

**THE EFFECT OF INTERLEUKIN 7 ON CD8⁺T CELL
ACCUMULATION AND DIFFERENTIATION IN THE
PRESENCE OF ADENOSINE SIGNALING**

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By

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December 2019

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

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ABSTRACT

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Cytotoxic T cells are important cytotoxic adaptive immune cells. Their role is to check every single cell for protecting the body against microbial infections. They are also the main players for antigen-specific recognition and elimination of cancer cells. The ability of the cancer cells to inhibit those cells, is very important to diagnose the severity of the tumors. Therefore, tumor microenvironment analysis is the key factor that need to be taken account before intervention to the tumor. Stress, cell damage, and ischemia, often seen in the tumor microenvironment, cause the release and accumulation of adenosine in the extracellular space. Therefore, extracellular adenosine levels are higher in the tumor microenvironments than adjacent normal tissues. Adenosine can target four subtypes of G protein-coupled receptors; A1, A2A, A2B and A3 adenosine receptors. Adenosine signaling causes inhibition of immune cells from both innate and adaptive compartments through adenosine A2A and A2B receptors (A2AR and A2BR, respectively). A2AR is the main receptor subtype expressed by cytotoxic T cells. Interleukin 7 (IL-7) is a cytokine expressed by immune cells and stromal cells. IL-7 is important for the regulation of T cell maintenance and memory differentiation. Previous studies have shown that global or T cell-specific deletion of *Adora2a* (A2AR gene) may unexpectedly promote growth of syngeneic B16 melanoma, which is associated with reduced T cell accumulation and IL7 receptor alpha (CD127) expression in tumor associated T cells. In this study we tested the effect of adenosine signaling in the presence or absence of IL-7 or in some cases IL-2 on CD8⁺ T cell accumulation and differentiation in vitro. Adenosine signaling inhibited accumulation and IL-2 receptor expression of CD8⁺T cells while partially sustaining the

IL-7 receptor expression. Addition of IL-7 but not IL-2 to the cell culture completely reversed the adenosine-mediated reduction in T cell accumulation. In addition, adenosine signaling reduced the expression of T cell exhaustion marker PD-1 in the presence of IL7 but not IL-2. Finally, adenosine signaling differentiated CD8⁺T cells towards central memory phenotype rather than effector/effector memory phenotype. Addition of IL-7 or IL-2 did not change this effect. In summary, adenosine cross-talk with IL-7 is important for CD8 T cell phenotypic differentiation and accumulation, which may have important implications in in vivo conditions.

Keywords: CD8⁺T cells, adenosine, adenosine receptor, IL-7, IL-7 receptor.

ÖZET

İNERLÖKİN 7 VE ADENÖZİN YOLAKLARININ CD8⁺T HÜCREİ UYARILMA VE BASKILAMASINA ETKİLERİ

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Sitotoksik T hücreleri, önemli sitotoksik adaptif bağışıklık hücreleridir. Görevleri, vücudun her bir hücrelerini kontrol ederek mikrobik enfeksiyonlara karşı korumaktır.

Ayrıca antijenleri tanınmasında ve kanser hücrelerinin yok edilmesinde ana oyuncular. Kanser hücrelerinin bu hücreleri engelleme yeteneği, tümörlerin ciddiyetini teşhis etmek için çok önemlidir. Bu nedenle, tümör mikro çevresel analizi, tümöre müdahale etmeden önce dikkate alınması gereken kilit faktördür. Tümör mikro ortamında sıkça görülen stres, hücre hasarı ve iskemi, hücre dışı alanda adenoziin salınımı ve birikmesine neden olur. Bu nedenle, hücre dışı adenoziin seviyeleri tümör mikro-çevresine bitişik normal dokulara göre daha yüksektir. Adenoziin, dört farklı G protein kenetli reseptör alt tipini hedefleyebilir; A1, A2A, A2B ve A3 adenoziin reseptörleri. Adenoziin sinyal yolağı, A2A ve A2B reseptörleri yoluyla hem doğal hem de adaptif bağışıklık hücrelerini engellemesine neden olur. A2AR, sitotoksik T hücreleri tarafından ifade edilen ana reseptör alt tipidir. İnterlökün 7 (IL-7), bağışıklık hücreleri ve stroma hücreleri tarafından ifade edilen bir sitokindir. IL-7, T hücresi bakımı ve hafıza farklılaşmasının düzenlenmesi için önemlidir. Önceki çalışmalar, *Adora2a*'nın (A2AR geni) global veya T hücresine spesifik olarak silinmesinin beklenmedik bir şekilde, genetik olarak özdeş B16 melanomunun büyümesini destekleyebileceğini göstermiştir ve buna bağlantılı olarak tümörle ilişkili T hücrelerinde ve alfa IL-7 reseptörü (CD127) ekspresyonunda azalma gözlenmiştir. Bu çalışmada, IL-7 varlığında veya yokluğunda adenoziin sinyalizasyonunun

veya bazı durumlarda IL-2'nin, CD8⁺ T hücre birikimi ve in vitro farklılaşması üzerindeki etkisini test ettik. Adenozin sinyali, kısmen IL-7 reseptör ifadesini korurken CD8 + T hücrelerinin birikimini ve IL-2 reseptör ifadesini engelledi. IL-2'nin değil de IL-7'nin hücre kültürüne eklenmesi, adenozin etkisine bağlı T hücre birikimindeki azalmayı tamamen tersine çevirdi. Ek olarak, adenozin uyarımı, IL-7 varlığında T hücrelerinde tükenme markörü PD-1'in ekspresyonunu azalttı, ancak IL-2 varlığında azaltmadı. Son olarak, adenozin sinyali CD8⁺ T hücrelerini efektör / efektör bellek fenotipinden ziyade merkezi bellek fenotipine doğru farklılaştırdı. IL-7 veya IL-2'nin eklenmesi bu etkiyi değiştirmede. Özetle, IL-7 ile adenozin birlikteliği, in vivo koşullarda önemli etkileri olabilecek CD8⁺T hücresi fenotipik farklılaşması ve birikmesi için önemlidir.

Anahtar kelimeler: CD8⁺T hücreleri, adenozin, adenozin reseptörü, IL-7, IL-7 reseptörü

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ABBREVIATIONS

Ab	Antibody
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Antigen presenting cell
AR	Adenosine receptor
ATP	Adenosine triphosphate
cAMP	cyclic Adenosine monophosphate
CD	Cluster of differentiation
CREB	cAMP response element binding protein
DC	Dendritic cell
ddH ₂ O	Double-distilled water
dH ₂ O	Distilled water
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
HBSS	Hank's balanced salt solution
IgG	Immunoglobulin G
IL	Interleukin
MHC	Major histocompatibility complex
NK	Natural killer
PD-1	Programmed death 1

PI	Propidium Iodide
PI ₃ K	Phosphoinositide 3-kinase
PKB	Protein kinase B (also known as Akt)
RPMI	Roswell Park Memorial Institute
SA	Streptavidin
Tfh	Follicular helper T cells
TGF	Transforming growth factor
Th	T helper cell
Treg	Regulatory T cell

CHAPTER 1

INTRODUCTION

1.1 THE IMMUNE SYSTEM

Organisms are exposed to different kinds of microorganisms through our interactions with the environment such as eating, touching and respiration. The main duty of the immune system is to recognize the self and non-self and to distinguish pathogenic vs. nonpathogenic structures, with the ultimate aim to eliminate disease-causing infections. The immune system is very complex and has different components including different organs, antibodies, cells, proteins. This system can be divided into two parts: innate immunity and adaptive immunity. Innate and adaptive immune cells communicate with each other to generate robust responses to infections and to establish tolerance to protect against uncontrolled inflammation and autoimmune reactions. [1][2]

1.1.1 Innate immunity

The innate immune system is evolved earlier than the adaptive immune system. Besides physical barriers innate immune system is the only host defense system of non-vertebrates. Pathogens, which are able to intrude physical barriers, will first encounter innate immune response [3].

The innate immune system is not a specialized to recognize specific antigens. They can recognize specific patterns on potential pathogens. Therefore, innate immune reactions

can occur rapidly within seconds or minutes. This is partly due to the fact that effector molecules and cytokines can be stored in innate immune cells and released rapidly upon recognition of a potential pathogen. Major innate-immune cell types are antigen presenting cells (macrophages and dendritic cells), natural killer cells and granulocytes (neutrophils, eosinophils and basophils). Antigen presenting cells can engulf pathogens; present antigens to adaptive cells; produce cytokine and chemokines to activate other immune cells and attract them to the sites of infection. Among antigen presenting cells dendritic cells are more specialized on antigen presentation. Natural killer cells recognize and kill infected cells to prevent spread of pathogens. Neutrophils where their number increase and decrease rapidly during infection, are important in phagocytosis, chemotaxis and production of anti-microbial peptides. Eosinophil and basophils are important in cytokines production and shaping the inflammatory milieu [4][5].

1.1.2 Adaptive immunity

After innate immune response to slow down the pathogen propagation, the adaptive immune system gets activated. Although it takes more time for adaptive immune cells to recognize pathogens and to reach enough numbers to eliminate the infection adaptive immune cells can recognize pathogens more accurately than innate immune cells. Also, when re-encountering a pathogen expressing an antigen, which was recognized by adaptive immune system before, adaptive immune cells act faster than the first encounter. This due to the fact that some of the adaptive immune cells can differentiate into long lasting memory cells. Therefore, reoccurring illnesses are less severe and sometimes asymptomatic [6].

Those cells that has never encountered a pathogen are called naive cells. To develop and mature the lymphocytic cells, the antigen presenting cells which are dendritic cells, macrophage cells, and B cells take part. The phenomenon is happening in the induction site like secondary lymphoid organs [7].

T and B lymphocytes are the white blood cells that play the major role in adaptive immunity. The adaptive immunity is divided into two: cell-mediated and humoral (antibody-mediated). T cells play the major role for cell-mediated adaptive immune

responses while B cells are the antibody-producers. Antibodies produced by B cells can interact directly with the pathogen by binding to their surface and blocking them or by tagging them to signal phagocytic cells to eliminate them. However, T cells attack indirectly to the pathogen, they rather kill the cells that are infected with the pathogen or might contain the pathogen [6].

