Comparison of CD28 and ICAM-1 signal transduction pathways, earliest signaling events involving CD45 and effect of statins and low pH on T cell function.

By

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Submitted to the graduate degree program in Molecular Biosciences and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date Defended: April 1, 2020

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Date Approved: April 1, 2020

Abstract

The multipotent naïve T cell of the immune system differentiates to mature, highly functional effector cells and differentiation is influenced by the microenvironment in which the T cell finds itself. Co-stimulatory molecules, local cytokines and other biological factors can tune the differentiation process and influence the differentiation outcomes. In this study, we investigated transcription factors and proteins activated downstream of co-stimulation through CD28 and ICAM-1. We observed that FAST-1, a DNA binding protein, activated only upon co-stimulation through ICAM-1. Co-stimulation through CD28 induced phosphorylation of receptor tyrosine kinases Csk, Dtk, FGFR1 and ROR2, while co-stimulation through ICAM-1 induced phosphorylation of Hgfr, IGF-1R, MuSK and EpHA8. Hence, specific sets of proteins were activated downstream of each co-stimulatory molecule leading to activation of unique signaling pathways.

We also investigated the early signaling process upon engagement of CD45 alone or in association with CD28. CD45 plays a crucial role in initiating T cell signaling by dephosphorylating a negative regulatory tyrosine residue in Src family kinases such as Lck. We observed that engagement of CD45 alone induced signaling in T cells. We also observed that TCR/CD3 stimulation with CD45 promoted prolonged Lck association with the TCR/CD3 complex and Lck remained associated during TCR/CD3+CD28+CD45 stimulation. We concluded that Lck association is dependent on TCR/CD3 and CD45 engagement.

Next, we examined the effect of statins on T cell function. We observed that upon CD28 co-stimulation, pravastatin induced increased T cell activation, proliferation and differentiation to effector and memory phenotypes, while upon ICAM-1 co-stimulation, pravastatin induced decreased T cell activation, proliferation and differentiation to effector and memory phenotypes.

Pravastatin treatment promoted increased differentiation to the Treg subset upon co-stimulation through both CD28 and ICAM-1. Hence, we concluded that the choice of co-stimulatory molecule influenced T cell function.

Finally, we investigated the influence of low pH on chemokine receptor expression on leukemic T cell lines (Jurkat and Molt-3) and primary T cells. Low pH treatment had no effect on chemokine receptor expression in primary T cells. Transient low pH resulted in an increase in CCR5 expression in Molt-3 but a decrease in Jurkat cells. In both Jurkat and Molt-3, transient low pH had a dramatic effect on CXCR4 expression and decreased CCR7 expression. In conclusion, low pH microenvironment influenced the expression of chemokine receptors differently in leukemic T cell lines and primary T cells.

Acknowledgements

I would like to use this platform to thank and appreciate my academia, professional and personal circles without whom I would not be in this position. First and foremost, I want to thank my mentor Dr. Stephen Benedict. I appreciate for the excellent example he has provided as a successful scientist, educator and mentor. I am also thankful for all his contributions of time, ideas, and insightful comments to make my Ph.D. experience productive and stimulating. He was a very supporting mentor who gave me the freedom to pursue various projects without objection. Without his guidance and constant feedback, this PhD would not have been achievable. The ecstasy and enthusiasm he has for his research were some of the motivational factor for me that helped progress one step at a time, even during tough times in the Ph.D. pursuit.

I would also like to express my gratitude to Dr. Marcia Chan, who was like a second mentor to me. I am very grateful to her for her scientific advice and insightful discussions and suggestions. I really appreciate the time and effort she put during my dissertation writing phase. Also, I would like to thank Dr. Susan Egan for being very supportive, especially during my last semester here at KU. I am grateful to my committee, Dr. Stephen Benedict, Dr. Marcia Chan, Dr. Susan Egan, Dr. David Davido, Dr. Kristi Neufeld and Dr. Helen Alexander for all the time, advice and support you have given me.

I would also like to thank former Benedict lab members- Amy Newton, Derek Danahy and Mandie Dunbar, who trained me during my first year in grad school. Mandie, thank you for being a wonderful friend over the years. Grad school experience would not be the same without you. Many of my experiments required peripheral blood from healthy donors. I would also like to acknowledge all generous blood donors and phlebotomist Laura Kimble, without whom most of my work would not have been possible.

Finally, I would like to thank my family. Thank you to my parents for your continuous love and support throughout my life. Anis, thank you for being a wonderful husband and my biggest cheerleader. Thank you for believing in me and for your words of encouragement all the time. At last, lots of love to my fur baby, Audio, for being very adorable and for your unconditional love.

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Chapter 1

Introduction

Adaptive immune response

The immune system can be divided into two main branches –innate immunity and adaptive immunity. The innate immune response is a non-specific, quick and robust response to pathogens. It includes (i) anatomical barriers such as skin and mucus membrane, (ii) physiological barriers such as temperature, acidic pH and lysozyme, and (iii) phagocytic barriers which are comprised of specialized cells that take up a foreign particle and degrade it. The adaptive immune response recognizes foreign antigens/threats with high specificity. It is comprised of B cells, T cells and antigen-presenting cells (APC). B cells and T cells undergo gene rearrangement giving rise to a diverse repertoire of B cell receptor (BCR) and T cell receptor (TCR) specificities respectively. B cells produce antibodies that bind to specific antigen and facilitate destruction and removal of foreign particles. T cells recognize cognate antigen presented on major histocompatibility complex (MHC) on antigen presenting cells. Antigen recognition leads to production of cytokines and soluble factors that act on other cells to promote or inhibit their activities. The adaptive immune response provides immunological memory for a more efficient secondary response.

T cells

Precursor T cells move from bone marrow to thymus, where they mature to give naïve $CD4^+$ or $CD8^+$ T cells. $CD4^+$ T cells engage with MHC class II molecules while $CD8^+$ T cells engage with MHC class I molecules. CD8+ T cells are the cytotoxic T cells that target and kill infected cells using perforin and granzyme pathways. CD4+ T cells are helper T cells that exert their effect by acting on other cells such as $CD8^+$ T cells, macrophages, natural killer cells, B cells, etc.

$CD4^+$ T cell subsets

$CD4^+$ T cells differentiate to give several subsets such as T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and regulatory T cells (Treg), each with specific functions. Th1 cells secrete interferon-γ (IFNγ) and interleukin-12 (IL-12), and activate macrophages and cytotoxic T cells to respond to viruses and other pathogens. Th2 cells secrete interleukin-4 (IL-4) and interleukin-10 (IL-10), promote B cell activity and help eliminate parasites. Th17 cells secrete interleukin-17 (IL-17) and interleukin-23 (IL-23) and activate inflammatory responses. Treg cells secrete interleukin-10 (IL-10) and TGF-β and have inhibitory functions. Treg cells suppress inflammation, protect against autoimmunity and inappropriate immune responses. Other $CD4^+$T cell phenotypes have been discovered such as T helper 9 cells (Th9). Th9 cells secrete interleukin-9 (IL-9) and interleukin-10 (IL-10) and promote cell proliferation in several hematopoietic cell types. Inappropriate immune responses can lead to various diseases, so understanding the mechanism of T cell differentiation to several subsets is important.

Naïve $CD4^+$ T cell differentiation

CD4+ T cells exit the thymus and migrate through the blood to secondary lymphoid organs where they interact with their cognate antigen. The cognate antigen is presented to the naïve T cell by an antigen presenting cell, that will result in activation, clonal expansion and differentiation of the naive T cell into effector and memory T cells (**Fig. 1.1)**. Effector T cells are highly activated T cells that secrete cytokines, chemokines, and other factors to elicit a primary response. Effector cells typically live for a short time as they undergo activation induced cell death (AICD) after clearance of the target antigen. Memory T cells differentiate directly from the naïve T cell or indirectly from the effector cells (**Fig. 1.1)**. Memory cells respond more quickly and strongly upon

secondary challenge with the same antigen. Memory T cells live for a long time and circulate through the secondary lymphoid tissues or home to specific tissues.

T cell signaling and activation

T cell activation requires two signals: Signal 1 is delivered by specific antigen via TCR and Signal 2 is delivered by a counter receptor on the APC through a resident molecule on the T cell as modeled in **Fig. 1.2**. Upon receiving Signal 1 + Signal 2, the naïve T cell differentiates into one of several possible phenotypes- Th1, Th2, Th17, Treg and others. In the absence of Signal 2, the cell can become anergic or apoptotic [1,2]. The second signal can be induced by different costimulatory molecules such as CD28, intercellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen-1 (LFA-1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) [3-5].

Influence of microenvironment on naïve T cell differentiation

Naïve T cell differentiation is influenced by the presence of specific cytokines. For example, IFNγ and IL-12 promote differentiation to Th1 subset and IL-4 promotes differentiation to Th2 subset. Our lab hypothesizes that naive T cell differentiation is influenced by the microenvironment in which the T cell finds itself. Microenvironmental influences that we study include co-stimulatory molecule interaction with counter receptors expressed on cells present in the local microenvironment during cell to cell contact. The costimulatory molecule is the second signal during T cell activation. In the absence of exogenous cytokines, stimulation through CD3+CD28 induces differentiation to Th2 (and a small number of Th1) effector and memory cells

Fig 1.1.

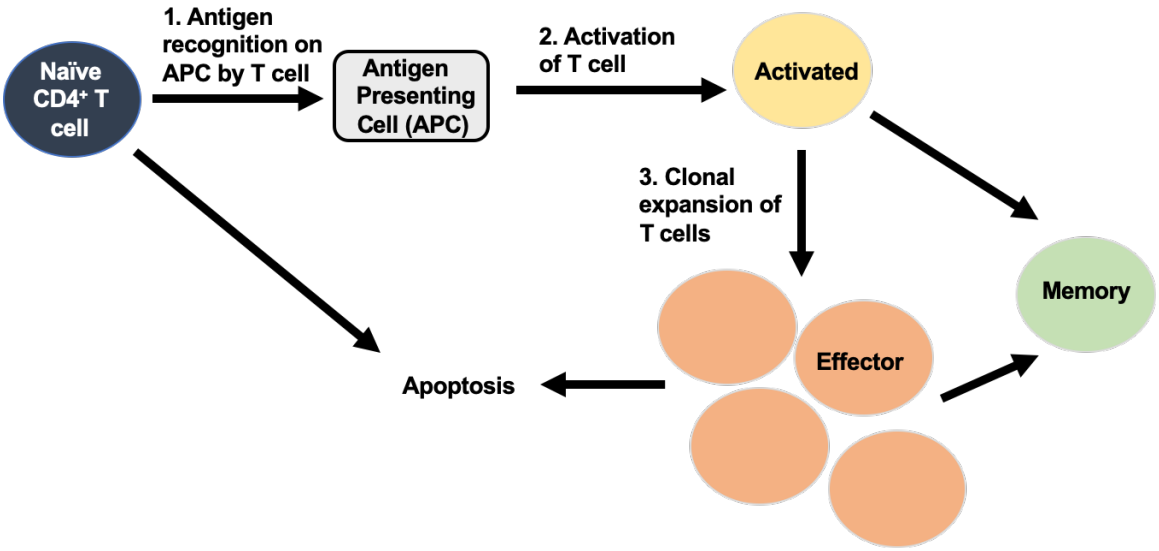
Naïve CD4+ T cell
1. Antigen recognition on APC by T cell
Antigen Presenting Cell (APC)
2. Activation of T cell
Activated
3. Clonal expansion of T cells
Memory
Effector
Apoptosis

Figure 1.1. Model of naïve $CD4^+$ T cell activation and differentiation to effector and memory T cells during a primary immune response.

Upon cognate antigen recognition, naïve $CD4^+$ T cells will activate, clonally expand, and differentiate into effector and memory T cells. Naïve T cells that do not encounter cognate antigen die by apoptosis. Effector T cells also undergo apoptosis after the immune response has taken place. Memory T cells arise directly from naïve T cells or from the effector population.

Fig 1.2.

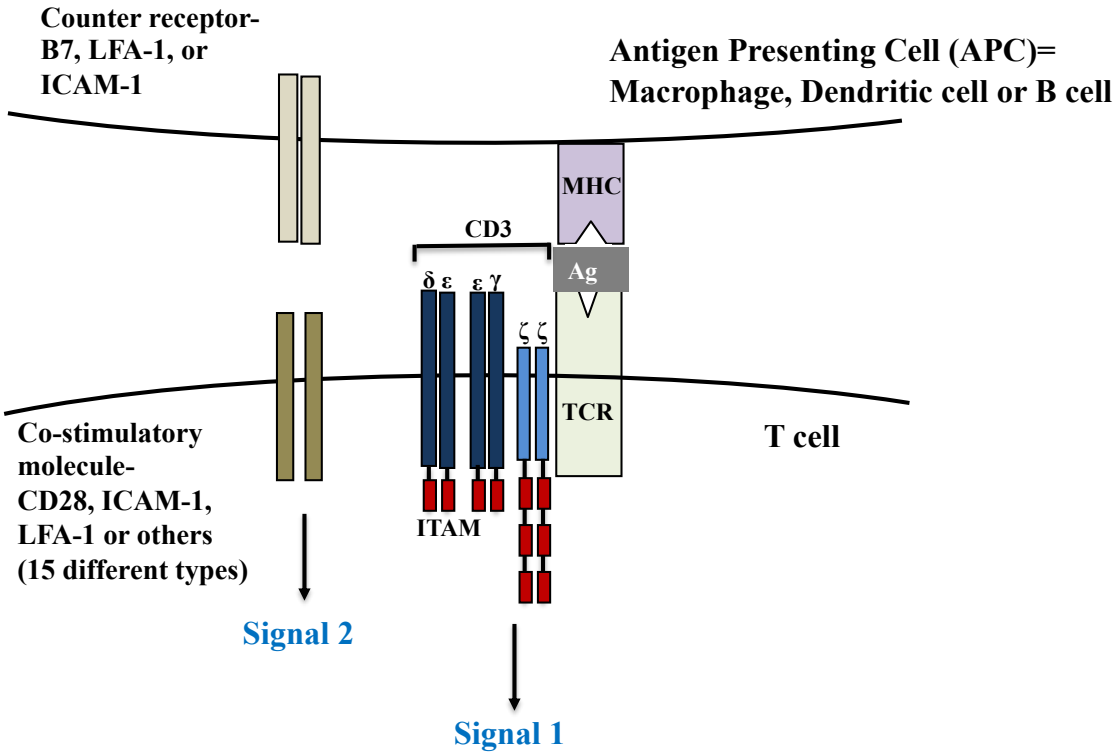
Counter receptor-
B7, LFA-1, or
ICAM-1
Antigen Presenting Cell (APC)=
Macrophage, Dendritic cell or B cell
MHC
CD3
Ag
δ ε
ε γ
ζ ζ
TCR
T cell
Co-stimulatory
molecule-
CD28, ICAM-1,
LFA-1 or others
(15 different types)
ITAM
Signal 2
Signal 1

Figure 1.2. Model of naïve T cell interaction with antigen presenting cell and signaling during T cell activation.

T cell activation requires two signals. The first signal is delivered by specific antigen (Ag) through the T cell receptor (TCR) and the second signal is delivered by a counter receptor on the Ag presenting cell (APC) through a co-stimulatory molecule on the T cell. Signal 1 and Signal 2 are required for T cell activation and differentiation. In the absence of Signal 2, with only Signal 1, the cell is triggered to die.

but not Treg cells, while stimulation through CD3+ICAM-1 induces differentiation to Th1 effector and memory cells and Treg cells but not Th2 cells [3,4,6,7]. The results of our previous studies have been summarized in **Table 1.1.**

Signal 1: TCR/CD3

Signal 1 during T cell activation is delivered through TCR/CD3 by a specific antigen presented on MHC molecule on an APC. The TCR/CD3 complex is a multi-subunit protein complex, composed of TCRαβ, CD3γε and CD3εδ heterodimers, and CD3ζζ homodimers. The TCR/CD3 complex is comprised of 10 immunoreceptor tyrosine-based activation motifs (ITAM). The cytoplasmic tails of γ, δ and ε subunits each have one ITAM and the ζ subunit have three ITAMs. ITAM is a signaling motif, composed of a conserved amino acid sequence- two repeats of YxxL/I (Y- tyrosine, L/I- Leucine/ Isoleucine) with a spacer of six to eight amino acids [8]. The ITAMs are not phosphorylated in a resting T cell. Upon TCR-MHC recognition, the Src family kinase Lck is activated which in turn phosphorylates the TCR/CD3 ITAMs, allowing for activation of downstream proteins and ultimately T cell activation.

Signal 2: CD28

CD28 is the best-characterized co-stimulatory receptor that provides the second signal necessary for T cell activation and function after stimulation through the TCR/CD3 complex. Co-stimulation through CD28 induces production of IL-2, an important cytokine for T cell activation and proliferation. CD28 has a single extracellular Ig variable-like domain. The counter receptors for CD28 are B7.1/CD80 or B7.2/CD86 expressed on the surface of antigen presenting cells [9]. CD28 is a 44 kDa transmembrane, glycosylated, disulfide linked homodimer, and is expressed on

Table 1.1

	TCR(CD3)+CD28	TCR(CD3)+ICAM-1	TCR(CD3)+LFA-1
Proliferation	+	+	+
Protection from apoptosis	+	+	-
Th1 subset differentiation	(-)	+	-
Th2 subset differentiation	+	-	-
Treg cell differentiation	-	+	-

Table 1.1 Comparison of co-stimulation through CD28, ICAM-1 or LFA-1.

Summary of our lab results contributed by Drs. Chintana Chirathaworn, Scott Tibbetts, Jacob Kohlmeier, Kelli Williams and Abby Dotson. Co-stimulation through CD28 promotes generation of Th2 and a small number of Th1 effector and memory cells but not Treg cells. Co-stimulation through ICAM-1 promotes generation of Th1 effector and memory cells and Treg cells but not Th2 cells. Co-stimulation through LFA-1 promotes T cell proliferation but not T cell differentiation.

most T cells [10]. Co-stimulation through CD28 activates PI 3-Kinase, NF-AT, NFκB, JNK and sphingomyelinase, that promote cell proliferation and survival [11-15].

Signal 2: ICAM-1

ICAM-1, also known as CD54, is expressed on different cell types such as endothelial cells, epithelial cells, monocytes, fibroblast and astrocytes. It is expressed at low levels on naïve and resting T cells but is upregulated upon activation [16]. ICAM-1 has five glycosylated extracellular Ig domains, a transmembrane domain and a short cytoplasmic tail [17]. Initially, the known role for ICAM-1 was to promote extravasation of leukocytes into inflamed tissues, by binding to its counter-receptor, LFA-1 on leukocytes. Few studies suggested that ICAM-1 induced signaling on cells as ICAM-1 stimulation induced phosphorylation of p38 MAPK in human airway epithelial cells and Src family kinase Lyn in a mouse B cell lymphoma [18-20]. Our lab has recently shown that ICAM-1 is a costimulatory molecule on T cells. The results are summarized in **Table 1.1** [3,4,7]. Co-stimulation of T cells through ICAM-1 provides protection from apoptosis as well as promotes differentiation to effector and memory subsets [4].

Dissertation: Study of signaling events in T cells (Chapters 2 and 3)

One of the primary goals of this dissertation was to investigate signaling events induced during stimulation through co-stimulatory molecules. In **Chapter 2**, we investigated signal transduction pathways induced upon co-stimulation through two co-stimulatory molecules - CD28 and ICAM-1. For this, we investigated proteins and transcription factors activated upon co-stimulation through CD3+CD28 and CD3+ICAM-1. In **Chapter 3**, we examined the role of tyrosine phosphatase, CD45, on T cell signaling during CD3+CD28 co-stimulation.

CD45

CD45 is a transmembrane glycoprotein, consisting of two intracellular phosphatase domains, a transmembrane domain and an extracellular domain (**Fig. 1.3**) [21]. Both intracellular domains are required for appropriate phosphatase activity [22]. It is highly expressed on leukocytes, making up to 10% of the cell surface area. CD45 gene consists of 33 exons. Various isoforms of CD45 are generated by alternative splicing of exons 4, 5 and 6 in the extracellular domain. Humans express 6 of the different isoforms with different frequency: RA, RO, RB, RAB, RBC and RABC, shown in **Fig. 1.4** [22,23]**.** Different subsets of T cells express different isoforms of CD45. Naïve T cells express the CD45RA isoform and mature cells express the CD45RO isoform [24]. However, the functions of different isoforms are not known. No ligands for CD45 have been identified.

Key substrate for CD45 is a tyrosine residue in the C-terminal region of Src family kinase (SFK) such as Lck and Fyn. SFK activity is controlled by phosphorylation at two tyrosine residues (eg. Lck Y394 and Y505). An intramolecular interaction between the phosphotyrosine (Y505) and SH2 domain results in an inactive conformation of Lck. Lck is specifically associated with the cytoplasmic region of CD4 and CD8 coreceptors. Upon antigen recognition by the TCR, CD4 binds to the MHC, bringing Lck close to the CD3 subunits in the TCR-MHC engagement site. CD45 dephosphorylates the negative regulatory tyrosine residue, Y505 in Lck and Lck auto-phosphorylates at the activating phosphate, Y394, resulting in an active conformation. Activated Lck phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) present in the CD3ζζ homodimer and CD3γε and CD3εδ heterodimers. Phosphorylated ITAMs promote recruitment and activation of Zap-70 protein tyrosine kinase, which leads to downstream signaling

[8] and modeled in **Fig.1.5**. Therefore, CD45 is a positive regulator of T cell activation since T cells without CD45 failed to proliferate [25] and CD45 is required for optimal TCR signaling [26].

Dissertation: Participation of other factors such as statins and low pH on T cell function (Chapters 4 and 5)

In **Chapter 4,** we investigated the role of lipid lowering drug- statins on T cell functions. We compared the effect of statin treatment on T cell viability, proliferation, activation and differentiation upon co-stimulation through two co-stimulatory molecules, CD28 and ICAM-1. In **Chapter 5,** we investigated the effect of low pH on the homing patterns of T cells assessed by analyzing the expression of chemokine receptors CXCR4, CCR7 and CCR5.

Statins

Statins are commonly used in the treatment of cardiovascular disease as they inhibit cholesterol synthesis by inhibiting HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase enzyme, which is a crucial step in the cholesterol biosynthesis pathway [27]. Several studies have suggested anti-inflammatory and immunomodulatory effects of statin treatment. Statin treatment promoted increased expression of TGF-β, IL-10 and IL-4, increased frequency of Treg cells and decreased levels of IL-1, IL-17 and IFN-γ in atherosclerotic plaques in mice *in vivo* [28,29]. Statin treatment also promoted increased frequency of circulating Treg cells in healthy individuals [30,31].

Fig. 1.3.

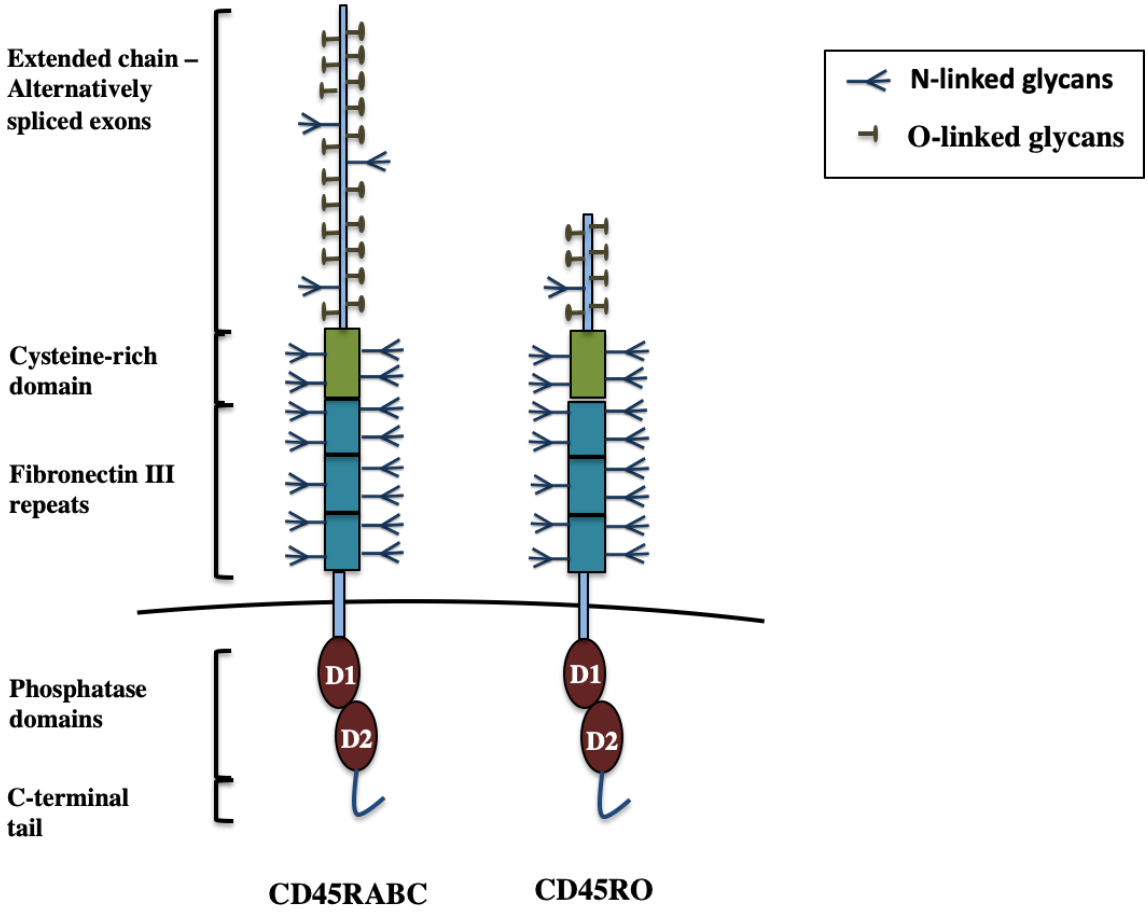
Extended chain – Alternatively spliced exons
Cysteine-rich domain
Fibronectin III repeats
Phosphatase domains
C-terminal tail
N-linked glycans
O-linked glycans
D1
D2
D1
D2
CD45RABC
CD45RO

Figure 1.3. Model of CD45.

The extracellular domain consists of extended chain, cysteine-rich domain and three fibronectin type III repeats. The extended chain consists of exons, which contain multiple sites for O-linked glycosylation. The cysteine-rich domain and fibronectin III repeats are heavily N-glycosylated, that are necessary for CD45 stability and its transport to the cell surface. CD45 has a single transmembrane domain and large cytoplasmic tail that consists of a tandem repeat of two phosphatase domains, D1 and D2. Only D1 has phosphatase activity while D2 acts as a regulator of D1 [23].

Fig. 1.4.

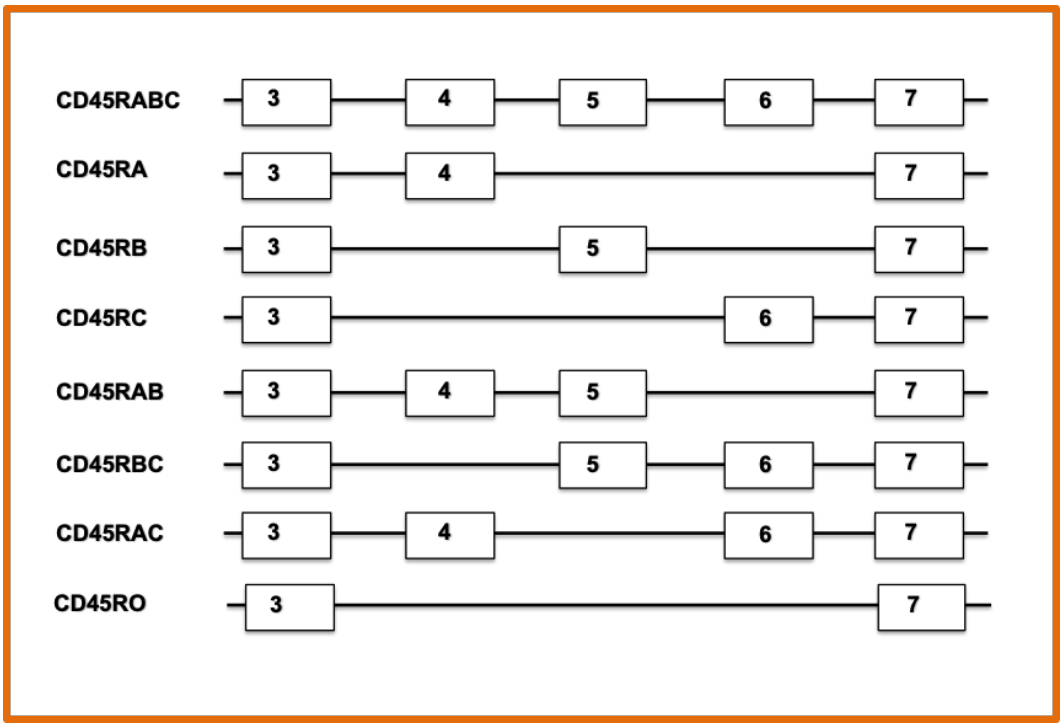
CD45RABC
3
4
5
6
7
CD45RA
3
4
7
CD45RB
3
5
7
CD45RC
3
6
7
CD45RAB
3
4
5
7
CD45RBC
3
5
6
7
CD45RAC
3
4
6
7
CD45RO
3
7

Figure 1.4. Model of all possible isoforms of CD45.

Different isoforms of CD45 generated by alternative splicing of exons 4, 5 or 6 in the extracellular domain. Letter A indicates presence of exon 4, letter B indicates presence of exon 5 and letter C indicates presence of exon 6. The most frequently expressed isoforms in humans are CD45 RA, RO and RB with minor expression of 3 others.

Fig. 1.5.

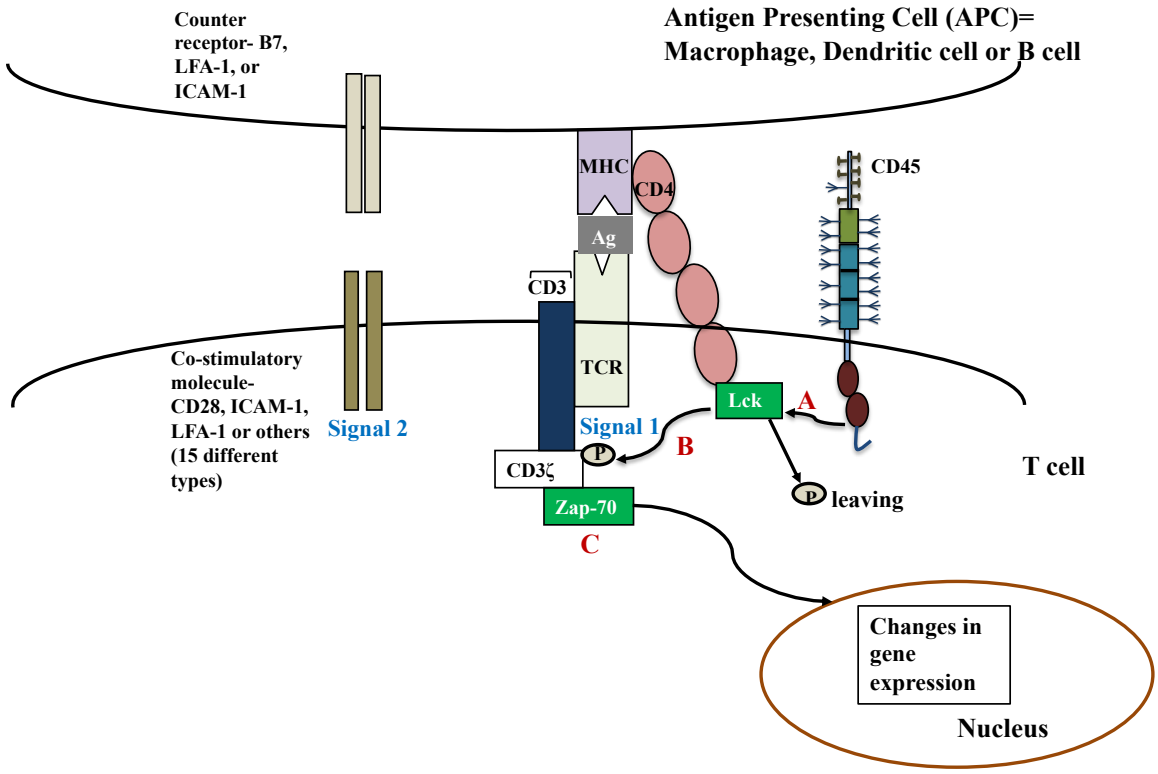
Counter receptor- B7, LFA-1, or ICAM-1
Antigen Presenting Cell (APC)= Macrophage, Dendritic cell or B cell
MHC
CD4
CD45
Ag
CD3
TCR
Co-stimulatory molecule- CD28, ICAM-1, LFA-1 or others (15 different types)
Signal 2
Signal 1
Lck
A
B
P
CD3ζ
Zap-70
C
P leaving
T cell
Changes in gene expression
Nucleus

Figure 1.5. Model of T cell signaling during activation.

Lck is a Src family tyrosine kinase that is specifically associated with the cytoplasmic region of CD4 and CD8 coreceptors. In a resting T cell, Lck is in an inactive conformation due to intramolecular interaction between phosphotyrosine, Y505 and an SH2 domain. A) Upon antigen recognition by the TCR, CD4 binds to the MHC and Lck accumulates at the TCR-MHC engagement. CD45 dephosphorylates Lck at Y505, which is the inactive conformation and Lck auto-phosphorylates at Y394, resulting in an active conformation. B) Activated Lck phosphorylates several ITAM motifs present in CD3 ζ, ε, δ and γ subunits of the TCR-CD3 complex. C) Phosphorylated ITAMs promote activation of Zap-70 protein tyrosine kinase and provides assembly points for nascent signaling complexes for downstream signaling.

Low pH microenvironment

Low pH microenvironment in the human body is found in the areas such as inflammatory loci, highly metabolic areas like lymph nodes and interstitial fluid of tumors [32]. Studies have shown that low pH altered motility and cytotoxic ability of immune cells, suggesting that low pH may influence T cell functions [33-35]. We investigated the effect of low pH on chemokine receptor expression to analyze homing patterns of T cells.

Overall, we were interested in studying how various cell factors and environments impact T cell signaling and functions. We studied signaling through two co-stimulatory molecules, CD28 and ICAM-1 and cell surface glycoprotein, CD45. We also studied the influence of soluble factor such as statin on T cell activation, proliferation and differentiation. Finally, we investigated the effect of low pH on T cell homing/migration.

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Chapter 2

Transcription factor activation and protein phosphorylation patterns are distinct for CD28 and ICAM-1 co-stimulatory molecules.

Introduction

Naïve T cell differentiation requires two signals: the first is delivered by antigen (Ag) via the T cell receptor (TCR), and the second is delivered by a counter-receptor on an Ag presenting cell via the corresponding co-stimulatory receptor on the T cell. Our lab hypothesizes that local microenvironment in which the T cell finds itself can influence the differentiation phenotypes. The microenvironmental influence includes the interaction between a co-stimulatory molecule on a T cell and its counter receptor on antigen presenting cell during cell-to-cell contact as well as naturally occurring soluble mediators such as cytokines, chemokines, or biological factors such as lipoproteins - LDL and HDL [1-4]. We have shown that, in the absence of exogenous cytokines, stimulation through CD3+CD28 induces differentiation to Th2 and some Th1 but not Treg, while stimulation through CD3+ICAM-1 induces differentiation to Th1 and Treg but not Th2 cells [1-3]. The question arises if each co-stimulatory molecule has unique signaling pathways and transcription factors activated downstream of them, then this may be the reason why the differentiation phenotypes differ between CD28 and ICAM-1. In this project, we intend to study protein phosphorylation and transcription factor activation downstream of each co-stimulatory molecule.

Preliminary work by a previous graduate student, Jake Kohlmeier using gene arrays showed that CD28 and ICAM-1 activated genes that were unique to CD28 or ICAM-1 [5]. Using cytokine/inflammatory response gene arrays, CD3+CD28 co-stimulation induced expression of CCR4, IL-10, IL5-Rα, IL-15, while CD3+ICAM-1 co-stimulation induced expression of CXCR4, MIP-1α, lymphotoxinα, RANTES, TGFβ1 [5]. Using extracellular matrix and adhesion molecule gene arrays, CD3+CD28 induced CEACAM1, PECAM1, ELAM1, Integrin α6, MMPs whereas CD3+ICAM-1 induced Caspase 8, cathepsin B and D, ICAM-1 and integrin β [5].

Hypothesis tested

The main goal of this project was to study different signaling factors, and transcription factors induced by two co-stimulatory molecules- CD28 and ICAM-1. We hypothesized that different co-stimulators induced activation of different proteins and transcription factors leading to activation of unique signaling pathways. This work will lead to more in-depth studies where specific molecular events can be identified that are crucial to pathways leading to different differentiation phenotypes. To investigate possible differences in the expression of different proteins between the two co-stimulatory molecules- CD28 and ICAM-1, we used transcription factor arrays and protein phosphorylation arrays.

