 3/28+N: CD4+ T cell group which cultured in polarizing conditions in the presence of 1 μM NECA. For Th2 polarization, Balb/c mice was used and for other polarizations, C57BL/6 mice was used. For statistical analysis, one-way ANOVA was performed (Experiment was performed as quadruplicate and validated with four independent experiments. Tukey's multiple comparison test was used. *p≤0.05, ***p≤0.001, ****p≤0.0001)*

4.3 Reduced T Cell Accumulation after Adenosine Receptor Signaling Correlates with Decreased Proliferation and Survival

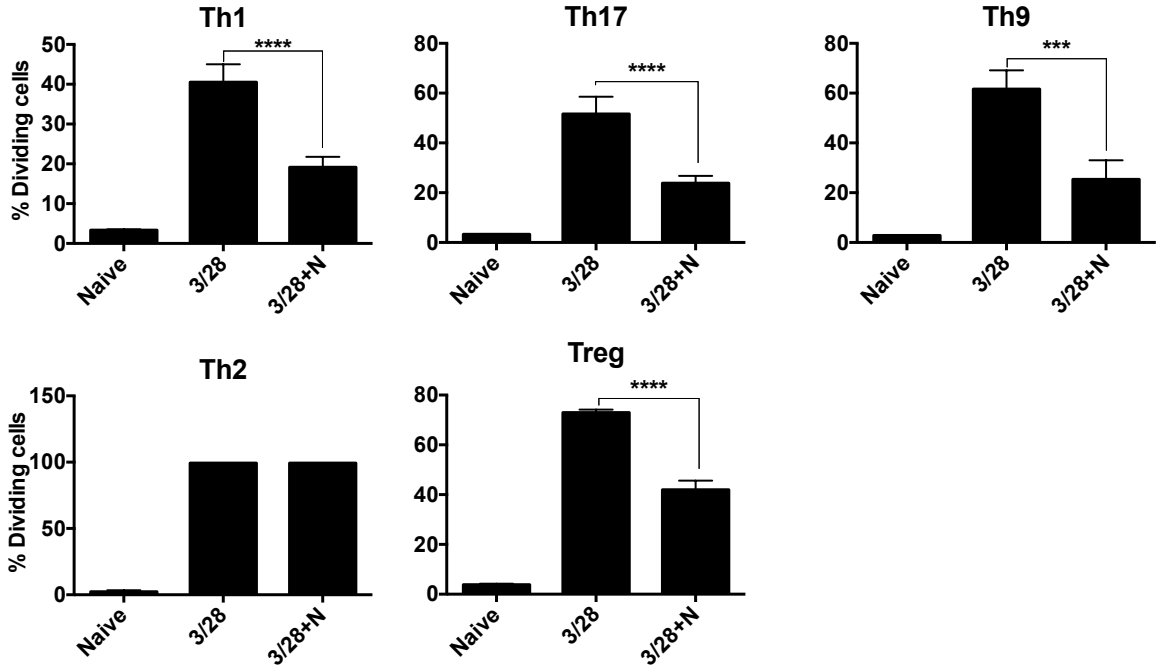
Hence the absolute numbers of cells are strongly decreased in the presence of NECA in Th polarization conditions (except for Th2 condition), we wanted to enlighten the effects of adenosine signaling to survival and proliferation. We labeled T cells with CFSE, a fluorescent dye that indicates proliferation of cells by emitting less fluorescent signal after each cycle of cell division due to getting diluted. Figure 4.3B suggested that, NECA

stimulation strongly decreased CFSE dilution of T cells in all the polarizing conditions except for Th2 condition suggesting that adenosine receptor stimulation inhibits proliferation in these conditions. We used Live/Dead cell viability assays to show that reduced numbers of T cells are also correlated with decreased survival in CD4+ Th subsets as shown in Figure 4.3A. In fact, NECA treatment also reduced the proportion of live cells suggesting adenosine receptor signaling suppress T cell survival in conditions other than Th2.

A.



B.



C.

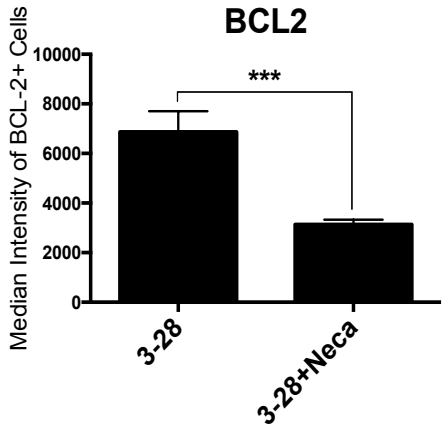


Figure 4.3 Adenosine receptor signaling functions as anti-inflammatory in CD4+ T cells effecting via reducing proliferation level together with survival. ***A)*** *Percentage of live cells,* ***B)*** *Percentage of dividing cells,* ***C)*** *Median Intensity of BCL-2+ CD4+ T cells.*

ADA pretreated CD4+ T cells were cultured in differentiating mediums in the presence or absence of NECA (1 μM). CFSE labelling is performed to monitoring the proliferation at the level of 5 μM. Both proliferation monitoring and viability screening was performed by flow cytometry. For BCL-2 detection, CD4+ T cells were stimulated with α-CD3ε (1ug/ml) and α-CD28(1ug/ml) in the presence or absence of NECA (1μM). For Th2 polarization, Balb/c mice was used and for other polarizations, C57BL/6 mice was used. For statistical analysis, one-way ANOVA was performed. (Experiment was performed as quadruplicate and validated with four

*independent experiments for A and B, with two independent experiments for C, Tukey's multiple comparison test was used. ***p≤0.001, ****p≤0.0001)*

T cell activation increases Bcl2 expression downstream of PI3K/Akt signaling pathway. It has been previously shown that A2AR activation in T cells causes reduced Akt activation [109]. BCL-2, an anti-apoptotic protein regulates the cell survival. [144] To test if adenosine receptor signaling influences anti-apoptotic Bcl-2 expression we activated T cells for 2 days in non-polarizing conditions and stained the cells intracellularly for Bcl-2 for flow cytometry. As shown in Figure 4.3C NECA treatment strongly suppressed Bcl2 expression suggesting that inhibition of TCR/Akt/Bcl2 pathway may be responsible for apoptotic effects of NECA.

4.4 Adenosine receptor signaling through $A_{2A}R$ causes activation of downstream cAMP signaling.

A_{2A} receptor is the major subtype in elevating the intracellular cAMP level in mouse splenocytes. [145]. ERK and CREB are two downstream targets of cAMP pathway regulated by EPAC and PKA respectively and adenosine signaling is suggested to be effective on lymphocytes predominantly via cAMP/PKA/CREB and ERK dependent manner. [102] However, cAMP/EPAC/ERK and Rap1 dependent cAMP pathway is also functional in adenosine receptor signaling in T cells. To readdress the effects of PKA and EPAC pathways in T cells downstream of adenosine receptor signaling we first tested the effects of NECA on downstream cAMP signaling by testing CREB and ERK phosphorylation in the presence or absence of NECA and A2A receptor specific antagonist SCH 58261. Figure 4.4A shows that in the presence of NECA intracellular cAMP level was elevated in CD4+ T cells. Moreover, Figure 4.4C and D show that NECA treatment increases ERK and CREB phosphorylation, which was reversed by SCH 58261. SCH 58261 also reversed the anti-proliferative effects of NECA as shown in Figure 4.4B. These results indicate that adenosine receptor signaling activates both pathways in T cells in A2AR dependent manner.