1.1.2.1 The T lymphocytes

In human and rodents $CD4^+$ T cells and $CD8^+$ T cells are the major T cell subtypes. They both mature in the thymus where their name came from. Those two types of cells are characterized according the surface antigen on their cell surface. The roles they play during adaptive immune responses are different.

The main function of $CD4^+$ T cells is to help other immune cells to generate a specific type of immune response; hence they are also named helper T cells. $CD4^+$ T cells have many subtypes with unique functions such as promoting humoral immune response, inducing immunological tolerance and causing chronic inflammation. $CD4^+$ T cells perform these functions by secreting unique cytokines and chemokines [8][9].

$CD4^+$ T cells polarize to T-helper 1 (Th1) subtype, in the presence of interleukin 12 (IL12) during antigenic stimulation. This subtype of $CD4^+$ T cells are important to fight against intracellular pathogens by producing cytokines such as lymphotoxin and IFN- γ . IFN- γ can have pleotropic effects such as stimulation of IL-12 production by antigen presenting cells as a positive feedback, increased antigen presentation by infected cells and antigen presenting cells and inhibition of proliferation of infected cells. All these events help cytotoxic natural killer cells and $CD8^+$ T cells to identify and to kill infected cells to limit the spread of invading pathogen.

T-helper 2 cells (Th2), which develop in the presence of interleukin 4 (IL-4), are important in fighting against extracellular parasites by the production of cytokines IL-4, 5, 13, which stimulates eosinophil, mast cells [10][11].

T helper-17 cells (Th17), which develops in the presence of IL-6, TGF β and IL-1 β , produce cytokine IL-17 and promote chronic inflammation. Th17 response is important to fight against persistent infections by microorganisms such as bacteria or fungi.

CD4⁺T cells polarize into regulatory subset called regulatory T cells (Treg) in the presence of TGF β . Tregs are important to protect the body from chronic inflammation and autoimmune reactions due to the recognition of cell-antigens.

Follicular helper T cells (Tfh) are important in formation of germinal centers where they promote humoral immunity by helping B cells produce high affinity antibodies [8].

CD8⁺T cells are also called cytotoxic T lymphocytes. As the name implies CD8⁺T can recognize target cells (infected cells or cancer cells with neoantigens) by antigen-specific manner and kill them. Activated CD8⁺T cells can directly interact with target cells through Fas/FasL interactions to induce apoptosis in these cells. They can also secrete granzymes and perforins that can form holes in the targets cells and cause programmed cell death by proteolytic cleavage of caspases. CD8⁺T cells can also produce cytokine such as IFN- γ to activate antigen-presenting cells and IL-10 to resolve detrimental inflammation [9][12].

1.1.2.1.1 Activation of T lymphocytes

T cells can bind to their antigen only when it is present on the surface of cells. The antigen presentation come from intracellular pathogens or pathogens that are endocytosed from outside the cells. The molecule that present antigens to T cells are called major histocompatibility complex (MHC). T cells bind to the antigen, presented by MHC, by their T cell receptor (TCR). TCRs are in complex with co-receptors called CD3 which contain 3 homodimers of ϵ , γ , δ chains and with ζ chain; these protein structures have a motif called immunoreceptor tyrosine-based activation motif (ITAM). When TCR binds to engages MHC-antigen complex the ITAMs become phosphorylated by Src family proteins, Fyn and Lck. After the phosphorylation the ITAMs become a binding target for tyrosine kinase ZAP-70, which in turn when bond to ITAMs, become phosphorylated by Lck. The activation of ZAP-70 lets the phosphorylation of LAT (linker of activated T cells) and SLP-76 which the later ones are together a binding site for phospholipase C- γ

(PLC- γ). PLC- γ catalyzes the formation of IP₃ and DAG from PIP₂. Those two molecules IP₃ increases cytoplasmic Ca²⁺ for and DAG activates protein kinase C and RasGRP. Ca²⁺ influx, PKC activation and activation of RasGRP cause the activation and nuclear translocation of transcription factors, NFAT, NF- κ B and AP-1 (figure1.1) [13][14]. These transcription factors turn on the target genes associated with T cell activation and effector function. Another important receptor in T cells is CD28 which is a co-stimulatory receptor that further strengthen the effects of TCR signaling. CD28 activation requires engagement of this molecule with CD80 and/or CD86 on antigen presenting cells. CD28 activates PI3 kinase pathway. This kinase turn phosphorylates PIP₂ to PIP₃. PIP₃ formation on cell membrane leads to the activation of Akt. Akt promotes T cell proliferation and survival after activation [13].

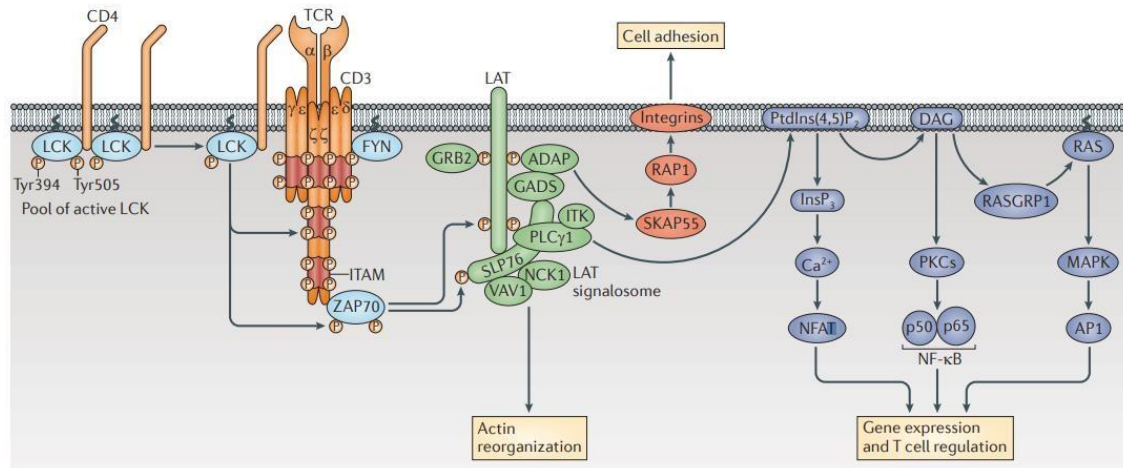


Figure1.1: The TCR signaling pathway of T cells [14]

1.1.3 Immunotherapy

Immune cells have the ability to recognize neoplastic growth and formation of new antigens (neoantigens) expressed by mutated endogenous genes. Neoantigens on tumor cells are presented to CD8⁺T cells directly or through antigen cross-presentation by antigen presenting cells. Therefore, they are one of the major players to fight cancer. Diagnosing the tumor responsiveness to immunotherapy by testing the immune cell/CD8⁺ T cell density became a very important key characterization of cancer tissues. In recent studies 4 major subtypes were proposed according to tumor immune cell profiling. Hot tumors basically are the ones enriched with immune cells, especially cytotoxic T cells whereas cold tumors are the ones with very few immune cells. Altered-immunosuppressed immune tumors are the ones where the immune cells are present but not functional, altered-excluded immune tumors are the ones where the immune cells cannot enter the tumors but they can accumulate around the tumors. Immune exclusion is achieved by the accumulation of stromal cells and extracellular matrix components that form physical barriers and constrict blood vessels and lymphatic vessels to prevent immune cell extravasation [15].

1.2 PURINERGIC SIGNALING

Purines have a significant role in the body as part of genetic material; DNA or RNA; energy currency, ATP or second messengers such as cAMP or GTP/GDP. ATP and backbone of ATP, adenosine, also has a signaling function, which plays an important role in nervous system, cardiovascular system and immune system [16].

The receptors involved in this purinergic signal can be grouped into 2: P1receptors with a family of 4 receptor subtypes and P2 receptors, which is divided into two subgroups P2X and P2Y. P2X subgroup contains 7 receptor subtypes and P2Y subgroup has 8 receptor subtypes[17].

The P2X receptors are ion channels. They are expressed throughout the body. ATP is the main ligand for P2X receptors. P2X receptors regulates smooth muscle contraction, cardiac rhythm, platelet aggregation, and help macrophages sense danger by activation of

a complex called inflammasome. Inflammasome activation is important for generation of inflammatory IL1 β [18][19].

The P2Y receptors are G coupled receptors that bind various nucleotides. P2Y receptors can regulate vasodilation, platelet aggregation, chemotaxis and activation of antigen presenting cells and neutrophils, secretion of water and chloride [19].

1.2.1 Adenosine signaling

Extracellular adenosine concentrations can be different in different tissues and also further changes in pathological conditions. Adenosine directly move from intracellular space to extracellular space from dead or damaged cells or through nucleotide transporters through facilitated diffusion. One major source of adenosine accumulation is hydrolysis of extracellular ATP by ecto-nucleotidases such as CD39 and CD73.

The adenosine outside of the cell can act as a local signal. Adenosine is important to protect cells and tissues from stress. This protection is achieved by adenosine by: 1. augmenting the percentage of oxygen in conditions where the cells need it, 2. vasodilation when there is limited blood supply, 3. formation of new blood or lymphatic vessels again to increase blood supply and tissue repair, 4. inhibiting inflammatory responses and autoimmune reactions to prevent tissue damage.