Materials and methods

Antibodies and reagents

Anti-CD28 (clone CD28.2) was purchased from BioLegend (San Diego, CA), anti-ICAM-1 (clone R6.5) was purchased from BioXCell (West Lebanon, NH), and anti-CD3 (clone OKT3) was purchased from eBioscience (San Diego, CA). Antibodies used for flow cytometry: anti-CD11a-FITC (clone MEM-25) was purchased from Novus Biologicals (Littleton, CO), anti-CD25-APC (clone MA251), anti-CD27-PE (clone O323) and anti-CD45RO-APC (clone UCHL1) were purchased from Biolegend (San Diego, CA) and anti-FoxP3-PE (clone PCH101) was purchased from eBiosciences (San Diego, CA). The transcription factor staining buffer set was purchased from eBioscience (San Diego, CA). Flow cytometry was performed with the Guava EasyCyte system (Millipore, Burlington, MA) and data analysis was done using Guava InCyte software.

Human subjects

Peripheral blood was obtained after informed consent of healthy adult volunteers of both genders. Procedures were approved by the University of Kansas Institutional Review Board.

Cell purification

Naïve $CD4^+$ T cells were isolated from peripheral blood of healthy donors. Whole blood was diluted 1:1 in sterile TC-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM glucose, pH 7.4) + 2% FBS + 1% penicillin/streptomycin and spun for 30 minutes at 1800 rpm using Ficoll density gradient

centrifugation. The buffy coat of PBMC was collected to isolate naïve $CD4^+$ T cell by negative selection using StemSep human naïve $CD4^+$ T cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Purity of the isolated naïve $CD4^+$ T cell was assessed for each isolation by staining for CD11a, CD27 and CD45RO and analyzing by flow cytometry. Purity of the isolated naïve CD4+ T cells was greater than 98%. The purified naïve T cells were $CD11a^{lo}$ $CD27^+$ $CD45RO^{(-)}$.

T cell stimulation

Each stimulating antibody was diluted in sterile PBS and titrated to the lowest effective concentration. Antibody solutions were added to tissue culture-treated plates (TPP, 6-well plates, Switzerland) by using anti-CD3 (OKT3) at 1ug/ml, anti-CD28 (CD28.2) at 2ug/ml or anti-ICAM-1 (R6.5) at 10ug/ml and incubated at 37^0C for 2 hours or overnight at 4^0C. Naïve $CD4^+$ T cells were added to each well at 1.5×10^6 cells/ml in RPMI 1640 (CellGro, Corning, New York), containing 10% FBS (Atlanta Biologicals, Atlanta, GA), 50 IU/ml penicillin/50 μg/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco) and incubated at 37°C with 5% CO_2 for the indicated times.

Flow cytometry

Cells were collected from the tissue culture plates, stained with fluorescently tagged antibodies and analyzed by the Guava EasyCyte system (Millipore, Burlington, MA) with Guava InCyte software. For Treg staining, cells were stained for anti-CD25-APC and anti-FoxP3-PE. First, cells were blocked with 0.5% BSA in PBS for 15 minutes on ice and stained for surface target CD25, and then fixed, permeabilized, and stained for transcription factor, FoxP3 using

eBioscience transcription factor staining buffer kit. The cells were analyzed immediately using the Guava EasyCyte system. Single color staining controls were used for compensation.

Protein Phosphorylation assay

Analysis of 71 different phosphorylated receptor tyrosine kinases was performed using Human RTK Phosphorylation Antibody Array C1 (RayBiotech Inc., GA) according to the manufacturer's protocol. Briefly, naïve T cells were stimulated through CD3+CD28 or CD3+ICAM-1 for 2 minutes and 5 minutes and protein lysate were prepared as per the manufacturer's instructions. 100ug-150ug protein lysate per sample was incubated with the array membranes and protein phosphorylation signals were visualized using UVP Biospectrum Chemi HR 410 imaging system. Relative density of specific protein expression was determined using ImageJ and each protein was normalized to the positive control.

Transcription Factor Activity Profiling Assay

Analysis of 48 transcription factors (TF) was performed using the TF Activation Profiling Plate Array I (Signosis, Santa Clara, CA) according to the manufacturer's protocol. Briefly, naïve T cells were stimulated through CD3+CD28 or CD3+ICAM-1 for 4 hours and nuclear extract were prepared using the nuclear extraction kit (Signosis, Santa Clara, CA) as per the manufacturer's instructions. 5ug of nuclear extract per sample was mixed with biotin labelled probes to form TF/probe mix and incubated on plates with complementary sequences of probes. Then, substrate was added and read using Bio-Tek Synergy HT Multi-Mode Microplate Reader. The reading was displayed in Relative light units, RLUs. The normalized RLU was calculated by subtracting the blank RLU value from each reading.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Statistical tests and significance are indicated in the figure legends.

Results

Co-stimulation through ICAM-1 promotes differentiation of naïve $CD4^+$ T cell to Treg cells.

Our lab has previously shown that naïve $CD4^+$ T cells differentiate to Treg phenotype upon co-stimulation through CD3+ICAM-1 and not through CD3+CD28 in the absence of exogenously added cytokines [3]. The naïve $CD4^+$ T cells used in this project were first investigated to study their differentiation to Treg cells after 9 days of co-stimulation through CD28 or ICAM-1. Treg cells were assessed by staining the cells for CD25 and transcription factor FoxP3 and analyzed by flow cytometry. We observed that only co-stimulation through ICAM-1 promoted differentiation to Treg cells ($CD25^+FoxP3^+$) after 9 days of stimulation (**Fig. 2.1-2.2**), thus confirming the results by Williams et al. [3].

Co-stimulation through CD28 or ICAM-1 induced differential phosphorylation of proteins.

Phosphorylation of proteins during cell signaling is a major mechanism that regulates protein function and promotes cellular signal transduction. To study what proteins are differentially phosphorylated by the two co-stimulating molecules, we analyzed the phosphorylation of 71 different receptor tyrosine kinases upon co-stimulation through CD28 or ICAM-1 for 2 minutes and 5 minutes. Receptor tyrosine Kinase (RTK) is a family of cell surface receptors with a ligand-binding extracellular domain, a single helix transmembrane domain and a cytoplasmic domain that mediate kinase activity. Several proteins of the RTK family mediate critical cellular events which include cell cycle, proliferation, metabolism, survival and migration [6].

Fig. 2.1.

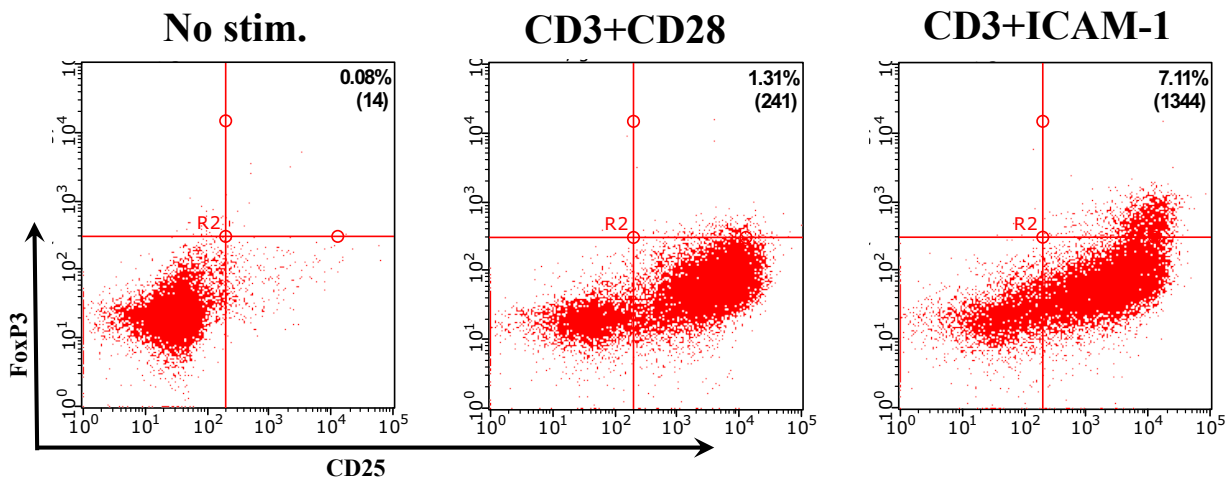
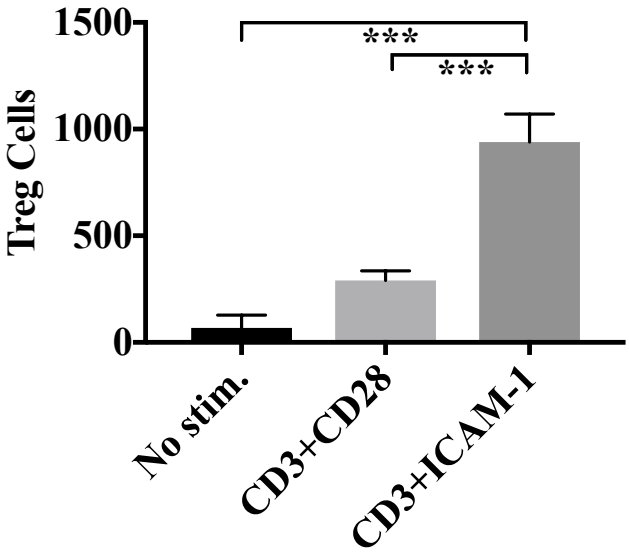

No stim.
CD3+CD28
CD3+ICAM-1
0.08%
(14)
1.31%
(241)
7.11%
(1344)
R2
FoxP3
CD25

Fig. 2.2.


1500
1000
500
0
Treg Cells
***
***
No stim.
CD3+CD28
CD3+ICAM-1

Figures 2.1-2.2. Co-stimulation through ICAM-1 promotes differentiation to Treg cells.

Naïve CD4+ T cells were left unstimulated or stimulated through CD3+CD28 or CD3+ICAM-1 for 9 days and stained with CD25 and FoxP3 and analyzed by flow cytometry. **Fig. 2.1:** Representative dot plots showing Treg cells ($FoxP3^+$ $CD25^+$) in upper right quadrant. Percent of Treg cells given in bold, with number of Treg cells in parentheses. **Fig. 2.2:** Number of Treg cells after 9 days of co-stimulation with error bars indicating standard error, n=7; ***$p<0.0009$ by two-way ANOVA test.

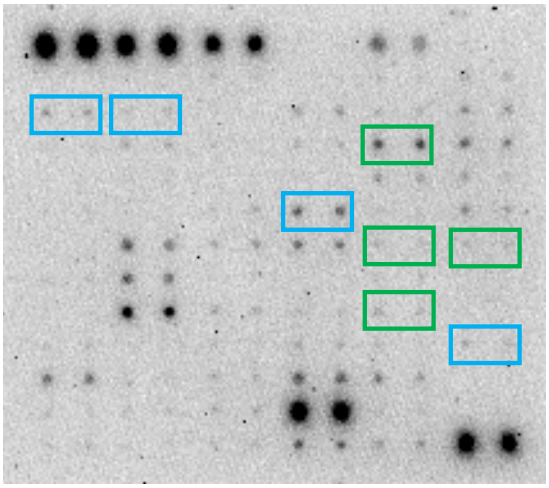
Fig. 2.3 shows blot images for unstimulated and 2 minute stimulation through CD3+CD28 and CD3+ICAM-1. **Fig. 2.4** and **Fig. 2.5** shows densitometry analysis for proteins with significant differences. Stimulation through CD3+CD28 increased phosphorylation of Csk (C-terminal Src kinase), Dtk (also known as Tyro3), FGFR1 (Fibroblast growth factor receptor-1) and ROR2 (Receptor Tyrosine Kinase Like Orphan Receptor 2) at 2 minutes of stimulation (**Fig. 2.4)**, and stimulation through CD3+ICAM-1 induced increased phosphorylation of Hgfr (Hepatocyte growth factor receptor), IGF-1R (Insulin-like growth factor 1 receptor), MuSK (Muscle specific tyrosine kinase) and EphA8 at 2 minutes of stimulation (**Fig. 2.5)**. Dtk and FGFR1, phosphorylated upon CD28 co-stimulation and IGF-1R and HGFR, phosphorylated upon ICAM-1 stimulation have been shown to play a crucial role for cell proliferation, differentiation and survival [7-11].

Increased FAST-1 activation upon co-stimulation through ICAM-1 but not by CD28 and increased NFκB activation by both CD28 and ICAM-1 co-stimulation.

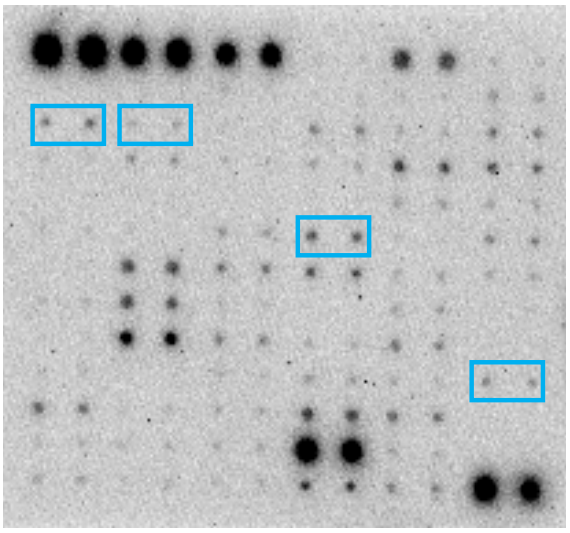
We compared the expression of 48 different transcription factors in response to co-stimulation through CD28 or ICAM-1 for 4 hours. Co-stimulation through ICAM-1 resulted in significantly increased activation of FAST-1 but not by CD28 co-stimulation (**Fig. 2.6)**. We also confirmed this observation by intracellularly staining for FAST-1 upon stimulation through CD28 and ICAM-1 (n=1) and observed increased FAST-1 activation by ICAM-1 co-stimulation compared to CD28 co-stimulation (data not shown). FAST-1, also known as Forkhead box H1 (FoxH1), is a nuclear protein that mediates TGFβ signaling pathway [12]. During the TGFβ signaling, the ligand binds to the receptors, TβRII and TβRI, activating the receptor complex. This allows phosphorylation of Smad2 and Smad3, which then associate with Smad4 forming a

Fig. 2.3.

No stimulation



2 min CD3+CD28



2 min CD3+ICAM-1

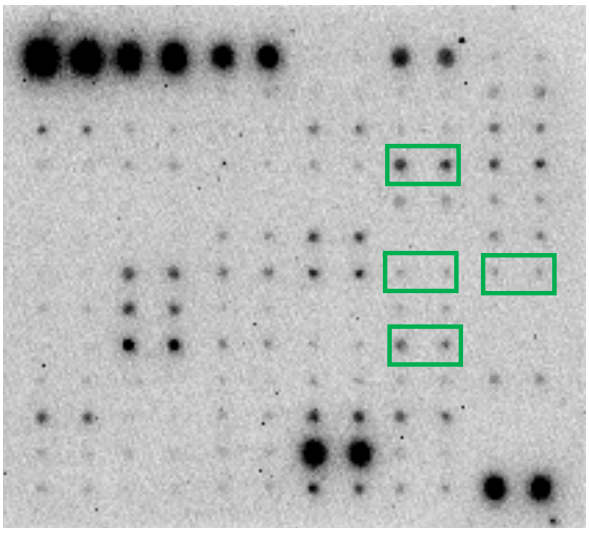


Fig. 2.4.

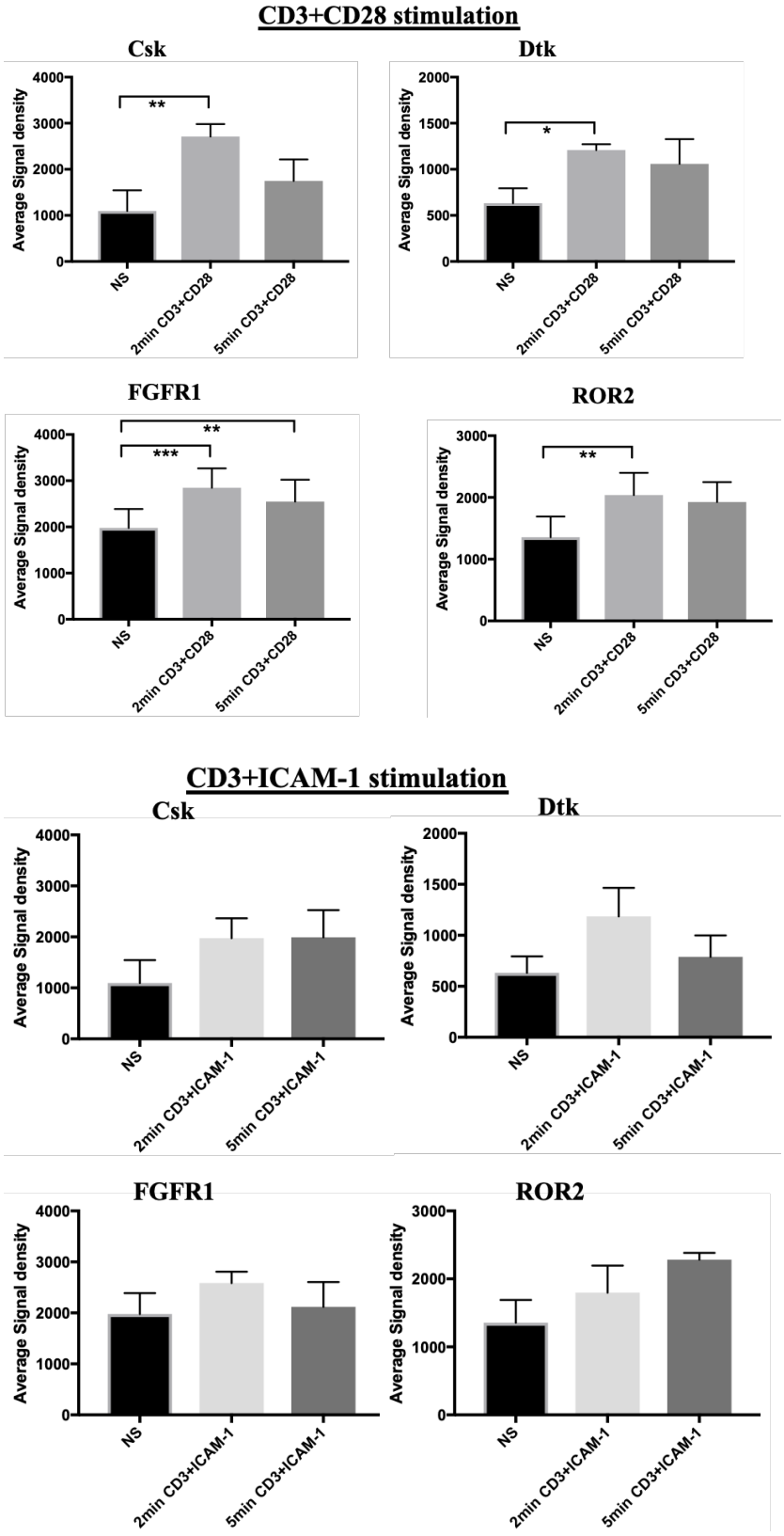
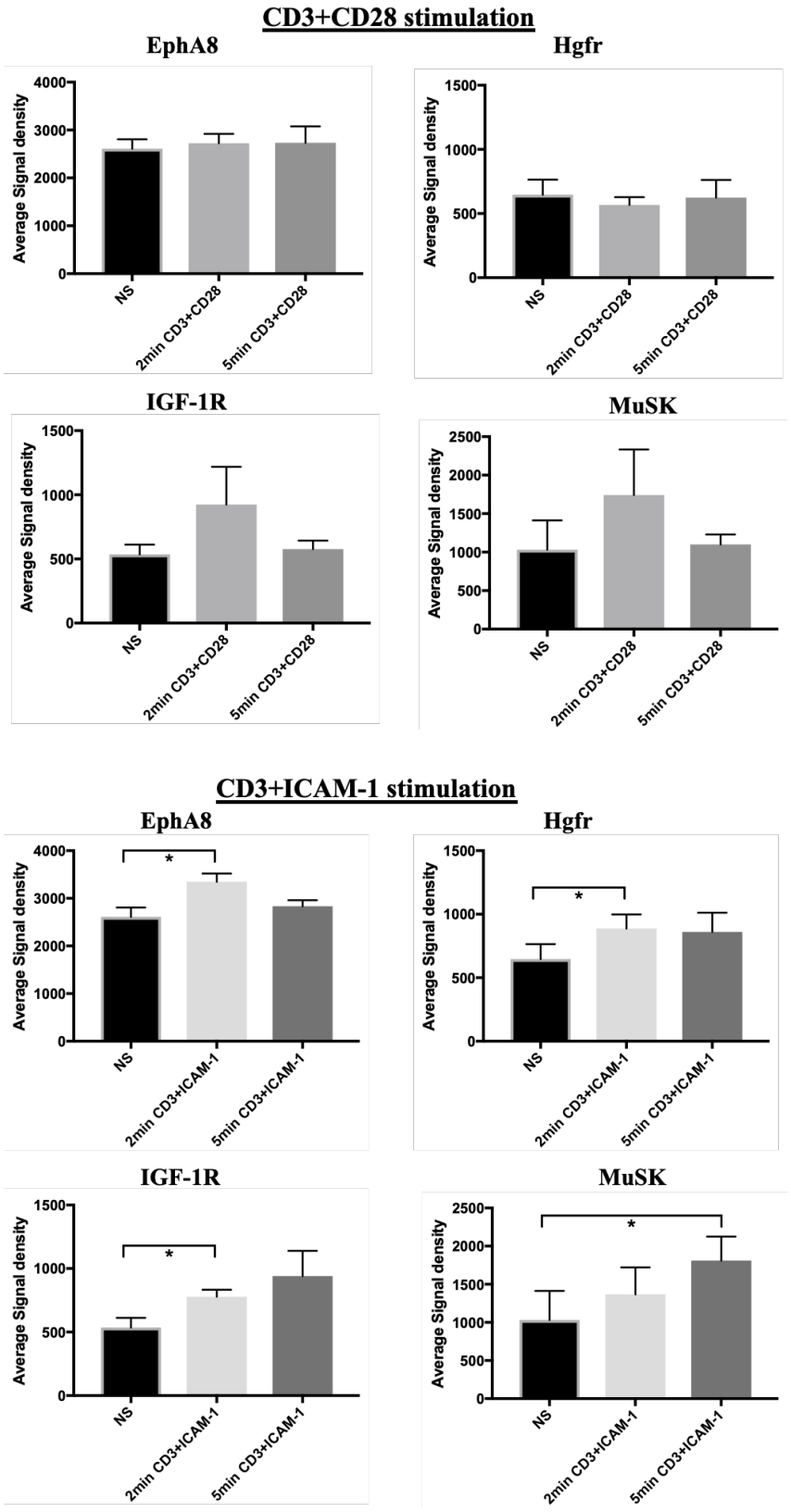

CD3+CD28 stimulation
Csk
Dtk
FGFR1
ROR2
CD3+ICAM-1 stimulation
Csk
Dtk
FGFR1
ROR2
Average Signal density
NS
2min CD3+CD28
5min CD3+CD28
2min CD3+ICAM-1
5min CD3+ICAM-1

Fig. 2.5.


CD3+CD28 stimulation
EphA8
Hgfr
IGF-1R
MuSK
CD3+ICAM-1 stimulation
EphA8
Hgfr
IGF-1R
MuSK
Average Signal density
NS
2min CD3+CD28
5min CD3+CD28
2min CD3+ICAM-1
5min CD3+ICAM-1
*

Figures 2.3-2.5. Co-stimulation through CD28 or ICAM-1 induced differential phosphorylation of proteins.

Naïve CD4+ T cells were left unstimulated or stimulated through CD3+CD28 or CD3+ICAM-1 for 2 minutes and whole cell lysates were prepared. Protein phosphorylation was analyzed by Human RTK Phosphorylation Antibody Arrays C1 from RayBiotech. **Fig. 2.3:** Representative blots for unstimulated, CD3+CD28 and CD3+ICAM-1 lysates. Each spot represents a protein and each protein was in duplicates. Blue boxes represent proteins with increased phosphorylation upon CD3+CD28 stimulation and green boxes represent proteins with increased phosphorylation upon CD3+ICAM-1 stimulation. **Fig. 2.4-2.5:** The signal intensities from **Fig. 2.3** were calculated using ImageJ and each spot was normalized to the positive control, with error bars indicating standard error, n=4; $*p<0.05$ by paired t test. **Fig. 2.4:** Proteins that showed statistically significant increases in phosphorylation upon CD3+CD28 stimulation. **Fig. 2.5:** Proteins that showed statistically significant increases in phosphorylation upon CD3+ICAM-1 stimulation.

heteromeric complex [12]. The heteromeric complex translocate to the nucleus and binds to the DNA binding target, FoxH1 to regulate gene expression. FoxH1 does not regulate transcription independently, but rather the transcriptionally active complex containing FOXH1/SMAD2/SMAD4 is required to activate transcription [13].

We observed that NFκB increased activation upon co-stimulation through both co-stimulatory molecules, CD28 and ICAM-1 (**Fig. 2.7)**. The increase in NFκB activation is greater in CD28 compared to ICAM-1 co-stimulation. The NF-κB family consists of five proteins- RelA/p65, RelB, c-Rel, NF-κB1 p50/p105, and NF-κB2 p52/p100 [14]. These proteins form a transcriptionally active homodimeric or heterodimeric complex. In an unstimulated cell, the NF-κB complex is in an inactive conformation in the cytoplasm due to its interaction with IκB proteins. In the presence of stimuli such as proinflammatory cytokines, LPS, growth factors, viral and bacterial infections and antigen receptors, the IKK complex (IKKβ, IKKα, and NEMO) is activated and phosphorylates IκB, promoting its degradation, and thus freeing the NF-κB complex. The active NF-κB complex translocates to the nucleus to induce gene transcription of chemokines, cytokines, cell adhesion molecules, regulators of apoptosis and proliferation; thus, playing a crucial role in cell proliferation, differentiation and survival [14]. It has been shown that NFκB is activated by CD28 which agrees with our finding as CD3+CD28 stimulation induced activation of NFκB [15]. Furthermore, we observed that NFκB is activated by another co-stimulatory molecule ICAM-1.

Co-stimulation through CD28 resulted in increased activation of other transcription factors such as CREB, E2F-1, ER, GR/PR, NF-1, NF-E2, Pax5, Pbx1, PPAR, PXR, Stat6, and TR compared to ICAM-1 co-stimulation (**Fig. 2.8)**. These transcription factors showed a pattern of increased activation with CD28 co-stimulation; however, the data were not statistically significant.

Fig. 2.6.

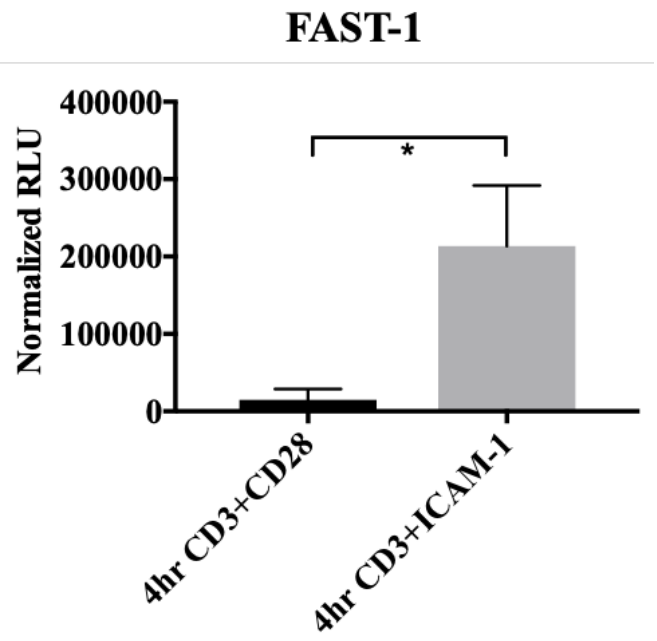
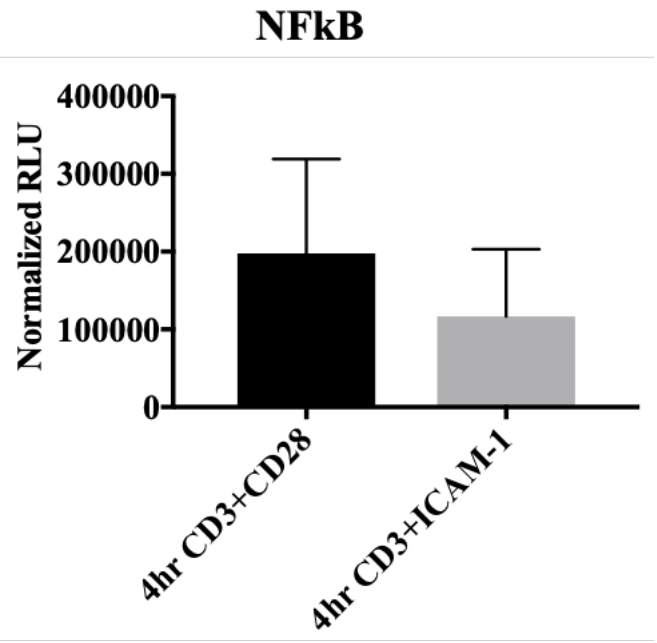

FAST-1
Normalized RLU
400000
300000
200000
100000
0
*
4hr CD3+CD28
4hr CD3+ICAM-1

Fig. 2.7.


NFkB
Normalized RLU
400000
300000
200000
100000
0
4hr CD3+CD28
4hr CD3+ICAM-1

Figures 2.6-2.7. Increased FAST-1 activation upon co-stimulation through ICAM-1 but not by CD28 and increased NFκB activation by both CD28 and ICAM-1 co-stimulation.

Naïve $CD4^+$ T cells were left unstimulated or stimulated through CD3+CD28 or CD3+ICAM-1 for 4 hours and nuclear extracts were prepared. Activation of 48 different transcription factors was analyzed using the TF Activation Profiling Plate Array I from Signosis. **Fig. 2.6:** Representative graph for transcription factor (FAST-1) with a significant increase in activation upon CD3+ICAM-1 stimulation. **Fig. 2.7:** Representative graph for transcription factor (NFκB) that showed increased activation upon co-stimulation through both CD28 and ICAM-1. Error bars indicate standard error, n=5; $*p<0.05$ by paired t test.

Fig. 2.8.

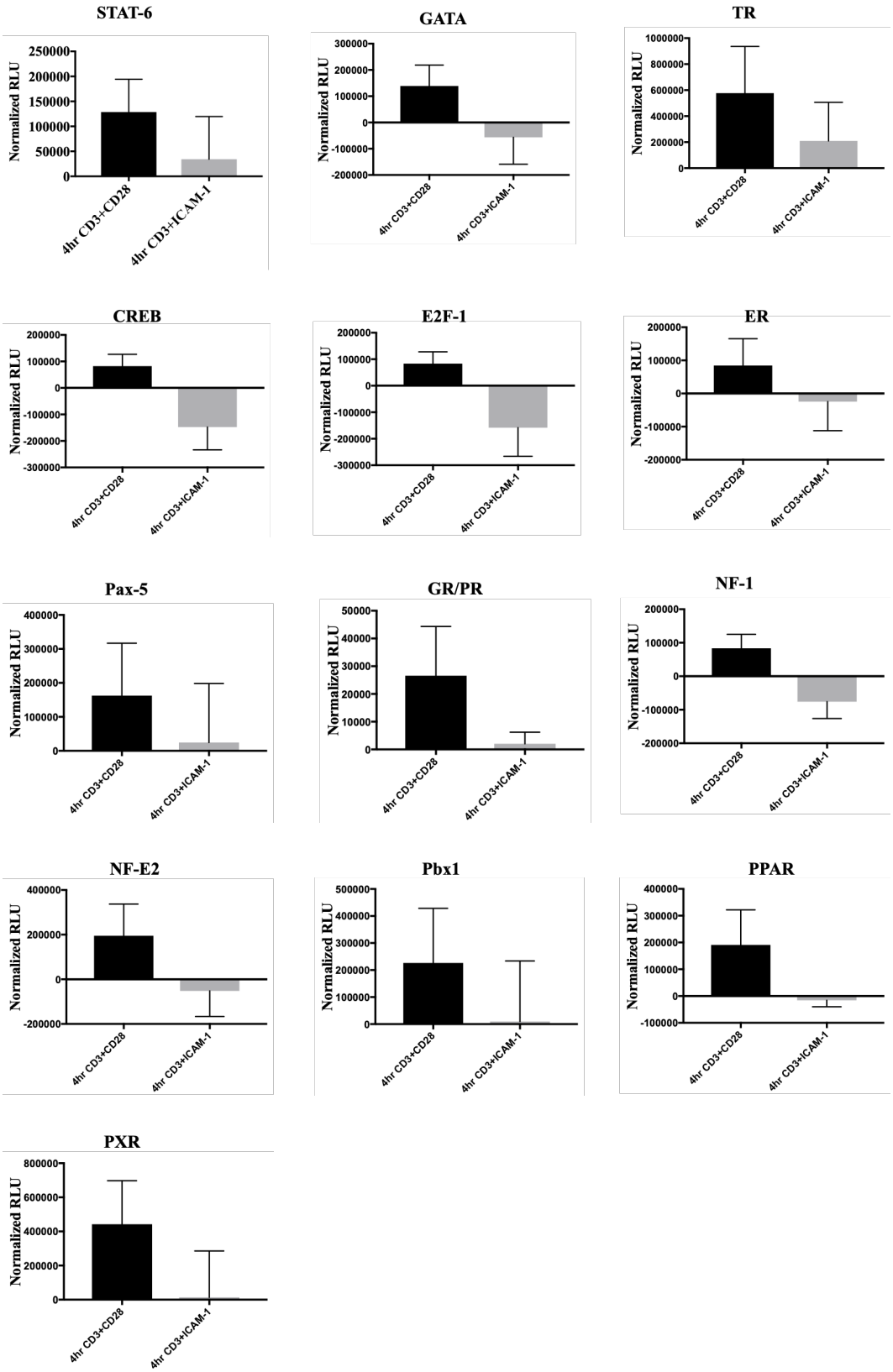

STAT-6
GATA
TR
CREB
E2F-1
ER
Pax-5
GR/PR
NF-1
NF-E2
Pbx1
PPAR
PXR
Normalized RLU
4hr CD3+CD28
4hr CD3+ICAM-1

Figure 2.8. Increased activation of STAT-6, GATA, TR, CREB, E2F-1, ER, Pax-5, GR/PR, NF-1, NF-E2, Pbx1, PPAR and PXR upon co-stimulation through CD28 compared to ICAM-1 co-stimulation.

Naïve $CD4^+$ T cells were left unstimulated or stimulated through CD3+CD28 or CD3+ICAM-1 for 4 hours and nuclear extracts were prepared. Activation of 48 different transcription factors activation was analyzed using the TF Activation Profiling Plate Array I from Signosis. Representative graph for transcription factors with increased activation upon CD3+CD28 stimulation compared to CD3+ICAM-1 stimulation. Error bars indicate standard error, n=5; $*p<0.05$ by paired t test.

Some of these transcription factors such as STAT6 and CREB have been previously reported to be activated upon stimulation through CD28 [16-17]. Stat6 is a member of the signal transducer and activator of transcription family of proteins that upon phosphorylation translocates to the nucleus and acts as transcription activators. Studies have shown that CD28 stimulation induced recruitment and phosphorylation of Stat6 [16]. CREB (cyclic AMP-responsive element binding protein) is transcriptionally activated by stimulation through CD3+CD28 in murine T cells [17].

Discussion

In this project, we investigated different protein phosphorylation and transcription factor activation upon co-stimulation through two co-stimulatory molecules, CD28 and ICAM-1. We studied 48 different transcription factors and 71 different receptor tyrosine kinases and observed that the two co-stimulatory molecules induced phosphorylation and activation of different proteins and transcription factors.

FAST-1 is a DNA-binding protein that plays a crucial role in the TGFβ signaling pathway. FAST-1 binds to the DNA sequence TGT/GT/GT/ATT to promote TGFβ signaling [13]. Studies have shown that TGFβ promotes FoxP3 expression in $CD4^+$ T cells as well as promoting Treg cell suppression of Th1 differentiation and proliferation [18-19]. We showed that ICAM-1 promoted differentiation to Treg phenotype and ICAM-1 promoted FAST-1 activation. We propose that ICAM-1 promotes differentiation to Treg phenotype by upregulation of FAST-1 activation (**Fig. 2.9**).

Our data show that NFκB is activated upon co-stimulation through both the co-stimulatory molecules. It has been shown that co-stimulation through CD28 induces downstream activation of PI3K, JNK, PLCγ1, NFAT and NFκB [23]. CD28 co-stimulation promotes NFκB activation in human and mouse T cells [15]. Our observation regarding NFκB activation by CD28 stimulation agrees with the literature, while NFκB activation by ICAM-1 stimulation is a novel observation. We also observed that Dtk and FGFR1 were activated upon CD28 stimulation while IGF-1R and HGFR were activated upon ICAM-1 stimulation.

Studies have shown that receptor tyrosine kinases Dtk, FGFR1, IGF-1R and HGFR activate PI3K/AKT/mTOR and MAPK/ERK pathways that are crucial for cell proliferation, differentiation and survival [7-11]. Dkt, also known as TYRO3 is a member of the TAM (TYRO3, AXL,

MERTK) family and ligands for TAM were identified as growth arrest-specific 6 (GAS6) and Protein S (Pros1) [20-21]. PI3K activation and downstream signaling through AKT failed in platelets derived from TRYO deficient mouse [7]. Studies have implicated IGF-1R in the activation of PI3K/Akt pathway in osteosarcoma [22]. IGF-1Ra promotes activation of PI3K-AKT and MAPK/ERK pathway in hematopoietic cells, thus promoting cell cycle progression and preventing apoptosis [10].

