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The Potential Mechanisms of Adenosine Regulating T cell Activation

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Potential Mechanisms of Adenosine Regulating T cell Activation" submitted by Ailian Yang in partial fulfillment of the requirements of the degree of Master of Science.

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ABSTRACT:

Adenosine has been regarded as a crucial anti-inflammatory agent that protects the host from excessive damage. However, it is a general observation that induction of T cell activation is an efficient event despite the high adenosine levels that are often present in the affected host due to injury or stress. The focus of this thesis was to determine the potential mechanisms of adenosine in regulating T cells and how T cells maintain activation in the high levels of adenosine during inflammation. This study revealed that prior to antigenic stimulation via TCR/CD3, exposure of T cells to adenosine desensitizes adenosine receptors, so as to create a window of several hours where T cells are "blind" to this ubiquitous suppressor. The results suggest that adenosine receptor desensitization may be an important mechanism for T cells to escape the general suppression during early points of T cell activation, and may present as a potential alternative of vaccine adjuvant.

ACKNOWLEDGEMENTS:

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DEDICATION:

To my father, a brave man who made all his efforts during his fight against cancer in his last days. He was diagnosed of cancer when I started my Graduate Program, and he left the world before I prepared my thesis. He always taught me to fulfill my dreams despite any difficulties. By facing his own challenge, he did set the best example for me. I would like to dedicate this thesis in memory of him.

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LIST OF ABBREVIATIONS:

Ab/mAb	Antibody/monoclonal antibody
Ado	Adenosine
ADA	Adenosine deaminase
A1	A1 Adenosine receptor
A1 KO	A1 adenosine receptor Knockout mice
A2A	A2a Adenosine receptor
A2B	A2b Adenosine receptor
A3	A3 Adenosine receptor
Ag	Antigen
BMDC	Bone marrow dendritic cells
cAMP	Cyclic adenosine-3',5'-monophosphate
CCM	Cell culture medium
CNS	Central nervous system
CGS	2-[p-(2-carboxyethyl) phenylethylamino]-5'-N-ethylcarboxamidoadenosine
CFA	Complete Freund's adjuvant
CPA	N^6-(cyclopentyl)-adenosine
DC	Dendritic Cell
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EHNA	Erythro-9-(2-hydroxy-3-nonyl) adenine
GPCR	G protein-coupled receptors

Hrs	Hours
5-HT	Hydroxytryptomine
IB-MECA	1-Deoxy-1-[6-[[(3-iodophenyl) methyl] amino]-9H-purin-9-y]-N-methyl-b-D-ribofuranuronamide
MECCPA	2-Chloro-N-cyclopentyl-2'-methyladenosine
NECA	5-(N-Ethylcarboxamido)
NTS	Nucleus Tractus Solitaris
OT-1	C57BL/6-Tg1100mjb/J Transgenic mice
OT-2	C57BL/6-Tg425Cbn/J Transgenic mice
OVA	Chicken Ovalbumin
P1R	P1 adenosine receptor
SIINFEKL	Chicken ovalbumin 257-264 residue peptide
Tc1	Type 1 cytotoxic T lymphocyte
Tc2	Type 2 cytotoxic T lymphocyte

CHAPTER I

INTRODUCTION AND LITERATURE

1.1 Introduction:

1.1.1 Adenosine physiology

Adenosine is a nucleoside that is composed of a single molecule of adenine attached to a ribose sugar molecule. It binds specific cell surface structures, termed adenosine receptors[1] on the surrounding cells. It is present in all body fluids and is generally maintained at levels below 1μM [2]. However, in response to cellular damage (e.g. inflammatory or ischemic tissue), adenosine concentrations can reach 10 μM or higher[3]. The concentration of free adenosine also increases during metabolic stress, such as muscle and nerve activities, or under pathological conditions such as hypoxia and ischemia.

The physiological effects of adenosine were first described in the cardiovascular system by Drury and Szen-Gyorgy in 1929[4]. They demonstrated that adenosine is both a negatively inotropic agent and a coronary vasodilator. Since then, adenosine has been known to play a major role in the central nervous system[5, 6], endogenous pain modulation [7], gastrointestinal tract [8] and the immune system[9].

Adenosine is an important nucleoside in biochemical processes, such as energy transfer – as it is present in adenosine triphosphate (ATP) and adenosine diphosphate (ADP). Adenosine participates in intracellular signal transduction in the form of cyclic adenosine monophosphate--cAMP. It is also an inhibitory neurotransmitter believed to play a role in promoting sleep and suppressing arousal[10]. Adenosine has tissue protection effects, and does so by two mechanisms. First, adenosine decreases the energy demand of the tissue by a direct inhibitory effect on parenchymal cell functions,

exemplified by the negative inotropic effect of adenosine on the heart muscle. Secondly, adenosine indirectly protects the tissue by providing a more favorable environment for parenchymal cells; the best example is adenosine—mediated augmentation of nutrient availability through vasodilation.

Adenosine is regarded as a regulator of innate immunity and it suppresses cellular activation by delivering negative signals to immune cells [9, 12]. While inflammation is a necessary part of host defense, by itself it is a measurable health threat that can lead to excessive tissue damage in the host. It is therefore an important question as to how the host controls this excessive tissue damage. Overall, the increased availability of adenosine in diseased tissues protects host cells from becoming over reactive. It has long been reasoned that some immune cells are also kept under control by the same mechanism in local and systemic pathologies in order to avoid undesirable autoimmune destruction [13].

1.1.2 Adenosine Metabolism

Immunologically relevant extracellular adenosine is produced from adenosine triphosphate (ATP). ATP is a multifunctional nucleotide that is important in intracellular energy transfer. It is produced as an energy source during the processes of photosynthesis, biosynthetic reaction and cell division [14]. It is present in extracellular fluids as a consequence of several mechanisms, including cell lysis and selective secretion through the plasma membrane [15]. In the immune system, extracellular ATP functions as a "natural adjuvant" that exhibits multiple proinflammatory effects. The

intracellular ATP is maintained at higher concentrations, and significant amounts of the nucleotide can be released upon cell damage. It is released by damaged cells as an indicator of trauma and cell death and can be inactivated by CD39 (nucleoside triphosphate diphosphohydrolase-1[NTPDase 1]), an ectoenzyme that degrades ATP to AMP [16]. (Figure 1.1)

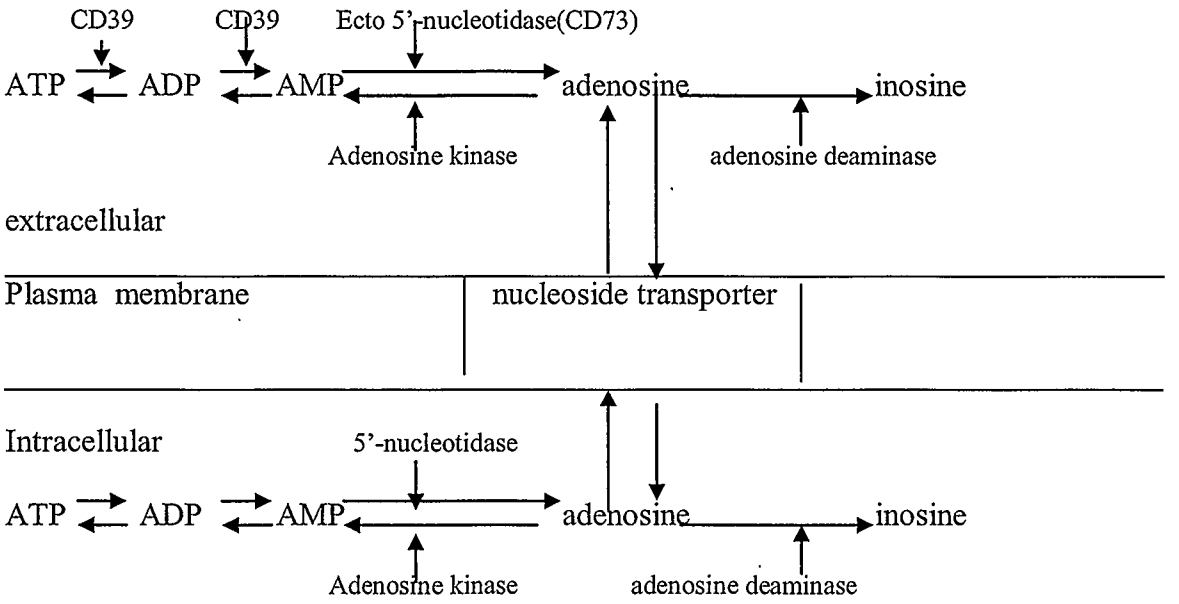


Figure 1.1: Schematic summary of the regulation of extracellular and intracellular adenosine

The pool of extracellular nucleoside (NTPs) is controlled by ectonucleoside triphosphate dephosphohydrolases (E-NTPDases)[17]. Members of this enzyme family are expressed on the surface of various cells and remove NTPs by degrading them to NMPs. Of this family, CD39 is the dominant ectoenzyme in the immune system[18]. It hydrolyzes ATP or ADP to AMP and is expressed by B cells, DCs, and subsets of T cells.

Besides removing a proinflammatory stimulus, it may also act in concert with CD73, another ectonucleotidase present on the surface of lymphocytes, to produce adenosine[19].

Intracellular adenosine is produced by the metabolic breakdown from adenosine 5' phosphates (AMP, ADP, ATP) [20]. This pool of adenosine can be transferred across the membrane into extracellular space. In normal conditions, intracellular ATP maintains a balance with adenosine by relevant nucleotidase and kinase activities [5]. In stress, the equilibrium is tilted towards adenosine, leading to its elevated secretion. The release of nucleic acids during cell death may potentially be a third source of extracellular adenosine [21]. Metabolism of adenosine by phosphorylation or degradation to inosine is catalyzed by adenosine kinase and adenosine deaminase respectively [3].

It is not entirely clear which cell types are the most important producers of extracellular adenosine, however, endothelial cells and neutrophils have both consistently been reported to release high levels of adenosine at sites of metabolic distress, inflammation and infection[9]. In addition, Sperlagh et al. demonstrated that nerve terminals are a major source of extracellular adenosine in the spleen in ischemia [22].

Adenosine signaling involving cAMP is a ubiquitous cellular second messenger. It is a critical component of a signal transduction pathway linking membrane receptors and their ligands to the activation of internal cellular enzymatic activities and gene expression. cAMP is synthesized from ATP by membrane bound adenylate cyclase. cAMP activates or inhibits various enzymes by promoting their phosphorylation or dephosphorylation. The cAMP signal is neutralized by hydrolysis of cAMP to AMP by

phodiesterases. Therefore, the concentration of cAMP in a cell is a function of the ratio between the synthesis from ATP by adenylate cyclase and its catabolism to AMP by specific phosphodiesterases[23].

1.1.3 Adenosine receptor

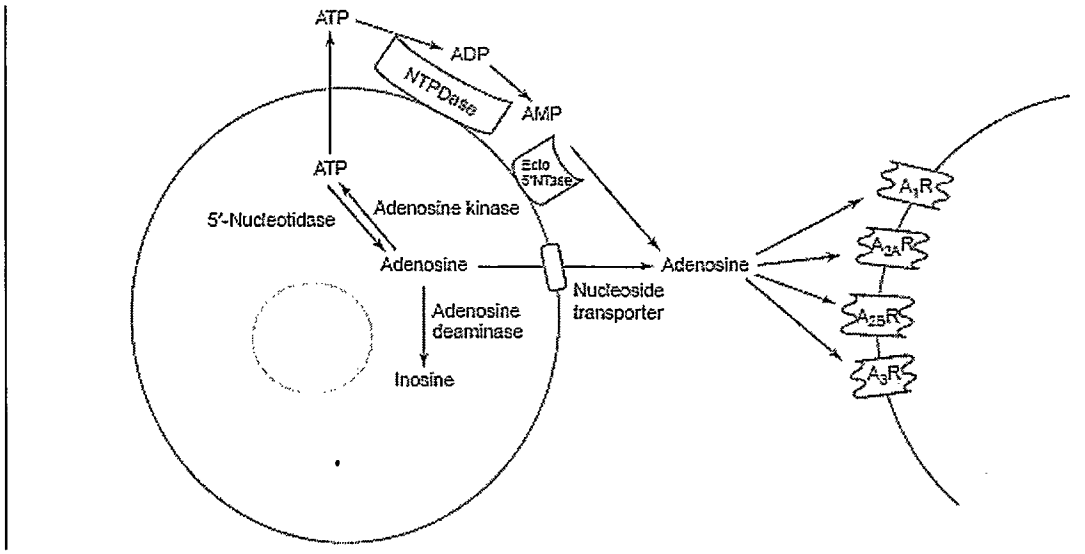


Figure1.2: adenosine metabolism and adenosine receptor (P1R)(Hasko G, 2004[11])

Purinergic receptors are a family of plasma membrane molecules involved in many cellular functions such as vascular reactivity and cytokine secretion. P1 receptors (P1Rs) recognize adenosine nucleoside, while P2 receptors likely see various forms of adenine nucleotides. In the interest of space, we will focus on adenosine/P1 receptors for this discussion. Adenosine receptors all belong to P1R and signal through the inhibition or stimulation of adenylyl cyclase that controls intracellular cAMP concentrations [24].

There are four membrane spanning adenosine receptors: A1, A2A, A2B and A3 [25, 26], all coupled to G proteins (GPCR).

The original classification of adenosine receptor subtypes in A1 and A2 receptors was based on the opposite effects of agonists on the levels of cAMP in diverse tissues: inhibitory and stimulatory effects for A1 and A2 receptors respectively[33]. A1 receptor is highly expressed in the brain (cortex, hippocampus and cerebellum) and A2 is highly expressed in the spleen, thymus, leukocytes and blood platelets [34]. The different families of G proteins are able to stimulate or inhibit different effector systems [5, 8]. The first G proteins that were described in the classical experiments by Gilman mediated the stimulation of cAMP production, and were thus called stimulatory G proteins or Gs. All of the Gs proteins can be activated by cholera toxin (CTX)[31] [32]. Subsequently, other G proteins were discovered that decrease cAMP levels by inhibiting adenylyl cyclase: the inhibitory Gi class of G proteins. All of the Gi proteins can be stimulated by the bacterial pertussis toxin (PTX).

	Affinity	Distribution (high expression)	Coupled G-protein	Function
A1	high	CN, brain, cardiovascular	Gi	cAMP↓
A2A	high	Spleen, leukocytes, CN	Gs	cAMP↑
A2B	low	colon, bladder	Gs	cAMP↑
A3	low	mast cells	Gi	cAMP↓

Table 1.1: Adenosine receptors: modified from Fredholm et al, Adenosine Receptor Review, 2000[45]

In adenosine mediated anti-inflammatory activities, the contributions of the specific adenosine receptor subtypes in various cells, tissues and organs are complex [27-30](Table 1.1). Moreover, adenosine receptor expression is regulated by numerous factors (Table 1.2).

Regulation Factors	Adenosine Receptor Expression	Cell types
Cytokines IL-1 β, TNF-α	→ increases A2A expression	THP-1 cells
Injury	→ induces A2B expression	Cells from Heart muscle
Or other stimulation	→ induces A2A expression	T cells
Oxygen	→ induces A1 expression	DDT-MF2

Table 1. 2 Factors regulating adenosine receptor expression

Khoa and Cronstein demonstrated that the inflammatory cytokines IL-1β and TNF-α increase the function and expression of adenosine receptors, especially A2A receptor, in what seems to be a feedback regulation of inflammation [35, 36]. However, cytokine-induced changes in A2A receptor numbers are probably insufficient to account for the increase in the receptor's activities, Khoa et al. reported that pretreatment of resting cells with CGS (A2A agonist) or NECA (pan-adenosine agonist) desensitized cAMP responses to CGS restimulation, but TNF-α treatment prevented A2A desensitization[37].

Signaling through adenosine receptors has multiple and varied effects on virtually all cells of the immune system, including neutrophils, monocytes, macrophages, T

lymphocytes and mast cells[38-40]. Agents or physiological conditions induce inflammatory responses from these cells have been found to influence ARs expression as well. Exposure to LPS, IL-1 or TNF-α trigger an up-regulation of A2AR mRNA and protein in the human monocytic cell line THP-1[35, 41]. Ischemia-reperfusion injury down regulates A3R, and induces A2BR transcript in mouse heart [42]. The reactive oxygen generating agent up regulates A1R expression in the testes of rats and DDT MF2 smooth muscle cells [43, 44].