Adenosine is the natural ligand for P1 receptors. As mentioned earlier P1 receptors consists of 4 subtypes: adenosine A1, A2A, A2B and A3 receptors. P1 receptors are G protein-coupled receptors. A1 and A3 receptors are coupled with Gi subunit and their activation leads to decreased cAMP. A2A and A2B receptors are Gs coupled; therefore, their activation leads to accumulation of cAMP. Major intracellular receptors for cAMP are protein kinase A and Epac molecules. These molecules can further influence activation of transcription factors and mitogen-activated protein kinases (MAPKs) such as CREB and ERK, respectively. A1, A2A and A3 receptors have higher affinities for adenosine as compared with A2B receptors. Besides regulating cAMP through Gi or Gs coupling, A1 and A2B receptors can also couple Gq subunit. Along with G α and G $\beta\gamma$ signaling, activation of these receptors can also lead to accumulation of Ca²⁺ and activation of

MAPKs such as p38[20][21][22][23]. A1 plays important roles in chemotaxis of immune cells, especially neutrophils while A2A and A2B receptors are important in resolution of excessive inflammation. A2B receptors also promote angiogenic inflammation and tissue healing processes. A3 receptors along with A2B receptors regulates mast cell degranulation [24].

The Figure 1.2 below shows the pathways used by different receptors.

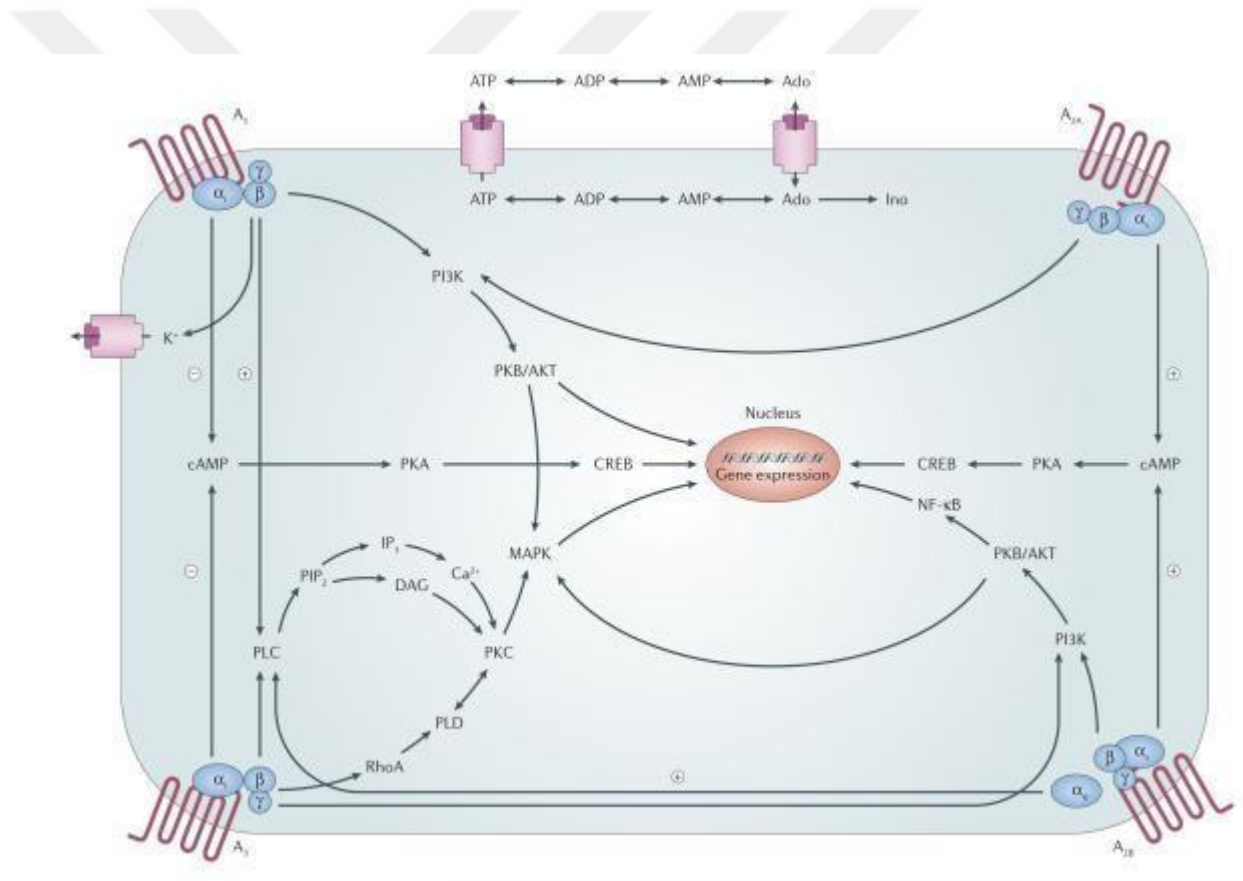


Figure 1.2: The adenosine signaling pathways used by the four classes of Ars [21].

1.2.2 The effect of adenosine signaling on T lymphocytes

The A2A receptors (A2AR) are the most abundant adenosine receptors in T lymphocytes. Accumulation of cAMP downstream of A2AR signaling can cause inhibition of TCR/CD28-induced stimulation phosphatidylinositide 3-kinase (PI3K)-AKT pathway, leading to decreased T cell proliferation and activation. A2AR stimulation have also shown to cause hypo-phosphorylation of TCR components. Interestingly TCR stimulation and Akt activation can cause down regulation of IL-7 receptor (CD127) while causing upregulation of IL-2 receptor (CD25). Strongly immunogenic tumors are more effectively rejected in A2AR-deficient mice, potentially due to the fact that T cells are more stimulated in these mice and received enough survival signals from their environment. However, certain tumors such as B16 melanoma or MB49 bladder carcinoma grows more rapidly in A2AR^{-/-} mice, which is associated with reduced T cell accumulation and strong IL-7R down regulation. Because IL-7 plays an important role in naïve and memory T cell maintenance there could be a potential link between reduced T cell accumulation and strong IL-7 receptor down regulation in the absence of A2AR signaling, which is poorly understood. [25][26][27].

1.3 AIM OF THE STUDY

Immunotherapeutic intervention to cancer has emerge as a promising area. Further characterization of potential interactions in the tumor microenvironment it is required to find novel strategies for cancer therapy. [15]. Adenosine is one of the hallmarks of the tumor microenvironment that can regulate both innate and adaptive immune responses. A2A receptors are the main adenosine receptors on CD8⁺T cells, A2AR signaling suppress CD8⁺T cell activation [28]. However, T cells can infiltrate tumors despite increased adenosine concentrations and blockade of A2AR signaling in clinic produce limited success for the control of tumor growth, suggesting depending on the cytokine milieu adenosine signaling may cause pleotropic effects on T cell activation and phenotypic

differentiation. One such interaction may stem from adenosine regulation of IL-7 signaling. Therefore, in this study the effect of the interaction between adenosine signaling and IL-7 signaling on CD8 T cells has been investigated [25][28].



CHAPTER 2

MATERIALS, SOLUTIONS and BUFFERS

2.1 CELL CULTURE REAGENTS AND BUFFERS

Table1.1: The list of the reagents and buffers used in cell culture media

Product	Cat. No.	Brand
RPMI 1640 Medium	21875-034	Gibco
Fetal Bovine Serum (FBS)	S181G-500	Biowest
Sodium Pyruvate (100 mM)	S8636	Sigma-Aldrich
Penicillin/Streptomycin	17-745E	Lonza
HBSS	10-547F	Lonza
HEPES Solution	H0887-100ML	Sigma-Aldrich
β -mercaptoethanol		AppliChem

2.2 T CELL ACTIVATION/STIMULATION MATERIALS

Table2.1: List of the cytokines and antibodies used to activate or stimulate naive CD8⁺T cells.

Product	Cat. No.	Brand
LEAF™ Purified Anti-mouse CD28	102112	BioLegend
LEAF™ Purified Anti-mouse CD3ε	100314	BioLegend
Goat IgG Fraction to Hamster IgG	55397	MP
Recombinant mouse IL-7	577802	BioLegend
Recombinant Mouse IL-2 (carrier-free)	21-8021-U005	Tonbobio

Table2.2: The list of the reagents used during T cells stimulation.

Product	Cat.No.	Brand
Adenosine Deaminase (ADA)	10102105001	Roche
NECA	35920-39-9	Tocris

2.3 CONSUMABLES AND GENERAL MATERIALS

Table2.3: The list of the consumables and general materials.

Product	Cat. No.	Brand
Filter tip p1000	TF-1000-R-S	Corning
Filter tip p200	TF-200-R-S	Corning
Filter tip p20	TF-20-R-S	Corning

Filter tip p10	T-300	Corning
Reaction tube 2 mL	MCT-200C	Corning
Reaction tube, 1.5 mL	MCT-150C	Corning
Reaction tube, 0.5 ml	PCR-05C	Corning
15 mL falcon tube	62.554.502.500	Sarstedt
50 mL falcon tube	62547254	Sarstedt
5 mL stripettes	4487	Corning
10 mL stripettes	4488	Corning
25 mL stripettes	4489	Corning
96 well plates	83.3920-005	Sarstedt
48 well plates	83.3923-005	Sarstedt
24 well plates	83.3922-50	Sarstedt
12 well plates	83.3921-50	Sarstedt
6 well plates	83.3920-50	Sarstedt
Minisart Syringe Filter pore size 0.2µm	16534-K	Sartorius
LightCycler 480 Multiwell plates 96, White	04729692001	Roche Molecular Systems
Nunc 96-well Polystyrene Conical Bottom plates	249944	Thermo Scientific
AccuGENE molecular biology water	51200	Lonza
EasySep Immunomagnetic Cell Separation equipment	18000	Stemcell
EasySep Mouse CD8 ⁺ T Cell Isolation Kit	19853A	Stemcell

2.4 EQUIPMENT

The flow cytometry used was CytoFlex purchased from Beckman Coulter. The analysis of raw data was done by GraphPad Prism 8.

