

**Novel roles for CD23, CTLA-4 and lipoproteins in human T cell function and differentiation**

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 15, 2014

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Date approved: April 15, 2014

## Abstract

The differentiation of multipotent naïve T cells is influenced by the microenvironment. Cytokines, costimulatory proteins and other biological factors can tune this differentiation process, influencing cell fate. Controlling naïve T cell differentiation can guide the type of immune response elicited since each T cell subset has a specific function in the immune system. In this dissertation, I examine the participation of costimulatory molecules expressed on the surface of naïve CD4<sup>+</sup> T cells in differentiation of these cells. I demonstrate that stimulation through the TCR and CTLA-4 directs naïve CD4<sup>+</sup> T cell differentiation to regulatory T cells in the absence of exogenous cytokines. I evaluate how combined stimuli influence differentiation and function in T cells. Combined stimulation through CTLA-4 and CD28 (CD3+CD28+CTLA-4) results in distinct functional outcomes from combined stimulation through CTLA-4 and ICAM-1 (CD3+ICAM-1+CTLA-4) or alternatively, ICAM-1 and CD28 (CD3+CD28+ICAM-1). Differentiation to Th1, Th2 or Treg cells has been evaluated in our previous studies. In this dissertation, I extend these studies by examining whether costimulation in the absence of exogenous cytokines can support Th17 differentiation. I found that costimulation through ICAM-1, CD28, LFA-1, or CTLA-4 did not activate naïve CD4<sup>+</sup> T cells to differentiate to Th17 cells. I also evaluated how the low affinity IgE receptor, CD23, expressed on T cells participates in naïve T cell differentiation and mature T cell function. CD23 functions as a negative regulator of Th2 responses by inhibiting differentiation of naïve T cells to Th2 cells and enhancing activation of Th1 cells in mature T cells. Next, I examined how lipoproteins relevant to atherosclerosis may guide differentiation of naïve CD4<sup>+</sup> T cells. Consistent with their known roles in promoting or inhibiting atherosclerosis, oxLDL activates differentiation to Th1 cells

whereas HDL inhibits T cell proliferation and viability. Different responses by naïve T cells to these lipoproteins may have direct implications in how atherosclerosis is exacerbated or attenuated during disease. Finally, I explore whether autoimmunity is involved in a mouse model of emphysema. I demonstrate that T cells adoptively transferred emphysema to naïve, immunodeficient mice, supporting a role for autoimmunity in an elastase-induced model. Peptide therapy targeting the interactions between ICAM-1 and LFA-1 was able to reduce severity of emphysema as well.

This dissertation is dedicated to my mom, Carol.

Thank you for always being my cheerleader.

## Acknowledgements

Words cannot convey how grateful and appreciative I am to all involved in the successful completion of my degree and my metamorphosis into an independent scientist. “It takes a village to raise a child”. Well, it also takes a village to train a scientist and help her complete a doctoral degree, especially a gal from Texas!

First, Steve, thank you for five years of coaching. I am truly grateful. Not only am I a confident and well-trained immunologist because of your “full-service” education, but I am a better speaker, writer, manager, teacher, and person. Some of the most difficult lessons were the most beneficial in helping me develop tougher skin so that I can take the harsh criticism of the world. Thanks for caring more about my future happiness and success than my momentary comfort to provide me with hard lessons. To Steve and Tom, thank y’all for the insanely quick edits and comments that you provided so that I could finish my dissertation in time for graduation. Tom, thank you for being like a second mentor to me, for your expertise, and for your encouragement. I appreciate the time and effort that you put into my graduate training. To Marci, you are so awesome. I am honored to have had the opportunity to collaborate and learn from you. Thank you, Marci, for all your assistance with the emphysema and CD23 projects and for the many years of helping us trouble-shoot experimental issues. It was always a joy to be in your presence.

To my wonderful labmates: Abby, Kelli, Mandie, Derek, Morgan, and Anuja, thank y’all for being my family during graduate school and making lab fun. Capsid tennis, Christmas parties, lab BBQs, throwing darts at each other, and getting a farmer’s tan for a year are among the many lab shenanigans that I will miss! Kelli and Abby, thank you for training me on impeccable tissue culture and sterile technique, and for answering so many questions during my

first years of graduate school. Abby, aka “Man Hands”, you are such an amazing person, and I have missed you dearly this past year. Thank you, Abby, for teaching me the art of letting things go. Mandie, I have so enjoyed getting to know you and work with you. Teaching immunology lab with you was a such a blast! Derek, you are an incredible friend and scientist. Thank you for the many times that you’ve watched my precious pups, Peaches and Leroy, and for all your contributions to the emphysema project including endless hours scoring pulmonary tissue sections.

Last but not least, I thank my family. Thank you for the love and support not just through graduate school but throughout my entire life. You played an essential role in getting me here. Mom, you have always and will continue to be my greatest fan. Thank you for believing in me even when I did not. Cameron, thank you for understanding and embracing all of who I am; thanks for being the perfect balance and grounding that I needed to keep me sane especially this last year.

To my village: I love y’all. Thank you. Thank you. Thank you.

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## **Chapter 1**

### **Introduction, Background, and Significance**

## **Overview of adaptive immune response and T cells**

The immune system can be divided into two main branches: the innate immune response and the adaptive immune response. The innate immune response elicits a quick, robust response and mostly recognizes pathogens nonspecifically. The adaptive immune system provides memory and can distinguish between non-self and self-antigens with high specificity. Adaptive immune cells include lymphocytes (T and B cells) and APCs (antigen presenting cells). Lymphocytes undergo gene rearrangement resulting in diverse repertoire of distinct BCR (B cell receptor) or TCR (T cell receptor) specificities to facilitate immune surveillance and clearance of pathogens. B cells produce antibodies which assist in targeting foreign antigens for clearance by complement or other immune cells such as neutrophils and macrophages. T cells, unlike B cells, require cognate antigen (Ag) to be presented in the context of MHC (major histocompatibility complex) on APCs (antigen presenting cells) to activate. Dendritic cells and activated macrophages and B cells can serve as APCs for T cells.

### *CD4+ and CD8+ T cells*

T cells can be divided into either CD4+ T cells (helper T cells) or CD8+ T cells (cytotoxic T cells) based on the type of MHC molecule that they engage. CD8+ T cells are MHC I-dependent and can interact with nearly all somatic cells. CD8+ T cells target and kill infected or altered cells (such as damaged or tumor cells). CD4+ T cells engage MHC II and therefore, are primarily limited to interactions with APCs. CD4+ T cells help orchestrate the immune response, activating several other types of immune cells such as CD8+ T cells, B cells, macrophages, etc. depending on the response necessary to protect the host.

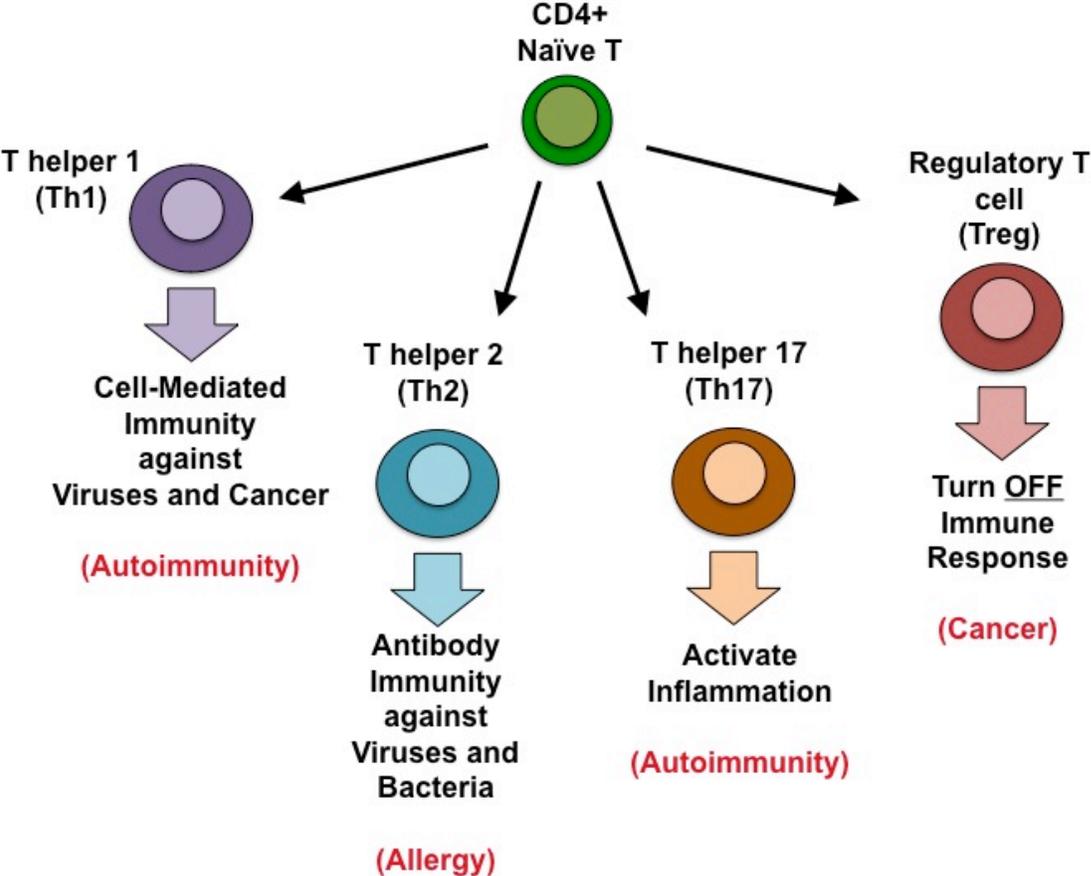
### *T helper subsets*

Several types of CD4<sup>+</sup> T cells subsets have been defined such as: T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T cells (Treg cells), each responsible for regulating a specific area of the immune system by different effector functions. See **Fig. 1.1** for brief description of the function of each subset in the immune response (black text) and the associated pathology associated with each subset when misdirected (red text). The balance of these subsets is important for protection against infection, cancer, allergies and autoimmunity. Therefore, understanding how these cells differentiate into the various subsets is important.

### *Naïve CD4<sup>+</sup> T cell differentiation*

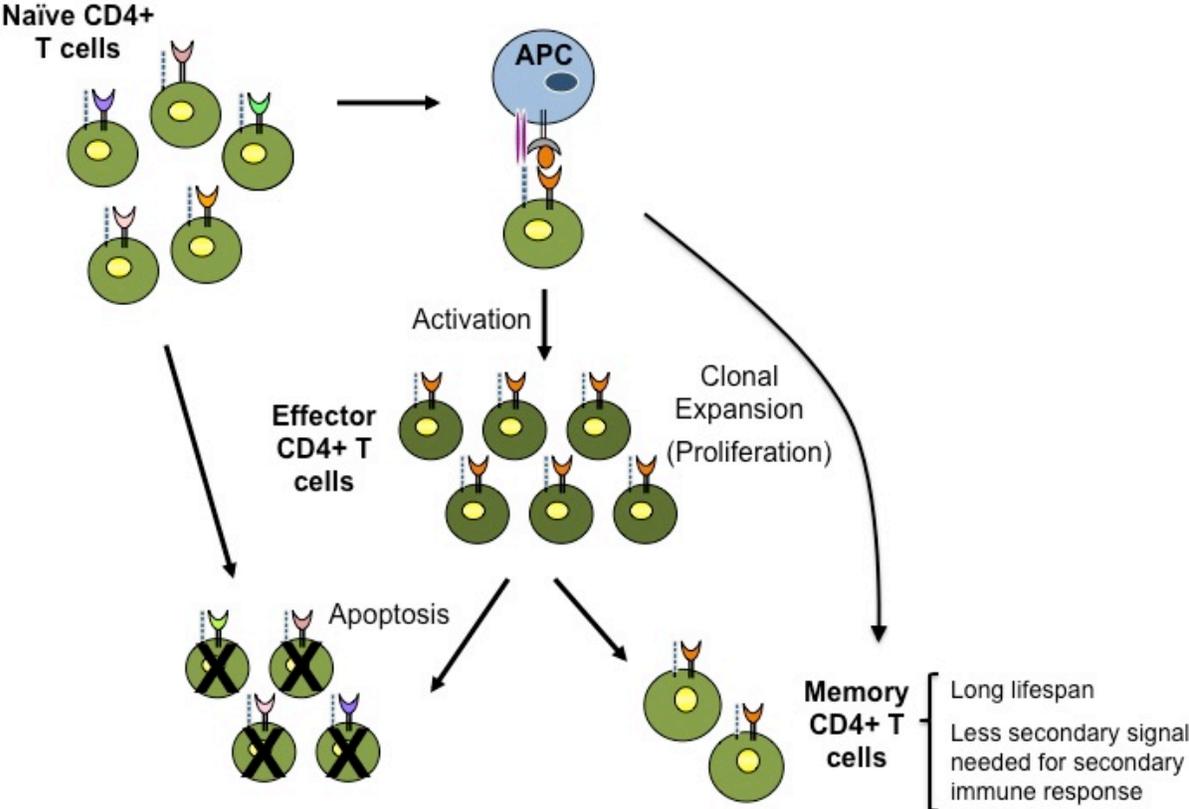
Naïve T cells represent a resting and less differentiated T cell subset that has recently emigrated from the thymus. Naïve cells circulate through the blood and secondary lymphoid organs in search for cognate antigen. If a naïve T cell does not find its specific antigen, it will undergo apoptosis in 6 weeks. If cognate antigen is appropriately presented by an APC, the naïve T cell will activate, clonally expand and differentiate into both effector and memory T cells. Effector T cells are highly activated, differentiated T cells that live for a short time, secreting cytokines, chemokines, and other factors to elicit a primary immune response. They typically live for a week or so, leaving behind memory T cells which can live for many years. See **Fig. 1.2** for model of naïve T cell differentiation to effector and memory T cells during a primary immune response. Memory T cells may differentiate indirectly through effector T cells or directly from naïve T cell populations and can remain in a resting state searching for cognate antigen for decades. Depending on expression of migration proteins, CD62L and CCR7,

Fig. 1.1



**Figure 1.1. Naïve CD4+ T cells can differentiate into various T helper subsets.** Th1 cells control the cell-mediated immune response which is important for controlling intracellular pathogens and cancer cells. Th2 cells mediate the humoral immune response during infection with various pathogens: viruses, bacteria and parasites. Th17 cells activate inflammatory responses and are particularly important in defense against pathogens in the mucosa. Treg cells help regulate the other immune cells to protect against autoimmunity and inappropriate immune responses. Normal functions of each subset are indicated by black text. Misdirected or inappropriate function of each subset can lead to various diseases indicated by red text.

Fig. 1.2



**Figure 1.2. Model of activation and differentiation of naïve CD4+ T cells to effector and memory T cells during a primary immune response.** Naïve CD4+ T cells will activate, clonally expand, and differentiate into effector and memory T cells in response to cognate antigen presented on MHC II of an APC. Naïve T cells that do not encounter cognate antigen eventually die by apoptosis. Effector T cells also die by apoptosis after carrying out their specific functions during the immune response. Memory T cells live for many years and respond more quickly and strongly during a secondary immune response than naïve T cells if antigen is encountered again.

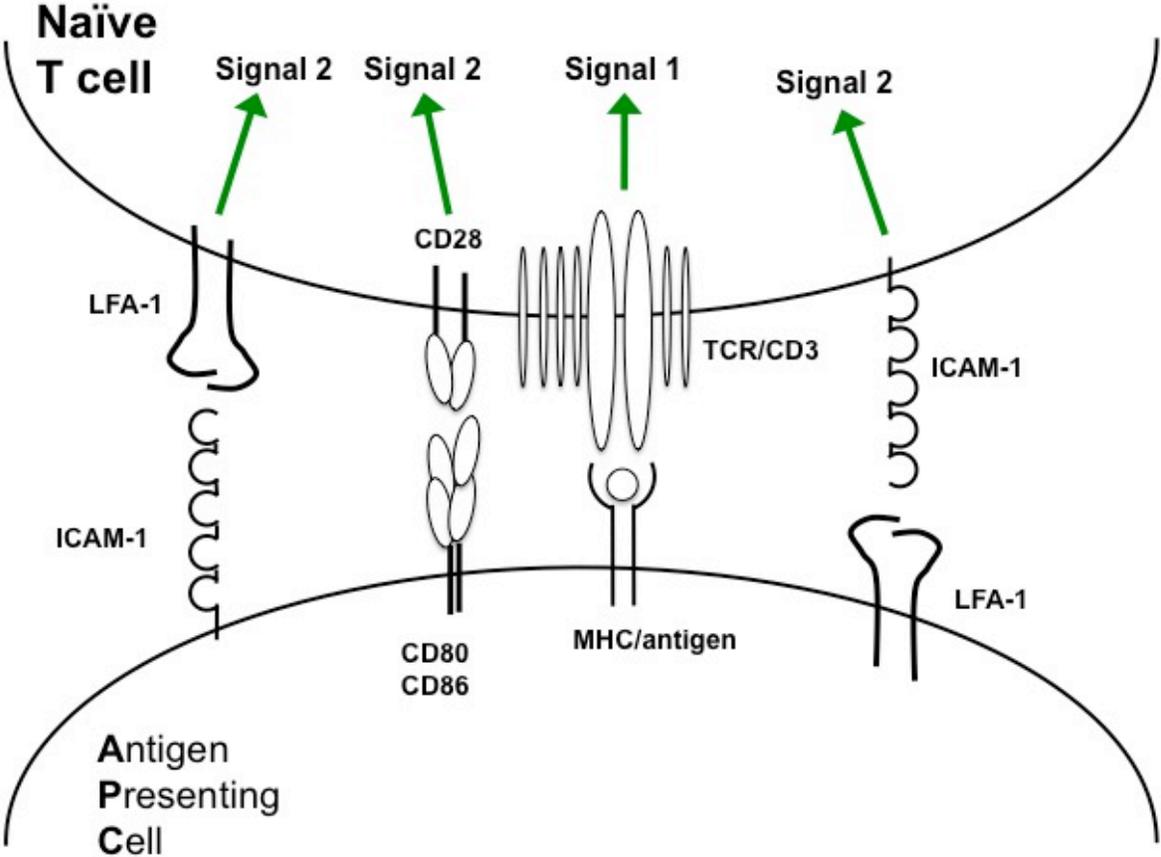
memory T cells may circulate through the secondary lymphoid tissues (central memory) or home to specific tissues (effector memory) (1). If a memory T cell encounters cognate antigen, a more robust, quicker immune response will be elicited, conferring an antigenic “memory” response to the host.