Activation of PI3K generates D3-lipids, PIP2 (phosphatidylinositol (3,4)- bisphosphate and PIP3 (phosphatidylinositol (3,4,5)-triphosphate), which recruit various molecules with pleckstrin homology domains such as PDK1 (phosphoinositide-dependent kinase 1), Akt, WASP (Wiskott Aldrich Syn- drome Protein), Itk and Vav [23]. PDK1 phosphorylates and activates Akt. Activated Akt has diverse downstream targets which leads to increased NFκB activation. Activated NFκB promotes transcription of Bcl-xL and IL-2, both of which promote cell survival and proliferation [23].

We propose that the two co-stimulatory molecules might use different pathways to activate NFκB that promotes cell survival (**Fig. 2.10)**. We propose that CD28 promotes NFκB activation by inducing phosphorylation of Dtk and FGFR1, while ICAM-1 promotes NFκB activation by inducing phosphorylation of IGF-1R and HGFR.

We observed increased Stat6 activation upon CD3+CD28 co-stimulation which agrees with Oki et. al. as they showed that CD28 stimulation induced recruitment and phosphorylation of Stat6. They also showed that Stat6 promoted transcriptional activation of IL-4 [16]. Zhu et al. showed that Stat6 was required for Th2 differentiation and cell expansion. They showed that Stat6 knockout CD4+T cells failed to differentiate to Th2 cells [24]. Activation of Stat6 led to upregulation of GATA-3 mRNA and GATA3 promoted T cell differentiation to Th2 [25-26]. Our

Fig. 2.9.

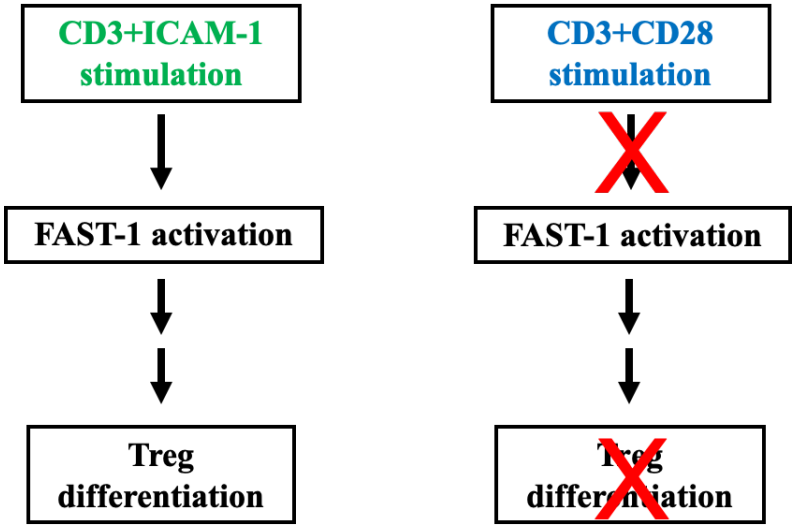

CD3+ICAM-1 stimulation
FAST-1 activation
Treg differentiation
CD3+CD28 stimulation
FAST-1 activation

Figure 2.9. Model for Treg differentiation upon ICAM-1 co-stimulation.

ICAM-1 co-stimulation promotes FAST-1 activation that leads to the differentiation to the Treg phenotype.

Fig. 2.10.

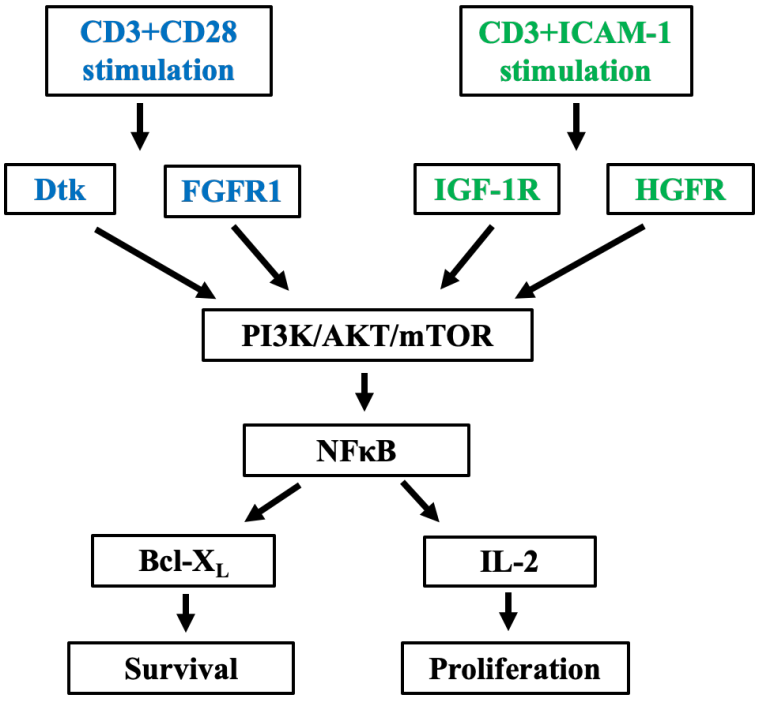

CD3+CD28 stimulation
CD3+ICAM-1 stimulation
Dtk
FGFR1
IGF-1R
HGFR
PI3K/AKT/mTOR
NFκB
Bcl-X$_L$
IL-2
Survival
Proliferation

Figure 2.10. Model for NFκB activation by CD28 and ICAM-1 co-stimulation.

Co-stimulation through CD28 promotes phosphorylation of Dtk and FGFR1, and co-stimulation through ICAM-1 promotes phosphorylation of IGF-1R and HGFR. Receptor tyrosine kinases Dtk, FGFR1, IGF-1R and HGFR activate PI3K. Activation of PI3K generates D3-lipids which recruit various molecules with pleckstrin homology domains such as PDK1 (phosphoinositide-dependent kinase 1), Akt, WASP, Itk and Vav1. PDK1 phosphorylates and activates Akt. Activated Akt has diverse downstream targets which leads to increased NFκB activation. Activated NFκB promotes transcription of Bcl-xL and IL-2, which promotes cell survival and proliferation.

lab has shown that CD3+CD28 promoted differentiation to Th2 phenotype [1-2]. Thus, this suggested that CD3+CD28 promoted Stat6 expression, which in turn led to differentiation to Th2 phenotype.

We learned that CD28 and ICAM-1 each activate specific sets of proteins suggesting that each co-stimulatory molecule has its own characteristic signaling profile. These results need additional verification at the mRNA level. Further investigation is required to identify specific signaling pathways and genes that are specific for T cell differentiation. Since choice of co-stimulatory molecule induced differentiation of naïve T cells to different phenotypes [1-3], identification of specific pathways or genes that tune differentiation phenotypes would provide a possibility for developing therapeutic approaches to control T cell differentiation.

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Chapter 3

Engagement of CD45 alters early signaling events in human T cells co-stimulated through TCR+CD28.

Introduction

Our lab has been developing the concept that co-stimulation of human naïve CD4+ T cells through different co-stimulatory molecules can tune differentiation to different phenotypes. Our lab hypothesizes that the differentiation outcome is influenced by the local microenvironment in which the T cell finds itself. Much of our work has compared co-stimulation through TCR/CD3+CD28 with co-stimulation through TCR/CD3+ICAM-1. In the absence of exogenous cytokines, stimulation through CD3+CD28 induces differentiation to Th2 (and a small number of Th1) effector and memory cells but not Treg cells, while stimulation through CD3+ICAM-1 induces differentiation to Th1 effector and memory cells and Treg cells but not Th2 cells [1-3]. An open question is where in the signaling pathways induced by the co-stimulation do differences occur that contribute to outcome of differentiation. So, we chose to investigate the earliest signaling events in T cell signaling, which is CD45. CD45 is a crucial transmembrane protein as it is the first step after antigen signal leading to T cell activation. We intend to learn whether the different differentiation outcomes influenced by the microenvironment begin at the early step of T cell activation and how these signals progress during the activation process.

CD45

CD45, also known as leukocyte common antigen, is a cell surface glycoprotein with tyrosine phosphatase activity [4]. It is highly expressed on leukocytes, occupying up to 10% of the cell surface area [4]. A key substrate for CD45 is a tyrosine residue in the C-terminal region of Src family kinases such as Lck [4]. However, a specific ligand for CD45 has not been identified and the functions of CD45 have not been fully described. For this project, we investigated the effect of CD45 engagement on signaling upon stimulation through TCR/CD3 alone and TCR/CD3+ a

co-stimulatory molecule, CD28 and tested the hypothesis that CD45 alters early signaling events induced by CD3+CD28.

CD45 consists of different isoforms generated by alternative splicing of three exons 4, 5 and 6 in the extracellular region, but functions of the different isoforms remains unclear [4]. Naïve T cells express the CD45RA isoform, which is the high molecular mass isoform and mature cells express CD45RO, which is the smallest isoform [5]. Cells expressing different isoforms show differential tyrosine phosphorylation patterns of proteins, suggesting that the extracellular domains of CD45 may regulate T cell signaling differentially [6,7].

CD45 in T cell activation

CD45 plays a crucial role in T cell signaling by dephosphorylating a negatively regulatory tyrosine residue in the C-terminal region of Src family kinases. Lck is a Src family tyrosine kinase that is specifically associated with the cytoplasmic region of CD4 and CD8 coreceptors. Before T cell activation, Lck is in an inactive conformation due to intramolecular interaction between phosphotyrosine and an SH2 domain. Upon antigen recognition by the TCR, Lck accumulates at the TCR-MHC engagement site due to CD4 binding to the MHC, bringing Lck close to the CD3 subunits. Lck is then dephosphorylated by CD45 at the inhibitory phosphate at Y505 and Lck autophosphorylates at the activating phosphate at Y394 [8-10]. Activated Lck then phosphorylates several immunoreceptor tyrosine-based activation motifs (ITAM) present in CD3 ζ, ε, δ and γ subunit of TCR-CD3 complex. Phosphorylated ITAMs promote activation of Zap-70 protein tyrosine kinase and provide assembly points for nascent signaling complexes for downstream signaling. Thus, the phosphatase activity of CD45 is crucial for and provides the first event during T cell activation.

Signaling through CD45 in T cells

CD45 in T cells is associated with proteins of sizes 116 and 80 kDa, Lck, CD4, CD3 zeta and CD28 [11-16]. While the intracellular tyrosine phosphatase activity of CD45 has been extensively studied, some studies suggest that CD45 induces signaling in T cells. CD45 engagement triggers early signaling events in human T cells shown by increase in phosphorylation of protein tyrosine kinases, adapter proteins and exchange factors and mouse splenic T cell shown by increase in phosphorylation of Grb2 protein [17,18]. Human T cells showed increased proliferation upon co-stimulation through CD3+CD45 [19,20]. In other cell types as well, CD45 engagement induced cytokine production including IL-1 and TNF- α production in monocytes, IFN-γ in NK cells, and TNF-α production in neutrophils [21-23].

Hypothesis tested

T cell activation requires two signals. The first signal is delivered by a specific antigen (Ag) through the T cell receptor (TCR) and the second signal is delivered by a counter receptor on the Ag presenting cell (APC) through a co-stimulatory molecule on the T cell. In the absence of the second signal, signal 1 alone can induce anergy or death [24,25]. Immediately after the T cell uses TCR/CD3 to recognize its cognate antigen as presented on the surface of an APC, CD45 initiates the signaling pathway leading to T cell activation. CD45 is active during antigen engagement and then exits the complex [26]. In this study, we are investigating the earliest events in the signaling process upon engagement of CD45 alone and in association with TCR/CD3 and TCR/CD3+ a co-stimulatory molecule: CD28. We hypothesize that during TCR recognition of cognate antigen, engagement of CD45 differentially guides signaling events in T cells, thus

altering the early signaling events induced by TCR/CD3+ co-stimulatory molecule, CD28 and possibly other costimulatory molecules.

Materials and methods

Antibodies and reagents

Anti-CD3 (clone OKT3) was purchased from eBioscience (San Diego, CA), anti-CD28 (clone CD28.2) was purchased from Biolegend (San Diego, CA), and anti-CD45 (F10-89-4) was purchased from SouthernBiotech (Birmingham, AL). The crosslinking goat anti-mouse F(ab)'$_2$ IgG antibody was purchased from Jackson ImmunoResearch (West Grove, PA) Antibody used for flow cytometry was anti-CD45-PE (clone HI30), purchased from Biolegend (San Diego, CA). Fixation/Permeabilization solution kit and PY20 antibody from BD were used for intracellular staining of phosphoproteins (San Jose, CA). Antibodies used for probing western blots were: anti-CD45 (polyclonal) from Bethyl Laboratories (Montgomery, TX), Lck (clone transduction 28) from BD Biosciences (San Jose, CA) and TCRβ (clone G-11) from Santa Cruz Biotechnology (Dallas, TX). Flow cytometry was performed with Guava EasyCyte system (Millipore, Burlington, MA) and data analysis using Guava InCyte software.

Cell stimulation

Each antibody was titrated to the lowest effective concentration. Jurkat cells were stimulated *in vitro* using: anti-CD3 (OKT3) at 1ug/ml, anti-CD28 (CD28.2) at 2ug/ml and CD45 (F1-89-4) at 10ug/ml and a crosslinking antibody goat anti-mouse F(ab)'$_2$ IgG at 4ug/ml for indicated times at 37^{0}C. Cells were lysed in triton lysis buffer containing 1% Triton X-100, 150mM NaCl, 10mM Tris pH7.4, 1mM EDTA, 1mM EGTA, 50mM NaF, 1mM Na_3VO_4 and 1mM PMSF. Lysed cells were incubated on ice for 30 minutes and centrifuged at 4^{0}C at 11,000 rpm for 30

minutes. Supernatants were collected and protein concentrations were determined by Bradford Protein Assay (BioRad). Cell lysates were stored at -70^{0}C until used.

Immunoprecipitation and Western blot

Anti-CD3 (OKT3) was used at 6 μg or anti-CD45 (F10-89-4) at 5 μg for immunoprecipitation. Protein G agarose beads (Millipore, Burlington, MA) were coated with anti-CD3 or anti-CD45 antibodies by incubating them with the antibodies for 1hr at 4^{0}C. Cell lysates (250-500ug protein) were immunoprecipitated with antibody-coated protein G agarose beads overnight at 4^{0}C. Immunoprecipitates were resuspended in 2X SDS sample buffer (69.5mM Tris pH6.6, 11% glycerol, 2.2% SDS, 0.028% bromophenol blue, and 5% β-mercaptoethanol) and heated at 100^{0}C for 5 minutes.

Samples were electrophoresed in 7% Tris-acetate gels in NuPAGE tris-acetate SDS running buffer (50mM Tricine, 50mM Tris base, 0.1% SDS) and transferred to PVDF membranes (Millipore) using a wet transfer device (Thermo Fisher Scientific) in Bolt transfer buffer (25mN Bicine, 25mM Bis-Tris, 1mM EDTA and 0.05mM Chlorobutanol) for 1 hr at 20V. Membranes were blocked in 3% BSA in TBS (44.5mM Tris HCL, 5.5mM Tris base, 150mM NaCl) for 1 hour at room temperature with gentle rocking. Membranes were washed in ddH20 and incubated with indicated primary antibody for 1 hour at RT or overnight at 4^{0}C. Membranes were washed 6 times in TBST (0.1% Tween-20 in TBS) and incubated with appropriate secondary antibody for 1 hour at RT. The blots were incubated with commercial enhanced chemiluminescence reagent (Thermo Fisher Scientific) and imaged using UVP Biospectrum Chemi HR 410 imaging system. For analysis, the band intensities were calculated using ImageJ and each band normalized to respective controls.

Statistical analysis

One-way ANOVA was used to determine statistical differences between two sets of data based on a 95% confidence interval. All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc. La Jolla, CA).

Results

CD45 engagement modulated phosphorylation in vitro.

We examined whether engagement of CD45 alone induced signaling in T cells. Spertini et al. have shown that protein tyrosine kinases Lck, ZAP-70 and Syk, adapter protein LAT, exchange factor Vav and CD3 ζ are phosphorylated in Jurkat cells upon epitope specific crosslinking of CD45 [17]. We observed overall cellular decrease in tyrosine phosphorylation upon CD45 engagement alone by intracellular phospho-tyrosine staining **(Fig. 3.1-3.2)**. Using protein blotting, we observed that engagement of CD45 alone induced changes in phosphorylation states of p110, p56, p54, and p41 proteins **(Fig. 3.3)**. The densitometry analysis indicates that the changes in the phosphorylation levels of p56 and p54 upon CD45 engagement is statistically significant **(Fig. 3.4)**. Thus, engagement of CD45 induced T cell signaling, as assessed by the phosphorylation/dephosphorylation state of several proteins.

Surface expression of CD45 was unaffected by CD3+CD28 stimulation.

The first signal for T cell activation is delivered through TCR/CD3 complex and the second signal is delivered through a co-stimulatory molecule on the T cell. So, we examined the surface expression of CD45 upon CD3 stimulation alone and CD3+CD28 stimulation. Upon stimulation through CD3 alone, surface expression of CD45 increased within 30 minutes of stimulation (**Fig. 3.5-3.6**). However, the increase was not statistically significant. Upon CD3+CD28 stimulation, the surface expression of CD45 remained unaffected (**Fig. 3.7-3.8**).

Fig. 3.1.

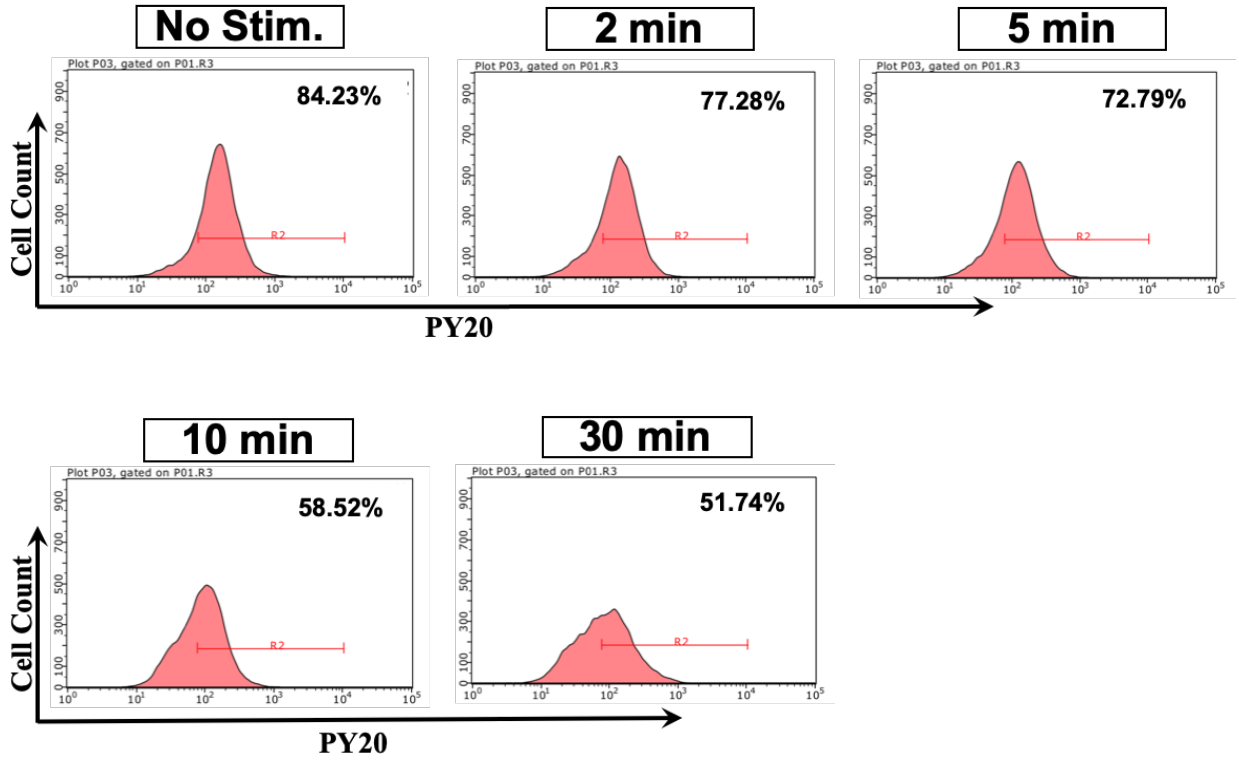

No Stim.
2 min
5 min
Plot P03, gated on P01.R3
84.23%
77.28%
72.79%
R2
Cell Count
PY20
10 min
30 min
58.52%
51.74%

Fig. 3.2.

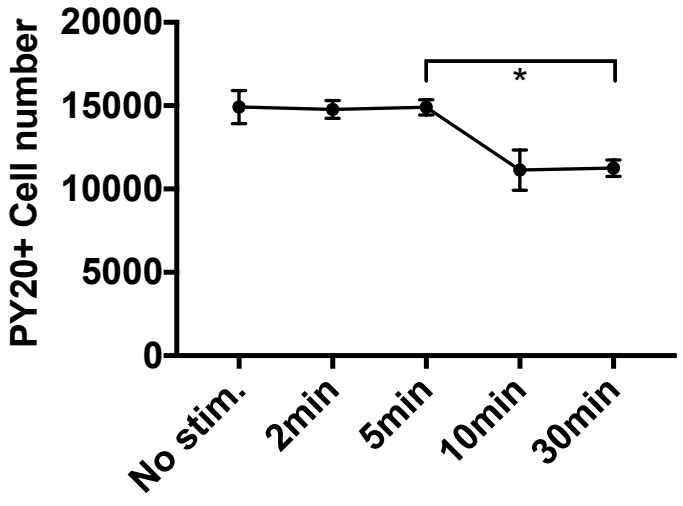

PY20+ Cell number
20000
15000
10000
5000
0
*
No stim.
2min
5min
10min
30min

Fig. 3.3.

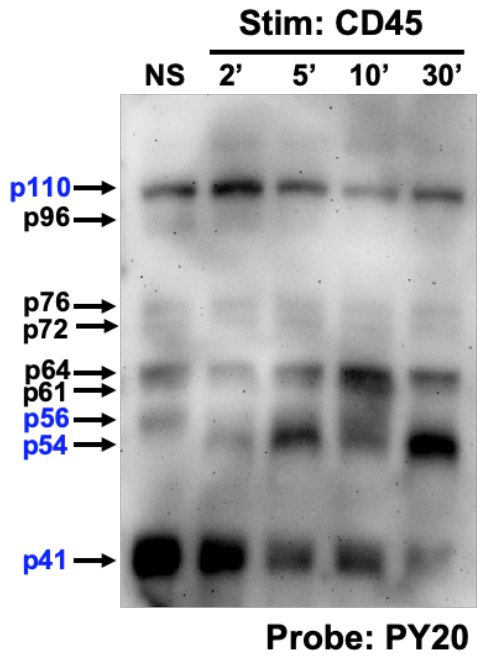

Stim: CD45
NS
2'
5'
10'
30'
p110
p96
p76
p72
p64
p61
p56
p54
p41
Probe: PY20

Fig. 3.4.

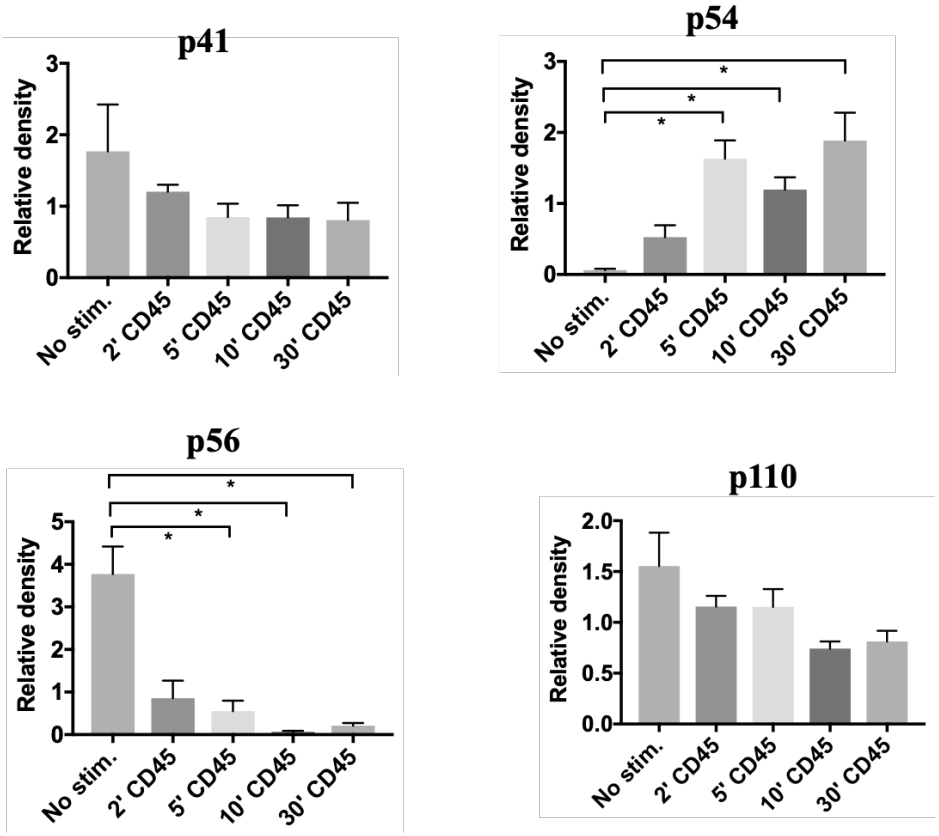

p41
Relative density
No stim.
2' CD45
5' CD45
10' CD45
30' CD45
p54
p56
p110

Figure 3.1-3.4. Engagement of CD45 alone modulated phosphorylation in vitro.

Jurkat T cells were stimulated *in vitro* using anti-CD45 and a crosslinking antibody for indicated times. **Fig.3.1:** Stimulated cells were intracellularly stained for anti-phosphotyrosine (PY20) and analyzed by flow cytometry for indicated times. Number in the right corner represents percentage positive and cell number in parentheses. Histogram plots representative of 3 independent experiments. **Fig.3.2:** Graph representing phospho-tyrosine positive cell numbers in **Fig.3.1**. **Fig.3.3:** Stimulated cells were lysed and whole cell lysates were resolved by SDS-PAGE, blotted to PDVF, and probed with anti-phosphotyrosine (PY20). Blots representative of 5 independent experiments. **Fig.3.4:** The band intensities from **Fig.3.3** were calculated using ImageJ and each band was normalized to the 76KDa band. Statistical analysis was performed by using the one-way ANOVA in GraphPad Prism. *$p<0.05$.

Fig. 3.5.

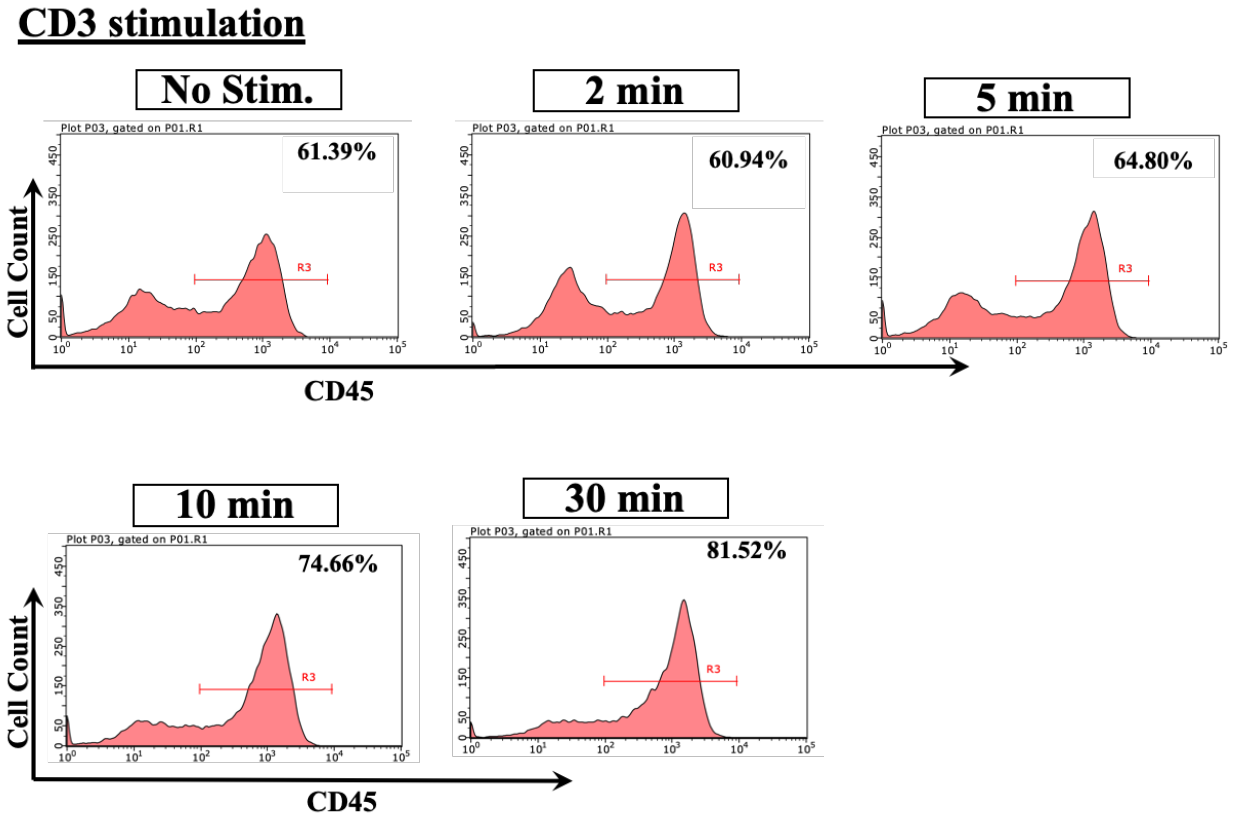
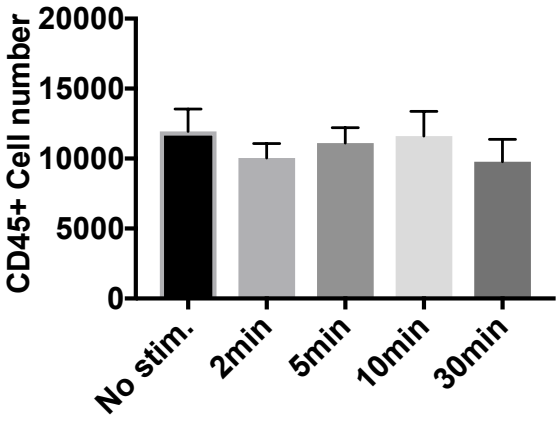

CD3 stimulation
No Stim.
2 min
5 min
10 min
30 min
Plot P03, gated on P01.R1
61.39%
60.94%
64.80%
74.66%
81.52%
R3
Cell Count
CD45

Fig. 3.6.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15000
10000
5000
0
No stim.
2min
5min
10min
30min

Fig. 3.7.

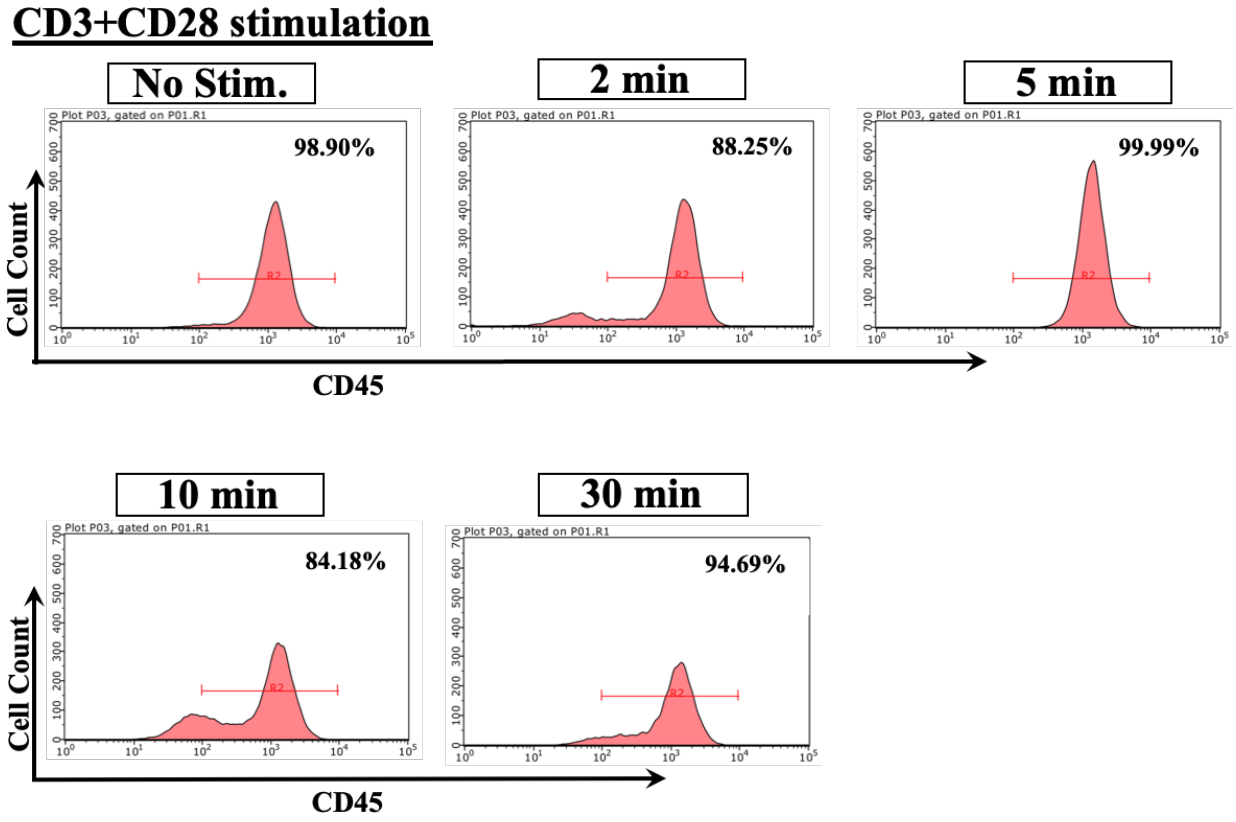

CD3+CD28 stimulation
No Stim.
98.90%
2 min
88.25%
5 min
99.99%
10 min
84.18%
30 min
94.69%
Cell Count
CD45
Plot P03, gated on P01.R1
R2

Fig. 3.8.

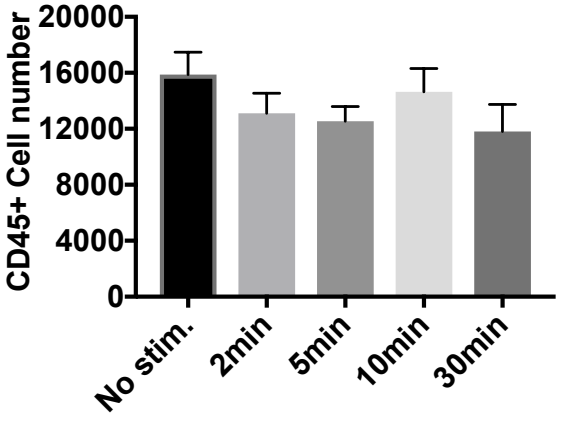

CD45+ Cell number
20000
16000
12000
8000
4000
0
No stim.
2min
5min
10min
30min

Figure 3.5-3.8. Surface expression of CD45 was unaffected by CD3+CD28 stimulation.

Jurkat cells were stimulated *in vitro* using anti-CD3 (**Fig. 3.5- 3.6**) or anti-CD3+anti-CD28 (**Fig 3.7- 3.8**) and a crosslinking antibody for indicated times and CD45 expression was analyzed by flow cytometry. **Fig. 3.5 and 3.7** are histogram plots representative of 4 independent experiments. Number in the right corner represents percentage positive and cell number in parentheses. **Fig. 3.6 and 3.8** are graphs representing CD45 positive cells in **Fig. 3.5 and 3.7.** Statistical analysis was performed by using the one-way ANOVA in GraphPad Prism.

CD45 expression altered upon CD45 co-engagement with CD3 and CD3+CD28 stimulation.

We introduced CD45 engagement to CD3 and CD3+CD28 stimulation to observe if co-engagement of CD45 influenced surface expression of CD45. Upon CD3+CD45 stimulation, surface expression of CD45 decreased at 2 minutes, returned to baseline at 5 minutes and decreased within 10-30 minutes (**Fig. 3.9-3.10**). Upon CD3+CD28+CD45 stimulation, the surface expression of CD45 decreased within 2-5 minutes and returned to baseline within 10-30 minutes (**Fig. 3.11-3.12**). From these data, we concluded that surface expression of CD45 was altered upon CD45 engagement during CD3 and CD3+CD28 stimulation and suggested that surface expression of CD45 is only responsive to CD45 engagement.

Lck co-immunoprecipitated with CD45 and decreased association within 5 to 10 min upon CD45 engagement alone.