2.5 FLOW CYTOMETRY ANTIBODIES AND CHEMICALS

Table2.4: The list of antibodies and chemicals used in Flow Cytometry

Product	Cat. No.	Brand
LIVE/DEAD Fixable Green Dead Cell Stain Kit	L34970	Thermo Scientific
Biotin anti-mouse/human CD44 antibody	103004	Biolegend
CD16/32 (Fc Block)	14-061-85	eBiosciences
CD4 PerCpCy5.5	65-0041-U100	Tonbobio
CD8a APC-Cyanine7	25-0081-U100	Tonbobio
Brilliant Violet 421 anti- mouse CD25	102034	Biolegend
Alexa Fluor 647 anti-mouse CD127	135020	Biolegend
APC anti-mouse CD127	135012	Biolegend
Anti-mouse CD25 PerCP- Cy5.5	45-0251-82	eBioscience
Brilliant Violet 421 anti- mouse CD185(CXCR5)	14551	Biolegend
CD69-APC	-	-

CD62L-PE	-	-
Sodium azide (NaN ₃)	71289-5G	Sigma
Streptavidin – BV510	563261	BD
PE/Cy7 anti-mouse CD39	143806	Biolegend
PE conjugated anti-mouse CD127(IL-7Ra)	12-1271-83	eBiosciences
PE/Cy7 anti-mouse CD279(PD-1)	109110	Biolegend
Anti-mouse TIM3 APC	17-5871-82	eBiosciences

2.6 CELL CULTURE MEDIA

- 10% FBS
- 0.11 mg/mL Na Pyruvate
- 50 g/mL Streptomycin/Penicillin
- 500 mL RPMI-1640 medium
- Storage temperature 4°C

2.7 FLOW CYTOMETRY BUFFER

- 2% FBS
- 0.05% NaN₃
- Ingredient dissolved in HBSS
- Storage temperature 4°C

2.8 LYMPH NODES AND SPLEEN COLLECTION BUFFER

- 2% of FBS dissolved in HBSS
- Storage temperature 4°C

CHAPTER 3

METHODS

3.1 CD8⁺ T CELL ISOLATION

Spleens of C57BL/6 mice were isolated and put into 2% FBS containing HBSS. Then the organs are mashed by the help of syringe and strained using 40 µm cell strainer. The single cell suspension is collected in a 50 mL falcon, then the cells are washed by HBSS and centrifuged at 300g for 5 mins. The pellet is resuspended in 10% FBS containing HBSS. Then 1×10^8 cells are taken into round bottom 5 ml polystyrene tube. CD8⁺ naïve T cell isolation is done according to the protocol of the kit (EasySep Mouse CD8⁺ T Cell Isolation Kit, Cat. no: 19853A). Firstly, Normal Rat Serum is added to the cells (50 µl for 1 ml of volume), later 50 µl/ml isolation cocktail is added with 3 µl of CD44 Biotin antibody. After waiting for 10 min at RT, 125 µl/ml of vortexed RapidSpheres are added and incubated for 5 min at RT. After the volume is completed to 2.5 ml with 10% FBS containing HBSS, the tube is placed into the magnet for 2.5 min. Then the supernatant is collected which contains the CD8⁺ T cells.

3.2 STIMULATION OF CD8⁺T CELLS

The protocol is written below and the groups are described in Table 3.5 and Table 3.6

Firstly, 96 well plate is coated by 50µL goat anti-hamster IgG (50µg/ml conc.) for each well. Then the plate is placed on a shaker for 2 hours at RT. After 2 hours, aspirate IgG and wash the wells with PBS, later wells on the plate are blocked by using cell culture medium for 10-15 min. Then, the medium is discarded and 75µL fresh medium containing the cytokines or molecules required are added to the wells according to the groups that are described in Table 3.5. Then, 30.000 cells per well is added in 75µL medium (in the experiment the total volume was 150µl and half was the medium containing the molecules and half was the medium contain cells). Then the plate is centrifuged at 1000-1100 rpm for 2.5 min at low break. Then, the cells in plate are incubated for 2 days in the 37°C incubator. After 2 days take 120µL from each well and add 120µL fresh media to each well that contain the molecules necessary described in Figure 3.6. And, incubate the cells for 2 days. All of the conditions have 3 replicas.

Table 3.5: Medium conditions used for the stimulation of CD8⁺T cells for Day 0-2

Groups	Condition
VC	Only media
group 1	CD3/CD28 con. 1µg/ml
group 2	CD3/CD28 con. 1µg/ml
group 3	CD3/CD28 con. 1µg/ml + IL-7 con. 10ng/mL
group 4	CD3/CD28 con. 1µg/ml + Neca con. 1µM
group 5	CD3/CD28 con. 1µg/ml + Neca con. 1µM
group 6	CD3/CD28 con. 1µg/ml + IL-7 con. 10ng/mL + Neca con. 1µM

Table 3.6: Medium conditions used for the stimulation of CD8⁺T cells for Day 2-4

Groups	Condition
VC	Only media
group 1	Only media
group 2	IL-2 con. 20ng/ml
group 3	IL-7 con. 10ng/mL
group 4	Neca con. 1μM
group 5	Neca con. 1μM + IL-2 con. 20ng/ml
group 6	IL-7 con. 10ng/mL+ Neca con. 1μM

3.3 FLOW CYTOMETRY

After the stimulation of CD8⁺T cells, they were transferred to Nunc™ 96 Well Polypropylene MicroWell™ Plates (Cat. No: 249944). Later, the cells were washed with cold HBSS and then with cold FACS buffer and centrifuged at 300g for 5 min. Later the cells were incubated with 25μL Fc block antibody (1:200) for 5 min and then 25μL antibody cocktail (1:200) is added and incubated for 30 min on ice at dark. After 30 min, the cells are washed with FACS buffer. Since, there is biotinylated antibody in the cocktail, the cells are incubated with 25μL streptavidin (1:400) for another 30 min on ice at dark. The cells are washed and dissolved with FACS buffer. Then the cells are analyzed with Flow Cytometry.

CHAPTER 4

RESULTS

4.1 ADENOSINE SIGNALING EFFECT ON CD8⁺T CELLS

A2AR is the main adenosine receptor subtype expressed in T cells. A2AR is important for the development and maintenance of T cells [25]. In this study we enriched naïve (CD8⁺CD44^{dim}) CD8⁺ T cells and exposed to NECA (stable, cell-impermeable analog of adenosine) before activation with anti-CD3 and anti-CD28 antibodies. For some groups we added IL-7 or IL-2 as well to test the interaction of these cytokines with adenosine signaling. Groups that did not receive NECA were used as positive control. Unstimulated T cells exposed to vehicle were used as negative control. We tested T cell accumulation and several key cell-surface markers to test the differentiation and activation of cultured cells (figure4.1).

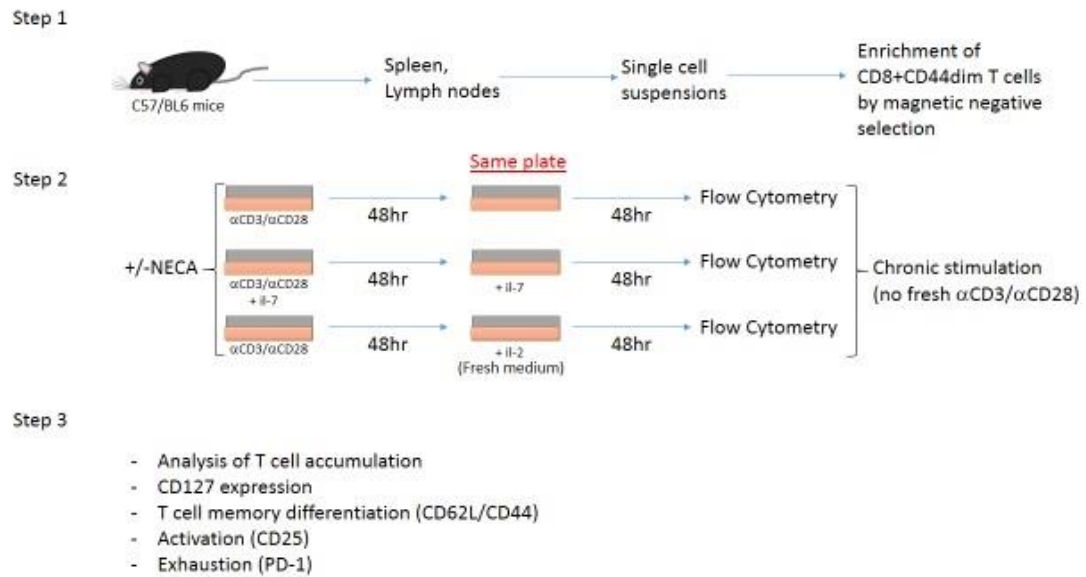


Figure 4.1 Graphical representation of experimental approach.

4.1.1 The effect of adenosine signaling on CD8⁺T cells accumulation and activation

Enriched CD8⁺T cells that were activated with, anti-CD3 and anti-CD28 in 96-well tissue culture plates. More numbers of T cells were accumulated (cell/ μ L) when compared with the control group, which were not stimulated with anti-CD3/anti-CD28. In the presence of NECA T cells accumulation substantially decreased (Figure 4.2).

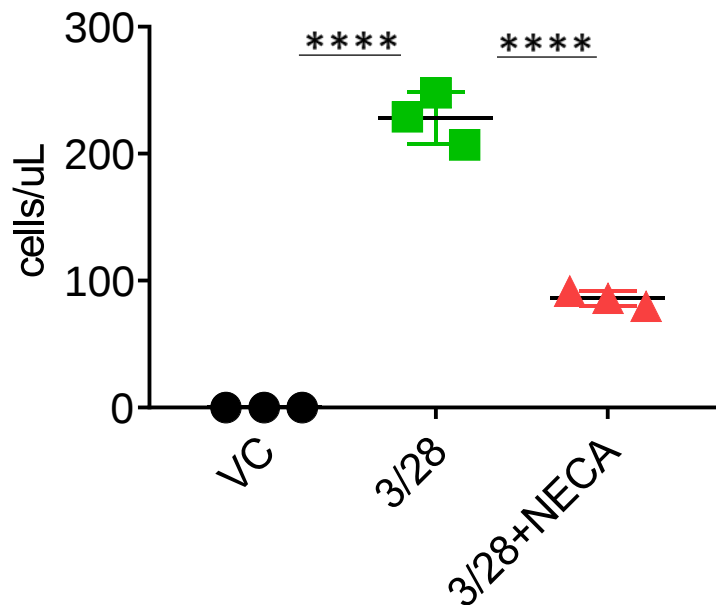


Figure 4.2 Adenosine signaling inhibits CD8⁺T cells accumulation in vitro. Flow staining was performed to find the cell density (cell/ μ l). VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1 μ M). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. **** $p \leq 0.0001$)

The effect of adenosine signaling on T cell effector differentiation was also investigated by testing the proportion of T cells expression CD44 and CD25 as activation markers. Figure 4.3 and figure 4.4 shows that once CD8⁺T cells were activated by the help of anti-CD3 and anti-CD28 antibodies proportion of CD25⁺CD44^{high} cells was substantially increased as compared to vehicle. Addition of NECA strongly suppressed the increase in CD25 expression. Collectively these results indicate that adenosine signaling suppresses T cells when stimulated with anti-CD3 and anti-CD28 in vitro.

