IN-VITRO GENERATED MEMORY-LIKE CD8^{+} T CELLS ELICITED PRONOUNCED CYTOTOXIC EFFECT UPON ANTIGEN SPECIFIC TCR GENE TRANSDUCTION

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By

Hakan Köksal

September 2016

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By Hakan Köksal

September 2016

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

İhsan Gürsel (Advisor)

Güneş Esendağlı

Özgür Şahin

Approved for the Graduate School of Engineering and Science:

Levent Onural

Director of the Graduate School

Abstract

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Hakan Köksal
M.S. in Molecular Biology and Genetics
Advisor: İhsan Gürsel
September, 2016

Cytotoxic T cells are the important arm of immune system against malignant and virus infected cells. APC activated cytotoxic T cells survey body for threats, recognize peptide-MHC class I molecules by TCR and eliminate targets by inducing apoptosis. Following a successful clearance of danger, these cells undergo a contraction phase which eliminates 90-95% of antigen specific $CD8^+$ T cells. At this stage, surviving cells are named as memory T cells. Maintenance of these cells are primarily depends on homeostatic cytokines, IL-7 and IL-15. Surviving memory $CD8^+$ T cells are especially important against recurrence of malignancies and reinfections due to their quick and potent immune response. Similar memory functions and characteristic can be achieved under lymphopenic

environment where abnormally low levels of lymphocytes present. This phenomenon is called homeostatic proliferation and has been shown to promote tumor clearance, reverse anergy and improve immune response against pathogens. Similar to natural generation of memory T cells, homeostatic proliferation fundamentally depends on homeostatic cytokines. Recent studies demonstrated memory-like phenotype ($CD44^{hi}CD62L^{hi}$) can be acquired *in vitro* by culturing naive $CD8^+$ T cell in the presence of anti-CD3/28 and recombinant IL-15. These *in vitro* generated phenotype when transferred to a mice proved to have an augmented antitumor response *in vivo*. In the light of these findings, we aimed to improve *in vitro* generation of memory-like phenotype and make it more practical and use identified improvements in tumor therapy. Our first approach is the utilization of DCs, known to be one of the most potent IL-15 secreting cells, in a co-culture setup with naive T cells. Identification of correct PRRs to trigger innate immunity and hence providing an environment suitable for transferred $CD8^+$ T cells, should improve overall immune response and antitumor potential of $CD8^+$ T cell. To test this idea, we made an *in vitro* co-culture setup. Stimulating DCs with potent IL-15 inducing ligands, c-GAMP and LPS, we achieved to improve expression of IL-15 but failed to induce memory-like phenotype in co-culture. Still we observed $CD8^+$ T cells acquire a memory-like phenotype with an increased cytotoxic potential in the presence of recombinant IL-15. As an alternative, $CD8^+$ T cells retrovirally transduced with an IL-15 vector to enable them to produce their own IL-15. Transduction managed to induce a memory-like progression without any additional supplementation. Similar to supplementation, IL-15 transduction improved cytotoxic potential of CD8+ T cell. Our approach can be used against any antigen with an improved immune response, eliminate the necessity of cytokine supplementation and enhance *in vivo* persistence of the transferred cells.

Keywords: Cancer Immunotherapy, CD8+ T cells, IL-15

Özet

İN-VİTRO ORTAMDA OLUŞTURULAN CD8$^+$ T HÜCRELERİNE ANTİJENE ÖZGÜ TCR GENİ TRANSFER EDİLDİĞİNDE GÖZLEMLENEN BELİRGİN SİTOTOKSİK ETKİLER

Hakan Köksal
Moleküler Biyoloji ve Genetik, Yüksek Lisans
Tez Yöneticisi: İhsan Gürsel
Eylül, 2016

Sitotoksik T hücreleri immün sistemin özellikle virüs enfekte olmuş ve malignan hücrelere karşı en önemli elemanlarındandır. Antijen sunucu hücrelerin aktive ettiği sitotoksik T hücreleri vücudu tehditlere karşı tarar ve TCR reseptörü yardımı ile tanıyıp hedef hücreyi hücrede apoptoz indükleyerek ortadan kaldırır. Tanınan tehditler ortadan kalktığında, antijene özgü olan T hücreleri kontraksiyon adı verilen bir faza girerler. Bu faz esnasında antijene özgü olan CD8$^+$ T hücrelerinde yaklaşık 90-95% oranında ölüm gözlenir. Hayatta kalan hücreler hafıza hücreleri olarak tanımlanır ve uzun vadeli immün sistemin en önemli uzantılarındandır. Hafıza hücrelerinin muhafazaları için homeostatik sitokinlerden olan IL-7 ve IL-15 en önemli unsurlardandır. Hafıza CD8$^+$ T hücreleri daha hızlı ve etkili yanıt

verebildikleri için tekrarlayabilecek rahatsızlıklara karşı önemli bir savunma potansiyeli sunmaktadır. Sitotoksik T hücreleri lenfosit miktarı düşük olan ortamlarda homeostazi sağlayabilmek amacı ile homeostatik proliferasyon adı verilen bir proliferatif tepki gösterirler. Bu tepki sonucu hafıza hücreleri fonksiyonu ve karakteristiği ($CD44^+CD62L^+$) gösterebilen hücrelere donüşebilirler. Geçtiğimiz yıllarda yapılan çalışmalar sonucu bu ortamın *in vitro* ortamda da taklit edilebileceği ve hafıza benzeri T hücrelerinin oluşturulabileceği gösterilmiştir. Doğal olarak üretilen hafıza hücrelerinde olduğu gibi hafiza benzeri hücrelerin üretiminde de en önemli unsur IL-15 olduğu gözlemlenmiş ve bu sayede üretilen hücrelerin kanser tedavisinde daha etkili olabileceği gösterilmiştir. Bu bulgular doğrultusunda yaptığımız araştırmalarda bu fenotipi oluşturma yöntemini daha pratik ve/veya daha etkili yapmayı hedefledik. Bulunacak potansiyel gelişmeler kanser tedavisinde test edilecek. İlk denenen yöntem, dendritik hücreleri çeşitli ligandlar ile stimüle ederek en efektif IL-15 salgılanmasına yol açan ligand tanımlanarak bunun *in vivo* ortamında verilen $CD8^+$ T hücrelerinin hafıza fenotipine dönüşmesine etkisi olup olamayacağını incelemekti. Fakat gözlemlerimiz IL-15 miktarı ile bağlantısız bir şekilde aktif edilmiş dendritik hücrelerinin gerek total salgıları ile gerek ise kendisi ile inkübe edilmiş T hücrelerinin hafıza benzeri fenotiplerinde düşüş gözlemlenmiştir. İkinci yöntemimizde ise IL-15 ifade geni retroviral olarak T hücrelerine transfer edilmiştir. Bu hücreler anti-CD3/28 ile aktive edildiğinde IL-15 takviyesi yapılan hücrelere yakın anti tümör sitotoksik etki gözlemlenmiştir. Bizim yöntemimiz ile üretilecek hafıza hücreleri herhangi bir ekstra takviyeye ihtiyaç olmadan istenilen antijene karşı dizayn edilebilir. Bunun yanı sıra burada belirtilerek üretilen hücreler transfer edilen canlıda çok daha uzun süreler dayanabilir ve uzun vadede daha etkili anti tümör fonksiyonu sergileyebilirler.

Anahtar sözcükler: Kanser İmmünoterapi, CD8+ T hücreleri, IL-15

To my precious family…

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testament in itself of her unyielding devotion. Thank you for transcending me into a new being with your love.

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Abbreviations

OAS1	2’-5’-oligoadenylate synthetase 1
AIM2	Absent in melanoma 2
ADAR1	Adenosine deaminase acting on RNA 1
ALR	AIM2-like receptors
ACK	Ammonium-Chloride-Potassium
Ab	Antibody
APC	Antigen presenting cell
AIF	Apoptosis-inducing factor
APAF1	Apoptotic protease-activating factor 1
BM	Bone marrow
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
JNK	c-jun N-terminal kinase
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid

CLR	C-type lectin receptor
cGAS	cyclic GMP-AMP synthetase
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DN	Double negative
DP	Double positive
ddH2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
EndoG	Endonuclease G
E.coli	Escherichia coli
EtOH	Ethanol
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FASL	FAS ligand
FADD	FAS-associated death domain protein

FBS	Fetal bovine serum
GMC-SF	Granulocyte macrophage colony-stimulating factor
RAC	Guanine triphosphatase
HSC	Hematopoietic stem cell
ISG	IFN stimulated gene
IRF3	IFN-regulatory factor 3
ITAM	Immunoglobulin family tyrosine-based activation motif
IFN	Interferon
IL	Interleukine
KIR	Killer inhibitory receptor
LPS	Lipopolysaccharide
LB	Lysogeny broth
MHC	Major histocompatibility complex
MDA5	Melanoma differentiation associated gene 5
mRNA	messenger ribonucleic acid
MAP	Mitogen-activated protein
NK	Natural killer
NLR	NOD-leucine rich repeat receptors
NF- κB	Nuclear factor- κB

NOD	Nucleotide-binding oligomerization domain
ODN	Oligonucleotide
PRR	Pattern recognition receptor
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinase
PLCγ1	Phospholipase Cγ1
PCR	Polymerase chain reaction
PD1	Programmed death 1
PI	Propidium Iodide
PKB	Protein kinase B
PKR	Protein kinase R
RAG	Recombination activating gene
RNA	Ribonucleic acid
RLR	RIG-I like receptor
RT	Room Temperature
RPMI	Roswell Park Memorial Institute
STING	Stimulator of interferon genes
Th	T helper
TCR	T-cell receptor

TLR	Toll-like receptor
TAE	Tris-Acetate-EDTA
TNF	Tumor necrosis factor

Chapter 1

Introduction

1.1 The immune system

The immune system is a vast network consisting of physical barriers, soluble factors and leukocytes which contribute to host resistance to both altered-self and non-self-threats [1]. According to specificity, there are two main branches of immunity named as innate and adaptive immunity. Innate immunity recognizes molecular patterns of pathogens (and in some cases even self danger signals) and initiates an orchestrated signaling cascades leading to primarily eradication of the insult. Another important feature of the innate immune system is to instruct effective adaptive immunity. Adaptive immunity, unlike innate doesn't rely on already established molecular patterns rather learns to recognize unique antigen(s) or antigenic epitopes and launches an immune response accordingly. One of the distinct differences between innate adaptive immunity is that the cells of the adaptive immunity are professional cells that can memorize the antigenic epitopes and remember same antigen upon second exposure [2].

1.1.1 Innate immune system

Innate immune system consists of variety of cell types such as neutrophils, macrophages, dendritic cells (DC), basophils, eosinophils, mast cells and natural killer (NK) cells. These cells provide the first line of defense against pathogens. These cells

express pattern recognition receptors (PRR) which enable them to recognize pathogen and danger-associated molecules. Therefore, innate immune system can be able to differentiate from self to non-self or altered-self [3]. PRR consist of several receptor types; Toll-like receptors (TLR), C-type lectin receptors (CLR), nucleotide-binding oligomerization domain-leucine rich repeat containing receptors (NLR), AIM2-like receptors (ALR), RIG-I like receptors and cytosolic DNA sensors [4-5]. Through their PRRs, innate cells are able to recognize microbial patterns, including lipids, glycoproteins, nucleic acids or polysaccharides that exist in fungi, parasites, bacteria and virus [4-7].

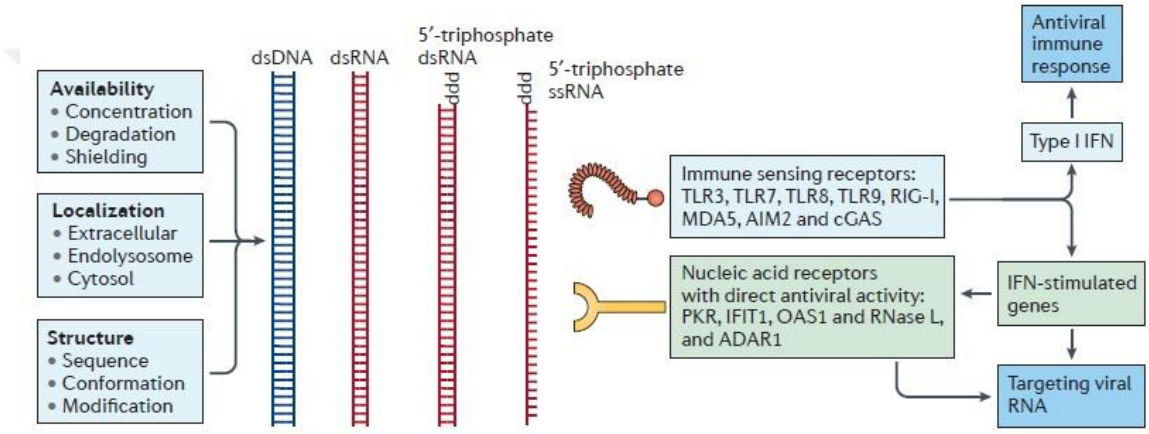


Figure 1.1: Nucleic acid recognizing receptors [12].

Nucleic acid sensors can be categorized as immune sensing receptors and nucleic acid receptors. The first category consists of TLR family, RIG-I like receptor (RLR) family, melanoma differentiation associated gene 5 (MDA5), absent in melanoma 2 (AIM2) and cyclic GMP-AMP synthetase (cGAS). Among TLR family, TLR3 recognizes dsRNA, TLR7-8 recognizes ssRNA and CpG-DNA recognized by TLR9. However, TLR family doesn't only recognize nucleic acids; TLR1-2, TLR2-6 responds to lipopeptides, TLR4 responds to lipopolysaccharides (LPS) and TLR5 recognizes flagellin [8]. In RLR family, RIG-I responds to 5'-triphosphate dsRNA, MDA5 also responds to dsRNA but if the length is greater than 2000 nucleotides [9]. Both AIM2 and cGAS recognizes dsDNA; AIM2 initiates inflammasome formation whereas cGAS responds through stimulator of interferon genes (STING) receptor [10-11]. Described

receptors either directly or indirectly promotes transcription factors such as nuclear factor- κB (NF- κB) and IFN-regulatory factor 3 (IRF3) which induces antiviral immune response by type I IFN and IFN stimulated genes (ISGs) [12]. Second category receptors, nucleic acid receptors, unlike PRRs they primarily depend on type I IFN and/or PRR signaling. Nucleic acid receptors have a more direct antiviral immunity and consists of protein kinase R (PKR), 2'-5'-oligoadenylate synthetase 1 (OAS1) and adenosine deaminase acting on RNA 1 (ADAR1) [12]. Similar to immune sensing receptors, nucleic acid receptors contribute to the antiviral immune response with a more direct approach such as translation inhibition or chemical modification and degradation of RNA threats [13].

Recognition of these patterns leads to an inflammatory response which aims to contain and eradicate the source of danger utilizing phagocytosis, pro-inflammatory mediators, recruitment and activation of innate and possibly the adaptive immune system [14].

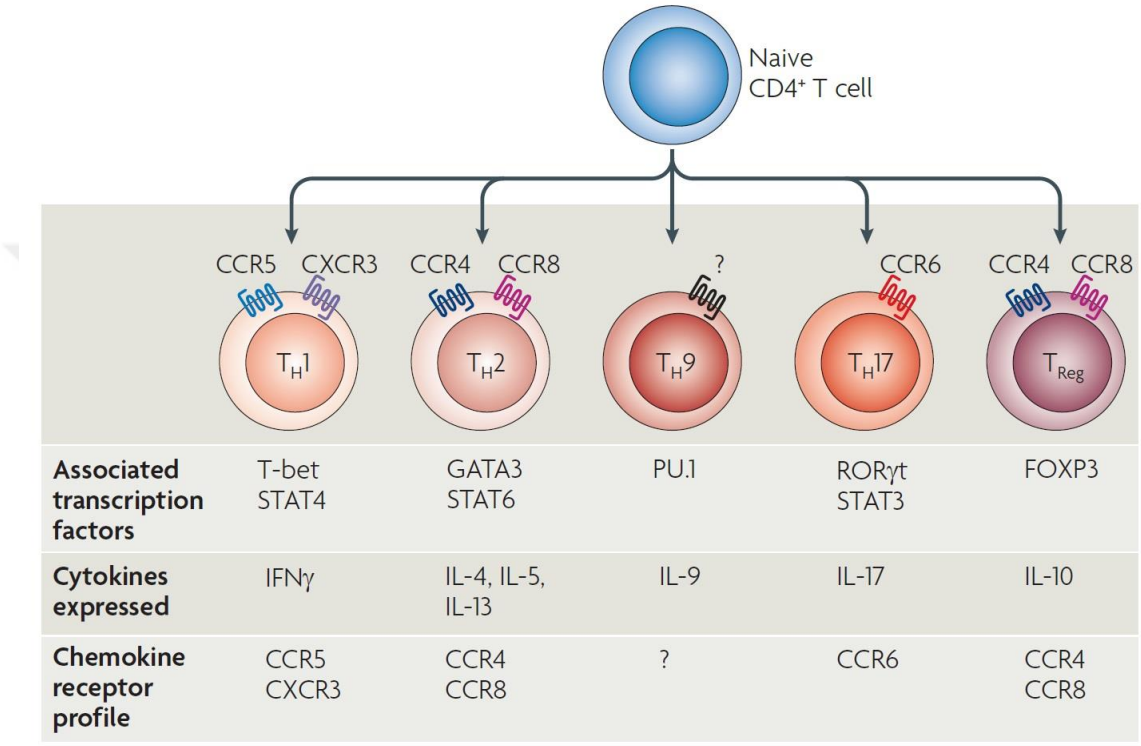
1.1.2 Adaptive immune system

An adaptive immune response is induced if innate immune system is overwhelmed by an infection. Even though, this happens very rarely innate defense mechanism cannot stop the replication of pathogen and antigen accumulates. The environmental changes by innate immunity combined with antigen accumulation induces adaptive immune system. Two major sets of cells, B and T lymphocytes, can induce antigen specific response in adaptive immune system.

B lymphocytes are the precursors of antibody-secreting plasma cells. Generally, naïve B lymphocytes are activated by antigen and T helper (Th) cells which then differentiated into plasma cells. Secreted antibodies have different ways to contribute adaptive immunity. Certain pathogens requires specific molecules on target cells to enter the cells. Antibodies can bind to pathogen and neutralize them by blocking the binding sites. Moreover, antibodies can coat the surface of a pathogen to make them a target for phagocytosis. Finally, antibodies can initiate complement system after coating the surface of a pathogen. Complement proteins opsonize the pathogen by interacting the complement receptors on phagocytic cells [15].

T lymphocytes completes their development in thymus and enters into circulation. Mature T cells that have not confront to an antigen are called naïve T cells. They

circulate back and forth between peripheral lymphoid tissues and blood. Upon antigen encounter, naïve T cells differentiate into several different functional subsets. Naïve $CD8^+$ T cells recognize antigen through pathogen peptides presented by MHC class I molecules. On recognition, naïve cells differentiate into effector $CD8^+$ T cells which are capable of killing the infected cells. Therefore, $CD8^+$ T cells play roles in limiting tumor development and growth and controlling intracellular pathogen infections [16].



	T_H1	T_H2	T_H9	T_H17	T_{Reg}
Associated transcription factors	T-bet STAT4	GATA3 STAT6	PU.1	RORγt STAT3	FOXP3
Cytokines expressed	IFNγ	IL-4, IL-5, IL-13	IL-9	IL-17	IL-10
Chemokine receptor profile	CCR5 CXCR3	CCR4 CCR8	?	CCR6	CCR4 CCR8

Figure 1.2: Naïve $CD4^+$ T cells differentiate into variety of subsets [17].

Upon activation, naïve $CD4^+$ T cells differentiate into several distinct subsets of Th cells; Th1, Th2, Th17 and iTreg. These subsets can be categorized according to their cytokine secretion patterns and associated transcription factors. (Figure 2) Th1 cells mainly contributes to macrophage and $CD8^+$ T cell mediated immunity whereas Th2 cells are related to humoral immune system where they stimulate B cells. On the other side, Th17 contributes to protection at mucosal surfaces and its dysregulation might lead to several autoimmune disorders. On the contrary, Treg cells demonstrate immosuppressive properties in order to prevent autoimmune diseases and keep the immune response in check. In general, $CD4^+$ T cells play roles in helping B cells in

antibody production, improve response of CD8 T cells and keep the autoimmunity in check [18].