1.2 Adenosine in the immune system

Although inflammatory and immunological reactions protect the host from invasion by microorganisms and eliminate debris at sites of tissue injury, they can also be responsible for significant tissue damage. Adenosine, elevated at injured and inflamed sites, has a central role in the regulation of inflammatory responses and in limiting inflammatory tissue destruction[11]. Sub-threshold doses of an inflammatory stimulus that caused minimal tissue damage in wild type mice were sufficient to induce extensive tissue damage, more prolonged and higher levels of pro-inflammatory cytokines, and death of animals deficient in the A2A adenosine receptor [47]. Ohta and Sitkovsky showed that the loss of A2A adenosine receptor functions was linked to massive tissue damage in host responses to inflammation and septic shock [46]. Adenosine signaling is also critically important in protecting against multiple organ failure in situations of trauma and shock [47, 48]. While there have been reports claiming that adenosine

signaling through adenosine receptor is anti-inflammatory, a great body of evidence have shown that adenosine might have counter-effect on cells (Table 1.3)

Cell types	Adenosine regulation
Neutrophils	low levels of adenosine: stimulation (A1) High levels of adenosine: inhibition (A2A)
PDCs	Immature: stimulation (A1) Mature: inhibition (A2A)
Mast Cells	Some report support A2B negative regulation Other report support A2B positive regulation
T cell	A1 not expressed on T cells A2A suppress T cell activation ??? counter-effect???

Table 1.3 Adenosine "counter-effect" regulation effect

1.2.1 Adenosine regulating innate immunity

Neutrophils are the first inflammatory cells to be recruited to sites of injury and inflammation [9, 11]. These cells kill microorganisms, eliminate debris and release factors responsible for the recruitment of other inflammatory cells. Although these cells are crucial for preventing dissemination of infections, if unchecked they can also be responsible for significant tissue injury.

Neutrophils release AMP during transmigration through the endothelial monolayer. Endothelial dephosphorylation of AMP to adenosine by ecto-5' nucleodidase promotes endothelial barrier function [53, 54]. A previous study showed that adenosine, via A2A receptor, inhibited neutrophil adhesion to endothelial and other cells. Adenosine also inhibited bactericidal activity, apoptosis, expression and shedding of adhesion molecules, secretion of cytokines and growth factors[55]. Adenosine suppresses the production of the superoxide anion, an effect that occurrs through cell surface receptors[56]. A1 and A2A receptors modulate neutrophil Fc gamma receptor function in opposing ways, allowing a concentration-dependent, adenosine-regulated feedback loop. At low concentrations, A1 receptor shows dominant activating effects, whereas at higher concentrations, the same cells are inhibited by A2A receptor [57]. This counter-regulatory effect suggests that low adenosine enhances the inflammatory responses, whereas in high concentrations it acts as a feedback inhibitor of inflammatory neutrophil functions by damaged tissues or cells [58, 59].

Macrophages and dendritic cells (DCs) are specialized phagocytes that have an important role in the clearance of apoptotic host cells, as well as in innate defense against infection. Adenosine receptor ligation on monocytes and macrophages strongly suppresses the production of IL-12 in response to TLR4 activation [60, 61]. Recent studies using knockout mice for the A2A and A3 receptors have illustrated that both of these receptors contribute to the adenosine suppression of proinflammatory mediator production following TLR stimulation [47, 62]. However, another report suggested that preconditioning by an A1R agonist promotes the resolution of inflammation by inducing

the production of A2AR, which exhibited decreased TNF-α and IL-6 sera levels and reduced leukocytes recruitment[63].

Interestingly, adenosine can also exert activating effect on dendritic cells. For plasmacytoid dendritic cells (PDC), adenosine was found to be a potent stimulus for immature PDCs via an A1 receptor-mediated mechanism. Upon maturation, PDCs down-regulate the A1 receptor, with a concomitant upregulation of A2A receptor [64]. Although, at first glance, this stimulation of immature DCs might indicate a potentially injurious role, this is not the case. The early accumulation of DCs at sites of microbial invasion is crucial for the initiation of an immune response [65]. However, in fully mature DCs that are encountered at the site of an established inflammatory and immune response, adenosine strongly suppresses the TLR-mediated production of IL-12[66].

Mast cells have traditionally been associated with IgE-mediated immune responses against parasites, and are now generally recognized as essential component of innate immune responses against bacteria and inflammatory arthritis[67]. Sergey Ryzhov and coworkers demonstrated that stimulation of A2B receptors in a human mast cell line increases production of Th2 cytokines IL-4 and IL-13[68-70]. Contrary to this body of evidence, a report by Hua suggested that A2B receptor functions as a negative regulator of mast cell activation[71]. The proinflammatory effects of mast cell adenosine receptor stimulation, especially in situations associated with long lasting high adenosine concentrations, seem to overwhelm the anti-inflammatory potential. Recent studies have revealed that mice, which accumulate high levels of endogenous adenosine due to a deficiency of the adenosine catabolizing enzyme, adenosine deaminase, develop a

pulmonary phenotype with mast cell mediated inflammation resembling the symptoms of asthma [72-74].

1.2.2 Adenosine regulation of T cells

The recognition of Ag by the TCR complex initiates a cascade of signaling events ultimately resulting in T cell activation. However, TCR-mediated IL-2 production, CD25 and CD69 expression, Fas ligand up regulation and cell proliferation are modulated by the activation of cell surface adenosine receptors [75, 76]. The literature suggests that A2A, A2B or A3 might be involved in this regulation, however, A1 is not expressed on T cells.

A2AR has long been regarded as an important receptor on T cells. Lappas et al. demonstrated that the A2AR is rapidly induced in $CD4^+$ T cells upon TCR activation, and that activation of the A2AR on $CD4^+$ T cells inhibits IFN-γ production in murine $CD4^+$ T cells [77]. The rapid induction of the A2AR mRNA in T cells provides a mechanism for limiting T cell activation and secondary macrophage activation in inflamed tissues [77, 78]. Some studies suggested A2AR is expressed on various T cell subsets and may regulate cytokine production in activated T lymphocytes [78]; and P1R agonist – mediated inhibition of IL-2 and TNF-α secretion occurred via A2AR on T cells, with no involvement of A1, A2B or A3 receptors. A2AR agonist also greatly reduced Th1 and Th2 cell IL-2 secretion [24, 77, 79]. In other studies, Northern Blotting analysis indicated that A2AR mRNA can be detected, but A1 or A3 mRNA can not be detected on T cells [24, 80]. In a study of anti-CD3 activated killer T cells (AK-T), AK-T cells express mRNAs for A2A, A2B and A3, but not for A1 receptor [81].

Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation as well[82]. Adenosine analogs have proven to be protective in a number of inflammatory disease models, including ischemia-reperfusion, colitis, autoimmune pneumonitis and type I diabetes [83-85]. In a T cell transfer model of colitis, both effector and regulatory T cells from A2AR deficient mice demonstrated that A2AR plays a novel role in the control of T cell mediated colitis by suppressing proinflammatory cytokines[83].

However, another report suggested that the A2B adenosine receptor is responsible for the accumulation of cAMP, as well as the inhibition of TCR-triggered IL-2 production in T cells. Activation of the CD3 complex by antibodies led to a significant increase in both the percentage of T cells expressing the A2B receptor and the intensity of the labeling. This receptor is functional since IL-2 production in these cells is reduced by the non-selective adenosine agonist, NECA, but not by A2AR agonist, CGS [86]. A recent report on A2BR -/- mice suggested that this receptor functions as a negative regulator of mast cell activation[71].

Inhibition of killer T cell activation by adenosine has also been attributed to signaling through the A3R. Adenosine acts through A3 receptor to prevent AK-T cell induction. Tumor associated adenosine may act through the same mechanism to impair the development of tumor-reactive T cells in cancer patients [81] .

1.3 Mechanisms of T cells escaping from adenosine suppression

1.3.1 Adenosine deamination reducing adenosine availability

Adenosine is a suppressive agent that protects cells from overreaction in stress conditions, such as inflammation or trauma. However, in cases of tissue distress, the immune system is almost inevitably activated, in response to either exogenous microbial assault or internal cell injury [25, 26]. It would be disadvantageous if negative regulatory mechanisms, including purinergic signaling were allowed to shut down the immune system during infections and diseases. It is crucial that the host maintains immunological diligence in stressed conditions because of concomitant infections. As T cells activation is affected by numerous factors, the mechanisms for T cell escaping from adenosine suppression highly variable.

Adenosine Deaminase (ADA) is a purine catabolic enzyme that regulates adenosine levels. When adenosine is catabolized, ADA removes an amine from the adenine base. The resulting inosine has no effect on immune system [51]. This deamination is crucial for immunogenesis. In the early immune system development, the lack of ADA function due to a genetic defect (an autosomal recessive mutation) leads to approximately 20% of all human Severe Combined Immune Deficiency (SCID), highlighted by the media report of the "bubble boy". The condition is fatal within infancy from infections if not intervened [88]. The pathology is believed, although not proven, to be caused by the toxicity of adenosine accumulation in immune cells [89-91].

Conspicuously, following the critical stages of early immune system development, ADA continues to be expressed in many tissues, despite its obscurity in biomedical research. Its levels in lymphoid organs are very high [92-94]. Under normal circumstances, extracellular adenosine is absorbed into the cytosol and converted into

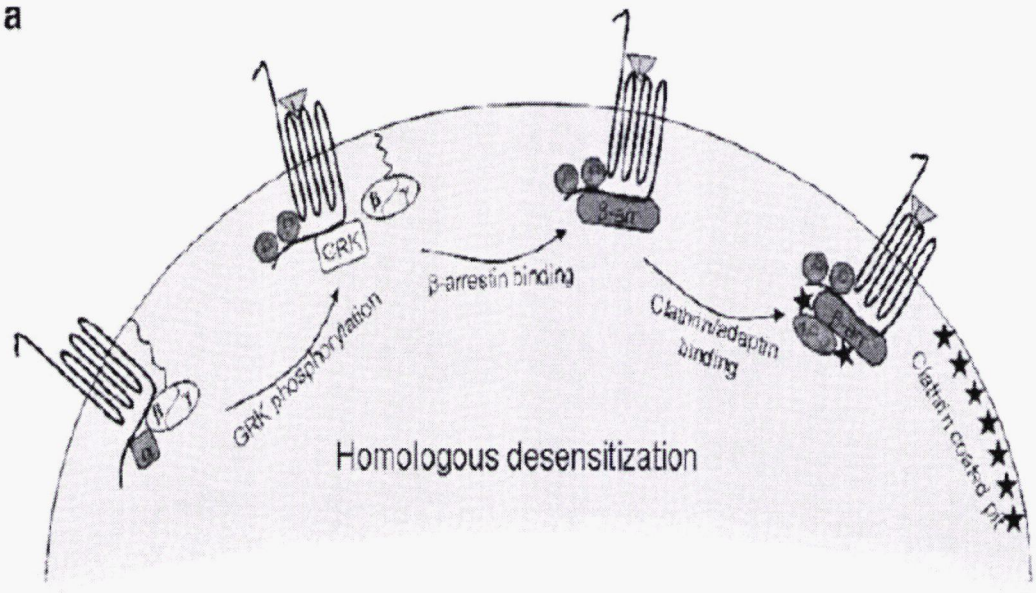
adenine nucleotides by adenosine kinases. In stressed conditions, extracellular ADA is responsible for the removal of increased amounts of adenosine [91, 95]. Linking this function to the biological effects of adenosine, it seems logical that the host uses ADA as a regulator of related cell activation. In reality, blocking extracellular ADA enhances adenosine signaling on T cells[76]. Sergey et al. demonstrated metabolic disturbances seen in ADA KO mice affected various signaling pathways that regulate thymocyte survival and function. Experiments with thymocytes in vivo confirmed that ADA deficiency reduces tyrosine phosphorylation of TCR associated signaling molecules and blocks TCR triggered calcium increase [96].

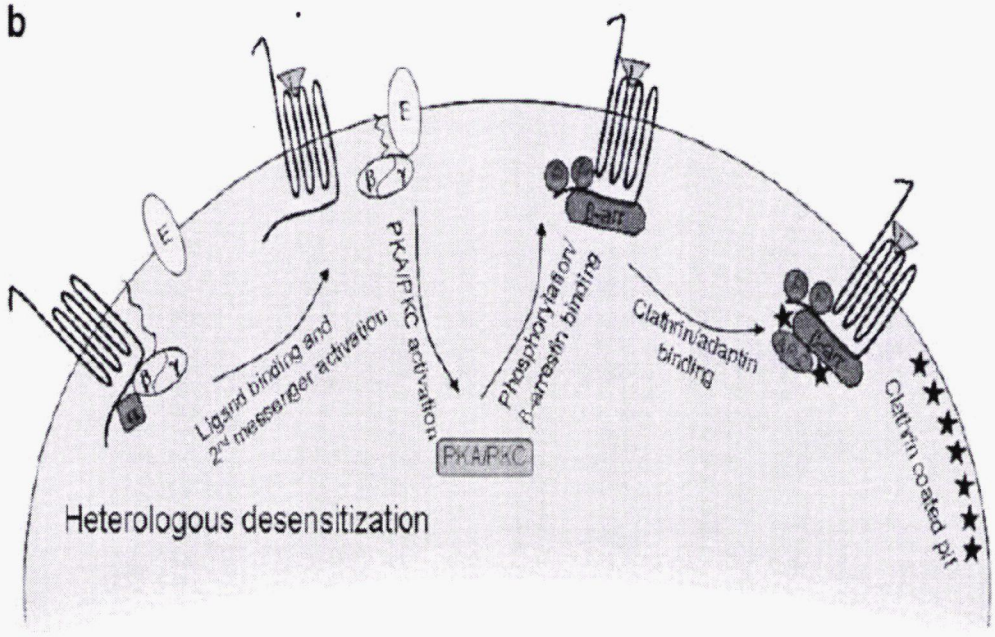
On some selected human cells, extracellular ADA is believed to be associated with CD26 [97-99], while engaging CD 26 delivers a co-stimulatory signal to T cells. In the CD26-ADA complex, the presence of ADA enzymatic activity has been suggested as ADA positive T-cells were insensitive to adenosine suppression. However, definitive evidence for this hypothesis remains scarce. Furthermore, unlike in humans, rodent CD26 is not known to be associated with ADA due to sequence variations [100]. The notion of CD26-ADA complex signaling would fail to explain the T cell activation. Previous research performed in our lab demonstrated that dendritic cells maintain their hyperreactive state in inflammation despite the general state of suppression [101]. However, it is still uncertain if ADA alone is sufficient to keep rodent T cells fully activated under high adenosine levels.

1.3.2 Adenosine receptor regulation

Since adenosine receptors are widespread throughout the body and involved in a variety of physiological processes and diseases, there is great interest in understanding how adenosine receptors are regulated. The major GPCR regulatory pathway involves phosphorylation of activated receptors by G protein coupled receptors kinases (GPK), a process that is followed by binding of arrestin proteins [102]. Upon agonist treatment, adenosine receptor subtypes are differently regulated [103-106].

Occupancy of a wide variety of hormone and neurotransmitter receptors by agonists leads to a loss of receptor-mediated responsiveness to a subsequent stimulation by the same agonist. This phenomenon is generally termed desensitization[107]. Desensitization reduces receptor activity and plays a role in signal duration, intensity and quality. Desensitization is initiated by phosphorylation of serine and threonine residues in the third intracellular loop and C terminus of the receptor. There are two types of desensitization, heterologous and homologous, and both are the result of receptor phosphorylation [108-110] (Figure 1.3a and 1.3b). Receptor desensitization, initiated by phosphorylation of the receptor by different protein kinases or GPKs, can be subsequently followed by receptor internalization (Figure 1.3c).


a
GRK
β-arrestin binding
GRK phosphorylation
Clathrin/adaptin binding
Clathrin coated pit
Homologous desensitization


b
PKA/PKC activation
Phosphorylation/ β-arrestin binding
Ligand binding and 2nd messenger activation
Clathrin/adaptin binding
Clathrin coated pit
PKA/PKC
Heterologous desensitization

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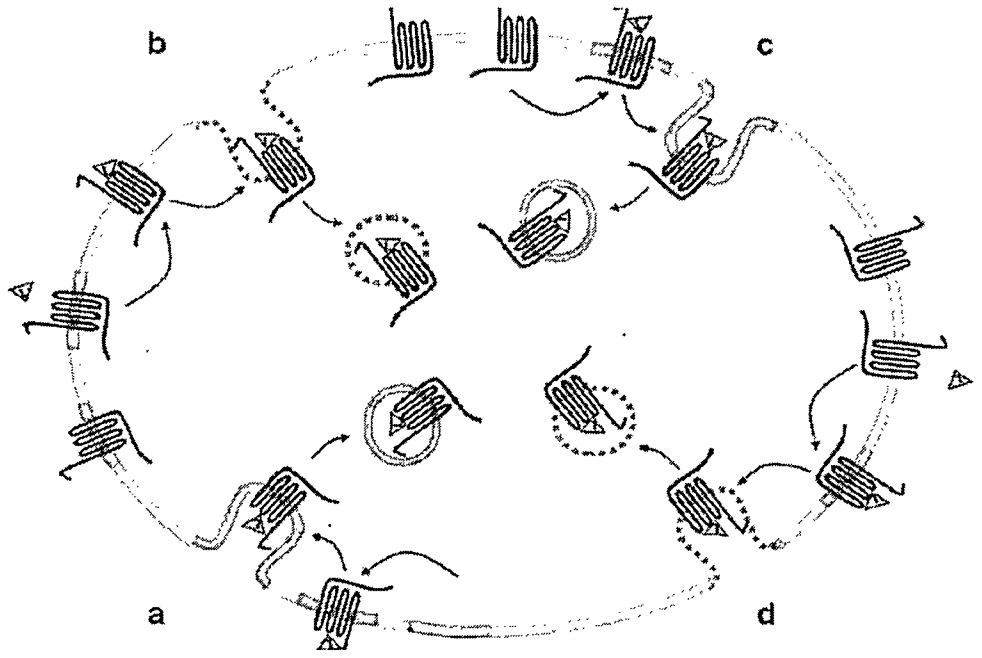


Figure 1.3: Adenosine receptor desensitization and internalization (Beukers etc 2008 [119])

For instance, adenosine, released under conditions of stress, confers cytoprotection in the cardiovascular and central nervous systems by activating cell surface adenosine receptors. This nucleoside provides rapid recovery of ventricular function induced after ischemia and reperfusion [111]. In the central nervous system, adenosine and adenosine analogs protect against transient ischemia. Rats treated with caffeine (A1 antagonist) were observed to have an increase A1R expression in the brain and were more resistant to ischemia[112]. In contrast, down regulation of the A1R by prolonged agonist treatment exacerbated the damage created by an ischemic episode[113].