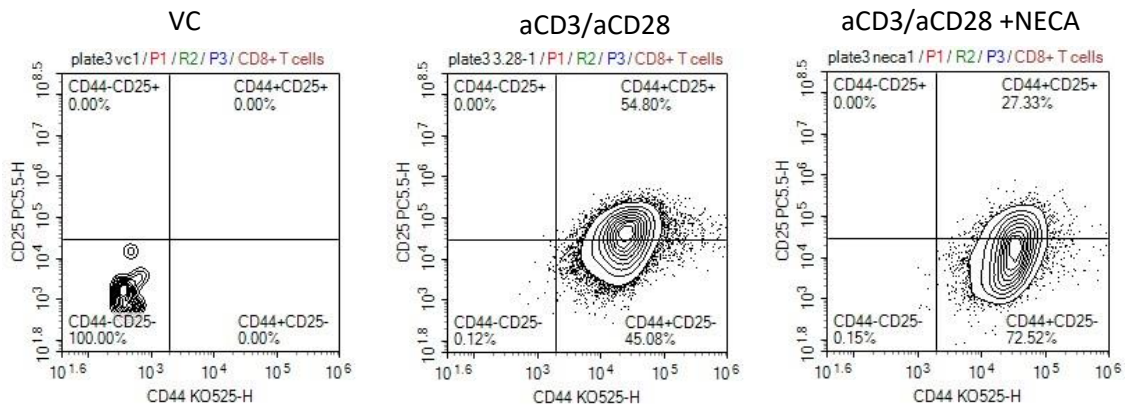


Figure 4.3: Adenosine signaling decrease the activation of CD8⁺T. Flow staining was performed to stain surface marker CD25 and CD44. VC group represent the naïve CD8⁺T activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1 μ M). One U/mL adenosine deaminase was added to the culture to prevent the

effect of endogenously generated adenosine (representative of one of three experiments with similar results).

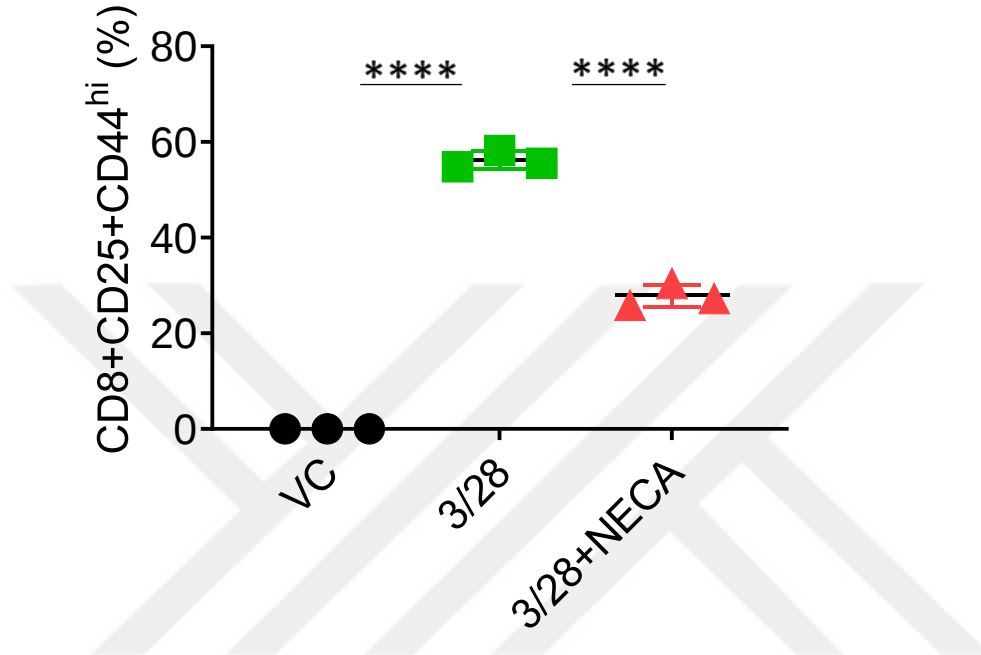


Figure 4.4: The adenosine signaling effect on CD8⁺ T cells activation. Flow staining was performed to find the cell density (cell/ μ l). VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1 μ M). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (Representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. **** $p \leq 0.0001$)

4.1.2 The effect of adenosine on the expression of surface IL-7 receptor in CD8⁺T cells

The effect of adenosine signaling as shown in figure 4.2 and 4.3, is reduction in T cell accumulation and activation respectively. Yet another effect of it was found to sustain IL7 receptor expression on the surface of the cell. Since activation of CD8⁺T cells decrease the cells IL-7 receptor expression, this effect was diminished by stimulating those cells with NECA. The data showing the result is in figure 4.5.

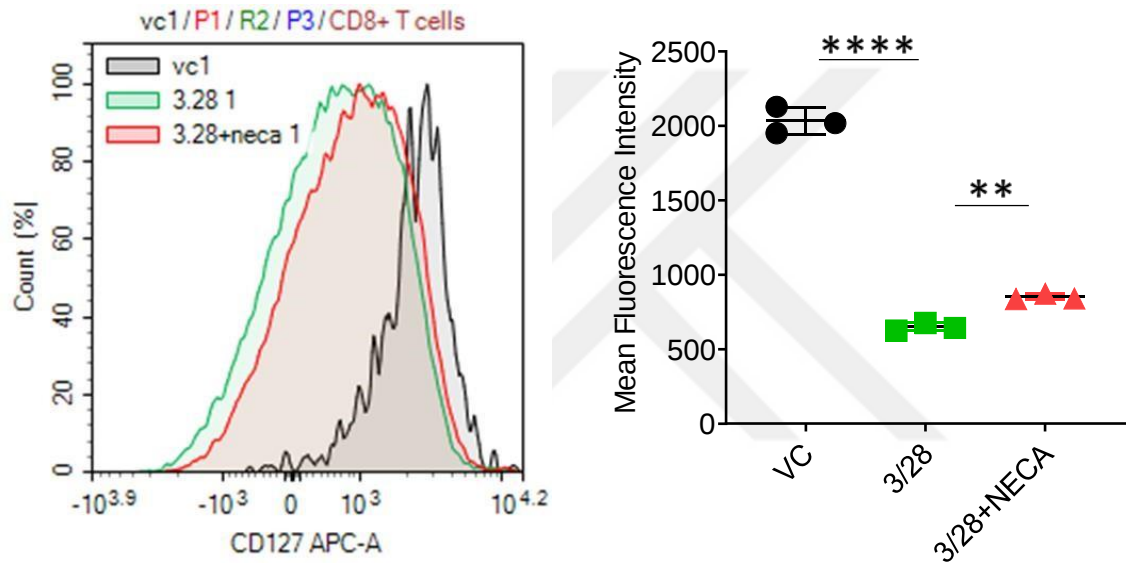


Figure 4.5: Adenosine signaling sustained the IL-7 expression on CD8⁺T cells. Flow staining was performed to stain surface marker IL-7 and the results were analyzed with MFI rates. VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1 μ M). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (Representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. ** $p \leq 0.01$, **** $p \leq 0.0001$)

4.1.3 The effect of IL-7 receptor signaling on NECA stimulated CD8⁺T cells

IL-7 is important not only for naïve T cell development and survival but also survival of long-lived effector T cells and memory T cells [29]. Adenosine signaling decreased IL-2 receptor expression while maintaining IL-7 receptor expression after CD8⁺ T cell stimulation. IL-7 is constitutively expressed by immune and non-immune cells while IL-2 is highly elevated only after strong activation of T cells, suggesting presence of IL-7 may contribute to the resistance of T cells to adenosine suppression in conditions where IL-7/IL-2 ratio is higher. Therefore, we investigated the effect of IL-7 or IL-2 on CD8⁺ T cell responses in the presence of adenosine signaling.

Addition of IL-7 cytokine during CD8⁺ T cell stimulation completely reversed NECA mediated inhibition of accumulation. However, IL-2 could not prevent NECA-mediated decrease in accumulation, suggesting IL-7 but not IL-2 could protect CD8⁺ T cells from adenosine-mediated inhibition of accumulation, which is well-correlated with the effect of adenosine signaling on these growth factor receptors after T cell stimulation.