### *Factors guiding naïve T cell differentiation within the microenvironment*

Signaling through the TCR is sufficient to activate and induce proliferation of memory T cells. Naïve T cells, however, must receive signals through the TCR (signal 1) and a costimulatory molecule (signal 2) to differentiate and avoid anergy induction or death (2-5) as modeled in **Fig. 1.3**. Many different costimulatory molecules can induce this second signal, but only one second signal is required in conjunction with the TCR signal. The microenvironment of an activating naïve cell may tune differentiation to a specific T helper subset such as Th1 or Th2 cells. Cytokines are well recognized as an essential part of tuning naïve T cell differentiation. The participation of costimulatory proteins has not been as well characterized. Data from the Benedict laboratory suggests that costimulatory proteins also tune differentiation, influencing cell fate. We have demonstrated that depending on the type of costimulation received by a naïve T cell, differentiation may be directed to Th1, Th2, or Treg cells in the absence of exogenous cytokines (6-8). The results from our previous studies comparing the participation of CD28, ICAM-1, and LFA-1 in T cell differentiation are summarized in **Table 1.1** and discussed below in each description of these costimulatory molecules.

Fig. 1.3

Signal 1 (TCR/CD3) + Signal 2 (LFA-1, CD28, or ICAM-1) = Differentiation



**Fig. 1.3. Model of naïve T cell and APC interaction and signaling during activation.** In naïve T cells, signaling through the TCR (signal 1) and a costimulatory molecule (signal 2) is required for full activation and differentiation. LFA-1, CD28, or ICAM-1 interact with their counter receptors on the APC to transmit signal 2.

**Table 1.1**

	<b>CD3+CD28</b>	<b>CD3+ICAM-1</b>	<b>CD3+LFA-1</b>
<b>Proliferation</b>	✓	✓	✓
<b>Protection from apoptosis</b>	✓	✓	×
<b>Memory T cell Differentiation</b>	✓	✓	×
<b>Effector T cell Differentiation</b>	✓	✓	✓
<b>Differentiation to Th1 cells</b>	✓	✓	✓
<b>Differentiation to Th2 cells</b>	✓	×	×
<b>Differentiation to Treg cells</b>	×	✓	×

**Table 1.1. Summarizes the findings from our lab comparing costimulation through CD28, ICAM-1 or LFA-1 in the absence of exogenous cytokines.** Drs. Jacob Kohlmeier, Scott Tibbetts, Chintana Chirathaworn, Kelli Williams and Abby Dotson have contributed to these observations.

## **Review of some of the 15 known costimulatory proteins**

### *CD28*

The best-characterized costimulatory molecule is CD28, which promotes T cell proliferation and production of IL-2, an important cytokine for T cell activation and proliferation. CD28 is highly expressed on most T cells, and as a homodimer interacts with one of two counter receptors: CD80 or CD86 (B7-1 and B7-2, respectively) (9-11). Signaling through CD28 activates PI-3K, JNK, NFAT and NF $\kappa$ B leading to expression of genes that promote cell survival, proliferation, and IL-2 production (12-15). In our studies, costimulation through CD28 supported the generation of effector and memory T cells with either Th1 or Th2 function but not suppressive function (Treg cells) (6, 7) (**Table 1.1**).

### *LFA-1 (Leukocyte function-associated antigen 1)*

LFA-1, a  $\beta$ 2 integrin, functions as an adhesion molecule supporting extravasation of leukocytes into tissues and promoting the stable interaction between a T cell and APC during activation. LFA-1 also functions as a costimulatory molecule and activates the MAPK and JNK pathways inducing T cell activation, IL-2 production, and Th1 differentiation (16-19). LFA-1 is a heterodimer that consists of two subunits: CD11a and CD18, and its expression is limited to leukocytes (white blood cells). Ligands of LFA-1 include the intercellular adhesion molecules: ICAM-1, ICAM-2, and ICAM-3. Costimulation through LFA-1 in the absence of exogenous cytokines induces differentiation to effector but not memory T cells with a Th1 but not Th2 or Treg phenotype (7) (**Table 1.1**).

### *ICAM-1 (Intercellular adhesion molecule 1)*

ICAM-1 is widely expressed on many cells including endothelial and epithelial cells, monocytes, leukocytes and low levels on naïve and resting T cells (20, 21). Expression of ICAM-1 is upregulated following T cell activation and can also function as a costimulatory molecule for T cells in addition to its many roles as an adhesion protein (6-8, 21, 22). The integrins, LFA-1, MAC-1, and gp150/95, and CD43 interact with ICAM-1. The signaling events activated by costimulation through ICAM-1 are still being characterized, but stimulation through ICAM-1 leads to activation of PKC, MAPK, and various other kinases (23-25). Efforts to define the signaling complex and pathway of ICAM-1 are ongoing in our laboratory. Thus far, the Benedict laboratory has determined that cdc2 kinase and MAPK are constitutively associated with the cytoplasmic tail of ICAM-1 and following crosslinking of ICAM-1, several proteins become quickly associated with ICAM-1: ZAP-70, Fyn, Lck, TCR $\beta$  and CD3 $\zeta$  chains and then dissociate 10 minutes later (26), unpublished data). Costimulation of naïve CD4<sup>+</sup> T cells through ICAM-1 stimulates differentiation to effector and memory T cells and Th1 and Treg cells but not Th2 cells (7, 8) (**Table 1.1**).

### *CTLA-4 (Cytotoxic T lymphocyte antigen 4)*

CTLA-4 is best known as a negative regulator of T cell activation since deletion of the *ctla4* gene causes a fatal lymphoproliferative disease in mice (27). CTLA-4 regulates T cell function by suppressing proliferation and the production of IL-2 and opposing the stimulatory actions of CD28 (28-31). CTLA-4 binds the same counter receptors, CD80 and CD86, and shares similar structure to CD28 (32, 33). Competition for these counter receptors is one proposed theory for CTLA-4 suppression since it binds the B7 ligands with higher affinity than

CD28 (34, 35). CTLA-4 may also capture CD80 or CD86 from APCs by trans-endocytosis into the T cell for degradation, limiting accessibility to these ligands by CD28 (36). Although early studies indicated an inhibitory function for CTLA-4 by transducing inhibitory signals directly into the T cell expressing CTLA-4, most functions of CTLA-4 are thought to be indirect and mediated by CTLA-4-expressing cells such as Treg cells (reviewed in 37). CTLA-4 is not expressed on resting T cells but can be detected 48 hours following activation (38).

CTLA-4 has been associated with function, maintenance and generation of regulatory T cells. Treg cells constitutively express CTLA-4, and CTLA-4 is important for the suppressive function of Treg cells and expression of the Treg transcription factor, Foxp3 (39-45). Foxp3 suppresses many genes associated with T cell activation including the genes that encode IL-2, JAK2 and ZAP-70 (46). Strong expression of Foxp3 by T cells is associated with suppressive function (47). Recently, participation of CTLA-4 in the differentiation of murine naïve CD4<sup>+</sup> T cells has been demonstrated by stimulation through CD3+CD28+CTLA-4 in the presence of exogenous IL-2 and TGF- $\beta$  (48) or by incubation with LPS-treated APCs and co-ligation of CTLA-4 into the TCR complex using a bispecific protein that binds MHC on the APC and CTLA-4 on the T cell (49).

### **Dissertation: Participation of costimulatory molecules in naïve CD4<sup>+</sup> T cell differentiation (Chapters 1-3)**

One primary goal of this dissertation work was to examine how costimulation through CTLA-4 guides differentiation of human naïve CD4<sup>+</sup> T cells in the absence of any other factors. This is addressed in **Chapter 2** of the dissertation. Also, in **Chapter 2**, we examine the role of CTLA-4 as an additional stimulus combined with costimulation through either ICAM-1 or CD28

and how signaling a naïve T cell through more than one costimulatory molecule influences differentiation. In **Chapter 3**, we examine the ability of CD28, LFA-1 and ICAM-1 to activate differentiation of human naïve T cells to Th17 cells, a helper T subset, associated with directing the inflammatory response.

**Dissertation: Participation of other biological factors in naïve T cell differentiation (Chapters 4 & 5)**

In addition to our overall goal to evaluate the participation of each costimulatory molecule in T cell activation and differentiation individually, we also seek to understand how other receptors or biological factors may guide cell fate. T cells express many other receptors on their surface. If one of these receptors happens to be engaged during activation and transmits signals into the T cells, this may influence differentiation. In **Chapter 4**, we examine the participation of the low affinity receptor for IgE, CD23, in naïve T cell differentiation and mature T cell activation.

Differentiation of naïve T cells typically occurs in secondary lymphoid organs. However, under certain chronic inflammatory conditions, naïve T cells can home to tissues and undergo activation and differentiation in extra-lymphoid tissues (50-52). In these situations, other biological factors that are normally not present in the microenvironment of a lymph node or spleen may also participate in tuning differentiation of naïve T cells. This is the premise of **Chapter 5** where we examine the role of lipoproteins in naïve CD4<sup>+</sup> T cell activation and differentiation.

### *CD23, the low affinity IgE Receptor*

CD23 is primarily associated with regulating IgE synthesis and B cell responses during an allergic or parasitic response (53-55). Many cells including activated T cells express CD23 (56-58). However, the function of CD23 on T cells has not been examined. The goal of Chapter 4 is to determine how stimulation through CD23 may regulate T cell differentiation and function in naïve CD4<sup>+</sup> T cells and mature (CD45RO<sup>+</sup>) T cells.

### *Lipoproteins in atherosclerosis and T cell responses*

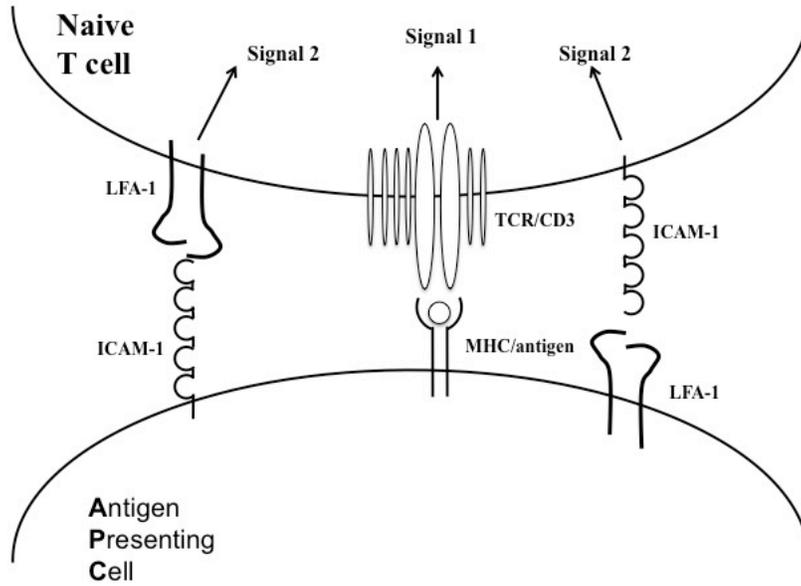
The lipoproteins, HDL (high density lipoprotein) and LDL (low density lipoprotein), exert opposing effects on the development and progression of atherosclerosis. LDL crosses the endothelium and becomes oxidized, forming oxLDL (oxidized LDL). This modified form of LDL is highly atherogenic and promotes the activation of platelets, endothelial cells, and macrophages (59-61). This leads to a robust inflammatory response and recruitment of other cells such as T cells, further exacerbating inflammation and progression of atherosclerotic plaque. HDL opposes many of these processes by inhibiting activation of platelets, macrophages, and endothelial cells, removing cholesterol from plaque, and inhibiting the oxidation of LDL (62-66). We have previously published the differential effects of lipoproteins on mixed T cell populations (naïve and mature effector and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells) in the context of either ICAM-1 or LFA-1 costimulation (67). We expand this investigation to purified naïve CD4<sup>+</sup> T cells comparing costimulation through CD28, ICAM-1 or CTLA-4 in Chapter 5.

## **Dissertation: Targeting costimulatory proteins for therapy (Chapter 6)**

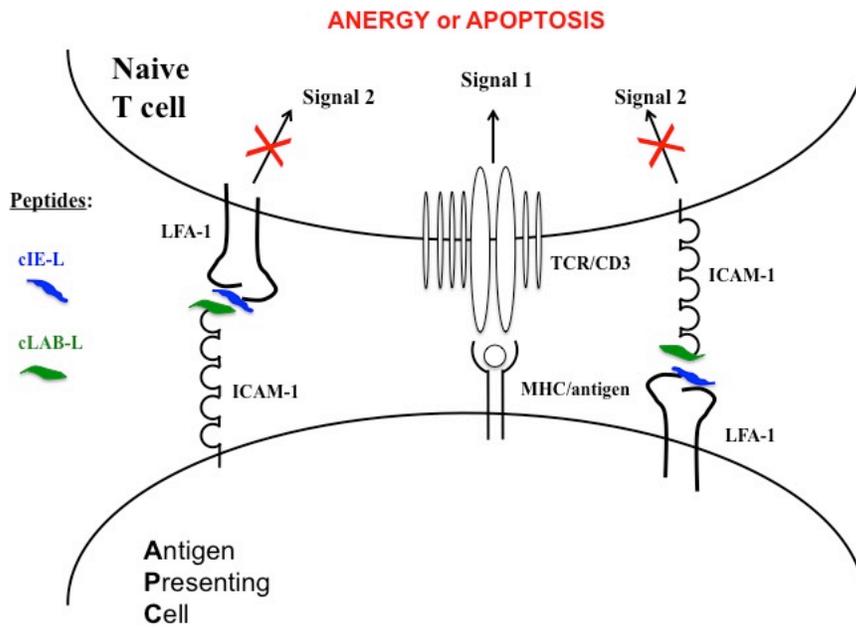
The immune system protects against pathogens and cancer and when misregulated can lead to autoimmunity or hypersensitivity (allergy). The normal protective immune response can also be harmful when elicited against a transplanted tissue or organ. T cells are the primary regulators of these immune responses. We seek to understand the molecular mechanisms guiding differentiation and function of T cells so that this knowledge can be used to develop effective and safe therapies to modulate the immune system to treat disease. By understanding the costimulatory proteins and other biological factors that guide T helper function and differentiation, we may be able to manipulate these processes *in vivo* for therapy. Our studies on the participation of ICAM-1 as a costimulatory molecule have led to the development of inhibitory peptides targeting ICAM-1:LFA-1 interaction (68-70). Each cyclic peptide consists of 10 amino acids; one binds to LFA-1 (cIE-L) and the other to ICAM-1 (cLAB-L), inhibiting T cell function and adhesion *in vitro* (68, 69). The peptides block the transduction of the second signal (generated by either ICAM-1 or LFA-1 on the T cell); without this second signal, naïve T cells become anergic or die (See **Fig. 1.4**). The combined use of these peptides attenuates murine models of autoimmunity, arthritis and diabetes (unpublished data, 71), by reducing leukocyte infiltration and suppressing antigen-specific responses by autoreactive T cells. We can treat autoimmune diseases in mice by inhibiting autoantigen-specific T cell responses through the use of this peptide therapy.

Fig. 1.4

**Without peptide therapy**



**Peptide therapy blocks second signal**



**Figure 1.4. Peptides (c-IE-L and c-LAB-L) block signal 2 which leads to anergy or apoptosis of autoreactive T cells.** The combined use of peptides, cIE-L and cLAB-L, inhibits the ability of ICAM-1 and LFA-1 to signal into the T cell expressing them, resulting in anergy or death of that cell.

### *Emphysema in COPD*

Emphysema represents one of the two major forms of chronic obstructive pulmonary disease (COPD). The other form is chronic bronchitis and not examined here. Destruction of the alveolar epithelia leads to enlarged airspaces and consequently, less efficient gas exchange making it more difficult to breathe. In patients with COPD, pulmonary inflammation and destruction continue even after smoking cessation and long after the noxious substances from cigarette smoke have been eliminated (72-74). This is suggestive of an autoimmune response against self-antigens. COPD has not been established as an autoimmune disease, but recent evidence supports a role for T cells in driving autoimmunity in this disease. CD4<sup>+</sup> T cell clones from patients with emphysema produce IL-6 (Th2 cytokine) and IFN- $\gamma$  (Th1 cytokine) in response to specific elastin peptide sequences, and the presence of these autoreactive T cells correlates with disease severity (75, 76). CD3<sup>+</sup> T cells from mice with smoke-induced emphysema transfer disease to naïve mice (77, 78). In **Chapter 6**, we evaluate the ability of T cells from an elastase-induced model of emphysema to transfer disease to immunodeficient mice and the efficacy of peptide therapy on disease severity.

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## **Chapter 2**

### **Role of CTLA-4 in T cell differentiation**

#### **Part I:**

**Costimulation through CTLA-4 induced differentiation of human naïve CD4+ T to regulatory T cells**

#### **Part II:**

**CTLA-4 enhanced naïve CD4+ T cell differentiation to memory or regulatory cells depending on the choice of costimulatory protein engaged during differentiation**

#### **Part III:**

**Costimulation through CTLA-4 did not induce differentiation of CD4+ T cells to regulatory T cells in mice**

## Introduction

CTLA-4 (cytotoxic T lymphocyte antigen-4), a member of the CD28 family of receptors, functions overall as a negative regulator of T cell activation in contrast to CD28 which induces T cell activation. Defining the precise mechanisms of T cell regulation by CTLA-4 has been challenging because CTLA-4 shares the same counter receptors, CD80 and CD86, with CD28. Multiple functions have been proposed, but there is still no consensus of how CTLA-4 functions *in vivo*. Most studies investigating the function of CTLA-4 have been performed in mice. Differences between human and mouse immunology and the complexities of studying CTLA-4 function prompted us to investigate how CTLA-4 costimulation would influence differentiation of human naïve CD4<sup>+</sup> T cells.