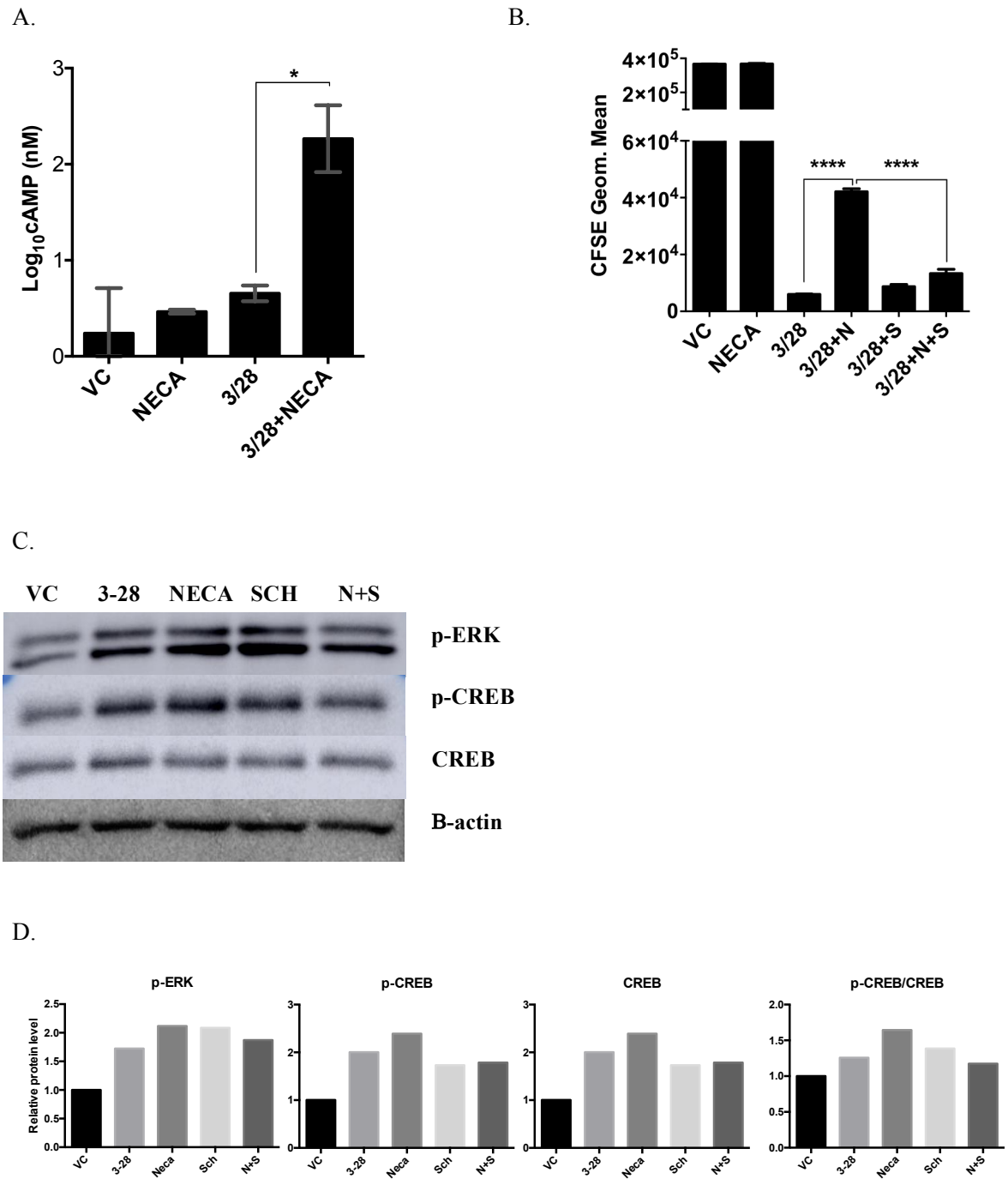


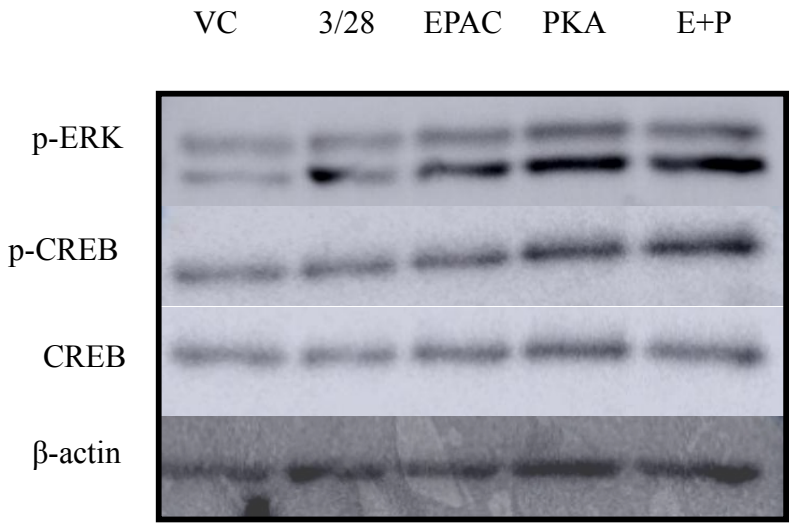
Figure 4.4 Adenosine signaling activates cAMP signaling and its downstream targets. ***A)*** *cAMP Accumulation Test* ***B)*** *Proliferation levels of lymphocytes were measured with CFSE labelling.* ***C)*** *Protein levels of p-ERK and p-CREB were detected with Western Blotting.* ***D)*** *Relative levels of ERK and CREB proteins. As a control, β-actin was used. ADA pretreated lymphocytes isolated from lymph node are stimulated with α-CD3e/CD28 (1ug/ml) in the presence or absence of NECA (1uM) and SCH 58261 (1uM). For cAMP test, naïve vs stimulated with α-CD3e/CD28 (1ug/ml) CD4+ T cells are incubated in the presence or absence of NECA (1uM).*

*For statistical analysis, one-way ANOVA was performed. (4.4A was performed as triplicate whereas 4.4B was performed as quadruplicate and they validated with two independent experiments, Tukey's multiple comparison test was used. *p≤0.05, ****p≤0.0001)*

4.5 Both PKA and EPAC pathways are important for inhibition of T cell activation by adenosine receptor signaling

To functionally test the effects of PKA and EPAC pathways on T cell activation downstream of adenosine receptors we used PKA and EPAC specific analogs. As shown in Figure 4.5A these analogs both increases ERK phosphorylation in T cells but only PKA specific analog can increase CREB phosphorylation, indicating their specificity. Figure 4.5B shows activation of both PKA and EPAC pathways together is required to phenocopy the effects of NECA on IFNγ production whereas PKA alone could induce comparable anti-proliferative effects to that of NECA as can be seen in Figure 4.5C. We observed similar patterns of additive effects on cell surface activation molecules as well (Data not shown). These results suggest that activation of both PKA and EPAC pathways fully simulate the suppressive effects of NECA in helper T cells.

A.