1.2 T cell development

Multipotent hematopoietic stem cells are the precursors of T lymphocytes located in bone marrow. Progenitor cells migrate from bone marrow to thymus for maturation. That is why name of these cells are T lymphocytes. Thymus supplies necessary survival environment to support the differentiation of T cells from Hematopoietic stem cell (HSC)-derived lymphocyte progenitors [19]. These cells don't express any of T cell surface markers when they enter into the thymus. Because they don't express CD8 and CD4, they are called double negative (DN). At this point DNs can give rise to two distinct lineage of T cells. They either don't express CD4 and CD8 at all and choose to become γ:δ T cells or express these markers and differentiate to α:β T cells [20]. DN stages takes place in outer cortex of the thymus and can be divided into four stages. (Figure 3) At the DN1 stage these cells express CD44 and Kit. When they acquire CD25 expression, they are called DN2 cells. Subsequently, expression of CD44 and Kit decreases and cells are named as DN3. At this stage if cells fail to rearrange T cell receptor β-chain locus, they can't move to DN4 stage and die. Otherwise, they lose CD25 surface expression and proceed to DN4 stage [21]. Upon expression of pre-TCR, additional rearrangement stops and expression of CD8 and CD4 surface markers are induced. CD8 and CD4 commitment takes place in inner cortex of the thymus.

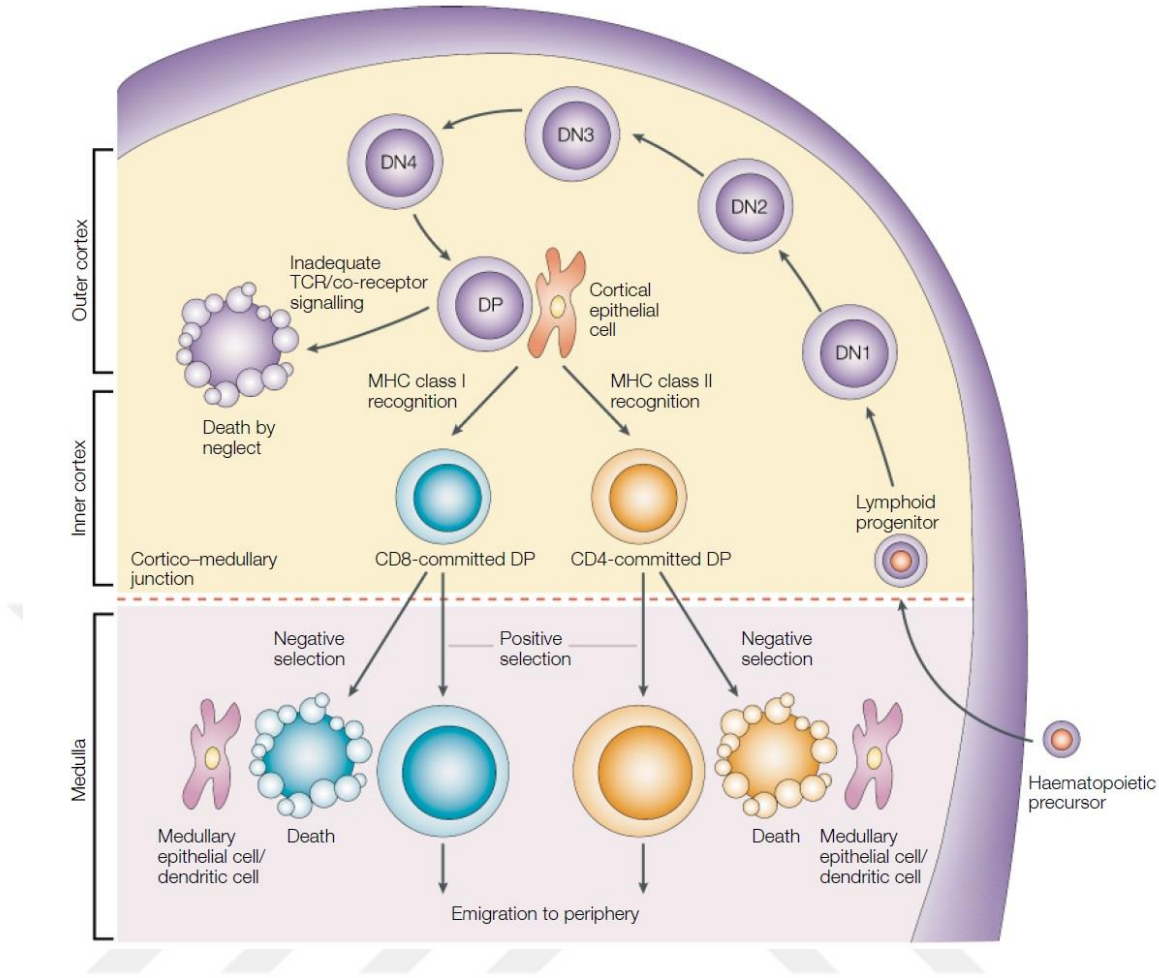


Figure 1.3: Diagram of T cell development [22].

At this step, cells are called double positive (DP) thymocytes. Initially, DP cells are large and have a huge proliferation capacity. Subsequently, proliferation rate decreases and cells become smaller. Consequently, α chain rearrangement begins which increases potential diversity. At this step small DP cells are tested against self-peptide: self-MHC complexes. If they don't recognize, cells die. If self-peptide complex was successfully recognized, small DP cells mature and lose one of the surface markers becoming either CD4 or CD8 single positive thymocytes [23]. The lineage commitment, whether these cells become helper $CD4^+$ or cytotoxic $CD8^+$, is regulated by series of mechanisms which include TCR and MHC interactions, transcriptional regulations and cytokine signaling [24].

1.3 $CD8^+$ T cell mediated immunity

1.3.1 Priming of naïve T cells by antigen presenting cells

Immune response of T lymphocytes is one of the most important arm of whole immune system. Antigen presenting cells (APCs) display antigen peptides of infected or transformed cells via MHC class I on their surface to specifically target and activate cytotoxic T cells. Antigen recognition is a must for T cells to undergo proliferation and differentiation. Naïve T cells can transiently bind mature APCs while migrating through cortical region of the lymph node. One of the most important APC in T cell priming is dendritic cells (DCs) these cells can attach to LFA-1 and CD2 on T cells via their ICAM-1, ICAM-2 and CD58 receptors [25]. Naïve T cells mainly receive two types of signal which are depicted in Figure 1.4. These signals are categorized as activation and survival.

First signal is the recognition of specific peptide: MHC class I complex by T-cell receptors (TCR) and co-receptor CD8. Upon this recognition, SRC kinase Lck is activated and immunoglobulin family tyrosine (Y)-based activation motifs (ITAMs) are phosphorylated. Then, ZAP-70 is recruited and phosphorylated which in turn phosphorylates adaptor proteins. Following activation of phospholipase Cγ1 (PLCγ1) and the guanine triphosphatase (RAC), several mitogen-activated protein (MAP) kinases; extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 together with phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt) are activated. All these pathways promote transcription factors related to T cell activation [26].

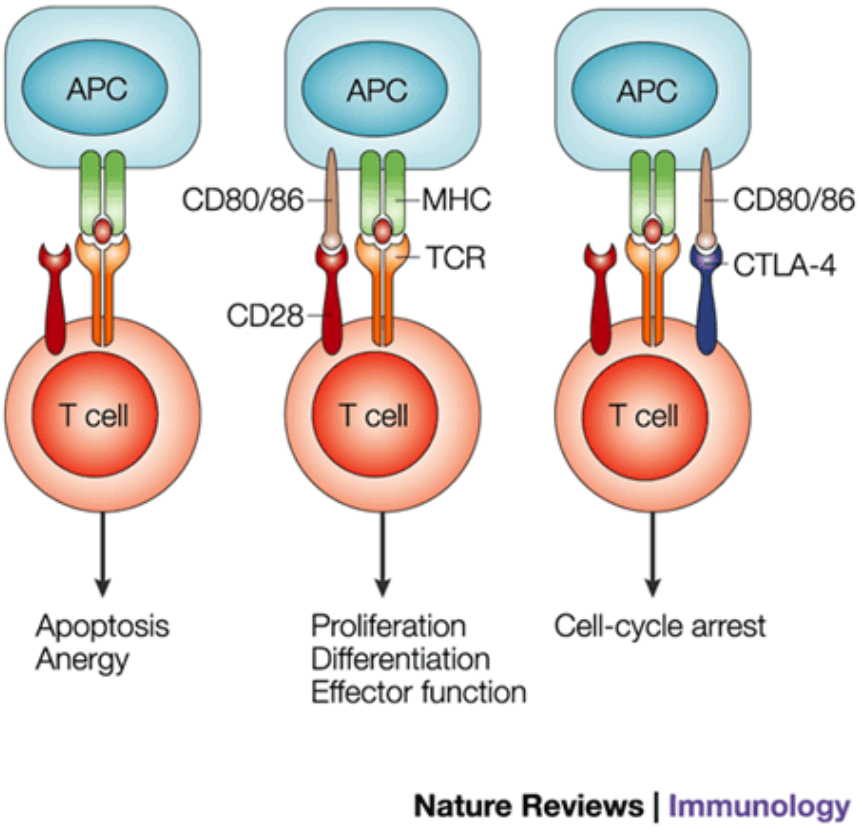


Figure 1.4: Under different conditions TCR engagement leads to separate T cell fate [26].

Second signal is co-stimulation, which is required for a pronounced immune response. One of the most well-known and effective co-stimulatory molecule is 44-kDa sized glycoprotein CD28. It is expressed by naïve and primed T cells and interacts with B7-1(CD80) and B7-2(CD86) on APCs. Co-stimulation is an indispensible since TCR recognition without a co-stimulator molecule leads to anergy (Figure 1). In general, anergic T cells aren't able to proliferate, secrete survival factors like IL-2 and are unresponsive to immune stimuli [27]. CD28 signal improves T cell response in many ways. Production of a panel of cytokines is improved in T cells receiving CD28 stimulation both by mRNA stabilization and transcriptional activity [28]. In addition to that CD28 promotes T cell survival by upregulating BCL-X_L, an anti-apoptotic BCL-2 family member [29]. Moreover, there are inhibitory signals that inactivate T cells such as cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed death 1 (PD-1) and killer inhibitory receptors (KIRs). Of all, CTLA-4 is the most studied receptor expressed on activated T cells. CTLA-4 competes with CD28 as it binds to same ligand and prevents

T cells from receiving co-stimulation. (Figure 1.4) T cell activation is inhibited by both blocking CD28 from promoting positive signals and arresting T cells at the G1 phase of the cell cycle [30].

1.3.2 T cell mediated cytotoxicity

Activation of $CD8^+$ T cells by APCs takes place in lymph nodes. Then, T cells enter into a rapid proliferation state and changes are observed in their surface markers. Initially, homing markers such as CD62L, a marker which allows T cells to recirculate through lymph nodes, is downregulated. Hence, T cells are allowed to leave lymph node and search for inflammation throughout the body. Moreover, integrin VLA-4 is expressed from activated T cells which allows T cells to recognize VCAM-1 expressed on inflamed tissues [31]. Therefore, activated T cells can identify and enter into the sites of inflammation.

$CD8^+$ cytotoxic T cells also known as cytotoxic T lymphocytes (CTLs) are especially important against malignant cells and intracellular pathogens such as viruses. When cells are infected or transformed, they display antigen fragments with MHC class I complex which are recognized specifically by $CD8^+$ cytotoxic T cells. APC activated T cells seek these cells and eliminate by inducing apoptosis. At first, discoveries led scientist to believe that changes in the cytoplasm of a CTL lead to granule exocytosis of a pore-forming protein perforin [32]. This idea is still valid today but more proteins are identified helping this phenomenon, such as granzymes.

Further studies revealed that CTLs can induce apoptosis not by just granule exocytosis of toxic materials. CTLs are also capable of inducing apoptosis through FAS, a tumor-necrosis factor receptor family member, pathway that doesn't require pore forming material secretion onto the target cell surface [33]. Upon activation, CTLs express a ligand named FAS ligand (FASL) which can interact with FAS receptor on target cell. Stimulation of FAS receptor leads to recruitment of caspase-8 with the help of an adaptor protein called FAS-associated death domain protein (FADD). (Figure 1.5) Caspase-8 either directly activates other caspase members such as caspase-3 or cleaves BID, a pro-apoptotic BCL-2 family member to induce apoptosis. After cleavage, BID and BAX are translocated to mitochondria and induce release of cytochrome c. Finally,

through apoptotic protease-activating factor 1 (APAF1) caspase-9 is activated which in turn activates caspase-3 to induce apoptosis [34].

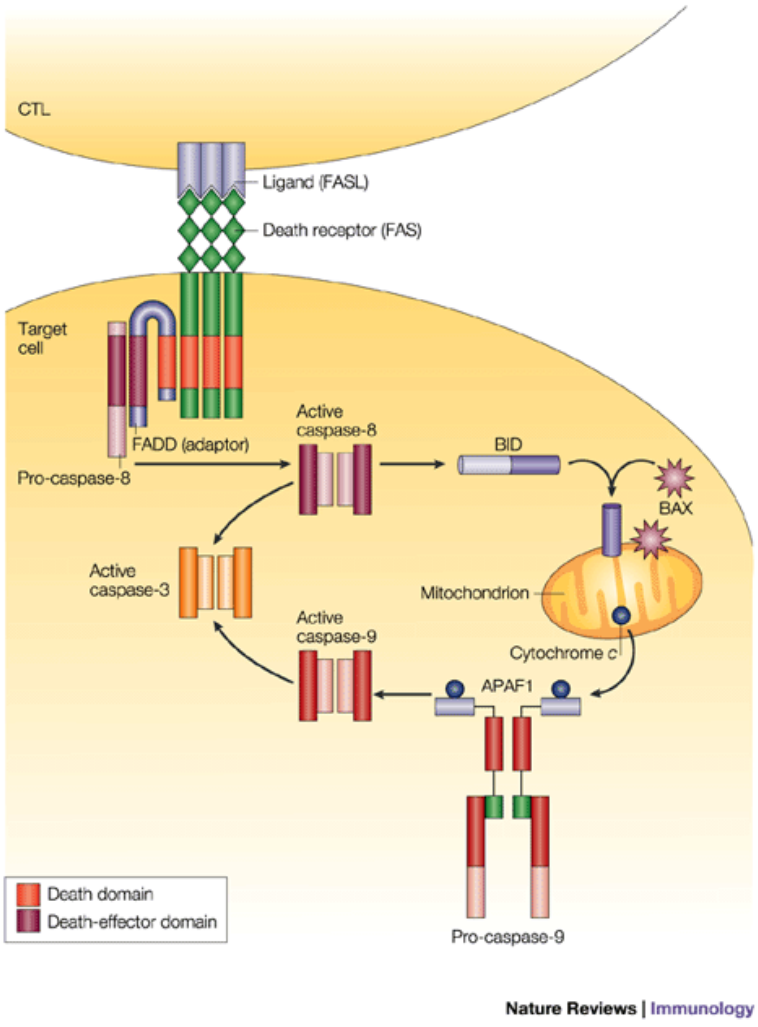


Figure 1.5: FAS ligand expressed on CD8 T cells induce apoptotic cell death upon interacting with FAS receptor (CD95) on target cell [33].

Perforin is exclusively expressed by CTLs and can be found in the cytoplasmic granules. Initially, perforin by itself is thought to be sufficient in killing cells due to its membrane damaging properties. However, it has been confirmed that even though perforin is capable of killing *in vitro*, they are not enough *in vivo* and requires other granule proteins including granzymes [35]. Granzymes are serine proteases released by cytoplasmic granules from CTLs. There are different types of granzymes that has been characterized, grazyme A,B, H, K and M. Granzyme B is the most studied and well-

characterized of all granzymes. It has been shown that granzyme B can be internalized by target cells via mannose-6-phosphate receptor with the help of perforin [36]. Once internalized, granzyme B can initiate cell death by variety of ways. (Figure 1.6) Similar to FAS-mediated cell death pathway, granzyme B can initiate apoptosis through caspase-3 directly or via caspase-8. Unlike FAS pathway, granzyme B promotes mitochondrial dysfunction which can potentially lead to necrosis, a more aggressive type of cell death which is not programmed unlike apoptosis [37-38]. Additionally, granzyme B can initiate a caspase-independent cell death through apoptosis-inducing factor (AIF) and endonuclease G (EndoG) [39].

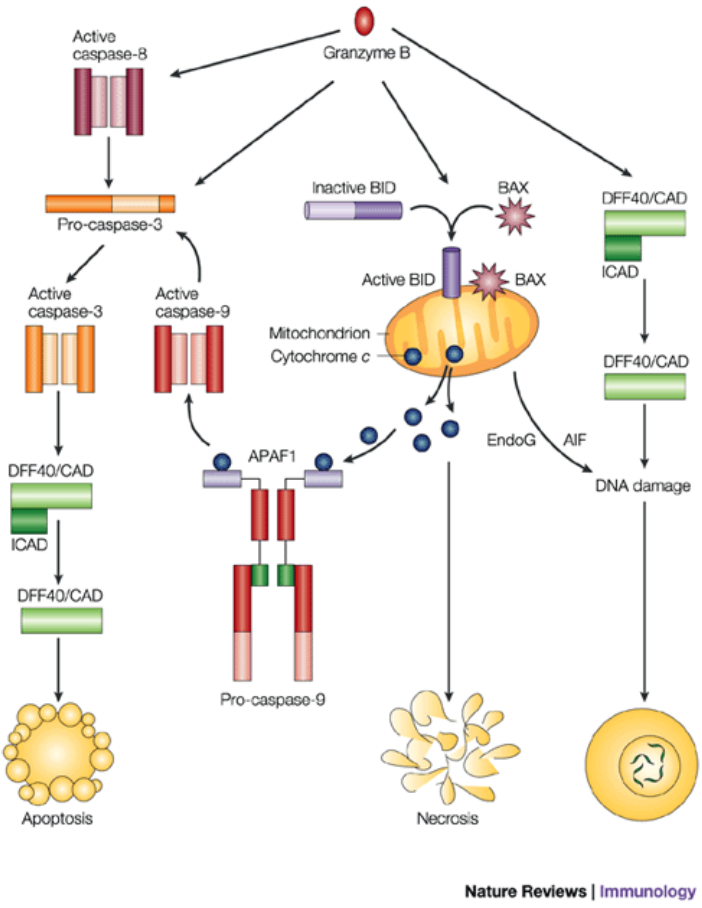


Figure 1.6: Granzyme B induced cell death pathways [33].

In addition to killing function, cytotoxic T cells by secreting specific cytokines can induce immune response. Even though main way is to induce apoptosis to obliterate target cells, cytotoxic T cells can secrete IFN-γ and TNF-α in order to contribute host

defense. IFN-γ can inhibit viral replication and increase the expression of MHC class I molecules on adjacent cells which improves the chance of recognition by CTLs. Together with TNF-α, IFN-γ promotes macrophage recruitment and activation [40].

1.4 Long term immunity

Upon activation antigen specific $CD8^+$ T cells initiate a proliferation cascade, only a single precursor is able to give rise to about 10,000 daughter cells in 5-8 days [41-42]. As explained in the previous section these cells leave the secondary lymphoid organs and seek for the signs of infection. Regardless of success in antigen specific $CD8^+$ T cell immune response, number of antigen specific $CD8^+$ T cells undergo a significant decline in all organs which is called contraction phase [41-42]. Mainly in contraction phase about 90-95% of antigen specific $CD8^+$ T cells are eliminated to prevent a potential immunopathology. Even though, most of the antigen specific portion is eliminated, a small percentage always survives from the contraction in all tissues [43-44]. At this stage, these surviving stable antigen specific cells are called memory $CD8^+$ T cells and they depend on homeostatic cytokines such as IL-7 and IL-15. (Figure 1.7) Memory T cells are able to induce a much more significant immune response to their specific antigen when it comes to recurrence of malignancies and reinfections [45].