*CD28 and CTLA-4 share the same counter receptors but have different roles in the immune response*

Naïve T cells that have recently exited the thymus, require signals from both the TCR (CD3) interacting with the antigen:MHC complex (signal 1) and a costimulatory molecule interacting with its counter receptor (signal 2) for activation and protection against anergy (1-3). The best characterized costimulatory molecule is CD28, which promotes T cell proliferation and production of IL-2 (4-7) by engaging either B7-1 or B7-2 (CD80 or CD86, respectively) (8). CD80 and CD86 also interact with CTLA-4 which shares a similar structure to CD28 but differs considerably in function (9, 10). CTLA-4 has a much higher affinity for these counter receptors than CD28 (11, 12). However, CD28 is expressed by resting and activated T cells in contrast to CTLA-4 which is not expressed on the surface of resting T cells (excluding Treg cells) but is upregulated following activation (13).

CTLA-4, a negative regulator of T cell activation, suppresses T cell proliferation, cell cycle progression, and production of IL-2 (14-17). Hypothesized mechanisms of CTLA-4 suppression included direct inhibitory signaling (15, 16), limiting access of CD28 to B7 ligands (CD80 and CD86) either through sequestration (18) or transendocytosis of the counter receptors from the surface of APCs (antigen presenting cells) (19). However, some evidence supports a positive effect on T cell proliferation by CTLA-4, opposing theories of CTLA-4 strictly as an inhibitory molecule (13, 20). Ultimately, the function of CTLA-4 was affirmed inhibitory since CTLA-4 knockout mice develop a fatal lymphoproliferative disorder (21), but this observation was made before the role and function of regulatory T cells (Treg cells) was well defined. Mice deficient in Foxp3, the transcription factor associated with Treg cells, develop a similar lymphoproliferative disease (22) to mice deficient in CTLA-4, suggesting a role for CTLA-4 in regulatory T cell function or development.

*CTLA-4 is important for the function of Treg cells and may play a role in Treg development or differentiation*

Although there has been some controversy over the role of CTLA-4 in Treg function in the past, it is becoming more widely accepted that CTLA-4 is vital for the suppressive function of Treg cells and possibly development. Suppressive function of Treg cells is largely dependent on constitutive expression of CTLA-4 (23-28). Deletion of CTLA-4 in Treg cells results in the loss of their suppressive function and the development of systemic lymphoproliferation (29), similar to what is observed in either Foxp3 or CTLA-4 deficient mice. Blocking CTLA-4 on Treg cells *in vitro* and *in vivo* eliminates their inhibitory activity (30-32). Furthermore, induced expression of CTLA-4 in T cells confers suppressive function (33). Currently, the thought is that

CTLA-4 mostly functions as an indirect regulator of T cell activation by inhibiting APC function, rather than transmitting direct inhibitory signaling into the T cell where it is expressed (34-36).

CTLA-4 may also play an important role in Treg differentiation. CTLA-4 promotes both *in vitro* and *in vivo* induction of Foxp3, the major transcription factor of Treg cells, and is required for TGF- $\beta$ -dependent induction of Foxp3 (37, 38). Engagement of CTLA-4 in the presence of IL-2 and TGF- $\beta$  or co-ligation of both the TCR and CTLA-4 through a bispecific protein induces differentiation of naïve T cells to adaptive CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg cells) in mice (38, 39). Whether CTLA-4 costimulation can induce human naïve CD4<sup>+</sup> T cell differentiation to Treg cells and do so independently of exogenous cytokines remains to be determined.

*In vitro model of differentiation to determine how CTLA-4 participates in the differentiation of human naïve CD4<sup>+</sup> T cells*

Defining the precise role of CTLA-4 in T cell differentiation is challenging because CTLA-4 and CD28 share the same counter receptors. Therefore, we used a reductionist approach as we previously described (40, 41) in which immobilized antibodies specific for the TCR and a costimulatory molecule model the activation of naïve T cells. Using this system, we have shown that the choice of costimulatory molecule differentially influences human naïve CD4<sup>+</sup> T cell differentiation (40, 41). We have shown that costimulation through CD28 induces differentiation to Th1 and Th2 cells but not Treg cells while costimulation through ICAM-1 promotes differentiation to Th1 and Treg cells not Th2 (40, 41). We used this *in vitro* system to investigate the effects of stimulation through CTLA-4 on human naïve CD4<sup>+</sup> T cell

differentiation in the absence of exogenous cytokines. We hypothesized that stimulation through CTLA-4 would support generation of Treg cells. Consistent with previous work in mice (39, 40), CTLA-4 and TCR (CD3) engagement of human naïve CD4<sup>+</sup> T cells induced differentiation to CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup> (Treg) cells capable of secreting IL-10, a suppressive cytokine secreted by Treg cells. Unique to other studies, we show that CTLA-4 costimulation induced Treg differentiation in human cells without the need for exogenous cytokines (See model of proposed CTLA-4 function as a costimulatory molecule in **Fig. 2.1-2.2**). In part I, we investigated the role of CTLA-4 as a costimulatory molecule (CD3+CTLA-4). In part II, the participation of CTLA-4 in conjunction with either CD28 or ICAM-1 costimulation was addressed (CD3+CD28+CTLA-4 and CD3+ICAM-1+CTLA-4) to determine how differentiation is modulated by stimulation through multiple costimulatory proteins. In part III, we examined whether costimulation through CTLA-4 could induce differentiation of murine CD4<sup>+</sup> T cells to regulatory T cells.

Fig. 2.1

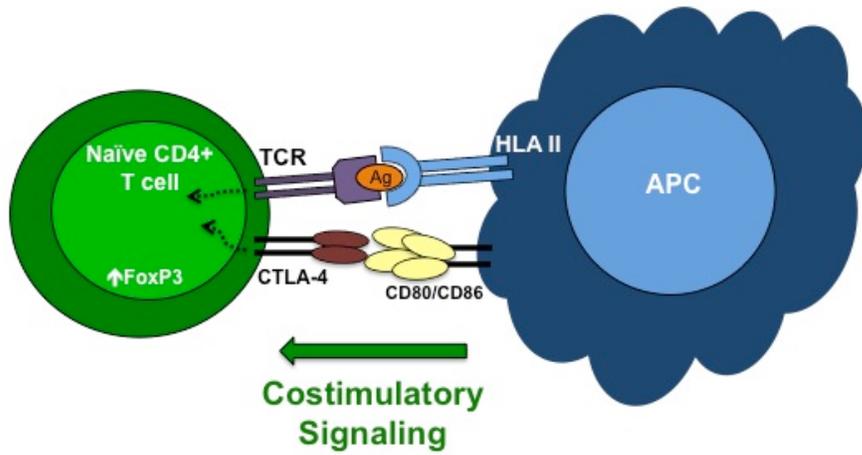
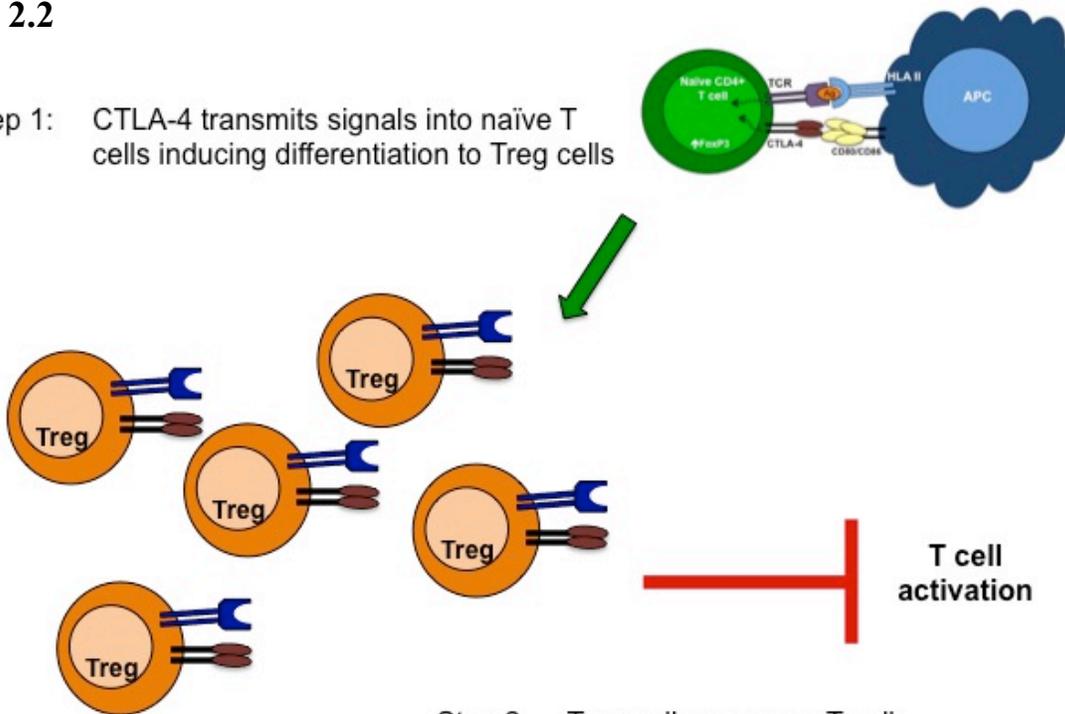


Fig. 2.2

Step 1: CTLA-4 transmits signals into naïve T cells inducing differentiation to Treg cells



Step 2: Treg cells suppress T cell activation

**Figures 2.1-2.2. Model of our hypothesis for a two-step process by which CTLA-4 functions as a negative regulator of T cell responses by inducing Treg differentiation. Fig. 2.1.** Step 1: CTLA-4 functions intrinsically by transmitting signals to induce activation and differentiation of human naïve CD4<sup>+</sup> T cells to regulatory T cells (Treg cells). **Fig. 2.2.** Step 2: CTLA-4 functions as a negative T cell regulator by generating a Treg population that suppresses T cell activation through various mechanisms.

## **Materials and Methods**

### *Antibodies and reagents*

Antibodies used to stain cells for flow cytometry were: anti-CD11a-FITC (BD Biosciences, San Jose, CA), anti-CD27-PE (Invitrogen, Carlsbad, CA), anti-CD27-PECy5 (eBioscience, San Diego, CA), anti-CD45RO-APC (Invitrogen), anti-CD45RO-Tri Color (Invitrogen), anti-Foxp3-PE (Miltenyi Biotec, Auburn, CA), and anti-CD25-Tri Color (Invitrogen). eBioscience Foxp3 buffers and protocol were used for intracellular staining of Foxp3. Cells were stained with 2.5  $\mu$ M 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen) on day 0 of stimulation for proliferation assays and stained on day 7 with 7-AAD (7-aminoactinomycin D, BD Biosciences) and Annexin V-PE (BD Biosciences) for cell death analysis. Flow cytometry was performed using an Accuri C6 (BD Accuri Cytometers, Ann Arbor, MI) and data analysis using CFlow (Accuri). Antibodies used for cytokine blocking were anti-IL-2 (5334; R&D Systems, Minneapolis, MN) and anti-TGF- $\beta$ 1 (9016; R&D Systems) with mouse IgG1 (eBioscience) as the isotype control. Clones and concentrations of stimulating antibodies used in the mouse studies are listed under *Murine CD4<sup>+</sup> T cell stimulation* (see below).

### *Human Subjects*

Peripheral blood (240 ml into heparin) was obtained after informed consent from healthy adult volunteers of both genders, ages 20-30 years, who had been free of infection for 14 days and did not have immune disorders. Procedures were approved by the University of Kansas Institutional Review Board.

### *Human naïve CD4+ T cell purification*

Human naïve CD4+ T cells were isolated from peripheral blood of healthy adult volunteers. Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) density gradient centrifugation yielded peripheral blood mononuclear cells (PBMCs), and then naïve T cells were negatively selected using the Human Naïve CD4+ T Cell Enrichment Kit from STEMCELL Technologies (Vancouver, BC) as previously described (40). Naïve T cells were defined as CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>(-)</sup>CD11a<sup>lo</sup> CD27<sup>+</sup>CCR7<sup>+</sup>CD62L<sup>+</sup>. Routinely, >98% CD45RO<sup>(-)</sup>CD11a<sup>lo</sup>CD27<sup>+</sup> naïve CD4+ T cell purity was obtained. Yields varied with human subjects but typically averaged 20 million naïve CD4+ T cells from 240 ml peripheral blood. Cells were cultured immediately in complete RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (Invitrogen), 50 units/ml penicillin, and 50 µg/ml streptomycin (P/S, Invitrogen) at 37°C, 5% CO<sub>2</sub>.

### *Human Naïve CD4+ T cell stimulation*

Human naïve CD4+ naïve T cells were stimulated through plate-bound antibodies as we have previously described (40). Each antibody was titrated to the lowest concentration that gave maximum T cell proliferation: anti-CD3 (OKT3) used at 1 µg/ml (eBioscience), anti-CD28 (ANC28.1/5D10) used at 2-3 µg/ml (Ansell, Bayport, MN), anti-ICAM-1 (R6.5) used at 10 µg/ml (BioXcell, West Lebanon, NH), and anti-CTLA-4 (BN13) used at 20 µg/ml (BD Biosciences or BioXcell). Stimulating antibodies in PBS were adhered to flat-bottom, 96-well plates for 2-3 hours at 37°C. Plates were washed three times with PBS to remove unbound antibody. Naïve CD4+ T cells were plated at 1.5 x10<sup>6</sup> cells/ml in 200 µL complete RPMI 1640 and stimulated 7-14 days using: anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-

CTLA-4, anti-CD3+anti-CD28+anti-CTLA-4, anti-CD3+anti-ICAM-1+anti-CTLA-4, or anti-CD3. In selected experiments where indicated either exogenous cytokines or blocking cytokine antibodies were added at the start of stimulation. Recombinant human cytokine TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN) was used at 10 ng/ml and IL-2 (Boehringer Mannheim, Germany) at 10 U/ml. To block endogenous cytokines, anti-IL-2 (R&D Systems), anti-IL-10 (R&D Systems) or isotype control (mouse IgG1 for anti-IL-2 or ratIgG1 for anti-IL-10) was added at 20  $\mu$ g/ml on day 0.

#### *Mice and cells*

10-week-old female C57Bl/6 mice (n =3) were used in part III. The University of Kansas Institutional Animal Review Board approved all animal experiments. Spleens were harvested, minced and pressed through a sterile 70  $\mu$ m nylon mesh cell strainer. Splenocytes were incubated at room temperature for 5 minutes in ACK lysis buffer (0.15 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA, pH 7.2) to remove erythrocytes. CD4<sup>+</sup> T cells were negatively selected using the Mouse CD4<sup>+</sup> T Cell Enrichment Kit from STEMCELL Technologies and were routinely  $\geq$ 98% pure.

#### *Murine CD4<sup>+</sup> T cell stimulation*

Murine CD4<sup>+</sup> naïve T cells were stimulated through plate-bound antibodies as we have previously described (40). Each antibody was titrated to the lowest concentration that gave maximum T cell proliferation: anti-CD3 (500A2, BD Biosciences) used at 0.5  $\mu$ g/ml, anti-CD28 (37.51, BD Biosciences) used at 2.5  $\mu$ g/ml, anti-ICAM-1 (KAT-1, eBioscience) or anti-ICAM-1 (YN1/1.7.4, eBioscience) used at 10  $\mu$ g/ml, and anti-human CTLA-4 (BN13, BD Biosciences or

BioXcell) or anti-murine CTLA-4 (9H10, BioLegend) used at 20 µg/ml. Stimulating antibodies were plated as described above for naïve CD4<sup>+</sup> T cell stimulations. Murine CD4<sup>+</sup> T cells were plated at 1.5 x10<sup>6</sup> cells/ml and stimulated 5 days using: anti-CD3, anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 (KAT-1), anti-CD3+anti-ICAM-1 (YN1.1.7.4), anti-CD3+anti-msCTLA-4 (9H10), anti-CD3+anti-huCTLA-4 (BN13), anti-CD3+anti-CD28+anti-msCTLA-4, anti-CD3+anti-CD28+anti-huCTLA-4, anti-CD3+anti-ICAM-1(KAT)+anti-msCTLA-4, anti-CD3+anti-ICAM-1(KAT)+anti-huCTLA-4, anti-CD3+anti-ICAM-1(YN1)+anti-msCTLA-4, or anti-CD3+anti-ICAM-1(YN1)+anti-huCTLA-4. Exogenous rh-IL-2 and rhTGF-β1 were added to select cultures stimulated through CD3+CD28 to serve as a positive control for Treg differentiation. Recombinant human cytokine TGF-β1 (R&D Systems) was used at 10 ng/ml and IL-2 (Boehringer Mannheim) at 10 U/ml.

### *ELISA*

Supernatant fluid from stimulated cells was collected at day 7 or 10 for analysis of IL-2 and IL-10 production using the Ready-Set-Go! ELISA kits and protocol (eBioscience).

Supernates from day 7 were collected to determine the production of TGF-β1 or soluble CD25 using Quantikine ELISA kits from R&D Systems. Plates were read at 450 nm using the EL311 Microplate Autoreader (Bio-Tek Instruments, Inc., Winooski, VT).

### *Statistical Analysis*

The one-way, paired student's *t*-test was used to determine statistical differences unless otherwise indicated. All statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

## Results

### Part I

#### *Costimulation through CTLA-4 activated differentiation of human naïve CD4+ T cells to regulatory T cells*

We observed previously that in the absence of exogenous cytokines, costimulation of human naïve CD4+ T cells through CD3+ICAM-1 induces differentiation to Treg cells in contrast to CD3+CD28 (41). CTLA-4 has been implicated in the maintenance of Treg function and the induction of Foxp3 expression in mice (30, 37, 42). Ligation of CTLA-4 in the presence of other stimuli has induced Treg differentiation in murine naïve T cells (38, 39). Therefore, we investigated whether stimulation of human naïve CD4+ T cells through the TCR (CD3) and CTLA-4 would promote differentiation to Treg cells. Treg cells can be identified by expression of CD25 (alpha subunit of the IL-2 receptor) and Foxp3 (Treg transcription factor). Naive CD4+ T cells stimulated for 7 or 10 days were assessed for the coexpression of these Treg markers: CD25 and Foxp3. Consonant with our previous work (41), ICAM-1 costimulation guided naïve T cell differentiation to cells with a Treg phenotype (CD25+Foxp3<sup>hi</sup>) (**Fig. 2.3-2.4**). As published previously (41, 43), stimulation through CD3 or CD3+CD28 did not induce differentiation to a Treg (CD25+Foxp3<sup>hi</sup>) population (**Fig. 2.3-2.4**). Stimulation of human naïve CD4+ T cells through CD3+CTLA-4 induced differentiation to a Treg population similar in manner to CD3+ICAM-1 (**Fig. 2.3-2.4**).