The early evidence for adenosine A1 receptor desensitization was largely obtained from primary cells and cell lines that were exposed to varying concentrations of adenosine receptor agonists, often examined over several time periods. Stiles and co-workers studied adenosine receptor desensitization in DDT MF-2 cells (smooth muscle cell line)[114]. They pretreated DDT MF-2 cells with R-PIA (A1R selective agonist) for 24hrs, after which adenylyl cyclase activity was reduced by approximately 50%. This was associated with a significant decrease in cell membrane bound A1 receptor and a concomitant increase of receptor number in intracellular compartments. The author also showed an increase in receptor phosphorylation, paralleling the time course of adenylyl cyclase modulation. Interestingly, pretreatment of rat pheochromocytoma PC12 cells with A2AR agonist resulted in a rapid loss of adenylate cyclase activity. A2A desensitization resulted in no change in receptor number or affinity [115]. Later, Nie et al. reported similar findings in the same cell line, although desensitization occurred at a somewhat faster speed [116].

The in vitro studies showed that chronic exposure of neuronal or smooth muscle cell cultures to adenosine A1 receptors agonists result in a reduced density of cell membrane A1 receptor[117]. The in vivo studies showed that injections of CPA (A1 agonist) caused a significant decrease in adenosine A1 receptor numbers in the hippocampus and somatosensory cortex [118].

Hettinger-Smith et al. reported that chronic exposure to adenosine receptor agonists and antagonists reciprocally regulates adenosine A1 receptor in cerebellar granule cells. Exposure to an A1 adenosine receptor agonist resulted in a reduction in the density of

receptors and a functional uncoupling of receptors from adenylyl cyclase. Antagonist exposure increased the A1 receptor in cerebellar granule cell membranes and the coupling of receptors to adenylyl cyclase [120]. In cardiac tissues, chronic exposure to A1R agonist produced a decrease in the density of A1R and a blunted sensitivity to inhibition of adenylyl cyclase produced by A1R agonists[121].

Although exposure of cerebellar granule cells to the A1R agonist led to a reversal of the inhibition of adenylyl cyclase. In this case, no change in mRNA level was observed with longer treatment (12 hrs), suggesting that post-transcriptional regulation underlies this receptor desensitization[122]. Hettinger et al. examined the A1R mRNA levels and reported that pre-exposure to the adenosine antagonist for 48 hrs did not alter A1R mRNA or transcript stability [123]. Fernandez et al. reported that there were no significant changes in A2AR mRNA measurements in brains from rats pre-exposed to adenosine agonist[124].

The study on rat adipocytes showed that chronic exposure to the A1 adenosine receptor agonist desensitized the A1R-adenylyl cyclase system. The regulation in this system did not occur by altering the levels of mRNA transcripts [125]. Ruiz et al. reported that chronic exposure of rat to NECA caused a significant loss of adenylyl cyclase activity in rat brain synaptic plasma membranes, suggesting desensitization of the adenosine A2 receptors mediated pathway [126].

Prolonged exposure of rat pheochromocytoma PC12 cells to adenosine agonist led to a fast and significant inhibition of A2A stimulation by the A2A selective agonist CGS. This effect appeared to occur at the level of adenylyl cyclase, since no change was

observed in receptor numbers or in CGS affinity for the receptor[115]. CHO cells exposed to NECA showed a rapid desensitization of A2AR stimulated adenylyl cyclase activity with no obvious difference between pretreatment of 30 minutes or 24h. This was associated with a slightly reduced affinity of the receptor for the radiolabelled CGS. Cell surface receptor numbers only diminished significantly upon long term pretreatment[127].

Conti et al. investigated whether prolonged exposure of A2A to the non-selective agonist NECA or A2AR selective agonist CGS influenced A2A desensitization. The authors used the porcine coronary artery as a sensitive vascular model expressing A2ARs. The results indicated that prolonged stimulation of A2A receptors did not lead to loss of functional response, suggesting that this receptor subtype is not desensitized after prolonged stimulation by agonists [128].In contrast, antagonist treatment may lead to a sensitization and an increase in receptor number [120]. Long term treatment with the adenosine receptor antagonist and agonist evoke an up-regulation and a down-regulate A1R in cerebellar granale cells, respectively [129].

The study of adenosine receptor desensitization is mostly limited on CNS cells, smooth muscle cells and selected cell lines. However as discussed earlier, there is no definitive mechanism governing this event. Previous work from our laboratory showed that DCs with a pre-exposure to adenosine had substantially higher responses to subsequent activation by PAMPs [130], however, no research has been performed on T cells.

1.4 Adenosine and its therapeutic aspects for diseases

Adenosine plays an important role in diseases. Animal models of asthma, ischaemia, arthritis, sepsis, inflammatory bowel disease and wound healing have helped elucidate the regulatory roles of the various adenosine receptors in the development and progression of the diseases.

Several lines of clinical and preclinical evidence support the notion that adenosine and its receptors are intimately involved in defining the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD). Adenosine receptors on immune cells in the lung appear to have particularly important roles. Most compelling are the observations that inhaled adenosine can induce bronchoconstriction in patients suffering from asthma or COPD but not in healthy individuals, and adenosine receptor blockade can prevent this response [131]. A2A receptor activation reduces lipopolysaccharide-induced neutrophil recruitment and release of cytokines. Pretreatment but not post treatment, also reduces the increase in vascular permeability. Furthermore, A2A agonists appear to be effective at curbing inflammatory lung tissue damage [132].

During reperfusion following ischemic injury, many tissues have been shown to be protected from injury owing to the activation of A2A receptor on bone-marrow-derived cells. These include liver [133], kidney [134], heart [135], skin [136], spinal cord [137], and lung [138]. Those findings suggest that reperfusion injury is initiated by CD1d dependent activation of NKT cells, and the activation of these cells is inhibited by A2A receptor activation.

Despite the introduction of a number of effective biological agents for the treatment of rheumatoid arthritis over the past decade, methotrexate remains one of the most effective and most commonly used therapies for inflammatory arthritis. Although originally introduced for the treatment of cancer as a folic acid analogue, the mechanism by which methotrexate, at very low dosages, diminishes inflammation differs from the anti-proliferative mechanism of the drug. In patients with rheumatoid arthritis treated with methotrexate, there is strong evidence that the therapy promotes adenosine release [139, 140]. Studies in mice and rats demonstrated that the anti-inflammatory effects of methotrexate are mediated by adenosine and the anti-inflammatory effect is lost if animals are treated with adenosine receptor antagonists or if their adenosine A2A or A3 receptors have been deleted [141-143].

Preclinical studies using both knockout and pharmacological approaches have provided insights into the role of the various adenosine receptors in the physiological response to sepsis [144]. Inactivation of A1 receptors increase the mortality in intra-abdominal sepsis induced by cecal ligation and puncture in mice, an effect that was correlated with a heightened inflammation-induced hepatic and renal injury [145]. Similar to results with A1 receptor blockade in cecal ligation and puncture, both knockout and pharmacological antagonism of A3 receptor increased mortality, whereas stimulation of A3 receptor with IB-MECA was protective [146]. A2A receptor inactivation by either gene deletion or administration of ZM241385 prevented cecal ligation and puncture induced mortality by a mechanism that involved decreased bacterial dissemination [147]. Paradoxically, A2A receptor activation, when combined with

antibiotics, has been noted to reduce mortality from sepsis induced by injecting live E. coli, possibly by suppressing an exaggerated inflammatory response that can result from the rapid drug-induced killing of large numbers of bacteria [148].

In the skin, inflammation is part of a continuum in which injured tissue is repaired and replaced resulting in wound healing. Montesinos et al. first reported that topical application of adenosine A2A receptor agonists increases the rate of wound healing in normal mice and in diabetic rats [149]. Sondenoson, an A2A agonist that regulates the inflammatory response and enhances tissue regeneration, is currently undergoing trials for the treatment of diabetic foot ulcers. The nonselective adenosine receptor agonist NECA prevented diabetes development in both MLDS-challenged mice and in cyclophosphamide-treated NOD mice [85].

1.5 Rationale and hypothesis

Adenosine has been regarded as a crucial anti-inflammatory agent that protects the host from excessive damage. It has been reported to play an important role in suppressing T cell activation. However, it is a general observation that induction of T cell activation is an efficient event despite the high adenosine levels that are often present in the affected host due to injury or stress. The suppression effect of adenosine should be reconciled with T cell robust activation in stress conditions. This necessity leads to an intriguing question as to how immune system activation can thrive under this general state of suppression. In neurological and cardiovascular studies, a concept of "desensitization" has been widely developed, but no evidence was obtained on T cell lines or immune tissues. It is not clear whether desensitization is one of the ways by which T cells escape adenosine suppression. As it remains unclear how T cells escape adenosine suppression in high adenosine level, **I hypothesize that adenosine mediated desensitization of cAMP signaling plays a regulatory role in T cell activation: Adenosine suppresses T cell activation, however, pretreatment with adenosine enhances the subsequent T cell activation.**

Objective 1: To investigate the effect of adenosine on T cells in different T cell stimulation assays; **Objective 2**: To determine whether pre-exposure of adenosine to T cells affects T cell activation; **Objective 3**: To investigate the mechanism of priming of T cells by adenosine; **Objective 4**: To investigate whether adenosine can be used to stimulate immune response when cells pretreated with adenosine.

CHAPTER II

MATERIALS AND METHODS

2.1 Animals and reagents

2.1.1 Mice

C57BL/6, OT-1 (C57BL/6-Tg (TcraTcrb) 1100Mjb/J) and OT-2 (C57BL/6-Tg (TcraTcrb) 425Cbn/J) mice were purchased from Jackson Laboratories. OT-1 mice express a transgenic TCR that recognizes a SIINFEKL peptide derived from residues 257-264 of ovalbumin. OT-2 mice express a transgenic TCR that is specific for chicken ovalbumin, 323-339. A1 knockout mice (A1R deficient C57BL/6) were a gift from Dr. Mickael R. Blackburn (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA). Animal protocols were approved by the University of Calgary Animal Care Committee and met the Canadian Guidelines for Animal Research.

2.1.2 P1R agonists and antagonists

Adenosine, 5-(N-Ethylcarboxamido) adenosine (NECA) and N^6-cyclopentyladenosine (CPA) were obtained from Sigma-Aldrich. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), CGS21680, MeCCPA, IB-MECA and DPCPX were purchased from Tocris Cookson. Most of these reagents were dissolved in DMSO as per manufacturer's instructions. All preparations were warmed to 37℃ and immediately added to assay wells to avoid crystallization.

NECA: non-selective adenosine agonist; CPA, MeCCPA: A1 adenosine agonist; CGS21680: A2A adenosine agonist; DPCPX: A1 adenosine antagonist; EHNA: adenosine deaminase inhibitor; IB-MECA: A3 adenosine agonist.

2.1.3 Reagents

Microbeads conjugated to monoclonal anti-mouse CD8a (ly-2, isotype, rat IgG2a), and anti-mouse CD4 antibody conjugated microbeads were from Miltenyi Biotec. Annexin V –FITC Apoptosis detection kit, mouse IFN-γ and IL-2 ELISA Ready-SET-Go kits were purchased from eBioscience. Purified anti-mouse CD3ε (clone 145-2c11) were from BD Bioscience. Chicken ovalbumin was from Sigma-Aldrich Company. Complete Freund's Adjuvant (CFC) was from Rockland Biotechnology Company. Cyclic AMP EIA kit was from Cayman Chemical Company. RT-PCR RNeasy Mini Kit was from QIAGEN.

2.2 Methods

2.2.1 Lymphocytes Isolation

In all spleen T cells assays, mice were euthanized by CO_2, and spleens were harvested and ground in the cell culture media (CCM) (10% FBS plus 1mM HEPES 25 μM 2-ME and penicillin/streptomycin antibiotics). Contaminating erythrocytes were lysed by incubation of cell suspensions in Hemolysis Buffer for 2 minutes at room temperature. After hemolysis, cells were washed with lymphocyte cell culture media. To purify $CD4^+$ T cells or $CD8^+$ T cells from the spleen of C57BL/6 or A1 knockout mice, splenocytes were washed with CCM and incubated with anti-CD4 or anti-CD8 antibody

conjugated microbeads (50μl microbeads per spleen) for 20 minutes at 4°C, then washed with MACs buffer comprised of 1% BSA and 2mM EDTA in phosphate buffered saline (PBS). $CD4^+$ or $CD8^+$ T cells were purified via positive selection in the MiniMACS system (Miltenyi Biotec Inc. Auburn, CA).

2.2.2 T cell stimulation assays

Anti-CD3e mouse antibody was diluted with PBS (5μg/ml) and coated at 37°C in the presence of 5% CO_2 for 6 hours in 96-well round bottom tissue culture plates (Falcon). The coated plates were washed twice with sterile PBS before use. Purified $CD4^+$ T cells from the spleen were isolated from the mice that had received different treatments in each experiment as indicated. These cells were then plated in triplicate wells and incubated for 24 hours or the indicated time in the experiment at 37°C in the presence of 5% CO_2. Supernatants were collected and cytokines were measured by ELISA.

In OT-1 mice experiments, splenocytes were harvested from OT-1 mice and suspensions were transferred to 24 wells plates. The SIINFEKL peptide was added to the corresponding wells. Cells were incubated at 37°C, 5% CO_2 in a humidified incubator for 24 hours. Supernatants were collected and cytokines were measured by ELISA.

In OT-2 mice experiments, splenocytes were harvested from OT-2 mice and suspensions were transferred to 24 wells plates. OVA(50μg/ml) was added to the corresponding wells with different treatments. Cells were incubated at a 37°C, 5% CO_2 humidified incubator for specified time periods.

2.2.3 Pre-injection with adenosine derivatives to mice

C57BL/6 mice and A1 KO mice were injected i.p.(intraperitoneal) with PBS, CPA (10^{-5} M/kg), NECA (10^{-6} M/kg) or DPCPX(10^{-6} M/kg), and then18 hrs later, reinjected with the same reagent. 24 hrs from the initial injection, mice were euthanized and splenocytes were harvested. $CD4^+$ or $CD8^+$ T cells were purified and activated with anti-cd3 mouse antibody.

2.2.4 Cyclic AMP measurement

Cayman's cAMP kit is a competitive EIA assay. It is based on the competition between free cAMP and a cAMP-acetylcholinesterase(AcHE) conjugate for a limited number of cAMP-specific rabbit antibody binding sites. The rabbit antibody cAMP complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then ELLMAN's reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color that absorbs strongly at 412 nm.