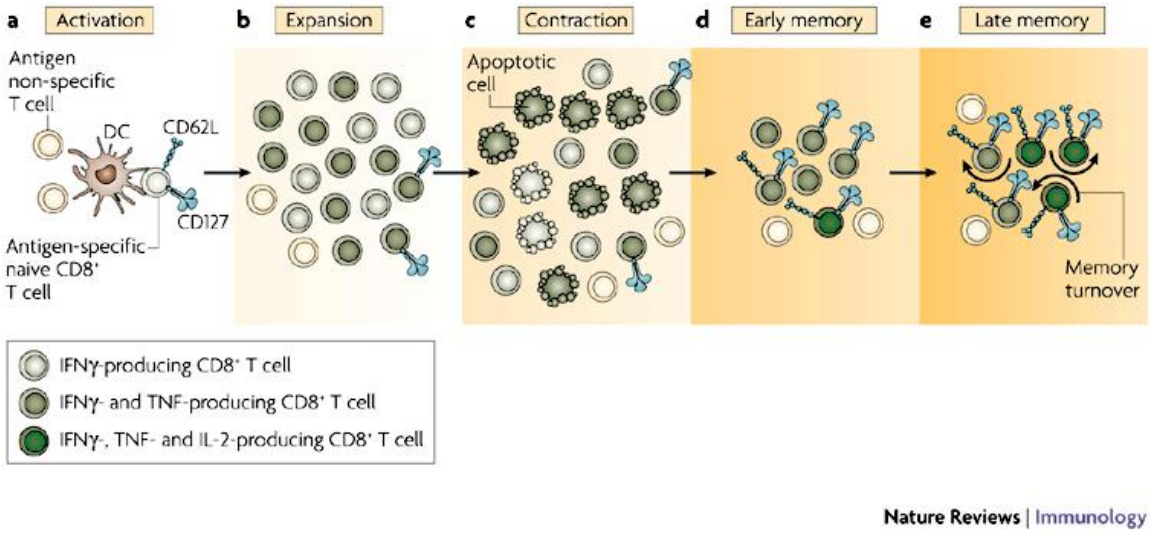


Figure 1.7: CD8+ T cell fate upon infection or vaccination [45].

1.4.1 Memory $CD8^+$ T cells

Although contraction phase clears most of the antigen specific $CD8^+$ T cells, number of surviving memory $CD8^+$ T cells are stable. To keep them in a stable state, memory $CD8^+$ T cells are maintained by a dynamic process depending on cytokine induced proliferation and a balanced death (memory turnover) [46-49]. Based on time and tissue which memory $CD8^+$ T cell resides, functional and phenotypic properties of these cells may vary [50-51]. Most of the time homing molecules are analyzed to differentiate memory $CD8^+$ T cell subsets. For effector memory T cells $CD62L^{low}CCR7^{low}$ phenotype is expected whereas in central memory phenotype expected phenotype is $CD62L^{hi}CCR7^{hi}$ [52-53].

Following a successful immune response from $CD8^+$ T cells may clear all pathogens and threats. Surviving contraction, cells acquire an early memory phenotype with low CD62L expression and IFN-γ secretion. (Figure 4) Eventually early memory cells are maintained by homeostatic proliferation related cytokines, IL-7 and IL-15 which are responsible from survival and proliferative signals respectively [49]. Depending on homeostatic proliferation related cytokines, cells acquire late memory phenotype with a higher CD62L expression and IFN-γ secretion. To keep the number of memory $CD8^+$ T cells at a stable level, homeostatic cytokines driven basal proliferation is accompanied by a similar rate of cell death. (Figure 4) In opposed to a successful immune response, if T cells had to undergo contraction without clearing the threats, this leads to significant alterations in both functionality and phenotype of memory $CD8^+$ T cells [54-55]. Chronic infections and inflammations might lead to deletion of $CD8^+$ T cells of certain specificities and/or exhaustion of these cells' effector functions [56].

1.4.2 Memory T cell generation under inflammation

Innate and adaptive system can't be separated from each other. In general, innate immune system recognizes danger signals through their PRRs and initiates APCs to promote adaptive immune system [57]. In the meantime, innate immune cells produce variety of proinflammatory cytokines like IL-12 by macrophages and DCs, type I IFN by plasmacytoid DCs and IFN-γ by NK cells and $CD8^+$ T cells [58]. It has been known that these proinflammatory signals are able to promote and sustain innate immune response but recently studies demonstrated that these cytokines can act directly on

$CD8^+$ T cells [59-60]. Reduced expansion of antigen specific $CD8^+$ T cells are observed when type I IFNs, IL-12 or IFN-γ receptors are deleted compared to wild-type $CD8^+$ T cells [61-63]. Most importantly, studies demonstrated that proinflammatory cytokines especially IL-12 and IFN-γ influence the contraction phase and memory T cell generation. In BALB/c IFN-γ deficient mice reduced contraction is observed after LCMV infection [64-65]. Upon IL-12 receptor deletion contraction phase is limited in antigen-specific $CD8^+$ T cells after infection [66]. Overall, these observations indicate that proinflammatory cytokine composition would affect contraction phase which would directly impact memory generation.

1.4.3 Homeostatic proliferation induced memory $CD8^+$ T cells

Under lymphopenic environment meaning abnormally low level of lymphocytes, T cells initiates a proliferative response to regain homeostasis. This phenomenon is called homeostatic proliferation and has been shown to promote tumor clearance, reverse anergy and improve immune response against pathogens [67-70]. Studies proved that when naïve $CD8^+$ T cells transferred to a lymphopenic host, recombination activating gene (RAG) deficient mice, these cells undergo homeostatic proliferation and acquire a central memory-like phenotype indicated as $CD44^{hi}CD62L^{hi}$. These memory-like $CD8^+$ T cells demonstrate functional characteristics of antigen specific memory $CD8^+$ T cells [71-72]. Conversion mechanism of naïve T cells into memory-like T cells are partially illuminated and categorized as two hits. First hit is the self-peptide-MHC recognition by TCR and second is the homeostatic proliferation related cytokines, IL-7 and IL-15 [73]. Adopting a memory-like phenotype following TCR recognition leads to improved immune response of $CD8^+$ T cells against foreign peptide-MHC molecules [74-75].

Generation of memory-like $CD8^+$ T cells would be helpful to induce a pronounced tumor rejection in adoptive cell therapies due to their augmented responsiveness [76-77]. Studies demonstrated that improving the degree of lymphopenia previous to adoptive transfer, allowing to T cells to acquire memory-like phenotype due to homeostatic proliferation, augments the clinical responses [78]. Even though these approaches are effective, they are fastidious especially if the person would like to collect these memory-like cells to use them directly in adoptive transfer. In order to improve the generation, Kaiser and his colleagues utilized many different approaches

to mimic this conversion *in vitro* [79]. Naturally, homeostatic proliferation is observable in secondary lymphoid tissues. For that reason, they incubated T cells on three-dimensional collagen coated ceramic replica of lymph node in the presence of IL-15 and IL-7. Hence, they discovered that IL-7 and lymph node are dispensable. Therefore, they claimed that culturing $CD8^+$ T cells in the presence of IL-15 would be suffice for them to acquire memory-like phenotype which improves their antitumor potential [79].

1.5 Cancer immunotherapy

In the industrialized countries cancer malignancies are one the most life-threatening diseases. Even though ingrained cancer therapies such as resection of primary tumor, chemotherapy and radiotherapy, still 1 out of 4 patients dies because of the cancer [80]. In addition to already established treatment methods, new methodologies arise such as cancer immunotherapy. In opposed to other therapeutic methods, immunotherapy aims to prevent the occurrence and the recurrence of the cancer. There are two major arms of immunotherapy, active and passive. Main aim of the active immunotherapy is the induction of long-lasting antigen specific immune response [81]. Mostly this approach involves a type of vaccination which induces host's immune response against a specific tumor antigen either an already established one or identified from patient's biopsy [82-84]. Additionally, active immunotherapy might be a more general approach involving unspecific stimulations induced by adjuvants and cytokines. On the other hand passive immunotherapy involves supplementation of one's own tumor specific effector cells. Unlike active immunization, passive immunotherapy is more potent but short lived and fastidious [80]. One of the most famous passive immunotherapy branch is the adoptive T cell therapy involves collecting patients' T cells, teaching them to recognize a specific antigen and transferring back to the patient. (Figure 1.8)

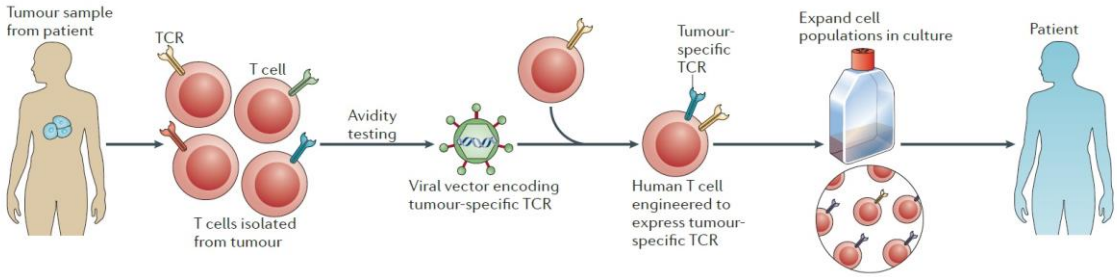


Figure 1.8: An approach to adoptive transfer of engineered T cells [85].

T cells can be engineered in many ways. An approach is the patient material, T cells collected from patient's cancer tissue are tested against that particular tumor. The ones with the best immune response are separated and their TCRs are cloned into a retroviral or lentiviral vector. In this approach, patient's T cells are transduced with this vector to enable autologous T cells to recognize patient's tumor. In another approach chimeric antigen receptor (CAR) vectors are utilized. These vectors consists of monoclonal antibody which enables T cells to recognize antigens without MHC restriction, TCR intracellular regions to induce T cell activation and activating motifs to eliminate the necessity of co-stimulatory molecules [85-86]. Considering immunotherapies can be used together with already established treatments such as chemotherapy and radiotherapy, novel immunotherapeutic approaches offer flexibility and a better treatment chances to the field.

1.6 Aim of the study

Considering the observation of Kaiser and his colleagues, we embarked on a project to improve the approach of memory-like T cells generation, make it more practical and then exploit the improvements in tumor therapy. Our first approach is to utilize bone marrow derived DCs, known to be one of the most potent IL-15 expressing cells, in a co-culture setup with T cells. Stimulating DCs with proper ligand or ligand combinations could induce pronounced IL-15 secretion that should then help to elicit memory-like T cells conversion. Therefore, we may use most potent ligands alongside of activated T cells *in vivo*. By this approach, we could both stimulate innate immune system to raise a response against cancer and create and environment which would help memory-like conversion and improve adoptively transferred T cells' antitumor

response. Next as an alternative approach, we utilized retroviral transduction to enable T cells to express their own IL-15 to induce memory-like conversion. Consequently, memory-like phenotype generation could be improved by transducing $CD8^+$ T cells with IL-15 expression vector without any extraneously secreted IL-15 or recombinant IL-15 usage. In future, this phenotype might be exploited in tumor therapy and might provide a viable alternative eliminating some of the limitations of classical adoptive T-cell therapy.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. General Laboratory & Cell Culture Reagents and Materials

Cell culture mediums DMEM and RPMI-1640, alongside of PBS, FBS, L-glutamine and 2-mercaptoethanol were purchased from Gibco, USA. For cell counting Zap-OGLOBIN® II Lytic reagent and Z-PAK Isoton II diluent used and purchased from Beckman Coulter, USA. Mouse Pan T, CD8 T cell magnetic isolation kits and all MACS equipments were purchased from Miltenyi Biotech, Germany. Other cell culture reagents Pen-Strep, Na-Pyruvate, HEPES, NEAA and Trypsin were purchased from Lonza, Switzerland. Another cell counting reagent was Trypan Blue and together with Hypure Molecular Biology Grade Water were purchased from Hyclone. Plastic materials utilized mainly in cell culture such as plates, flasks, scrapers, serological pipettes and filters purchased from Corning Life Sciences Inc., USA. In order to freeze and store the cells, Mr. Frosty container was used and purchased from Thermo Scientific, USA and 2 ml cryovials were purchased from Greiner bio-one, Austria.

2.1.2 Recombinants and Other Agents

Recombinants used in bone marrow derived dendritic cell generation, mouse IL-4 and GM-CSF were purchased from Tonbo, USA. Recombinant used in T cell culture, mouse IL-15 was purchased from Biolegend, USA. T cell activation materials such as anti-CD3 and anti-CD28 were purchased from Tonbo, USA.

2.1.3 CpG ODNs

Sequences of CpG ODNs supplied from Alpha-DNA. Their length and sequences were listed in Table 2.1.

ODN	**Brand**	**Length**	**Sequence**
1466-Acore-PO	Alpha-DNA, Canada	16mer	TCAACGTTGATTCAAA
D35-3CG	Alpha-DNA, Canada	20mer	GGTCGATCGATCGAGGGGGG
K23-PS	Alpha-DNA, Canada	12mer	TCGAGCGTTCTC

Table 2.1: Commercial name of the CpG ODN names and their properties.

2.1.4 PRR Ligands

Ligands used for DC stimulations were listed in Table 2.2.

Ligand	**Source**	**Brand**
c-di-GMP	Synthetic	Biolog, Germany
3’3’ cGAMP	Synthetic	Invivogen, USA
LPS	*E. coli*	Sigma, USA
3’3’ cGAMP	Synthetic	Invivogen, USA

Poly(da:dt)	Synthetic	Invivogen, USA
R848	Synthetic	Invivogen, USA

Table 2.2: Commercial name of the ligands and their sources.

2.1.5 Flow Cytometry

Staining antibodies used in flow cytometry analysis were listed in Table 2.3. In order to perform cell fixation Medium A was used and purchased from Invitrogen, USA.

Dye	**Species**	**Brand**	**Cat #**
Anti-CD3-PE	Mouse	Biolegend, USA	100206
Anti-CD8-FITC	Mouse	Biolegend, USA	6003
Anti-CD44-FITC	Mouse	Biolegend, USA	103005
Anti-CD44-APC-eFluor780	Mouse	eBioscience, USA	47-4031
Anti-CD62L-PE/Cy5	Mouse	Biolegend, USA	104410

Table 2.3: Commercial name of the antibodies and their properties.

2.1.6 Determination of Gene Expression

In order to perform RNA isolation, Trizol reagent were used and purchased from Life Technologies, USA. For cDNA synthesis, ProtoScript M-MulV cDNA Synthesis Kit and OneTaq Quick-Load 2x Mastermix were used and purchased from NEB, USA. High fidelity polymerases used in cloning procedure such as Phusion HF DNA polymerase and purchased from NEB, USA. DNA laders and 6X loading dye were used in PCR analysis and purchased from Fermentas, USA.

2.1.6.1 Primers

Primers used to confirm gene expression were listed in Table 2.4 and designed as follows. Mouse cDNA sequences at Ensembl™ database were used and primers were designed by using Primer3 Input v.0.4.0 program (http://frodo.wi.mit.edu/primer3/input.htm). Primer sequences designed to perform cloning were listed in Table 2.5. Original murine OT-I TCR retroviral vector (Plasmid #52111) was purchased from Addgene, USA.

Gene Name	**Direction**	**Sequence**	**Product size (bp)**
IL-15	F	CATCCATCTCGTGCTACTTGTGTT	491
IL-15	R	CATCTATCCAGTTGGCCTCTGT	
β-actin	F	GTATGCCTCGGTCGTACCA	105
β-actin	R	CTTCTGCATCCTGTCAGCAA	

Table 2.4: Primers used in confirming gene expression.

Sequence Name	**Direction**	**Sequence**
EcoR-I/IL-15	F	GCGCCAGAATTCACCATGAAAATTTTGAAAC CATATATG
Xho-I/IL-15	R	GCGTCGCTCGAGTCAGGACGTGTTGATGAAC
EcoR-I/TCR-α	F	CAGCCCTCACTCCTTCTCTAGGCGCCGGAATT CACCATGGACAAGATCCTGACAGCATC
TCR-β/T2A	R	CCAGCCTGCTTCAGCAGGCTGAAGTTAGTAG CTCCGCTTCCGGAATTTTTTTTCTTGACC

T2A/IL-15	F	CTGCTGAAGCAGGCTGGAGACGTGGAGGAG AACCCTGGACCTACCATGAAAATTTTGAAAC CATATATGAGGAATACATC
IL-15/Xho-I	R	GCGTCGCTCGAGTCAGGACGTGTTGATGAAC

Table 2.5: Primers used in cloning procedure.

2.1.7 Reagents for ELISA

To perform ELISA, 2HB plates were used and purchased from SPL Life Sciences, Korea. ELISA antibodies were purchased either from Biolegend, USA or Mabtech, Sweden. SA-ALP was purchased from Mabtech, Sweden as well. To develop PNPP Tablets and 5X Diethanolamine Buffer were used and purchased from Thermo Scientific, USA. Detailed information of antibodies and recombinant proteins were listed on Table 2.6.

Antibody Name	**Brand**	**Cat #**	**Working concentration**
Anti-IFN-γ Ab	Mabtech, Sweden	3321-1a-20	1 μg/ml in PBS
IFN-γ Biotinylated Ab	Mabtech, Sweden	3321-1a-20	1:1000 diluted in T cell buffer
Recombinant IFN-γ	Biolegend, USA	575309	80 ng/ml in Blocker
Anti-IL-15 Ab	LSBio, USA	LS-C104510	1 μg/ml in PBS
IL-15 Biotinylated Ab	LSBio, USA	LS-C104416	1 μg/ml in T cell buffer

Recombinant IL-15	Biolegend, USA	566302	200 ng/ml in Blocker

Table 2.6: List of mouse ELISA antibodies and recombinants.

2.2 Solutions, Buffers and Culture Media

2.2.1 Cell Culture Media

High Glucose DMEM and RPMI-1640 (Lonza)

- 2, 5 or 10% FBS inactivated at 55 °C
- 50 g/ml Penicillin/Streptomycin
- 10 mM HEPES
- 0,11 mg/ml Na Pyruvate
- 1% Non-Essential Amino Acids Solution
- 2 mM L-Glutamine
- Ingredients dissolved in 500 ml medium
- Storage temperature: +4 °C

2.2.2 Flow Cytometry Buffers

PBS-BSA-Na azide Buffer

- 500 ml 1x PBS
- 5g BSA (1%)
- 125 mg Sodium Azide (0.25%)
- Storage temperature: +4 °C

2.2.3 Agarose Gel Electrophoresis

50X TAE (Tris-Acetate-EDTA)

- 242 g Tris ($C_4H_{11}NO_3$)
- 37.2 g Tritiplex 3 (EDTA= $C_{10}H_{14}N_2Na_2O_2 \cdot 2H_2O$)

- 57.1 ml Glacial acetic acid
- Ingredients dissolved in 1 lt ddH_2O and autoclaved
- Storage temperature: +4 °C
- Working dilution: 1X

2.2.4 ELISA Buffers

Blocking Buffer

- 500 ml 1x PBS
- 25 g BSA (5%)
- 250 µl Tween20 (0,025%)
- Storage temperature: -20 °C

T-cell Buffer

- 500 ml 1x PBS
- 25 ml FBS (5%)
- 250 µl Tween20 (0,025%)
- Storage temperature: -20 °C

Wash Buffer

- 500 ml 10x PBS
- 2.5 ml Tween20
- 4.5 lt ddH_2O
- Storage temperature: +4 °C

10X PBS (Phosphate Buffered Saline)

- 80 g NaCl
- 2 g KCl
- 8,01 g Na_2HPO_4 . $2H_2O$
- 2 g KH_2PO_4
- 1 lt ddH_2O
- pH adjustment: 6.8

- Storage temperature: Room temperature

2.2.5 MACS Buffer

- 0.5% BSA
- 2mM EDTA
- Ingredients dissolved in 1X PBS
- Storage temperature: +4 °C

2.2.6 Bacteria Solutions

2.2.6.1 Liquid Broth

- 10 grams Tryptone
- 5 grams Yeast Extract
- 5 grams NaCl
- Ingredients dissolved in 1lt ddH_2O and autoclaved.
- Storage temperature: +4 °C

2.2.6.2 Agar Plate

- 10 grams Tryptone
- 5 grams Yeast Extract
- 5 grams NaCl
- 10 grams of Agarose
- Ingredients dissolved in 1lt ddH_2O and autoclaved.
- Chilled to ~50 °C and distributed to 20 ml per petri dish
- Storage temperature: +4 °C

2.3 Methods

2.3.1 Maintenance of cell lines

2.3.1.1 PHOENIX-ECO

PHOENIX-ECO (ATCC®, CRL-3214) is a human kidney epithelial derived cell line. Cells were sustained in a DMEM media with 10% regular FBS. Cells cultured for retrovirus packaging and production are seeded as $3x10^6$ cells on 10cm plates. These cells are stated as adherent but they are very fragile and can detach from the plate very easily. Sub-culturing performed at 90% confluence with 1X Trypsin. Cells collected at 300 g with 5 min centrifugation were seeded on appropriate density back to new plate.