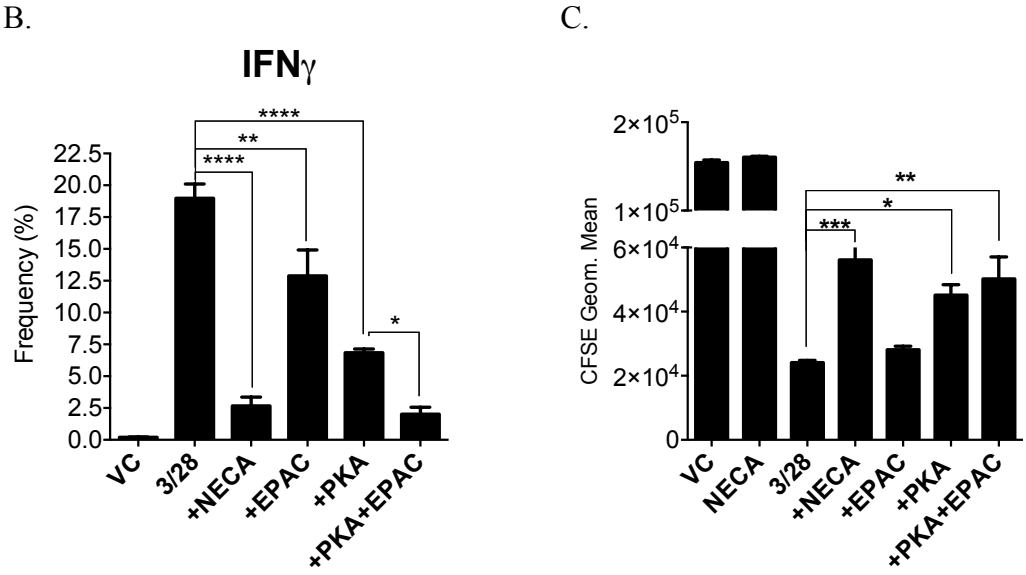


Figure 4.5 Both PKA- and EPAC- dependent pathways are important in regulating the Adenosine/cAMP signaling during inhibition of T cell activation. ***A)*** *Protein levels of pERK and pCREB were measured by western blotting* ***B)*** *Flow cytometry was performed to see the percentage of IFN γ^+ cells.* ***C)*** *Proliferation levels were measured with CFSE labelling by flow cytometry. ADA pretreated lymphocytes isolated from lymph node are stimulated with α-CD3e/CD28 (1ug/ml) together with EPAC-specific analog (8-pCPT-2-O-Me-cAMP-AM) at 3 uM and PKA-specific analog (6-Bnz-cAMP sodium salt) at 30 uM. For figure B and C, ADA pretreated CD4+ T cells were used. For statistical analysis, one-way ANOVA was performed. (4.5B and 4.5C were performed as quadruplicate and validated with two independent experiments, Tukey's multiple comparison test was used. ***p≤0.001, ****p≤0.0001)*

PKA/CREB signaling has been demonstrated with the inhibition of the major pro-inflammatory transcription factor NF-κB [146] and also effective on K_{Ca} 3.1 potassium channels in human CD4+ T cells are effective in reducing the IL-2 secretion and STAT5 activation [147, 148]. Furthermore, phosphorylation of nuclear factor of activated T cells (NF-AT) together with CREB is also effective in regulating the cytokine secretion and proliferation of T cells [103]. In general, intracellular cAMP is the main mediator of A_{2A} signaling and its influence on cytokine secretion, activation and proliferation of T cells, regulated by PKA-dependent and in lesser extent EPAC-dependent manners.

4.6 Adenosine Receptor Signaling Reduces the Phosphorylation of Akt and Its Downstream Target Foxo1

Adenosine signaling provides its effects in activated T cells, through inhibition of AKT in PI3K pathway following by TCR stimulation in A_{2A}-dependent manner [109]. Foxo1 is shown to regulate several different aspects of T cell responses, such as Treg differentiation, naïve T cell maintenance and T cell survival [128, 135, 140]. AKT is one of the key upstream factors essential in Foxo1-dependent regulation in cell metabolism. Akt phospho-activation causes Foxo1 phospho-inactivation and Foxo1 transcriptional activity. Therefore, we first wanted to check the effect of adenosine signaling on phosphorylation of Akt and Foxo1 on Akt target site. Indeed, as shown in Figure 4.6A and B, NECA stimulation strongly reduced both Akt phosphorylation and phosphorylation of Foxo1. Suggesting some of the effects of NECA on T cell responses can occur due to its effects on Akt/Foxo1 pathway.

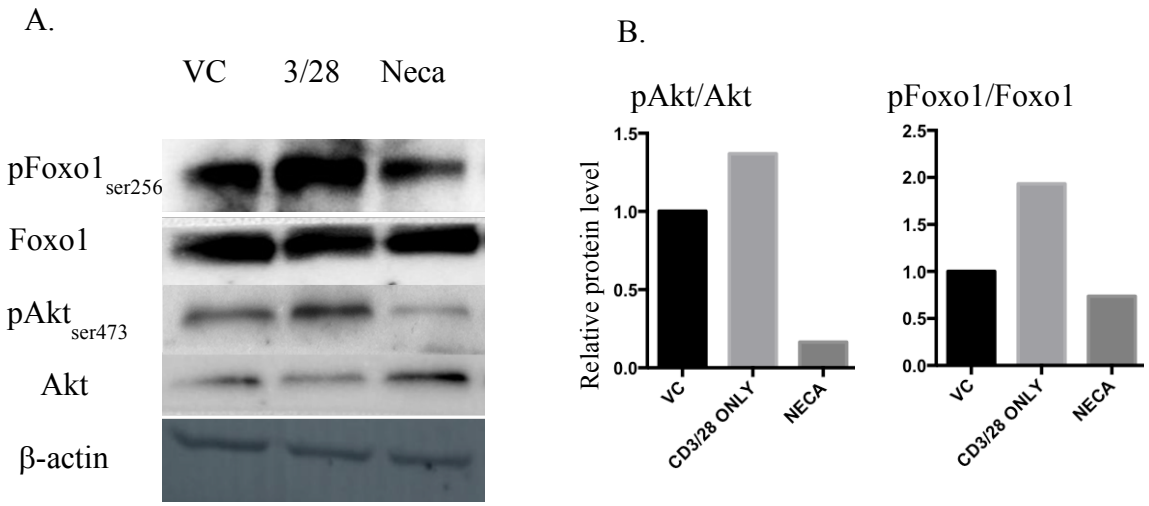


Figure 4.6 AKT phosphorylation and its downstream target Foxo1 are diminished due to adenosine receptor signaling. ***A)*** *Western Blot images of protein levels* ***B)*** *Analyzed relative levels of AKT and Foxo1 proteins. ADA pretreated lymphocytes isolated from lymph node are stimulated with α-CD3ε (1ug/ml) and α-CD28(1ug/ml) in the presence or absence of NECA (1μM). Western Blotting was performed to detect the pFoxo1, Foxo1, pAKT, Akt levels. As a control, β-actin was used.*

4.7 Foxo1 inhibition reverses decrease in T cell accumulation by adenosine receptor signaling in Th1 and Th17 conditions.

Adenosine receptor stimulation decreased the inhibitory Foxo1 phosphorylation after T cell stimulation suggesting that adenosine receptor signaling sustains Foxo1 activation downstream of Akt pathway. By using Foxo1 inhibitor, AS1842856, we tested if reversing sustained Foxo1 activation will also reverse some of the effects of adenosine receptor signaling on T cell activation. We confirmed specific activity of Foxo1 inhibitor, AS1842856, by testing CD127 expression of naïve T cells (Appendix Figure 7). Foxo1 inhibitor decreased naïve T cell expression of CD127, which is similar to what is observed elsewhere in T cell specific Foxo1 deletion models [128, 135]. These studies also indicated the transcriptional activity of Foxo1 in CD127 promoter in naïve T cells. As can be seen in Figure 4.7, foxo1 inhibition reversed decreased accumulation of Th1 and Th17 cells by NECA. We also observed a slight but significant increase in Th2 cells by NECA, which is also reversed by Foxo1 inhibition. Interestingly, Foxo1 inhibition did not influence the effect of NECA on T cell accumulation in Th9 and Treg polarizing conditions.

A.