One mechanism by which Treg cells inhibit other T cells is through the secretion of suppressive cytokines, TGF- $\beta$  and IL-10. IL-10 can limit the production of IL-2, thereby suppressing T cell activation (44, 45). These cytokines also participate in Treg differentiation

Fig. 2.3

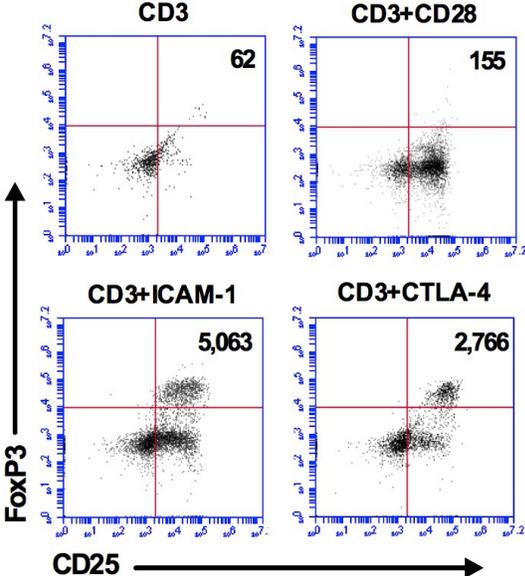


Fig. 2.4

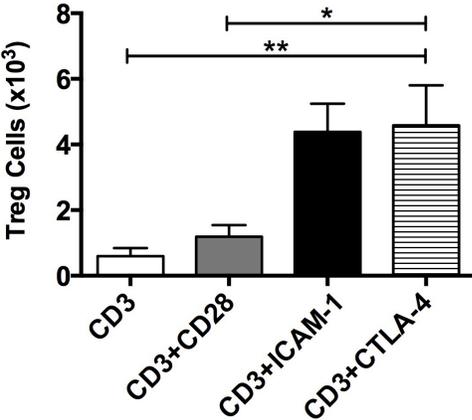
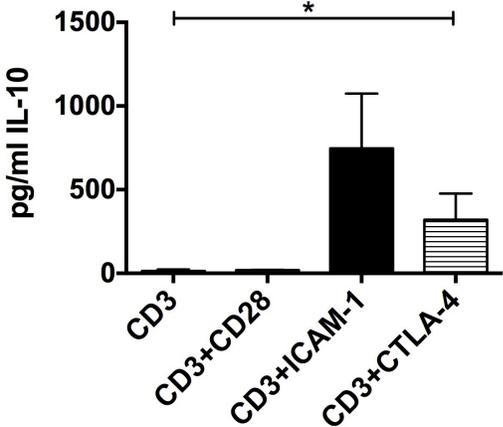


Fig. 2.5



**Figures 2.3-2.5. Costimulation through CTLA-4 induced differentiation of human naïve CD4+ T cells to regulatory T cells.** Human naïve CD4+ T cells stimulated for 7 or 10 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 were stained for CD25 and strong Foxp3 and analyzed by flow cytometry. Regulatory T cells (Treg cells) are defined as CD4+CD25+Foxp3<sup>hi</sup>. Supernates were collected for analysis of IL10 production by ELISA. **Fig. 2.3**, Upper right quadrant identifies regulatory T cell population with cell numbers indicated. **Fig. 2.4**, average number of CD4+CD25+Foxp3<sup>hi</sup> T cells ± SEM at days 7 or 10. Representative of fifteen (CD3+CD28; CD3+ICAM-1) or thirteen (CD3+CTLA-4; CD3) experiments. **Fig. 2.5**, Average production of IL-10 pg/ml ± SEM at days 7 or 10. Representative of two (CD3+CD28) or nine (CD3+ICAM-1; CD3+CTLA-4; CD3) experiments.

\* p < 0.05, \*\* p < 0.01

(46-48). TGF- $\beta$  and IL-10 production was measured to determine whether Treg phenotype (expression of CD25 and strong Foxp3) was accompanied with Treg function. Supernates from the various stimuli were collected on day 7 or day 10 for analysis by ELISA. Consistent with our previous work (41), stimulation through CD3+CD28 yielded minimal IL-10 ( $19 \pm 2$  pg/ml) whereas CD3+ICAM-1 generated substantial IL-10 secretion ( $746 \pm 328$  pg/ml) (**Fig. 2.5**). High levels of IL-10 were also produced by cells costimulated through CD3+CTLA-4 ( $321 \pm 157$  pg/ml) and did not statistically differ from CD3+ICAM-1 (**Fig. 2.5**;  $p = 0.06$ ). As expected, we detected negligible amounts of IL-10 ( $14 \pm 9$  pg/ml) by cells stimulated through only the TCR (CD3). Thus, in this system, the appearance of a Treg phenotype was accompanied by secretion of IL-10, supporting a role for CTLA-4 in guiding differentiation of human naïve T cells to Treg cells.

TGF- $\beta$  production was also measured but was not a reliable indicator of Treg function in this *in vitro* system as we previously reported (41). Although CD28 costimulation failed to support Treg differentiation, cells costimulated through CD28 secreted high levels of TGF- $\beta$  similar to costimulation through ICAM-1 or CTLA-4 (data not shown).

#### *CTLA-4 costimulation of naïve CD4<sup>+</sup> T cells generated effector and memory T cell populations*

In addition to the Treg population induced by CTLA-4, we observed a CD25<sup>+</sup> Foxp3<sup>lo</sup> population at days 7 and 10, suggesting that costimulation through CTLA-4 may also induce differentiation of naïve T cells to classically defined memory and effector T cells (49). Consistent with our previous work (40), human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days through either CD3+CD28 or CD3+ICAM-1 differentiated to both effector [CD45RA(-)RO<sup>+</sup>, CD11a<sup>hi</sup>, CD27<sup>hi</sup>] (upper right quadrants in **Fig. 2.6, 7 days** and **Fig. 2.9, 14 days** summarized in

**Fig. 2.7, 7 days** and **Fig. 2.10, 14 days**) and memory [CD45RA(-)RO+CD11a<sup>hi</sup>CD27<sup>lo</sup>] (lower right quadrants in **Fig. 2.6, 7 days** and **Fig. 2.9, 14 days** summarized in **Fig. 2.8, 7 days** and **Fig. 2.11, 14 days**). Stimulation through CD3+CTLA-4 promoted differentiation to both effector and memory T cells that was comparable to ICAM-1 costimulation on day 7 ( $p = 0.13$ ) but significantly higher than stimulation through CD3 ( $p < 0.05$ ) and lower than CD3+CD28 ( $p < 0.01$ ) (**Fig. 2.6-2.8**). As we have previously observed (40, 41), costimulation through CD28 demonstrates faster kinetics of activation than ICAM-1. The greater number of effector and memory T cells generated by stimulation through CD28 on day 7 (**Fig. 2.7-2.8**) is consistent with these studies, and by day 14, costimulation through ICAM-1 or CTLA-4 have attained comparable levels of differentiation to CD28 (**Fig 2.10-2.11**).

#### *Costimulation through CTLA-4 supported the generation of central memory T cells*

Memory T cells [CD45RO+CD45RA(-)CD11a<sup>hi</sup>CD27<sup>lo</sup>] can be classified into two major subtypes: effector memory or central memory, which differ in their activation states and homing patterns (50). Central memory T cells express CCR7 and CD62L allowing them to home to secondary lymphoid organs such as lymph nodes in contrast to effector memory cells that express lower levels of CCR7 and CD62L and wander widely through the body (51). We compared the ability of CTLA-4 to generate central memory T cells to CD28 and ICAM-1 by staining for CD27, CD62L and CD45RO on days 7 and 14 (**Fig. 2.12-2.13**). Costimulation through CTLA-4 supported a slightly elevated frequency of central memory T cells (CD45RO+CD27(-)CD62L<sup>hi</sup>) compared to CD28 ( $p < 0.01$ , 7 days;  $p < 0.05$ , 14 days) but did not differ from ICAM-1 ( $p = 0.06$ , 7 days;  $p = 0.48$ , 14 days). Although statistically different,

Fig. 2.6

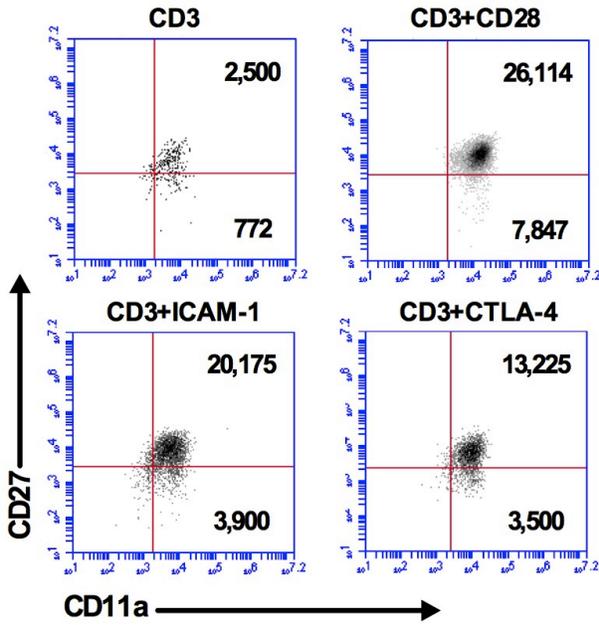


Fig. 2.7

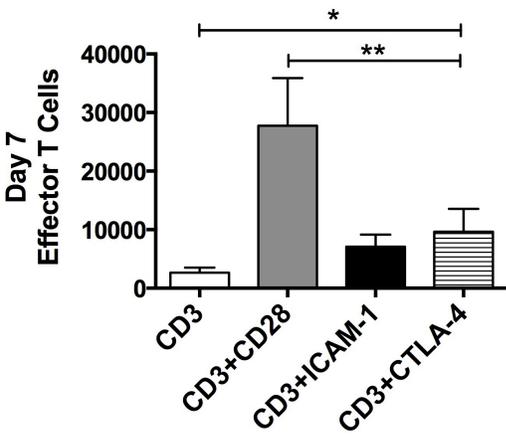


Fig. 2.8

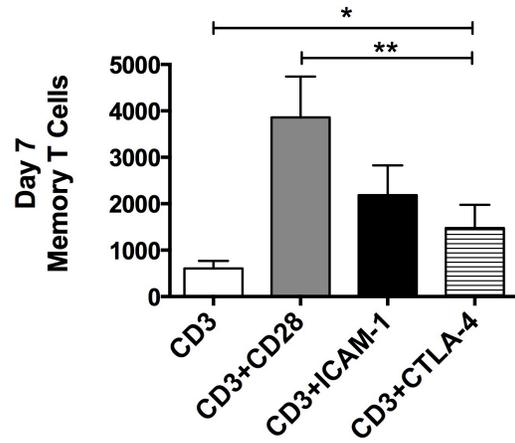


Fig. 2.9

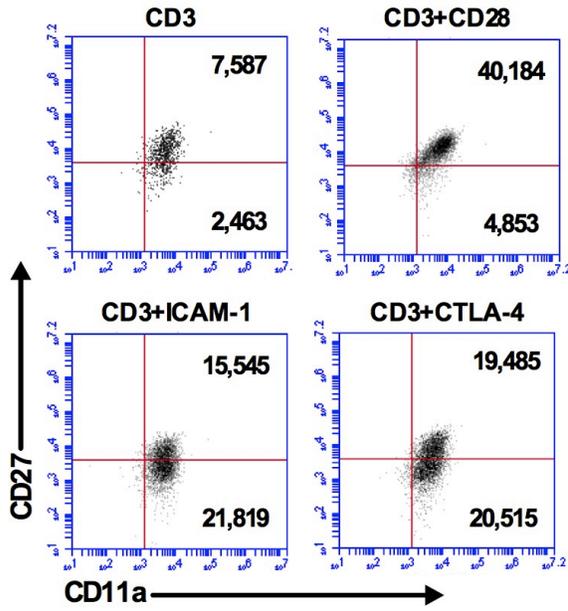


Fig. 2.10

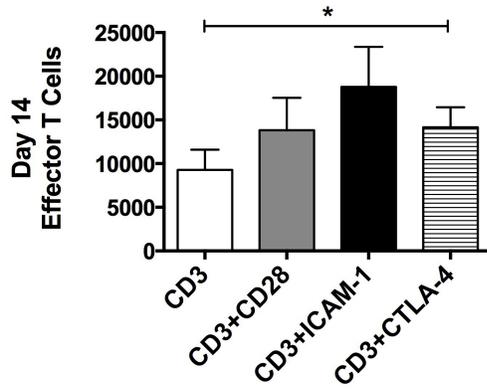
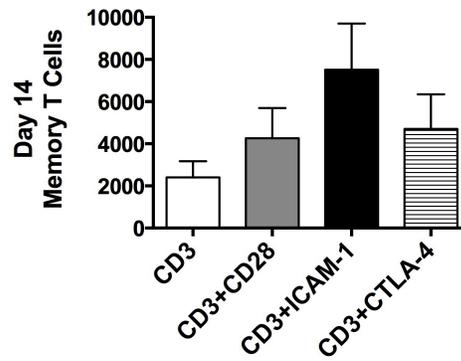


Fig. 2.11



**Figures 2.6-2.11. Costimulation through CTLA-4 supported differentiation of human naïve CD4<sup>+</sup> T cells to effector and memory T cells.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 were stained for expression of CD11a, CD27 and CD45RO and analyzed by flow cytometry. Representative of fourteen (CD3+CD28), thirteen (CD3+ICAM-1), or twelve (CD3+CTLA-4; CD3) experiments. **Fig. 2.6 and 2.9.** representative figure of the number of CD4<sup>+</sup>CD45RO<sup>+</sup>CD11a<sup>+</sup>CD27<sup>hi</sup> T cells (effector T cells, upper right quadrant) and CD4<sup>+</sup>CD45RO<sup>+</sup>CD11a<sup>+</sup>CD27<sup>lo</sup> T cells (memory T cells, lower right quadrant) on day 7 (**Fig. 2.6**) or day 14 (**Fig. 2.9**). **Fig. 2.7 and 2.10,** average number of effector T cells  $\pm$  SEM on day 7 (**Fig. 2.7**) or day 14 (**Fig. 2.10**). **Fig. 2.8 and 2.11,** average number of memory T cells  $\pm$  SEM at day 7 (**Fig. 2.8**) or day 14 (**Fig. 2.11**).

\* p < 0.05, \*\* p < 0.01

Fig. 2.12

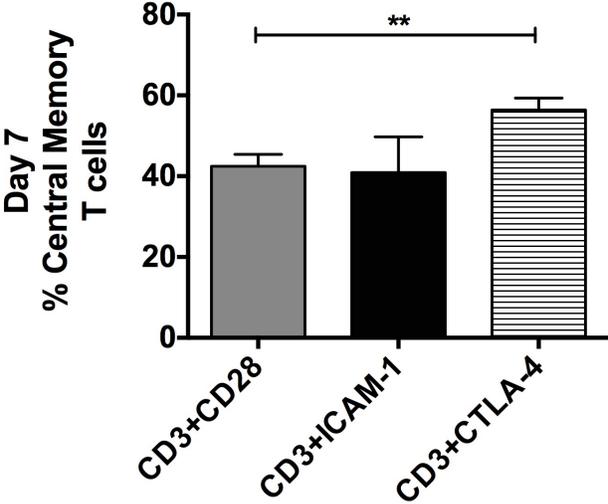
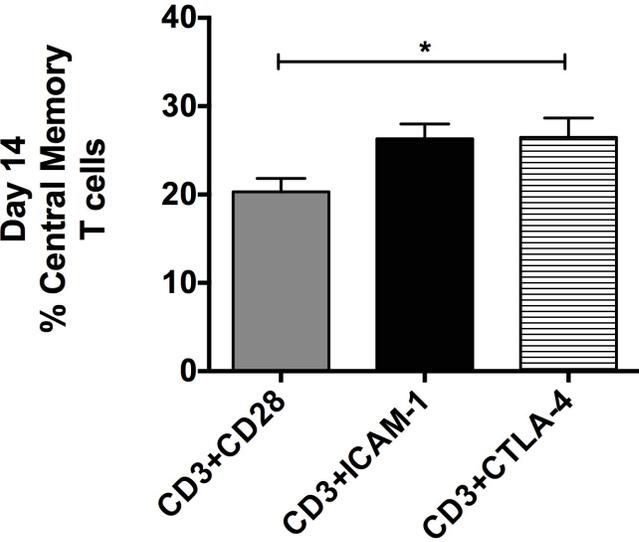


Fig. 2.13



**Figures 2.12-2.13. Costimulation through CTLA-4 supported central memory T cells.**

Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, or anti-CD3+anti-CTLA-4 were stained for CD45RO, CD27 and CD62L expression and analyzed by flow cytometry. Central memory T cells were defined as [CD4<sup>+</sup>CD45RO<sup>+</sup>CD27(-)+CD62L<sup>+</sup>]. Representative of four (CD3+CD28) or three (CD3+ICAM-1 and CD3+CTLA-4) experiments. **Fig. 2.12**, Average percentage of central memory T cells ± SEM on day 7. **Fig. 2.13**, Average percentage of central memory T cells ± SEM on day 14.

\* p < 0.05, \*\* p < 0.01

the changes in percentage of central memory T cells are modest and may not support a biologically relevant difference among the three costimulatory proteins.

*Costimulation through CTLA-4 promoted human naïve CD4+ T cell proliferation without impairing cell viability*

Cell proliferation is an integral part of the differentiation process. CTLA-4 inhibits proliferation of mixed murine T cell populations consisting of CD8+ and CD4+ memory, effector, and naïve T cells at 72 hours of *in vitro* stimulation (14-17). Other studies support a positive role for CTLA-4 in T cell proliferation (13, 20, 52). We hypothesized that costimulation through CTLA-4 would support proliferation of naïve T cells. In contrast to work of others (14-17), we studied proliferation of purified human naïve CD4+ T cells (representative data in **Fig. 2.14**, summarized in **Fig. 2.15-2.17**). Costimulation through CD3+CTLA-4 induced proliferation on days 7, 10, and 12 in contrast to stimulation through the TCR (CD3). CTLA-4 failed to induce the same robust proliferation observed with CD28 or ICAM-1 by day 7 (**Fig. 2.15**), however, by days 10 (**Fig. 2.16**) or 12 (**Fig. 2.17**), proliferation generated by CTLA-4 costimulation was indistinguishable from ICAM-1 or CD28.