2.3.1.2 B16-F10

B16-F10 (ATCC®, CRL-6475) is a murine skin melanoma cell line. These cells are strongly adherent. Sub-culturing of these adherent cells performed at %90 confluence with 1X Trypsin following fresh RPMI1640 supplemented with %10 FBS. Cells are centrifuged at 300g for 5 min and seeded on appropriate concentration back to new plate. If necessary, cells are counted by haemocytometer.

2.3.1.3 B16-OVA

B16-OVA is a murine skin melanoma cell line. This cell line is a variant of B16-F10 which stably expresses ovalbumin protein. Similar to B16-F10 these cells are strongly adherent. Sub-culturing of these adherent cells performed at %90 confluence with 1X Trypsin following fresh RPMI1640 supplemented with %10 FBS. Cells are centrifuged at 300g for 5 min and seeded on appropriate concentration back to new plate. If necessary, cells are counted by haemocytometer.

2.3.1.4 B16-Blue IFNα/β

B16-Blue IFNα/β is a murine type I IFN sensor cell line. This line has the origin of C57BL/6 murine b16 melanoma cell line. Cells can respond to type-I IFN because this line is stably transfected by a SEAP reporter gene under the control of the IFNα/β-inducible ISG54 promoter. B16-Blue IFNα/β cell line can monitor the activation of JAK/STAT/ISGF3 and IRF3 pathway. Culturing and sub-culturing are performed in

RPMI1640 media supplemented with 10% FBS. Sub-culturing of these adherent cells performed at %90 confluence with 1X Trypsin following fresh RPMI1640 supplemented with %10 FBS. Cells are centrifuged at 300g for 5 min and seeded on appropriate concentration back to new plate. If necessary, cells are counted by haemocytometer.

2.3.2 Thawing of cells and cryopreservation

Cryovials are taken from liquid nitrogen and immediately put in 37 °C pre-heated water bath. Vials are gently swirled in a circular motion until they are melted. After the ice in the vials melted, cells are immediately transferred into a 15 ml falcon tube and supplemented with appropriate medium until the total volume is 15 ml. Afterwards, the falcon is centrifuged at 300 g for 5 min. Supernatant aspirated and the pellet is re-suspended with appropriate fresh complete medium. Then, cells are seeded on preferred plate or flask and placed into the cell culture incubator, which is set for 37 °C with 5% CO_2.

Cryopreservation can be performed when cells reach around 60-80% confluency and 80-90% viability. Cells are collected with an appropriate method and centrifuged to remove the supernatant. Then the cell pellet is re-suspended in FBS which has a 10% DMSO content. Cell mixture is immediately transferred into labeled cryovials. Cryovials are placed in Ms. Frosty Freezing Container and placed to -80 °C fridges. At minimum 24 hours later cells are transferred into liquid nitrogen tanks to be able to store them for longer periods of time.

2.3.3 Cell counting

2.3.3.1 Haemocytometer

After the primary cells and adherent cell lines are washed, centrifuged and re-suspended in 1-10 ml of fresh media. 10 µl from cell mixture are mixed with 10 µl Trypan Blue to separate live cells from dead ones. 10 µl from this Trypan Blue-cell mixture are loaded on haemocytometer and cells are counted under light microscope. Live cells can be observed as bright dots and only these bright dots are counted to calculate the total number of living cells by considering the dilution factor.

2.3.3.2 Flow cytometer

After the primary cells and suspension cell lines are washed, centrifuged and re-suspended in 1-10 ml of fresh media. Generally, 20 µl of cell suspension are mixed 10 ml Isoton II Diluent buffer and 2-3 drops of ZAP-OGLOBIN II Lytic Reagent was added to lyse erythrocytes if necessary. This mixture was analyzed under Flow Cytometry. Live cells were gated, apoptotic cells or cell clusters were ignored. Total number of cells are calculated considering live cell gate and dilution factor.

2.3.4 Primary murine cell suspension preparation

2.3.4.1 Mouse spleen cell suspension preparation

Cervical dislocation used to sacrifice mice (C57/BL6). The left side of the sacrificed mouse wet by using %70 ethanol. Cut open the left side of the mouse, nearly half-way between front and back legs. Spleen removed by using forceps and scissors and transferred into %2 FBS supplemented complete RPMI-1640 media. The spleen was mashed using the syringe plunger into a petri dish and washed twice with 10ml fresh RPMI-1640 medium followed by a centrifugation step at 300 g for 5 minutes. At the final step, cells were resuspended in 1ml of %5 FBS supplemented RPMI-1640 to count by Flow Cytometry.

2.3.4.2 Bone marrow suspension preparation

Cervical dislocation used to sacrifice mice (C57/BL6). Sacrificed mice pinned down to dissection board and sterilized with ethanol. Incisions made on thigh and ankle, the skin was peeled down to ankle and removed from the leg. Muscle tissue was removed from each leg by using a surgical blade. Each leg completely separated and placed into ethanol for sterilization. Bones are further cleaned from remaining muscle tissues and separated from knee joints as femur and tibia. By holding each bone fragment with a tweezer bone marrow containing red part was identified and introduced a cut immediately before the identified part. Bone marrow was flushed out with 26g insulin syringe into the 15ml Falcon tube. %2 FBS supplemented RPMI-1640 media passed through the bone until the color turns from red to white. Collected marrow cells were centrifuged at 300g for 5 minutes. Red blood cells were lysed by resuspending marrow in 1ml of ACK lysis buffer and incubated for 2 minutes. Cells were washed with %2

FBS supplemented RPMI-1640 media and centrifuged at 300g for 5 minutes. Cells were resuspended in %10 FBS supplemented RPMI-1640 media to count by Flow Cytometry.

2.3.5 Bone marrow derived dendritic cell generation

Bone marrow cells are seeded as $1x10^6$ cells/ml into $10cm^2$ plate in RPMI-1640 supplemented with 10% FBS, 20ng/ml mGM-CSF and 10ng/ml mIL-4 recombinant proteins. Three days after initial seeding half of the medium aspirated from plate and replenished with fresh medium and supplements. Six days after initial seeding cells are collected. At this time point cells can be a bit adherent in order to detach them cold 1X PBS supplemented with 2mM EDTA can be used. Then, cells are stained with CD11b/CD11c and analyzed under Flow Cytometry to confirm their dendritic cell phenotype.

2.3.6 $CD8^+$ T cell separation from mouse spleen

After mouse spleen suspension prepared and counted, ACK lysis performed. Generally, single spleen re-suspended and incubated for 2 minutes in 2 ml of ACK lysis buffer. In order to dilute lysis buffer 15 ml falcon filled with fresh RPMI-1640 and centrifuged at 300 g for 5 minutes. Most of the pellet should be observed as white colored. There might be a thin red layer above the white pellet that wouldn't create a problem since all other cells are seperated. Supernatant was aspirated, re-suspended in fresh RPMI-1640 and cells are counted again with the help of Flow Cytometry. After calculating total number of splenocytes, pellet can be re-suspended in MACS buffer as 40µl buffer per 10^7 splenocytes. Then, 10µl of Biotin-Antibody Cocktail was added to per 10^7 total cells and incubated for 5 minutes in the refrigerator. After incubation, 30µl MACS buffer per 10^7 splenocytes was added to mixture. On top of that 20µl Anti-Biotin Microbeads are added to per 10^7 total cells and incubated for 10 minutes again in the refrigerator. Afterwards, cells are washed with 2 ml of MACS buffer and centrifuged and supernatant removed to rid of excess magnetic beads. Pellet was re-suspended in 500µl MACS buffer. In the meantime an MS column was prepared by washing with 500µl buffer. After washing was finished, splenocyte suspension were loaded on the MS column attached to a magnet. At this step cells are separated by magnetic force and drop down with the help of gravity. After the last drop of cell suspension, 500µl MACS

buffer was loaded on MS column in order to wash the column. This step performed twice. Then, flow through was centrifuged and cells are re-suspended in 1ml of RPMI-1640 supplemented with 50µM 2-mercaptoethanol in order to count them via Flow Cytometry. A percentage of separated cells are used in FACS staining, specifically for CD3 and CD8 markers, to calculate the separation efficiency.

2.3.7 $CD8^+$ T cell activation

$CD8^+$ T cells are activated through anti-CD3 and anti-CD28 murine antibodies. This is an artificial activation mimicking the APC cell contact. Activation of $CD8^+$ T cells are a crucial part of retroviral transduction. It has been known that anti-CD3 is better when coated whereas anti-CD28 is better in soluble form. Therefore, anti-CD3 prepare in 1X PBS at 2µg/ml and discard on the wells in appropriate amounts. Plate can be coated overnight at 4°C or 1-2 hours at 37°C. Later on wells were flooded with 1X PBS two times to remove excess antibodies. Finally, PBS were removed and separated $CD8^+$ T cells were placed on coated wells. As indicated above, anti-CD28 was added in soluble form at 1µg/ml concentration.

2.3.8 Retrovirus collection from Phoenix-Eco cells

At Day 0 cells are collected from the plate and counted via haemocytometer. For 10cm2 plates cells are seeded as 3x106 cells/plate in 9 ml DMEM supplemented with 10% FBS. On the next day, transfection mixture is prepared. 12µg retroviral vector, 3µg pCL-Eco helper plasmid and 50µl 2.5M $CaCl_2$ are mixed. Total volume is adjusted to 500µl ddH_2O. Then, while vortexing the CaCl2 mixture, 500µl 2X HEPES are added drop wise. Total mixture incubated for 20 minutes at room temperature. Afterwards, mixture transferred on seeded Phoenix-Eco cells drop wise. After about 2 hours, small crystal like structures can be observed via 20X light microscope. If there are no small dots but rather big clumps, solutions must be checked and most probably the pH of the solutions are not proper. At Day 2, transfection mixture is aspirated, plate is washed with warm 1X PBS and 10 ml of fresh DMEM with 10% FBS is added on cells for virus production. At Day 3, exactly 24 hours later medium is collected, filtered via 0.45µm and stored at -80°C. Another set of virus production can be made with a lower titer. To do that cells are feeded with another set of fresh DMEM with 10% FBS and on the next day can be collected, filtered and stored. Collected Phoenix-Eco supernatant

can be subjected to ultracentrifugation to condense virus particles. Exactly at $1x10^5$ g supernatant can be centrifuged for 2 hours at 4°C. Pellet is visible if supernatant is more than 30ml. Supernatant is aspirated and pellet can be re-suspended in either 1X PBS or DMEM with %10 FBS according to the volume of choice. After this step dense virus aliquots can be stored at -80°C.

2.3.9 Generation of memory-like $CD8^+$ T cells

$CD8^+$ T cells are separated from spleen and seeded on anti-CD3 coated wells. Soluble anti-CD28 is added to each well. IL-15 mouse recombinant protein is added in designated wells as 200ng/ml concentration. Cells are incubated for 7 days in total when medium turns to yellow cells are diluted in 1:3 ratio and supplemented with previously indicated conditions. On day 7, cells are collected from wells. In general, T cells are suspension cells meaning their detachment can be simply performed by a 1ml pipet. If cells don't detach from the well properly, cold 1X PBS or cold 1X PBS supplemented with 2mM EDTA can be used. After collection, cells are either fixed and stained or directly stained with CD3, CD44 and CD62L FACS antibodies to determine their phenotype. In general, articles suggest many other markers alongside of CD44 and CD62L such as PD-1, CD127, Ly6c and CD25. One of the most used combination determined for memory-like phenotype is $CD44^{hi}CD62L^{hi}$ whereas effector phenotype accepted as $CD44^{hi}CD62L^{lo}$ and naïve phenotype as $CD44^{lo}CD62L^{hi}$.

2.3.10 Retroviral transduction of $CD8^+$ T cells

$CD8^+$ T cells separated from mouse spleen is seeded on coated 2µg/ml anti-CD3 wells and supplemented with 1µg/ml anti-CD28 in RPMI-1640 with 10% FBS and 50µM 2-mercaptoethanol. For 24-well plate, cells are seeded in 500µl medium. Cells are activated for 2 days with antibodies. On day 2, cells are collected and counted with Flow Cytometry to calculate total cell number. After calculation, cells are separated $5x10^5$ cells/well in 500µl retrovirus containing medium supplemented with 10µg/ml Polybrene. For optimum efficiency each vector should be subjected to titration. After seeding the cells, plate is placed in the incubator to allow cells to regain optimal temperature and CO_2 percentage. In the meantime, Nüve NF 1200R centrifuge is set to 32°C. After about an hour, plate is removed from the incubator and immediately wrapped with parafilm to keep the pH stable. Plate can be centrifuged at 1500 g for 1.5

hours, if the temperature of the centrifuge is about 28°C or more. After centrifugation plate is placed into incubator for an hour to improve transduction efficiency. Because viral particles either are internalized or attached to cell membrane with the help of Polybrene. Incubating cells a little longer gives time for already attached viral particles to internalize, otherwise they can be washed away while replacing the medium. After incubation, medium is aspirated and replenished with 10% FBS and 50μM 2-mercaptoethanol supplemented RPMI-1640.

2.3.11 $CD8^+$ T cell:B16 co-culture and antigen specific lysis

$CD8^+$ T cells were activated and transduced as indicated. After confirming CD8+ T cell phenotype, co-culture ratio was calculated. Ratio can range between 1:1 and 1:10 meaning ten times more $CD8^+$ T cells can be added on top of the B16 cell line. Due to the low amount of $CD8^+$ T cell acquisition, 1:5 ratio was our limit. In this method, B16-F10 cell line and B16-OVA variant were used to show antigen specific lysis. For 24-well plate B16 cells were seeded as $5x10^5$ cells/well. Exactly one hour after B16 seeding, $CD8^+$ T cells were added on B16 cells in preferred ratios. Plate was placed in the incubator for 48 hours. After incubation supernatants and cells were collected. Supernatants were stored at -20°C for ELISA analysis. Collected cells were stained with Propidium Iodide to observe whether they are alive or not by testing their membrane integrity. Comparison between B16-F10 PI positive cells and B16-OVA PI positive cells indicates antigen specific cytotoxicity. Additionally, IFN-γ response acquired from ELISA analysis could be used to support antigen specific response of $CD8^+$ T cells.

2.3.12 Flow cytometry

2.3.12.1 Fixation of cells

Collected cells are centrifuged at 300 g for 5 minutes. Then, supernatant removed and cells are washed with 1X PBS. 50μl of Fixation Medium A used for 1x106 cells added on centrifuged cells while they are being subjected to mediocre vortexing. Immediately after, cells are incubated at room temperature for 15 minutes. After incubation, cells are washed with 1 ml of PBS-BSA-Na-Azide buffer and centrifuged at 500 g for 5 minutes.

100ul of PBS-BSA-Na-Azide buffer is used to re-suspend the cells. At this time point, cells can be either stored 4°C up to a week or directly stained with FACS antibodies.

2.3.12.2 Surface marker staining of cells

Cells can be stained after fixation or without fixation. After fixation protocol, certain markers are reduced considering this fixation can be avoided in suggested markers staining. After cell fixation or simply centrifugation cells are re-suspended in 100 µl PBS-BSA-Na-Azide buffer. FACS antibodies are used in 1µg/ml concentration and cells are stained in PBS-BSA-Na-Azide buffer in dark at room temperature for 30 minutes. After staining cells are washed with 2 ml of PBS-BSA-Na-Azide buffer. Then, cells are subjected to centrifugation at 500 g for 5 minutes. Finally, cells are re-suspended in 1X PBS and analyzed with Flow Cytometer.

2.3.13 Gene expression determination

2.3.13.1 Total RNA isolation

Cells were detached and collected in eppendorf tubes. Cells were centrifuged at 300 g, 4 °C for 5 minutes. After centrifugation, supernatant was discarded and pellet re-suspended in 1 ml of TRIzol. This suspension can be stored at -80°C for long term. Afterwards, TRIzol suspension kept on ice during isolation. To each TRIzol containing eppendorfs 200 µl chloroform was added and shaken vigorously for 15 seconds and left for 3 minutes incubation at room temperature. Then, tubes are subjected to centrifugation at 13200 rpm for 17 minutes. After centrifugation, layers were formed and upper clear aqueous layer collected and 500 µl isopropanol added on collected layer to precipitate the RNA. At this step, suspension was gently inverted up and down to mix and then left for 10 minutes incubation at room temperature. After incubation, suspension centrifuged for 12 minutes at 13200 rpm. Supernatant was discarded and in order to wash pellet was re-suspended in 1 ml of 75% EtOH. Then, tubes are subjected to centrifugation for 7 minutes at 13200 rpm. Another washing step was performed with 1 ml of >99,8% EtOH and centrifuged at 8000 rpm for 7 minutes. After final centrifugation, supernatant was removed and pellet was left to air-dry. Finally, pellet was re-suspended in 20 µl of RNase/DNase free water. Purity and concentration of the isolated samples were carried out by using NanoDrop® ND-1000 spectrophotometer. A260/280 ratio indicated whether there is a chemical contamination or not which

normally ranges between 1.8 and 2.0. Final, isolated product can be stored at -80 °C for long term use.

2.3.13.2 cDNA synthesis

To synthesize cDNA from previously isolated total RNA ProtoScript First Strand cDNA synthesis kit was used. For each reaction, 1 µg of RNA mixed with 2 µl oligo dT and RNase/DNase free water added to the mixture until total volume is 8 µl. In order to perform pre-denaturation step, tubes were transferred to MJ Mini thermocycler (Bio-Rad, USA) which was set to 70 °C and left there for 5 minutes. Afterwards, 2 µl M-MulV enzyme mix and 10 µl M-MulV reaction mix were added to the previously completed reaction. For the final PCR reaction, tubes are again transferred to the MJ Mini thermocycler and program was set according to the following conditions; 42 °C for 60 minutes, 80 °C for 5 minutes and 4°C for 10 minutes. After this reaction, cDNA samples if preferred can be measured like RNA products. Otherwise, they can be stored at -20 °C.

2.3.13.3 PCR

In order to detect gene expression level, PCR can be performed. A specific primer pairs (Table 2.7) were designed and optimal PCR conditions were optimized for each gene analysis. Reaction mixture prepared according to the following recipe; 12,5 µl of Quick Load Taq 2X Master Mix, 9,5 µl of Nuclease-free H_2O, 1 µl of 10pmol Forward Primer, 1 µl of 10pmol Reverse Primer, 1 µl of cDNA. Total 25 µl volume of mixture transferred to the MJ Mini thermocycler. As indicated PCR reaction protocol might vary both according to the gene of interest and primer sets which is shown in Table 2.7. Final PCR products were kept at 4°C if products were to be analyzed with agarose gel electrophoresis. Otherwise, products can be stored at -20°C for future analysis.

PCR Steps	Temperature	Duration	Cycle
Initial Denaturation	-	-	-
Denaturation	94 °C	30 sec	35x
Annealing	β-actin 55 °C	30 sec	

	IL-15 60 °C		
Elongation	72 °C	1 min	
Final Extension	72 °C	5 min	1x
Hold	4	∞	

Table 2.7: PCR protocol for gene expression determination.

2.3.14 Gene cloning

2.3.14.1 2-step PCR

This cloning technique is generally used for incorporation of new sequences to the already established vector. Basically, primers were designed to cover about 8-10 base pairs from the restriction site of the both products that are going to anneal together with a linker site. Designs were made assuming linker sequences are already present right after the restriction site. There should be about 15 base pairs of overlapping sequences between forward and reverse linker primers. Primers were designed not just according to the base pair requirements, annealing temperatures considered as well. After primers designs two products which are going to be annealed together amplified in different reactions. To do that 2-step PCR approach was utilized (Table 2.8). In first few cycles annealing temperatures were kept lower than the suggested temperatures to improve binding efficiency. On the next step annealing temperatures were raised exactly 5 °C to improve the precision. Overall, on the second step there would be increased number of correct precursors which significantly improve the yield. After amplifying products which should have linker protruding sites were being subjected to another set of 2-step PCR procedure after confirming their size via agarose gel electrophoresis. In order to anneal these two products from the overlapping linker sites 2-step PCR procedure was utilized. To validate the correct annealing agarose gel electrophoresis were utilized.