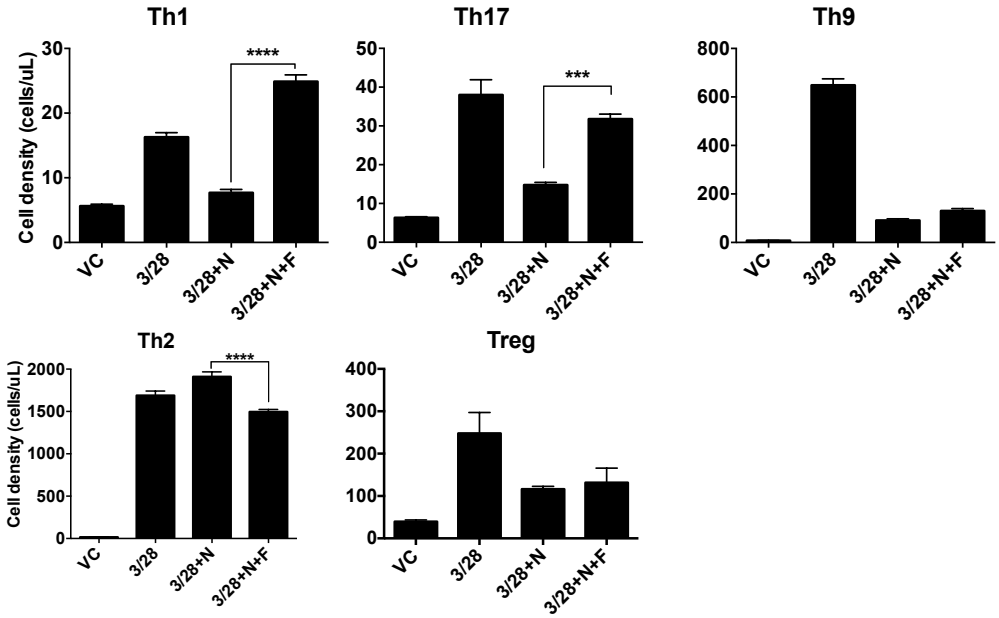


Figure 4.7 Diminished T cell accumulation caused by NECA can be reversed by Foxo1 Inhibitor *ADA pretreated CD4+ T cells were cultured in differentiating with NECA (1 μM) in the presence or absence of Foxo1 inhibitor, AS1842856 (50 nM). For Th2 polarization, Balb/c mice was used and for other polarizations, C57BL/6 mice was used. Flow staining was performed to show the absolute CD4+ T cell numbers. For statistical analysis, one-way ANOVA was performed. (Experiment was performed as quadruplicate and validated with four independent experiments, Tukey's multiple comparison test was used. ***p≤0.001, ****p≤0.0001)*

4.8 Foxo1 does not change anti-proliferative effect of adenosine receptor signaling in T cells

To see the effect of Foxo1 inhibition on T cell proliferation in the presence or absence of NECA, cells were labeled with CFSE dye at the beginning of polarizing conditions. Even though the inactivation of Foxo proteins are associated with the induction of T-cell proliferation, [128] we did not observe any change in proportion of T cells proliferating after Foxo1 inhibition in the presence of NECA, suggesting that sustained Foxo1 activation is not responsible from anti-proliferative effect of adenosine receptor signaling as can be seen in Figure 4.8.

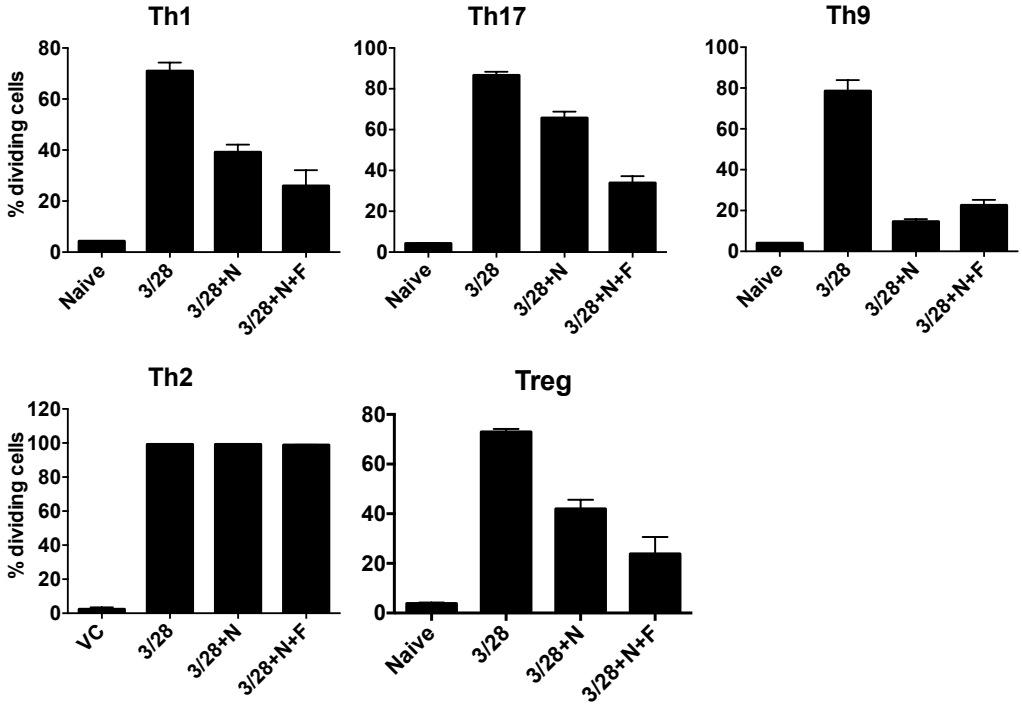


Figure 4.8 AS1842856 is failed to reverse the anti-proliferative effect caused by NECA in CD4+ Th subsets. *ADA pretreated CD4+ T cells were cultured in differentiating with NECA (1 μM) in the presence or absence of Foxo1 inhibitor, AS1842856 (50 nM). For Th2 polarization, Balb/c mice was used and for other polarizations, C57BL/6 mice was used. CFSE labelling is performed to monitoring the proliferation at the level of 5 μM. Proliferation screening was performed by flow cytometry. Experiment was performed as quadruplicate and validated with four independent experiments.*

4.9 Foxo1 inhibition promotes survival of Th1 and Th17 cells in the presence of adenosine receptor signaling

There are previous reports indicating that T cell survival plays a more important role in T cell accumulation after activation especially in conditions immunostimulatory adjuvants are used [149-151]. Therefore, we tested if Foxo1 inhibition will promote T cell survival. As can be seen in Figure 4.9. AS1842856 increased the proportion of live cells when added with NECA treated group as compared to NECA only group in Th1, Th17 and in Th9 conditions. Although we observed increased survival in Th9 polarizing conditions by Foxo1 in inhibition this effect was not sufficient to restore decreased T cell accumulation, which may be due to the fact that in Th9 condition the effect of NECA on T cell proliferation was more obvious than its effect on T cell survival.