It has been extensively shown that CD45 initiates the first step in T cell activation by dephosphorylating a negatively regulatory tyrosine residue in the C-terminal region of the Src family kinase member, Lck. Upon dephosphorylation by CD45 at its inhibitory phosphate and autophosphorylation at the activating phosphate, activated Lck phosphorylates ITAM subunits of CD3-TCR complex, allowing for downstream T cell signaling [9,10]. In the absence of CD45, T cells failed to proliferate in response to antigen or CD3 stimulation [27]. Studies have reported that Lck is associated with CD45 in both resting and activated human T cells [11], as well as in resting and peptide activated mouse T cells [28]. As Lck is an important Src family kinase and we observed that CD45 induced signaling in T cells as shown in **Fig. 3.1-3.4**, we examined if CD45 engagement induced any effect on Lck association with CD45. We stimulated the cells through

Fig. 3.9.

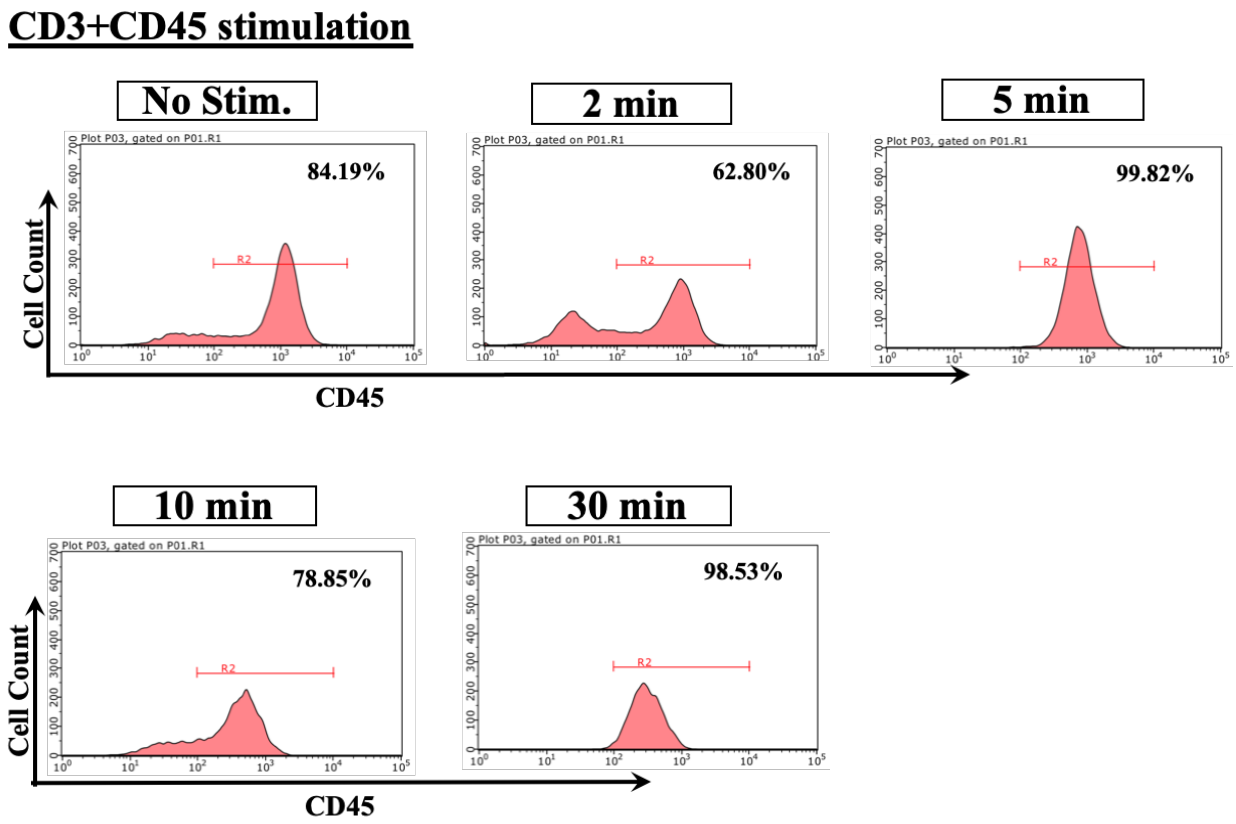


Fig. 3.10.

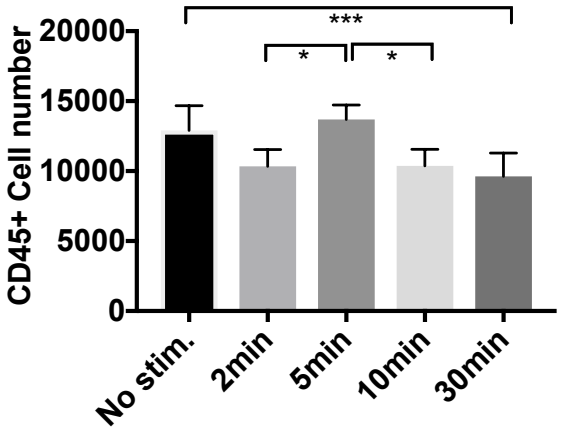


Fig. 3.11.

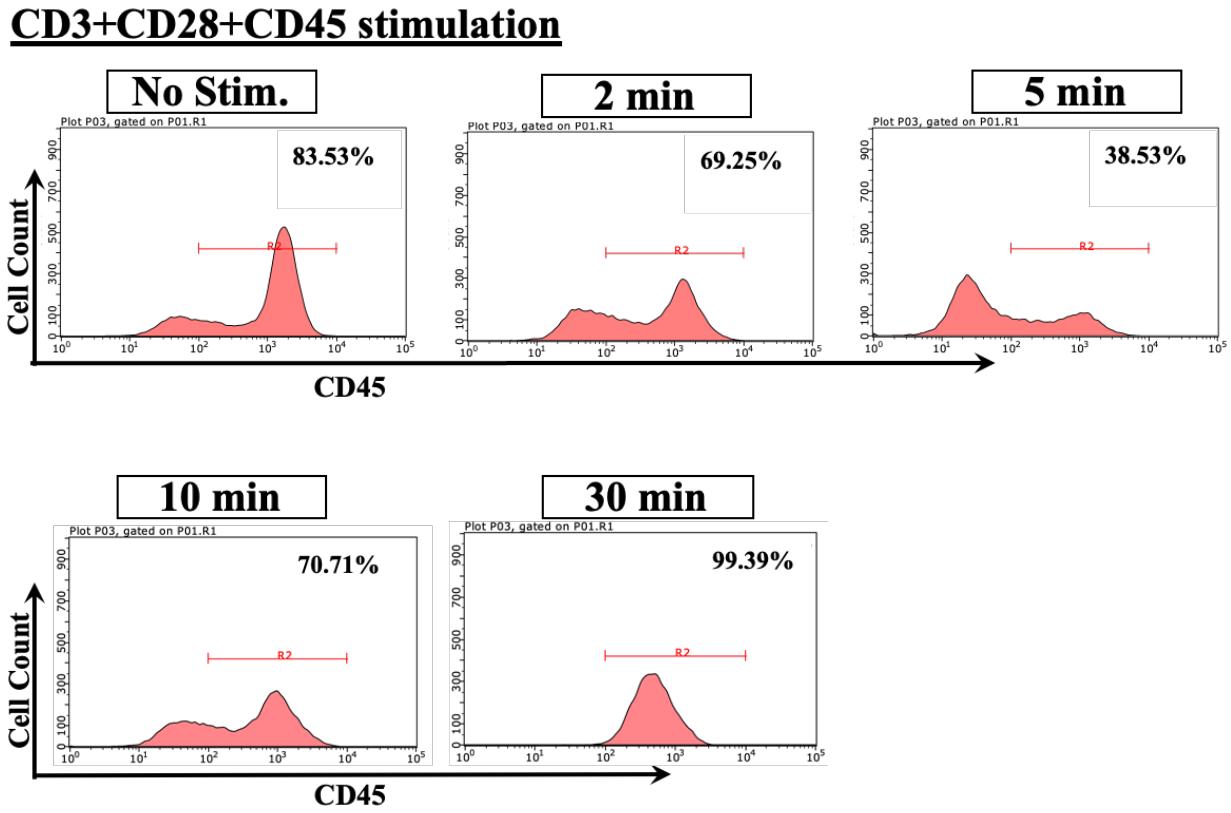


Fig. 3.12.

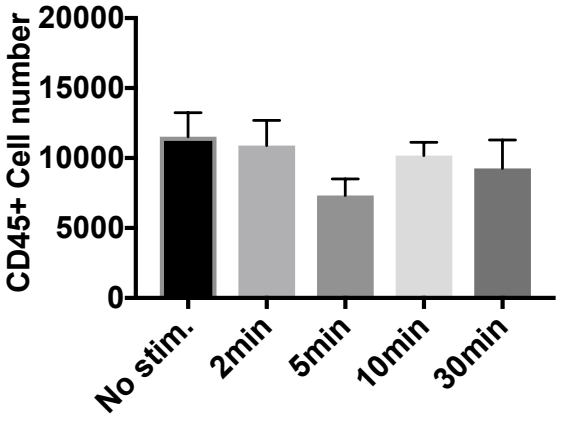


Figure 3.9-3.12. CD45 expression decreased upon CD45+CD3 or CD45+CD3+CD28 engagement.

Jurkat cells were stimulated *in vitro* using anti-CD3+anti-CD45 (**Fig. 3.9-3.10**) or anti-CD3+anti-CD45+anti-CD28 (**Fig. 3.11-2.12**) and a crosslinking antibody for indicated times and CD45 expression was analyzed by flow cytometry. **Fig. 3.9 and 3.11** are representative histogram plots. Representative of 4 independent experiments. **Fig. 3.10 and 3.12** are graphs representing CD45 positive cells. Statistical analysis was performed by using the one-way ANOVA in GraphPad Prism. *$p<0.05$, ***$p<0.001$

Fig. 3.13.

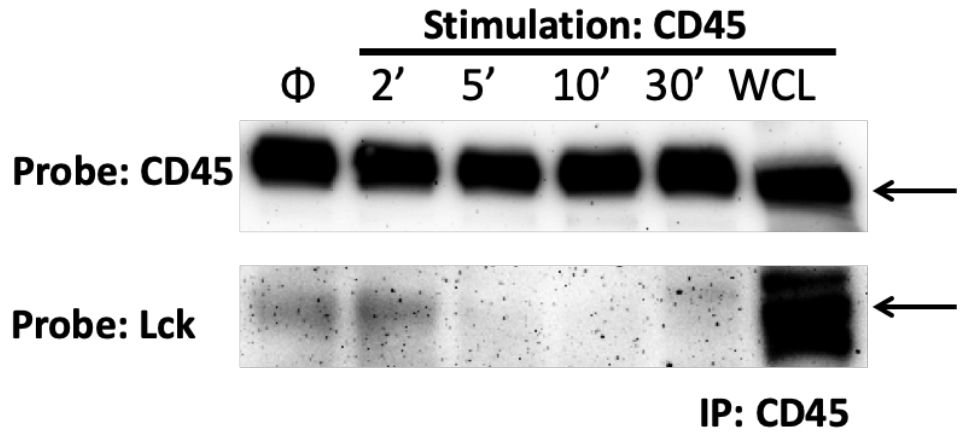


Fig. 3.14.

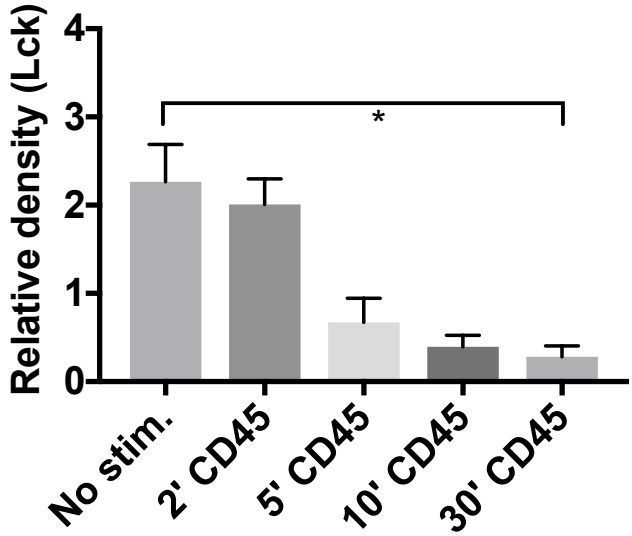


Figure 3.13-3.14. Lck co-immunoprecipitated with CD45 and decreased association within 5 to 10 min upon CD45 engagement alone.

Jurkat cells were stimulated *in vitro* using anti-CD45 and a crosslinking antibody for indicated times and lysates were immunoprecipitated with anti-CD45. **Fig. 3.13:** Immunoprecipitates were resolved by SDS-PAGE, blotted to PDVF, and probed as indicated. Representative of 4 independent experiments. Φ- Non-stimulated control, WCL- whole cell lysate. Arrow indicates CD45 (top) and Lck (bottom) proteins. **Fig. 3.14:** The band intensities from **Fig. 3.13** was calculated using ImageJ and each band was normalized to CD45. Statistical analyses were performed by using the one-way ANOVA in GraphPad Prism. *$p<0.05$

CD45 and immunoprecipitated with CD45. We observed that Lck immunoprecipitated with CD45. However, Lck association significantly decreased within 5-10 min of CD45 engagement (**Fig 3.13-3.14**).

Lck maintained association with CD45 upon TCR+CD45 and TCR+CD45+CD28 engagement.

Next, we investigated if CD3 or CD3 plus co-stimulatory molecule, CD28 had any effect on Lck association with CD45 in the context of CD45 engagement. It has been shown that co-stimulation through CD3+CD45 induces increased proliferation in human T cells [19,20]. Upon CD3+CD45 stimulation, Lck immunoprecipitated with CD45 and remained associated with CD45 for 30 minutes of stimulation **(Fig. 3.15 and 3.16).** Similarly, Lck remained associated with CD45 when cells were stimulated through CD3+CD28+CD45 **(Fig. 3.17 and 3.18).** Thus, Lck associated with CD45 for a brief 2 minutes upon CD45 engagement alone. However, upon TCR engagement along with CD45, with or without co-stimulatory molecule CD28, Lck remained associated with CD45. These results suggest that Lck dissociation is responsive to CD45 unless cells are stimulated through TCR/CD3 or TCR/CD3+CD28

Lck associated with TCR for 2-5 minutes and left the complex within 10-30 min of CD28 engagement with TCR.

As mentioned earlier, during the TCR/CD3 engagement with antigenic MHC, Lck phosphorylates ITAM subunits of TCR/CD3 complex, which become the docking site for ZAP-70, and activates ZAP-70. Zap-70 along with Lck phosphorylate downstream signaling molecules to activate TCR-CD3 controlled signaling cascade. Since, Lck is crucial for TCR/CD3 signaling, we examined if Lck remained associated with TCR/CD3 complex upon CD3/TCR engagement. We observed that

Fig. 3.15.

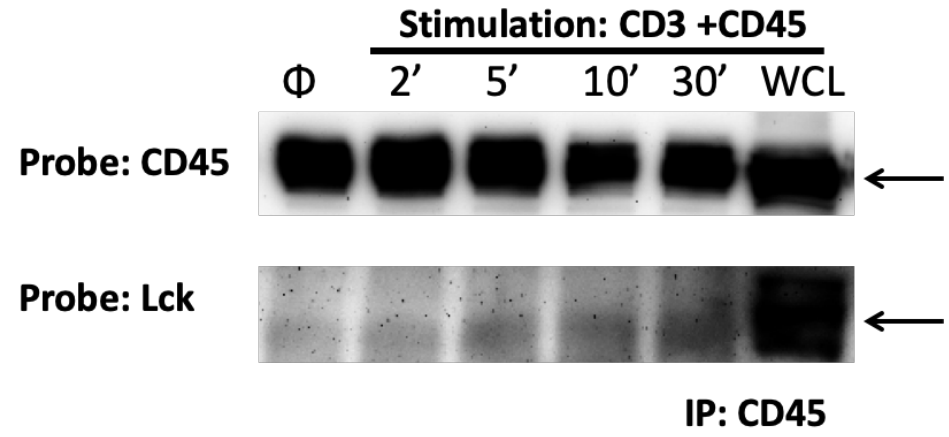


Fig. 3.16.

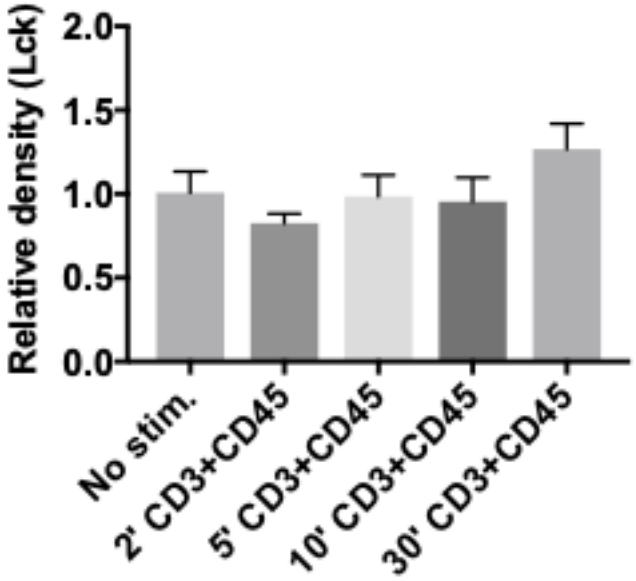


Fig. 3.17.

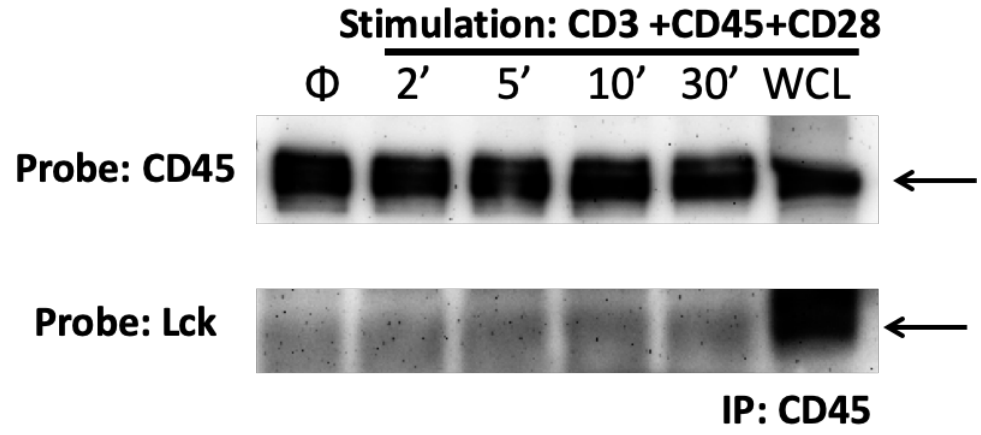


Fig. 3.18.

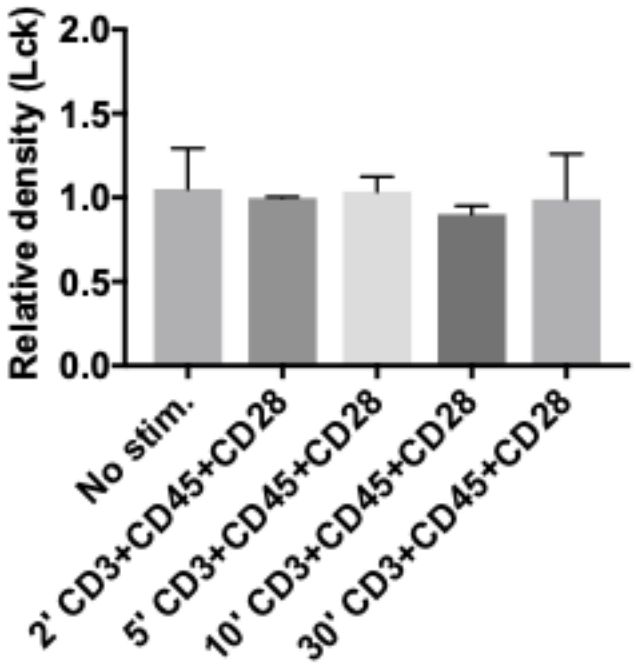


Figure 3.15-3.18. Lck maintained association with CD45 upon TCR+CD45 and TCR+CD45+CD28 engagement.

Jurkat cells were stimulated *in vitro* using anti-CD3+anti-CD45 (**Fig. 3.15**) or anti-CD3+anti-CD45+anti-CD28 (**Fig. 3.17**) and lysates were immunoprecipitated with anti-CD45. **Fig. 3.15 and 3.17:** Immunoprecipitates were resolved by SDS-PAGE, blotted to PDVF, and probed as indicated. Representative of 4 independent experiments for **Fig. 3.15** and 3 independent experiments for **Fig. 3.17**. Φ- Non-stimulated control, WCL- whole cell lysate. Arrow indicates CD45 (top) and Lck (bottom) proteins. **Fig. 3.16 and 3.18:** The band intensities from **Fig. 3.15 and 3.17** were calculated using ImageJ and each band was normalized to CD45. Statistical analyses were performed by using the one-way ANOVA in GraphPad Prism.

CD45 did not associate with TCR/CD3 complex when cells were stimulated through CD3 alone (**Fig. 3.19)** or CD3+CD28 (**Fig. 3.21**). Lck immunoprecipitated with TCR/CD3 complex and remained associated with TCR/CD3 during 30min of TCR/CD3 engagement (**Fig. 3.19**) and no statistically significant differences were observed among different timepoints for Lck association with TCR/CD3 complex **(Fig. 3.20).** Next, we examined Lck association with TCR/CD3 upon CD3+CD28 stimulation. CD28 is the second signal which amplifies weak TCR signal into strong signal [29-30]. No unique signaling pathways have been identified for CD28, nor is CD28 an alternative for TCR signaling [29]. Lck association with CD28 and the kinetics of association during CD3+CD28 stimulation have not been studied. Thus, we examined Lck association with CD3/TCR upon CD3+CD28 stimulation and observed that Lck associated with CD3/TCR complex for the first 2-5 minutes of stimulation and then left the complex (**Fig. 3.21**). The decrease in Lck association with TCR/CD3 at 30 minutes stimulation was statistically significant **(Fig. 3.22).**

Lck association with CD3 was sustained upon CD45 engagement with TCR or TCR+CD28

We examined the effect of CD45 engagement during TCR/CD3 alone and CD3+CD28 stimulation. We observed that CD45 inducibly associated with TCR/CD3 upon CD3+CD45 engagement (**Fig. 3.23**) and CD3+CD45+CD28 engagement (**Fig. 3.26**). In both cases, CD45 association with TCR/CD3 increased with increasing time of stimulation (**Fig. 3.24, 3.27)**. Next, we examined Lck association with TCR/CD3 complex and observed that Lck associated with TCR/CD3 complex at all timepoints when cells were stimulated through CD3+CD45 (**Fig. 3.23, 3.25**). Although not statistically significant, a decrease in Lck association was observed within 2 to 5 minutes when cells were stimulated through CD45+CD3+CD28 and returned to baseline by

10-30 minutes of stimulation (**Fig. 3.26, 3.28**). From these observations, we concluded that the engagement of CD45 dominates the effect of CD3+CD28 stimulation such that Lck remained associated with TCR/CD3 complex upon CD45+CD3+CD28 stimulation.

Fig. 3.19.

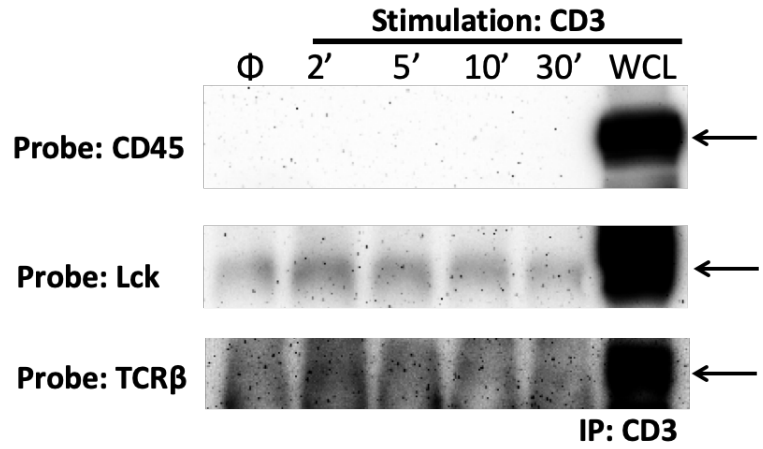


Fig. 3.20.

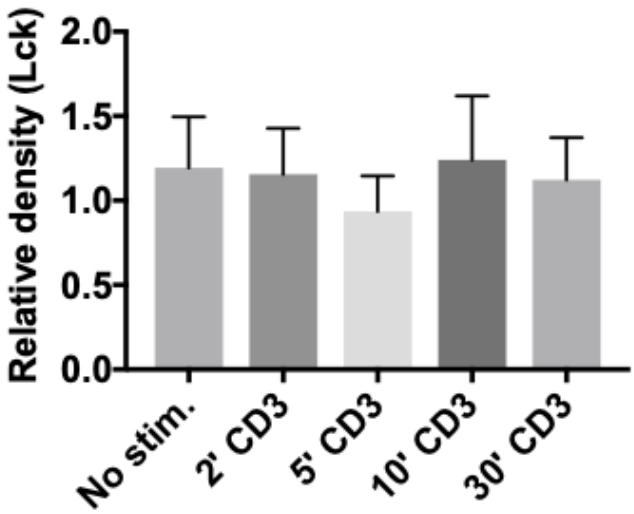


Fig. 3.21.

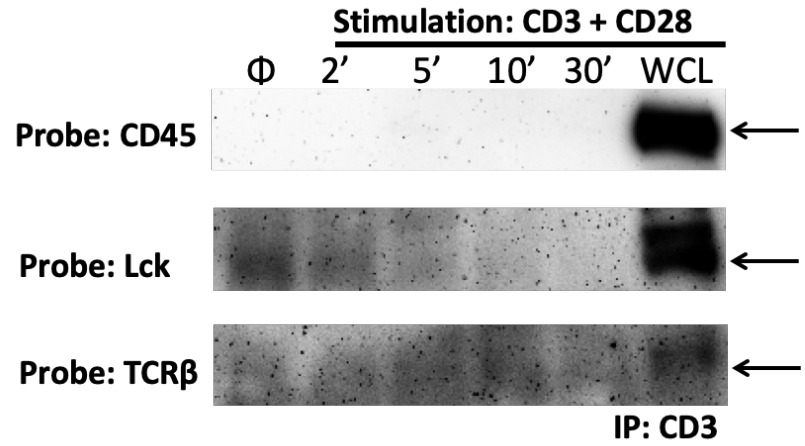


Fig. 3.22.

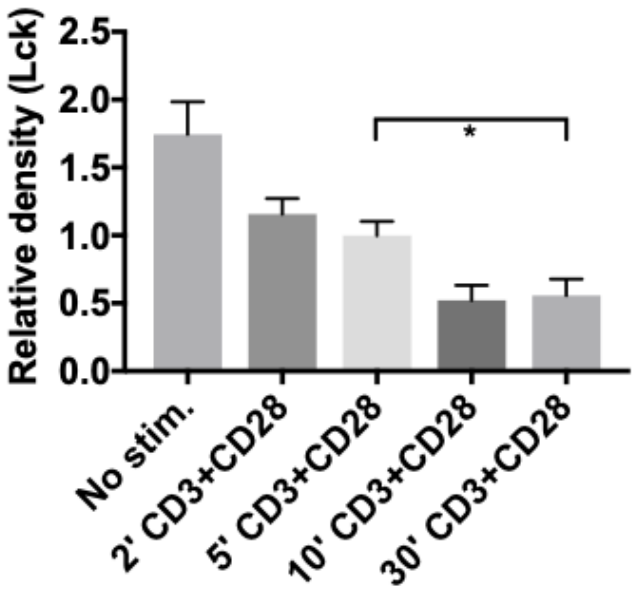


Figure 3.19-3.22. Lck associated with TCR for 2-5 min and eventually left the complex within 10-30 min of CD28 engagement with TCR.

Jurkat cells were stimulated *in vitro* using anti-CD3 (**Fig. 3.19**) or anti-CD3+anti-CD28 (**Fig. 3.21**) and lysed and immunoprecipitated with anti-CD3. **Fig 3.19 and 3.21**: Immunoprecipitates were resolved by SDS-PAGE, blotted to PDVF, and probed as indicated. Representative of 3 independent experiments. Φ- Non-stimulated control, WCL- whole cell lysate. Arrow indicates CD45 (top), Lck (middle) and TCRβ (bottom) proteins. **Fig 3.20 and 3.22**: The band intensities from **Fig 3.19 and 3.21** were calculated using ImageJ and each band was normalized to TCRβ. Statistical analyses were performed by using the one-way ANOVA in GraphPad Prism. *$p<0.05$

Fig. 3.23.

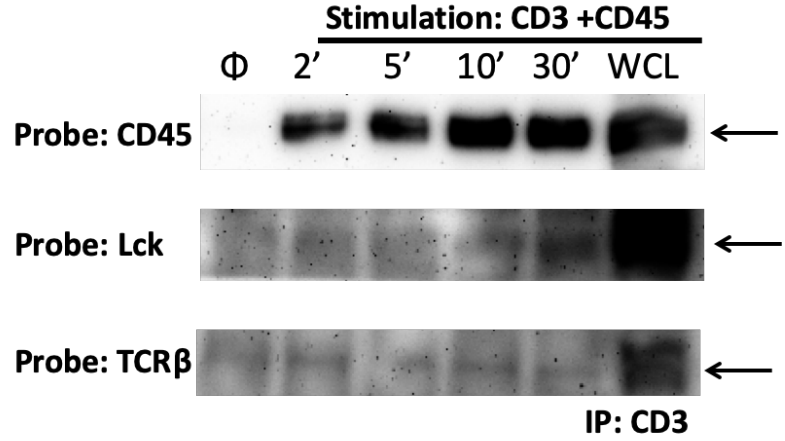


Fig. 3.24.

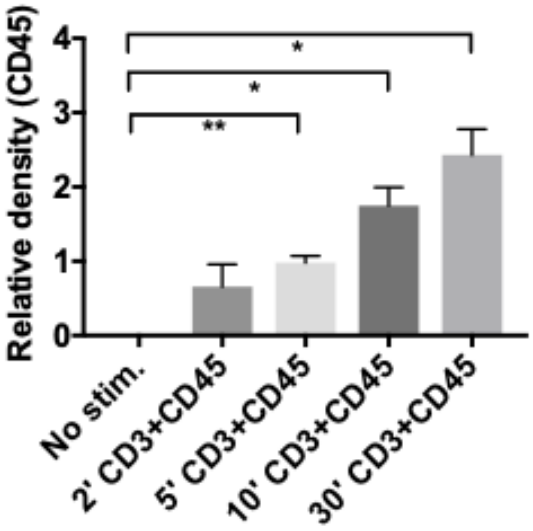


Fig. 3.25.

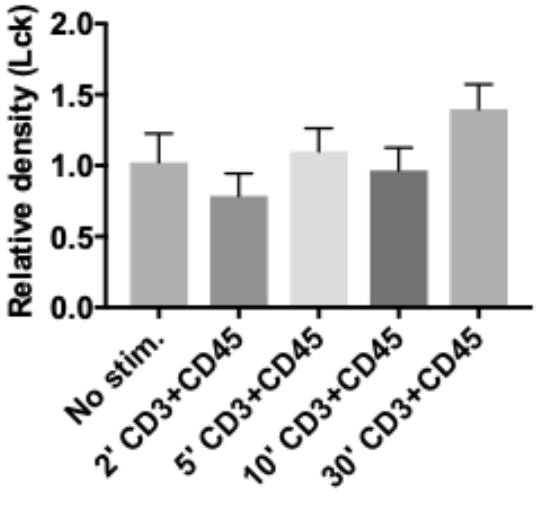


Fig. 3.26.

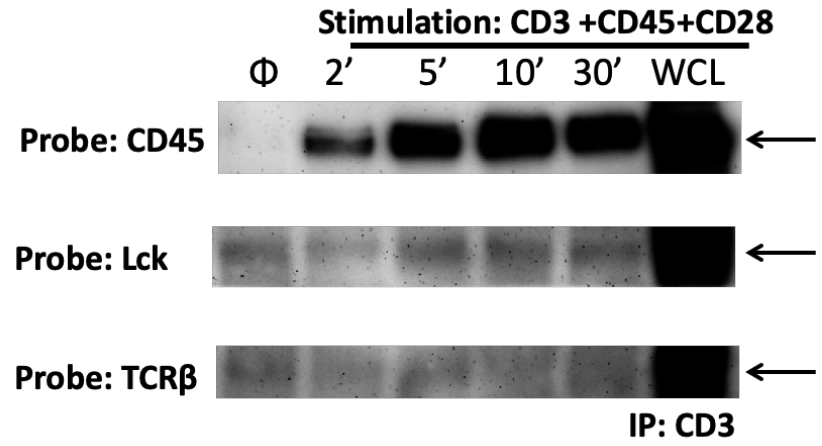


Fig. 3.27.

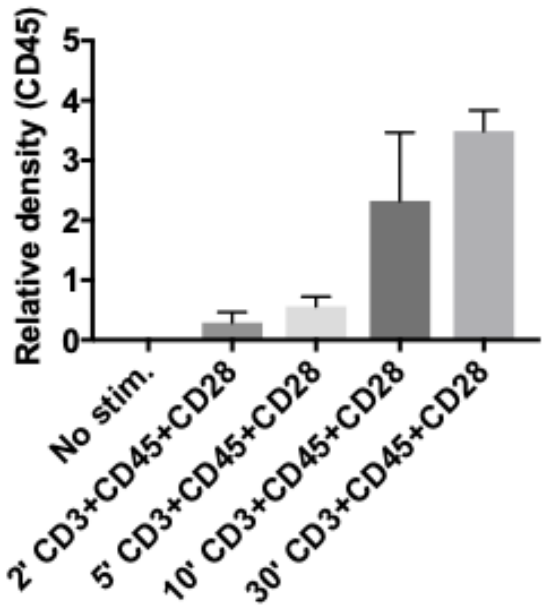


Fig. 3.28.

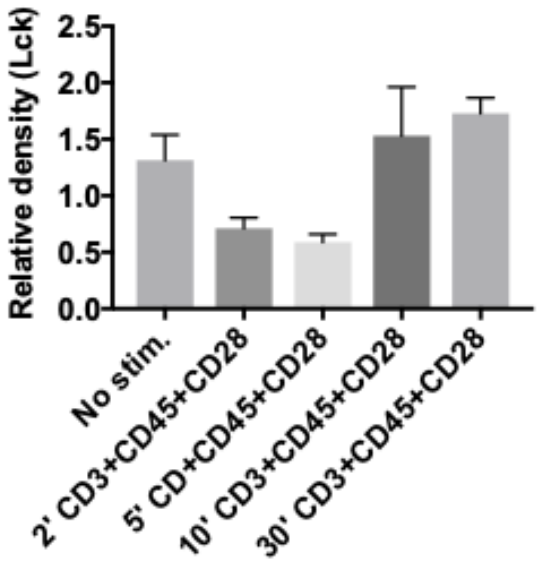


Figure 3.23-3.28. Lck association with CD3 was sustained upon CD45 engagement with TCR or TCR+CD28.

Jurkat cells were stimulated *in vitro* using anti-CD3+anti-CD45 (**Fig. 3.23**) or anti-CD3+anti-CD28+anti-CD45 **(Fig. 3.26)** and lysed and immunoprecipitated with anti-CD3. **Fig. 3.23 and 3.26:** Immunoprecipitates were resolved by SDS-PAGE, blotted to PDVF, and probed as indicated. Representative of 4 independent experiments for **Fig. 3.23** and 3 independent experiments for **Fig. 3.26**. Φ- Non-stimulated control, WCL- whole cell lysate. Arrow indicates CD45 (top), Lck (middle) and TCRβ (bottom) proteins. **Fig. 3.24, 3.25, 3.27 and 3.28**: The band intensities from **Fig. 3.23 and 3.26** were calculated using ImageJ and each band was normalized to TCRβ. Statistical analyses was performed by using the one-way ANOVA in GraphPad Prism. *$p<0.05$, **$p<0.01$

Discussion

It is well known that CD45 initiates the first step in T cell activation by dephosphorylating a negatively regulatory tyrosine residue in the c terminal region of the Src family kinase member, Lck. In this study, we observed that engagement of CD45 alone induced signaling in T cells as indicated by overall decrease in phosphorylation by flow cytometry and phosphorylation or dephosphorylation of proteins by protein blotting upon 30 minutes of CD45 engagement. Our results agree with Spertini et al., as they showed that stimulation through CD45 induced increased phosphorylation of proteins and concluded that CD45 induced signaling through T cells [17].

The surface expression of CD45 remained unaffected by CD3+CD28 stimulation. However, the engagement of CD45 decreased the surface expression of CD45. This could be suggestive of CD45 being capped. The phenomenon of capping has been studied in lymphocytes. Crosslinking of specific receptor by ligands form aggregates of the receptors called patches, which coalesce to form cap and this process is called capping. The process of capping requires energy and involves actin and myosin [31]. Upon ligand binding, Peck et al., showed that CD45 is capped in mouse splenocytes treated with fatty acid [32]. Other possibilities of the decreased surface expression could be due to internalization/ endocytosis or shedding upon CD45 engagement.