	Temperature	Time	# of cycles
Initial denaturation	94	1min	1
Denaturation	94	30s	2
Annealing	EcoR-I/Xho-I IL-15 54 °C EcoR-I TCRa/TCRb-T2A 56 °C T2A-IL-15/Xho-I IL-15 55 °C	1min	
Elongation	72	4min	
Denaturation	94	30s	33
Annealing	EcoR-I/Xho-I IL-15 59 °C EcoR-I TCRa/TCRb-T2A 61 °C T2A-IL-15/Xho-I IL-15 60 °C	1min	
Elongation	72	4min	1
Final elongation	72	10min	
Cooling	4	Hold	

Table 2.8: 2-step PCR protocol for gene cloning.

2.3.14.2 Agarose Gel Electrophoresis

Agarose gel was prepared in different concentrations according to the necessity. The gel was either used for the confirmation of a PCR product or gel extraction procedure, %1 gel was prepared for the former and 0.5% for the latter. The gel was prepared with 1X TAE buffer, the volume of the buffer depends on the size of the electrophoresis tank. For small tank, 60 ml of 1X TAE dissolved in 0.5% agarose and boiled via microwave. Then, 1 µl of 1 mg/ml ethidium bromide solution was added and the solution was poured down in to the gel casting. Lanes are loaded with PCR products with the minimum concentration of 500 ng products per lane and maximum volume of 25 µl. DNA ladders are loaded in another lane according to the size of the product. Gels were ran for 20-45 minutes at 80-120 V power. After run, gels were visualized under UV transilluminator (Vilber Lourmat, France) with BIO-PROFILE Bio-1D V 11.9 software.

2.3.14.3 Restriction enzyme digestion

In order to clone a sequence or confirm the final product, vectors were digested from a specific sites via restriction enzymes. To double digest a DNA product of 1 µg in 50 µl of total reaction volume, 5 µl of NEB Cutsmart buffer and 1 µl from each restriction enzyme were used. The rest of the volume completed via ddH_2O. Digestion reaction performed at 37 °C for 1 hour. Additionally, NEB Cutsmart buffer didn't induce start activity overnight so safe to perform this digestion overnight.

2.3.14.4 Gel extraction

In order to isolate the correct DNA fragment from the agarose gel, gel extraction was performed. Correct size fragment was decided under UV light and removed from the rest of the gel with the help of a razor blade. Removed gel fragment were placed in an eppendorf tube and weight of the fragment was measured. During extraction procedure Macherey-Nagel PCR clean-up/ Gel extraction kit was used. For each 100 mg of gel fragment, 200 µl of Buffer NTI was added to the PCR tube. The tube was placed in a heat-block which was preheated to the 50 °C and incubated for about 5 minutes. After the gel fragment was completely dissolved, the solution was transferred to the Nucleobind column which was subjected to the 30 seconds centrifugation at 11,000g. Flow-through was discarded and column was washed with 700 µl of Buffer NT3 and

was subjected to the same centrifugation step. This washing step was repeated two times in total. Then, to get rid of all the excess NT3 buffer tube was centrifuged at 11,000g for 3 minutes. In order to elute the DNA, 15-30 µl of Buffer NE was loaded on to the column and incubated for 1 minute preferentially in the preheated heat-block. Finally, tube was centrifuged at 11,000g for 1 minute for elution. Flow-through collected and then measured via a nanodrop.

2.3.14.5 Ligation

In order to ligate two fragment following protocol was performed. Ligation ratios might change according to each product or backbone. However, in general 50 ng of vector with a 3 or 7-fold molar excess of insert was used. Molar ratios were calculated by NEBBioCalculator. Total volume was adjusted to 10 µl with ddH_2O. On top of that 10 µl of 2X Quick Ligation Buffer and 1 µl of T4 Quick Ligase added. Mixture was incubated at 25 °C for 30 minutes in a PCR machine. The end product are either immediately subjected to the transformation protocol or kept at -20 °C for further use.

2.3.14.6 Transformation

Competent *E.coli* strain was retrieved from -80 °C and defrosted on ice. Then, 50 µl ultra-competent cells are mixed with 5 µl of ligation product in an Eppendorf tube and incubated on ice for 30 minutes. After incubation, tube was placed in a heat-block or water-bath pre-heated to exactly 42 °C and kept there exactly 1 minute. Then, the tube was immediately placed on ice. After two minutes, 200 µl of Liquid Broth (LB) was added and tube was placed into shaker at 37 °C for an hour. Finally, 250 µl of solution was transferred on top of the antibiotic containing agar plate. Solution was uniformly distributed on plate and incubated for overnight at 37 °C.

2.3.14.7 Plasmid DNA purification

After a successful transformation, there would be observable bacterial colonies on agar plate. These colonies were taken via a pipette tip and placed into antibiotic containing LB for overnight growth at 37 °C. On the next day LB containing 15 ml falcon was subjected to centrifugation at 4500g for 15 minutes. Throughout the DNA purification protocol, Macherey-Nagel Plasmid DNA purification kit was used. After centrifugation, supernatant was discarded and cell pellet was lysed via 250 µl of Buffer

A1. On top of that 250 µl of Buffer A2 was added. The tube was inverted 6-8 times and kept at room temperature for 5 minutes. Then, 300 µl of Buffer A3 added and the tube was again inverted 6-8 times. Afterwards, the tube was centrifuged at 11,000g for 5 minutes at room temperature. Carefully, collected supernatant was loaded on to the NucleoSpin column and another centrifugation step performed at 11,000g for 1 minute. Then, 600 µl of Buffer A4 loaded on to the column and again centrifuged at 11,000g for 1 minute. To get rid of remaining buffer tube centrifuged for 2 minutes at 11,000g. In order to elute the DNA 50 µl of Buffer AE added and the tube was incubated at heat-block preheated to 70 °C for 2 minutes. Finally, the tube was centrifuged at 11,000g for 1 minute. The end product was collected and measured via a nanodrop. In order to confirm the cloned vector, restriction enzyme digestion was performed and the product run on agarose gel.

2.3.15 Cytokine ELISA

After any type of stimulation or incubation, culture mediums are separated from the cells and stored at -20 °C until further use. ELISA Immunoplates were coated with 50 µl/well monoclonal antibodies. Antibody dilution prepared with PBS and coated plate were incubated at +4 °C overnight. On the next day, coating solution was discarded and wells were flooded with 200 µl/well blocking buffer for 2 hours at room temperature. Then, blocking solution was discarded and plates were washed. Washing was performed in two steps. In the first step, plates were washed with washing buffer and buffer discarded for 5 times, each consist of 3 minutes of incubation. In the second step, plates were washed with dH_2O again 5 times without any incubation. After washing, cell supernatants were thawed and added to desired wells as 50 µl/well. In order to determine the concentration of cytokines, recombinant cytokines were used and prepared in blocking buffer at preferred concentrations. At this point plates were incubated overnight at +4 °C. On the next day, plates were washed as previously indicated. Then, 1:1000 diluted biotin conjugated antibodies in T-cell buffer added to wells as 50 µl/well. After 2 hours of incubation at room temperature, plates were washed again and 1:1000 diluted streptavidin-ALP solution in T-cell buffer were added to wells as 50 µl/well. After 1 hour of incubation at room temperature, plates are washed again. At this step PNPP substrate solution was prepared according to its datasheet and

added to wells as 50 µl/well. After PNPP addition, wells were read at ELISA reader (Biotek) at 405 nm periodically.

2.3.16 B16 IFN-α/β reporter assay

After any stimulation or incubation, cell culture medium are separated from cells and stored at -20 °C until further use. Previously cultured B16 derived Blue™ IFN-α/β cells were collected from culture plate and seeded as $8x10^5$ cells/well of 96-well plate. Cells were seeded in 180 µl RMPI-1640 supplemented with 5% FBS. Culture supernatants were thawed and added 20 µl to each well and incubated for 24 h at 37°C in a cell culture incubator. On the next day, supernatant of B16 derived Blue™ IFN-α/β cells were collected. 20 µl collected supernatant are mixed with 50 µl of prepared PNPP solution in another 96-well plate. Plate was incubated for 5 hours at 37 °C in total but periodically read at ELISA reader (BioTek) at 405.

Chapter 3

Results

3.1 Efforts to induce memory-like phenotype by DC co-culture

3.1.1 Identification of potential ligands to induce IL-15 secretion from DCs

Lymphopenia-induced homeostatic proliferation of $CD8^+$ T cells promotes tumor rejection. In lymphopenic host, T cells adopt a central memory-like phenotype ($CD44^+$ $CD62L^+$) T cells switch from naïve to memory-like phenotype by self-peptide-MHC recognition and homeostatic signals such as IL-7 and IL-15 [72]. Homeostatic proliferation of T cells transpire in secondary lymphoid organs where DCs can interact with T cells. Additionally, one of the most potent IL-15 secreting cells are DCs [87]. We thus hypothesized that when DCs are properly induced via pathogen recognition receptor ligands, they could create an environment where naïve T cells can convert to memory-like phenotype.

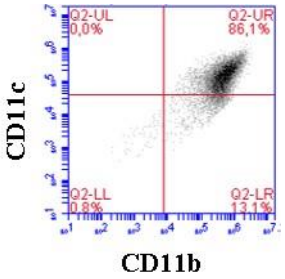


Figure 3.1: BMDC generation efficiency. *After a week of culture in IL-4 (10ng/ul) and GM-CSF (20ng/ul), cells are stained with CD-11b/CD11-c to measure generation efficiency.*

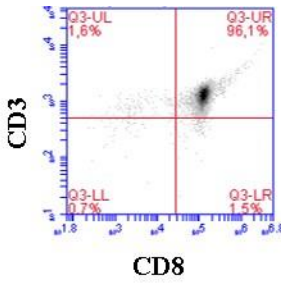


Figure 3.2: CD8⁺ T cell isolation efficiency. *CD8⁺ T cells isolated from murine spleen by MACS CD8a T cell isolation kit, stained with CD-3/CD-8 to measure isolation efficiency.*

In order to establish a co-culture, DCs were generated from mouse bone marrow via GM-CSF and IL-4 supplementation. After a week, generation efficiency was measured with flow cytometry (Figure 3.1). T cells were magnetically isolated from murine spleen with CD8a MACS separation kit and isolation efficiency was measured right after the isolation (Figure 3.2) IL-15 expression significantly increases when splenic DCs treated with Type-I IFN inducing ligands [88]. We made a similar observation, generated DCs stimulated with several pathogen recognition receptor ligands and their IL-15 expressions were measured (Figure 3.3.A) We identified that LPS, cGAMP, c-di-GMP and D3CG induced the highest IL-15 expression which are also known as inducers of Type-I IFN. A CpG-ODN categorized as K-type ODN 1466-Acore-PO sequence induced the lowest IL-15 expression (Figure 3.3.B). Highest two, LPS and cGAMP, were selected as positive groups. Since it can still activate DCs even though it promoted the lowest IL-15 expression, 1466-Acore-PO was selected as a negative control for further experiments. Activation of DCs was confirmed by both ELISA and flow cytometry. Selected ligands induced variety of cytokines, LPS promoted both IL-6 and IL-12 greatly whereas cGAMP highly improved Type-I IFN secretion. 1466-Acore-PO didn't significantly improved any screened cytokine in the indicated concentration (Figure 3.4). Increase in DC activation markers like MHC-II and CD86 were confirmed by flow cytometry after stimulations. According to our observations, LPS activated DCs to a greater extend compared to cGAMP and 1466-Acore-PO (Figure 3.5). We have identified the most potent IL-15 inducers and their overall DC activation capacities.

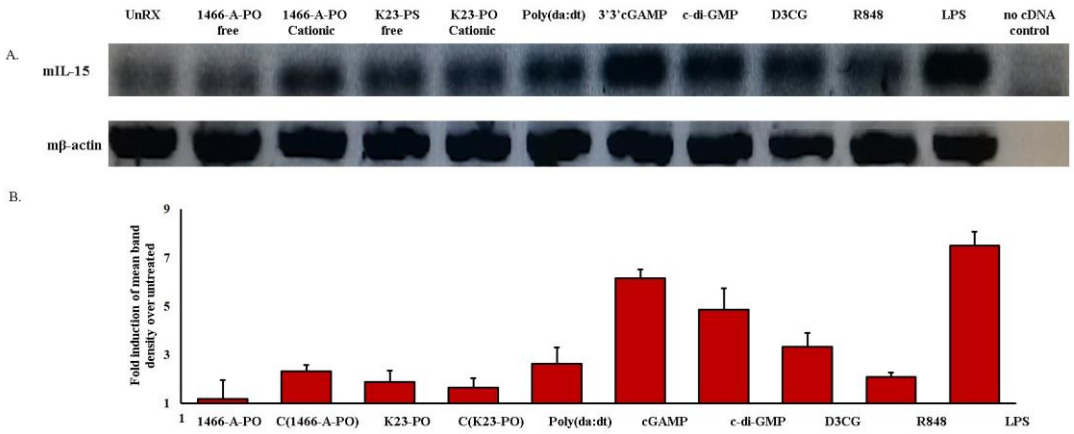


Figure 3.3: Comparison of IL-15 mRNA transcripts in total RNA of CD11c^{+} BMDC. *A. Total RNA harvested from stimulated DCs at 5h. IL-15 expression was then analyzed by RT-PCR. B. Density of bands are quantified by using ImageJ and fold inductions are calculated.*

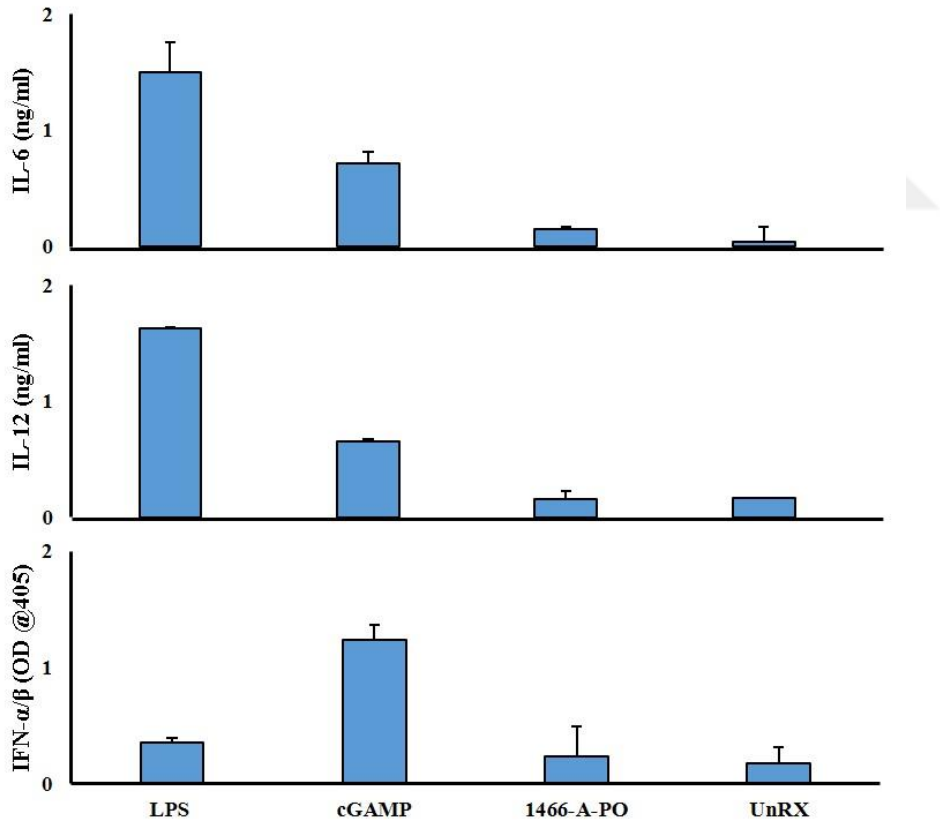


Figure 3.4: IL-6, IL-12 and IFN-α/β production from stimulated DCs. *Cytokine productions were detected from supernatant of 24 hours stimulated DCs by ELISA. IFN-α/β measurement was analyzed with B16 IFN-α/β reporter cell line.*

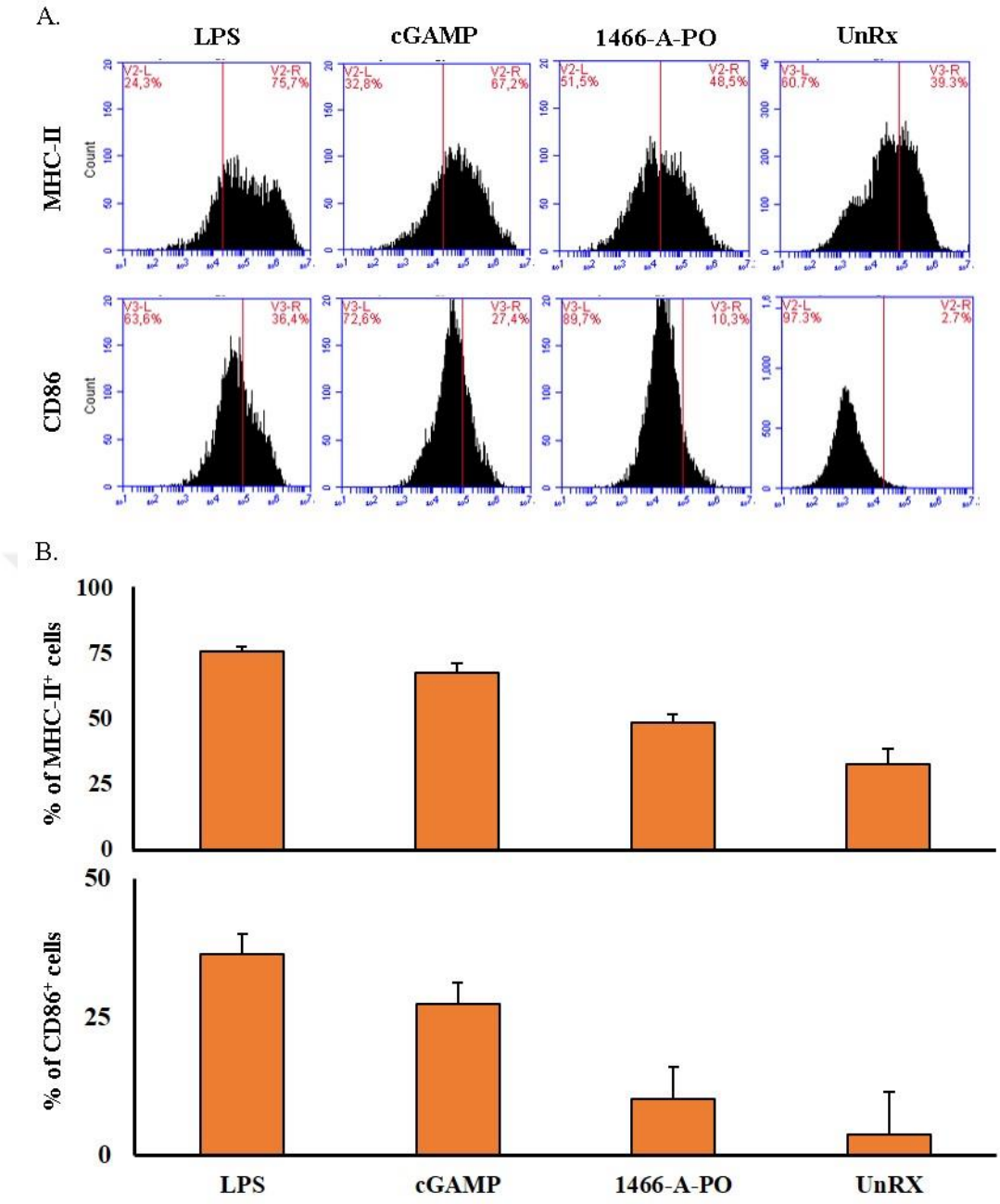


Figure 3.5: DC activation is confirmed by FACS analysis. *A. Co-stimulatory molecules are analyzed by staining collected cells with MHC-II and CD-86 antibodies. B. Percentages of active DCs improves upon PRR ligand stimulation.*

3.1.2 Regardless of IL-15 expression, DCs doesn't support memory-like progression in co-culture setup

LPS and cGAMP were identified as the most potent inducers of IL-15 from DCs and their overall activation potentials were confirmed. These activated DCs were co-cultured with magnetically isolated $CD8^+$ T cells for a week. In order to claim IL-15 is the most important variable in memory-like progression each group were also supplemented with mouse recombinant IL-15. Since our overall aim is to induce an antigen specific memory-like progression, DCs were pulsed with ovalbumin specific peptide SIINFEKL. After a week cells were collected from the U-bottom 96 well plates and their phenotypes were analyzed by flow cytometry. In order to separate T cells from DCs, cells were stained with and gated on CD8 receptor (Figure 3.6.A). However, DCs started to enter apoptosis during co-culture and caused a high autofluorescence background which made harder to perform analysis. On top of that we observed that T cells didn't proliferate very well and mostly they were in very bad condition. Still we performed CD44 and CD62L flow cytometry analysis and compared their phenotype. According to our observations, LPS, the most potent IL-15 inducer, didn't contribute to memory-like progression at all. Even when co-culture was supplemented with recombinant IL-15, memory-like phenotype wasn't affected. Another interesting observation was to see 1466-Acore-PO improved the phenotype alongside of cGAMP. Overall, phenotype of T cells didn't change when DCs were pulsed with SIINFEKL (Figure 3.6.B). Having the lowest IL-15 inducing potential didn't stop K-type ODN from inducing the memory-like phenotype. However, the conditions of cells were not good and T cells couldn't survive from further usage. Downsides of co-culture setup convinced us to utilize a different approach.