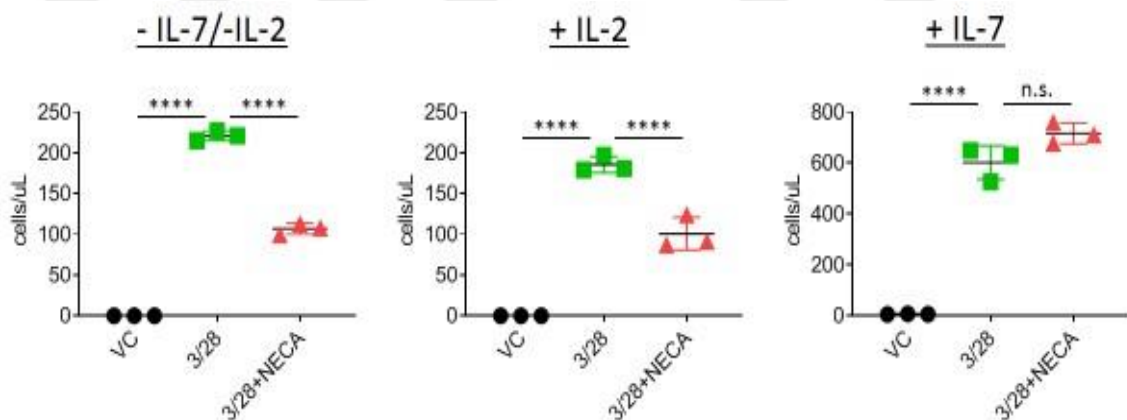


Figure 4.6: IL-7 and IL-2 effect on NECA stimulated CD8⁺T cells. Flow staining was performed to find the cell density (cell/μl). VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1μM). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously

generated adenosine (representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. **** $p \leq 0.0001$)

4.1.5 The effect of adenosine signaling on exhaustion marker PD-1

Naïve $CD8^+$ T cells when cultured in vitro in the presence of activation antibodies, CD3 and CD28, they become exhausted and begin to express the PD-1 (programmed cell death1) receptor. The role of this receptor is to suppress T cell activation [30]. This receptor is expressed generally at the late stage of differentiation. Binding of PD-1 to PD-L1 and PDL2 expressed on antigen presenting cells or cancer cells inhibits T cell effector function by suppressing division, cytokines production, and cytotoxic activity [31][32].

PD-1 is upregulated in stimulated $CD8^+$ T cells and addition of NECA to the cell culture inhibits PD-1 expression (figure 4.7).

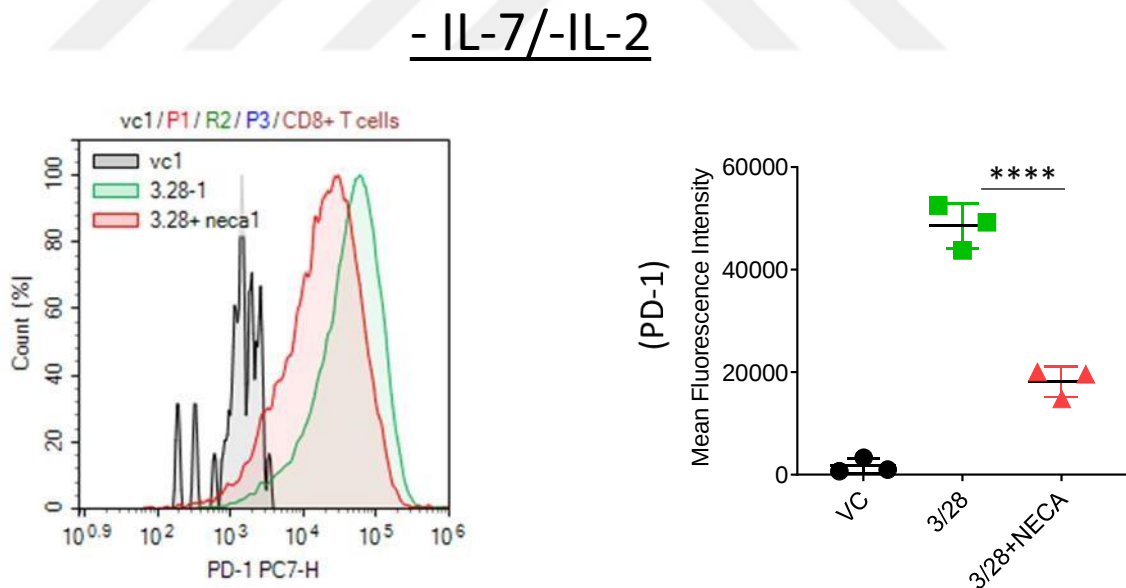


Figure 4.7: Adenosine signaling decrease the expression of exhaustion marker PD-1 on $CD8^+$ T cells. Flow staining was performed to stain surface marker PD-1 and the results were analyzed with MFI rates. VC group represent the naïve $CD8^+$ T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the $CD8^+$ T cells activated by antiCD3 and anti-CD28 antibodies, 3/28+NECA group represent the $CD8^+$ T cells that

are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1 μ M). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. **** $p \leq 0.0001$)

Addition of IL-7 sustained the NECA-induced suppression of PD-1 expression (figure 4.8). However, in the presence of IL-2, NECA did not change PD-1 expression (figure 4.9), suggesting cytokine milieu may influence T cell exhaustion in the presence of adenosine.

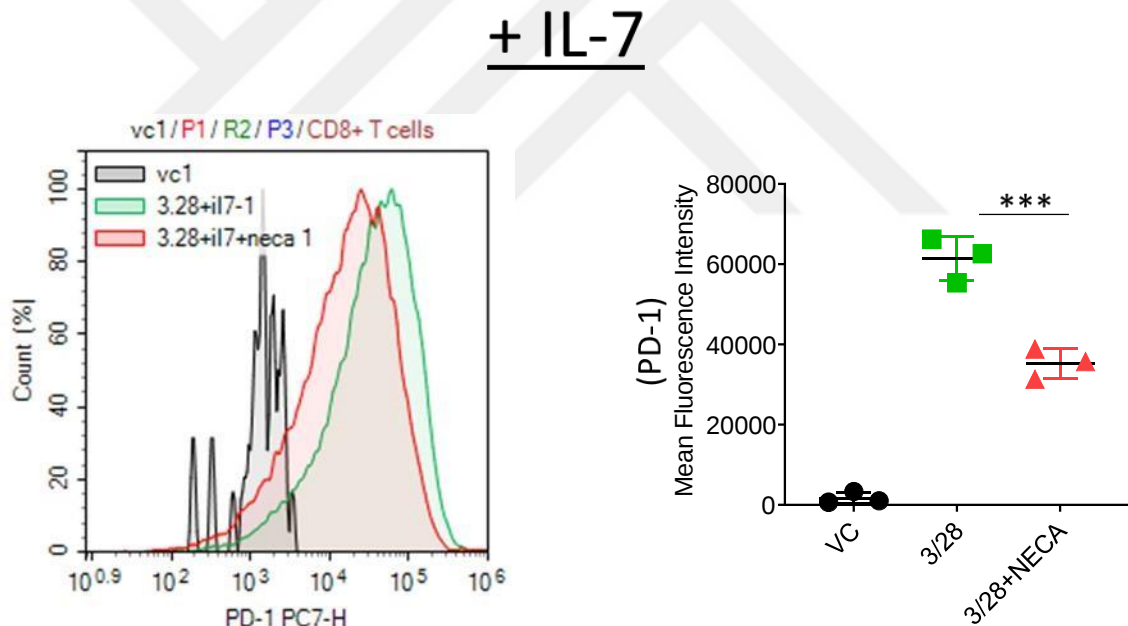


Figure 4.8: IL-7 has little effect on PD-1 expression of NECA stimulated CD8⁺T cells.

Flow staining was performed to stain surface marker PD-1 and the results were analyzed with MFI rates. VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1 μ M). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (representative of one of three experiments with similar results. One-way

ANOVA with Post-hoc Tukey's test was used for statistical analysis. *** $p \leq 0.001$)

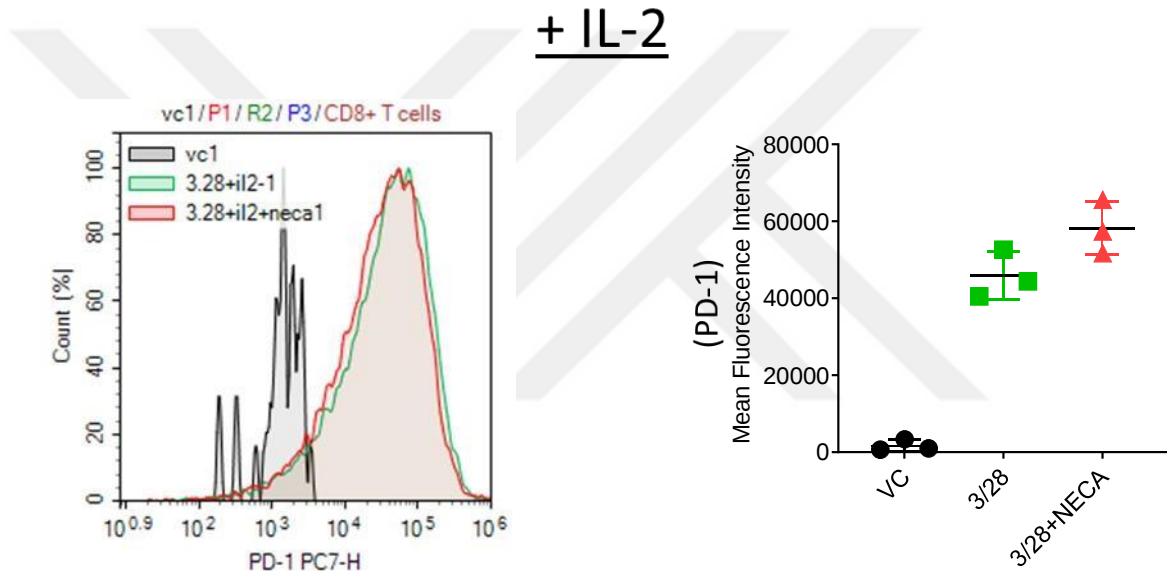


Figure 4.9: IL-2 has strong effect on PD-1 expression of NECA stimulated CD8⁺T cells. Flow staining was performed to stain surface marker PD-1 and the results were analyzed with MFI rates. VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1 μ M). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis)

4.1.6 The effect of adenosine signaling towards polarizing activated CD8⁺T cells to effector or central memory cells

The long-lasting antigen-specific protection against pathogens depends on differentiation of T and B lymphocytes into memory. When the body experience a pathogen that was previously encountered memory cells have the ability to react faster, more effectively and more strongly as compared with naïve cells. Therefore, we tested if adenosine signaling plays a role in memory differentiation of CD8⁺ T cells. There are two major memory T cell compartments: Effector memory and central memory. Effector memory cells (CD62L^{low}CD44^{high}) can respond to infections very quickly and very strongly; however, they can get exhausted and lose their self-renewal capacity. Central memory cells expressing high CD62L and CD44, have the capacity to self-renew and have resistance to apoptosis due to exhaustion or activation-induced death [33].