Our lab observed (40) that CD28 or ICAM-1 costimulation supports T cell proliferation while protecting against cell death but LFA-1 induces strong proliferation accompanied by considerable cell death and only modest differentiation. Here (representative data in **Fig. 2.18**, summarized in **Fig. 2.19**), stimulation through CD3+CTLA-4 did not increase cell death beyond that caused by CD28 and ICAM-1. Thus, stimulation through CD3+CTLA-4 promoted both differentiation and proliferation of human naïve CD4+ T cells in much the same manner as either CD28 or ICAM-1.

Fig. 2.14

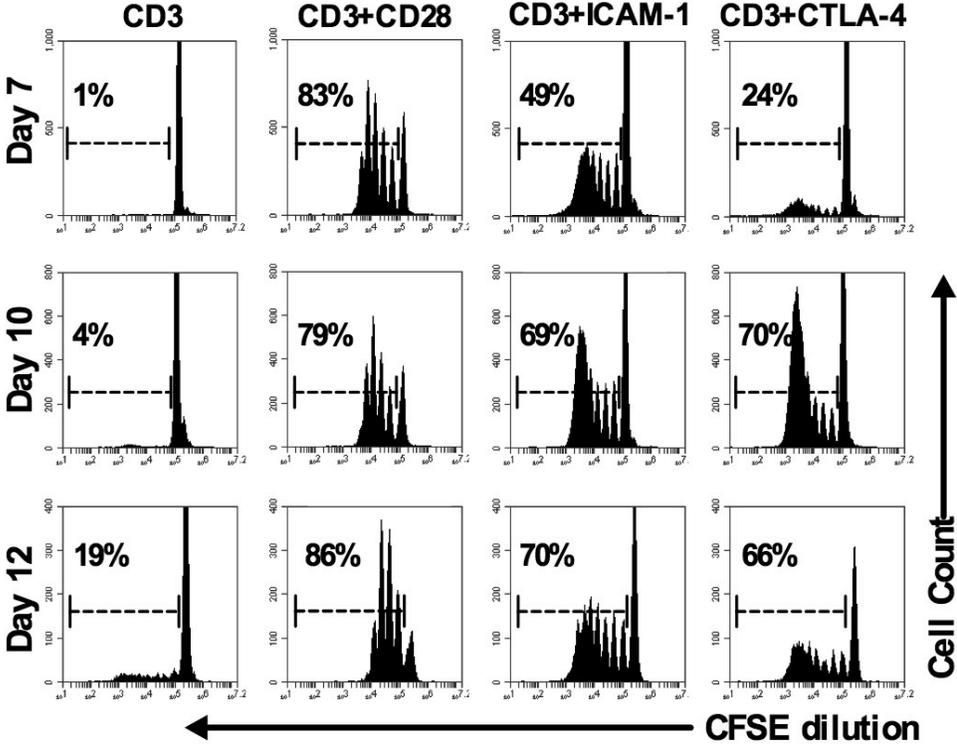


Fig. 2.15

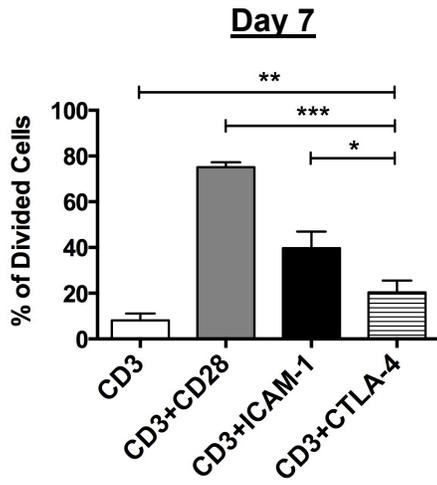


Fig. 2.16

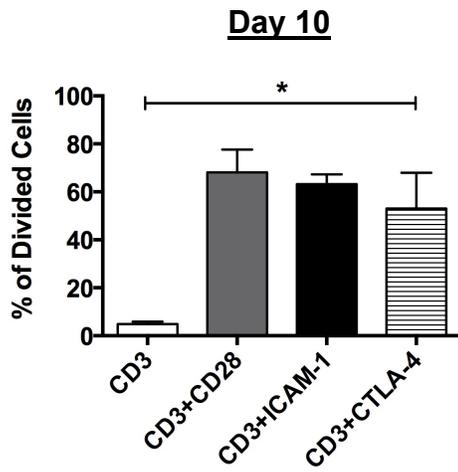
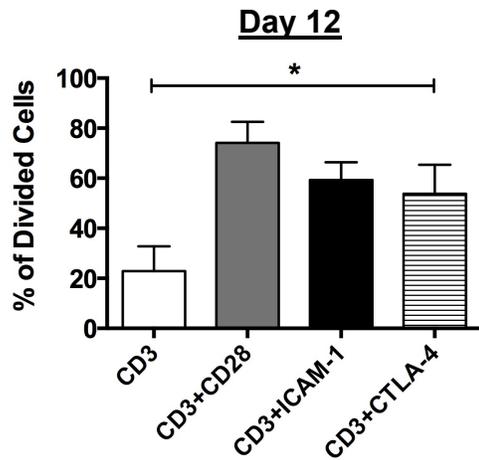


Fig. 2.17



**Figures 2.14-2.17. Costimulation through CTLA-4 supported proliferation of naïve CD4+ T cells.** Human naïve CD4+ T cells were stained with 2.5  $\mu$ M CFSE on day 0 and stimulated for 7, 10 or 12 days using anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3. CFSE dilution was analyzed by flow cytometry. **Fig. 2.14**, Proliferation on days 7 (upper panels), 10 (middle panels), and 12 (lower panels). The percentage of divided cells is indicated in each histogram plot. **Fig. 2.15**, Average percentage of total divided T cells  $\pm$  SEM at day 7. Representative of 6 experiments. **Fig. 2.16**, Average percentage of total divided T cells  $\pm$  SEM at day 10. Representative of 4 experiments. **Fig. 2.17**, Average percentage of total divided T cells  $\pm$  SEM at day 12. Representative of 4 experiments.

\*  $p < 0.05$ , \*\*  $p < 0.01$

Fig. 2.18

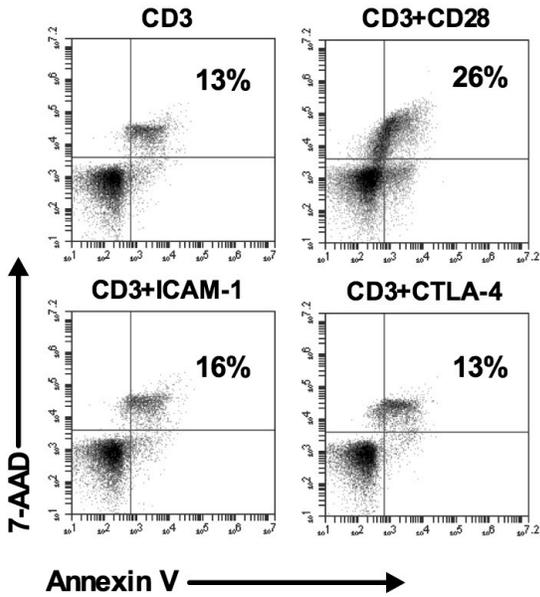
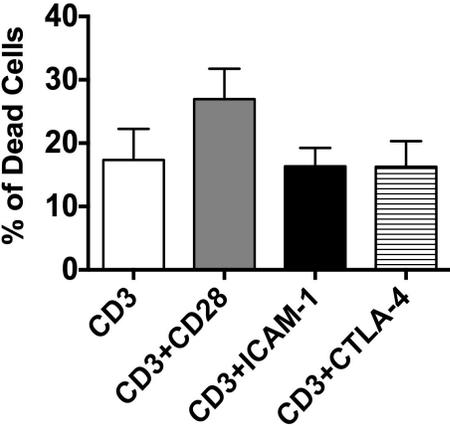


Fig. 2.19



**Figures 2.18-2.19. Costimulation through CTLA-4 promoted proliferation and differentiation of naïve T cells without affecting cell survival.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days using anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3. Cells were stained for Annexin V and 7-AAD and analyzed by flow cytometry. Dead cells were indicated by expression of both Annexin V and 7-AAD. Representative of nine separate experiments. **Fig. 2.18**, Upper right quadrant of each plot identifies the percentage of Annexin V+7AAD<sup>+</sup> cells. **Fig. 2.19**, Average percentage of Annexin V+7AAD<sup>+</sup> cells  $\pm$  SEM on day 7.

### *Costimulation through CTLA-4 favored differentiation to Treg cells*

Although costimulation through CTLA-4 resulted in less proliferation on day 7 than either CD28 or ICAM-1, CTLA-4 generated numerous Treg cells. This suggests that CTLA-4 may favor differentiation to Treg cells rather than inducing widespread T cell activation, a function that would be consistent with its role as a negative regulator. We examined the relative percentage of Treg cells in the divided and CD25<sup>+</sup> (Treg and activated cells) populations to determine whether costimulation through CTLA-4 induces a larger representative Treg population compared to ICAM-1 (**Fig. 2.20-2.25**). Although costimulation through CTLA-4 generated a larger CD25<sup>+</sup> population than stimulation through only the TCR (CD3) ( $p < 0.01$ ) (representative data in **Fig. 2.20**, summarized in **Fig. 2.21**,  $p < 0.01$ ), this CD25<sup>+</sup> population was much smaller than the CD25<sup>+</sup> populations observed with CD3+CD28 ( $p < 0.05$ ) or CD3+ICAM-1 ( $p < 0.001$ ). This indicates that CTLA-4 does not induce the same degree of activation as the other two stimuli. If we examine the fraction of the CD25<sup>+</sup> T cell population that are Treg cells, the Treg population represented on average 23%, 13%, and 1% of the CD25<sup>+</sup> T cells induced by stimulation through CD3+CTLA-4, CD3+ICAM-1, and CD3+CD28, respectively (**Fig. 2.22**). Thus, stimulation through CTLA-4 favored differentiation to Treg cells greater than ICAM-1 by inducing less activation but greater percentages of Treg cells.

Costimulation through CTLA-4 induced overall less T cell proliferation than ICAM-1 or CD28 on day 7 but appeared to favor proliferation of Treg cells (**Fig. 2.23-2.25**). We examined the percentage of Treg cells with the T cell population that divided to evaluate whether CTLA-4 favored proliferation of Treg cells (**Fig. 2.25**). Stimulation through CD3 did not activate proliferation as expected. Costimulation through CD28 induced robust proliferation but no

Fig. 2.20

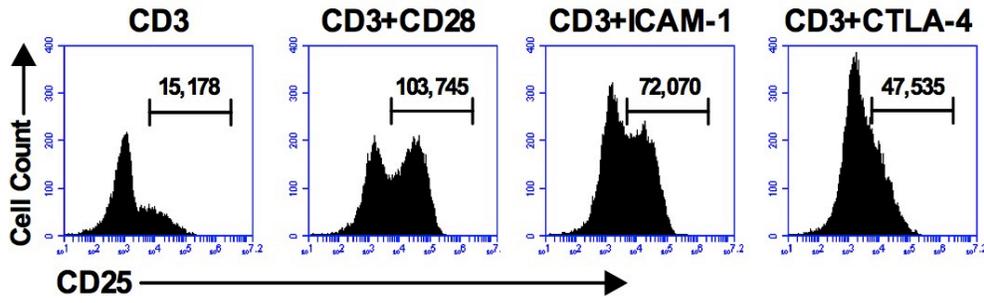


Fig. 2.21

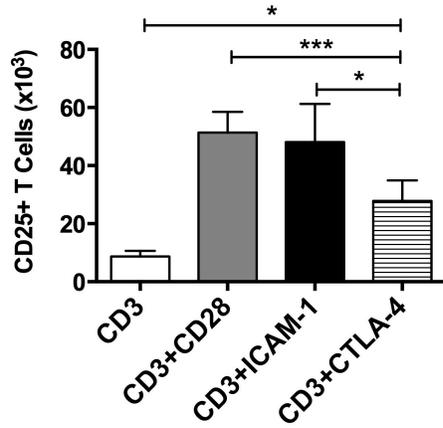
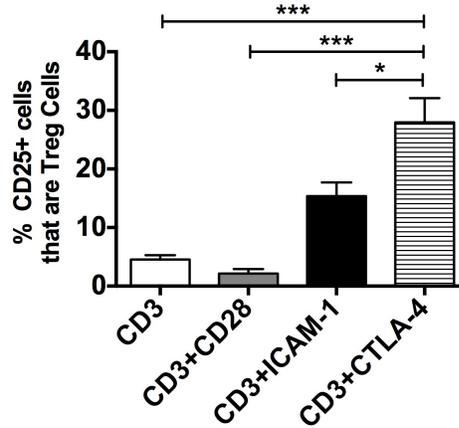


Fig. 2.22



**Figures 2.20-2.22. Costimulation through CTLA-4 induced less T cell activation than ICAM-1 or CD28 and favored differentiation to Treg cells.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Representative of fifteen (CD3+CD28; CD3+ICAM-1) or thirteen (CD3+CTLA-4; CD3) separate experiments. **Fig. 2.20**, Histogram plots identify the CD4<sup>+</sup>CD25<sup>+</sup> T cell population with cells numbers indicated. **Fig. 2.21**, Average number of total CD4<sup>+</sup>CD25<sup>+</sup> T cells ± SEM on day 7 (gated on total population). **Fig. 2.22**, Average percentage ± SEM of total CD4<sup>+</sup> CD25<sup>+</sup> T cells that also strongly express Foxp3 (gated on CD25<sup>+</sup> population only).

\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

Fig. 2.23

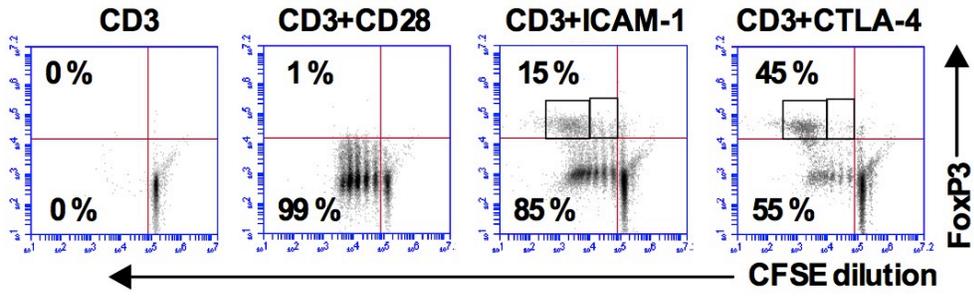


Fig. 2.24

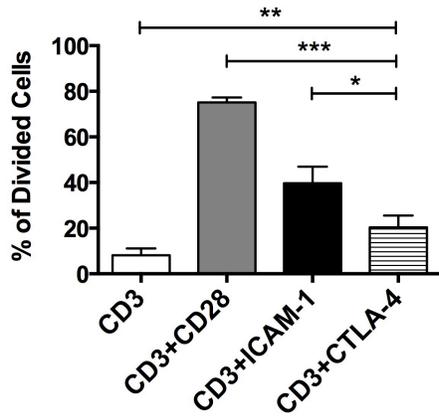
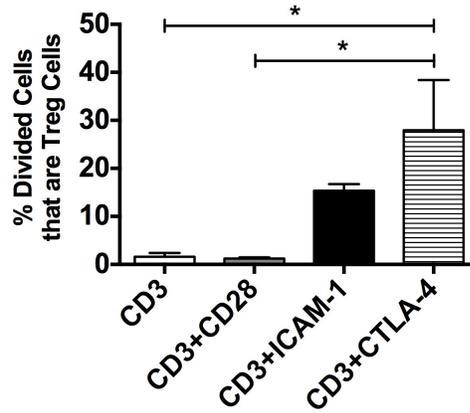


Fig. 2.25



**Figures 2.23-2.25. Costimulation through CTLA-4 induced overall less T cell proliferation than ICAM-1 or CD28 and favored proliferation of Treg cells.** Human naïve CD4<sup>+</sup> T cells were stained with 2.5  $\mu$ M CFSE at day 0 and stimulated for 7 days through anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3. Cells were stained for CD25 and strong Foxp3 (**Fig. 2.23** and **Fig. 2.25**) on day 7 and analyzed for CFSE dilution and expression of Treg markers by flow cytometry. Representative of 10 experiments (**Fig. 2.24**) or 3 experiments (**Fig. 2.23** and **Fig. 2.25**). **Fig. 2.23**, Upper left quadrant identifies the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup> cells that had divided, with percentages indicating the percentage of Treg cells within the divided population. **Fig. 2.24**, Average number of divided T cells  $\pm$  SEM. **Fig. 2.25**, Average percentage of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>hi</sup> T cells (Treg cells)  $\pm$  SEM within the population of divided cells.

\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

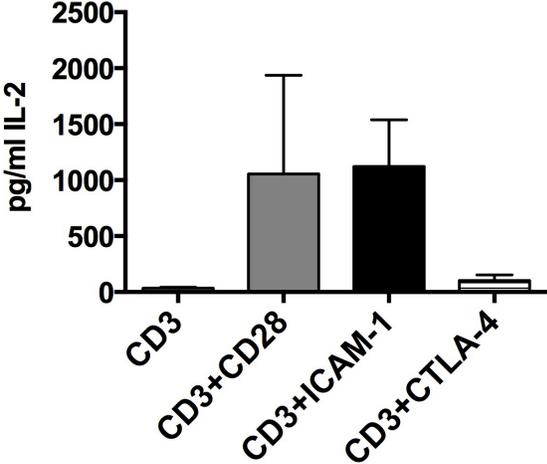
Treg differentiation (**Fig. 2.23-2.25**). ICAM-1 also activated a greater proliferative response compared to CTLA-4 (**Fig. 2.24**); however, costimulation through CTLA-4 favored proliferation to Treg cells.