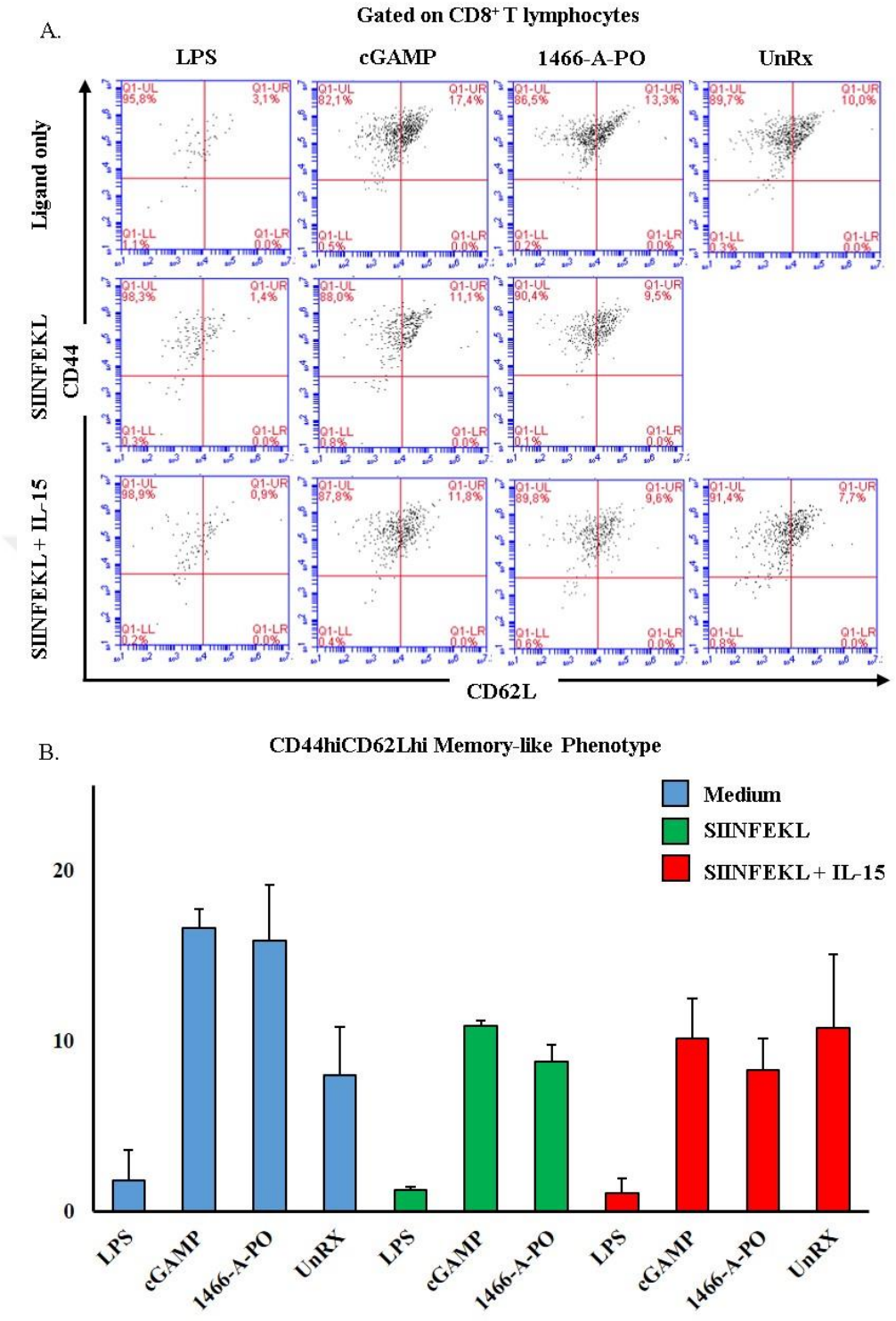


Figure 3.6: Strongest IL-15 inducer decreases memory-like T cell phenotype regardless of peptide pulse or IL-15 supplementation. *A. Surface expression of CD44 and CD62L among $CD8^+$ lymphocytes was determined after 7 days of culture. B. Percentages of central memory-like phenotype ($CD44^{hi}$ $CD62L^{hi}$).*

3.2 Efforts to induce memory-like phenotype by DC conditioned medium

3.2.1 Regardless of IL-15 amount, DC conditioned medium supports effector phenotype progression rather than memory-like phenotype

Stimulated DCs regardless of their IL-15 secretion potential failed to promote an efficient memory-like progression and T cell proliferation. As a result, we sought another approach where we stimulated T cells with anti-CD3 and anti-CD28 to activate and proliferate in the presence of DCs conditioned medium. Hence, we would be able to see that whether environment created by DCs would be enough to promote memory-like progression of $CD8^+$ T cells. Bone marrow derived DCs were stimulated with previously established the most potent IL-15 inducing ligands. Then, conditioned medium was separated from cells and used in $CD8^+$ T cell culturing. T cells were layered on anti-CD3 and anti-CD28 added in soluble form. In order to test the effect of IL-15, all groups also had IL-15 supplemented counterparts. After a week of T cell culturing, cells were collected and analyzed under flow cytometry. Groups that are indicated with ligand names are DC conditioned mediums. DC conditioned medium wasn't used in untreated group and neither DC conditioned medium nor activating antibodies used in uncoated group (Figure 3.7). Top panel, shows the memory-like phenotype ($CD44^{hi}$ $CD62L^{hi}$), and indicates that DC conditioned medium regardless of IL-15 amount doesn't promote the memory-like progression. Bottom panel, shows the effector phenotype ($CD44^{hi}$ $CD62L^{lo}$), and indicates that DC conditioned medium groups support effector phenotype rather than memory-like phenotype, regardless of IL-15 amount. Compared to DC conditioned medium, only antibody activated $CD8^+$ T cells had a better memory-like phenotype percentage which further increased in the presence of recombinant IL-15. In addition to that $CD8^+$ T cells cultured without any activating antibodies demonstrated an increase in the presence of recombinant IL-15 (Figure 3.7).

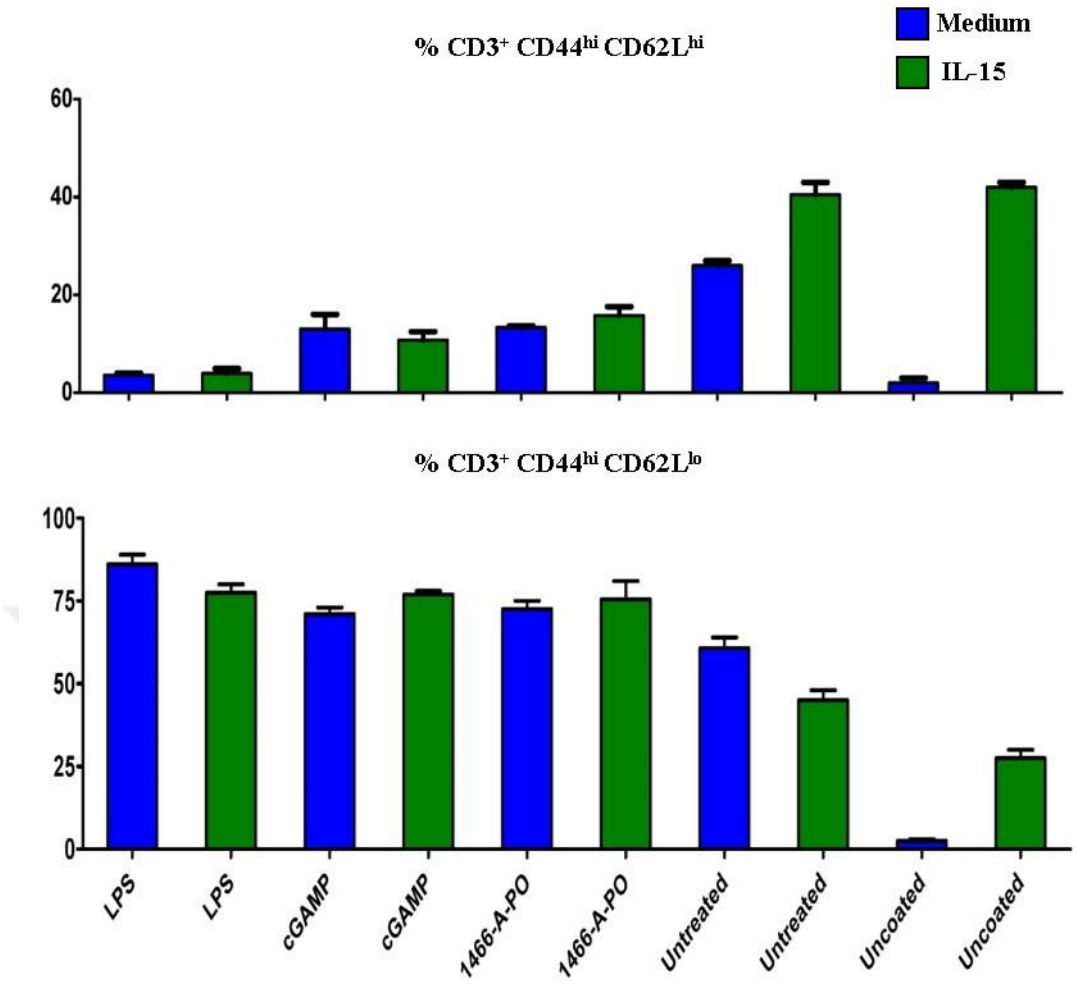


Figure 3.7: DC conditioned mediums favored effector phenotype regardless of IL-15 supplementation. *Surface expression of CD44 and CD62L among $CD8^+$ lymphocytes was determined after 7 days of culture. Top panel: Percentages of central memory-like phenotype ($CD44^{hi}$ $CD62L^{hi}$). Bottom panel: Percentages of effector phenotype ($CD44^{hi}$ $CD62L^{lo}$). All $CD8^+$ seeded on CD3/CD28 antibody coated wells except uncoated.*

3.2.2 Antibody activated $CD8^+$ T cells elicit pronounced antigen specific cytotoxic activity upon TCR gene transduction

We observed memory-like percentages of groups and decided to test their antigen specific cytotoxic potential. Since DC conditioned mediums failed to promote memory-like phenotype, we only selected cGAMP for further comparison. Our focus has shifted from DC conditioned medium to only antibody activated $CD8^+$ T cell. In order to promote T cells to recognize a specific antigen, we utilized retroviral transduction

approach. Cells were activated with antibodies transduced with commercially acquired OT-I TCR expressing EGFP tagged retroviral vector. Transduction efficiency were measured by analyzing $EGFP^+$ cells. Our transduction protocol yielded an efficiency between 50-55% (Figure 3.8). After transduction, T cells were cultured for a week according to previously indicated conditions. OT-I TCR transduction should allow T cells to recognize ovalbumin antigen. In order to test this, we co-cultured transduced T cells with B16-F10 a melanoma cell line and its ovalbumin expressing counterpart B16-OVA. After 36 hours of co-culture, cells and supernatant were collected. IFN-γ secreted from activated T cells and the total amount can give us a mean to compare their state of activity. IFN-γ ELISA performed from co-culture supernatant proved that all T cells cultured in the presence of antibodies were active and secreting IFN-γ except uncoated group. Even though, uncoated group did improve memory-like phenotype in the presence of IL-15, group didn't show a cytotoxic potential and lost its usefulness for tumor therapy. Overall, upon transduction each group's IFN-γ response increased, indicating the effect of antigen recognition. Highest IFN-γ secretion observed from the same group with the highest percentage of memory-like phenotype indicating that IL-15 supplementation improves both the phenotype and cytotoxic response (Figure 3.9). Then, collected cells were stained with propidium iodide (PI) an intercalating agent which can stain cells only when the membrane integrity of cell disrupted. Therefore, cells stained with PI would indicate the number of dead or dying cells. Then, cells were analyzed under flow cytometry (Figure 3.10.A). To clearly see antigen specific response fold lysis change and fold change in IFN-γ secretion were drawn. From the mock control group we could see that without transduction T cells still could kill a percentage of cells because they were highly active due to antibody stimulation. However, there was no significant difference between B16-OVA and B16-F10. For the other groups, we could clearly see an increase in terms of fold change of IFN-γ and lysis, indicating the antigen specific response of these cells. We could clearly confirm that $CD8^+$ T cells activated with anti-CD3/28 in the presence of recombinant IL-15 have a better overall antigen specific cytotoxic potential (Figure 3.10.B).

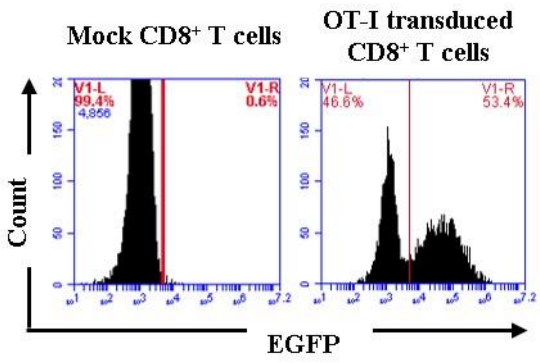


Figure 3.8: OT-I TCR retroviral vector can efficiently be transduced to CD3/CD28 antibody activated CD8$^+$ T cell. *Efficient transduction of EGFP expressing OT-I TCR chains to CD8$^+$ T cells confirmed by FACS analysis at 48h.*

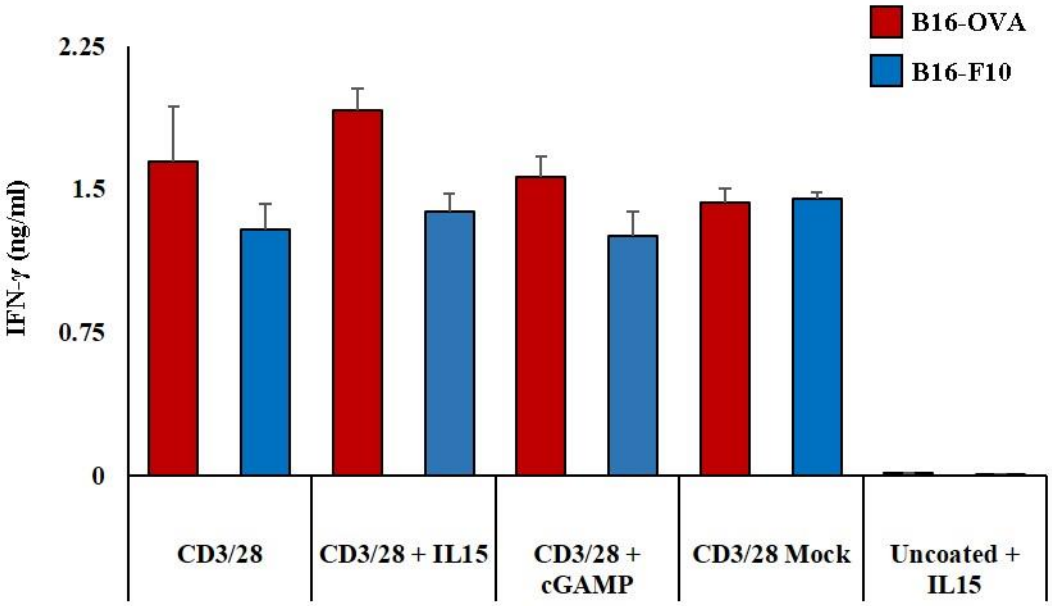


Figure 3.9: rIL15 supplementation improves IFN-γ secretion in CD8$^+$ T cell : B16 co-culture. *All CD3/28 antibody activated CD8$^+$ T cells secreting IFN-γ indicating they were in active state except uncoated. CD3/28 Mock was not transduced and used as a control.*

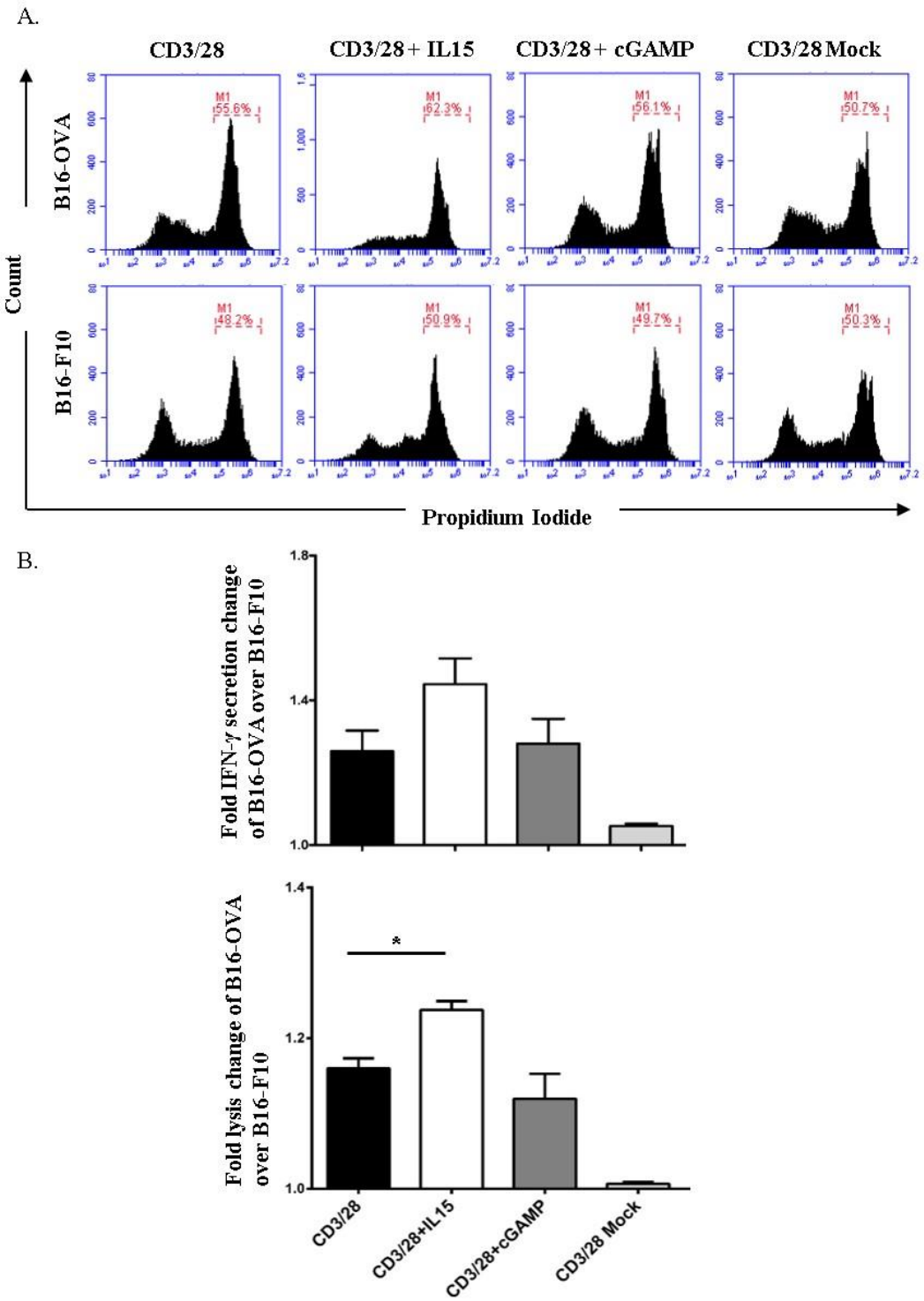


Figure 3.10: rIL15 enhences overall cytotoxicity potential of CD8$^+$ T cells. *A. B16 cells and CD8$^+$ T cells were co-cultured in a 1:1 ratio. B16 melanoma cell line and ovalbumin expressing variant used to confirm antigen specificity. At 36 hours of co-culture, cells were stained with PI to measure lysis via FACS analysis. B. Data are mean±sem of three independent experiments. Statistical analysis by unpaired Student's t-test: *P<0.05.*

3.3 Efforts to induce memory-like phenotype by transducing CD8$^+$ T cells with IL-15 expression vector

3.3.1. Antibody activated CD8$^+$ T cells without additional IL-15 supplementation elicit pronounced antigen specific cytotoxic activity upon dual transduction of OT-I TCR gene and IL-15 expression vector

DCs conditioned medium failed to improve memory-like phenotype and we observed that highest cytotoxic potential can be achieved only with recombinant IL-15. Therefore, we hypothesized that if we could transduce T cells with both OT-I TCR and IL-15 gene, they could be able to produce their own IL-15. Hence, we don't need additional IL-15 supplementation, we could reduce the culture period by simply transfusing them and let them convert into memory-like phenotype in vitro and we could improve their in vivo persistence since IL-15 transduced primary human T cells can survive up to 180 days in the absence of exogenous cytokine [89]. We aimed to clone IL-15 expression sequence right after OT-I TCR gene via ribosomal skipping sequences. Therefore, we could achieve to express them from the same promoter. However, we failed to clone such vector in time. Therefore, as a proof of concept, we wanted to transduce both OT-I TCR and IL-15 genes with separate vectors and called them as dual transduction group. Since we couldn't determine which percentage of transduced cells received which vector because they have the same backbone with EGFP tag, we wanted to improve transduction efficiency by concentrating viral supernatant via ultracentrifugation. We concentrated viral supernatant by the factors of 2X, 5X and 10X. At 10X, efficiency reach to a plateau and remained between 50-55% (Figure 3.11).