The figure 4.10 shows the expression of CD62L and CD44 in naïve CD8⁺T cells. It can be seen that naïve cells express high CD62L and low CD44.

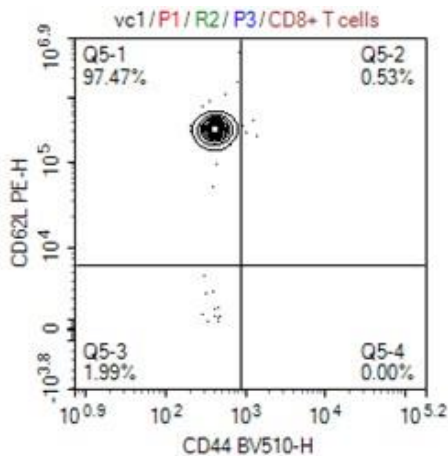


Figure 4.10: The expression of CD62L and CD44 on naïve CD8⁺T cells. *The cells were ADA pretreated and cultured without any stimulation. Flow data staining done according CD62L and CD44.*

When CD8⁺T cells were stimulated they began to differentiate mostly to effector/effector memory T cells, expressing low CD62L and high CD44 surface markers. In the presence of NECA proportion of T cells with central memory (CD62L^{high}CD44^{high}) increased (figure 4.11). Addition of IL-7 (figure 4.12) or IL-2 (figure 4.13) did not influence the NECA-mediated increases in the proportions of central memory cells, suggesting that adenosine signaling can directly influence T cell memory differentiation.

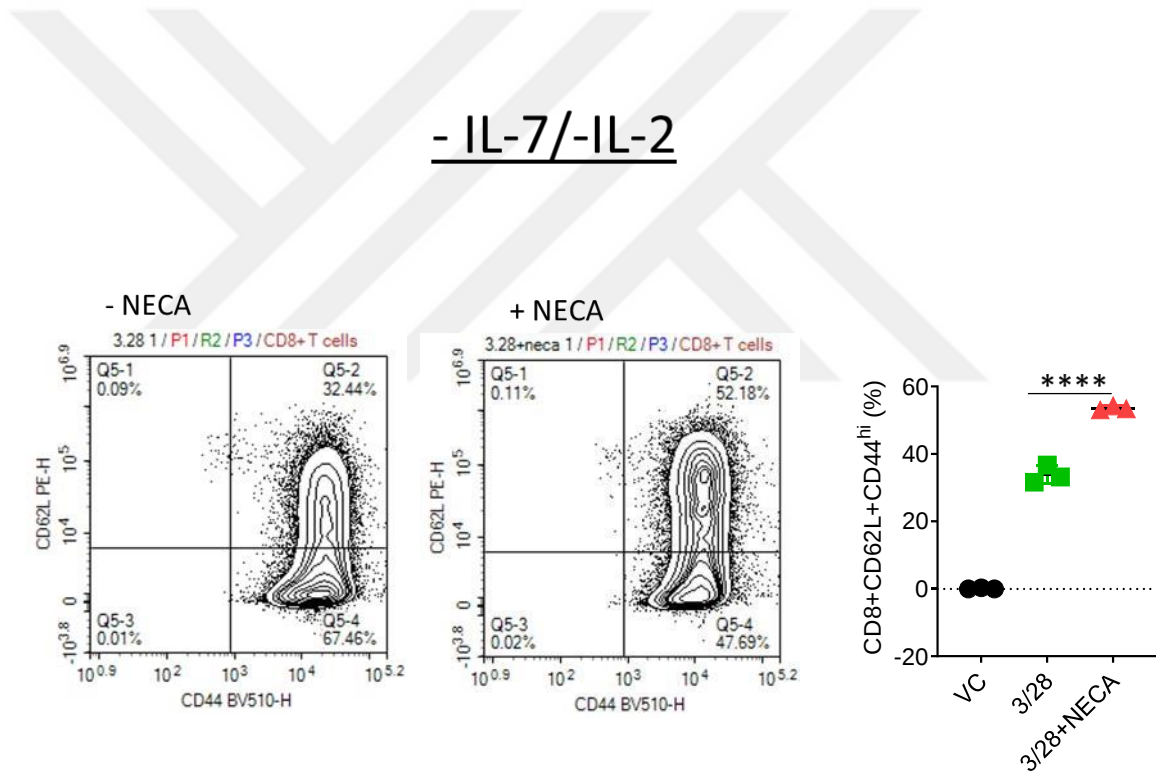


Figure 4.11: Adenosine signaling effect on CD8⁺T cell differentiation into memory cells. Flow staining was performed to stain surface marker CD62L and CD44, the results were analyzed with MFI rates. VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1μM). One

U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. **** $p \leq 0.0001$)

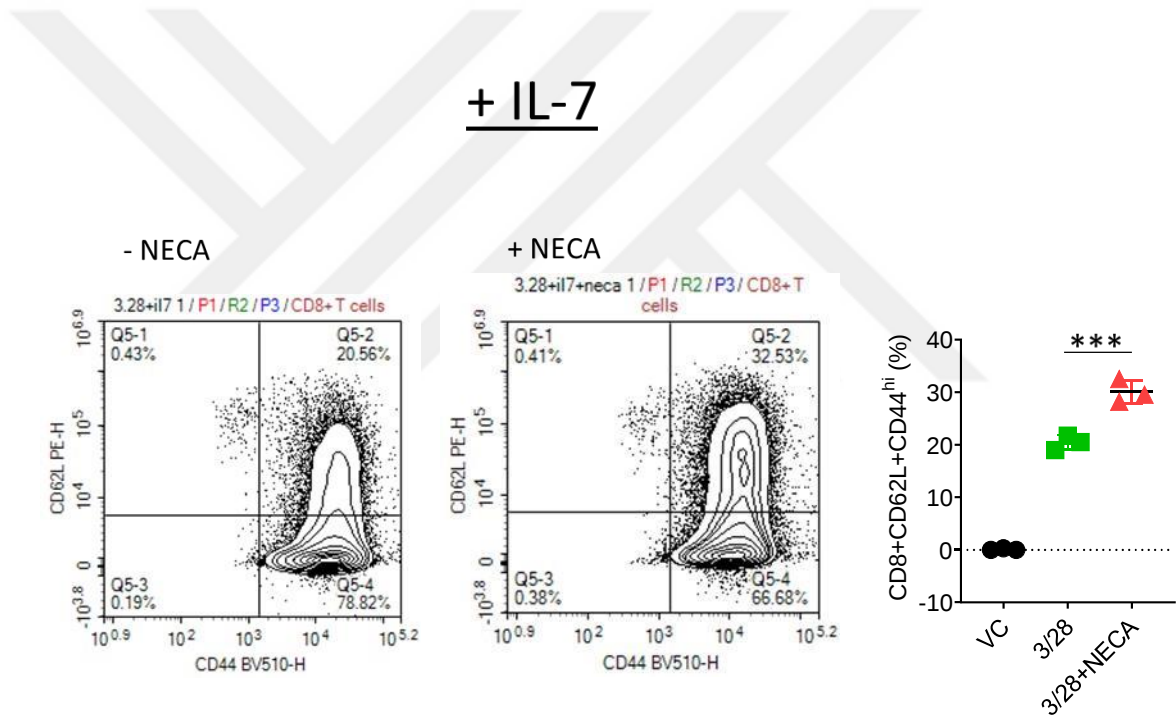


Figure 4.12: IL-7 has no influence on the memory differentiation of NECA stimulated CD8⁺T cells. Flow staining was performed to stain surface marker CD62L and CD44, the results were analyzed with MFI rates. VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1μM). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. *** $p \leq 0.001$)

+ IL-2

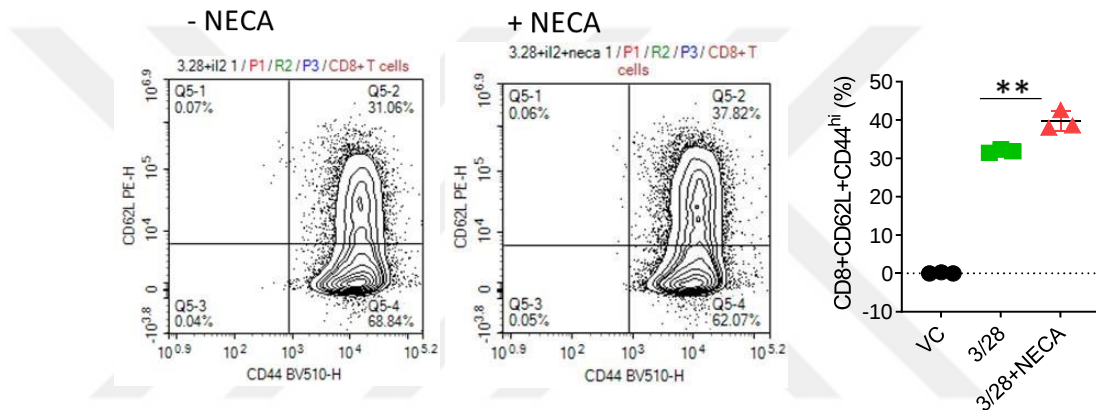


Figure 4.13: IL-2 has no influence on the memory differentiation of NECA stimulated CD8⁺T cells. Flow staining was performed to stain surface marker CD62L and CD44, the results were analyzed with MFI rates. VC group represent the naïve CD8⁺T cells receiving PBS and DMSO as vehicles, the 3/28 group represent the CD8⁺T cells activated by anti-CD3 and anti-CD28 antibodies, 3/28+NECA group represent the CD8⁺T cells that are activated by anti-CD3 and anti-CD28 antibodies in the presence of NECA (1μM). One U/mL adenosine deaminase was added to the culture to prevent the effect of endogenously generated adenosine (representative of one of three experiments with similar results. One-way ANOVA with Post-hoc Tukey's test was used for statistical analysis. ** $p \leq 0.01$)

CHAPTER 5

DISCUSSION

The immune system is very important for the defense of the body from the invasion of pathogens and emergence of neoplastic formations within itself. The immune system can be divided in two categories which is the innate and adaptive immune system, and by two different mechanism of actions, humoral and cellular. T lymphocytes take part in the adaptive immune system and they are part of cellular immunity because of their mechanism utilize to fight the danger. The T cells that has a direct role in cellular immunity are CD8⁺T cells (cytotoxic T cells).