In our study, we showed that engagement of CD45 alone promoted Lck association within the first 2 minutes, followed by a decrease in Lck association within 5-30 minutes of engagement. Marie-Cardine et al. investigated Lck expression upon CD45 engagement and showed that in the absence of CD45 engagement, Lck was detected in the cell membrane. However, Lck internalized after 15 minutes of CD45 engagement as visualized by fluorescence microscopy [33]. Thus, the decrease in Lck association with CD45 could be the result of Lck internalization due to CD45 engagement. The results for this study have been summarized in **Table 3.1.**

Table 3.1

Treatment →	CD3	CD3+CD28	CD45	CD3+CD45	CD3+CD45 +CD28
CD45 Expression	++	++	↓↓	↓↓	↓↓
Lck:CD45	ND	ND	↓↓	++	++
Lck:CD3	++	↓↓	ND	++	++

Table 3.1: Summary of the results for surface expression of CD45, and association of Lck with CD45 and CD3/TCR complex.

For CD45 expression, ++ represents that the surface expression of CD45 remains unaffected and ↓↓ represents the decrease in surface expression of CD45 upon indicated engagement/treatment. For Lck:CD45 and Lck:CD3, ++ represents that Lck is associated at all timepoints, while ↓↓ represents that Lck left the complex upon indicated engagement/treatment. (ND- Not Determined)

As CD45 is crucial for T cell activation, several studies have been conducted to examine the association of CD45 at TCR/MHC interaction site. Shaw et al. in a review discussed a model for T cell activation and CD45 association at the TCR-MHC complex. They discussed that TCR engagement alone was not enough for T cell activation, rather CD45 exclusion-initiated T cell activation. They hypothesized that due to large size and strong negative charge, CD43 and CD45 moved out of the interaction site between T cell and APC, allowing for downstream signaling and T cell activation [34]. Leupin et al. showed that CD45 excluded from TCR signaling area in stimulated human T cell by using confocal microscopy. They used an extensive panel of anti-CD45 antibody to rule out artifacts such as conformational changes or steric hindrance within the molecular cluster and concluded that CD45 excluded for TCR-MHC contact [35]. Another group, Chang et al. also showed that CD45 is excluded from sites of TCR-MHC engagement [26]. In this study, we observed that CD45 did not associate with TCR/CD3 complex upon CD3 or CD3+CD28 stimulation, thus agreeing with the observations of Leupin et al. that CD45 only associated with TCR/CD3 in an inducible manner when CD45 was engaged. He et al. showed that the exclusion of CD45 from proximal TCR signaling components is required for TCR signaling as inclusion of CD45 activity in lipid rafts diminished TCR-initiated signaling [36]. However, Sperling et al. showed that TCR signaling was sufficient to induce CD43 exclusion but not sufficient to induce CD45 exclusion at TCR-MHC site using mouse cell lines [37]. The differences among various studies could be due to use of different cells and different staining conditions.

TCR/CD3 alone or CD28 alone is not sufficient for T cell signaling. Under normal conditions, only few TCRs are ligated that generate incomplete activation events, and fail to induce proliferation or differentiation of T cells. This rather induces T cell anergy. TCR/CD3 signaling complex includes Lck, Zap70, LAT-SLP76, PI3K, TEC, ITK (IL-2 inducible T cell kinase), VAV1

and AKT [29]. CD28 stimulation alone results in the transient expression of only few genes and no obvious biological consequences. CD28 signaling complex includes PI3K, TEC, ITK, VAV1 and AKT, which is a small subset of proteins that are included in the TCR signaling pathway [29]. Upon co-stimulation through TCR/CD3+CD28, CD28 amplifies a weak TCR signal resulting in T cell activation. Hence, CD28 is not an alternative for TCR/CD3 signaling, rather CD28 is a quantitative supporter for TCR signaling [29].

Lck is a Src-family kinase associated in the cytoplasmic tail of CD4 and CD8 co-receptors. Upon TCR-MHC recognition, the co-receptors (CD4 or CD8) bind to their specific MHC molecule, bringing the Lck associated to it closer to the TCR-MHC interaction site. Hutchcroft et. al. studied Lck association with CD28 in human T cells and they showed that Lck is associated with CD28 upon stimulation through CD28 [38]. They looked at the Lck association at an early time point of 5 minutes. Viola et al. showed that in T cells, overall Lck decreased upon CD3+CD28 stimulation for 1 hr [39]. Bachmann et al. showed that CD8 associated Lck decreased upon 15 minutes of stimulation. Both these groups suggested that Lck is consumed by the triggered TCR, hence there is decrease in Lck association as the time of stimulation increases [40]. These studies suggest that Lck is associated at an early time and then decrease in Lck association is observed during CD3+CD28 stimulation. Our data suggest that Lck associates with TCR/CD3 complex at the early time of 2 to 5 minutes and then Lck association decreased within 10-30 minutes of CD3+CD28 stimulation.

We propose a model to summarize our results (**Fig. 3.29-3.33)**. While looking at the overall picture, for a resting T cell, Lck is associated with CD45 and TCR/CD3 and Lck could be in an inactive state (**Fig. 3.29**). Upon 2 minutes of CD45 engagement, Lck is activated and associated with TCR/CD3 complex (**Fig. 3.30**). However, in the absence of cognate Ag, the complex

Fig. 3.29.

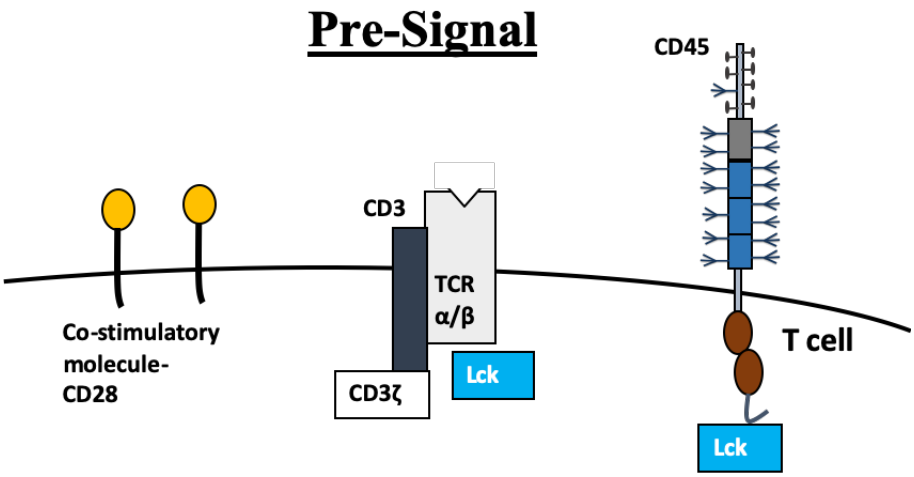

Pre-Signal
CD45
CD3
TCR
α/β
Co-stimulatory
molecule-
CD28
CD3ζ
Lck
T cell
Lck

Fig. 3.30.

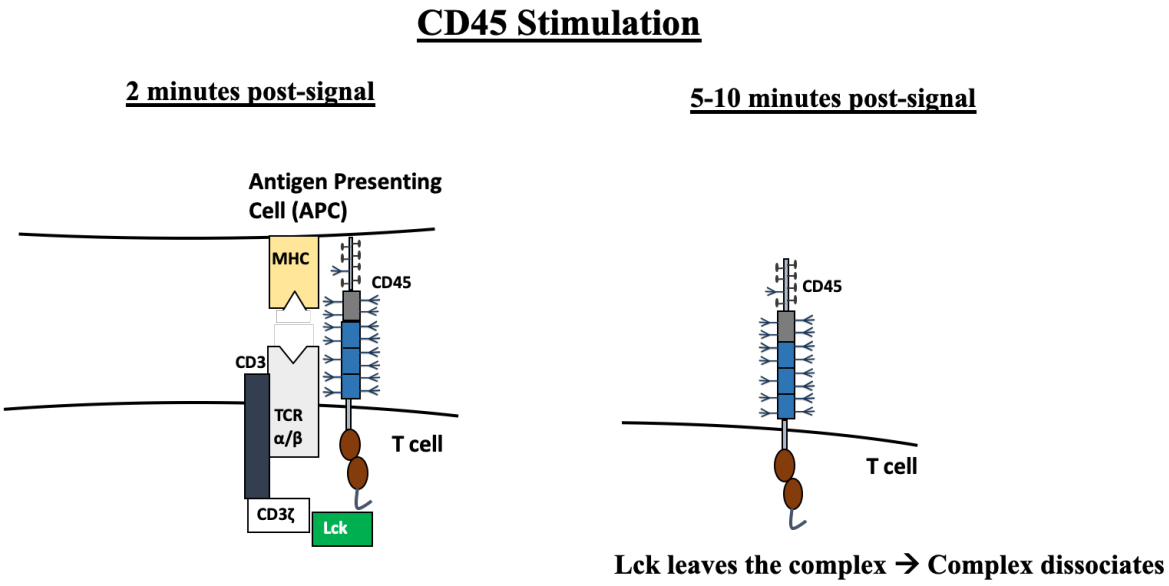


Fig. 3.31.

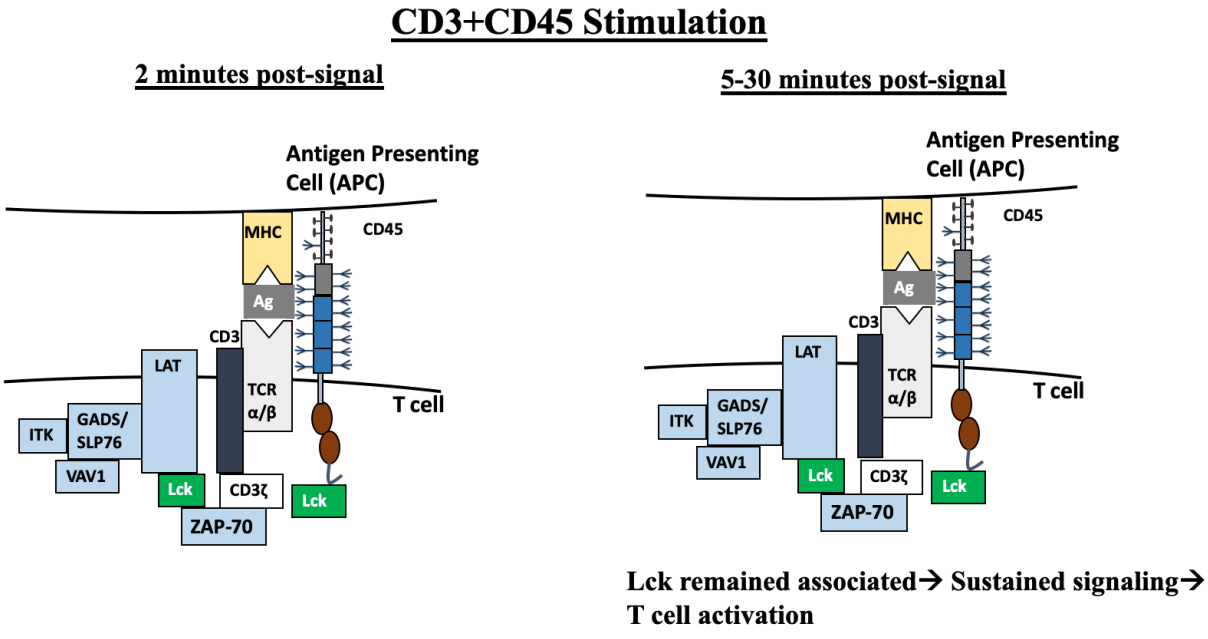


Fig. 3.32.

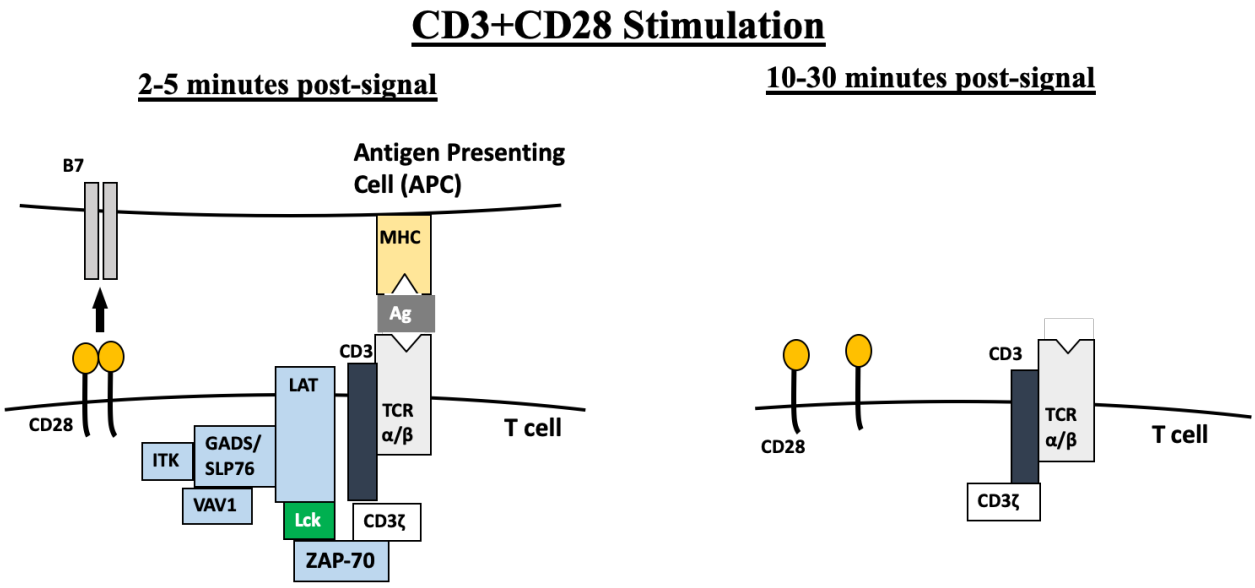
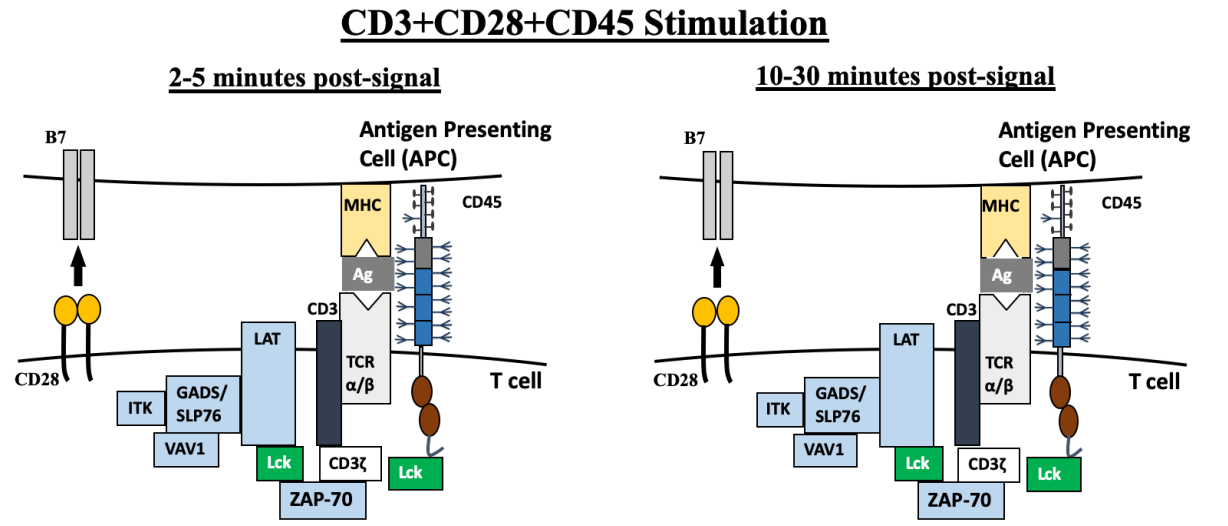


Fig. 3.33.



Lck remained associated→ Sustained signaling→ T cell activation

Figure 3.29-3.33. Model for Lck association with CD45 and TCR/CD3 complex.

Lck is associated with CD45 and CD3 in a resting T cell (**Fig. 3.29**). **Fig. 3.30**. Upon CD45 engagement, Lck activates and associates with CD45 and TCR/CD3 for the first 2 minutes and then the complex dissociates. **Fig. 3.32**. Upon stimulation through CD3+CD28, Lck activates and remains associated with TCR/CD3 for 2-5 minutes and then leaves the complex. **Fig.3.31** engagement of CD3+CD45 and **Fig.3.33** engagement of CD45 along with CD3+CD28 prevents Lck from leaving the complex.

dissociates within 5-10 minutes, indicating that signaling upon CD45 engagement alone is not sufficient for downstream signaling required for T cell activation. Similarly, upon CD3+CD28 stimulation, within 2-5 minutes, Lck is activated and associated with TCR/CD3 complex with other proteins required for T cell activation. However, the complex gets dissociated within 10-30 minutes of stimulation (**Fig. 3.32**). This suggests that if cognate antigen is recognized by TCR in the first 2-5 minutes, the T cell will receive signal 1 and 2 and will provide downstream signals for T cells activation and proliferate. Upon CD3+CD45 and CD3+CD45+CD28 engagement **(Fig. 3.31 and Fig. 3.33)**, within 2-5 minutes, Lck is activated and associated with TCR/CD3 along with other proteins required for activation, and the signaling is sustained for 10-30min as Lck remains associated. As CD3/TCR is the first signal during T cell activation and signaling, it seems to play a crucial role during CD45 engagement as well, as CD3/TCR signaling prevents Lck from leaving the CD45 complex. CD45 engagement forces Lck to remain associated with CD3, even in the presence of a full CD3+CD28 co-stimulation. Hence, Lck association is dependent upon TCR/CD3 stimulation and CD45 engagement, thus promoting sustained T cell signaling. We conclude that CD45 altered early signaling events in T cells induced upon co-stimulation through particular costimulatory protein, CD28.

Originally, we wanted to compare the two different co-stimulations in this project to study the differences in signaling induced by two co-stimulatory molecules- CD28 and ICAM-1. But we were limited by the choice of cell line that we used in the study. Jurkat, the T cell line we used in this study does not express ICAM-1. Another T cell line we tested - Molt-3 does not express CD28. So, for this project we just focused on one co-stimulatory molecule CD28.

Future studies will investigate if CD45 alters early signaling events induced by another co-stimulatory molecule that the lab studies, ICAM-1. Comparing the signaling events induced by

CD45 engagement + the two costimulatory molecules CD28 and ICAM-1 would be interesting and may be suggestive of beginning mechanisms of T cell differentiation. We intend to learn whether the early events in T cell signaling influence the different effects on naïve T cell differentiation of co-stimulation through ICAM-1 or CD28. If true, then the differentiation differences begin at the earliest steps of T cell activation. While it has been extensively studied that CD45 is essential for T cell activation, the role of CD45 in signaling has not been fully explored. Determining the role of CD45 in signaling could explain molecular mechanism for T cell differentiation.

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Chapter 4

The influence of statins on T cell proliferation, activation, and differentiation is dependent on the microenvironment.

Introduction

Statins

Statins are lipid-lowering drugs, used in the treatment of cardiovascular diseases. Statins inhibit cholesterol synthesis by inhibiting HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase enzyme. HMG-CoA reductase is involved in the early steps of the cholesterol synthesis pathway by converting HMG-CoA to mevalonate [1]. Mevalonate is a precursor for cholesterol and an essential metabolite in the formation of isoprene. Isoprene is essential for prenylation of numerous signaling molecules. The cholesterol synthesis pathway is demonstrated in **Fig. 4.1** and shows the inhibition of cholesterol biosynthesis by statins [1]. All statins share a common moiety, which is structurally similar to HMG- Co A and is present in an inactive lactone form [2]. In vivo, the pro-drugs are enzymatically hydrolyzed to active hydroxyl-acid forms that competitively bind HMG-Co A reductase and displace the substrate HMG-Co A. This inhibits catalytic conversion of HMG-Co A to L- mevalonate [2] and ultimately inhibits cholesterol synthesis. Generally, statins are divided into two types- naturally occurring compounds derived from fungal fermentation, which are type 1 and synthetic compounds, which are type 2. Type 1 includes mevastatin, lovastatin, simvastatin and pravastatin and type 2 includes fluvastatin and atorvastatin [2]. **Fig. 4.2** demonstrates structures of HMG-Co A and two statins- Pravastatin and Atorvastatin [2].

Immunomodulatory effect of statins

Besides lowering lipids, statins exert anti-inflammatory and immunomodulatory effects. Statins inhibit IFN-γ induced expression of MHC II molecules and inhibit MHC II mediated T cell activation [3]. Atorvastatin, lovastatin and simvastatin treatment promote increased frequency of

Fig. 4.1.

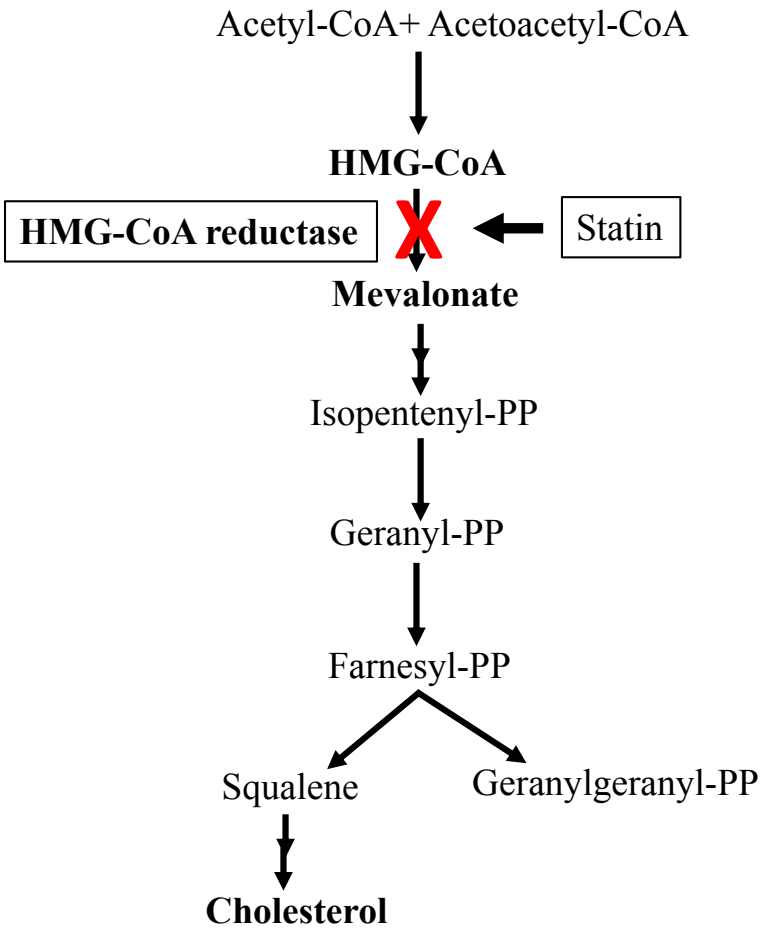
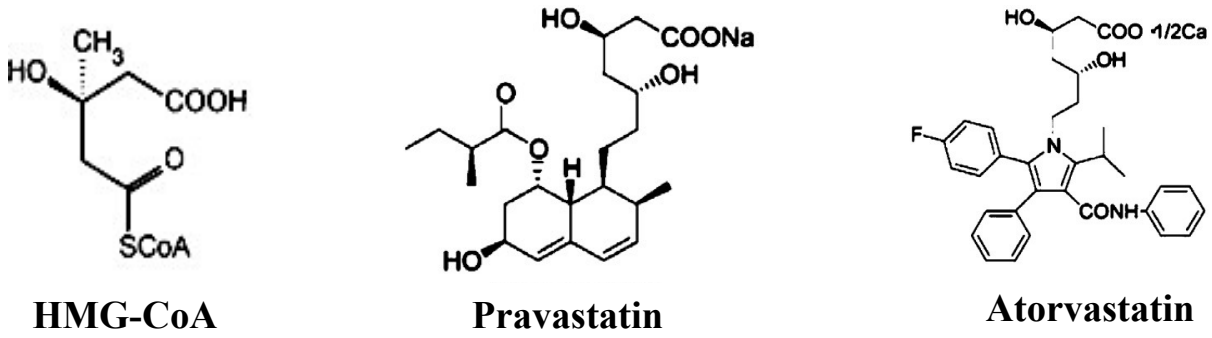

Acetyl-CoA+ Acetoacetyl-CoA
HMG-CoA
HMG-CoA reductase
Statin
Mevalonate
Isopentenyl-PP
Geranyl-PP
Farnesyl-PP
Squalene
Geranylgeranyl-PP
Cholesterol

Figure 4.1. Cholesterol biosynthetic pathway.

The cholesterol biosynthesis pathway is also called the mevalonate pathway which leads to cholesterol synthesis as well as several isoprenes that are essential for prenylation of numerous signaling molecules. Statins share a common moiety that is structurally similar to HMG- Co A. Statins competitively bind to HMG-Co A reductase and displace the substrate HMG-Co A, thus inhibiting cholesterol biosynthesis and several isoprenes.

Fig. 4.2.



HMG-CoA **Pravastatin** **Atorvastatin**

Figure. 4.2. Structural formula of HMG-Co A molecule and statins.

Pravastatin is type 1 natural statin and Atorvastatin is type 2 synthetic statin.

regulatory T cells (Treg) in healthy individuals, patients with acute coronary syndrome and atherosclerotic plaques in mice [4-7]. Simvastatin promotes Th2 phenotype as indicated by increased IL-4 mRNA and protein expression and decreased levels of IL-1, IL-17 and IFN-γ in atherosclerotic plaques in mice *in vivo* [7].

As mentioned in earlier chapters, our lab has studied the influence of microenvironment on the differentiation outcomes of T cells. The microenvironmental influence includes the co-stimulatory molecule interaction with its counter receptor on the antigen presenting cell in the local microenvironment during cell to cell contact. Our lab has shown that co-stimulation through different costimulatory molecules leads to different differentiation phenotypes [8-10]. Briefly, we have shown that, in the absence of exogenous cytokines, stimulation through CD3+CD28 induces differentiation to Th1 and Th2 effector and memory cells but not Treg cells, while stimulation through CD3+ICAM-1 induces differentiation to Th1 effector and memory cells and Treg but not Th2 cells [8-10]. Co-stimulation through CD3+CTLA4 induces differentiation to Treg cells but not Th1 or Th2 [11]. Besides the co-stimulatory molecule: counter receptor interaction during cell to cell contact, our lab has also studied the influence of naturally occurring soluble compounds such as HDL and LDL on T cell differentiation. Differentiation in the presence of the lipoprotein oxidized LDL favors differentiation to Th1 cells [12]. These studies support that naïve T cell differentiation is influenced by the different microenvironment in which the T cell finds itself. In this project, we wanted to investigate the effect of statins on T cell differentiation and survival. The main goal of the project was to analyze the effect of statins on T cell proliferation, activation, viability and differentiation in association with co-stimulation through different co-stimulatory molecules.

Hypothesis tested

Statins inhibit cholesterol synthesis, resulting in a significant reduction in cholesterol levels and preventing cardiovascular disease. We were curious to know if the survival and differentiation of human T cells upon statin treatment, is influenced by the microenvironment, i.e., different co-stimulatory molecules. We chose one of each type 1 and type 2 statins for the study. We used pravastatin for type 1 and atorvastatin for type 2 statin. We studied the effect of statin treatment on total T cells as well as naïve $CD4^+$ T cells during co-stimulation through CD28 or ICAM-1. We hypothesized that statins influence T cell activation, proliferation and differentiation and these effects are dependent on the microenvironment.

Materials and methods

Antibodies and reagents

Anti-CD3 (clone OKT3) was purchased from eBioscience (San Diego, CA), anti-CD28 (clone CD28.2) was purchased from BioLegend (San Diego, CA), and anti-ICAM-1 (clone R6.5) was purchased from BioXCell (West Lebanon, NH). Pravastatin and atorvastatin were purchased from EMD Millipore (Burlington, MA). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Carlsbad, CA), Annexin V-PE and 7-Amino-Actinomycin D (7AAD) were purchased from BD Pharmingen (San Jose, CA). Anti-CD11a-FITC (clone MEM-25) was purchased from Novus Biologicals (Littleton, CO), anti-CD25-APC (clone MA251), anti-CD27-PE (clone O323) and anti-CD45RO-APC (clone UCHL1) was purchased from Biolegend (San Diego, CA). Anti-FoxP3-PE (clone PCH101) and transcription factor staining buffer set were purchased from eBiosciences (San Diego, CA). Flow cytometry was performed with Accuri C6 (BD Accuri Cytometers, Ann Arbor, MI) and data analysis was done using CFlow software.

Human Subjects

Human peripheral T cells and naïve $CD4^+$ T cells were isolated from blood of healthy volunteers after informed consent and approval by the University of Kansas Institutional Review Board

Cell purification

Peripheral blood was diluted 1:1 in sterile TC-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM glucose, pH 7.4) + 2%

FBS (Atlanta Biologicals, Atlanta, GA) + 1% penicillin/streptomycin (Gibco), and mononuclear cell layer was separated using Ficoll density gradient centrifugation for 30 minutes at 1800 rpm. The PBMC (peripheral blood mononuclear cell) layer was collected and washed in TC-PBS to remove residual Ficoll. Total T cells were isolated by E-rosetting, in which PBMC suspension was mixed with 10% sheep red blood cells at a ratio of 10:1, incubated for 10 minutes at 37°C, centifuged for 10 minutes at 2000 rpm, incubated on ice for 30 to 60 minutes and separated using Ficoll density gradient centrifugation for 30 minutes at 1800 rpm. Purity was immediately assessed for each isolation by staining for CD3 and gating on $CD3^+$ population. Purity for total T cells was greater than 90% as assessed by flow cytometry.

For naïve $CD4^+$ T cells, whole blood was diluted 1:1 in sterile TC-PBS + 2% FBS + 1% penicillin/streptomycin and centrifuged for 30 minutes at 1800 rpm using Ficoll density gradient centrifugation and the PBMC layer was collected. Naïve T cells were negatively selected using the Human Naïve $CD4^+$ T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Naïve $CD4^+$ T cells were defined as $CD45RO^{(-)}$ $CD11a^{lo}$ $CD27^+$ and the purity of the naïve $CD4^+$ T cells were greater than 98% as assessed by flow cytometry.

After isolation, cells were resuspended in complete RPMI 1640 (CellGro, Corning, New York), containing 10% FBS, 50 IU/ml penicillin/50 μg/ml streptomycin, and 2 mM L-glutamine (Gibco) and incubated overnight at 37°C with 5% CO_2.

Cell stimulation

Human peripheral T cells or naïve $CD4^+$ T cells were stimulated by plate-bound antibodies as described previously [9]. Each stimulating antibody was titrated to the lowest effective

concentration and added to tissue culture-treated plates (TPP, 96-well plate, Switzerland). Anti-CD3 (OKT3) was used at 1 μg/ml, anti-CD28 (CD28.2) was used at 2 μg/ml and anti-ICAM-1 (R6.5) was used at 10 μg/ml. Stimulating antibodies were incubated at 37^0C for 2 hours or overnight at 4^0C and washed three times with PBS to remove unbound antibody. T cells were added to each well at 1.5 x10^6 cells/ml in 200 μl complete RPMI 1640. Pravastatin and atorvastatin were dissolved in appropriate diluent as per the manufacturer's instructions and added to the indicated wells at 55 ng/ml for pravastatin and 65 ng/ml for atorvastatin as described previously [13]. The plates were then incubated for 7-10 days at 37°C with 5% CO_2.

Flow cytometry

Cell proliferation was measured by CFSE dilution as previously described [14]. Cells were stained with 2.5 μ M CFSE for 7 minutes at 37°C in serum-free RPMI 1640 prior to stimulation. After 7-10 days of stimulation, cells were stained to analyze cell death, and differentiation to effector/memory or Treg subset. Cells were removed from the tissue culture plate by gentle pipetting. For evaluation of cell death, cells were were washed in cold PBS, stained in Annexin V binding buffer (0.01 M HEPES pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) with Annexin V-PE and 7AAD, washed in Annexin V binding buffer and analyzed by Accuri C6 flow cytometer with CFlow software.

For evaluation of T cell differentiation to effector/memory phenotype, cells were blocked with 0.5% BSA in PBS, stained with CD11a-FITC, CD27-PE, and CD45RO-APC for 15 minutes on ice in the dark, washed in cold PBS and analyzed by flow cytometry. For analyzing Treg differentiation, cells were stained with anti-CD25-APC and anti-FoxP3-PE. Cells were blocked with 0.5% BSA in PBS, stained for surface target CD25, fixed and permeabilized using

eBioscience transcription factor staining buffer kit and stained for transcription factor, FoxP3. Stained cells were then analyzed immediately by flow cytometry. Single color staining controls were used for compensation and gating.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Statistical tests used and significance are indicated in figure legends.

Results *(Preliminary data)*

Pravastatin and atorvastatin did not affect cell viability during CD28 or ICAM-1 co-stimulation of T cells.

At first, we examined if statin treatment affected the viability of T cells. For that, cell death was examined by Annexin V and 7-aminoactinomycin D (7AAD) staining. Annexin V is tagged to a fluorophore PE and binds to the phospholipid phosphatidylserine. In a healthy cell, phosphatidylserine is found only on the inner surface of the cell membrane but in an apoptotic cell, phosphatidylserine is exposed on the outer surface. Annexin V only binds to the cell that is apoptotic. 7AAD is a fluorescent molecule that binds nucleic acids. 7AAD does not pass through cell membranes in healthy cells. In dead or dying cells, the cells have lost membrane integrity, exposing nucleic acids that bind to 7AAD. The dead cells are positive for both Annexin V and 7AAD, while viable cells are double negative.

We stimulated the cells through CD3+CD28 or CD3+ICAM-1 in the absence (stimulation only control) or presence of two different statins- pravastatin and atorvastatin for 7 days and stained the cells using Annexin V and 7AAD and analyzed the cells by flow cytometry. **Fig. 4.3** and **Fig. 4.5** represent cell death for $CD4^+$ T cells (top row) and $CD4^-$ T cells (bottom row) during CD3+CD28 or CD3+ICAM-1 stimulation respectively, in the absence (control) or presence of pravastatin and atorvastatin. The upper right quadrant represents dead cells. Upon co-stimulation through the two co-stimulatory molecules, the number of dead cells (Annexin V^+ $7AAD^+$) with pravastatin and atorvastatin treatment was not different from the number of dead cells in stimulation only control for both $CD4^+$ and $CD4^-$ T cells (**Fig. 4.3-4.6)**. It should be noted that this result is representative of only two independent experiments.

Fig. 4.3.

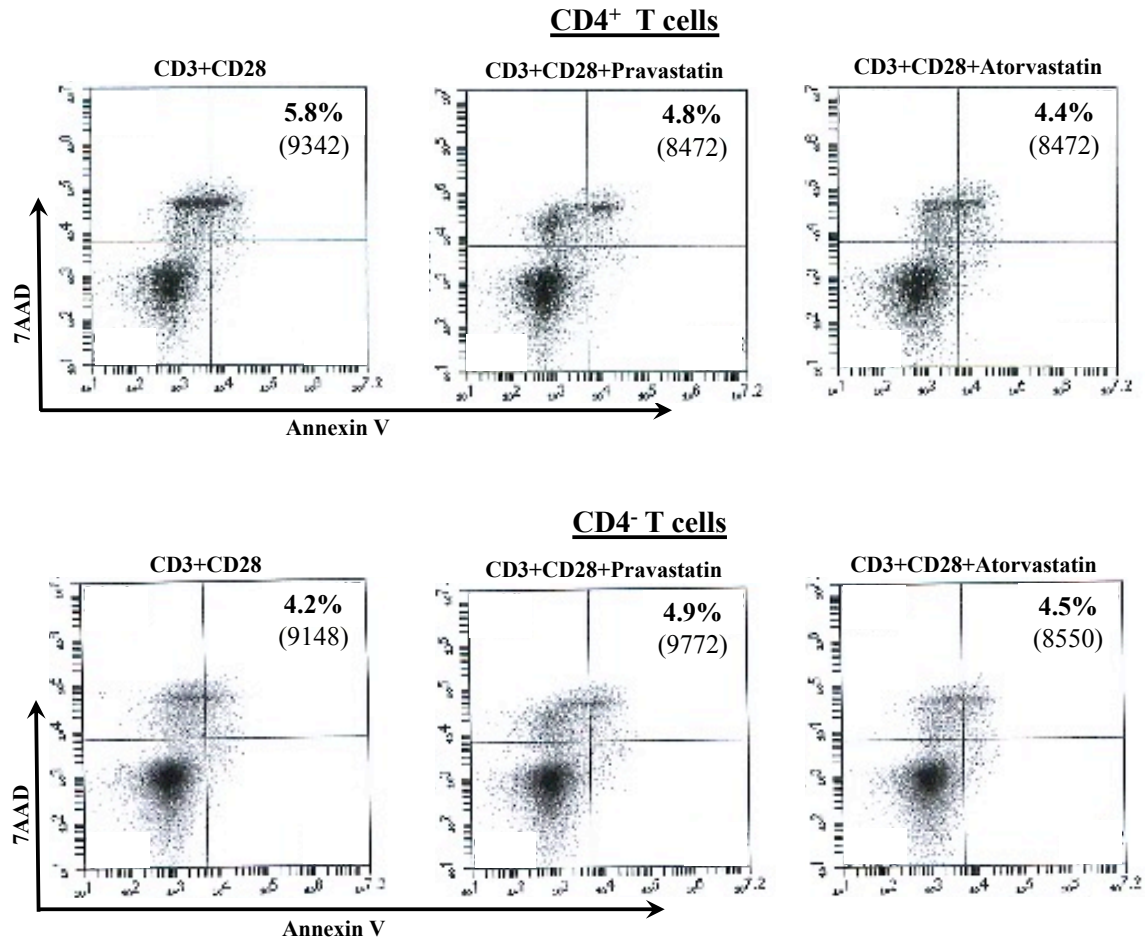

CD4+ T cells
CD3+CD28
5.8%
(9342)
CD3+CD28+Pravastatin
4.8%
(8472)
CD3+CD28+Atorvastatin
4.4%
(8472)
7AAD
Annexin V
CD4- T cells
CD3+CD28
4.2%
(9148)
CD3+CD28+Pravastatin
4.9%
(9772)
CD3+CD28+Atorvastatin
4.5%
(8550)
7AAD
Annexin V

Fig. 4.4.