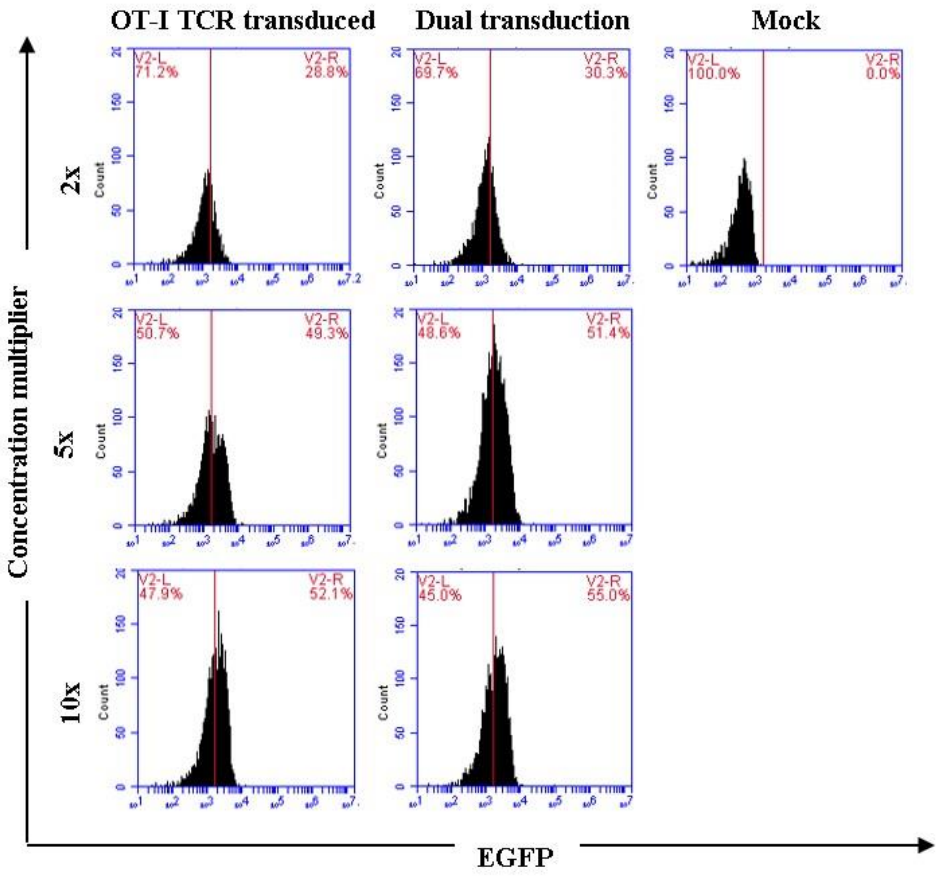


Figure 3.11: Retroviral vector transduction efficiency can be improved by concentration of virus through ultra-centrifugation. *CD3/CD28 antibody activated CD8$^+$ T cells were transduced by OT-I TCR only and in combination with IL-15 retroviral vector at indicated concentrations. Transduction efficiency was determined by measuring EGFP expressing cells via FACS analysis at 48 h. (Dual transduction: Transduction of both OT-I TCR chains and IL-15 expression sequence in separate retroviral backbones)*

Further transductions were all performed with 10X concentrated viral supernatant. IL-15 known to be inducer of T cell proliferation and anti-apoptotic pathways [89]. Therefore, we wanted to track down each groups' proliferation capacity by simply counting them periodically. At Day 10, we observed that antibody activated CD8 T cells in the presence of recombinant IL-15 had the highest proliferation capacity which is followed by dual transduction. Therefore, we observed IL-15 induced proliferation both in supplementation and transduction (Figure 3.12).

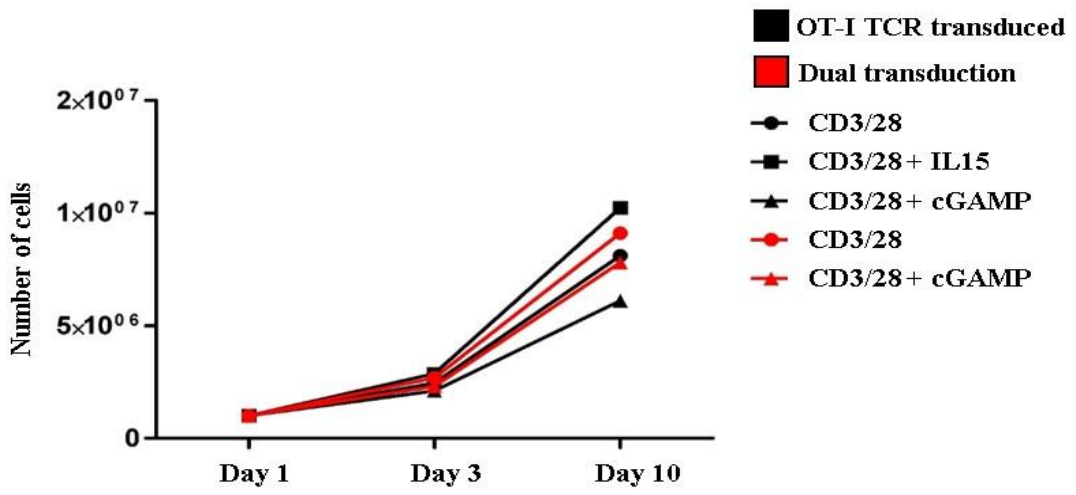


Figure 3.12: Induction of CD8$^+$ T cells proliferation by rIL-15. *1x106 cells were seeded on CD3/28 antibodies at Day 1. Total cell numbers counted on Day 3 and 10. Proliferation rate improved the most when cells were supplemented with 200 ng/ml of rIL-15, followed by IL-15 retroviral transduced CD8$^+$ T cells. (Dual transduction: Transduction of both OT-I TCR chains and IL-15 expression sequence in separate retroviral backbones)*

Then, we wanted confirm whether IL-15 really secreted from cells or not. In order to do that we performed IL-15 ELISA with the culture medium. Considering, cells secreting IL-15 also uses IL-15 which makes it harder to detect real concentration of secreted cytokine. Nevertheless, we performed ELISA and observed a very low amount of IL-15 from transduced T cells confirming IL-15 secretion and of course a higher concentration observed from the IL-15 supplemented group (Figure 3.13). Consequently, we wanted to test whether IL-15 transduced T cells would also acquire a memory-like phenotype or not. To that end, we collected cells after culture and stained with CD3, CD44 and CD62L. We observed that cells transduced with IL-15 have also an improved percentage of memory-like phenotype but not as well as IL-15 supplemented cells. We also observed that again DC conditioned medium didn't support memory-like progression (Figure 3.14). As we did before, we again wanted to confirm their antigen specific cytotoxic potential. Similar to previous attempt CD8$^+$ T cells co-cultured with B16-OVA and B16-F10. The only difference is we established two different co-culture ratio to prove improving T cell count would in turn improve the overall cytotoxic response. Initially, we performed IFN-γ ELISA to compare their secretion levels. As we observed before all groups are highly active due to antibody

stimulation. Overall, IFN-γ secretion increased when T cells recognized their antigen. IL-15 transduced $CD8^+$ T cells had an improved IFN-γ secretion but again highest group was the IL-15 supplemented counterpart (Figure 3.15.A).

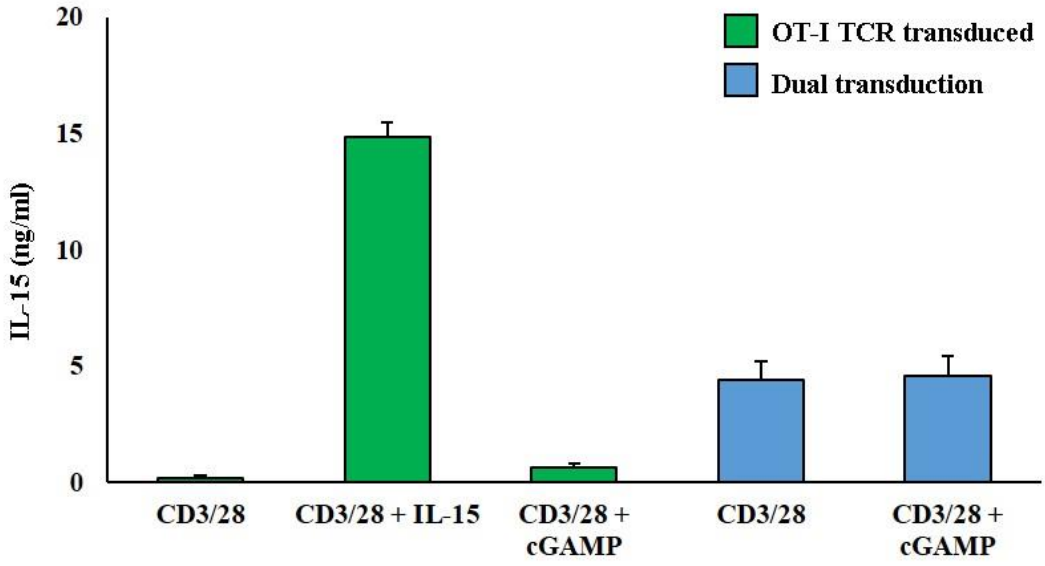


Figure 3.13: Dual transduction allows $CD8^+$ T cells to produce rIL-15. *Following retroviral transduction cells kept under indicated conditions. Three days after transduction cells are collected for counting and supernatant collected as well. Specifically, IL-15 ELISA performed on collected supernatants to confirm whether cells are capable of producing their own IL-15. (Dual transduction: Transduction of both OT-I TCR chains and IL-15 expression sequence in separate retroviral backbones)*

To clearly demonstrate the antigen specific IFN-γ response, fold change graph of B16-OVA over B16-F10 was drawn. Observations were quite the same, highest response detected from the IL-15 supplemented $CD8^+$ T cells followed by IL-15 transduced counterpart. DC conditioned mediums' potential to induce IFN-γ secretion were very low. As expected, increasing the number of $CD8^+$ T cells in co-culture ameliorated the IFN-γ response indicating that optimizing ratio would also improve the cytotoxic potential (Figure 3.15.B). Similar to previous attempt, we performed PI staining to measure lysis of B16 cells. We again observed a huge amount of cell lysis even from B16-F10 cells indicating cells are highly active due to antibody activation, background increases when the number of $CD8^+$ T cells increased in co-culture (Figure 3.16.A). Our observations were exactly the same, highest antigen specific lysis induced from the

IL-15 supplemented group and followed by IL-15 transduced counterpart. Number CD8+ T cells positively correlated with general cytotoxic potential. Therefore, increasing the number improves the antigen specific percentage of lysis (Figure 3.16.B). As indicated slight improvement in terms of antigen specific lysis were better depicted in Figure 17. Compared to the other groups increasing the number of $CD8^+$ T cells in IL-15 supplemented group improves the antigen specific lysis the most (Figure 3.17.A). Increasing the number of CD8+ T cells in IL-15 transduced group also improves the antigen specific lysis but not as well as supplementation (Figure 3.17.B).

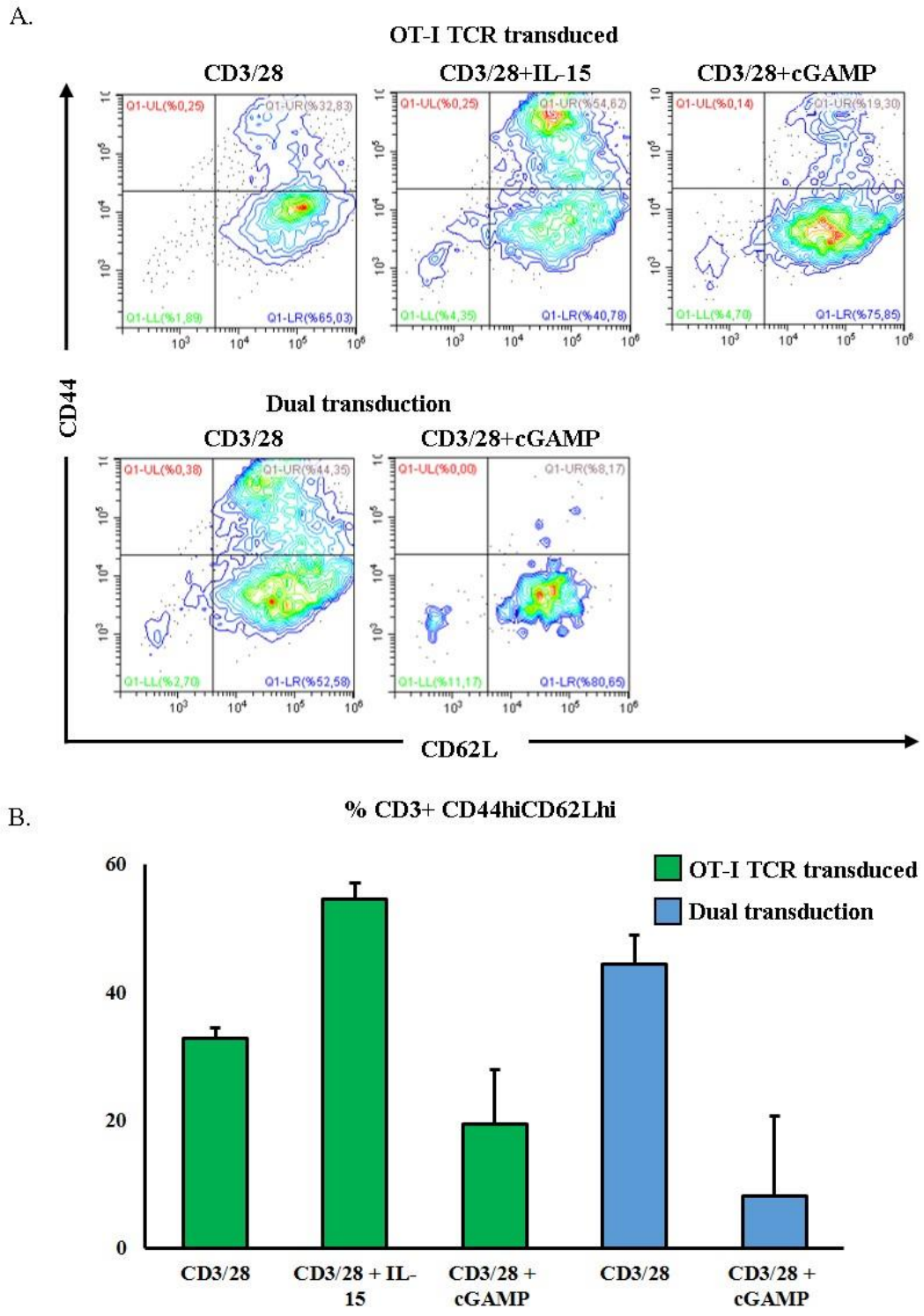
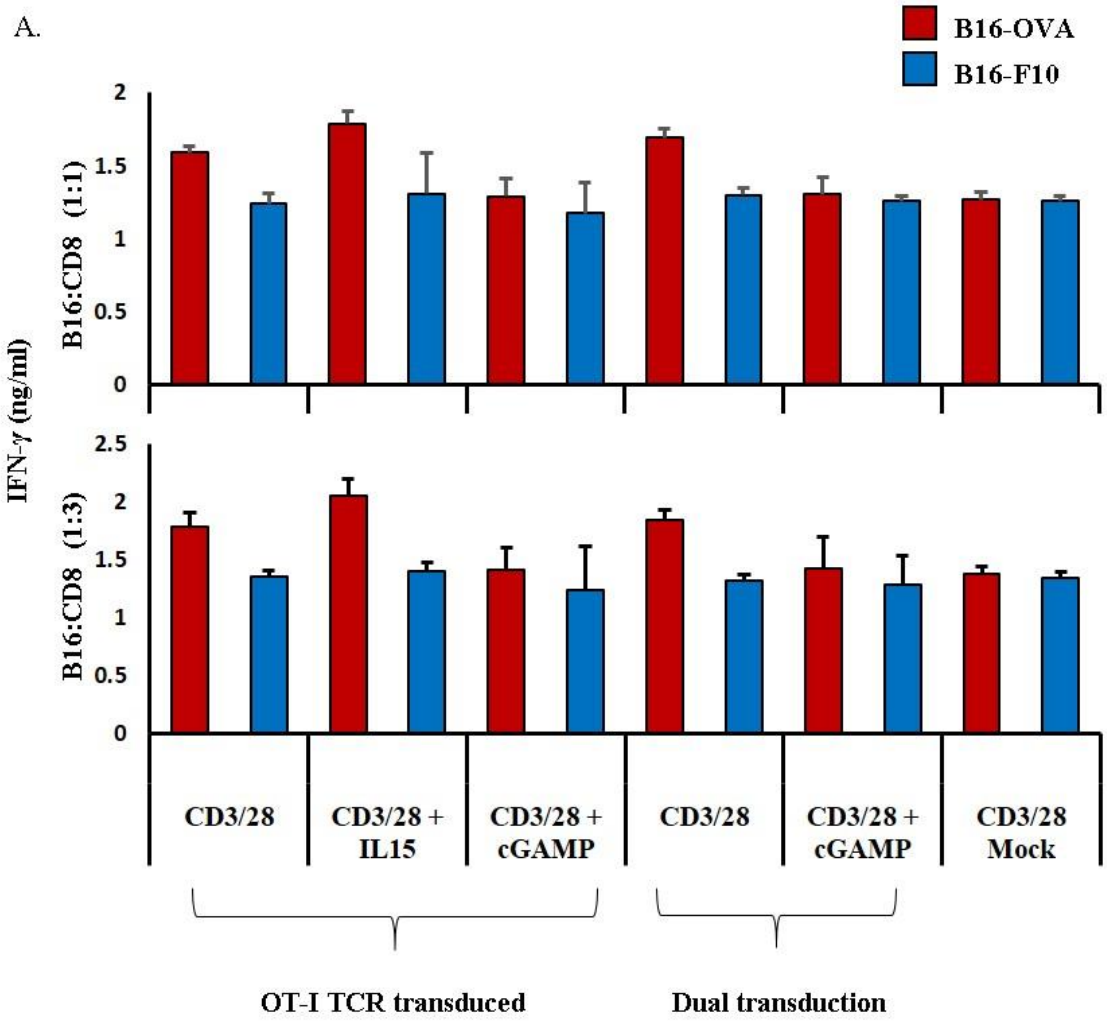


Figure 3.14: Transduction of IL-15 expression retroviral vector improves memory-like phenotype. *A. Cells were collected 7 days after transduction and subjected to phenotype analysis via Flow Cytometry. B. Percentages of memory-like phenotype acquired CD8+ T cells. rIL-15 supplemented CD8+ T cells showed a better improvement in terms of memory-like*

phenotype and followed by IL-15 transduced cells. (Dual transduction: Transduction of both OT-I TCR chains and IL-15 expression sequence in separate retroviral backbones)



B.