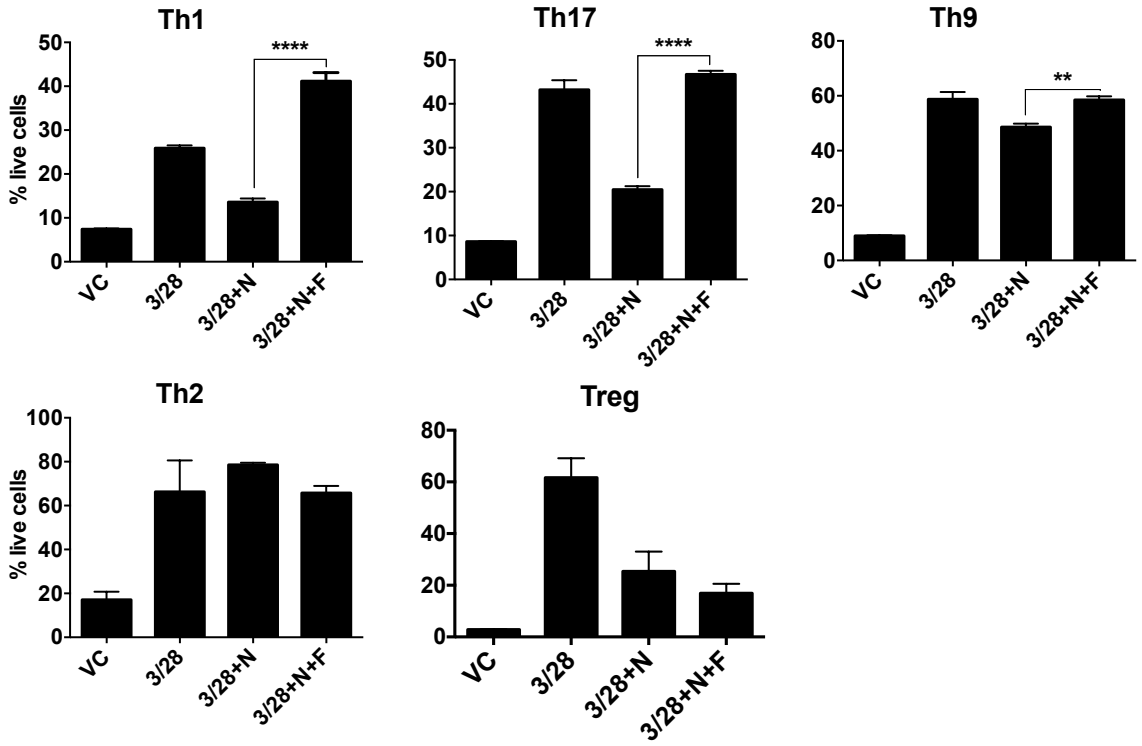


Figure 4.9 Decreased survival in Th1 and Th17 by NECA is reversed in the presence of AS1842856

*ADA pretreated CD4+ T cells were cultured in differentiating conditions with NECA (1 μM) in the presence or absence of Foxo1 inhibitor, AS1842856 (50 nM). For Th2 polarization, Balb/c mice was used and for other polarizations, C57BL/6 mice was used. Viability screening was performed by flow cytometry. For statistical analysis, one-way ANOVA was performed. (Experiment was performed as quadruplicate and validated with four independent experiments, Tukey's multiple comparison test was used. **p≤0.01, ****p≤0.0001)*

Overall these results suggest that sustained activation of Foxo1 signaling prevents T cell accumulation in Th1 and Th17 conditions by decreasing T cell survival.

Chapter 5

Discussion

Immune system is essential for the protection of the body from any harmful organisms and molecules. However, it must be tightly regulated for self-protection from excessive immunogenic response. Many physiological networks take part in this process. Purinergic signaling, as one of major mechanisms for regulating the immune response, is initiated in the presence of extracellular ATP, secreted either from dying/injured cells or from activated immune cells. This chemical, besides its fundamental function in energy supplying, is also included in various physiological processes. In immune system, it acts as chemotactic for neutrophils, and its release to immune synapse causes the initiation of purinergic signaling by conversion into AMP and adenosine by CD39 and CD73 respectively. Numerous studies have performed in adenosine signaling to monitoring the precise mechanisms in immune system regulation. Adenosine receptor signaling in T cells causes cAMP accumulation and inhibition of immediate downstream signaling events such as Akt phosphorylation. In this study, we studied the effects of adenosine receptor signaling on T helper cell polarization into different functional subsets because these cells are important promoters of both humeral and cellular adaptive immune responses and their functional polarization is indicated to be important in many disease conditions [152-155].

The importance of adenosine signaling, has been demonstrated in the development, maintenance and survival of naïve T cells [109]. In addition to that, there are studies pointed out the importance of adenosine signaling in inhibiting cytokine production in CD4+ T cells and CD8+ T cells in A_{2A} dependent manner [156, 157]. According to various studies, in the presence of adenosine, activation of Th cell subsets, Th1, Th2, Th17 are inhibited due to their reduced cytokine secretion [142, 158]. Moreover, adenosine dependent suppression of Th1 and Th2 cells are occurred within both early development and late effector stages. [92] However, most of the studies were performed with CGS 21680, an A_{2A} specific agonist while we used NECA, a general adenosine analog has affinity against all adenosine receptor subtypes. Controversial results in regulating the immune response are shown depending on the cell types and ligands during adenosine signaling as well as the time of the cells are exposed to the adenosine analogs. For example, upregulating effect of adenosine on IL-4 and IL-13 secretion from mast cells is shown in A_{2A} dependent manner by NECA treatment [159]. In another study, CGS-mediated A_{2A} agonism had nominal effect on IL-4 secretion from Th2/Tc2 population, while effecting IL-10 production instead [156]. In addition to that, polarization conditions might be effective in this controversial result as well. For instance, IL-6, together with TGF-β are key factors in Th17 polarization process. A_{2A} stimulation might be effective in Th17 polarization by targeting IL-6 *in vivo*, however, A_{2A} signaling by CGS21680 did not alter the Th17 polarization *in vitro* when IL-6 was added to culturing conditions, similar to our results [106]. Some of the studies examining adenosine effects on T cell also employed whole splenocytes rather than isolated T cells. In these studies it was not clear whether the seen effects are through APCs or directly on T cells. One caveat of testing Th polarization is also using ELISA assays without taking cell number into account. In this study we used highly purified T cells, and tested polarization of all the major Th subsets in the presence of adenosine receptor signaling, including for the first time Th9 subset, which is important in allergic reactions and anti-tumor immunity. Our results indicated that adenosine selectively inhibits accumulation and Th1 polarization of Th cells while having almost no effect on Th2 polarization. In other polarizing conditions adenosine did not change their polarization in to Th9, Th17 or Treg phenotype; however, adenosine strongly inhibited T cell accumulation.

Our results suggested that, diminished T cell accumulation in the presence of adenosine signaling, is correlated with decreased proliferation and survival. For further confirmation of anti-survival effect of adenosine, we stained T cell for monitoring BCL-2 expression, an anti-apoptotic protein that involves in the regulation of cell survival. Indeed, adenosine profoundly suppresses the BCL-2. Moreover, the reduced survival effect of adenosine has pointed out in another study, suggesting that extracellular adenosine is effective in T cell death via inducing internucleosomal DNA cleavage [160]. On the other hand, decreased proliferation of T cells, might be associated with IL-2 signaling. Adenosine pathway is shown to effective in reducing the IL-2 dependent proliferation via inhibition of STAT5 phosphorylation [161].

Although it was shown that adenosine signaling elevates cAMP levels to regulate T cell responses. After confirming previous studies showing that adenosine receptor signaling can activate downstream of cAMP signaling we tested which intracellular receptors of cAMP plays the major role for adenosine-inhibition of T cell responses by using specific analogs, Our studies indicated that both PKA and EPAC pathways downstram of cAMP play important roles in inhibiting Th1 polarization of T cells, while PKA alone was responsible for the anti-proliferative effect. These results are particularly important because in situations where agonists or antagonists of adenosine receptor signaling cannot compete with the natural ligands or outnumbered by adenosine receptors, therapeutic targeting of both of these signaling pathways rather than one can bring full benefit.

As indicated adenosine receptor signaling inhibits the immediate downstream signaling events after TCR stimulation. One of the major pathway regulating T cell activation, proliferation and survival is the PI3K/Akt signaling pathway. PI3K-AKT signaling, one of major factors that regulate the cell response followed by TCR stimulation, has control over Foxo1 nuclear localization to regulate its effects. Our previous studies have shown that adenosine A2A receptor stimulation suppresses activation of Akt pathway. In this study, we have shown that adenosine receptor stimulation alos reduced the phosphorylation of Foxo1 downstream of Akt pathway. Foxo1 is known to regulate both survival and proliferation. However, most studies dissecting the roles of Foxo1 activation in T cells employed genetic depletion of this transcription factor [128, 135]. In this study for the first time we exclusively performed our experiments by using a pharmacological approach

because in transgenic models, chronic absence of target gene may cause activation of feedback mechanisms. Which may obscure the results. Our results indicated that sustained Foxo1 activation by adenosine receptor stimulation strongly reduces the survival rather than proliferation, suggesting that other mediators of cell cycle downstream of Akt pathway play important roles driving T cell proliferation, which is suppressed by adenosine. Interestingly Foxo1 inhibition promoted T cell accumulation and survival after adenosine receptor stimulation particularly in Th1, Th9 and Th17 conditions suggesting that Foxo1 regulation by adenosine particularly effective in these conditions. This also suggests that some of adenosine's effect on immediate downstream TCR signaling is compensated by addition of cytokines unique to these polarization conditions.