$CD4^+$ T cells were isolated from splenocytes from mice with MACS microbeads. Cells were activated with anti-CD3, with or without adenosine agonists. In some assays, Forskolin was added to the plate. At different time points (10 mins, 15 mins, 20 mins, 30mins, 1 hrs, 4 hrs and 24 hrs), cell suspension was aspirated and cells were lysed with 0.1M HCL by incubation at room temperature for 20 minutes. The lysates were centrifuged at 1000*g for 10 minutes and the supernatant was transfered to a clean tube. The samples were acetylated with 4 M KOH (100µl/500 µl sample) and Acetic Anhydride(25 µl/500 µl sample) in quick succession. The mixture was vortexed for 15 seconds and an additional 25 µl KOH was added followed by vortexing again. The

mixture was then incubated at 4°C overnight. The developing and the reading of the plate were carried out according to the manufacturer's protocol. The plate was read with a visible light 96 well plate reader at a wavelength between 405 and 420 nm. The raw data points were converted into cAMP units using a spreadsheet program provided by the manufacturer (Cayman Chemical Company cAMP kit).

2.2.5 Apoptosis assay

Splenocytes from OT-1 or OT-2 (C57BL/6 background) mice were treated with or without adenosine agonists for various times. After 6 and 24 hrs, cells were stained for annexin V and propidium iodide using a kit from eBioscience. Cells were washed and resuspended in 100 μl of annexin V binding buffer containing 5μl of annexin-V-FITC, incubated at room temperature for 15 minutes. After washing with binding buffer, cells were resuspended in 100 μl buffer with 5 μl propidium iodide. Flow cytometry was used to determine the apoptosis of cells.

2.2.6 Adjuvant effect assay

C57BL/6 mice were injected i.p. with CPA (10^{-5} M/kg) or PBS overnight. The next day, the mice were immunized s.c. with 1mg of soluble OVA. The same amount of OVA mixed with Complete Freund's adjuvant (50 μl) was used as the positive control. One week later, the splenocytes were harvested and activated with soluble OVA and the IL-2 production was measured after 48 hours. In the OT-1 mouse experiment, mice were injected i.p. with CPA (10^{-5} M/kg), NECA (10^{-6} M/kg) or PBS overnight. The next day, the mice were immunized with 5μg of OVA coated on latex beads. The same beads were mixed with CFA (50 μl) as the positive control. Two days later, splenocytes from treated

mice were stimulated with B6 BM DCs pulsed with SIINFEKL peptide. The cytokine production was measured after 48 hours.

2.2.7 RNA isolation and DNA analysis

Total RNA was isolated from spleen $CD4^+$ T cells through purification as described in the lymphocyte isolation. mRNA was purified using the RNeasy Mini kit from QIAGEN. cDNA from C57BL/6 mice was synthesized from the total isolated RNA by reverse transcription using a cDNA kit from Invitrogen. Real-time PCR and two steps RT-PCR were run by using Applied Biosystems and SYBR Green I. The primers used in the experiments were as follows:

	5'primer	3'primer
A1A	GTGATTTGGGCTGTGAAGGT	CAAGGGAGAGAATCCAGCAG
A2A	GCTATTGCCATCGACAGATACATC	AATGACAGCACCCAGCAAATC
A2B	TGGCTGTCGACCGATATCTG	CTCGCTCGTGTCCCAGTGA
A3	CAGTCAGATATAGAACGGTTACCACTCA	GTTGGCTTTTCTATTCCAGCCAAA

Table 2.1 Adenosine receptors primers

For Real Time PCR, total RNA was isolated from purified CD4+ T cells from PBS or NECA injected C57BL/6 mice using the RNeasy Mini Kit supplied by QIAGEN. 5 to 10 million cells were homogenized using QIAshredder spin columns. The RNA quality and harvest efficiency were determined with Nanodrop 1000. cDNA synthesis was performed

using reagents supplied from Invitrogen. The reaction mixture, consisting of 1 ul random primers, 9 ul RNA, 1 ul 10 mM dNTP, and 1 ul sterile H2O, was heated at 65 °C for 5 minutes. The reaction mixtures were then placed on ice and centrifuged to ensure all the contents were on the bottom. 4 ul 5X first strand buffer, 2 ul 0.1 M DTT, and 1 ul RNase out were added to the reaction mixture and incubated at 37 °C on a heating block for 2 minutes. 1 ul of M-MLVRT was added and mixed by pipetting up and down and left at room temperature for 10 minutes. The reaction mixture was then incubated at 37 °C for 50 minutes and then the reaction was inactivated by heating the mixture to 70 °C for 15 minutes. The cDNA was then stored at -20 °C until real time PCR. For the QuantiTect SYBR Green PCR Kit (AB), mouse GAPDH was used as the internal control while the targets were mouse A1, A2A, A2B and A3 receptors.

Real time PCR was performed in the ABI PRISM 7000 using QuantiTect SYBR Green PCR protocol per manufacturer's instructions. Using the MicroAmp optical 96-well reaction plate, 20ul of master mix and 5 ul of cDNA were added to the appropriate wells. Non-template controls, along with RNA controls, were run in order to determine the background noise. The total reaction volumes consisted of 12.5ul of 2XQuantiTect SYBR Green PCR Master Mix, 6 ul of RNase-free water, 0.75 ul of Primer A and Primer B (final concentration 0.3 uM), and 5ul of a 1 in 5 dilution of cDNA. The optical 96-well reaction plate was then sealed with an optical adhesive cover and placed in the centrifuge for 5 minutes. The ABI PRISM 7000 was setup to have 2 min at 50 °C, 15 min at 95 °C for the PCR initial activation step, and 40 cycles of 3 steps consisting of 15 sec at 94 °C, 30 sec at 60 °C and 30 sec at 72 °C. A dissociation step was also added after the 40 cycles were completed. Real Time PCR

results were obtained using the ΔCT (Cycle Threshold) method as follows: ΔCT = Target CT - Normalizer CT (GAPDH).

Standard rodent GAPDH primers(Applied Biosystems) were used as a quantitative control.

2.2.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software. Data are represented as mean +/- SEM. For comparisons between two means, a Student t-test was used. Comparisons among three or more experimental groups were performed using a One-Way analysis of variance (ANOVA) followed by Bonferroni post-tests. The differences were considered to be significant if a p value < 0.05 was achieved.

CHAPTER III

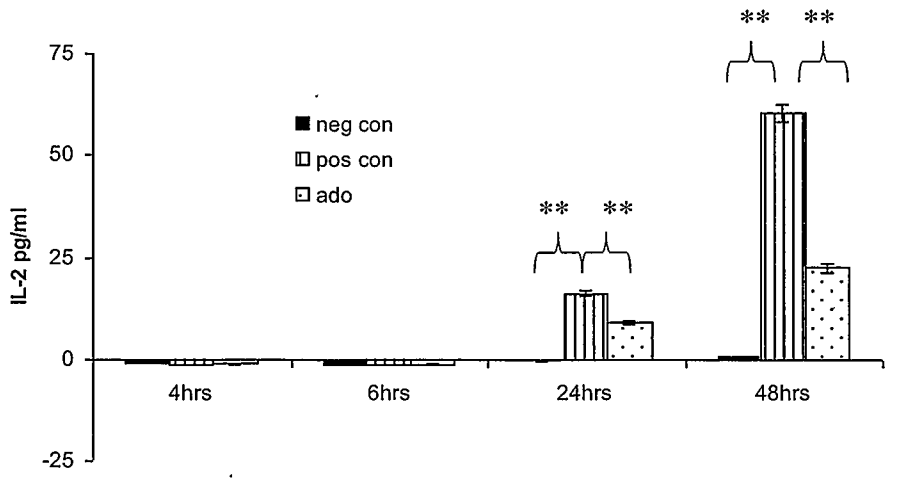
ADENOSINE MEDIATED DESENSITIZATION OF cAMP SIGNALING ENHANCING T CELL RESPONSES

3.1 Results:

3.1.1 Adenosine suppresses $CD4^+$ T cell activation

While adenosine mediated suppression of T cell activation is well documented, the report of individual adenosine receptor function has varied from assay to assay [24, 47, 77, 79, 82]. To examine the effect of adenosine suppressing T cells activation, we first treated purified splenic $CD4^+$ T cells from OT-2 mice (Figure3.1A) or C57BL/6 mice (Figure 3.1B) with plate bound anti-CD3 mAb and measured their IL-2 production as an indicator of T cell activation. In order to determine the best time point for measuring the effect of activation, supernatants were collected at different time after activation. Anti-CD3 mAbs induces T cell activation and IL-2 production was detected at 24 hrs and 48hrs, while there was lower IL-2 secretion at 4hrs or 6hrs activation [Figure3.1A: positive control (labeled as pos con)]. When high levels of exogenous adenosine were present in the cell culture, the IL-2 production was reduced [Figure 3.1A: adenosine treatment (labeled as ado)]. Since the adenosine concentration suppressing effect was varied from assay to assay [24, 75], different concentrations of adenosine were examined, and the result showed that both of the high exogenous adenosine levels (5μM or 10μM) has similar suppressing effect on T cell activation (Figure 3.1B)

A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75
50
25
0
-25
IL-2 pg/ml
neg con
pos con
ado
4hrs
6hrs
24hrs
48hrs

B

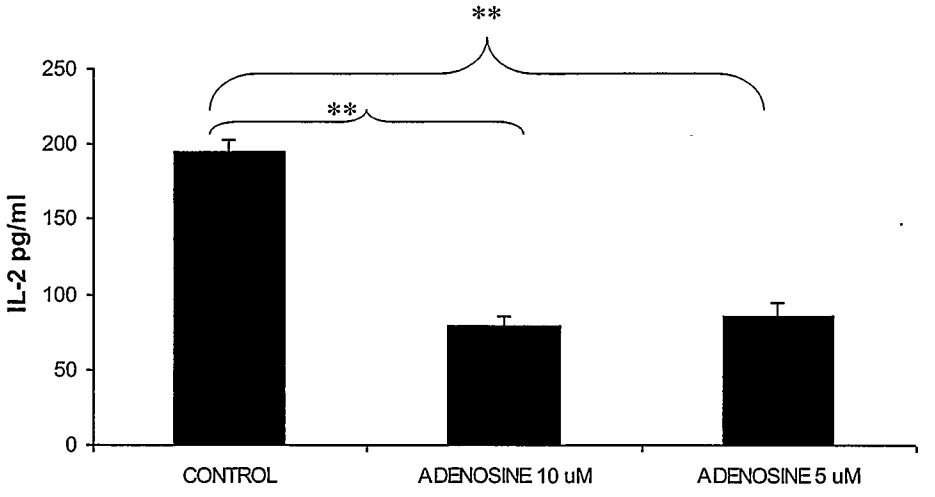
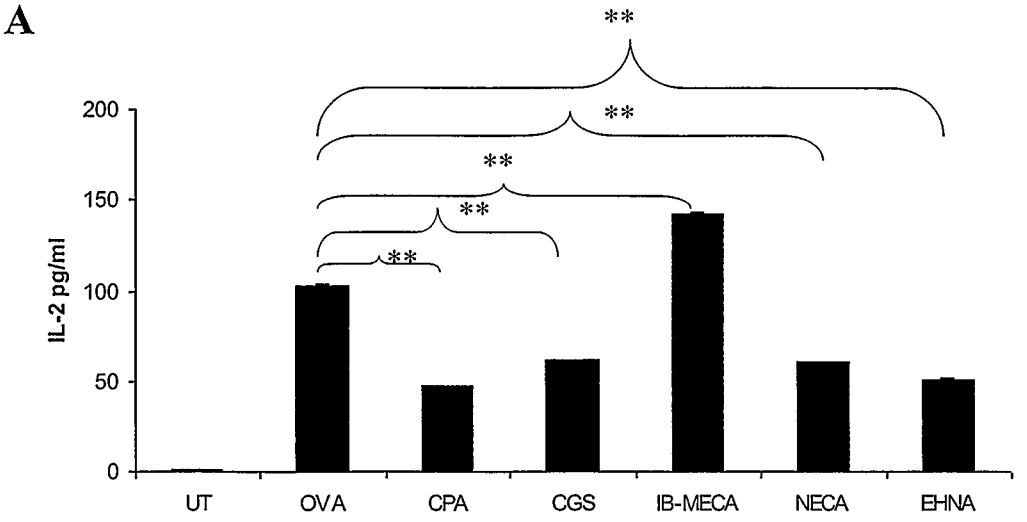

**
**
250
200
150
100
50
0
IL-2 pg/ml
CONTROL
ADENOSINE 10 uM
ADENOSINE 5 uM

Figure 3.1: Adenosine suppresses $CD4^+$ T cell activation: (A): Purified $CD4^+$ T cells from OT-2 mice were loaded into empty plate (neg con) or anti-CD3 (25ng/well) coated plate (pos con), incubated with (ado) or without adenosine in the incubator. Supernatants were collected at varying lengths of time and IL-2 production was measured. (B): As previously described, purified $CD4^+$ T cells from C57BL/6 mice were stimulated with anti-CD3. Following their activation, various concentrations of adenosine were added to the culture, after 24 hours incubation, IL-2 was measured by an ELISA kit. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post-tests. $^{**}P<0.01$

3.1.2 $CD4^+$ T cells are sensitive to the suppressive effect of multiple adenosine derivatives

Since adenosine in serum media could be rapidly converted to inosine by adenosine deaminase [101], we measured the effect of various non-degradable adenosine derivatives on T cell activation. Anti-CD3 mAb serves as a model of TCR mediated activation and it is also a direct assay to activate T cell, however, it is an artificial activation. To determine a more specific method of stimulation and also determine whether adenosine plays the same role in different ways of stimulation, we tested the OT-2 splenocytes' response to ovalbumin, in the presence or absence of specific P1R agonists (Figure 3.2A). First, we harvested the spleen from OT-2 mice and loaded splenocytes (2 x 10^6/ml) mixed with BMDC (0.2 million/ml) into 12 well plates, followed by addition of OVA (1mg/ml). After 24 hrs of incubation, supernatants were collected and IL-2 production was measured. We confirmed previous reports that CGS (A2A agonist) and NECA (pan P1R agonist) inhibited T cell antigen specific responses. IB-MECA (A3R agonist), enhanced T cell activation, which was found to be consistent with a previous report [11], but contradicts with another [81]. CPA (A1 agonist) also down regulated T cell activation, which contradicts the general consensus of A1R as an activating signal in contrast to A2AR[11]. EHNA (adenosine deaminase inhibitor) also inhibited T cell activation, which was consistent with our previous report [101]. To confirm that this response was not limited to this specific antigen reaction, we also purified $CD4^+$ T cells from C57BL/6 mice and stimulated them with anti-CD3 coated plate (Figure 3.2B). The suppressive effect of CPA was still evident, similar to NECA. These results showed that

non-selective adenosine agonist NECA reduces T cell activation, although the effect of individual receptor agonists might not be consistent with other reports.