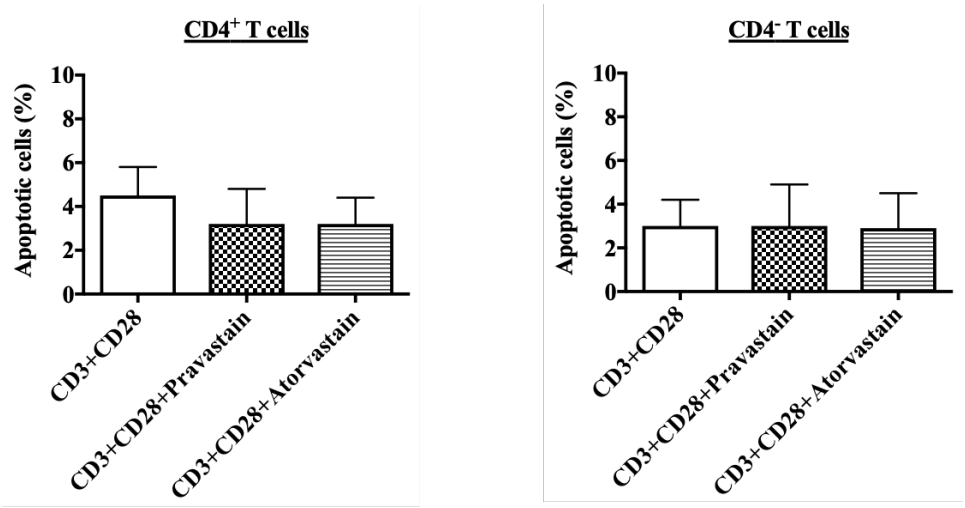

CD4+ T cells
Apoptotic cells (%)
10
8
6
4
2
0
CD3+CD28
CD3+CD28+Pravastain
CD3+CD28+Atorvastain
CD4- T cells
Apoptotic cells (%)
10
8
6
4
2
0
CD3+CD28
CD3+CD28+Pravastain
CD3+CD28+Atorvastain

Fig. 4.5.

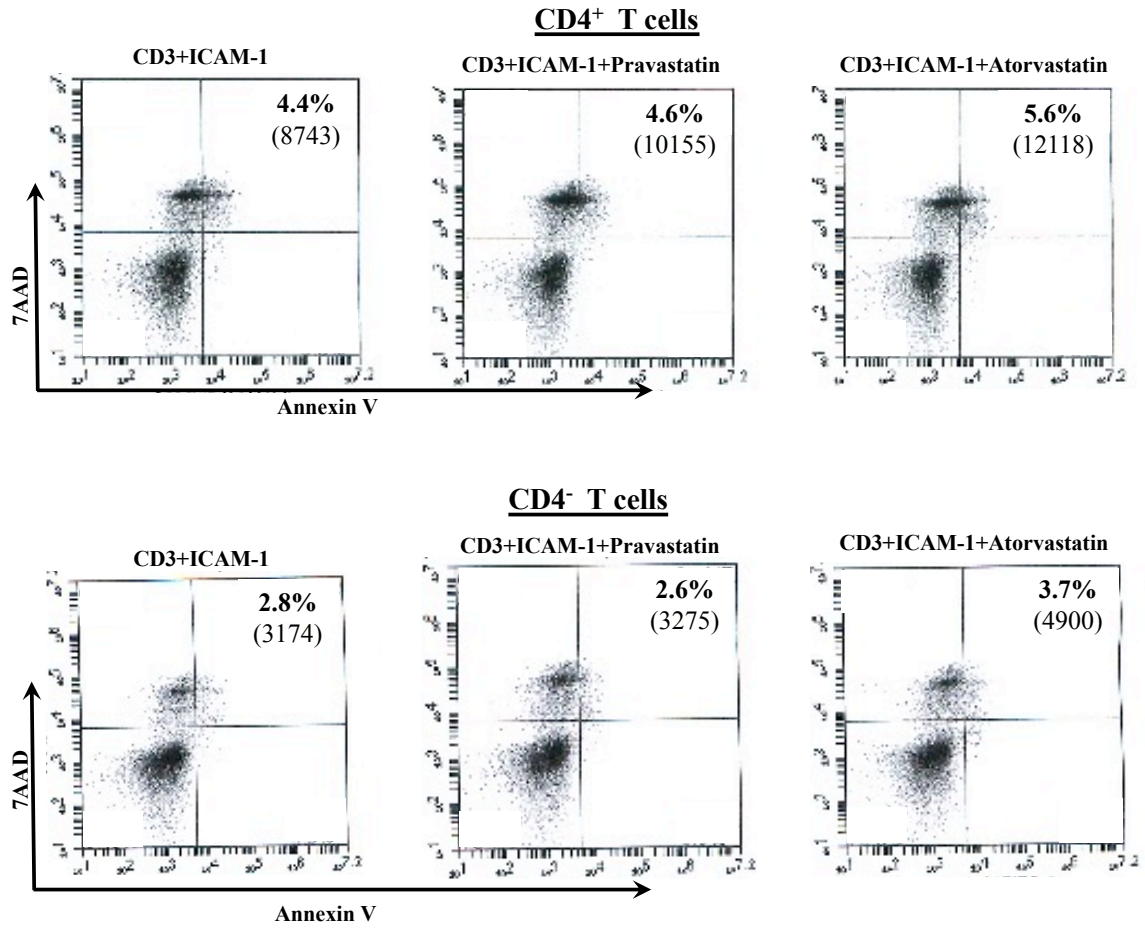
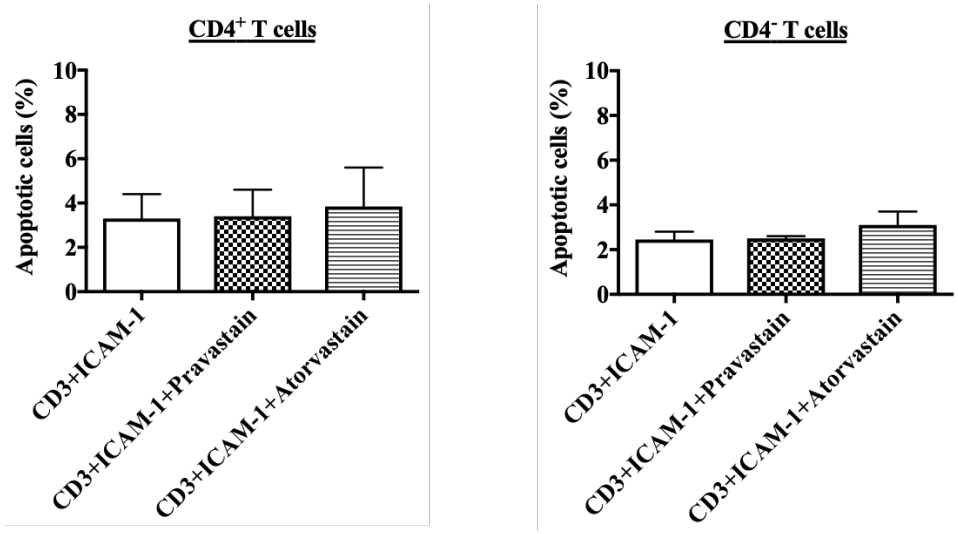

CD4+ T cells
CD3+ICAM-1
CD3+ICAM-1+Pravastatin
CD3+ICAM-1+Atorvastatin
4.4%
(8743)
4.6%
(10155)
5.6%
(12118)
7AAD
Annexin V
CD4- T cells
CD3+ICAM-1
CD3+ICAM-1+Pravastatin
CD3+ICAM-1+Atorvastatin
2.8%
(3174)
2.6%
(3275)
3.7%
(4900)
7AAD
Annexin V

Fig. 4.6.


CD4+ T cells
Apoptotic cells (%)
10
8
6
4
2
0
CD3+ICAM-1
CD3+ICAM-1+Pravastain
CD3+ICAM-1+Atorvastain
CD4- T cells
Apoptotic cells (%)
10
8
6
4
2
0
CD3+ICAM-1
CD3+ICAM-1+Pravastain
CD3+ICAM-1+Atorvastain

Figures 4.3-4.6. Pravastatin and atorvastatin did not affect cell viability during CD28 or ICAM-1 co-stimulation of T cells.

Primary T cells were stimulated through CD3+CD28 (**Fig. 4.3-4.4)** or CD3+ICAM-1 **(Fig. 4.5-4.6)** in the absence (stimulation alone control) or presence of pravastatin or atorvastatin for 7 days and stained with Annexin V and 7AAD and analyzed by flow cytometry. **Fig. 4.3 and Fig. 4.5:** Representative dot plots showing Annexin V/7AAD stain with apoptotic cells in upper right quadrant. Percent of apoptotic cells (Annexin V^+ $7AAD^+$) in the upper right quadrant given in bold and number of apoptotic cells in parentheses. **Fig. 4.4 and Fig. 4.6:** Percent of apoptotic cells after 7 days of co-stimulation with error bars indicating standard error. Representative of two independent experiments.

Pravastatin treatment promoted increased T cell activation upon stimulation through CD3+CD28 but decreased T cell activation upon stimulation through CD3+ICAM-1.

We analyzed T cell activation upon co-stimulation through CD28 or ICAM-1 in the absence (control) or presence of pravastatin and atorvastatin. After 7 days of stimulation, cells were stained with CD25 (IL-2Rα), which is considered a classic T cell activation marker. **Fig. 4.7** represents a dot plot for CD28 co-stimulation, in which the upper right quadrant represents activated $CD4^+$ T cells and lower right quadrant activated $CD4^-$ T cells. These preliminary data suggested that upon stimulation through CD3+CD28, pravastatin treatment promoted increased T cell activation for both $CD4^+$ and $CD4^-$ T cells, while atorvastatin treatment did not (**Fig. 4.7-4.8).** **Fig. 4.9** represents a dot plot for ICAM-1 co-stimulation. Upon stimulation through CD3+ICAM-1, both pravastatin and atorvastatin treatment induced decrease T cell activation for both $CD4^+$ and $CD4^-$ T cells (**Fig. 4.9-4.10).** Hence, T cell activation during statin treatment is dependent on the choice of co-stimulatory molecule.

Pravastatin treatment promoted increased proliferation of T cells upon CD3+CD28 stimulation but not upon CD3+ICAM-1 stimulation.

We examined the effect of statin treatment on T cell proliferation by staining cells with CFSE at day 0 and then treating the cells with statins and stimulating the cells through CD3+CD28 or CD3+ICAM-1 for 7 days. CFSE is a dye that readily passes through the cell membrane and is crosslinked to intracellular proteins. It is fluorescent when cleaved by the cell's esterase. The dye levels are divided in half each time the cell divides, and fluorescence is measured by flow cytometry. **Fig. 4.11** represents a CFSE dot plot for CD28 co-stimulation, in which the upper left quadrant represents $CD4^+$ T cells and lower left quadrant represents activated $CD4^-$ T cells. Upon

Fig. 4.7.

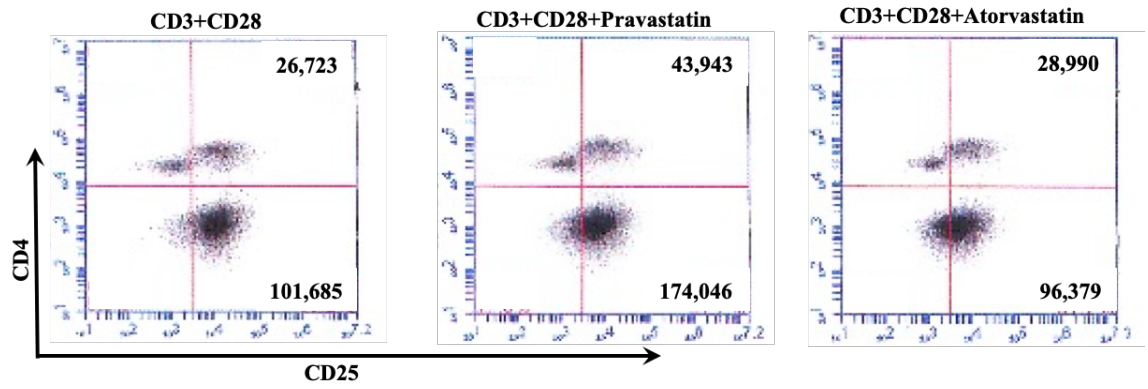

CD3+CD28
CD3+CD28+Pravastatin
CD3+CD28+Atorvastatin
26,723
101,685
43,943
174,046
28,990
96,379
CD4
CD25

Fig. 4.8.

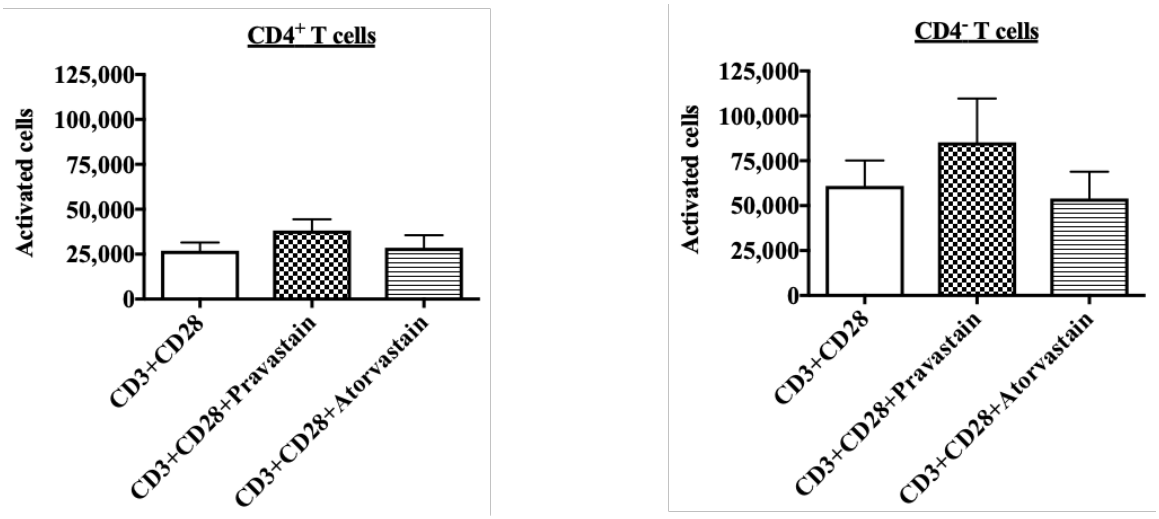

CD4+ T cells
Activated cells
125,000
100,000
75,000
50,000
25,000
0
CD3+CD28
CD3+CD28+Pravastain
CD3+CD28+Atorvastain
CD4- T cells
Activated cells
125,000
100,000
75,000
50,000
25,000
0
CD3+CD28
CD3+CD28+Pravastain
CD3+CD28+Atorvastain

Fig. 4.9.

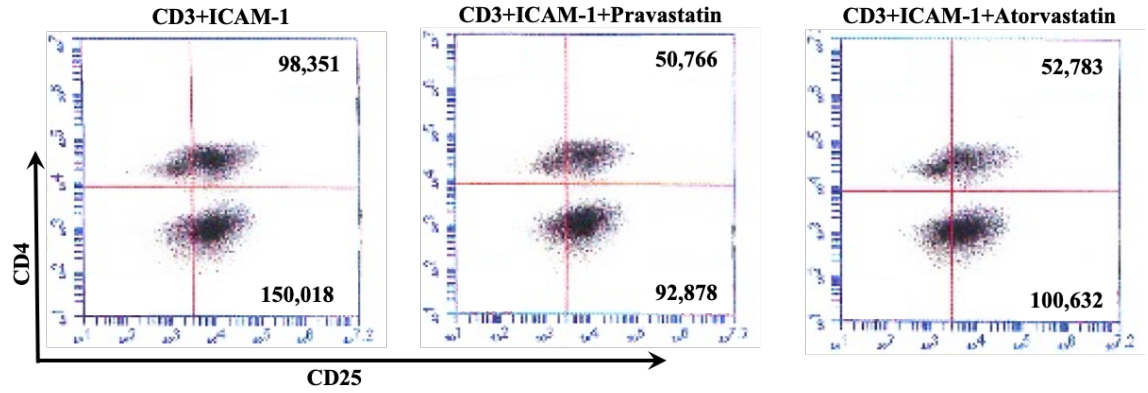
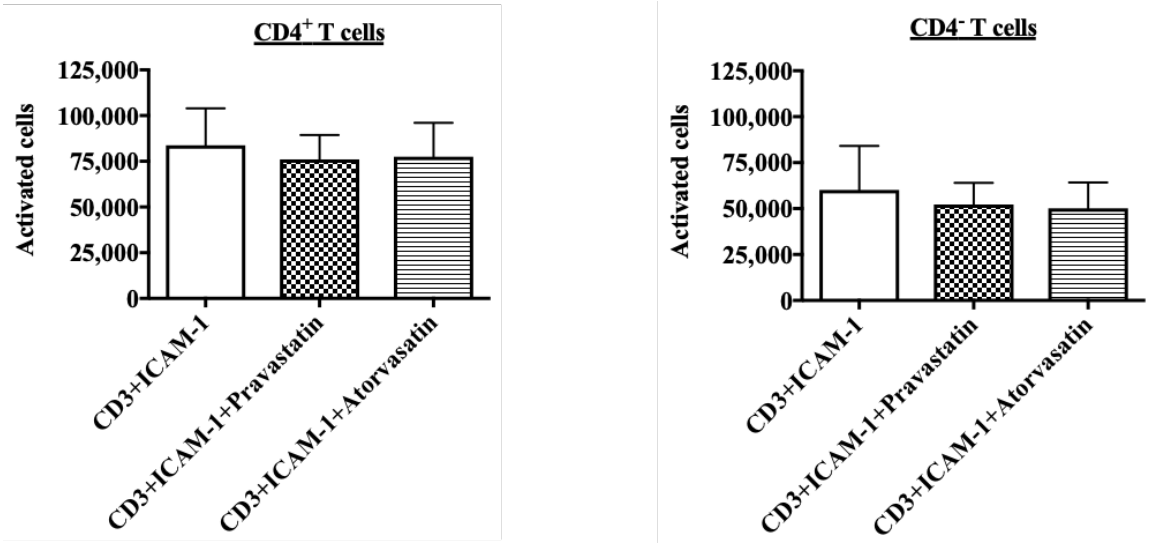


Fig. 4.10.



Figures 4.7-4.10. Pravastatin treatment promoted increased T cell activation upon stimulation through CD3+CD28 but decreased T cell activation upon stimulation through CD3+ICAM-1.

Primary T cells were stimulated through CD3+CD28 (**Fig. 4.7-4.8**) or CD3+ICAM-1 (**Fig. 4.9-4.10**) in the absence (stimulation alone control) or presence of pravastatin or atorvastatin for 7 days and stained with CD25 and analyzed by flow cytometry. **Fig. 4.7 and Fig. 4.9**: Representative dot plots showing $CD4^+$ activated cells in upper right quadrant and $CD4^-$ activated cells in the lower right quadrant. Number of $CD4^+$ activated cells is given in the upper right quadrant and number of $CD4^-$ activated cells is given in the lower right quadrant. **Fig. 4.8 and Fig. 4.10**: Number of activated cells after 7 days of co-stimulation with error bars indicating standard error. Representative of five independent experiments.

CD3+CD28 stimulation, pravastatin induced increased cell proliferation for both $CD4^+$ and $CD4^-$ T cells while atorvastatin did not (**Fig. 4.11-4.12)**. **Fig. 4.13** represents a dot plot for ICAM-1 co-stimulation. Both pravastatin and atorvastatin treatment decreased T cell proliferation for both $CD4^+$ and $CD4^-$ T cells upon stimulation through CD3+ICAM-1 (**Fig. 4.13-4.14).** In conclusion, we observed that T cell proliferation was promoted by CD28 co-stimulation but not by ICAM-1 co-stimulation upon statin treatment. This indicated that choice of co-stimulatory molecule influenced T cell proliferation during statin treatment.

Pravastatin treatment increased effector and memory cell differentiation upon stimulation through CD3+CD28 and may reduce effector and memory cell differentiation upon stimulation through CD3+ICAM-1.

In the past, our lab has shown that co-stimulation through CD3+CD28 or CD3+ICAM-1 promote T cell differentiation to effector and memory phenotype [9]. To study the effect of statin treatment on T cell differentiation, we analyzed the differentiation to effector and memory phenotype in the presence of statins. The cells were treated with statins and co-stimulated through CD3+CD28 or CD3+ICAM-1 for 7 days and analyzed by flow cytometry. The cells were gated on $CD4^+$ $CD45RO^+$ population to remove any undifferentiated cells from the analysis. The effector cells are $CD45RO^+CD11a^{hi}CD27^{hi}$ and the memory cells are $CD45RO^+CD11a^{hi}CD27^{lo}$. **Fig. 4.15** is a representative dot plot for CD3+CD28 stimulation, showing effector cells in the upper right quadrant and memory cells in the lower right quadrant. We observed that both pravastatin and atorvastatin treatment induced increased effector and memory cell differentiation (**Fig. 4.15). Fig. 4.16** represents a summary bar graph for effector (left) and memory (right) cells. The increase in effectors cells upon pravastatin treatment was statistically significant (**Fig. 4.16,** $p<0.05$ by paired

Fig. 4.11.

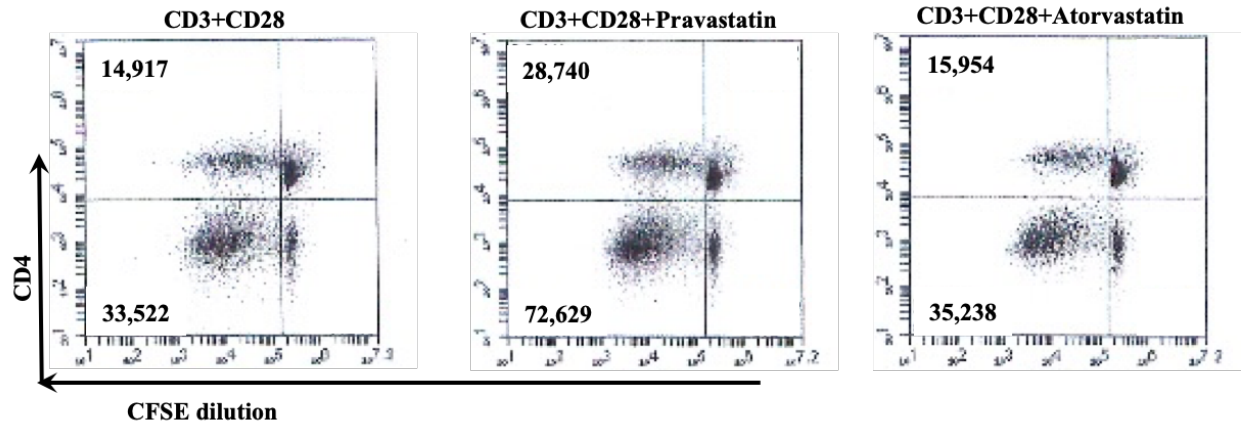
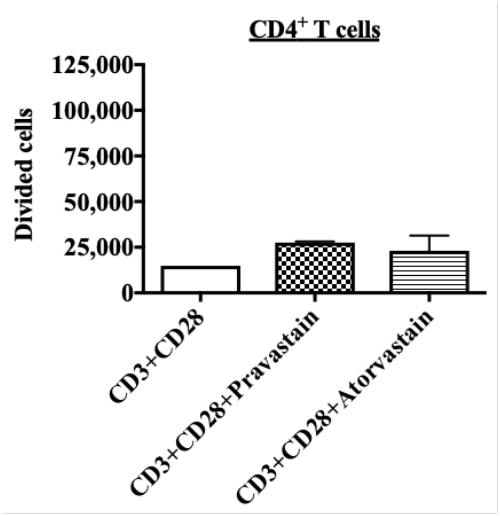


Fig. 4.12.



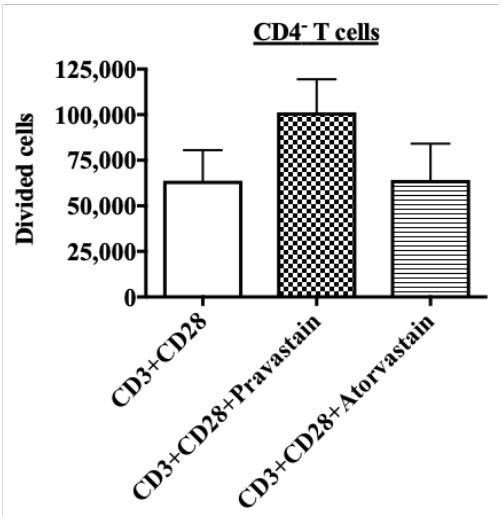
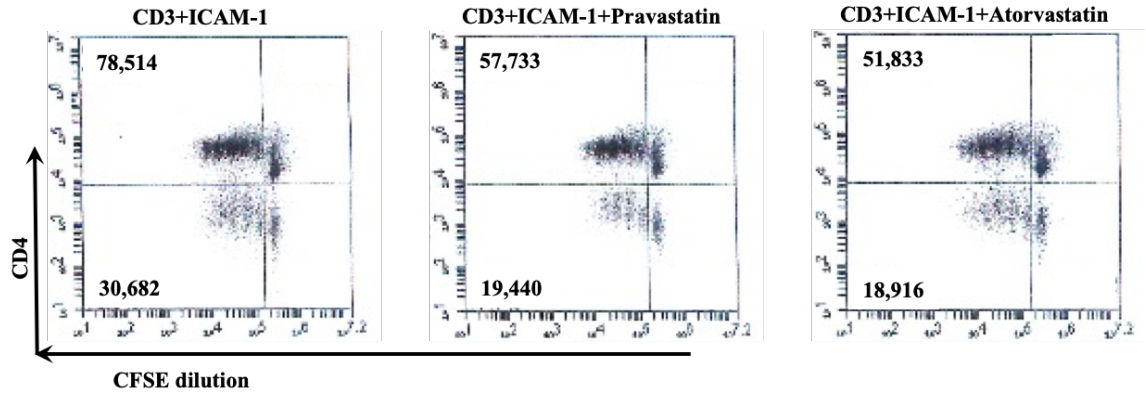


Fig. 4.13.



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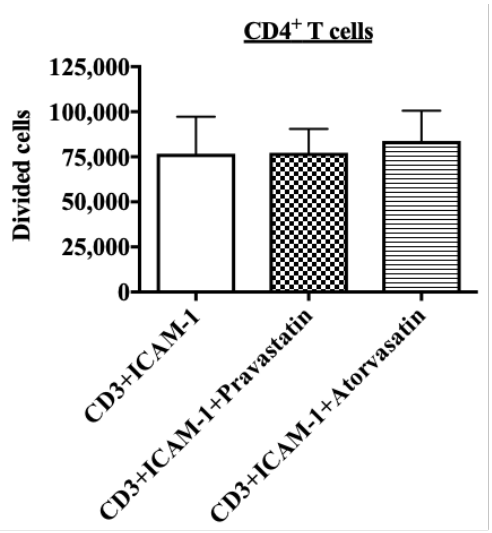
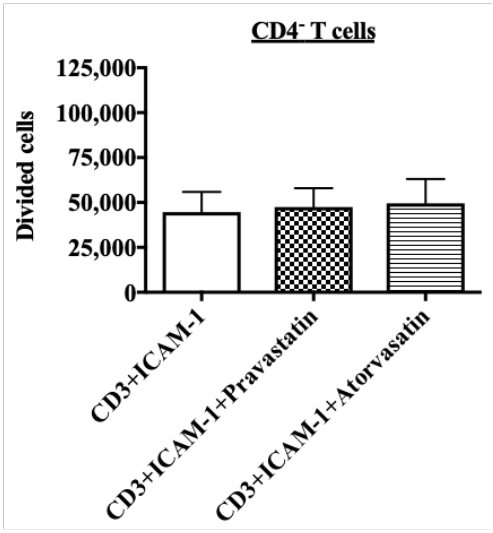




Figures 4.11-4.14. Pravastatin treatment promoted increased proliferation of T cells upon CD3+CD28 stimulation but not upon CD3+ICAM-1 stimulation.

Primary T cells were stained with CFSE at day 0 and then stimulated through CD3+CD28 (**Fig. 4.11-4.12)** or CD3+ICAM-1 **(Fig. 4.13-4.14)** in the absence (stimulation alone control) or presence of pravastatin or atorvastatin for 7 days and analyzed by flow cytometry. **Fig. 4.11 and Fig. 4.13:** Representative dot plots showing $CD4^+$ divided cells in upper left quadrant and $CD4^-$ divided cells in the lower left quadrant. Number of $CD4^+$ divided cells is given in the upper left quadrant and number of $CD4^-$ divided cells is given in the lower left quadrant. **Fig. 4.12 and Fig. 4.14:** Number of divided cells after 7 days of co-stimulation with error bars indicating standard error. Representative of four independent experiments.

two-tailed t test**). Fig. 4.17** is a representative dot plot for CD3+ICAM-1 stimulation, with effector cells in the upper right quadrant and memory cells in the lower right quadrant. Pravastatin treatment induced a decrease in effector and memory cells upon CD3+ICAM-1 stimulation (**Fig. 4.17-4.18).** Atorvastatin treatment also induced a decrease in effector cells but had no effect on the memory cell population during CD3+ICAM-1 stimulation (**Fig. 4.17-4.18).** It should be noted that the decrease was not statistically significant.

Pravastatin treatment increased effector and memory cell differentiation of naïve CD4$^+$T cells upon stimulation through CD3+CD28 but not through CD3+ICAM-1 stimulation.

Total T cell populations are comprised of diverse mature CD4$^+$ T cell subsets and CD8$^+$ T cells. Using total T cells requires more than 3 to 5 repetitive experiments due to inter-human differences and cell complexity. So, we wanted to look at a specific T cell population which we could isolate, and we decided to look at the effect of statin treatment on naïve CD4$^+$ T cells, which seemed to perform in a homogenous manner. We observed that pravastatin treatment induced an increase in effector and memory cells upon CD3+CD28 stimulation (**Fig. 4.19-4.20).** Atorvastatin treatment also induced an increase in effector cells but had no effect on the memory cell population during CD3+CD28 stimulation (**Fig. 4.19-4.20).** It should be noted that the increase was not statistically significant. For CD3+ICAM-1 stimulation, both pravastatin and atorvastatin had no effect on effector and memory T cell populations (**Fig. 4.21-4.22)**. It should be noted that this result is representative of only two independent experiments.

Fig. 4.15.

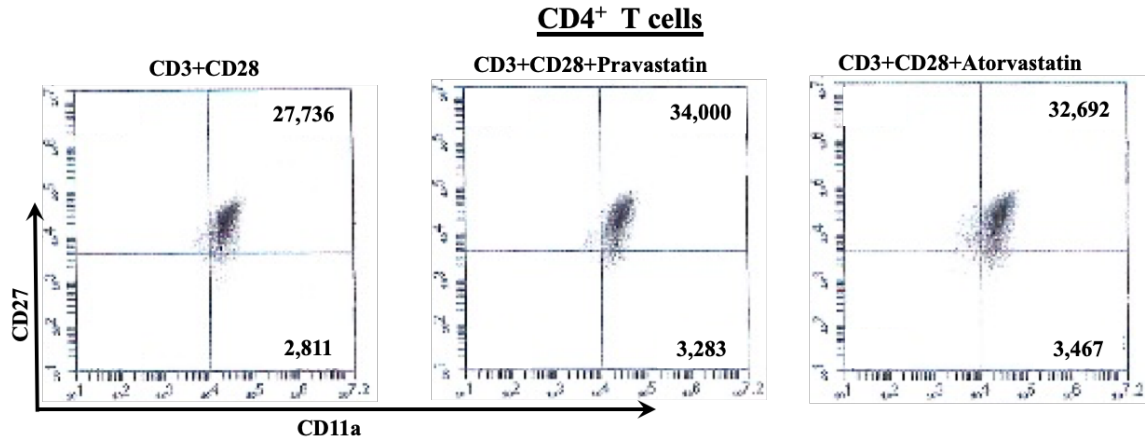
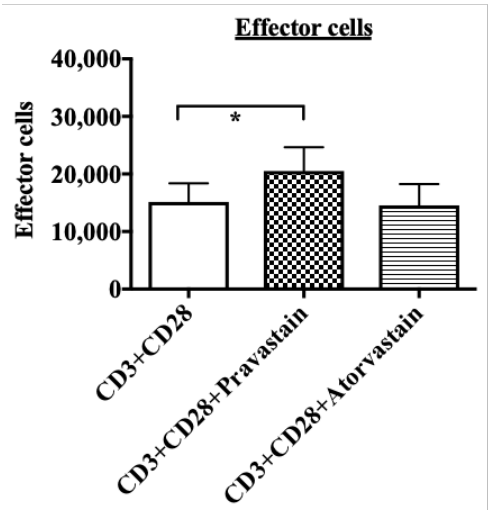


Fig. 4.16.



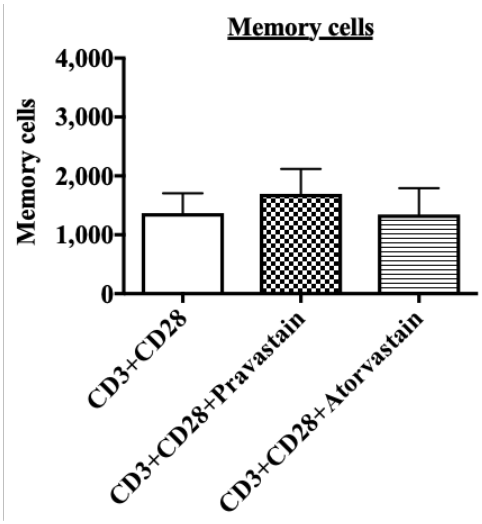


Fig. 4.17.

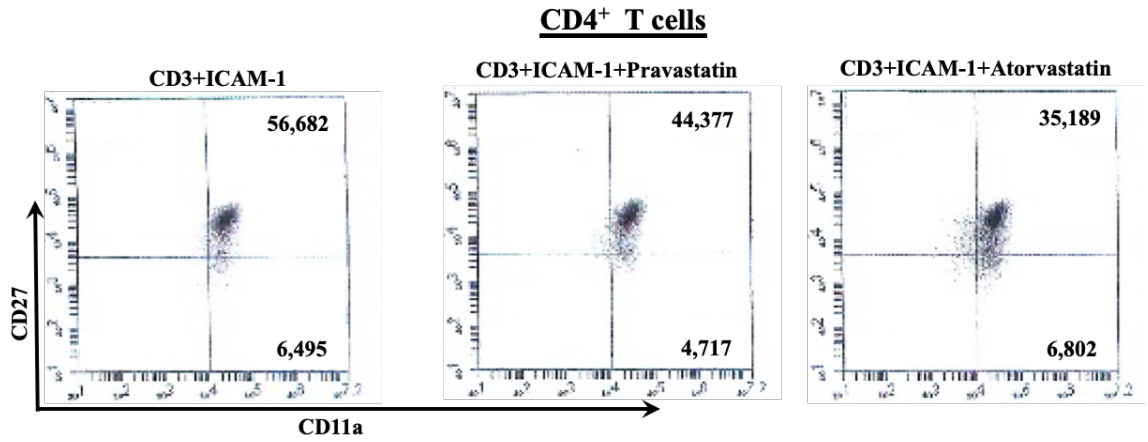
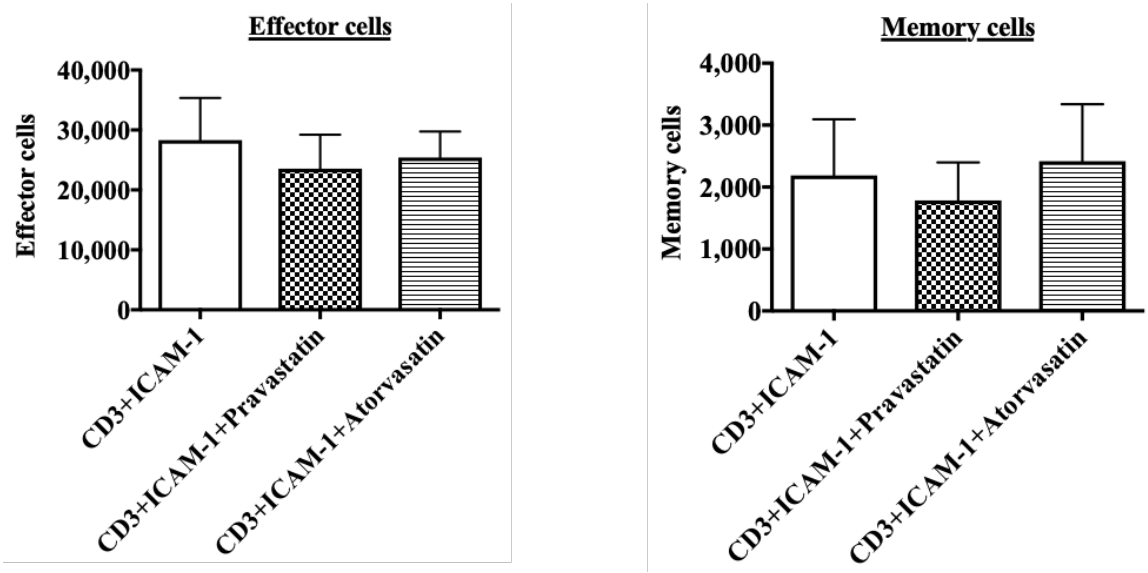


Fig. 4.18.