Although costimulation through CTLA-4 supported production of IL-10 (a suppressive cytokine secreted by Treg cells) (**Fig. 2.5**), it did not support substantial production of IL-2 as seen with CD28 or ICAM-1 costimulations (**Fig. 2.26**). Costimulation through CTLA-4 modestly increased IL-2 production from  $34 \pm 9$  pg/ml (CD3) to  $103 \pm 51$  pg/ml (CD3+CTLA-4). IL-2 supports general T cell activation and proliferation and is an index of Th1 function in conjunction with IFN- $\gamma$ . Therefore, we also tested whether costimulation through CTLA-4 induced production of IFN- $\gamma$  on day 7. Average production of IFN- $\gamma$  induced by costimulation through CTLA-4 (174 pg/ml) was slightly lower than CD28 (273 pg/ml) or ICAM-1 (285 pg/ml), but CTLA-4 failed to induce any IFN- $\gamma$  in 67% of the experiments (not shown). CTLA-4, therefore, did not appear to support Th1 function based on the low production of IL-2 and inability to consistently induce IFN- $\gamma$  production. These data suggest that CTLA-4 induces differentiation and proliferation of naïve CD4<sup>+</sup> T cells but strongly favors differentiation to Treg cells, a role that would be consistent with its status as an inhibitory protein.

*Differentiation of naïve T cells to Treg cells by CTLA-4 was dependent on a low level of endogenous IL-2*

In our previous study (41), costimulation through ICAM-1 guided naïve CD4<sup>+</sup> T cell differentiation to Treg cells by an IL-2-dependent mechanism. In the present study, CTLA-4 costimulation yielded 10-fold lower levels of IL-2 production ( $103 \pm 51$  pg/ml) in contrast to ICAM-1 costimulation ( $1,122 \pm 417$  pg/ml) suggesting a possible mechanistic difference in

Fig. 2.26



**Fig. 2.26. Costimulation through CTLA-4 induced low levels of IL-2 production.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3. On day 7, supernate was collected for analysis by ELISA. **Fig. 2.26,** Average production of IL-2 pg/ml  $\pm$  SEM. Representative of four experiments (CD3+CD28) or five experiments (CD3+ICAM-1, CD3+CTLA-4, CD3). **Fig. 2.27,** Average production of IFN- $\gamma$  pg/ml  $\pm$  SEM. Representative of four experiments (CD3+CD28), five experiments (CD3+ICAM-1), or three experiments (CD3+CTLA-4).

induction of Treg differentiation between the two stimuli. We tested whether induction of Treg differentiation by CTLA-4 required endogenous IL-2 (**Fig. 2.27-2.28**). An isotype control or blocking antibody against IL-2 or IL-10 were added at the start of stimulations, and the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup> T cells (Treg cells) was assessed by flow cytometry on day 7. Blocking IL-2 inhibited differentiation to Treg cells by ICAM-1 and CTLA-4 (representative figures in **Fig. 2.27**, summarized in **Fig. 2.28**). *In vivo* studies indicate that Treg cells require IL-2 for maintenance and survival (53, 54) consistent with our findings here.

TGF- $\beta$  induces differentiation of naïve T cells to Treg cells that is further supported by IL-2 addition (41, 55, 56). In Dr. Kelli Williams's dissertation (58), the addition of exogenous IL-2 and TGF- $\beta$  enhanced differentiation of naïve T cells to Treg cells by ICAM-1 costimulation. Here, we compared the effect of adding exogenous IL-2, TGF- $\beta$ , or both IL-2 and TGF- $\beta$  on Treg differentiation induced by ICAM-1 and CTLA-4. Adding exogenous IL-2, TGF- $\beta$ , or both IL-2 and TGF- $\beta$  showed an overall trend of increasing the number of Treg cells generated through CD3+ICAM-1 (**Fig. 2.29-2.30**). Addition of TGF- $\beta$  or TGF- $\beta$ +IL-2 augmented differentiation to Treg cells 2.5- or 2-fold, respectively, by CTLA-4 (**Fig. 2.29-2.30**). Exogenous IL-2 consistently had little effect on Treg induction by CTLA-4 in contrast to costimulation through ICAM-1, which may suggest different mechanistic pathways by which ICAM-1 and CTLA-4 induce Treg differentiation. Overall, differentiation to Treg cells by costimulation through CTLA-4 was dependent on low levels of endogenous IL-2 and was enhanced by addition of exogenous TGF- $\beta$  but not IL-2.

Fig. 2.27

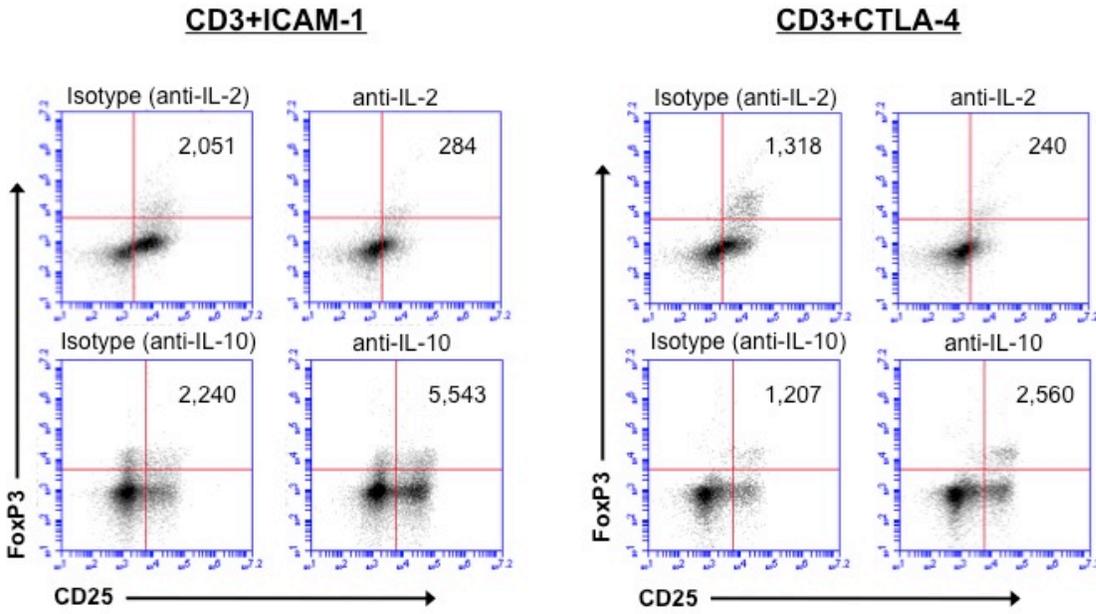
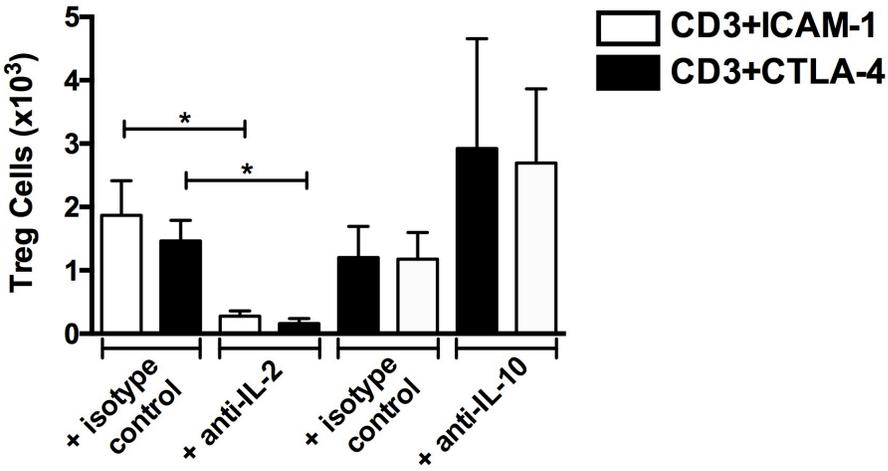


Fig. 2.28



**Figures 2.27-2.28. Differentiation of naïve T cells to Treg cells by CTLA-4 was dependent on a low level of endogenous IL-2.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4. Isotype controls, anti-IL-2 antibody, or anti-IL-10 antibody were added to medium at the start of stimulations as indicated. On day 7, T cells stained for CD25 and Foxp3 were analyzed by flow cytometry. Treg cells were defined as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup>. Representative of 4 experiments. **Fig. 2.27**, Upper right quadrant identifies the Treg population with cell numbers indicated. **Fig. 2.28**, Average number of Treg cells  $\pm$  SEM with added isotype control, anti-IL-2, or anti-IL-10.

\*  $p < 0.05$

Fig. 2.29

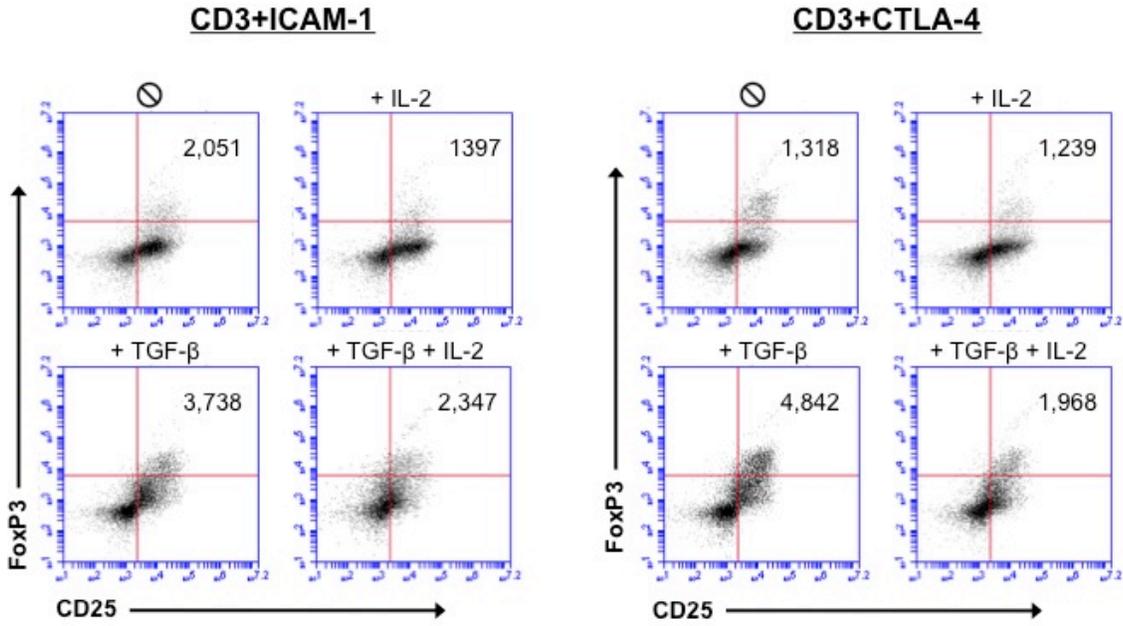
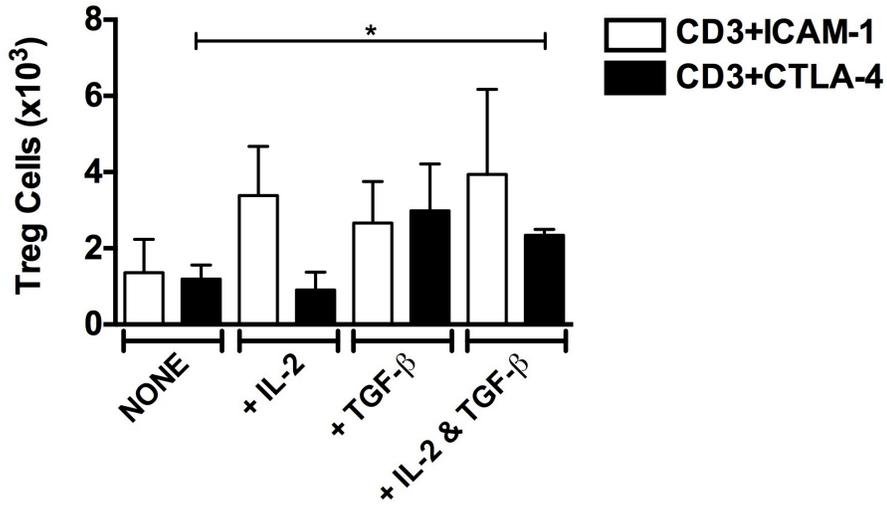


Fig. 2.30



**Figures 2.29-2.30. Differentiation to Treg cells by CTLA-4 costimulation was enhanced by exogenous TGF- $\beta$  but not IL-2.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4. Recombinant human IL-2 (rhIL-2), recombinant human TGF- $\beta$  (rhTGF- $\beta$ ), or the combination of rhIL-2 and rhTGF- $\beta$  were added to medium at the start of stimulations as indicated. On day 7, T cells stained for CD25 and Foxp3 were analyzed by flow cytometry. Treg cells were defined as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup>. Representative of 4 experiments. **Fig. 2.29**, Upper right quadrant identifies the Treg population with cell numbers indicated. **Fig. 2.30**, Average number of Treg cells  $\pm$  SEM with added medium (NONE), rhIL-2, rhTGF- $\beta$ , or combination of rhIL-2 and rhTGF- $\beta$ .

\*  $p < 0.05$

*Naïve T cells costimulated through CTLA-4 released soluble CD25 at a higher frequency than those costimulated through ICAM-1*

CD25, a marker of activated and regulatory T cells, can be released as a soluble form (sCD25 or sIL-2R $\alpha$ ) by both types of T cell subsets and is capable of binding IL-2 (58, 59). sCD25 can suppress effector T cell proliferation and enhance suppressive function of Treg cells (60). We have observed so far that costimulation through CTLA-4 favored differentiation to Treg cells that is dependent on IL-2; yet, minimal IL-2 is produced in these cultures. We hypothesized that cells costimulated through CTLA-4 may release more soluble CD25 than cells costimulated through ICAM-1, thus, sequestering secreted IL-2. Evidence of a higher frequency of soluble CD25 in cells costimulated through CTLA-4 may explain dependence on IL-2 for Treg induction and low endogenous IL-2 production. Both ICAM-1 and CTLA-4 caused high levels of soluble CD25 production, averaging 23,604 and 26,001 pg/ml, respectively (**Fig. 2.31**); however, there were significantly fewer activated (CD25+) T cells generated by CD3+CTLA-4 compared to CD3+ICAM-1 (**Fig. 2.32**). The amount of soluble CD25 (pg/ml) secreted per CD25+ T cell was determined to normalize this observed difference in CD25+ T cell numbers. CD25+ T cells stimulated through CD3+CTLA-4 secreted sCD25 at a nearly three-fold higher frequency than those through CD3+ICAM-1, averaging  $1.08 \pm 0.36$  pg/ml sCD25 per cell compared to  $0.38 \pm 0.09$  pg/ml ( $p < 0.05$ ) (**Fig. 2.33**). The increased rate of sCD25 production in the supernate from cells costimulated through CTLA-4 may sequester IL-2, reducing the level of IL-2 being detected by ELISA.

Fig. 2.31

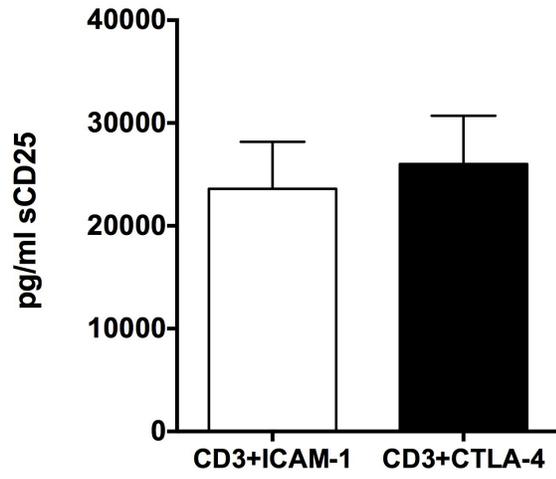


Fig. 2.32

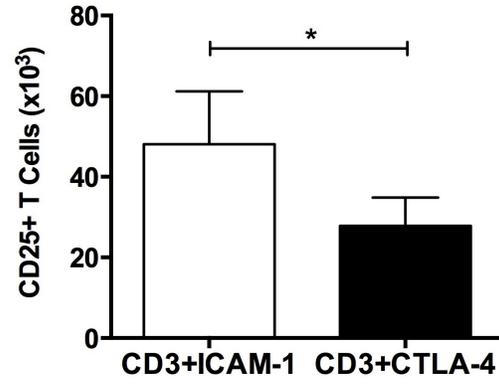
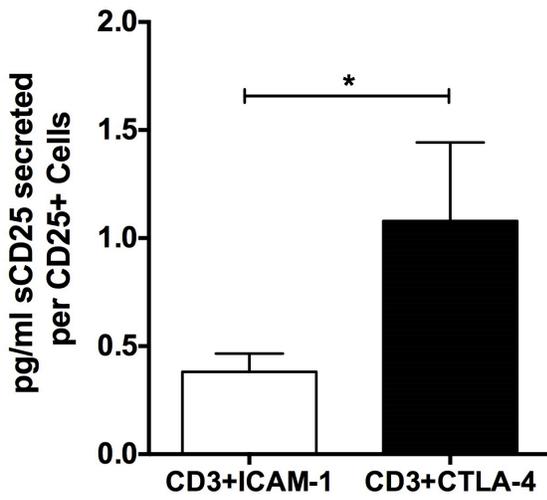


Fig. 2.33



**Figures 2.31-2.33. Costimulation through CTLA-4 induced the release of soluble CD25 at a higher frequency than ICAM-1.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4. On day 7, supernate was collected and analyzed for sCD25 (sIL-2R $\alpha$ ) by ELISA (**Fig. 2.31** and **Fig. 2.33**). T cells were stained for CD25 expression and analyzed by flow cytometry (**Fig. 2.32**). Representative of 7 experiments. **Fig. 2.31.** Average sCD25  $\pm$  SEM (pg/ml) released in supernates. **Fig. 2.32.** Average CD25<sup>+</sup> T cells  $\pm$  SEM on day 7. **Fig. 2.33,** Average sCD25 (pg/ml) produced per CD25<sup>+</sup> T cell  $\pm$  SEM.