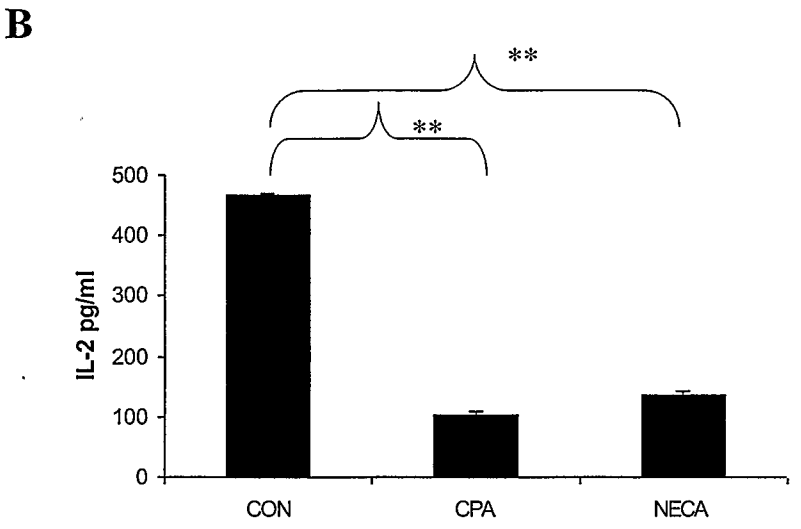
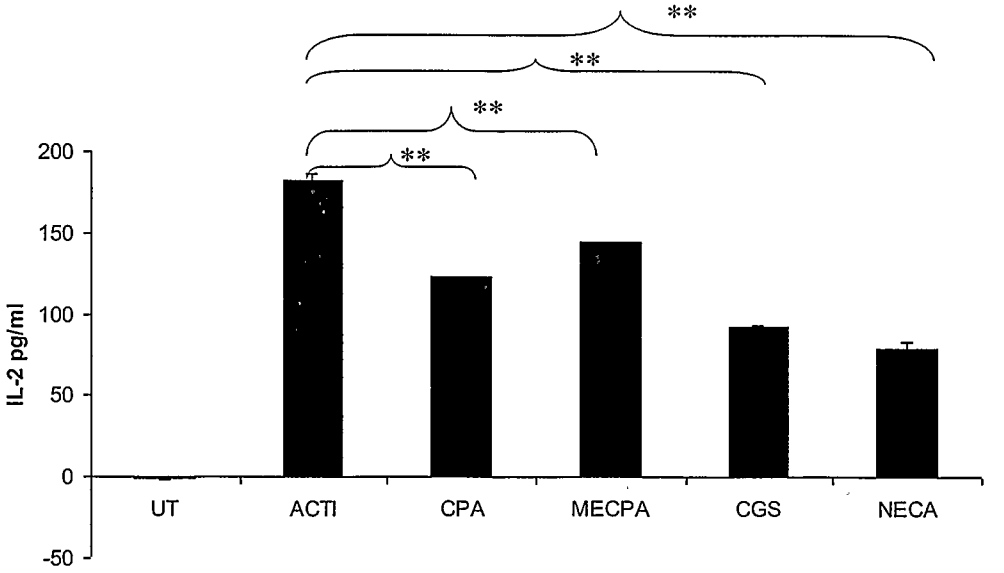


Figure 3.2 $CD4^+$ T cells are sensitive to the suppressive effect of multiple adenosine derivatives. (A): Spleens were harvested from OT-2 mice and splenocytes (2 million/ml) were loaded into 12-well plates, mixed with DC (0.2 million/ml) from bone marrow. The CD4+ T cells were activated with soluble OVA (1mg/ml). Various adenosine derivatives were added to the solution: CPA (10μM), A1 agonist; CGS (10μM), A2A agonist; IB-MECA (10μM), A3 agonist; NECA (10μM), non-selective adenosine agonist; EHNA (10μM): ADA inhibitor. After a 24 hrs of incubation, supernatants were collected and IL-2 production was measured. (B): As previous described, purified $CD4^+$ T cells from C57BL/6 were activated with anti-CD3 (25 ng/ml), followed by the addition of adenosine derivatives CPA or NECA. After 24 hrs of incubation, IL-2 was measured by an ELISA kit. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post-tests. **P<0.01

3.1.3 $CD8^+$ T cells are sensitive to the suppressive effect of multiple adenosine derivatives

The previous result showed that adenosine A1 agonist CPA suppress $CD4^+$ T cell activation, which is contrary to other reports [13, 30, 79]. A2A agonist CGS and non-selective adenosine agonist NECA suppressed $CD4^+$ T cells, which is consistent with other reports. Since it was possible that this phenomenon was limited to $CD4^+$ T cells, we repeated the assay with $CD8^+$ T cell and examined the OT-1 splenocytes response to SIINFEKL peptide (Ovalbumin 257-264), in the presence of specific P1R agonists (Figure 3.3A). After 24 hrs stimulation, supernatants were collected and IL-2 production was measured. We confirmed previous reports that CGS (A2A agonist) and NECA (pan P1R agonist) inhibited $CD8^+$ T cell antigen specific responses. CPA and MECCPA (A1 agonists) also down regulated $CD8^+$ T cell activation, consistent with the results of $CD4^+$ T cells. To confirm that this response was not limited to this specific antigen reaction, we also purified $CD8^+$ T cells from C57BL/6 mice and stimulated with an anti- CD3 coated plate (Figure 3.3B): both adenosine and pan P1R adenosine agonist NECA suppressed $CD8^+$ T cell activation.

A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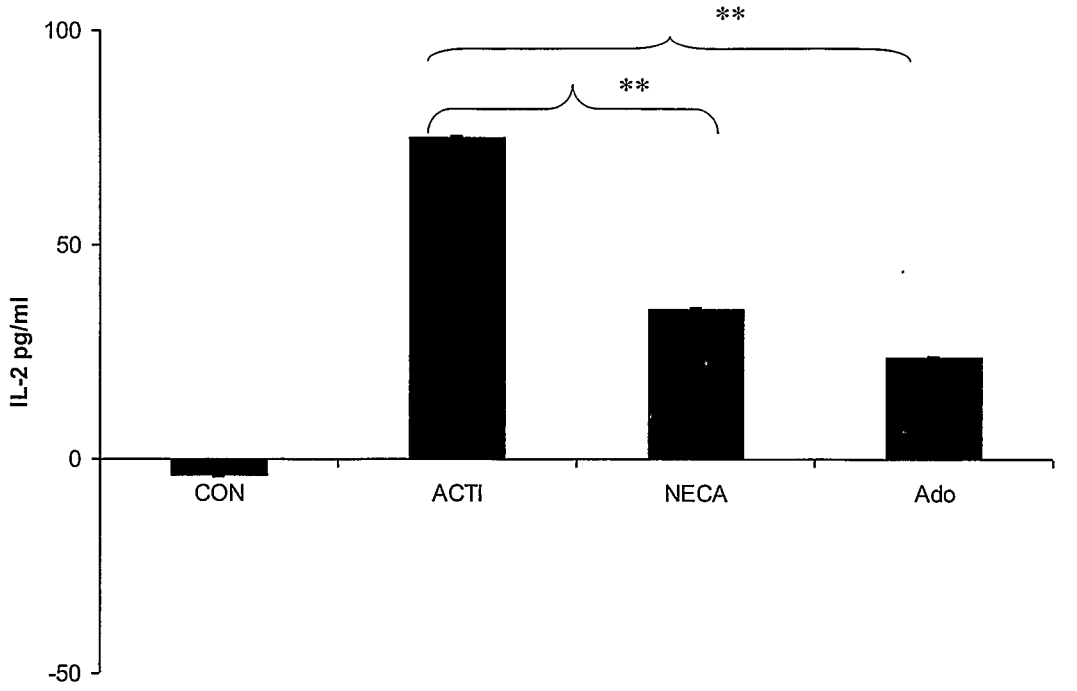
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IL-2 pg/ml
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NECA
Ado

Figure 3.3 $CD8^+$ T cells are sensitive to the suppressive effect of multiple adenosine derivatives. (A): Splenocytes (2 millions/ml) from OT-1 mice were stimulated with the SIINFEKL peptide (10^{-6} M), in the absence or presence of various adenosine derivatives (CPA and MECCPA: A1 agonist; CGS: A2A agonist; NECA: non-selective adenosine agonist). Supernatants were collected after 24 hrs and IL-2 levels were measured. (B): Purified $CD8^+$ T cells from C57BL/6 were activated with anti-CD3 in the presence or absence of adenosine derivatives CPA or NECA. Supernatants were collected and IL-2 was measured by an ELISA kit. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by One-Way ANOVA followed by Bonferroni post-tests. **P<0.01

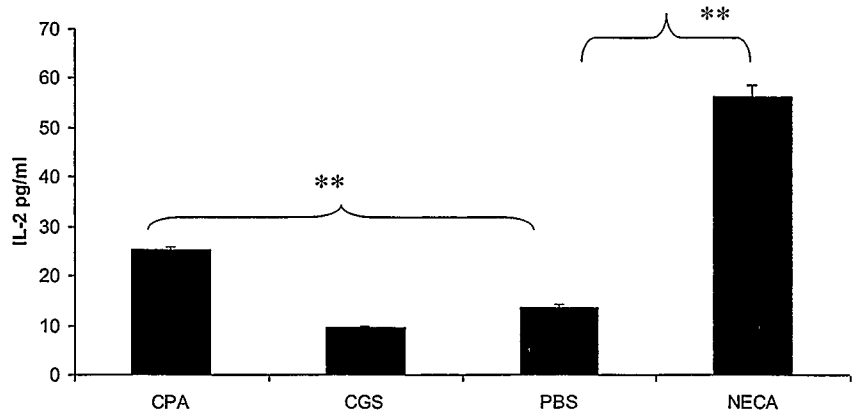
3.1.4 Pre-exposure to adenosine renders T cell hyper-reactive to antigen stimulation

Our central question is whether there are any balancing mechanisms to the seemingly dominant effect of adenosine suppression. Adenosine deaminase catalyzes serum adenosine to inosine, which down regulates the effect of adenosine [101]. It is not clear whether this is the only mechanism for T cells to escape from adenosine suppression. Tissue stress and inflammation lead to higher adenosine levels, which happened before any antigenic encounter by T cells, as the latter event only follows antigen presenting cell migration to the draining LNs, some point after 24 or 48 hrs. In other words, T cells are likely exposed to adenosine in injury or infection prior to their encounter with antigens presented by DCs. We pre-injected mice with adenosine derivatives overnight and studied their T cell responses after pretreatment. The design was to mimic T cell responses in relationship with adenosine regulation in vivo. Surprisingly, the in vivo pre-exposure to NECA or CPA up regulated the subsequent $CD4^+$ T cell activation via anti-CD3 stimulation. IL-2 production from the mice pretreated with NECA or CPA was elevated when compared with the control (PBS treatment). However, CGS pre-injection never achieved the same effect (Figure 3.4 A). This result showed that pre-treatment with adenosine agonist NECA or CPA enhanced the subsequent $CD4^+$ T cell activation. To see if this observation was limited to $CD4^+$ T cells, we again purified $CD8^+$ T cells from splenocytes and performed an identical stimulation assay (Figure 3.4 B). It showed a similar result: NECA pre-injection vastly up regulated the subsequent $CD8^+$ T cell response to the anti-CD3 stimulation. The pre-

injection of CGS did not have the same effect. While mice were injected with adenosine agonists, a cascade of biological systems was affected. To confirm that this effect was not caused by other organs or systems, an in vitro assay was performed: splenocytes were harvested and incubated with or without NECA for 24hrs, and $CD4^+$ T cells were purified and activated with anti-CD3. The in vitro experiment also showed that NECA treatment $CD4^+$ T cell had higher IL-2 production when compared to the control (Figure 3.4 C).

One possible complication in this study was that since adenosine reduces cellular activation, it was very possible that T cell treated with adenosine might have changed cell's apoptosis in comparison with regular T cell activation. The difference in apoptosis might have affected cell life span and it contributed to the amounts of cytokine measured. To rule out that the heightened T cell activation was due to a prolonged cell life or reduced cell death which can occur in T cells, we checked T cell apoptosis with annexin V and propidium iodide staining following the treatment of NECA and CPA. The result showed that NECA and CPA pretreatment did not change the state of cell apoptosis at 24 hrs incubation (Figure 3.4 D).

A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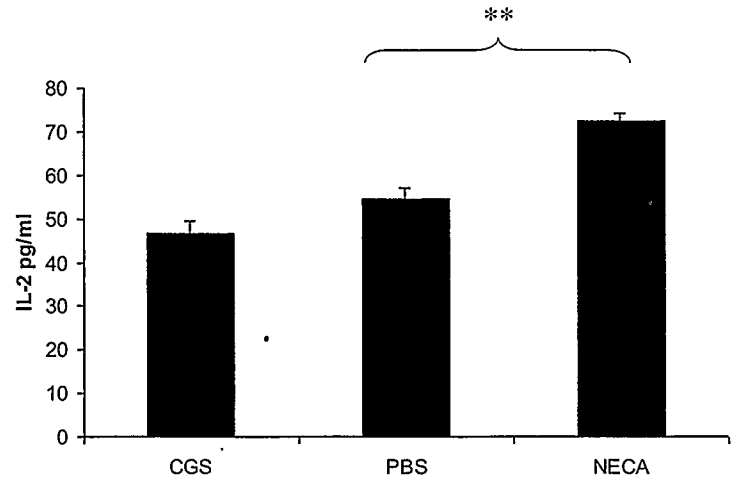
IL-2 pg/ml
CPA
CGS
PBS
NECA

B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80
70
60
50
40
30
20
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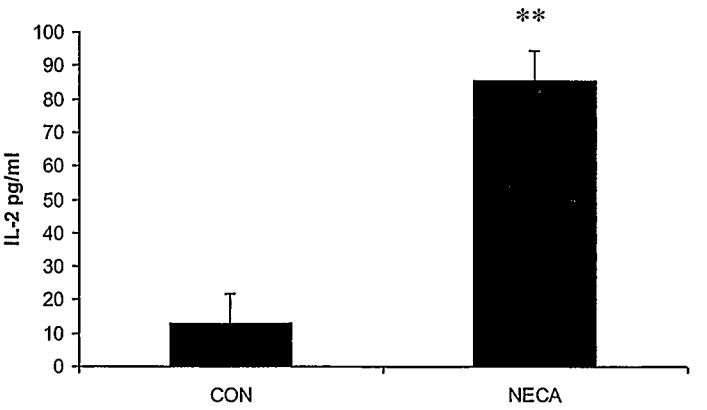
0
IL-2 pg/ml
CGS
PBS
NECA

C



D

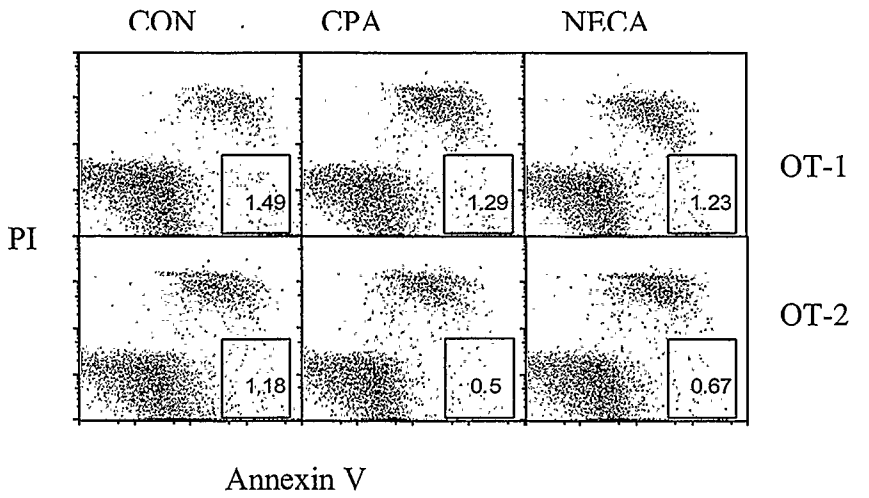


Figure 3.4: Pre-exposure to adenosine renders T cells hyper-reactive to antigen stimulation: (A): C57BL/6 mice were injected i.p. with PBS, CPA (10^{-5} M/kg), CGS (10^{-6} M/kg) or NECA (10^{-6} M/kg), and the same treatment was repeated at 18 hrs. After 24 hours, mice were euthanized and splenocytes were harvested. Purified $CD4^+$ T cells were activated with anti-CD3.After 24 hrs of incubation, supernatants were collected and IL-2 was measured. (B): In a similar method to A, except $CD8^+$ T cells were purified and

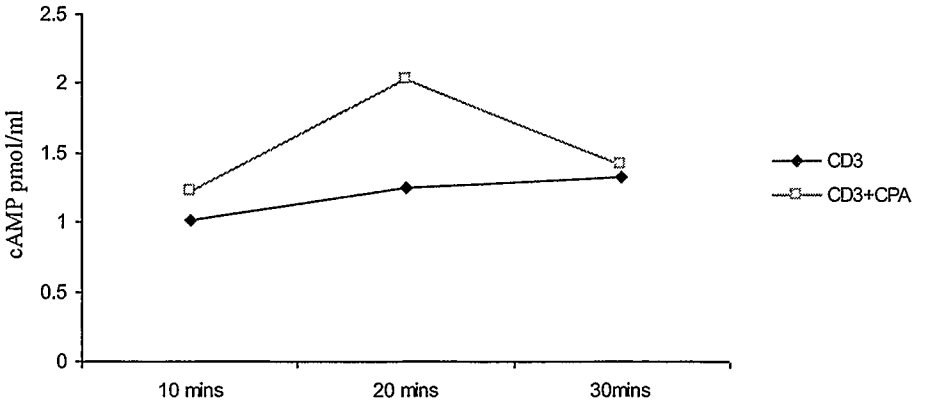
activated with anti-CD3. (C): Splenocytes were harvested and incubated with or without NECA (10μM) for 24hrs in vitro in an incubator, then washed with warm cell culture medium. $CD4^+$ T cells were purified to measure its activation via anti-CD3 stimulation. Supernatants were collected and IL-2 was measured. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post-tests (A, B) or a Student's t-Test (C). **P< 0.01 (D): Splenocytes from OT-1 or OT-2 mice were incubated with or without NECA (10μM) or CPA for 24 hours and then stained with annexin V and propidium iodide according to the manufacturer's protocol. Cells were analyzed by FACS to determine their apoptosis.