PKA, EPAC and Foxo1 pathways can be regulated by other GPCRs or metabolic sensing mechanisms. For example, depending on the nutrient availability or growth factor signaling Akt/Foxo1 pathway can be affected. Our study is particularly important showing that in complex microenvironment where adenosine is present but Foxo1 activity is limited due to increased growth factor signaling or increased nutrient availability T cell accumulation may not change by adenosine receptor stimulation. Also for adenosine rich, T cell suppressive microenvironments, such as tumor microenvironments, targeted Foxo1 inhibition may reverse adenosine's detrimental effects on T cell accumulation. To better dissect if adenosine receptor signaling is causing apoptotic or necrotic cell death, we are in the process of performing annexin V / PI staining in different polarization conditions. Further studies either using pharmacological inhibitors or conditional, cell specific knock-out systems is needed to observe the true functional effects of these interactions *in vivo*.

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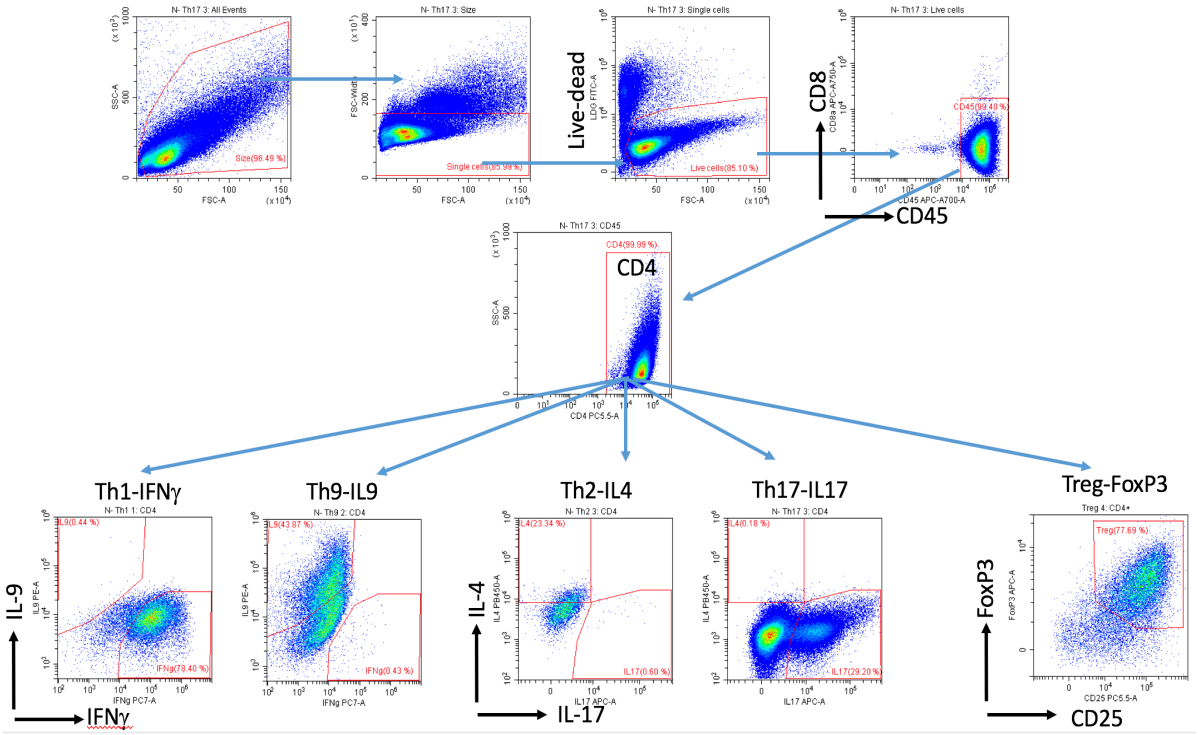
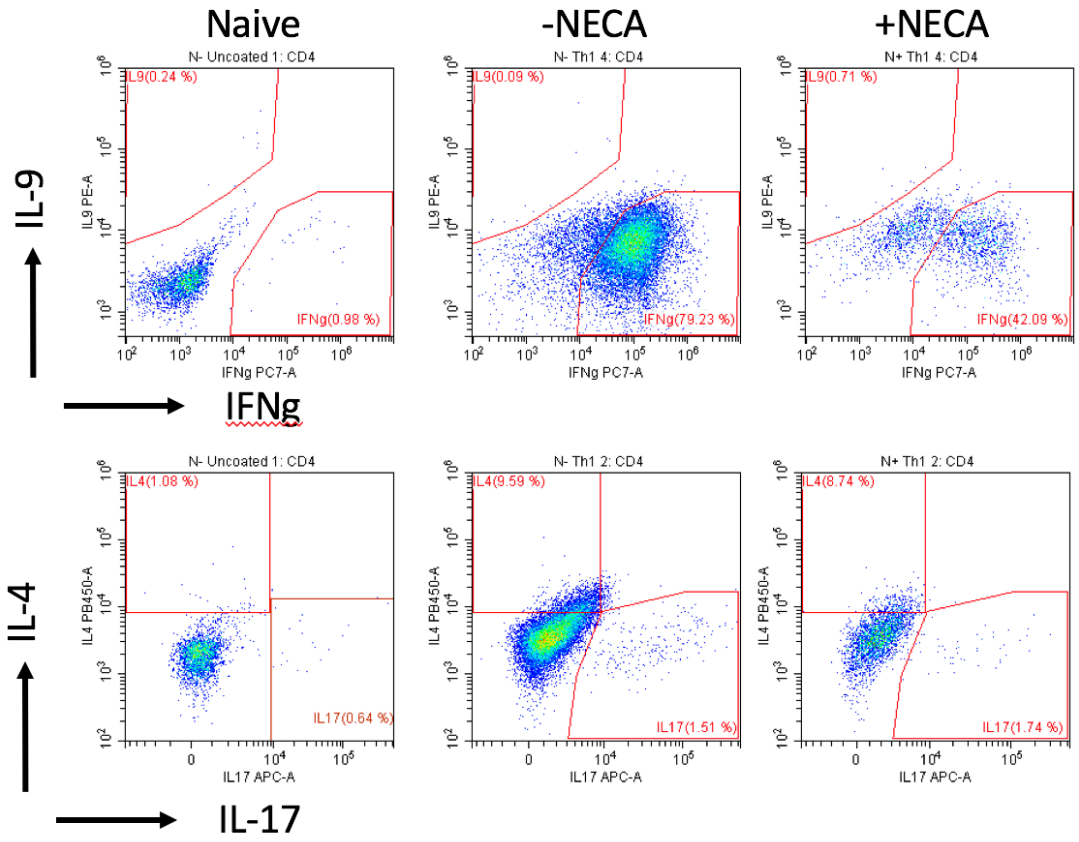
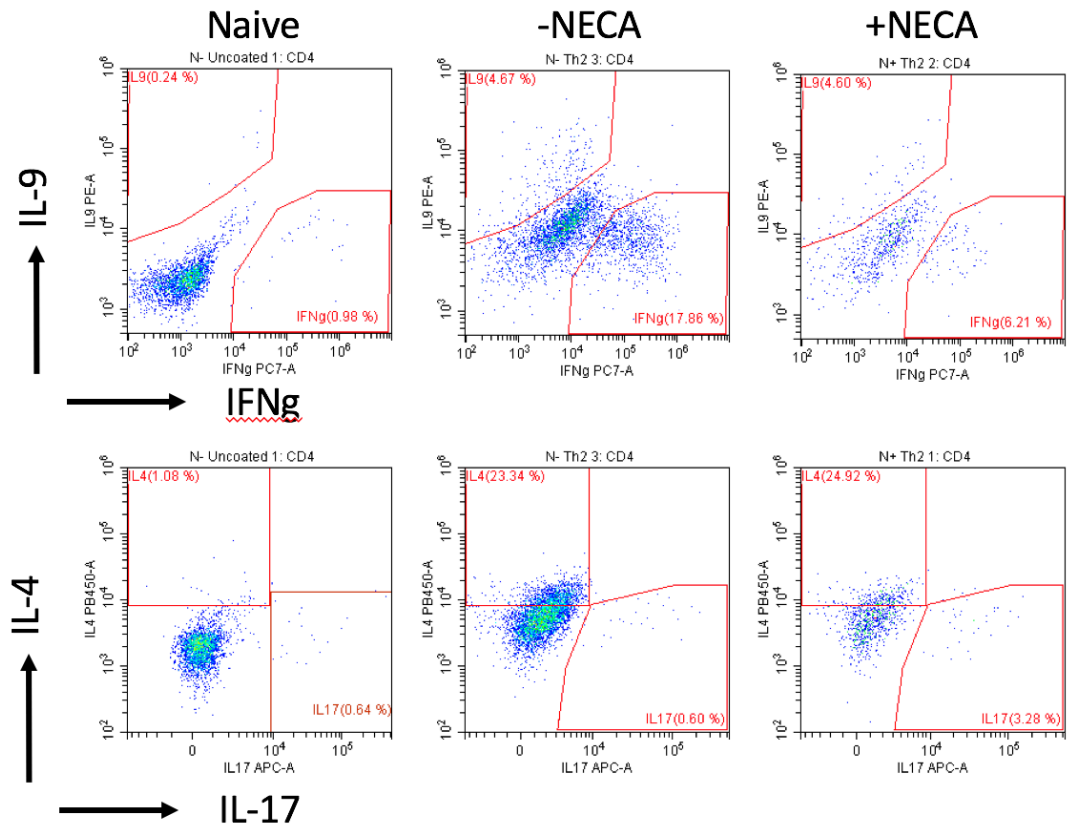
APPENDIX