\* p < 0.05

*Stimulation through CD28 suppressed Treg cell induction by CTLA-4 but not ICAM-1*

CD28 and CTLA-4 compete for the shared counter receptors, CD80 and CD86. CD28 is expressed by resting and activated T cells in contrast to CTLA-4 which is constitutively expressed by Treg cells or upregulated in activated T cells. CTLA-4, however, has a much higher affinity for both CD80 and CD86 than CD28 (11, 12). Depending on the relative expression levels of CD28, CTLA-4 and their shared counter receptors, CD80 and CD86, in some cases CD28 may be the primary signaling protein and in other cases, the balance of CD28 and CTLA-4 signaling may shift favor to CTLA-4. We wondered how differentiation to Treg cells would be affected by costimulating cells through both CD28 and CTLA-4, giving each receptor equal opportunity to signal without competition for CD80 or CD86. In **Fig. 2.34**, anti-CD28 was added to cultures stimulated through CD3+CTLA-4 (closed bars), or alternatively, CD3+ICAM-1 (open bars). The number of Treg cells in each culture was assessed on day 7. CD28 signaling reduced differentiation to Treg cells in a dose-dependent manner in cells stimulated through CD3+CTLA-4 (closed bars) but not CD3+ICAM-1 (open bars) (**Fig. 2.34**). All stimuli were added at day 0 which may have given CD28 a bit of a head start since CTLA-4 expression was usually not detectable by flow cytometry until 24-48 hours after stimulation in this system (See **Fig. 2.39-40** later in Chapter 2). Inhibition of Treg differentiation began at 0.25  $\mu\text{g/ml}$  and was maximal when the level of anti-CD28 reached 1.0  $\mu\text{g/ml}$ . In contrast, even at maximum concentration (2  $\mu\text{g/ml}$ ), CD28 did not inhibit differentiation to Treg cells induced by ICAM-1 costimulation (**Fig. 2.34**, open bars).

Varying the concentration of anti-CTLA-4 in conjunction with constant stimulation with anti-CD3 (1  $\mu\text{g/ml}$ ) + anti-CD28 (2  $\mu\text{g/ml}$ ) did not reverse the inhibition of anti-CD28 on Treg

Fig. 2.34

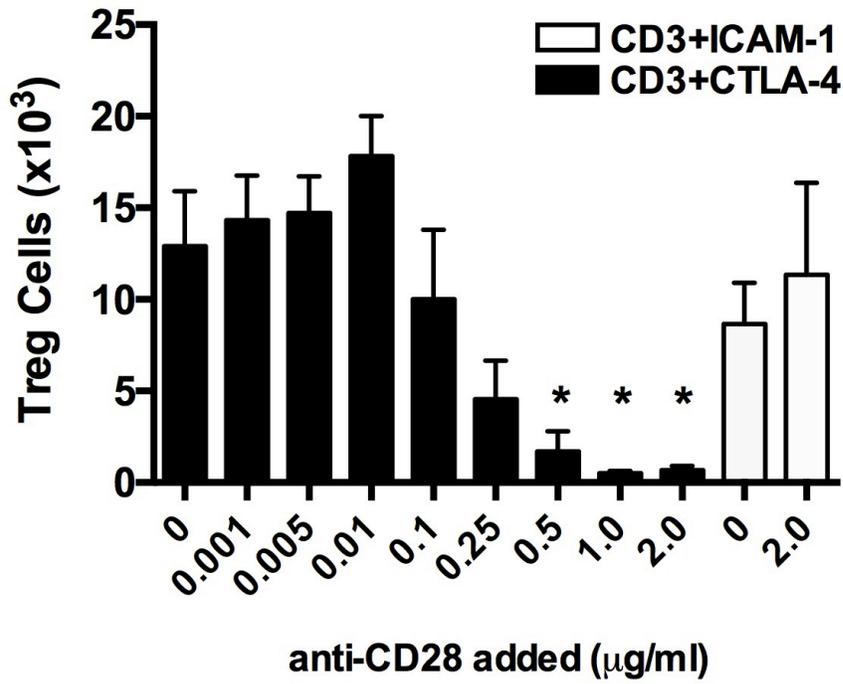
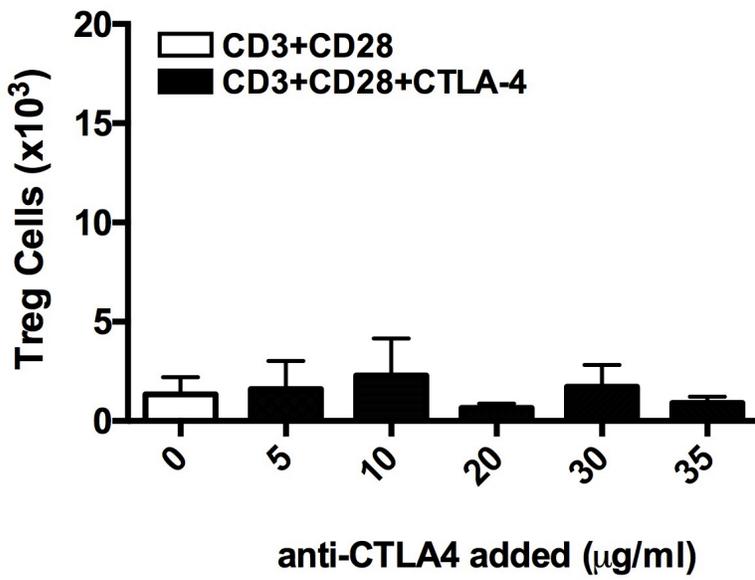


Fig. 2.35



**Figures 2.34-2.35. Costimulation through CD28 suppressed differentiation to Treg cells induced by CTLA-4 but not by ICAM-1.** Human CD4<sup>+</sup> naïve T cells were stimulated with anti-CD3+anti-ICAM-1, anti-CD3+anti-ICAM-1+anti-CD28 (2 µg/ml), anti-CD3+anti-CTLA-4 (20 µg/ml), anti-CD3+anti-CD28 (2 µg/ml), anti-CD3+varying [anti-CD28]+anti-CTLA-4 (20 µg/ml), or anti-CD3+anti-CD28 (2 µg/ml)+varying [anti-CTLA-4] for seven days. Anti-CD3 and anti-ICAM-1 were used at 1 µg/ml and 10 µg/ml, respectively. On day 7, cells were stained for CD25 and Foxp3 and analyzed via flow cytometry. Representative of 3 experiments each performed in duplicate except for the addition of 0.1 µg/ml anti-CD28 (**Fig. 2.34**), which is representative of 2 experiments each performed in duplicate. **Fig. 2.34**, Average number of CD25<sup>+</sup> Foxp3<sup>hi</sup> T cells ± SEM generated at day 7. Closed bars indicate cells that were stimulated with anti-CD3 (1 µg/ml) + anti-CTLA-4 (20 µg/ml) with varying concentrations of anti-CD28 stimulating Ab shown in **Fig 2.34**. Bar 1 indicates cells that were stimulated only with anti-CD3 (1 µg/ml) + anti-CTLA-4 (20 µg/ml). The open bars indicate cells stimulated with anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD28. **Fig. 2.35**, Average number of CD25<sup>+</sup> Foxp3<sup>hi</sup> T cells ± SEM generated at day 7. Closed bars indicate cells that were stimulated with anti-CD3 (1 µg/ml) + anti-CD28 (2 µg/ml) with varying concentrations of anti-CTLA-4 stimulating Ab as shown in **Fig. 2.35**. Bar 1 indicates cells that were stimulated only with anti-CD3 (1 µg/ml) + anti-CD28 (2 µg/ml).

\*p < 0.05

induction (**Fig. 2.35**). However, the effect of CTLA-4 concentrations greater than 35  $\mu\text{g/ml}$  was not determined and may warrant further investigation.

Next, we addressed the kinetic profile for how late after initiation of the CTLA-4 costimulation that anti-CD28 could be added and still affect inhibition of differentiation to Treg cells. As seen in **Fig. 2.36-2.37**, stimulation through CD28 completely inhibited Treg induction by CTLA-4 if added in soluble form within the first 24 hours and partially inhibited if added at 48 hours. This inhibition by CD28 was not observed in cultures stimulated through CD3+ICAM-1 (**Fig. 2.38**) as was consistent with our findings in **Fig. 2.34**. Thus, it appears that stimulation through CD28 can suppress differentiation to Treg cells by CTLA-4 but not ICAM-1.

#### *Surface expression of CTLA-4 was induced upon stimulation of naïve CD4+ T cells*

As mentioned earlier, CD28 is expressed on both resting and activated T cells including naïve T cells. CTLA-4, however, is not detected on the surface of conventional T cells until 48 hours after activation (13). Human naïve CD4+ T cells did not express CTLA-4 on their surface, as expected (data not shown). In **Fig. 2.39-2.42**, we assessed the ability of each stimulus to induce CTLA-4 expression in naïve CD4+ T cells at 2, 4, 24, 48, 72, and 96 hours and days 7 and 14 to determine the onset, peak, and duration of CTLA-4 expression (**Fig. 2.39-2.42**). As seen in **Fig. 2.39** (MFI of CTLA-4) and **Fig. 2.40** (% CTLA-4+ cells), CTLA-4 expression was detected by flow cytometry as early as 24 hours and peaked at 72 hours following stimulation. At peak expression (72h), costimulation through CD28, ICAM-1 or CTLA-4 induced elevated levels of CTLA-4 compared to stimulation through only the TCR (CD3). Although CTLA-4 expression declined at 96h (**Fig. 2.39-2.40**), later in differentiation (days 7 and 14) the

Fig. 2.36

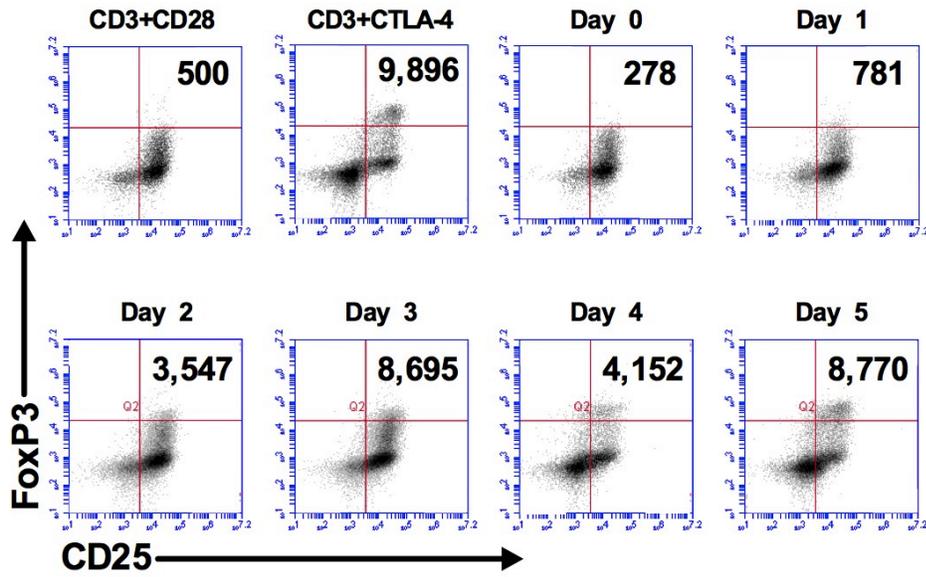


Fig. 2.37

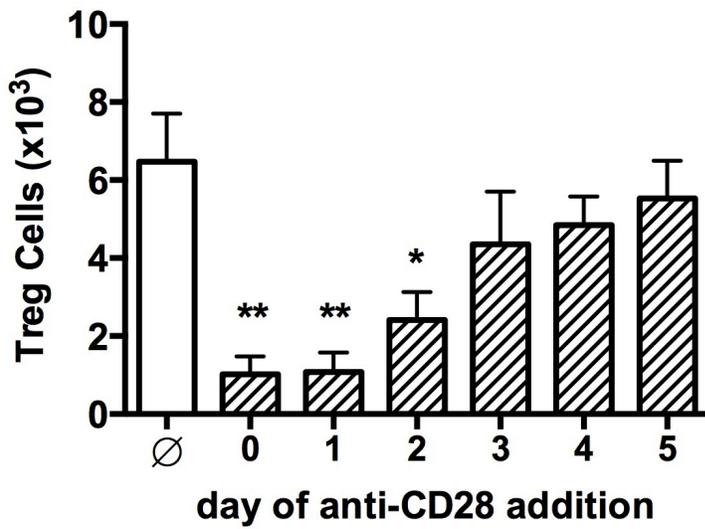
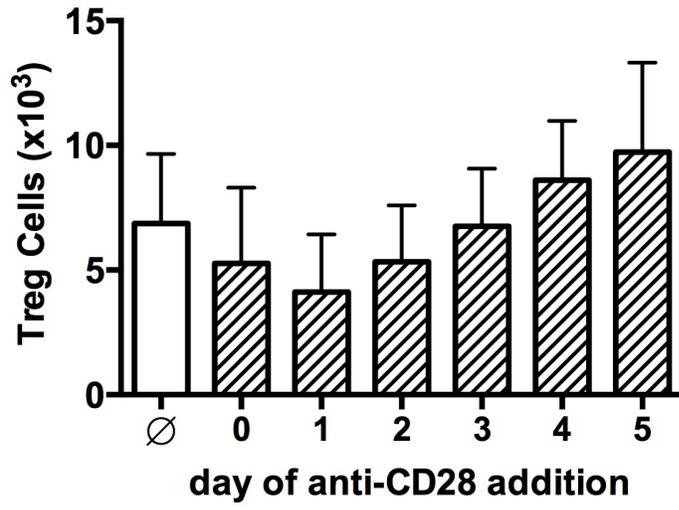


Fig. 2.38



**Figures 2.36-2.38. CTLA-4 required at least 48 hours of stimulation without signaling through CD28 to induce regulatory T cells *in vitro*.** Human CD4<sup>+</sup> naïve T cells were stimulated with anti-CD3+anti-CTLA-4 or anti-CD3+anti-ICAM-1 for seven days. Anti-CD28 (2 µg/ml) was added on days 0, 1, 2, 3, 4, or 5 in solution. On day 7, cells were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Representative of 5-6 experiments (CD3+CTLA-4) or 4 experiments (CD3+ICAM-1). **Fig. 2.36**, Upper right quadrants identify the CD25<sup>+</sup> Foxp3<sup>hi</sup> (Treg) population with cell numbers indicated. The days indicate when anti-CD28 was added. **Fig. 2.37**, Average number of CD25<sup>+</sup> Foxp3<sup>hi</sup> T cells ± SEM generated at day 7 through CD3+CTLA-4 with varying the timing of anti-CD28 addition. **Fig. 2.38**, Average number of CD25<sup>+</sup> Foxp3<sup>hi</sup> T cells ± SEM generated at day 7 through CD3+ICAM-1 with varying the timing of anti-CD28 addition.

\*p < 0.05, \*\* p < 0.01

Fig. 2.39

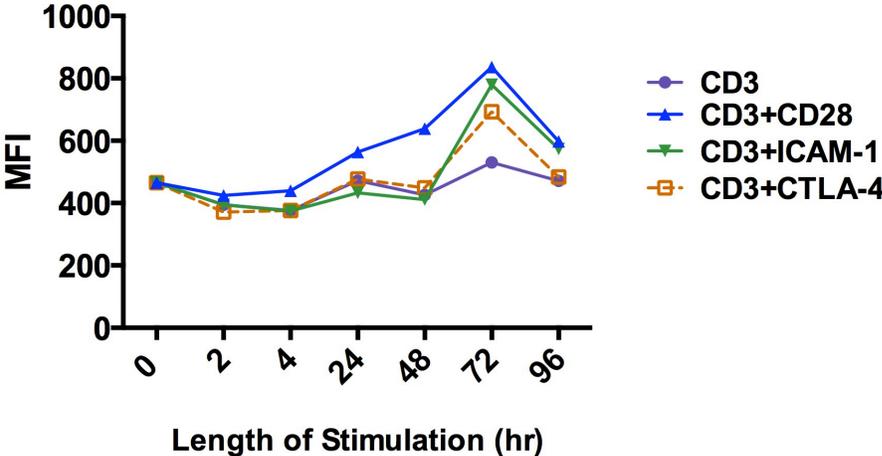


Fig. 2.40

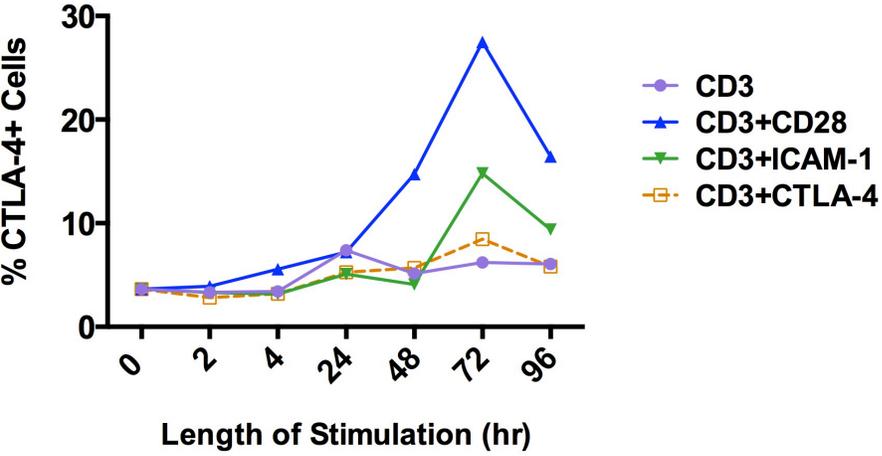


Fig. 2.41

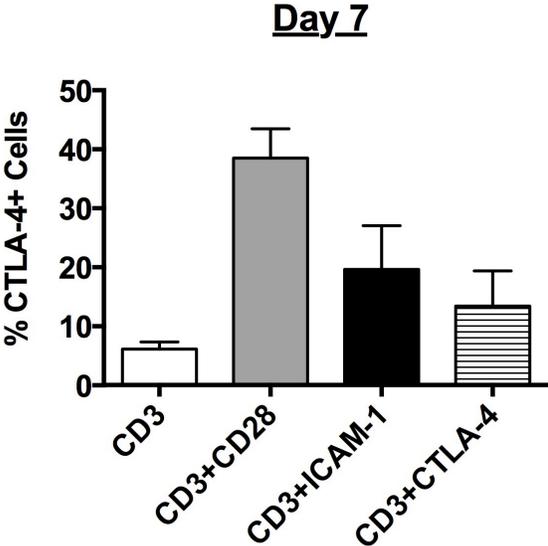
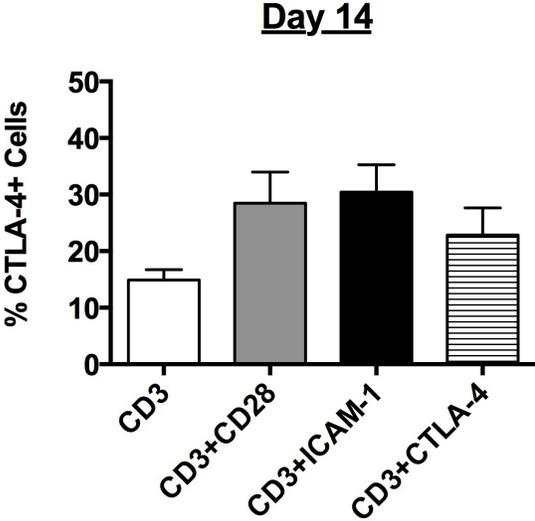


Fig. 2.42



**Figures 2.39-2.42. Surface expression of CTLA-4 peaked at 72 hours after activation and was detected on differentiated cells on days 7 and 14.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 for 2, 4, 24, 48, 72 and 96 hours and 7 and 14 days after stimulation began, cells were analyzed for surface expression of CTLA-4. For **Fig. 2.39-2.40**, representative of 4-5 experiments for all timepoints except 96 hour (n = 2). For **Fig. 2.41-2.42**, representative of 7 experiments. **Fig. 2.39**, Average MFI of CTLA-4. **Fig. 2.40**, Average percentage of CTLA-4<sup>+</sup> cells. **Fig. 2.41**, Average percentage CTLA-4<sup>+</sup> cells ± SEM on day 7. **Fig. 2.42**, Average percentage CTLA-4<sup>+</sup> cells ± SEM on day 14.

percentage of cells expressing CTLA-4 increased (**Fig. 2.41-2.42**). Thus, it appeared that stimulation of naïve T cells induced a transient increase in CTLA-4 expression at 72 hours as cells became activated. The timing of this peak in CTLA-4 expression corresponded with the ability of CD28 to inhibit Treg differentiation by CTLA-4 if present during the first 48 hours of stimulation (**Fig. 2.37**). At 72 hours, addition of CD28 stimulation no longer inhibited the generation of Treg cells by CTLA-4.