Figures 4.15-4.18. Pravastatin treatment increased effector and memory cell differentiation upon stimulation through CD3+CD28 and may reduce effector and memory cell differentiation upon stimulation through CD3+ICAM-1.

Primary T cells were stimulated through CD3+CD28 (**Fig. 4.15-4.16)** or CD3+ICAM-1 **(Fig. 4.17-4.18)** in the absence (stimulation alone control) or presence of pravastatin or atorvastatin for 7 days and stained with CD4, CD11a, CD27 and CD45RO and analyzed by flow cytometry. **Fig. 4.15 and Fig. 4.17:** Representative dot plots gated on $CD4^+$ $CD45RO^+$ population showing effector cells in upper right quadrant and memory cells in the lower right quadrant. Number of effector cells is given in the upper right quadrant and number of memory cells is given in the lower right quadrant. **Fig. 4.16 and Fig. 4.18:** Number of effector cells (left) and memory cells (right) after 7 days of co-stimulation with error bars indicating standard error. Representative of six independent experiments; *$p<0.05$ by paired two-tailed t test.

Fig. 4.19.

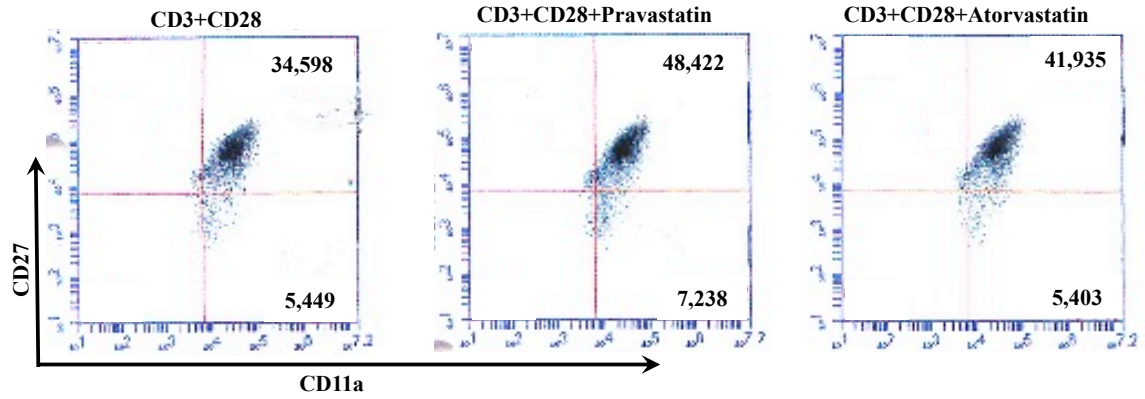


Fig. 4.20.

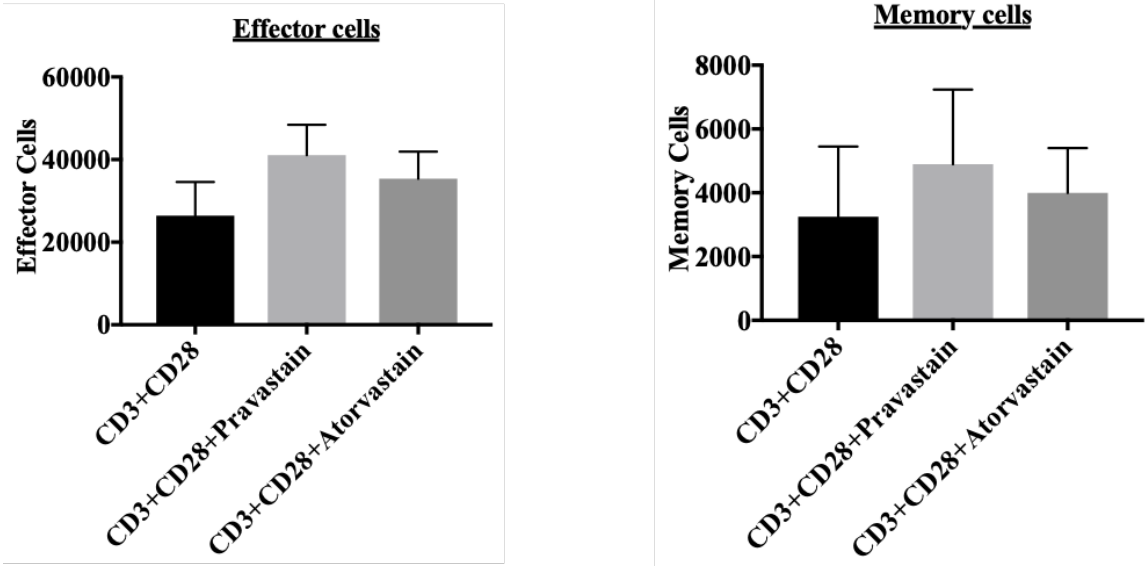


Fig. 4.21.

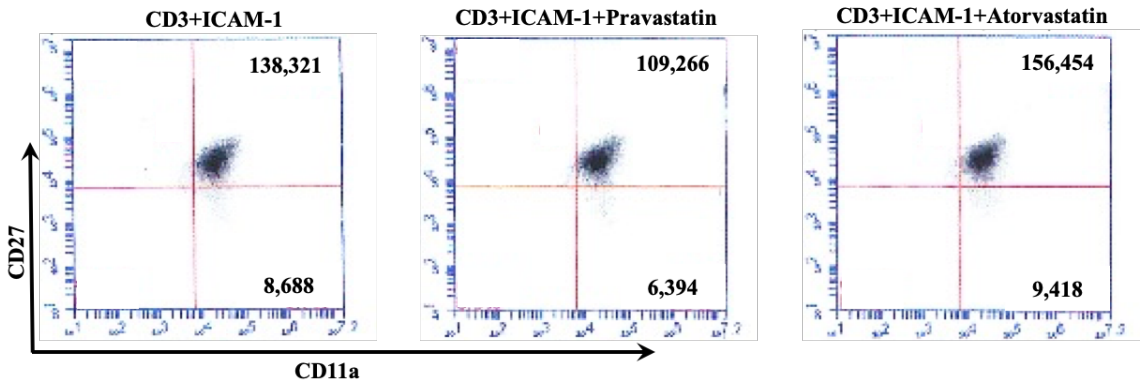
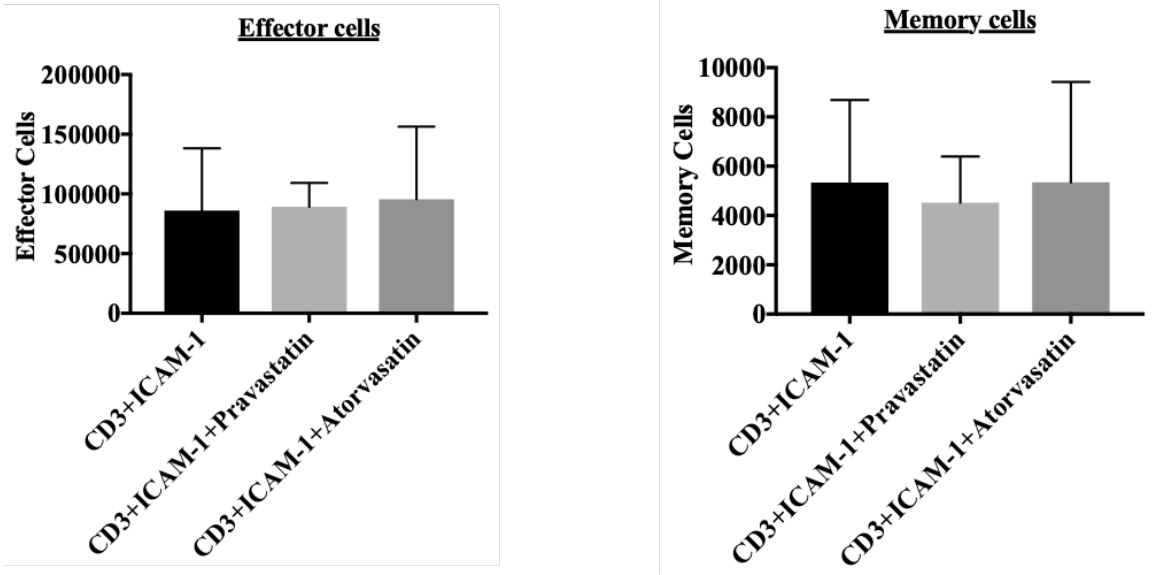


Fig. 4.22.



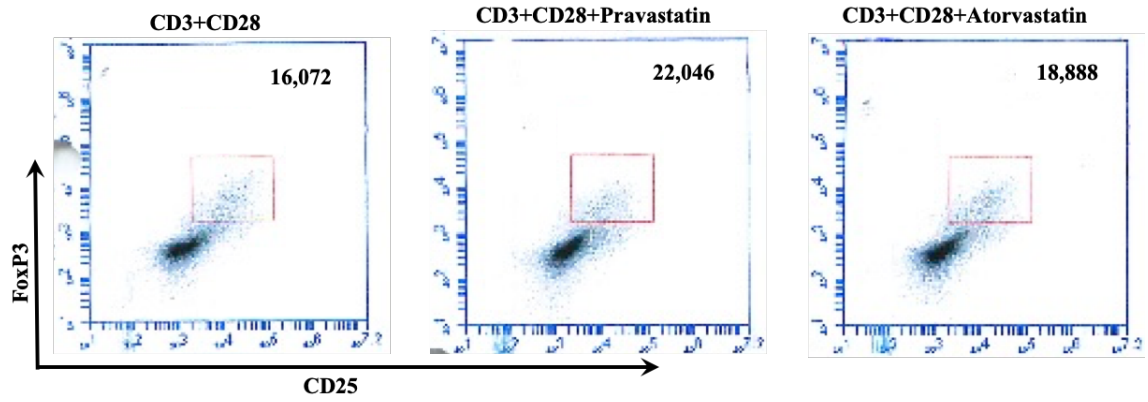
Figures 4.19-4.22. Pravastatin treatment increased effector and memory cell differentiation of naïve $CD4^+$ T cells upon stimulation through CD3+CD28 but not by CD3+ICAM-1 stimulation.

Naïve CD4+ T cells were stimulated through CD3+CD28 (**Fig. 4.19-4.20)** or CD3+ICAM-1 **(Fig. 4.21-4.22)** in the absence (stimulation alone control) or presence of pravastatin or atorvastatin for 7 days and stained with CD11a, CD27 and CD45RO and analyzed by flow cytometry. **Fig. 4.19 and Fig. 4.21:** Representative dot plots gated on $CD45RO^+$ population showing effector cells in upper right quadrant and memory cells in the lower right quadrant. Number of effector cells is given in the upper right quadrant and number of memory cells is given in the lower right quadrant. **Fig. 4.20 and Fig. 4.22:** Number of effector cells (left) and memory cells (right) after 7 days of co-stimulation with error bars indicating standard error. Representative of two independent experiments.

Pravastatin treatment promoted naïve T cell differentiation to regulatory T cells.

Next, we investigated the effect of statins on differentiation of naïve CD4+T cells to different subsets. Our lab has shown that ICAM-1 co-stimulation promoted T cell differentiation to Treg phenotype, while CD28 co-stimulation did not [10]. Studies have shown that statin treatment promoted increased frequency of Treg cells in mice with atherosclerotic plaques, patients with acute coronary syndrome as well as in heathy individuals [4-7]. So, we investigated the effect of statins on naïve T cell differentiation to Treg phenotype. The cells were treated with statins and co-stimulated through CD3+CD28 or CD3+ICAM-1 for 10 days, stained for transcription factor, FoxP3 and surface protein, CD25 and analyzed by flow cytometry. Treg cells are $FoxP3^+$ $CD25^+$ cells. **Fig. 4.23** is a representative dot plot for CD3+CD28 stimulation and **Fig. 4.25** is a representative dot plot for CD3+ICAM-1 stimulation, with Treg cells in the square gate. For both CD28 and ICAM-1 co-stimulation, pravastatin treatment promoted naïve T cell differentiation to Treg phenotype, while atorvastatin treatment did not (**Fig. 4.23-4.26**). Pravastatin treatment promoted significant increase in Treg subset during ICAM-1 co-stimulation (**Fig. 4.26,** $p<0.05$ by paired two-tailed t test).

Fig. 4.23.



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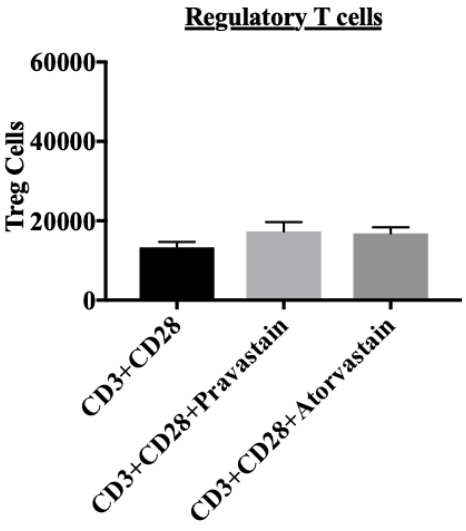

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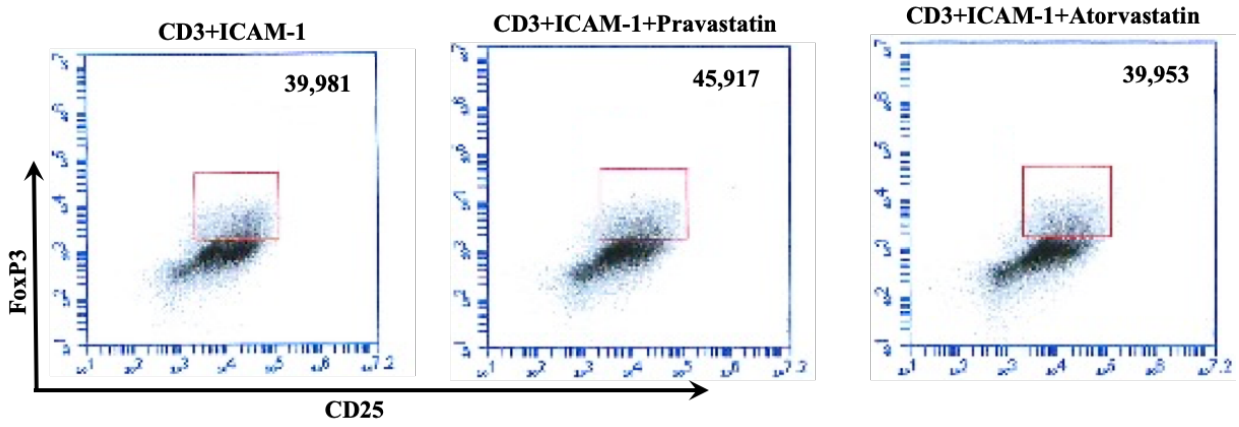
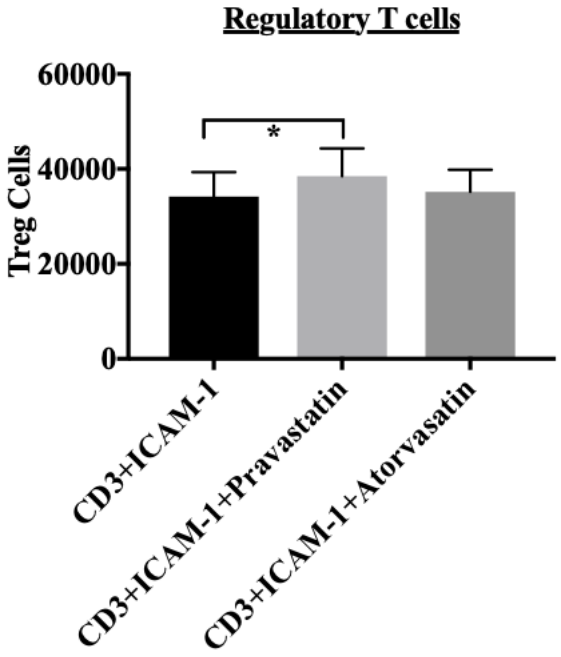


Fig. 4.26.



Figures 4.23-4.26. Pravastatin treatment promoted naïve T cell differentiation to regulatory T cells.

Naïve CD4+ T cells were stimulated through CD3+CD28 (**Fig. 4.23-4.24)** or CD3+ICAM-1 **(Fig. 4.25-4.26)** in the absence (stimulation alone control) or presence of pravastatin or atorvastatin for 10 days and stained with CD25 and FoxP3 and analyzed by flow cytometry. **Fig. 4.23 and Fig. 4.25:** Representative dot plots showing regulatory T cells ($FoxP3^+$ $CD25^+$) in square gate. Number of regulatory T cells is given in the upper right corner. **Fig. 4.24 and Fig. 4.26:** Number of regulatory T cells after 10 days of co-stimulation with error bars indicating standard error. Representative of three independent experiments; *$p<0.05$ by paired two-tailed t test.

Discussion

Statins are HMG-CoA reductase inhibitors, extensively used in the treatment of cardio-vascular diseases. Statins have been shown to exhibit immunomodulatory and anti-inflammatory effects [3]. Khaw et al. showed that statins such as pravastatin, atorvastatin and lovastatin inhibited IFN-γ induced expression of MHC class II molecules in antigen presenting cells and act as a repressor of MHC class II mediated T cell activation [3]. Several studies have reported the benefits of statin treatment on autoimmune disease conditions such as multiple sclerosis [15,16]. Atorvastatin treatment inhibited the progression of the disease, reduced the inflammation in the central nervous system and prevented chronic and relapsing paralysis in experimental autoimmune encephalomyelitis (EAE) murine models [15,16].

In this project, we investigated the effect of statins- pravastatin and atorvastatin on T cell functions. We showed that statin treatment did not affect cell viability. However, T cell activation, proliferation and differentiation upon statin treatment was dependent on co-stimulation through CD3+CD28 or CD3+ICAM-1.

We observed that upon CD3+CD28 stimulation, pravastatin induced increased T cell activation, proliferation and differentiation to effector and memory phenotypes, while atorvastatin did not have any effect. Upon CD3+ICAM-1 stimulation, both pravastatin and atorvastatin induced decreased T cell activation, proliferation and differentiation to effector and memory phenotypes. It has been shown by Blank et al. that atorvastatin treatment of PBMCs (peripheral blood mononuclear cells) inhibited T cell activation and proliferation during CD3+CD28 stimulation [17]. The difference in the results might be due to the use of different cells. PBMCs comprise of a mix of cell population such as B cells, T cells and monocytes. So, the observation on statin

treatment by Blank et al. may be contributed by cells other than T cells, while our observation specifically showed effects of atorvastatin on T cells.

Regulatory T cells are suppressor T cells that play a role in preventing various inflammatory and autoimmune diseases. In this project, we observed that both CD28 and ICAM-1 co-stimulation induced increased T cell differentiation to Treg phenotype in the presence of pravastatin. Our lab has previously shown that co-stimulation through ICAM-1 only promotes naïve T cell differentiation to Treg phenotype but not through CD28 co-stimulation [10]. We observed that CD28 co-stimulation in the presence of pravastatin induced increased Treg subsets. This observation suggests that upon stimulation through either co-stimulatory molecule- CD28 or ICAM-1, pravastatin may reduce the inflammatory response due to Treg mediated suppression of the immune system. However, it should be noted that the result is representative of only three independent experiments. Several studies using statins such as atorvastatin, lovastatin and simvastatin have shown that statins induce increased Treg subset in atherosclerotic plaques, patients with coronary heart disease as well as in healthy individuals [4-7]. Another study by Kim et al. showed that simvastatin treatment during CD28 co-stimulation promoted increased $FoxP3^{+}$ Treg subset using murine cells [18]. Together, our data suggest that immunosuppressive effects of pravastatin treatment may be contributed by increased Treg population with both CD28 and ICAM-1 co-stimulation.

The two statins are different in their lipophilic (atorvastatin) and hydrophilic (pravastatin) properties [19]. Atorvastatin can be easily taken up by the cells while pravastatin has reduced ability [19]. This property of the two statins might contribute to their differential effects on T cell functions.

Most studies of the immunomodulatory effects of statins have been conducted using atherosclerotic mouse models, patients with coronary heart diseases and human PBMCs. Hence, this study was conducted to study the effect of stain treatment using human peripheral T cells and human naïve $CD4^{+}$T cells. The effects of pravastatin on T cell function has not been conducted, so our observations regarding the effect of pravastatin are novel. We observed that the choice of co-stimulatory molecules influenced the outcome T cell activation, proliferation, and differentiation, without affecting T cell viability.

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Chapter 5

Transient decrease in pH reprograms T cell homing characteristics of leukemia T cells and normal T cells.

Introduction

T cells of the immune system differentiate from multipotent, naïve cells to mature, highly functional "effector" cells. Some effector cells create an immune response by using several forms of attack whereas others suppress an immune response when it is no longer needed. T cells also are capable of "tuning" their response based on needs of the system at the time. T cells can leave one compartment such as a lymph node and travel specifically to another compartment such as the gut by changing gene expression for the long term. Our lab studies the influence of microenvironment on T cell differentiation using an in vitro system to mimic cell contacts provided by different microenvironments.

Microenvironment influences T cell differentiation. Microenvironmental influences that we studied include (A) proteins expressed on other cells in the area and that interact with T cell counter receptors during cell to cell contact; (B) naturally occurring soluble mediators such as lipoproteins- LDL and HDL; and (C) foreign compounds introduced by environmental influence such as polycyclic aromatic hydrocarbons (PAHs). Our lab has shown that naïve CD4+ T cell stimulation through CD3+CD28 induces Th1 and Th2 effector and memory cells, while stimulation through CD3+ICAM-1 induces effector and memory Th1 and Treg cells without exogenously added cytokines [1-3]. We also have studied the influence of factors other than cell contact such as lipoproteins. Previously our lab has shown that lipoproteins affect naïve CD4+ T cell differentiation. Oxidized LDL influences naïve T cell differentiation to Th1 and promotes plaque progression while HDL opposes this activity [4]. In this project, we investigated the effect of physical environment: pH on expression of chemokine receptors and homing pattern using leukemic T cells and primary T cells.

Low pH microenvironment

Local acidosis occurs at inflammatory loci resulting in lower pH than normal tissue [5]. Interstitial fluid of tumors and abscesses shows pH values of less than 6, averaging .2-.6 units lower than the mean extracellular pH of normal tissue [5]. The lowest pH reported at the site of inflammatory locus is 5.5 [6]. Also, highly metabolic areas such as lymph nodes have low pH. A few studies have demonstrated that low pH influences the motility of lymphocytes as well as neutrophils and cytotoxic ability of killer T cells [7-9]. Not much has been studied on the effect of altered pH microenvironment on homing patterns of immune cells and their functions.

Chemokine and chemokine receptors

Chemokines are chemoattractant cytokines consisting of 70-125 amino acids that range from molecular mass of 6 to 14 kDa [10,11]. Chemokines are categorized into four different classes based on the arrangement of cysteine residues in the N-terminal region [12]. The different classes of chemokines are C, CC, CXC and CX3C [12]. C represents the number of cysteine residue in the N-terminal region and X represents the number of amino acids between the cysteine residue [10-12]. Chemokines bind to G protein-coupled cell surface receptors, known as chemokine receptors, to mediate cell adhesion and chemotactic migration of cells. Chemokine receptors are seven transmembrane GTP-binding protein (G-protein) coupled receptors expressed on the immune cells [12].

Each chemokine receptor interacts with and responds to a specific chemokine [13]. A variety of chemokine receptors and chemokines direct cells to all parts of the body. Each tissue expresses different chemokine patterns that, in conjunction with adhesion molecules, guide migrating cells to that specific tissue type. When a chemokine receptor detects the presence of a

chemokine, the cell migrates along an increasing gradient of chemokines until it reaches the desired location. Expression of chemokine receptors provides homing guidance during migration.

Hypothesis tested

In this project, we have modeled the activities of T cells when they encounter the reduced pH of an area where immune cells are actively dividing such as areas of the lymph node or active inflammation. The main objective of this project was to learn if low pH microenvironment could change homing patterns of T cells and whether T cell lines representing acute lymphoblastic leukemia (ALL) can be tuned by the microenvironment in the same way as normal cells or in a different manner. We tested expression of chemokine receptors using Jurkat and Molt-3, which are human lymphoma derived T cell lines and normal T cells taken from human tonsil or peripheral blood. We applied low pH (pH=5.7) for 30 minutes to normal and leukemic T cells to learn effect on expression of chemokine receptors and homing pattern. We hypothesized that transient decrease in pH differentially affects the homing patters of normal and leukemic T cells.

Materials and Methods

Antibodies

Anti-CXCR4-PE (Clone: 12G5), anti-CCR7-PE (Clone: G043H7) and anti-CCR5-PE (Clone: J418F1) and anti-CD3-APC (Clone: UCHT1) were purchased from Biolegend (San Diego, CA). Annexin V-PE, and 7-Amino-Actinomycin D (7AAD) were purchased from BD Pharmingen (San Jose, CA).

T cell isolation and culture

Jurkat and Molt-3 cells were grown in RPMI 1640 (CellGro, Corning, New York), containing 10% FBS (Atlanta Biologicals, Atlanta, GA), 50 IU/ml penicillin/50 μg/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco).

Primary T cells were isolated from human tonsil or peripheral blood of healthy donors. Tonsils were minced in TC-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM glucose, pH 7.4) + 2% FBS + 1% penicillin/streptomycin, and cells in suspension were separated from tissue using a strainer. Peripheral blood was diluted 1:1 in sterile TC-PBS + 2% FBS + 1% penicillin/streptomycin. Mononuclear cell layer from minced tonsil and peripheral blood was separated using Ficoll density gradient centrifugation for 30 minutes at 1800 rpm. The buffy coat of mononuclear layer was collected, washed in TC-PBS to remove residual Ficoll and total T cells was isolated by E-rosetting. Briefly, the mononuclear layer suspension was mixed with 10% sheep red blood cells at the ratio of 10:1, incubated for 10 minutes at 37°C, spun for 10 minutes at 2000 rpm and incubated on ice for 30 to 60 minutes. Total T cell was separated using Ficoll density gradient centrifugation

for 30 minutes at 1800 rpm. Freshly isolated T cells were rested overnight at 37° before use. Purity for total T cells was greater than 90% as assessed by flow cytometry.

Low pH cell treatment

Hank's Balanced salt solution, HBSS (Cellgro, Corning, New York) was adjusted to a pH of 5.7. Jurkat or Molt-3 or Primary T cells were treated with low pH HBSS solution for 30 minutes at 37^0C. Cells were washed and resuspended in RPMI 1640, containing 10% FBS, 50 IU/ml penicillin/50 μg/ml streptomycin, and 2 mM L-glutamine. Cells were added to 96-well tissue culture plates and incubated at 37°C with 5% CO_2 for 6 hours, 16-18 hours and 7 days.

Flow cytometry

Cells were collected from the tissue culture plate and stained with fluorescent tagged antibodies to be analyzed by Accuri C6 flow cytometer with CFlow software (Accuri Cytometers). For Annexin V/ 7AAD staining, cells were washed twice in cold PBS, stained in Annexin V binding buffer (0.01 M HEPES pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) with Annexin V-PE and 7AAD (7-aminoactinomycin D), washed in Annexin V binding buffer and analyzed by flow cytometry. For chemokine receptor staining, cells were blocked with 0.5% BSA in PBS for 15 minutes on ice, stained with anti-CXCR4-PE, anti-CCR7-PE and anti-CCR5-PE antibodies for 15 minutes on ice, washed with 0.5% BSA in PBS, and analyzed by flow cytometry.

Results (*The data presented here are preliminary data)*

Treatment of human T cells with low transient pH did not cause toxicity

At first, we examined whether brief treatment of cells with low pH of 5.7 caused toxicity. We treated human leukemic T cell line, Molt-3 cells with pH 5.7 HBSS for 30 minutes and incubated the cells at 37^0C for 6 hours, 16-18 hours and 7 days. Cell viability was analyzed by staining cells with fluorochrome-tagged Annexin V and 7-aminoactinomycin D (7AAD) via flow cytometry as published previously [4]. **Fig. 5.1** is a representative dot plot for cell death by transient low pH treatment at 6 hours, 16-18 hours and 7 days. The double positive cells, Annexin V^+ $7AAD^+$ are the dead cells, which are present in the upper right quadrant and the double negative cells, Annexin V^- $7AAD^-$ are viable cells, which are present in the lower left quadrant in **Fig. 5.1**. **Fig. 5.2** is bar graph representing apoptotic cell numbers. The number of dead cells in low pH treated samples were not different from culture medium pH (control sample) for 6 hours. At 16-18 hours and 7 days, dead cell number increased for culture medium pH. However, treatment of low pH resulted in fewer cell death at 7 days of treatment compared to the culture medium pH. We observed that low pH treatment did not affect the viability of cells at early time point and low pH is slightly protective against cell death up to 7 days of treatment.

Transient low pH caused dramatic effect on CXCR4 expression for up to 7 days post treatment in leukemia T cells.

We studied the expression of certain chemokine receptors upon transient low pH treatment using two leukemia T cell lines, Jurkat and Molt-3 and normal primary T cells. CXCR4 is a homeostatic receptor that is ubiquitously expressed in embryonic as well as adult tissues [14].

Fig. 5.1.

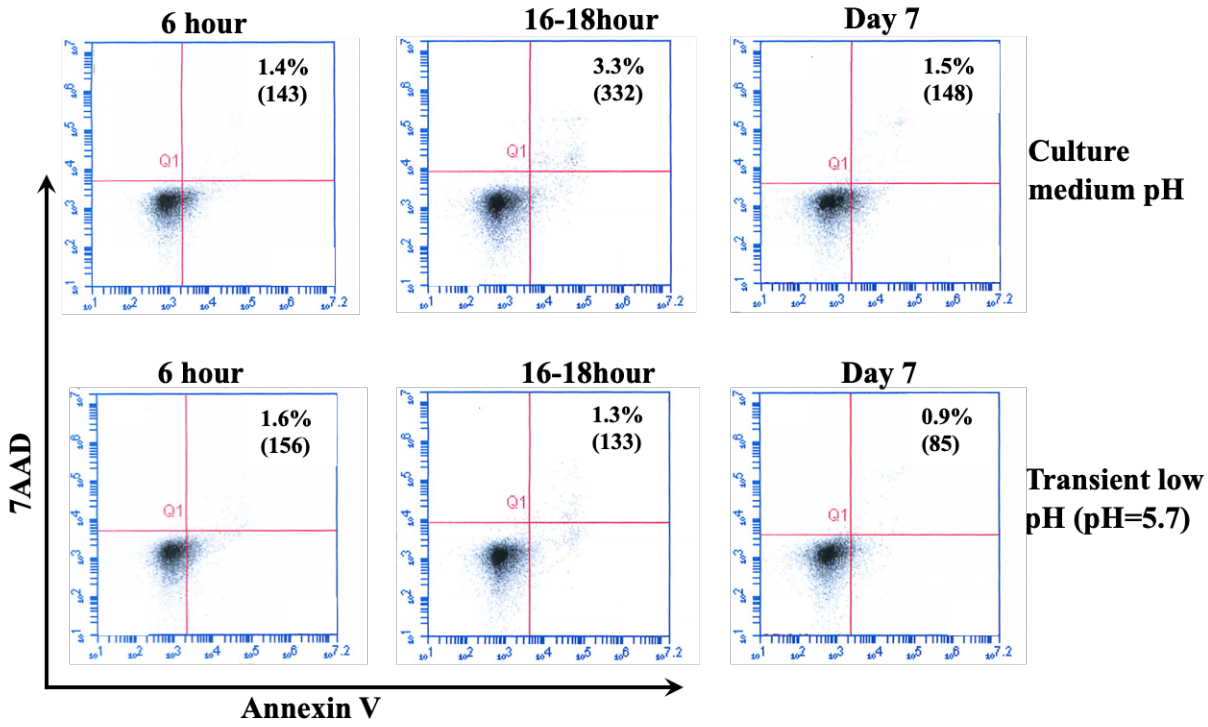
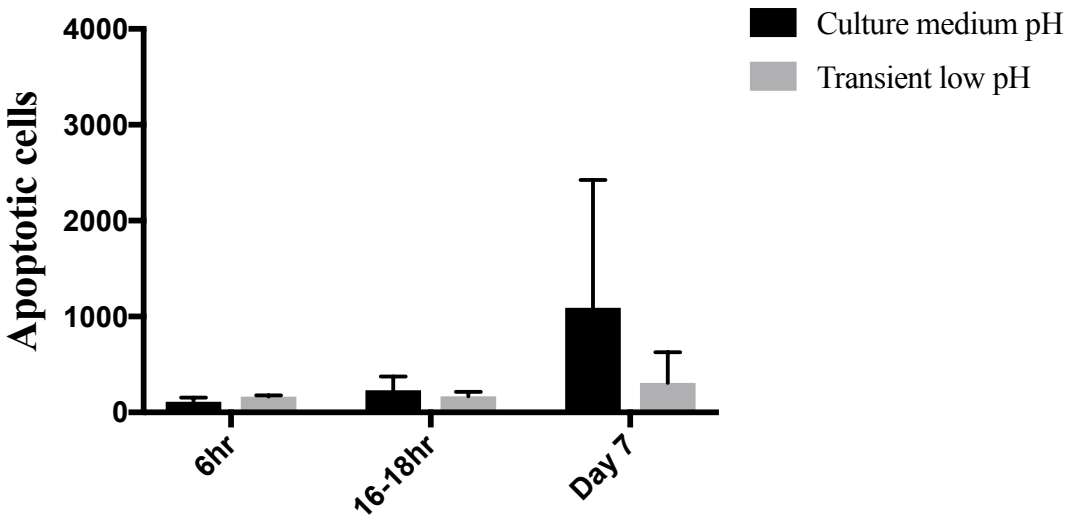

6 hour
16-18hour
Day 7
1.4%
(143)
3.3%
(332)
1.5%
(148)
Culture
medium pH
6 hour
16-18hour
Day 7
1.6%
(156)
1.3%
(133)
0.9%
(85)
Transient low
pH (pH=5.7)
Q1
7AAD
Annexin V

Fig. 5.2.


Culture medium pH
Transient low pH
Apoptotic cells
4000
3000
2000
1000
0
6hr
16-18hr
Day 7

Figures 5.1-5.2. Treatment with low pH for 30 minutes did not affect cell viability.

Leukemic T-cell line, Molt-3 cells were treated with pH 5.7 HBSS for 30 minutes at day 0 and incubated at 37^0C for 6 hours, 16 hours and 7 days and stained with **Annexin V** and **7AAD** and analyzed using flow cytometry. **Fig. 5.1.** Representative dot plots showing apoptosis. Percent of apoptotic cells (Annexin V^+ $7AAD^+$) with number of apoptotic cells in parentheses in upper right quadrant. **Fig. 5.2.** Bar graph for number of apoptotic cells after 6 hours, 16 hours and 7 days of treatment with error bars indicating standard error. Representative of 2 independent experiments.

CXCR4 plays a crucial role in T cell migration by recruiting cells to infected tissue and lymphoid tissue [14]. CXCR4 has a unique ligand, stromal cell-derived factor-1 (SDF1). SDF1 is produced in multiple tissues including primary and secondary lymphoid tissue and peripheral tissue [14]. It has been shown that CXCR4/SDF1 deficient mice show defect in hematopoiesis, and in cardiovascular and neural development [10]. Thus, studies suggest that CXCR4 is very essential for hematopoietic cell migration as well as homing of T cells [14].

In this project, upon low pH treatment, CXCR4 expression in Molt-3 did not change at the early time-points, 6 hours and 16-18 hours compared to the control culture medium pH. However, CXCR4 expression decreased by day 7 as shown in **Fig. 5.3**. In Jurkat, CXCR4 expression decreased at 6 hours upon low pH treatment, while at 7 days, we observed a dramatic effect of low pH on CXCR4 expression similar to that of Molt-3 as shown in **Fig. 5.4**. In primary T cells, low pH treatment did not have an effect on CXCR4 expression compared to the control as shown in **Fig. 5.5**. Thus, transient low pH (pH 5.7) had a dramatic effect on CXCR4 expression in both leukemia T cell lines- Molt-3 and Jurkat that was observable at day 7 but not on the primary T cells.