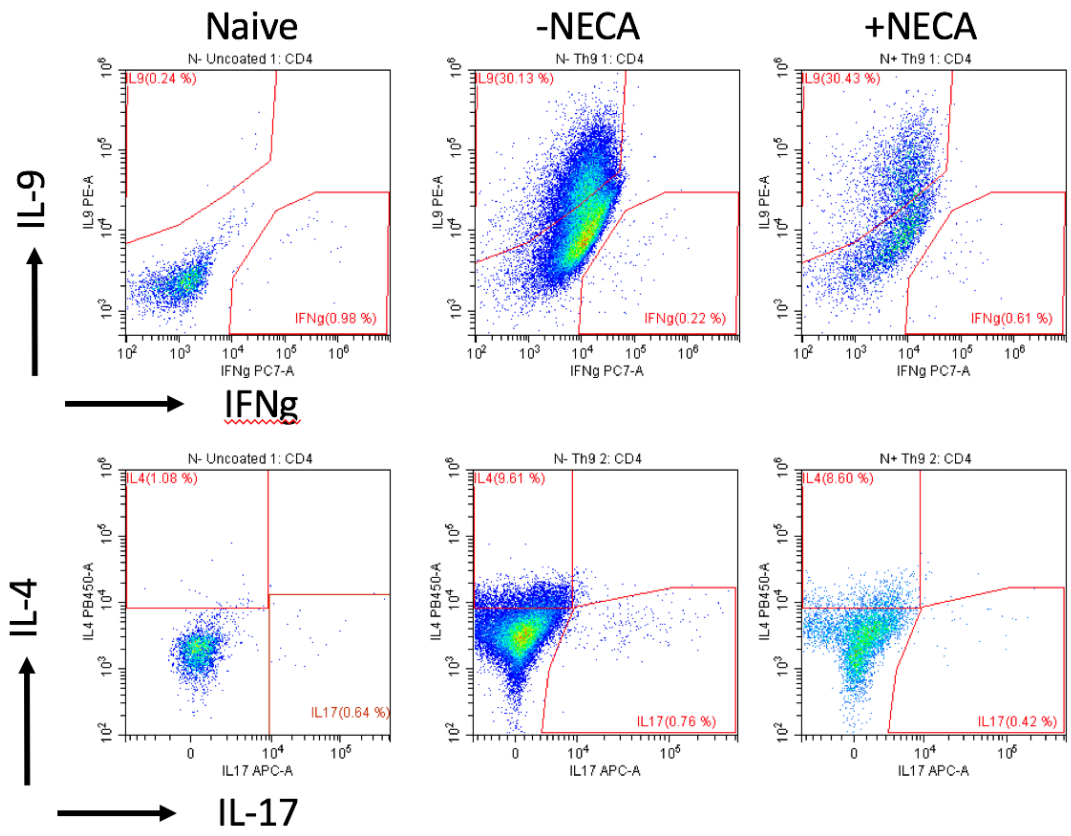
Appendix Figure 1: Main gating strategy to target CD4+ Th subsets in flow cytometry. *Firstly, desired population was selected based on their size via SSC/FSC graph. To eliminate the clusters, duplets, etc and take only single cell suspension, FSC-Width/FSC graph was used. Live/Dead staining graph in FITC channel allow us to select the live cells only. Among live cells, $CD8^{-}CD45^{+}$ cells were chosen for further conformation to monitor activated CD4+ T cells with high purify level. Indeed, SSC/CD4+ gating as a next step to select activated CD4+ cells only. In next step, gating strategies were chosen depending on the characteristic phenotypes of interested Th subsets.*



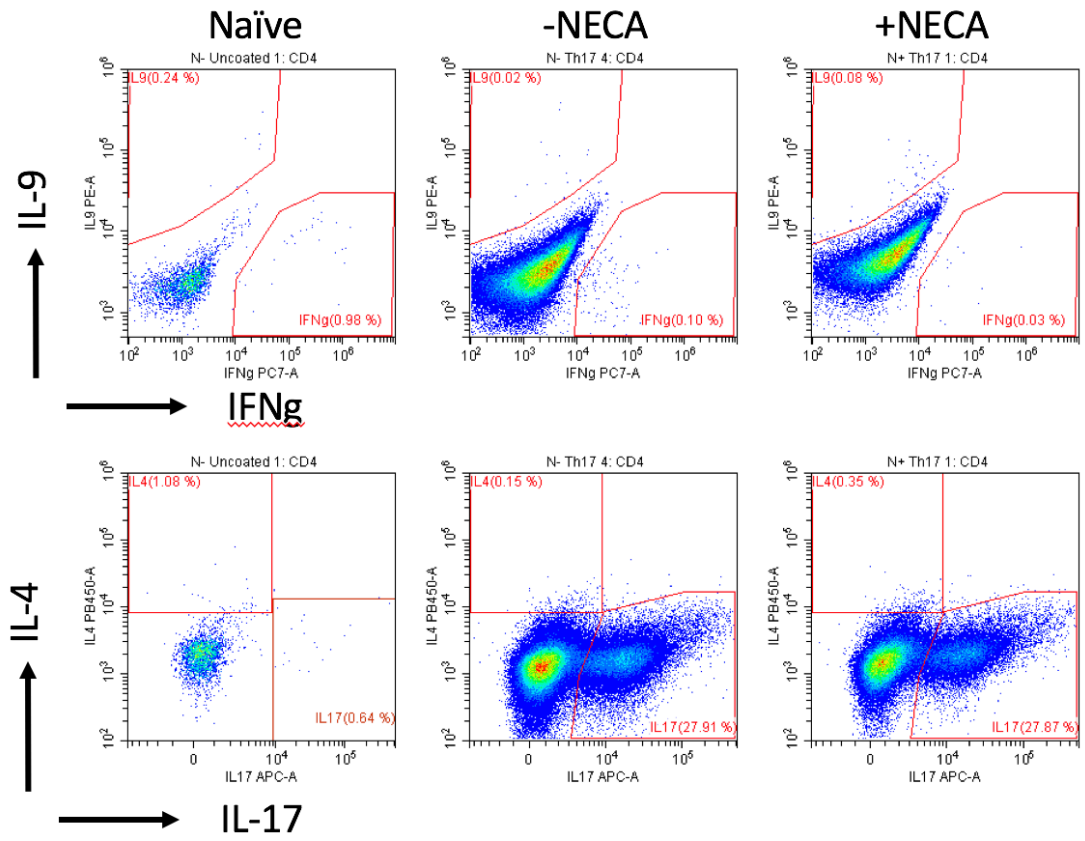
Appendix Figure 2: Gating Strategy for Th1 polarization in flow cytometry. After the selection of $CD8^{-}CD4^{+}CD45^{+}$ cells, cytokine production profile was detected based on the intracellular staining.