The T cell progenitors are produced in the bone marrow, but they mature in the thymus where they get their name came from. Each T cell has a unique T cell receptor (that can recognize antigen) which are produced by a gene rearrangement process. Once antigens are detected cytotoxic T cells act directly on the infected cells by Fas/FasL or perforin pathway, but can also produce cytokines such as interferons that lead indirect protection against the pathogen [9].

Immunotherapy has recently become a trend topic on the cure for cancer. By changing the tumor microenvironment, it is possible to improve immune infiltration to tumors. Also categorizing and investigating cancer according the immune infiltration can have an important prognostic value [34][35][15].

Extracellular adenosine accumulates in the tumor microenvironment as a result of stress, cell damage, ischemia and inflammation. Adenosine is directly released from cells or generated due to the dephosphorylation of ATP. Extracellular adenosine signals through

adenosine receptors to regulate both immune cells and in some cases tumor cells [36]. Because adenosine negatively regulates immune cells cancer cells may evolve to express ATP catabolizing enzymes CD39 and CD73 to generate adenosine to suppress immune cells [26]. Although, targeting adenosine signaling may be an attractive strategy to cure cancer, sometimes inhibiting a single receptor may cause more progression of the tumor. For example, in preclinical tumor models, global deletion of A2AR is effective for some syngeneic tumors such as sarcoma and highly immunogenic melanoma but not the others such as MC38 colon carcinoma, B16 melanoma and MB49 bladder carcinoma. Even blocking that signal only in T cells have the consequences such as reduced tumor accumulation. For A2AR deletion a large number of engineered T cells should be given in the presence of checkpoint blockade to observe a substantial effect suggesting adenosine signaling may suppress T cells but their deletion may cause T cell exhaustion. Therefore, a little amount of adenosine may be necessary for cytotoxic T cells to efficiently differentiate into memory phenotype. Also, the tumor microenvironment is also an important factor [37][28][38]. Other explanation for such results is that adenosine signaling is very important during early stage development of T cells where without that signal a decrease in naïve T cell population is observed [25]. Our results suggest that adenosine signaling can directly influence IL-7 responsiveness of naïve T cells and their memory differentiation while reducing expression of T cell exhaustion marker PD-1, suggesting adenosine in the tumor microenvironment can have direct anti-tumoral effects depending on the cytokine milieu, which is independent of its effects on T cell development.

One possible approach to have a cure in those tumors is to find a solution that can reverse the unwanted effect of adenosine but preserving the beneficial ones. The signaling from IL-7 receptor promotes the ability to stay alive for T cells [39]. Our recent observations suggest that in the presence of IL-7 receptor signaling adenosine signaling may have an anti-tumoral effect but without IL-7 signaling adenosine signaling has a pro-tumoral effect. (Koyaş A., Kayhan M., Tüçer S. and Çekiş Ç. unpublished manuscript). Our current study provides an important rationale for this observation since we obtained complete reversal of adenosine-mediated inhibition of T cell accumulation in the presence of IL-7. Naïve T cell need to be activated to generate an immune response [2]. In the

absence of IL-7 or in the presence of IL-2 adenosine significantly limited T cell activation and accumulation. Therefore, in preclinical models where cell-intrinsic adenosine signaling in CD8⁺T cells is immunosuppressive, the tumor microenvironment may provide signals such as IL-2 to promote survival of effector cells differentiated in the absence of adenosine signaling.

Another effect we observed after adenosine signaling is the suppression of exhaustion marker PD-1 receptor. This marker is a very important mechanism that protect normal tissue from possible harm due to uncontrolled inflammation and immune reaction. Cells expressing the ligand for this receptor inhibits T cells to express inflammatory cytokines and to show cytotoxic effect [40]. Effect of adenosine signaling on PD-1 expression is not influenced by IL-7 signaling or by vehicle. Interestingly, in the presence of IL-2 adenosine signaling did not influence the PD-1 expression suggests that PD-1 induction in the presence of IL-2 may not be suppressed by adenosine signaling.

The central memory cells express CD62L receptor on their surface as naïve cell do, which helps them to migrate to the lymphoid organs and lymphoid structures. They also express CD44 receptor, which is a marker for effector cells, memory cells and antigen-experienced T cells. Different from central memory cells the effector/effector memory T cells express CD62L in low amount. Effector/effector memory cells reside mostly in tissue and peripheral circulation. In addition, by expressing adhesion receptors they can migrate to inflamed tissues. The effector memory cells are well known for their ability to migrate inflamed area, and react faster to the antigen than central memory cells. The effector cells highly express effector proteins such as perforin as highly cytotoxic cells and produce effector cytokines to stimulate other innate and adaptive immune cells for a rapid immune response. Effector/effector memory cells are more prone to exhaustion. The central memory on the other hand, has an increased cell renewal capacity and they are less prone to exhaustion. Therefore, they can resist apoptosis better then effector memory cells [33][41][42]. Our results suggest that adenosine stimulation can promote central memory formation independent of IL-2 or IL-7 stimulation suggesting in situations where effector

memory cells encounter exhaustive T cell signaling adenosine signaling may provide an advantage to polarize them into a phenotype with more self-renewal capacity.

Overall our study suggests that adenosine signaling may inhibit the direct effective immune reaction against tumor by reducing effector memory differentiation. The consequence of such an effect can be that the tumor is well protected for a while since effector memory cells are very cytotoxic; however for longer time periods central memory cell differentiation is more beneficial since they have strong ability to resist apoptosis, they have a strong ability to propagate upon re-stimulation, and also they have the ability of self-renewal [33], which may let them persist better in adenosine-rich tumor microenvironments especially in the presence of IL-7.

Bibliography

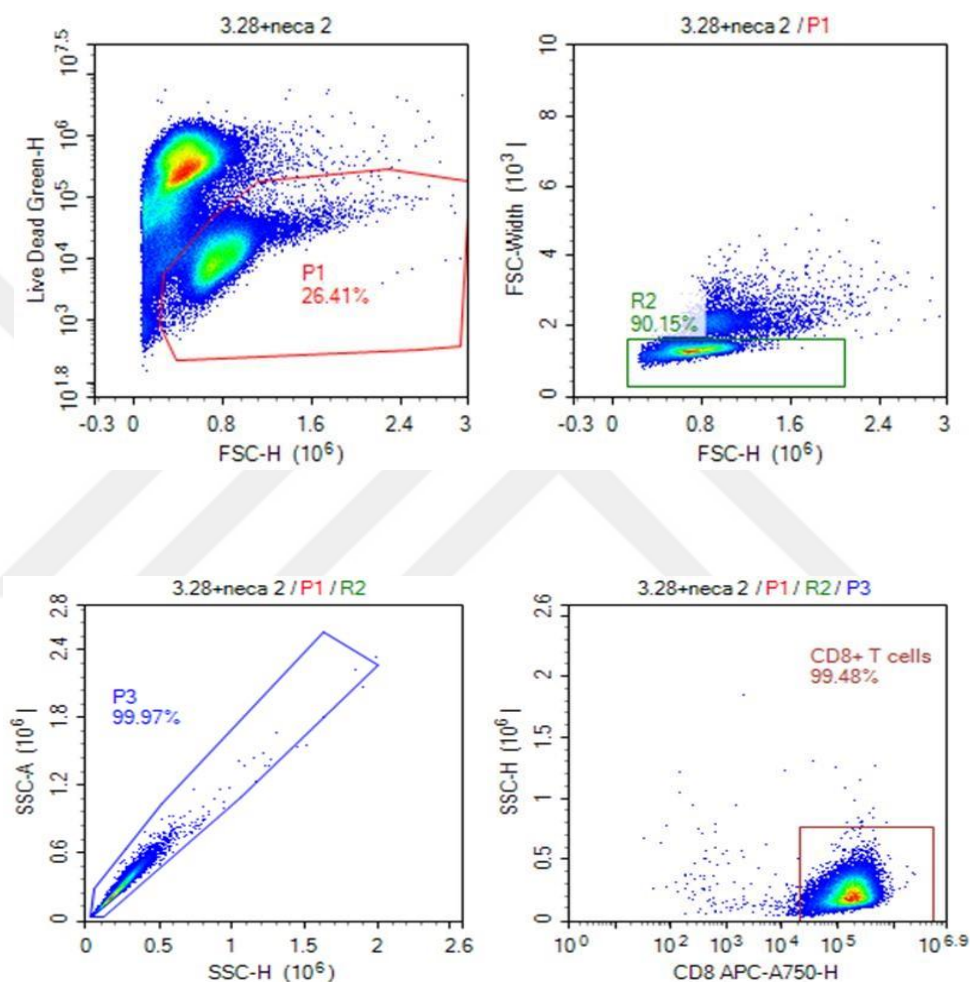
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APPENDIX



Appendix Figure 1: Gating strategy used for CD8⁺T cells flow cytometry analysis.

Firstly, live cells were selected from Live-Dead green-H/FSC-H. Then, by using FSC-Width/FSC-H and SSC-A/SSC-H graphs single cell population was taken. Later, SSC-H/CD8 APC-A750-H graph was used for selecting CD8⁺ T cell population. For next steps, the gating strategy was performed and was done according to the phenotype investigated.

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