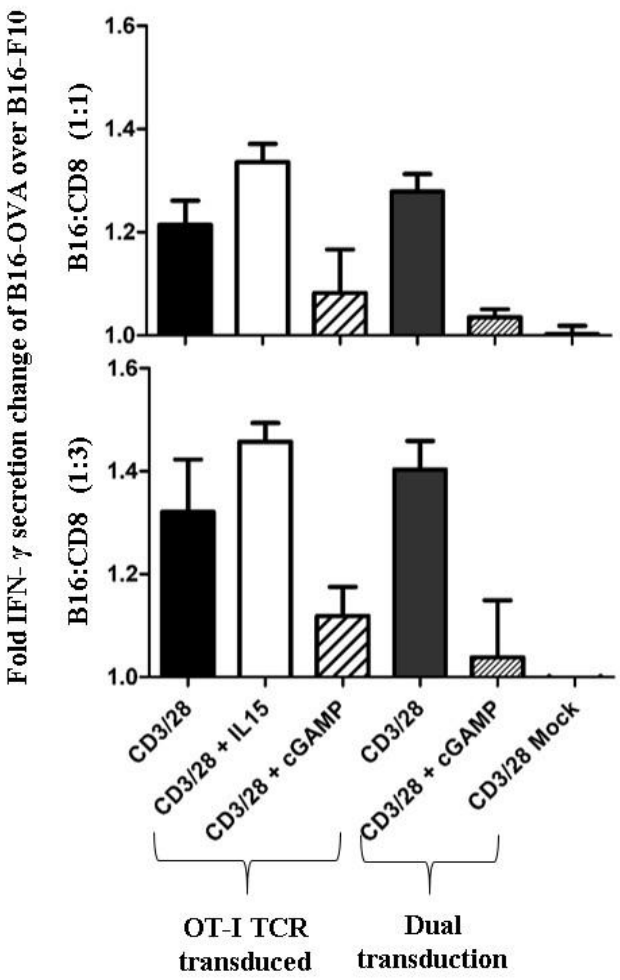
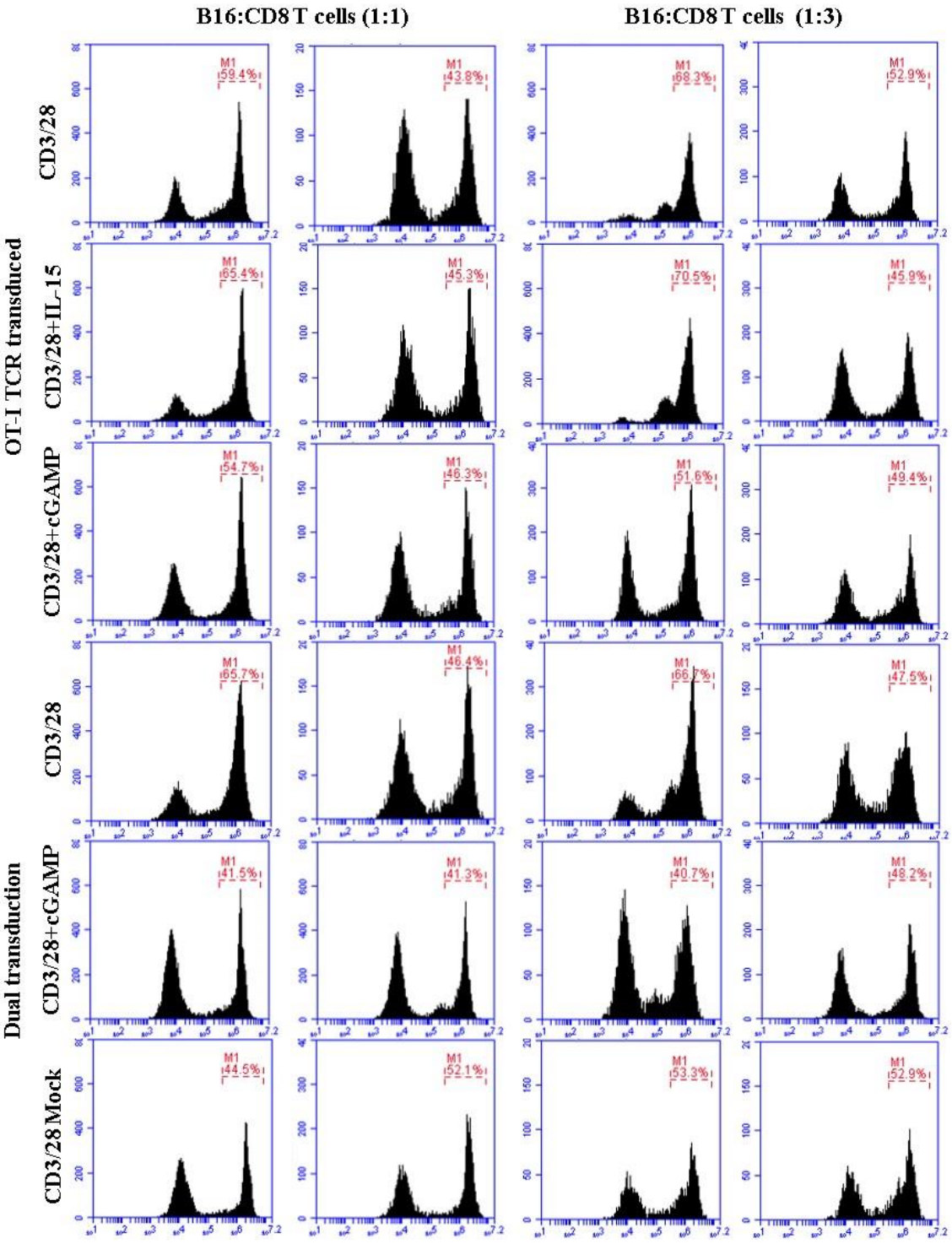


Figure 3.15: Transduction of IL15 retroviral vector improves IFN-γ secretion in CD8+ T cell : B16 co-culture indicated ratios. *A Supernatants collected at 36 hours of co-culture and subjected to ELISA analysis. All CD3/28 antibody activated CD8+ T cells secreting IFN-γ indicating they were in active state. CD3/28 Mock is not transduced and was used as a control. B Fold change of B16-OVA over B16-F10 co-culture. IL-15 transduced cells demonstrated an increased antigen specific IFN-γ secretion.*

A.


B16:CD8 T cells (1:1)
B16:CD8 T cells (1:3)
OT-I TCR transduced
Dual transduction
CD3/28
CD3/28+IL-15
CD3/28+cGAMP
CD3/28
CD3/28+cGAMP
CD3/28 Mock
M1 59.4%
M1 43.8%
M1 68.3%
M1 52.9%
M1 65.4%
M1 45.3%
M1 70.5%
M1 45.9%
M1 54.7%
M1 46.3%
M1 51.6%
M1 49.4%
M1 65.7%
M1 46.4%
M1 66.7%
M1 47.5%
M1 41.5%
M1 41.3%
M1 40.7%
M1 48.2%
M1 44.5%
M1 52.1%
M1 53.3%
M1 52.9%

B.

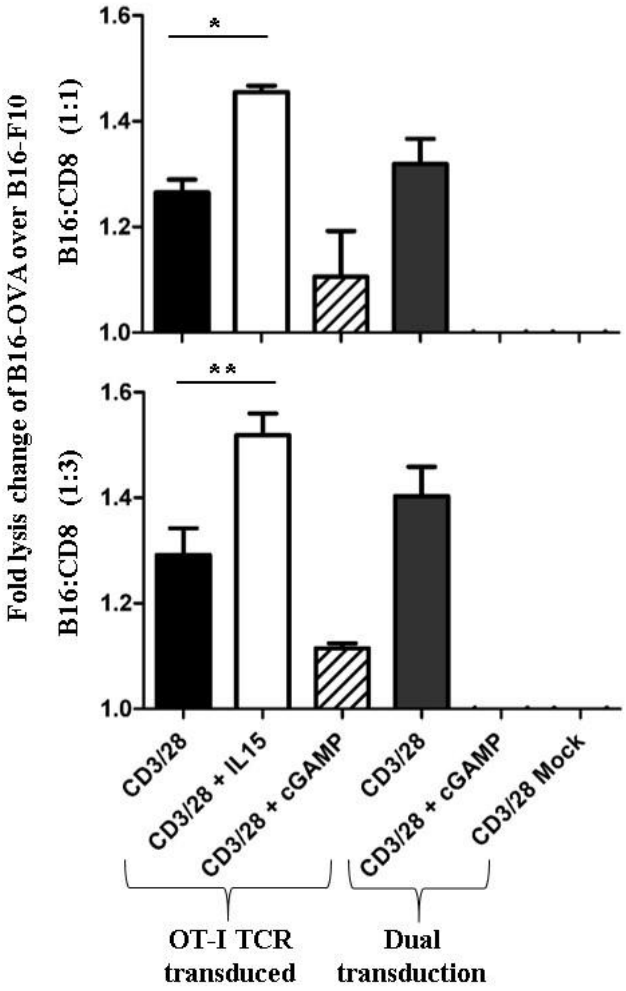


Figure 3.16: Transduction of IL15 retroviral vector improves antigen specific lysis. *A. B16 cells and CD8$^+$ T cells were co-cultured in indicated ratios. B16 melanoma cell line and ovalbumin expressing variant used to confirm antigen specificity. At 36 hours of co-culture, cells were stained with PI to measure lysis via FACS analysis. B. Data are mean±sem of three independent experiments. Statistical analysis by unpaired Student's t-test: *P<0.05, **P<0.01*

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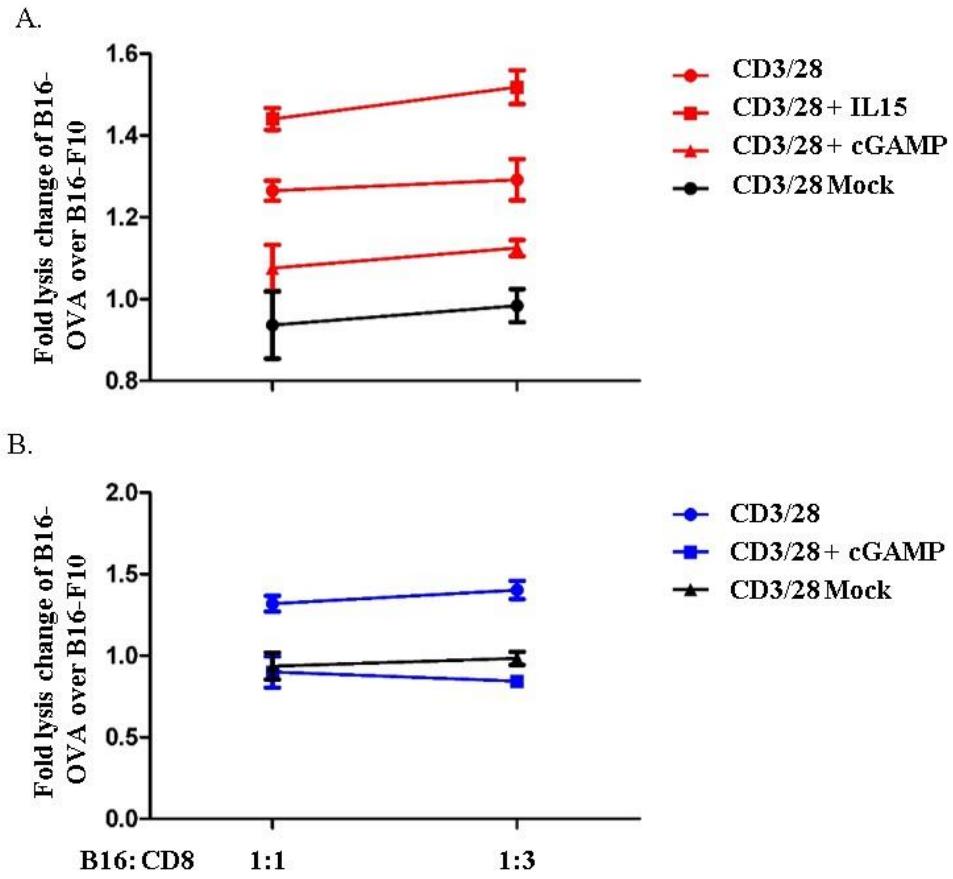

Figure 3.17: Antigen specific lytic capacity was analyzed by staining B16 cells. *At 36 hours of co-culture, cells were stained with PI to measure lysis via FACS analysis. Increasing co-culture ratio improves overall antigen specific lysis of B16-OVA cells.*

Chapter 4

Discussion

Host defense system recognizes self or non-self danger signals through innate immunity. If innate immunity is overwhelmed or can't clear the problem efficiently, it induces adaptive immune system. Unlike innate immune system, adaptive arm demonstrates a more specific immune response. One of the most important cell type from adaptive immune system is $CD8^+$ T cells which primarily recognize malignant and/or infected cells through MHC class-I depended manner [16]. Upon antigen recognition, $CD8^+$ T cells acquire effector phenotype and initiate several signaling cascades through either granzyme or FAS pathway that leads to apoptosis of the target cell [33]. In order to eliminate a potential immunopathology, number of antigen specific $CD8^+$ T cells significantly decreases over time [41-42]. This phase is called contraction phase and in this phase ~95% of antigen specific $CD8^+$ T cells from all tissues are eliminated [43-44]. Surviving cells are called memory $CD8^+$ T cells and they primarily depend on homeostatic signals, IL-7 and IL-15. Memory $CD8^+$ T cells have a significant contribution to long term immunity, they are able to respond very quickly and aggressively due to increased IFN-γ secretion [45]. This aggressive phenotype can be utilized in adoptive cancer therapy approach where these cells are infused to patient's tumor site. In order to acquire this phenotype, researchers create a lymphopenic environment (RAG-/- mice) to induce homeostatic proliferation and promote memory progression which highly depend on IL-15 and IL-7 [67-70]. Recent studies indicated that this approach can be mimicked *in vitro* by activating naïve CD8+ T cells with anti-CD3/28 in the presence of recombinant IL-15 [79]. In this thesis, we aimed to improve

memory-like progression by utilizing DCs ability to produce IL-15 and/or transducing CD8+ T cells with retroviral IL-15 expression vector to enable them to produce their own IL-15. Hence, all approaches aimed to induce memory-like progression and utilization of this phenotype in cancer treatment.

Bone marrow derived DCs stimulated with several PRR ligands showed differential expression of IL-15 (Figure 3.3). Our observations indicated IL-15 expression can be improved by type I IFN inducing ligands and LPS (Figure 3.4). This observation was also supported by previous studies which DC mediated IL-15 expression was improved by LPS and type I IFN [88]. By analyzing DCs' activation markers, selected ligands were able to activate DCs with different efficiencies (Figure 3.5). Then, activated DCs were co-cultured with magnetically separated $CD8^+$ T cells to test whether they are able to activate T cells and promote memory-like progression by IL-15. However, co-culture setup was not able to promote T cell proliferation so numbers remained low so most of the T cells were either dead or dying (Figure 3.6.A). Still, we compared their memory-like phenotype ($CD44^{hi}CD62L^{hi}$) and observed that the most potent IL-15 inducer, LPS, led to the lowest memory-like phenotype. DCs stimulated with cGAMP or 1466-Acore-PO improved the memory-like phenotype progression. Additionally, all these observations were not affected by either pulsing DCs with a target peptide (SIINFEKL) or supplementing cells with additional recombinant IL-15 (Figure 3.6.B). The state which $CD8^+$ T cells in after co-culture convinced us to change the approach. Therefore, we wanted to use DCs conditioned medium and stimulate $CD8^+$ T cells with anti-CD3/28 so that DC mediated environment was going to be tested and rather than activating T cells with DCs, artificial co-stimulatory molecules were going to be used. Similar to previous results, we observed that $CD8^+$ T cells cultured in any type of DC conditioned medium failed to acquire memory-like phenotype. On the contrary, additional IL-15 supplementation didn't change memory-like percentage and DC conditioned medium promoted effector phenotype ($CD44^{hi}CD62L^{lo}$) rather than memory-like phenotype (Figure 3.7). We were able to observe the memory-like promoting effect of IL-15 on $CD8^+$ T cells which only activated with anti-CD3/28 without DC conditioned medium. In addition to that a negative control group which $CD8^+$ T cells were cultured only in the presence of IL-15 demonstrated an increase in terms of memory-like phenotype (Figure 3.7). Since DC conditioned medium promoted effector phenotype, we wanted to compare their cytotoxic potential with the memory-

like phenotype acquired groups. Groups were transduced with OT-I TCR retroviral vector to enable $CD8^+$ T cells to recognize ovalbumin cancer antigen. Transduced $CD8^+$ T cells co-cultured with B16-F10 and ovalbumin expressing counterpart B16-OVA. Even though uncoated group demonstrated an increase in terms of memory-like phenotype, our observations demonstrated that they are not cytotoxically active at all (Figure 3.9). In addition to that IL-15 supplementation improved antigen specific IFN-γ response supporting memory-like phenotype have a better cytotoxic potential compared to effector phenotype (Figure 9). Complementary to previous observation, antigen specific lysis measurement proved that $CD8^+$ T cells cultured in the presence of IL-15 acquire memory-like phenotype and improved antigen specific cytotoxic potential (Figure 3.10).

In other approach, we aimed to transduce $CD8^+$ T cells with a retroviral vector which has both OT-I TCR chains and IL-15 expression sites linked with T2A ribosomal skipping sequences. However, we failed to clone such a vector rather we were only able to clone IL-15 expression sequence from cDNA to the same retroviral backbone. Therefore, as a proof of concept, we performed dual transduction of OT-I TCR and IL-15 in separate vectors just to show without any additional recombinant IL-15 whether only transduction is able to induce memory-like progression. Our observations indicated that anti-CD3/28 activated $CD8^+$ T cells have the highest proliferation rate followed by transduced $CD8^+$ T cells (Figure 3.12). Phenotype analysis also confirmed memory-like phenotype can be acquired by transduction without any additional recombinant IL-15 but with a bit less efficiency compared to IL-15 supplemented counterpart (Figure 3.14). Comparison of their antigen specific cytotoxic potential confirmed that transduced $CD8^+$ T cells acquire an improved cytotoxic potential. Similar to previous observation, transduction improvement was a bit lower than the IL-15 supplemented counterpart (Figure 3.16).

Chapter 5

Discussion

Our transduction approach would become an alternative to the classical adoptive therapy in fighting cancer. In previous models, $CD8^+$ T cells are separated from OT-I mice which are transgenically have the potential to recognize model antigen [79]. Our approach especially if one could clone both antigen specific TCR and IL-15 domains into a single vector, could be used against any identified antigen. Additionally, previous studies indicated that $CD8^+$ T cells transduced with IL-15 can survive up to 180 days *in vivo* [89]. Therefore, our approach not only improves cytotoxic potential of $CD8^+$ T cells but also contributes to their survival *in vivo* and might also be a potential solution to persistence problem of adoptive therapy.

The exact mechanism of how memory-like progression is induced has not been fully elucidated. Literature provides only a single study where they were able to confirm IL-15 induces this phenotype through eomesodermin transcription factor [90]. Therefore, as a future perspective signaling mechanism leads to memory-like phenotype formation might be further studied and identified genes or signaling factors can be exploited to induce a more efficient and effective conversion. Another future perspective is that we were unable to test the efficacy of transduced cells *in vivo* tumor treatment. IL-15 transduction induced memory-like phenotype's efficacy in cancer treatment, potential of *in vivo* survival and long term effect in preventing recurrence can be tested.

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