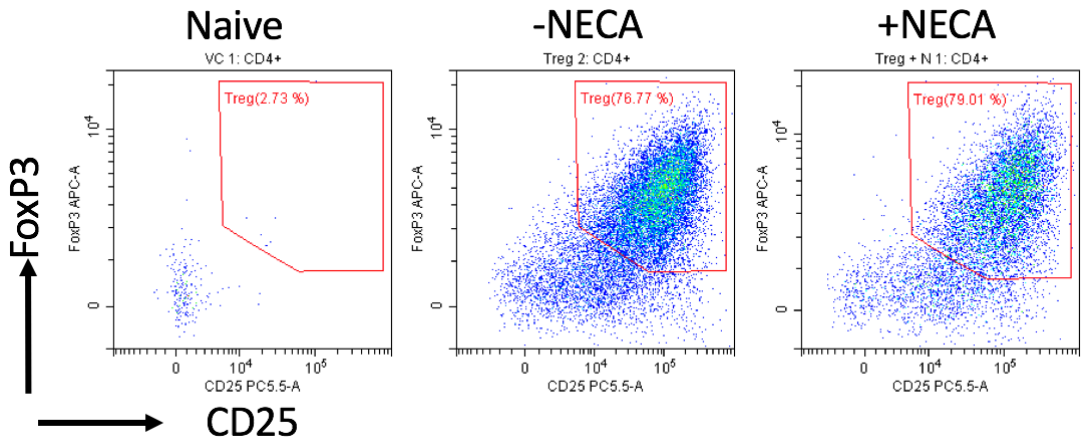
Appendix Figure 3: Gating Strategy for Th2 polarization in flow cytometry. After the selection of $CD8^{-}CD4^{+}CD45^{+}$ cells, cytokine production profile was detected based on the intracellular staining.



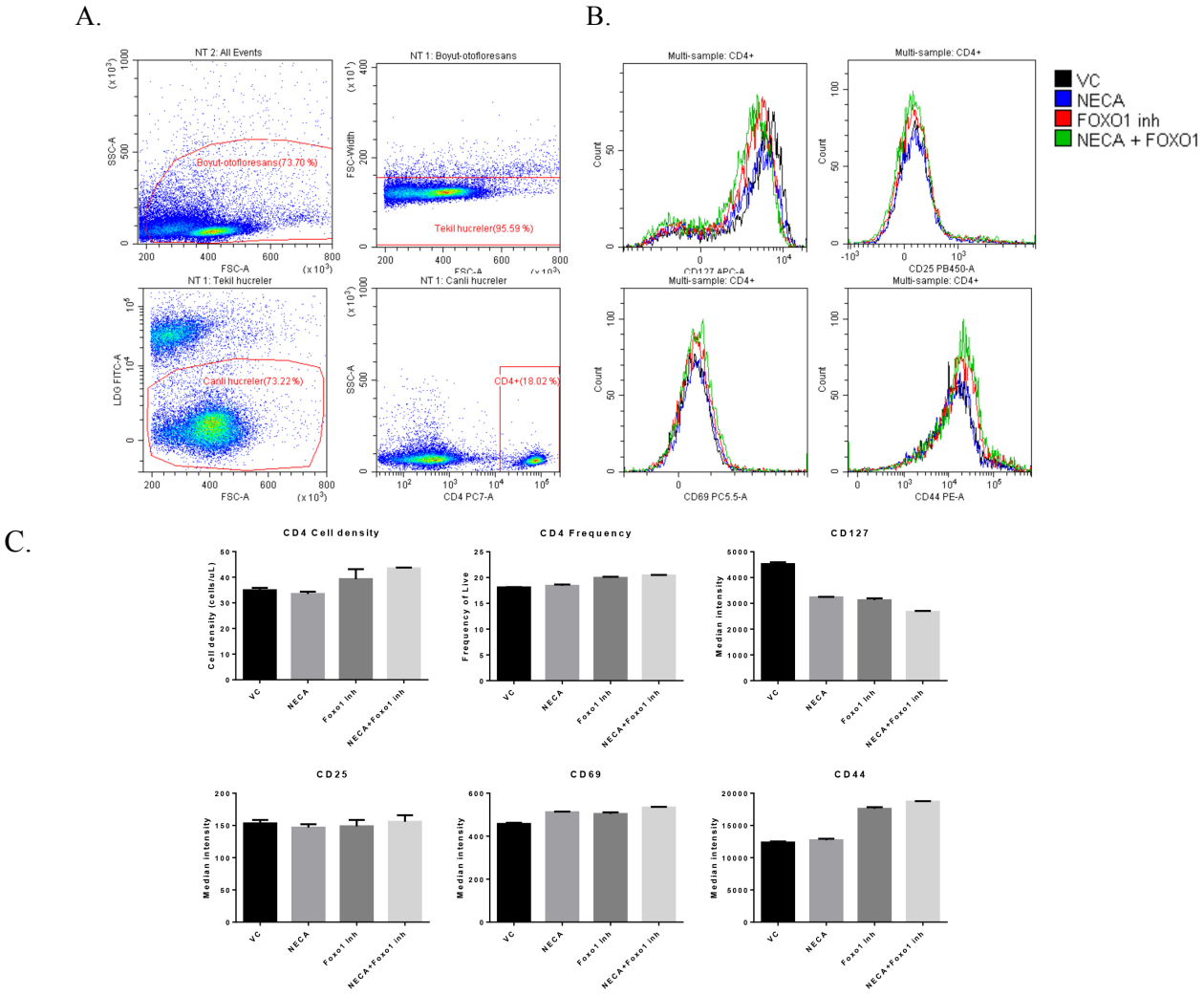
Appendix Figure 4: Gating Strategy for Th9 polarization in flow cytometry. After the selection of $CD8^{-}CD4^{+}CD45^{+}$ cells, cytokine production profile was detected based on the intracellular staining.



Appendix Figure 5: Gating Strategy for Th17 polarization in flow cytometry. After the selection of $CD8^{-}CD4^{+}CD45^{+}$ cells, cytokine production profile was detected based on the intracellular staining.



Appendix Figure 6: Gating Strategy for Treg polarization in flow cytometry. After the selection of $CD8^{-}CD4^{+}CD45^{+}$ cells, cytokine production profile was detected based on the intracellular staining.



Appendix Figure 7: The effects of adenosine analog and Foxo1 inhibitor on naïve T cell phenotype. ***A)*** *Gating strategy of T cells isolated from lymph nodes by flow cytometry,* ***B)*** *Expression of naïve T cell markers with histogram overlay,* ***C)*** *Bar graphs of those stimulated T cell number after treatment.*

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