Transient low pH induced decrease in CCR7 expression in leukemia T cells

CCR7 guides naïve and memory T cells to the lymph nodes where cells undergo activation if they encounter specific antigen [15]. All naïve T cells express chemokine receptor CCR7 that interacts with two chemokines CCL19 and CCL21 [15]. Naive T cells enter the lymph node through the walls of high endothelial venules (HEV). HEV produce chemokine CCL21 that interacts with CCR7 on T cells and mediates entry into the lymph node. Inside the lymph node, CCR7 binds and responds to CCL19 located in specific areas in the lymph node. Then, the naïve

Fig. 5.3.

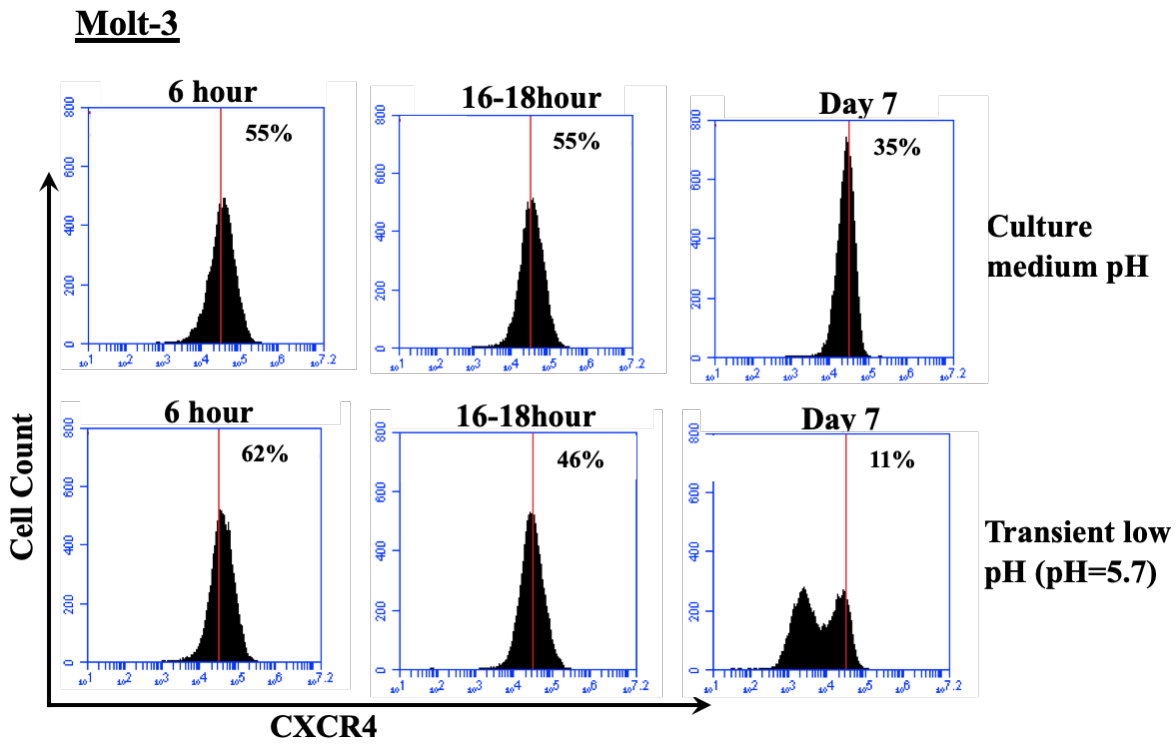

Molt-3
6 hour
55%
16-18hour
55%
Day 7
35%
Culture medium pH
6 hour
62%
16-18hour
46%
Day 7
11%
Transient low pH (pH=5.7)
Cell Count
CXCR4

Fig. 5.4.

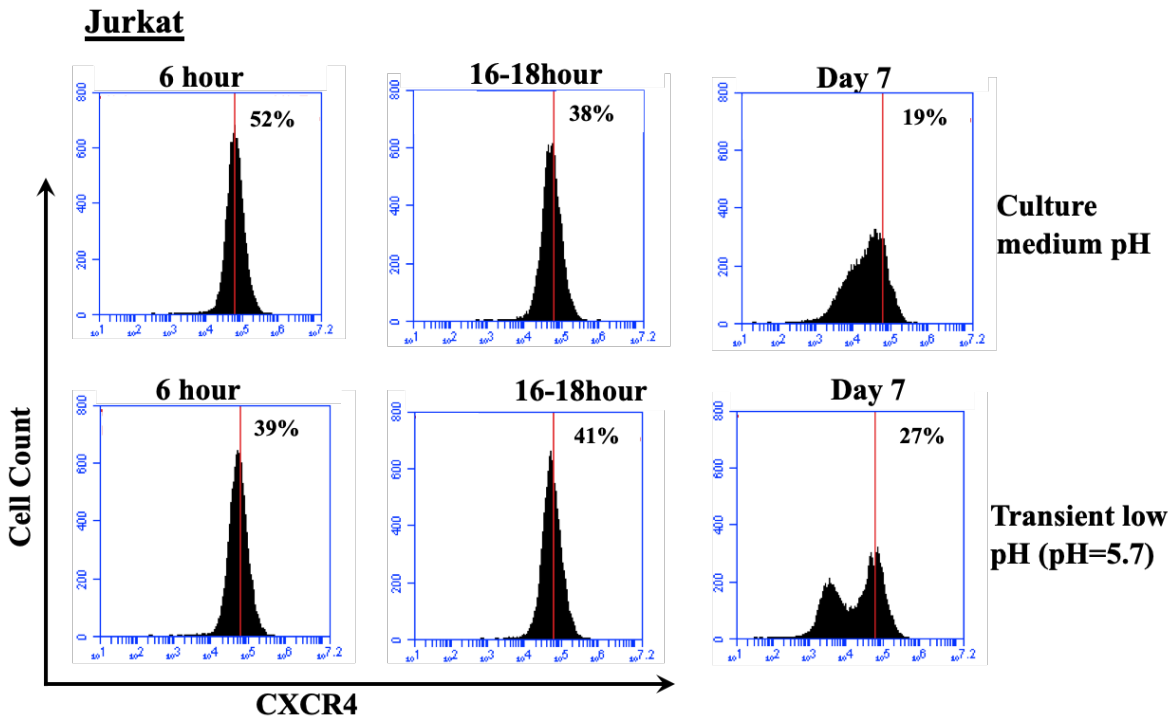
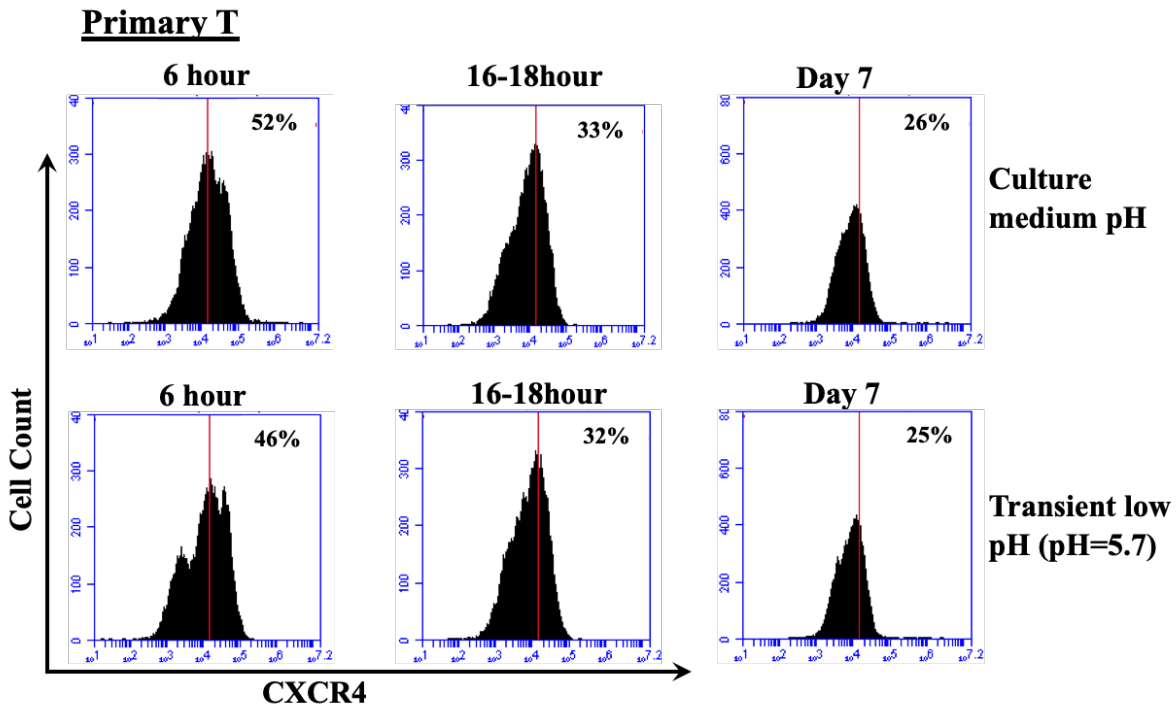

Jurkat
6 hour
52%
16-18hour
38%
Day 7
19%
Culture medium pH
6 hour
39%
16-18hour
41%
Day 7
27%
Transient low pH (pH=5.7)
Cell Count
CXCR4

Fig. 5.5.


Primary T
6 hour
16-18hour
Day 7
52%
33%
26%
Culture medium pH
6 hour
16-18hour
Day 7
46%
32%
25%
Transient low pH (pH=5.7)
Cell Count
CXCR4

Figures 5.3-5.5. Transient low pH caused dramatic effect on CXCR4 expression for up to 7 days post treatment in leukemia T cells.

Leukemic T-cell lines, Molt-3 and Jurkat and normal primary T cells were treated with pH 5.7 HBSS for 30 minutes at day 0 and incubated for 6 hours, 16 hours and 7 days. Cells were stained for **CXCR4** chemokine receptor and analyzed by flow cytometry. Representative histogram for CXCR4 expression at 6 hours, 16-18 hours and day 7 for Molt-3 (**Fig. 5.3**), Jurkat (**Fig. 5.4**) and Primary T cells (**Fig. 5.5**) at culture medium pH and transient low pH respectively.

Representative of 1 experiment for Molt-3, 4 independent experiments for Jurkat and 2 independent experiments for primary T cells.

T cell is activated by specific antigen in the lymph node. If the T cell fails to receive any antigen signal, it returns to circulation through efferent lymph vessels and thoracic duct [16]. CCR7 is a crucial chemokine receptor for T cell migration and homing to lymph nodes.

We studied CCR7 expression upon low pH treatment. In Molt-3 and Jurkat, **Fig. 5.6-5.7**, CCR7 expression decreased upon low pH treatment at all time-points- 6 hours, 16 hours and day 7. However, CCR7 expression remained unaffected by low pH treatment in primary T cells, **Fig. 5.8**. This indicates that low pH treatment induced the change in phenotype of leukemia T cell causing decrease in CCR7 expression as early as 6 hours.

Normal cells and tumors showed different patterns of CCR5 expression upon transient low pH treatment.

CCR5 plays a crucial role in T cell migration by recruiting cells to the sites of inflammation [13]. The ligands for CCR5 are RANTES, MIP-1α, MIP-1β and MCP-2 [13]. Upon low pH treatment, CCR5 expression increased in Molt-3 at 16-18 hours and day 7 (**Fig. 5.9),** while CCR5 expression decreased in Jurkat at all time-points- 6 hours, 16-18 hours and day 7 (**Fig. 5.10)**. The CCR5 expression remained unchanged by low pH treatment in primary T cells (**Fig. 5.11)**. We observed that Jurkat, Molt-3 and Primary T cells respond to transient low pH differently in terms of CCR5 expression.

Fig. 5.6.

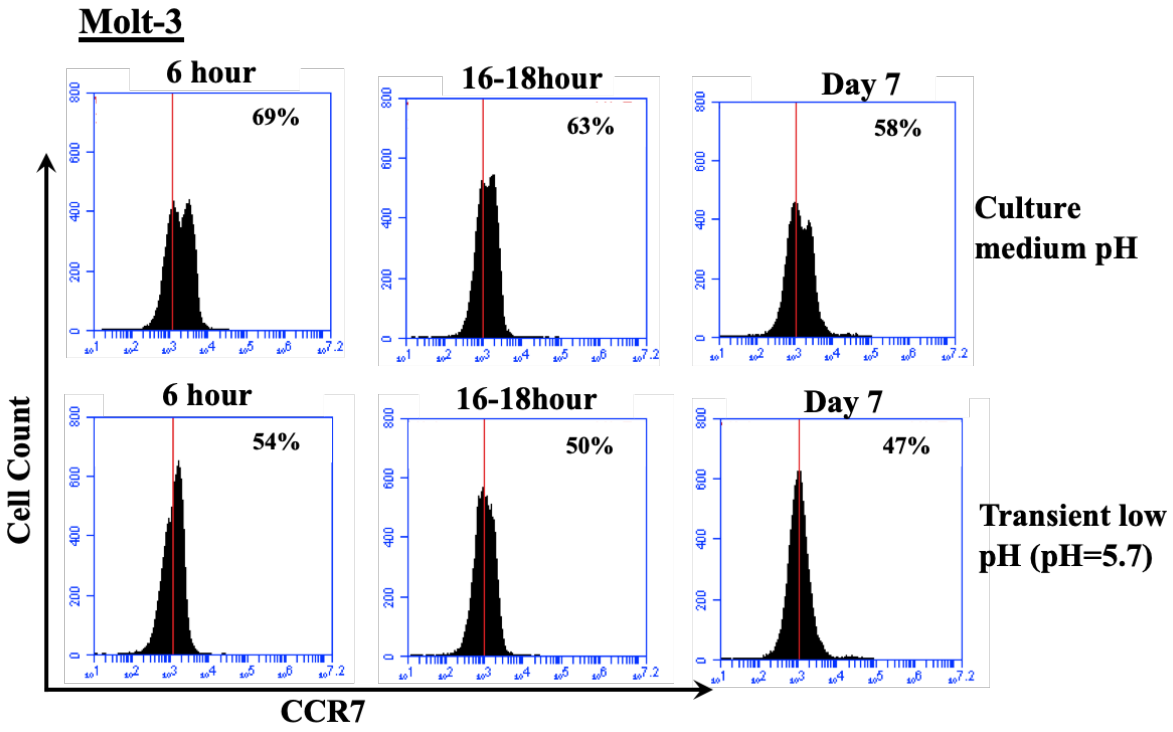

Molt-3
6 hour
69%
16-18hour
63%
Day 7
58%
Culture medium pH
6 hour
54%
16-18hour
50%
Day 7
47%
Transient low pH (pH=5.7)
Cell Count
CCR7

Fig. 5.7.

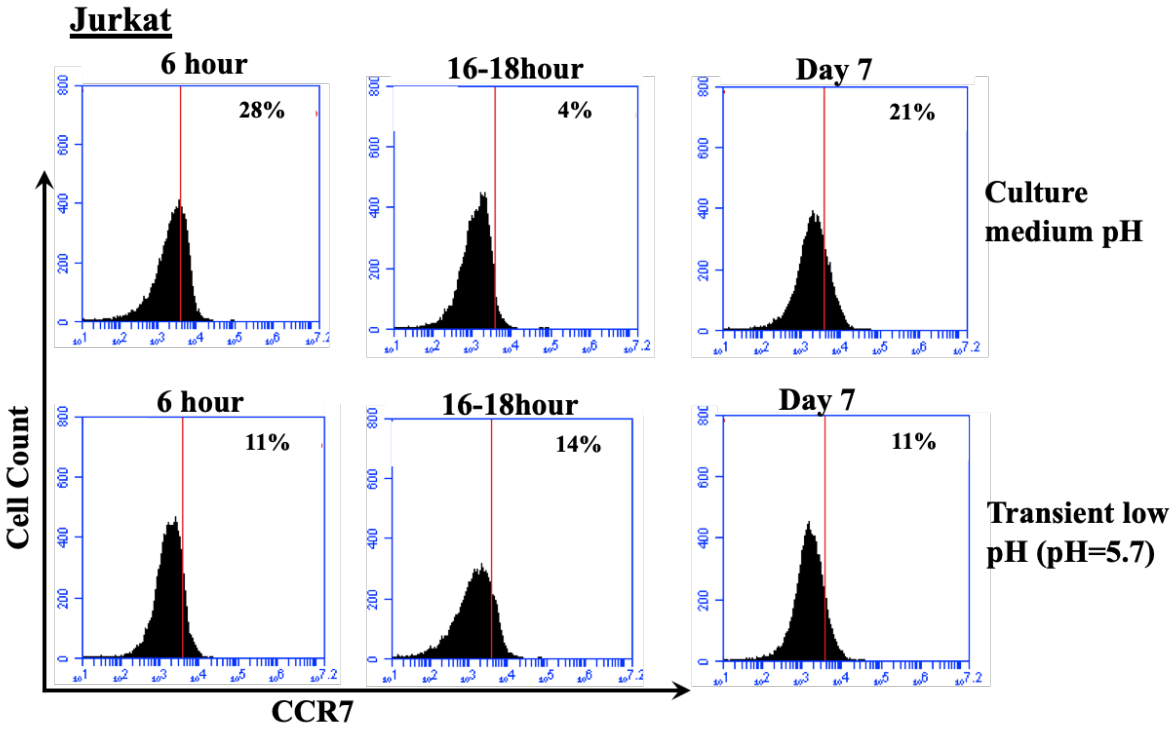
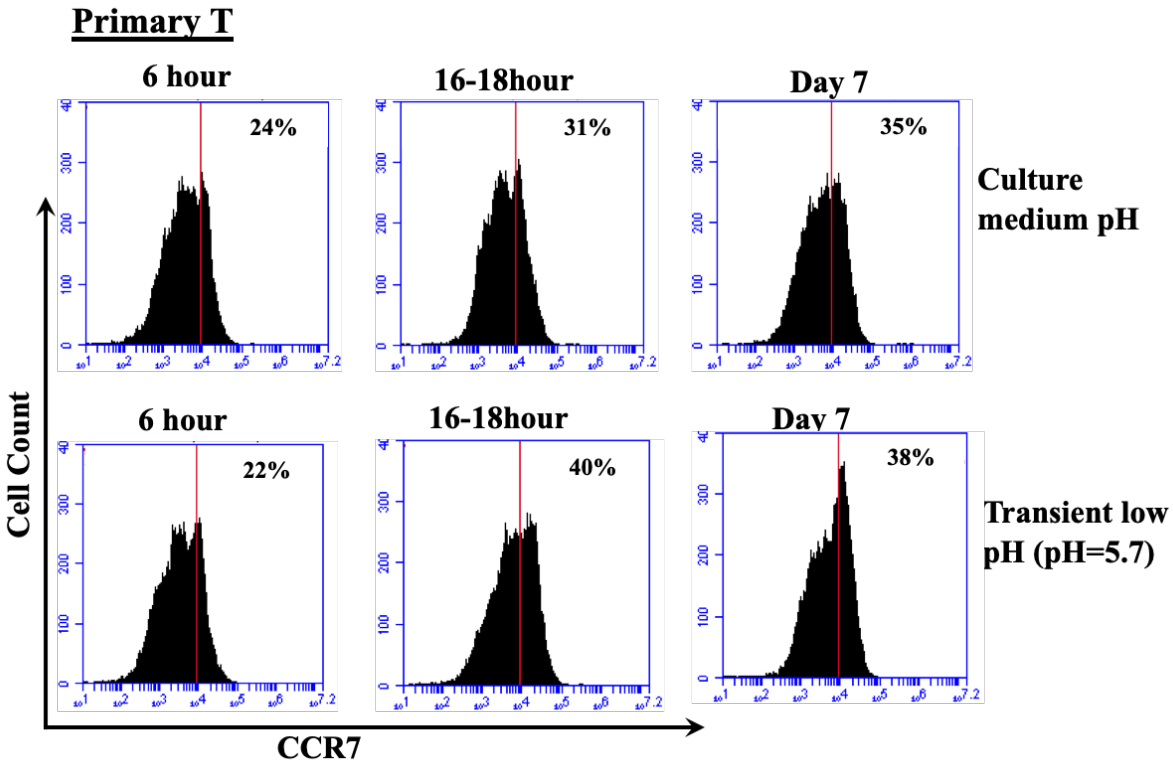

Jurkat
6 hour
28%
16-18hour
4%
Day 7
21%
Culture medium pH
6 hour
11%
16-18hour
14%
Day 7
11%
Transient low pH (pH=5.7)
Cell Count
CCR7

Fig. 5.8.


Primary T
6 hour
16-18hour
Day 7
24%
31%
35%
Culture medium pH
6 hour
16-18hour
Day 7
22%
40%
38%
Transient low pH (pH=5.7)
Cell Count
CCR7

Figures 5.6-5.8. Transient low pH induced decrease in CCR7 expression in leukemic T cells. Molt-3, Jurkat and primary T cells were treated with pH 5.7 HBSS for 30 minutes at day 0 and stimulated for 6 hours, 16-18 hours and 7 days. Cells were stained for **CCR7** chemokine receptor and analyzed by flow cytometry. Representative histogram for CCR7 expression at 6 hours, 16 hours and day 7 for Molt-3 (**Fig. 5.6**), Jurkat (**Fig. 5.7**) and Primary T cells (**Fig. 5.8**) at culture medium pH and transient low pH respectively. Representative of 1 experiment for Molt-3, 4 independent experiments for Jurkat and 2 independent experiments for primary T cells.

Fig. 5.9

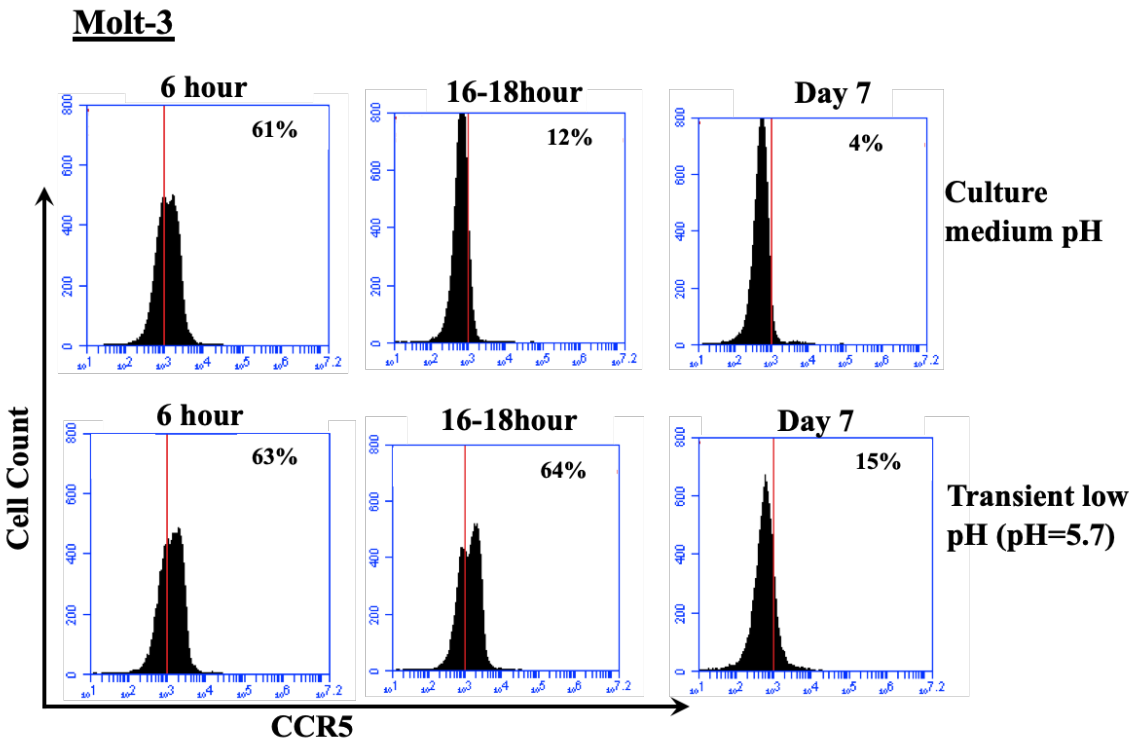


Fig. 5.10.

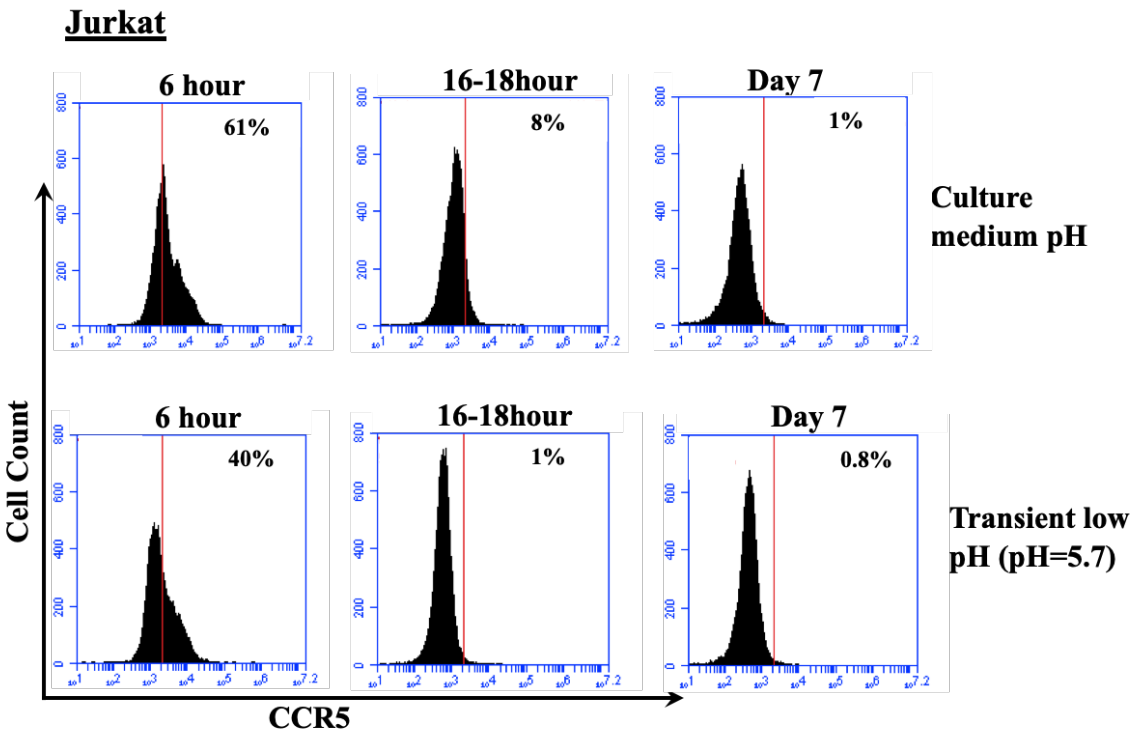
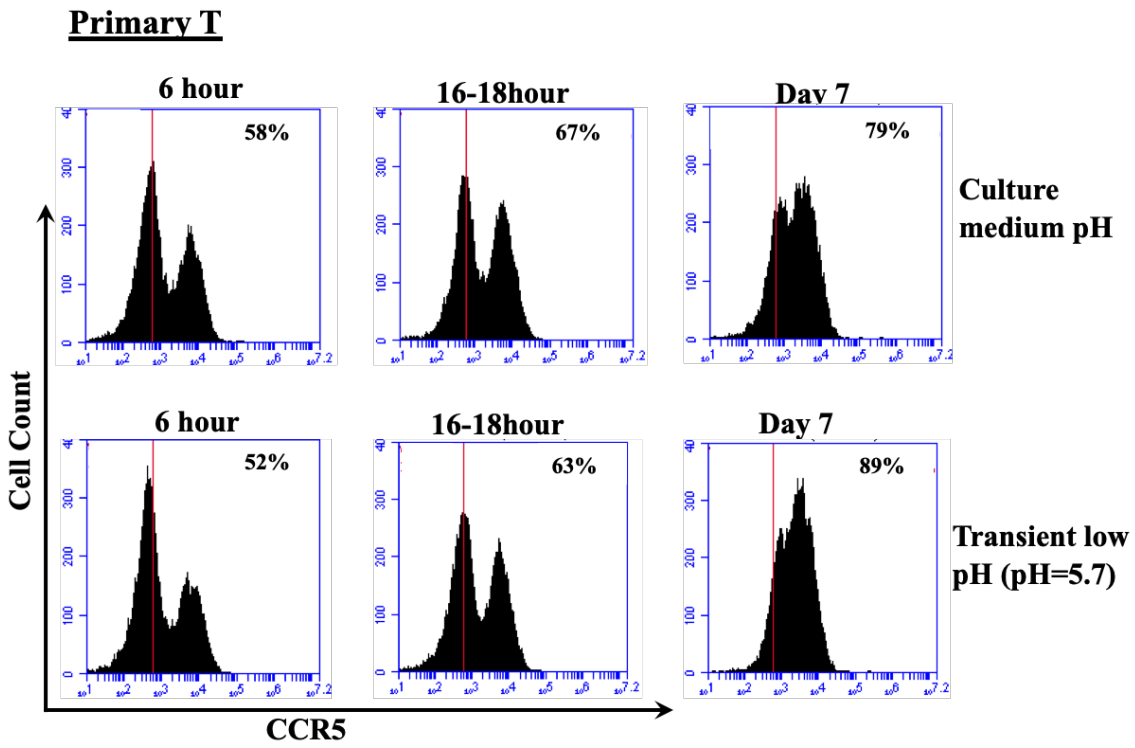


Fig. 5.11.


Primary T
6 hour
16-18hour
Day 7
58%
67%
79%
Culture medium pH
6 hour
16-18hour
Day 7
52%
63%
89%
Transient low pH (pH=5.7)
Cell Count
CCR5

Figures 5.9-5.11. Primary T cells and leukemic T cell lines showed different patterns of CCR5 expression upon transient low pH treatment.

Molt-3, Jurkat and primary T cells were treated with pH 5.7 HBSS for 30 minutes at day 0 and stimulated for 6 hours, 16 hours and 7 days. Cells were stained for **CCR5** chemokine receptor and analyzed by flow cytometry. Representative histogram for CCR5 expression at 6 hours, 16 hours and day 7 for Molt-3 (**Fig. 5.9**), Jurkat (**Fig. 5.10**) and Primary T cells (**Fig. 5.11**) at culture medium pH and transient low pH respectively. Representative of 1 experiment for Molt-3, 4 independent experiments for Jurkat and 2 independent experiments for primary T cells.

Discussion

Previously, our lab has shown that microenvironment influences naive T cell differentiation [1-4]. The choice of co-stimulatory molecules- CD28 and ICAM-1 can influence naïve T cell differentiation outcomes [1-3]. Similarly, different lipoproteins- oxidized LDL and HDL result in different differentiation phenotypes [4]. The goal of this project was to investigate the effect of low pH microenvironment using leukemic T cell lines and primary T cells. We investigated the expression of chemokine receptors on the cell surface upon low pH treatment to study the homing patterns of T cells. Low pH microenvironment is commonly found in the body parts such as lymph node, inflammatory locus, or interstitial fluid of tumors and abscess.

Regarding the effects of pH on immune cell functions, most studies have focused on the role of intracellular pH in the regulation of various cellular activities such as increase in cytosolic pH increased DNA and protein synthesis, enhanced metabolic rate, cell proliferation and mitosis [5]. However, very few studies have focused on the effect of altered extracellular pH on lymphocyte functions. Ratner et al. showed that low pH (pH 6.7) microenvironment induced an increase in the motility of murine lymphocytes [7]. Rabinovich et al. studied the movement of mouse bone marrow neutrophils in agarose at pH 6 and concluded that movement of neutrophils increased upon lowered pH [8]. These studies indicate that low pH microenvironment may influence lymphocyte migration. Another study by Severin et al. showed decreased cytotoxic activity of human lymphokine activated killer cells in acidic pH (pH 6.8,6.3, and 5.8), indicating that low pH microenvironment influenced the cytotoxic activity of immune cells [9]. However, the effect of low pH on the expression of different chemokine receptors has not been studied.

The data presented in this chapter are preliminary. We report here that a brief (30 min) burst of decreased pH induces altered migration patterns as inferred from changes in expression of

chemokine receptors that last for 7 days. In leukemia T cells, transient low pH has dramatic effect on CXCR4 expression as seen in **Fig. 5.3-5.4**. Low pH treatment decreased CCR7 expression in both the leukemia cells at the early time point of 6 hours (**Fig. 5.6-5.7)**. The different leukemic T cells responded to transient low pH differently as seen in CCR5 expression (**Fig. 5.9-5.10)**. Transient low pH resulted in an increase in CCR5 expression in Molt-3 but a decrease in Jurkat cells (**Fig. 5.9-5.10)**. However, low pH treatment had no effect on chemokine receptor expressions or homing patterns in primary T cells. Finally, it seemed that in this limited sample, normal cells and leukemia derived cells can be tuned differently by the same microenvironmental stimuli.

As a next step in the project, we wanted to investigate if chemokine receptor or homing patters in the leukemia cells could be altered by co-stimulation through different co-stimulatory molecules- CD28 and ICAM-1, at the low pH microenvironment. However, the leukemia cells we used could not be used for this purpose as Jurkat does not express ICAM-1 and Molt-3 does not express CD28. As we observed in our preliminary data chemokine receptor expression is not affected in primary T cell by low pH treatment, but we were interested in investigating if low pH influenced naïve T cell differentiation. However, the StemSep naïve T cell isolation kit that the lab had been using for many years was discontinued and replaced by a new EasySep naïve T cell isolation kit. With the EasySep naïve T cell isolation kit, we failed to isolated naïve T cells that proliferated and differentiated. Hence, we lost the ability to isolate functional naïve T cell at that time and had to discontinue the project.

To summarize, these data suggest that transient decrease in pH modulate homing and migration of cells. Studying homing patterns of T cells in low pH microenvironment would help predict T cell migration patterns in tumor or inflammatory locus and this information could play an important role in development of possible immunotherapies for cancer and infections.

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Chapter 6

Summary and future directions

In **Chapter 2** of this dissertation, we examined signaling differences between two co-stimulatory molecules by analyzing transcription factors and proteins that are activated downstream of these co-stimulations. Our lab has previously shown that the choice of co-stimulatory molecule influences the differentiation outcome. The main goal of this project was to analyze if the different co-stimulatory molecules utilized different signaling pathways to tune differentiation outcomes of naïve T cells.

In this project, we observed that the two co-stimulations, CD28 and ICAM-1, induced activation of different sets of proteins, suggesting that each co-stimulatory molecule has its own characteristic signaling profile. For protein phosphorylation, we observed that receptor tyrosine kinases Dtk and FGFR1 were phosphorylated upon CD28 co-stimulation and IGF-1R and HGFR were phosphorylated upon ICAM-1 stimulation. All four proteins play crucial roles in cell proliferation, differentiation and survival. For transcription factor activation, we observed that FAST-1, a crucial protein in TGFβ signaling pathway, was activated by only ICAM-1 co-stimulation, and not by CD28. In the past, we have shown that ICAM-1 promoted differentiation to Treg phenotype. This project was an exploratory project to identify differences in signaling between CD28 and ICAM-1 co-stimulatory molecules.

We need to further investigate specific signaling pathways and genes for each co-stimulatory molecule. We could use pathway analysis software to investigate specific pathways downstream of each co-stimulatory molecule. Further analyses may provide in-depth studies where specific molecular events can be identified that are crucial to one pathway of differentiation. This study would provide the possibility of devising additional therapeutic approaches for controlling T cell differentiation.

In **Chapter 3**, we investigated the role of CD45 in T cell signaling. CD45 is a tyrosine phosphatase and the phosphatase activity has been extensively studied. This project focused on the role of CD45 signaling in T cells. At first, we observed that CD45 engagement induced signaling in T cells. During CD3+CD28 stimulation, Lck associated with TCR/CD3 complex for 2-5 minutes of stimulation and then the association decreased. We observed that CD45 and TCR/CD3 engagement induced prolonged Lck association during CD3+CD28+CD45 stimulation, suggesting that CD45 engagement altered T cell signaling through CD28.

It would be of interest to investigate if CD45 engagement influenced T cell differentiation outcomes. As it has been shown in the past that CD3+CD45 promote T cell proliferation, the question here arises if CD45 is a co-stimulatory molecule and how does it influence T cell differentiation. We need to further investigate if CD45 alters early signaling events induced by another co-stimulatory molecules such as ICAM-1. Comparison of the two costimulatory molecules CD28 and ICAM-1 during CD45 engagement might reveal mechanisms for differential T cell differentiation phenotypes.

In **Chapter 4**, we investigated the effect of statins on T cell functions. Based on the preliminary data, we observed that pravastatin treatment on T cell activation, proliferation and differentiation differed depending on the co-stimulation: CD28 or ICAM-1. Future directions would be to collect more n values for these observations and also analyze other T cell subsets upon statin treatment. In **Chapter 5**, we investigated the effect of low pH on chemokine receptor expression. We observed that a transient decrease in pH modulated chemokine receptor expression in leukemic cells, suggesting that low pH altered homing and migration of cells. We need to further investigate if chemokine receptor expression is altered by co-stimulation through different co-stimulatory molecules- CD28 and ICAM-1 at the low pH microenvironment.