3.1.5 Adenosine receptor signaling controls T cell activation by regulating cAMP

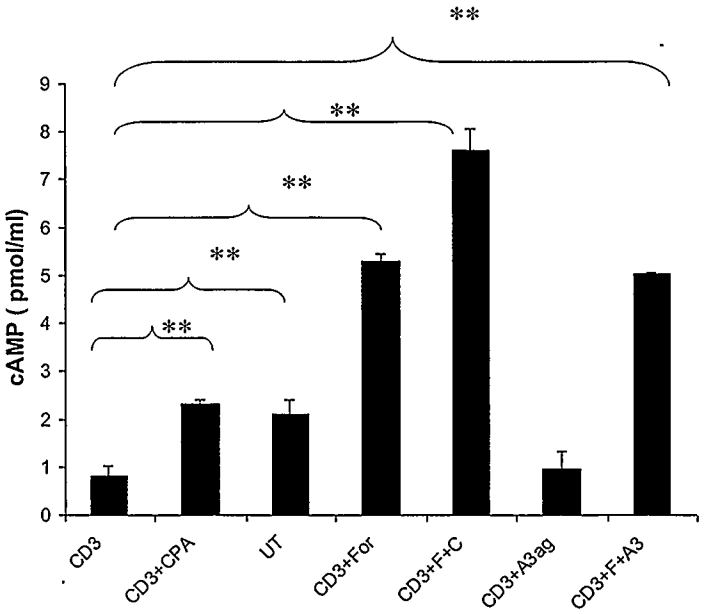
Traditionally, adenosine receptor signaling is thought to occur through the inhibition or stimulation of adenylyl cyclase with a concomitant decrease or increase in intracellular cyclic AMP concentrations [150]. We stimulated purified $CD4^+$ T cells with anti-CD3 in the presence or absence of adenosine derivatives and measured the levels of cAMP. Because the timing of this assay, following P1R engagement, varied among different reports [71, 77, 86], we firstly measured several time points and determined that a 15 to 20 minute period of exposure is optimal (Figure 3.5A). The addition of A1 adenosine agonist CPA increased the cAMP level, which contradicts with report that the stimulation of A1 receptor decreased the cAMP level on central nervous system or smooth muscle cells [45]. We compared the literature about adenosine receptor expression on different systems and found out that A1 is not expressed on T cells [24, 77, 78, 80]. In order to investigate whether this agonist is non-specific on T cells, we further designed experiments (The result is shown on result 3.1.10) (Figure 3.10). We found out that A1 agonist which is widely used in the neuroscience study is non-specific on T cells. The data from 4hrs, 6hrs and 24 hrs stimulations were not shown because the cAMP levels from longer stimulations were very close to the basal line. The reports on adenosine receptors A1, A2A, A2B and A3 regulating cAMP were not always consistent. Some reports showed that A2A ,A2B or A3 receptor activation increased cAMP [77, 81, 86], while other reports showed different results [11]. Forskolin is regarded as a reagent that stimulates cAMP directly, therefore, we used Forskolin and other adenosine

derivatives to measure cAMP following adenosine receptor activation (Figure 3.5B). The result showed that stimulation with anti-CD3 decreased cAMP in the cell's suspension, but CPA (A1 agonist) and Forskolin (cAMP stimulant) increased the cAMP level. As a control, A3R engagement showed a decrease in cAMP level. In order to determine whether pre-exposure of adenosine to $CD4^+$ T cells affected subsequent stimulation, we pretreated $CD4^+$ T cells with or without NECA for 24 hours, and stimulated with anti-CD3 in the presence or absence of NECA (Figure 3.5 C). In the presence of NECA, the cAMP level was elevated. Interestingly, the pre-treatment of $CD4^+$ T cells with NECA had different results: the pretreatment T cells with NECA did not increase cAMP level. In other words, after pretreatment with NECA, $CD4^+$ T cells seemed to have lost the ability to sense NECA.

A


2.5
2
1.5
1
0.5
0
cAMP pmol/ml
10 mins
20 mins
30mins
CD3
CD3+CPA

B


**
**
**
**
**
9
8
7
6
5
4
3
2
1
0
cAMP (pmol/ml)
CD3
CD3+CPA
UT
CD3+For
CD3+F+C
CD3+A3ag
CD3+F+A3

C

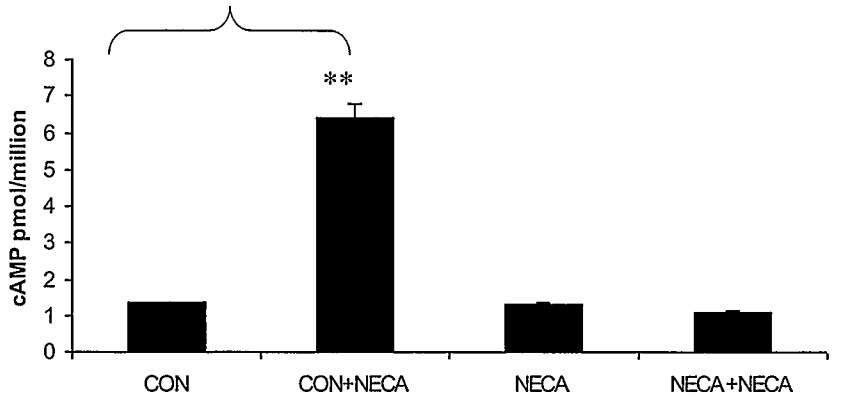


Figure 3.5: Adenosine receptor signaling T cells activation by regulating cAMP: (A): Purified $CD4^+$ T cells from C57BL/6 were suspended in the cell culture medium and stimulated with immobilized anti-CD3 with or without adenosine agonist, CPA. Cell suspension was collected at different time points: 10 mins, 20 mins and 30 mins (data of 4 hrs, 6hrs and 24hrs are not shown). Cells were then lysed with 0.1M HCL (20µl in 200 µl medium) and incubated at room temperature for 20 minutes. The concentration of cAMP was measured according to the protocol and the data were derived from the raw data following manufacturer's instruction. (B): Purified $CD4^+$ T cells from C57BL/6 mice were treated the same as A, except cells were activated with anti-CD3 in the presence of different adenosine agonists or Forskolin. The basal level of cAMP in untreated $CD4^+$ T cells were also measured (labeled as UT). (C): Splenocytes from C57BL/6 mice were injected with PBS (labeled as CON) or NECA for 24 hours, and then washed with warm cell medium, followed by stimulation with anti-CD3 in the presence or absence of NECA (10 µM). Cell suspension was collected at 15 minutes of stimulation and cAMP was measured according to the protocol. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post-tests. **$P<0.01$

3.1.6 T cell adenosine receptors mRNA transcription message

Adenosine, as a signal of stress and injury, has been regarded as an important suppressor to reduce the threshold of T cell activation. However, T cells activation was enhanced when T cells pretreated with adenosine agonist NECA. The signaling on T cells triggered by adenosine agonist NECA leads to increased cAMP rather quickly, reaching a maximum in 15 minutes, yet the increased level of cAMP is not sustained. Another observation is that T-cell pre-exposure to adenosine agonist NECA for a longer time period does not increase the cAMP level. In other words, NECA treatment caused a significant loss of NECA-stimulated adenylyl cyclase activity.

The reason why pre-treatment with adenosine agonists caused the loss of adenosine regulating cAMP level was not clear. One possible reason is that adenosine receptors transcription is altered when they are treated with adenosine agonists. To determine whether pretreatment with NECA affected adenosine receptor mRNA, we treated splenocytes from C57BL/6 with or without NECA for 24hrs, purified the $CD4^+$ T cells, and performed a real time PCR to measure the mRNA relative levels (Figure 3.6). It showed that, after the pre-treatment with adenosine agonist NECA, A2A, A2B receptor mRNA increased slightly, but was not statistically significant ($P>0.05$). The A3 receptor decreased slightly but was not statistically significant ($P>0.05$).

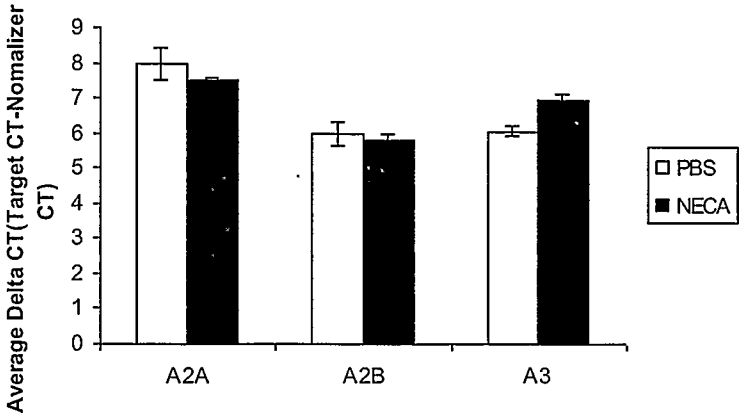


Figure 3.6: T cell adenosine receptors mRNA transcription message: Splenocytes from C57BL/6 were harvested and CD4+ T cells were purified. T cells were treated with or without NECA for 24 hours. mRNA was purified using the RNeasy Mini kit from QIAGEN. cDNA was synthesized from the total isolated RNA by reverse transcription using a cDNA kit from Invitrogen. Real-time PCR (two-step RT-PCR) was run by using Applied Biosystems and SYBR Green I. The primers used in the experiments were described in the Material and Methods. Standard rodent GAPDH primers (Applied Biosystems) were used as a quantitative control. Real Time PCR results were obtained using the ΔCT (Cycle Threshold) method as follows: ΔCT = Target CT - Normalizer CT (GAPDH). Statistical analyses were performed by Student's T-Test

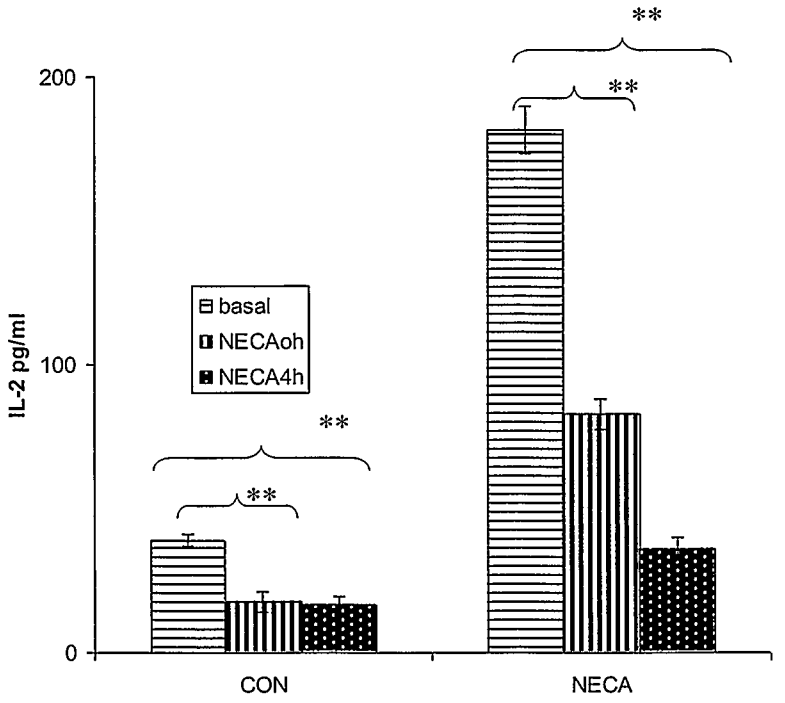
3.1.7 The priming effect is due to T cell P1R desensitization

We continued to explore other possibilities to explain how T cells escape from adenosine suppression when they are pre-exposed to adenosine. In neurological tissues, the adenosine receptor may become desensitized, removing its negative feedback in subsequent cell activation. Our previous result also showed that pre-exposure of adenosine agonist NECA to T cells for a longer time period decreased T cell sensitivity to subsequent treatment of adenosine agonist NECA.

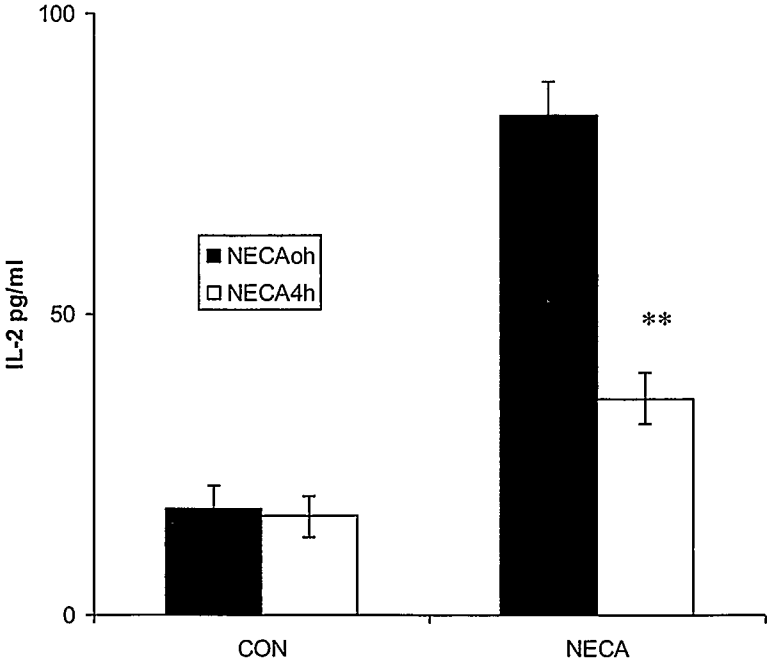
In a neurological tissue study, the pretreatment of adenosine agonists desensitize cell response to adenosine, but this tapers off after 12 hours [92]. To examine whether desensitization occurs on T cells, we performed both in vivo and in vitro assays to test this possibility. C57BL/6 mice were injected with NECA, and after 24 hours, splenocytes were harvested and CD4+ T cells were purified. T cells were then stimulated with plate bound anti-CD3. At various time points (0 hour and 4 hours), NECA was added to the culture to examine whether these T cells were still sensitive to the suppression. T cells from mice pre-injected with NECA produced several fold higher IL-2 levels compared to untreated T cells (Figure 3.7A). The addition of NECA at the time of CD3 stimulation reduced IL-2 production, but the T cells from the treated mice showed great resistance to the suppression in comparison to the control. The addition of NECA 4 hours after anti-CD3 stimulation further reduced the response, suggesting that the desensitization effect was being lost over time and the difference is statistically significant ($P<0.05$). To make the comparison clearer, we compared the effect of the addition of NECA at different time points (Figure 3.7 B): for T cells from the control

group, the addition of NECA at stimulation or 4 hours later produced the same effect in IL-2 production ($P>0.05$), however, for T cells from the pretreatment group, the addition of NECA at 4 hrs further reduced IL-2 production. The result suggested that T cells regained the sensitivity to adenosine agonist NECA after 4 hrs stimulation ($P<0.05$). We applied the experiment in vitro and showed a nearly identical effect (Figure 3.7 C, D).

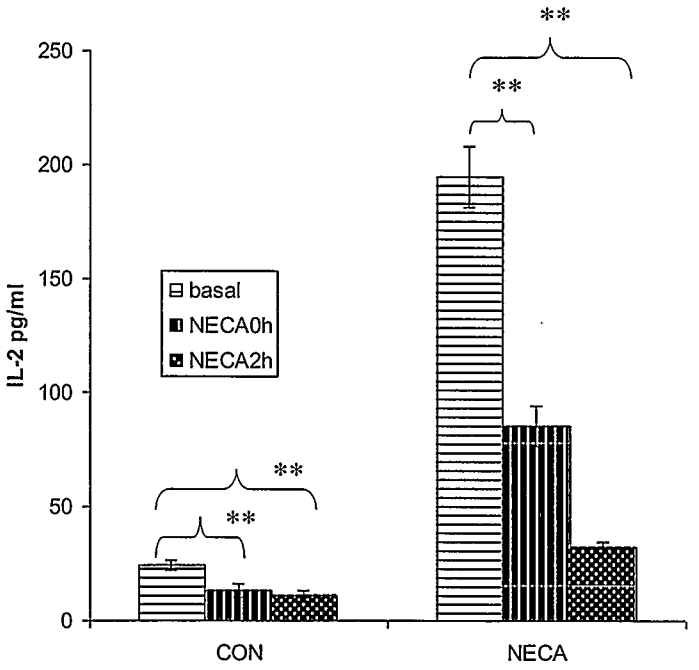
A


200
100
0
IL-2 pg/ml
basal
NECAoh
NECA4h
**
**
**
CON
NECA

B


100
50
0
IL-2 pg/ml
NECAoh
NECA4h
**
CON
NECA

C


250
200
150
100
50
0
IL-2 pg/ml
basal
NECA0h
NECA2h
**
**
**
**
CON
NECA

D

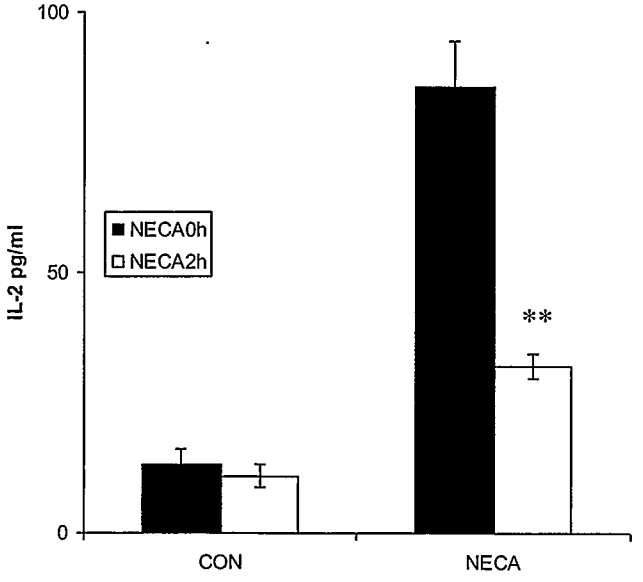

100
50
0
IL-2 pg/ml
NECA0h
NECA2h
**
CON
NECA

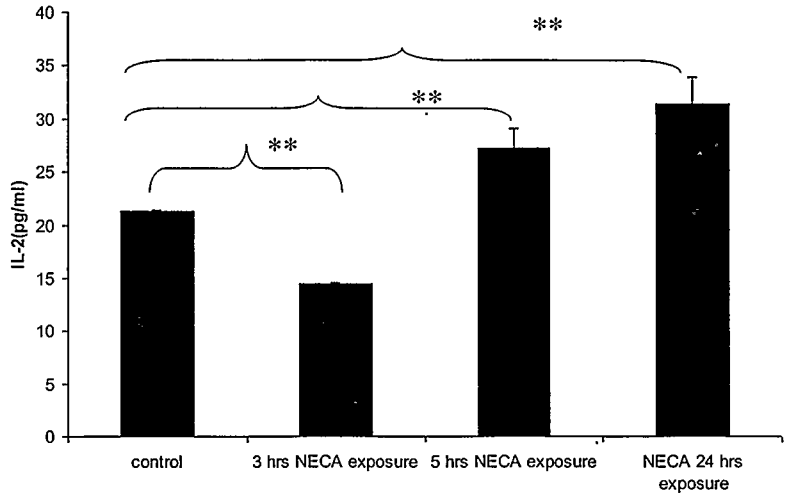
Figure 3.7: The priming effect is due to desensitization: (A, B): C57BL/6 mice were injected with or without NECA (10^{-6} M/kg) overnight (The sample without NECA treatment is labeled as CON). Splenocytes were harvested and $CD4^+$ T cells were purified followed by stimulation with plate bound anti-CD3. At 0 hours and 4 hours, NECA was added to the culture. After 24 hrs of incubation, supernatant were collected and IL-2 production was measured. (C, D): Splenocytes were harvested from C57BL/6 mice and treated with or without NECA for 24 hours. After washing with warm cell culture medium, $CD4^+$ T cells were purified and activated with anti-CD3. NECA was added at various time points after activation. After 24 hrs of incubation, supernatants were collected and IL-2 production was measured. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post test (A, C) or Student T-test (B, D) $^{**}P< 0.01$

3.1.8 The duration of desensitization and resensitization

Adenosine levels fluctuate in vivo. Constantly elevated levels of adenosine indicate tissue stress and inflammation. Conversely, if adenosine levels decrease in the host, one would expect that T cells should quickly regain their sensitivity to adenosine to avoid a sustained T cell activation under physical conditions. The desensitization mechanism is a clear advantage for immune activation. However, it is also evident that such an event must be short lived to permit the protective effect of adenosine. First, we measured how quickly T cells were desensitized in the presence of adenosine. Purified $CD4^+$ T cells from OT-2 mice splenocytes were cultured with adenosine agonist NECA for various time points. The cells were washed with warm cell medium culture and activated with anti-CD3. 24 hours later, supernatants were collected and IL-2 production were measured (Figure 3.8A). The result showed that a shorter time exposure to adenosine suppressed T cells activation; however, longer time exposure to the adenosine agonist enhanced T cell activation. Next, we measured the duration after which the treated T cells regained the sensitivity to adenosine suppression. We injected C57BL/6 mice with CPA overnight. The $CD4^+$ T cells were then purified and stimulated with plate coated anti-CD3. At various points, NECA was added into the culture (Figure 3.7 B). It appeared that the desensitization occurred after the removal of CPA. T cells retained full activation even when the adenosine analog was introduced concomitantly with anti-CD3. However, after a rest period of 2 hours, they became fully sensitive to adenosine inhibition. This information is important in that it provides a logical explanation that may bridge various

reports in T cell responses to adenosine, and points out that the desensitization is a transient effect. Physically, since high adenosine levels persist, T cells in fact may be constantly desensitized until immediately after convalescence. Paradoxically, as long as adenosine is constantly present, T cells would be "blind" to its presence.

A


40
35
30
25
20
15
10
5
0
IL-2(pg/ml)
**
**
**
control
3 hrs NECA exposure
5 hrs NECA exposure
NECA 24 hrs exposure

B

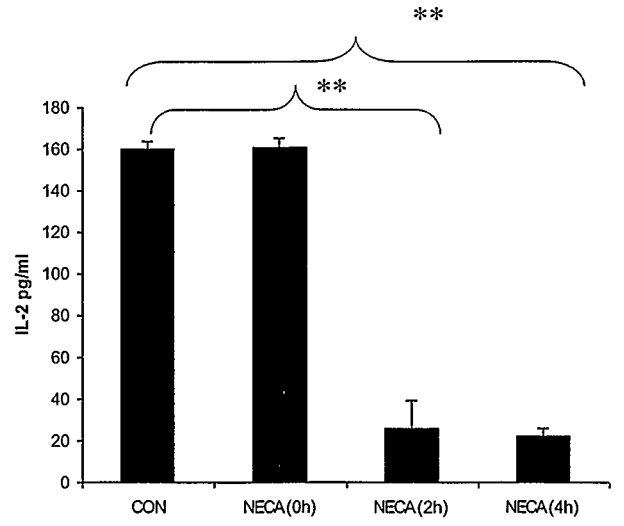
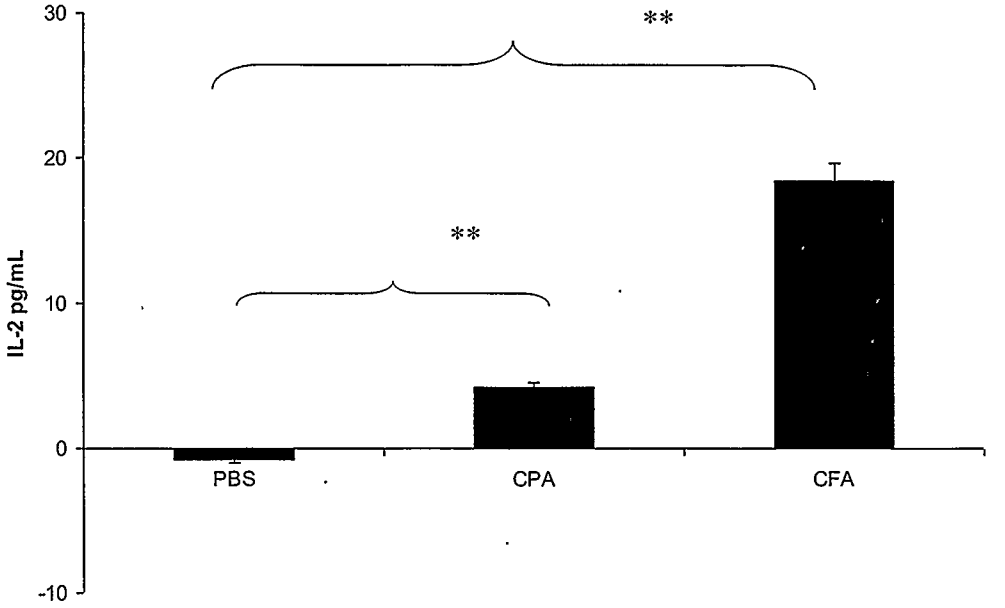

180
160
140
120
100
80
60
40
20
0
IL-2 pg/ml
**
**
CON
NECA(0h)
NECA(2h)
NECA(4h)

Figure 3.8 The duration of desensitization and resensitization: (A): Purified $CD4^+$ T cells from OT-2 mice splenocytes were cultured with adenosine agonist NECA for various time periods (The sample without NECA treatment is labeled as control). Cells were washed with warm cell medium culture and activated with anti-CD3. Supernatants were collected after 24 hrs and IL-2 production was measured. (B): C57BL/6 mice were injected with CPA overnight. After washing with cell culture medium, $CD4^+$ T cells were purified and stimulated with plate coated anti-CD3. At various time points, NECA was added into the culture (The sample without the addition of NECA is labeled as CON). After 24 hrs of incubation, supernatants were collected and IL-2 production was measured. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post-tests. **$P<0.01$

3.1.9 The desensitization of P1R exerts a strong adjuvant-like effect on $CD8^+$ T cells

The surprisingly robust T cell response following adenosine desensitization seemed to implicate a potential adjuvant-like effect. We further investigated this observation and tested whether the pre-treatment of adenosine could be used similarly to an adjuvant to enhance T cell antigen specific responses. We injected C57BL/6 mice with CPA or PBS overnight. The next day, the mice were immunized s.c. with 1mg of soluble OVA. A same amount of OVA mixed with Complete Freund's adjuvant was used as the positive control. One week later, the splenocytes were harvested, activated with soluble OVA and IL-2 production was measured after 48 hours. The pre-injection of CPA increased the cytokine production although the intensity was lower than the effect of Complete Freund's adjuvant (CFA) (Figure 3.9A). To analyze if the desensitization had a strong effect on $CD8^+$ T cell response, we injected OT-1 mice with NECA, CPA and PBS overnight. The next day, the mice were immunized with 5μg of OVA coated on latex beads. The same beads were mixed with CFA as the positive control. Two days later, splenocytes from treated mice were stimulated with C57BL/6 BM DCs pulsed with the SIINFEKL peptide and the cytokine production was measured after 48 hours. To our surprise, both CPA and NECA exerted strong adjuvant-like effect, very similar to that of CFA (Figure 3.9B).

A


30
20
10
0
-10
IL-2 pg/mL
**
**
PBS
CPA
CFA

B

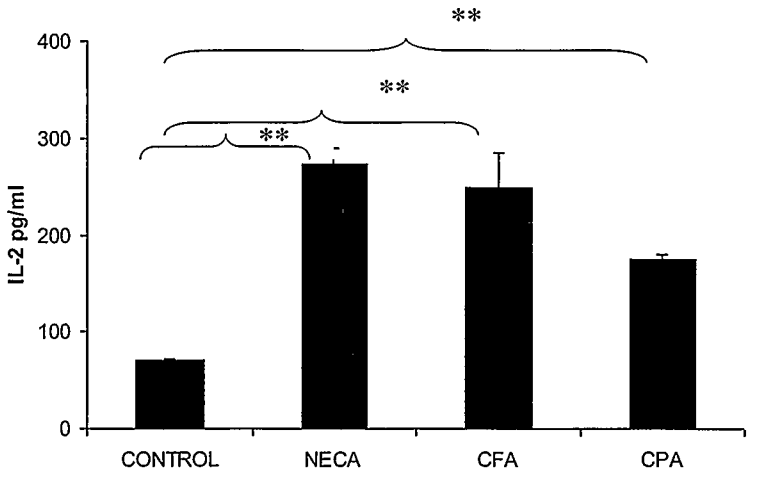
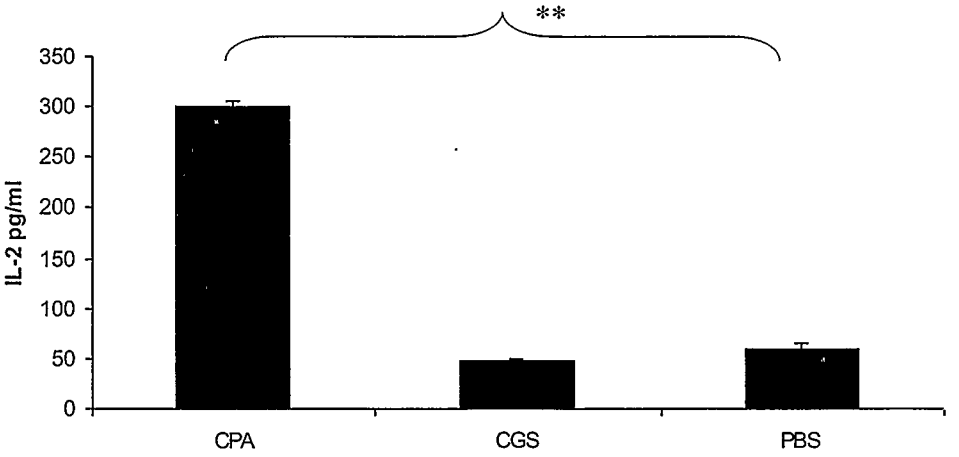

400
300
200
100
0
IL-2 pg/ml
**
**
**
CONTROL
NECA
CFA
CPA

Figure 3.9 The desensitization of P1R exerts a strong adjuvant effect on CD8+ T cells: (A): C57BL/6 mice were injected i.p. with PBS or CPA (10^{-5} M/kg) overnight. The next day, the mice were immunized s.c. with 1 mg of soluble OVA. A same amount of OVA mixed with Complete Freund's adjuvant was used as the positive control. One week later, the splenocytes were harvested, activated with soluble OVA and the IL-2 production was measured after 48 hours. (B): OT-1 mice were injected i.p. with PBS (labeled as CONTROL), CPA (10^{-5} M/kg) or NECA (10^{-6} M/kg) overnight. The next day, the mice were immunized with 5 μg of OVA coated on latex beads. The same beads were mixed with CFA as the positive control. 2 days later, splenocytes from treated mice were stimulated with C57BL/6 BM DCs, pulsed with the SIINFEKL peptide and the cytokine production was measured after 48 hours. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post-tests. **P<0.01

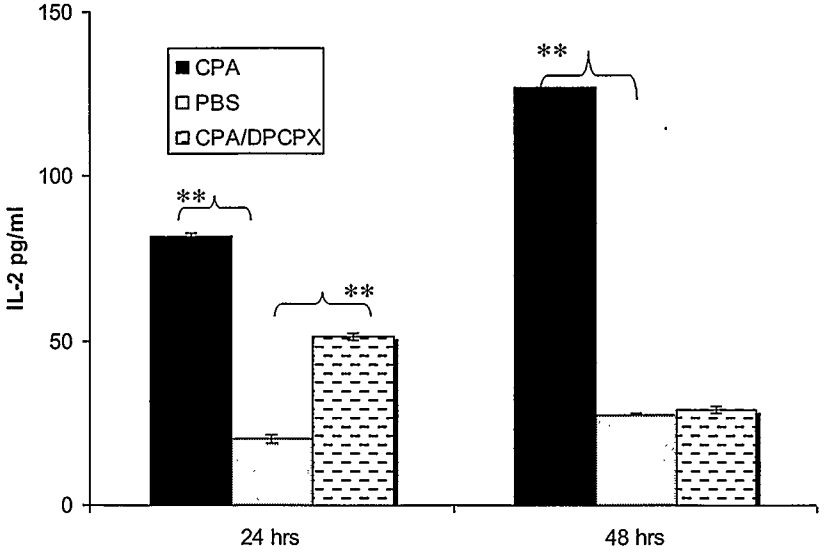
3.1.10 Desensitization is mediated by a structure targeted by CPA and DPCPX

The effect of CPA displayed in the previous assays presented implicates an A1 adenosine receptor involvement in desensitization. This is consistent with previous literature which illustrates that CPA has been historically used to target this particular receptor with assumed specificities [111-114]. However, previous reports showed that the A1 receptor is not expressed on T cells [75-78]. To determine the structure targeted on the T cells, we pretreated C57BL/6 mice with A1 agonist, CPA, and A2A agonist, CGS. Surprisingly, CPA enhanced the following T cell activation while CGS did not alter the subsequent T cell activation (Figure 3.10A). To ascertain that this result was not due to the reagent CPA's pharmacological specificity, we injected CPA and DPCPX (A1 antagonist) into the mice. It showed that the A1 antagonist blocked the effect of desensitization (Figure 3.10 B). To confirm the results with a genetic approach, we tested A1R KO mice. The result suggested that CPA still had the same effect on A1A KO mice, and that A1 antagonist blocked the NECA effect on T cells (Figure 3.10 C).

A


**
350
300
250
200
150
100
50
0
IL-2 pg/ml
CPA
CGS
PBS

B


150
100
50
0
IL-2 pg/ml
CPA
PBS
CPA/DPCPX
**
**
**
24 hrs
48 hrs

C

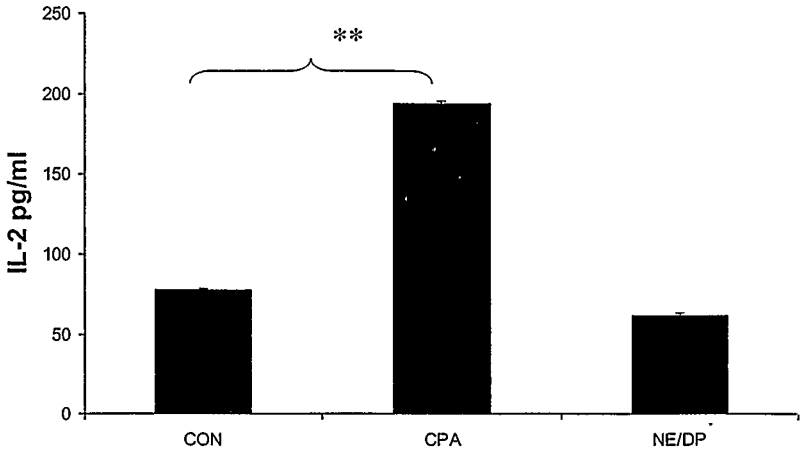


Figure 3.10 Desensitization is mediated by a structure targeted by CPA and DPCPX: (A): C57BL/6 mice were injected i.p. with PBS, CPA (10^{-5} M/kg) or CGS (10^{-6} M/kg), and the same treatment was repeated at 18 hrs. After 24 hours, mice were euthanized and splenocytes were harvested. Purified $CD4^+$ T cells were activated with anti-CD3. After 24 hrs of incubation, supernatants were collected and IL-2 levels were measured. (B): The same assay was applied except DPCPX (10^{-6} M/kg) and CPA were injected at the same time. (C): The same assay was applied except A1 KO mice instead of BL6 mice were treated with PBS (labeled as CON), CPA or NECA+DPCPX. After 24 hrs of incubation, supernatant were collected and IL-2 production was measured. Data shown are the mean±SEM, and were performed in triplicate. This panel is representative of three independent experiments. Statistical analyses were performed by a One-Way ANOVA followed by Bonferroni post-tests. **P<0.01

3.2 Discussion

Adenosine, a nucleoside which is present in all body fluids, has been regarded as an important regulator in immune system. Elucidating the mechanisms that adenosine regulation of immune system is of importance not only for understanding of normal physiology, but also for devising treatments for pathological situations such as asthma, ischemia or other inflammatory diseases.

The loss of adenosine receptor was linked to massive tissue damage in host responses to inflammation and septic shock [46]. Adenosine signaling is also critically important in protecting against multiple organ failure in situations of trauma and shock [47,48]. The recent studies demonstrated that many diseases are related with adenosine levels in the body fluids. For example, Severe Combined Immunodeficiency (SCID) (also known as Boy in the Bubble syndrome), is a severe form of immunodeficiency. These babies, if untreated, usually die within 1 year due to severe infections. One of most common forms of SCID is caused by a defective enzyme, adenosine deaminase (ADA). Lack of ADA causes accumulation of adenosine and inhibits lymphocyte proliferation, leading to a compromised immune development [88-91]. A large body of evidence demonstrated that adenosine is involved in defining the pathophysiology of asthma and chronic obstructive pulmonary disorder (COPD).

Since the effect of adenosine has been studied, adenosine and adenosine analogs have been used in the clinical research and in curing diseases. Methotrexate remains one of the most effective and most commonly used therapies for inflammatory arthritis due to

the therapy promoting adenosine release. Adenosine analogs have proven to be protective in a number of inflammatory diseases models, including ischemia-reperfusion, colitis and type I diabetes. The use of adenosine analogs as a drug in clinic has triggered a question: Have we known all of adenosine effects on most organs or systems?

In immune system, adenosine is an important regulator of inflammatory and immune responses, and recent studies have clearly shown that this includes negative regulation of T cell responses [24, 47, 77, 79, 82]. While it is important to appreciate the protective/suppressive effect of adenosine on the immune system, it should be reconciled with the robust T cell activation under high levels of adenosine during infection and injury. Prior to our study, this issue had not been directly assessed on T cells. Our study demonstrated the mechanisms that illustrate how T cells temporarily escape the general state of suppression mediated by adenosine. T cells use a common method of impediment: the ligand induced receptor desensitization, a prevalent feature of P1Rs in neuronal cells, adipocytes and smooth muscle cells [120-125].

As one of the essential tools to fend off the excessive inflammation and cell activation that are often harmful to the host during infection or injury, adenosine is very important to the host's well being [47]. This necessity leads to an intriguing question as to how immune system activation can thrive under this general state of suppression. Surface ADA has been studied in recent years and believed to be the mechanism in which T cells escape adenosine suppression. On some human cells, extracellular ADA is associated with CD26, delivering a co-stimulatory signal to T cells [98-100]. However, rodent CD26 is not associated with ADA due to the sequence variability [101]. This

findings lead to a question: If "ADA-CD26 co-stimulatory signal" cannot fully explain rodent T cell activation under high adenosine level, are there any alternative mechanisms to explain it?

Adenosine level fluctuates in body fluids under different conditions. Tissue stress and inflammation lead to higher adenosine levels [5, 9], which are present before any antigenic encounter by T cells, as the latter event only follows antigen presenting cell migration to the draining LNs, some point after 24 or 48 hours. In other words, T cells are likely exposed to adenosine in injury or infection prior to their encounter with antigens presented by DCs. To mimic the stress condition, we pretreated T cells in vitro with the adenosine agonist NECA or injected mice with NECA in vivo. Both results demonstrated that pre-exposure of pan-P1R adenosine agonist NECA to T cells enhances the subsequent T cell activation. This surprising result contradicts with the predominant notion that adenosine suppresses T cell activation. However, it might explain why T cells retain robust activation under high adenosine levels.

Is it possible that adenosine might play different roles in different stages of T cell activation? Even though similar studies of the desensitization effect on T cells have not been reported, a body of evidence on other cells has demonstrated that adenosine is often dichotomous. For example, adenosine has a concentration dependent effect on neutrophils: adenosine enhances the inflammatory responses at sites where adenosine is present in low concentrations, whereas in high concentrations acts as a feedback inhibitor of inflammatory [58,59]. For Dendritic Cells, adenosine was found to be a potent chemotactic stimulus for immature PDCs, but an inhibitor for mature PDCs. The

stimulation of immature DCs is crucial for the initiation of an immune response [66]. Similar to neutrophils and DCs, adenosine might play different roles on T cells under different circumstances. We proposed that adenosine regulation of T cells is not an invariable process: during stimulation, T cells activation is suppressed by adenosine; however, when T cells pretreated with adenosine, its subsequent stimulation will be enhanced due to an adenosine "priming" effect (Table 3.1).

TREATMENT			EFFECT
Adenosine pretreatment → (24 hrs prior to stimulation)	stimuli → (stimulation with or without adenosine)	Adenosine	T cell activation
No	No	No	No
No	Yes	No	++
No	Yes	Yes	+
Yes	Yes	No	++++

Table 3.1 Adenosine regulation T cell activation

There are several possible scenarios underlying this phenomenon, because T cell activation is affected by numerous factors. One potential complication in this study was that since adenosine pretreatment affect the subsequent T cell activation, T cells treated with adenosine might have changed their rate of programmed cell death (apoptosis) in comparison with regular T cell activation. Our result demonstrated that the addition of NECA did not change cell apoptosis in mouse splenocytes.

Another possible explanation was that T cell adenosine receptor expression was changed because T cell activation has been associated with the level of adenosine receptor transcription or mRNA stability. TCR activation induces adenosine receptor A2A on T cell and the activation of A2A inhibits IL-2 [75-77]. Lappas et al. also reported that the increase of A2AR mRNA resulted in the suppression of T cell activation [26, 27]. To assess whether the pretreatment led to the alteration of adenosine receptor mRNA, we examined A2A, A2B, A3 adenosine receptor mRNA via quantitative RT-PCR. It showed that pretreatment T cells with adenosine (NECA) resulted in no significant changes in the adenosine receptor mRNA level.

Our findings showed that adenosine "priming" effect is not associated with cell apoptosis or the change of cell adenosine receptor mRNA transcription message. Is P1R desensitization associated with this priming effect? In the study of neural and smooth muscle cells, Hettinger et al. and other groups proposed that adenosine receptor desensitization is one of most important mechanisms in understanding the "adenosine priming effect" and post-transcriptional regulation underlies receptor desensitization since no change in mRNA level was observed on pre-exposure of neuron cells to adenosine antagonist [119-121].

Desensitization has been regarded as an important mechanism to reduce adenosine sensitivity in some tissues. The early evidence for adenosine receptor desensitization was largely obtained from primary cells, cell lines and tissues that were exposed to varying concentrations of adenosine receptor agonists, often examined over several time periods. However, no evidence was obtained on T cell lines or immune tissues. Our results

indicate that adenosine receptor desensitization observed on neurological tissue occurs in the immune system as well.

Adenosine receptor signaling is thought to occur through the inhibition or stimulation of adenylyl cyclase with a concomitant decrease or increase in intracellular cyclic AMP concentrations [150]. In T cell activation, the addition of adenosine agonist induces cAMP, which suppresses T cell activation. cAMP level is an important and direct yardstick to understand adenosine receptor desensitization. The data herein show that the pretreatment induces adenosine receptor desensitization, which mediates stimulation of cAMP. Following the desensitization, the overall ability to sense adenosine (NECA) is reduced, blocking cAMP production. Our result, consistent with other reports in the brain and other tissues, demonstrated the desensitization of adenosine receptor resulted in the incapability of adenylyl cyclase to generate cAMP [120-125].

Our work also points out such a scheme of adenosine mediated T cell regulation. In inflammation and tissue injury, the stress leads to higher adenosine level. This high level of adenosine desensitizes T cells adenosine receptors, rendering them unable to sense the extracellular adenosine. The second messenger cAMP, remains low. T cells keep a robust activation profile after encountering antigenic presentation. However, the desensitization effect tapers off rapidly. Within two hours, T cells completely regain their sensitivity to adenosine inhibition. This observation suggests that in vivo, once adenosine levels are falling back to normal, T cells are under the protection of adenosine. Conversely, it appears that as long as adenosine remains high, T cells are likely insensitive to the adenosine suppression. It may indicate that during infection and tissue

stress, the generally assumed immune suppressive effect by adenosine is minimal, particularly on T cells.

One practical aspect of our work is the finding that adenosine desensitization demonstrates a strong adjuvant-like effect for CD8+ T cells. This is consistent with another observation that T cell activation, in response to either anti-CD3 or antigens following NECA pre-exposure, is stronger than that of untreated controls. These results suggest that untreated T cells, upon activation, produce adenosine by themselves in order to serve as a negative regulator. Pre-exposure to adenosine removes this mechanism and in turn manifests itself as an immune enhancer.

It was a surprise that in our settings, the desensitization effect is mediated by CPA and blocked by DPCPX, yet not via A1R. Although both Northern Blotting and RT-PCR analysis of mRNA from purified mouse T cells revealed expression of mRNA transcript for A2A, A2B and A3 subtypes but little or no message of the A1 subtypes [24, 80, 81], our finding showed that A1 agonist CPA and MECCPA also suppressed T cell activation. This result along with the confirmation that A1 receptor is not expressed on T cells is difficult to comprehend under the general notion that A1 agonist enhances cell activation by reducing cAMP level.

Both CPA (A1 agonist) and DPCPX (A1 antagonist) trace their specificity origins to adenosine signaling studies in non-immune cells [120, 151]. The A1R deficient mice used in our assays were known to have A1 signaling defects in other physiological functions, such as urine retention in the kidneys and cardiovascular outputs [152, 153]. Our work seems to indicate that in the immune cells, CPA lacks its proclaimed specificity

in other tissues and targets multiple P1Rs, similar to NECA. This is not surprising as P1R agonists demonstrate vastly different targeting specificities and efficiencies in different tissue [154], and the specificity issues of A1 agonists and antagonists in the immune system had been discussed in some early reports[63, 68, 155]. However, prior to our study, no effort had been made on the issue of A1 agonist specificity on T cells. Our finding was the first study disclose the possibility that A1 agonist and antagonist lack their specificity on T cells.

Although A2A is the predominant signaling receptor on T cells [24, 80, 81] and A2A specific adenosine agonist CGS suppresses T cell activation [24, 77, 79], our finding demonstrated that pre-exposure adenosine agonist CGS (specific to A2A receptor) to T cells did not affect the subsequent T cell activation. In other words, A2AR does not mediate this desensitization effects, we propose that multiple P1Rs participate in the process. Following desensitization, the overall ability to sense adenosine (NECA) is reduced. Our results suggest that the overall adenosine feedback is blocked. This cross inhibition may have important biological implications in terms of total adenosine signaling via all P1Rs on T cells under high adenosine levels.

Our work points out one of the important mechanisms which regulates T cell activation in the high adenosine levels. This is in addition to adenosine deamination by ADA on T cells and other immune cells. We would like to argue that this short lived desensitization effect is necessary for T cell activation to overcome the initial hurdle of activation induction, while not interfering with the general protective effect of adenosine at resting times.

CHAPTER IV

CONCLUSIONS AND FUTURE STUDIES

4.1 Conclusions and Summary of this Study

In this study, we investigated the potential mechanism by which adenosine regulating T cell activation. The negative feedback caused by adenosine signaling has been reported by various groups. However, this feedback notion contradicts with the observation that T cells keep robust activation under high levels of adenosine during infection and injury. Our results point out one of the mechanisms that illustrate how T cells temporarily escape the general state of suppression mediated by adenosine. Prior to antigenic stimulation via TCR/CD3, exposure of T cells to adenosine desensitizes adenosine receptors, so as to create a window of where T cells are insensitive to this ubiquitous suppressor. This desensitization occurs at a post-transcriptional level and regulates T cell activation by mediating cAMP production.

Our works also point out that adenosine mediated immune cell suppression is not an invariable process. Under inflammation or injury condition, the stress leads to higher adenosine level. This high level of adenosine desensitized T cells adenosine receptor signaling cAMP. Following desensitization, the overall ability to sense adenosine is reduced and T cells keep a robust activation after encountering antigenic presentation. However, the desensitization effect tapers off rapidly. Adenosine regains its suppressive regulation of T cells, in order to protect T cells from over-reaction.

4.2 Future Studies

Our studies indicated that adenosine receptor desensitization might be one of important mechanisms for T cells to escape the general suppression during early points of T cell activation. Pre-exposure adenosine to T cells desensitizes T cells response to further adenosine. The desensitization effect tapers off rapidly. This observation suggests that in vivo, once adenosine levels are falling back to normal, T cells are under the protection of adenosine. However, local adenosine levels fluctuate in vivo. The relationship of local adenosine levels and adenosine inducing desensitization effect would be an important part in the future study, and this study will disclose more details in studying adenosine regulation of immune system.

One practical aspect of our work was the finding that T cells from mice that were pre-exposed to adenosine showed stronger responses to antigenic stimulation. The desensitization demonstrates a strong adjuvant like effect for CD8+ T cell. In immunology, an adjuvant is an agent that may stimulate the immune system and increase the response to a vaccine, without having any specific antigenic effect in itself. There are many known adjuvants in widespread use, including oils, aluminum salts, and virosomes, although precisely how they work is still not entirely understood. It will be a topic of future study whether adenosine priming effect, which is presumably safe and less invasive, will have any impact in vaccine development. Adenosine might emerge as a potential alternative for vaccine adjuvant.

In our study, one of the surprising findings is that A1 agonist and antagonist, which specific to A1 receptor in non-immune cells, are non-specific on T cells. On T cells, A1 agonist has the same effect as a pan-adenosine agonist NECA. Moreover, although A2A has been regarded as the predominant adenosine receptor on T cells, the desensitization is not mediated by A2A adenosine receptor. It is possible that there are some other adenosine receptors play major roles in regulating T cells. This possibility leads to the necessity to study how adenosine receptors are regulated on T cells. Because of the limitation of the reagents, we could not explore this possibility deeper in this project. The further study of adenosine receptors on T cell might be very important in disclosing the mechanism of adenosine regulation of T cells.

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Certification of Animal Protocol Approval

Applicant: Dr. Y. Shi

Faculty/Department: Microbiology and Infectious Diseases

Project Title: Endogenous immune regulators

Sponsoring Agency(s): Canadian Institutes of Health Research

Effective: January 19, 2010 Expires: January 31, 2011 (1st renewal date)

The Animal Care Committee,
having examined the animal care and treatment protocol,
approves the experimental procedures proposed and certifies
with the applicant that the care and treatment of animals
used will be in accordance with the principles
outlined in the most recent policies and
"Guide to the Care and Use of Experimental Animals"
By The Canadian Council on Animal Care.

Applicant — Date: Jan 19, 2010

Chair of Animal Care Committee or University Veterinarian — Date: Jan 26 2010

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