*Costimulation through CTLA-4, ICAM-1, or CD28 influenced surface expression of CD28 and ICAM-1 differently*

We sought to characterize the expression of the other two costimulatory proteins during the first 96 hours of stimulation to better understand the timing of activation and differentiation induced by CD28, ICAM-1, and CTLA-4. Low levels of ICAM-1 are expressed by naïve T cells with increased expression following activation (61-63). As seen in **Fig. 2.43-2.44**, CD3+CD28 induced high levels of ICAM-1 expression in comparison to all other stimuli. CD3+CTLA-4 caused slightly increased percentages of ICAM-1+ cells at 24, 48 and 96 hours compared to CD3+ICAM-1 (**Fig. 2.44**). Upon activation, CD28 costimulation induced a considerable decline in the percentage and intensity of CD28 expression through 48 hours of stimulation, but then sharply increased CD28 expression from 72-96 hours (**Fig. 2.45-2.46**). Overall, the extent and pattern of ICAM-1 and CD28 expression generated by costimulation through CD28 differed greatly from that observed with costimulation through ICAM-1 or CTLA-4. Stimulation through ICAM-1 and CTLA-4 reflected similar patterns and levels of ICAM-1 and CD28 expression.

Fig. 2.43

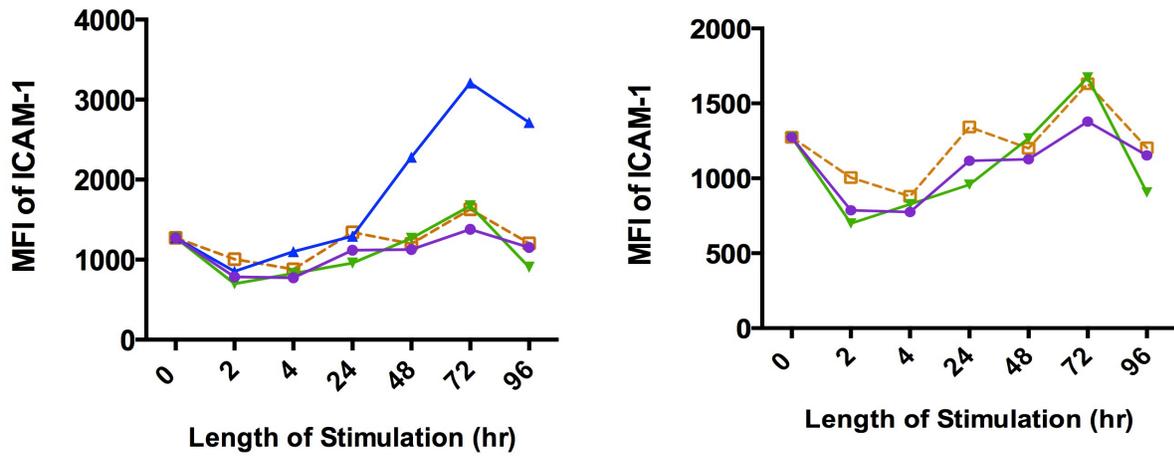
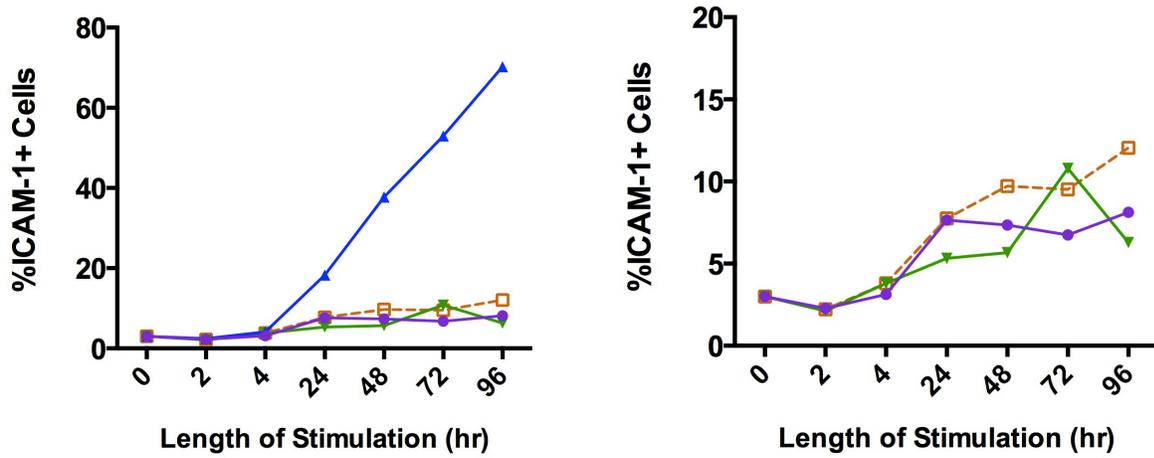


Fig. 2.44



- CD3
- ▲ CD3+CD28
- ▼ CD3+ICAM-1
- ◻ CD3+CTLA-4

**Figures 2.43-2.44. Costimulation through ICAM-1 or CTLA-4 induced similar intensities of ICAM-1 expression but differed slightly in the percentage of cells expressing ICAM-1.**

Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 for 2, 4, 24, 48, 72 and 96 hours after stimulation began, cells were analyzed for surface expression of ICAM-1. Graphs on the right are duplicates of the graph to the left and exclude stimulation through CD3+CD28 so that differences among CD3, CD3+ICAM-1, and CD3+CTLA-4 can be observed. Representative of 3-4 experiments for all timepoints. **Fig. 2.43**, Average MFI of CTLA-4. **Fig. 2.44**, Average percentage ICAM-1<sup>+</sup> cells.

Fig. 2.45

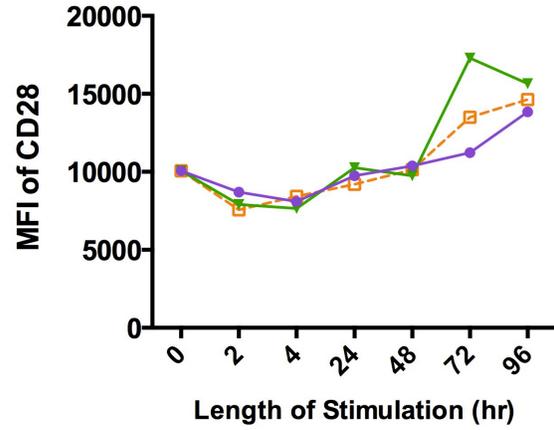
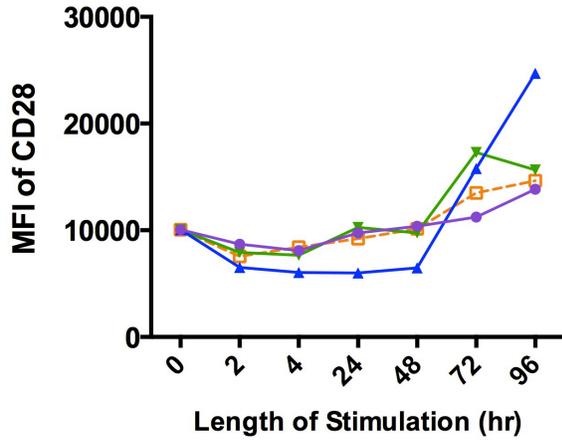
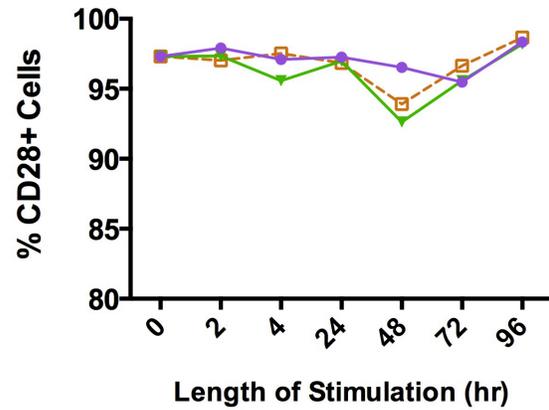
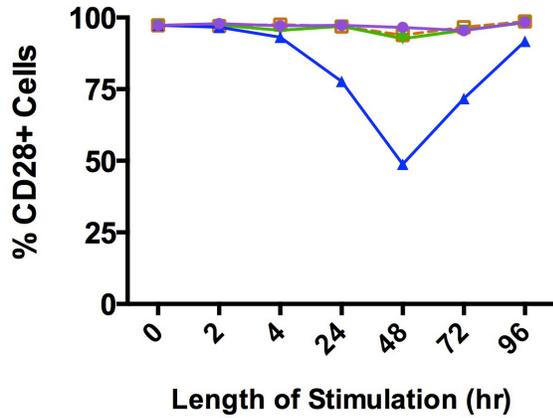


Fig. 2.46



- CD3
- ▲ CD3+CD28
- ▼ CD3+ICAM-1
- ◻ CD3+CTLA-4

**Figures 2.45-2.46. Costimulation through CD28 altered expression of CD28 greater than costimulation through either ICAM-1 or CTLA-4.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 for 2, 4, 24, 48, 72 and 96 hours after stimulation began, cells were analyzed for surface expression of CD28. Graphs on the right are duplicates of the graph to the left and exclude stimulation through CD3+CD28 so that differences among CD3, CD3+ICAM-1, and CD3+CTLA-4 can be observed. Representative of 3 experiments for all timepoints. **Fig. 2.45,** Average MFI of CD28. **Fig. 2.46,** Average percentage CD28<sup>+</sup> cells.

*Expression of CD62L, CD45RO, and CD27 but not CD69, CCR7, or CD25 reflected early differentiation events induced by costimulation through CTLA-4*

(Summarized in **Table 2.1** on page 66)

The expression of several surface proteins was assessed to determine whether activation and differentiation induced by costimulation through CTLA-4 could be detected at early timepoints and whether expression patterns induced by CTLA-4 differed from costimulation through ICAM-1 or CD28. Activation markers (CD69 and CD25), migration markers (CCR7 and CD62L), and differentiation markers (CD45RO and CD27) were assessed at 48 and 96 hours of stimulation. CD69 and CD25 were induced by costimulation through CTLA-4 but did not differ significantly from levels induced by stimulation through the TCR (CD3) (**Fig. 2.47-2.50**). However, stimulation through CD3+ICAM-1 did not induce high levels of activation early during the differentiation process either, consistent with the slightly delayed kinetics we have observed with ICAM-1 compared to CD28 costimulation (40). The expression of migration markers, CD62L and CCR7, induced by costimulation through CTLA-4 were comparable to ICAM-1 (**Fig. 2.51-2.54**). Cells stimulated through CD28 expressed higher levels of CCR7 and lower levels of CD62L compared to cells stimulated through CTLA-4 or ICAM-1. The differences in CCR7 and CD62L expression may reflect the variation in timing of the transition of naïve T cells (which express high levels of CCR7 and CD62L) to effector, memory, and regulatory T cells by the various stimuli.

CD45RO and strong expression of CD27 were determined to assess how the transition from naïve to differentiated T cells is influenced by the various costimulatory proteins (**Fig. 2.55-2.58**). Although not statistically supported, the expression of CD45RO by the four different stimulations (**Fig. 2.55-2.56**) reflected the degree of effector T cells observed on day 7 and was

Fig. 2.47

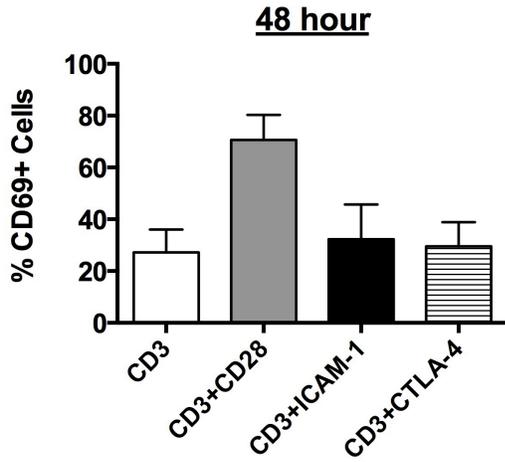


Fig. 2.48

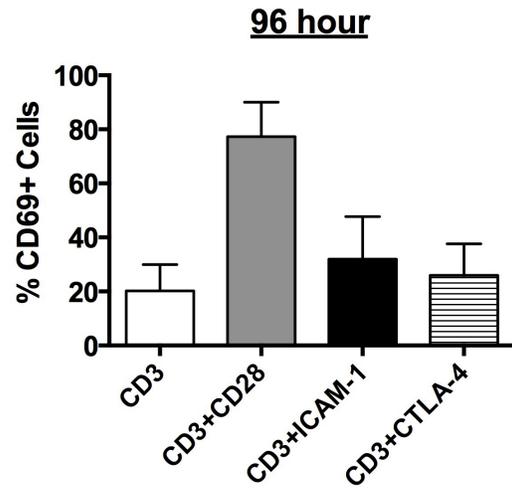


Fig. 2.49

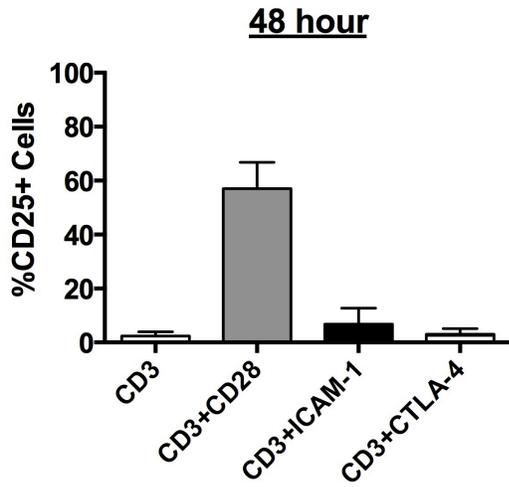


Fig. 2.50

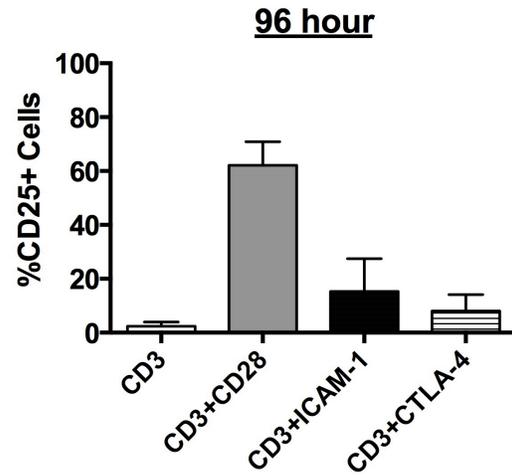


Fig. 2.51

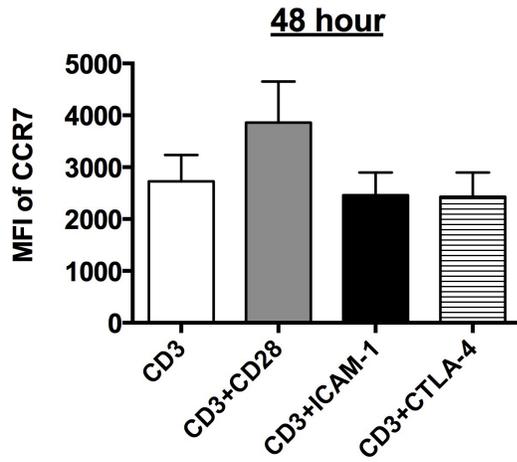


Fig. 2.52

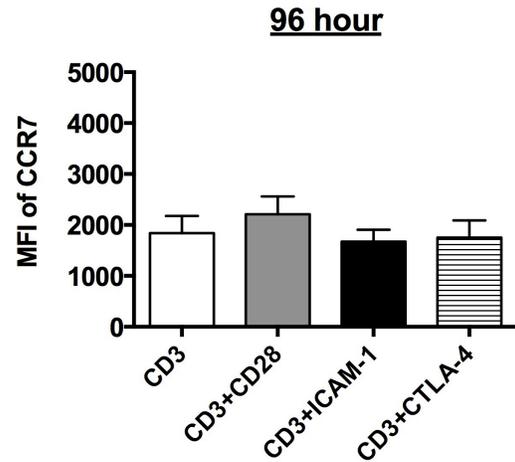


Fig. 2.53

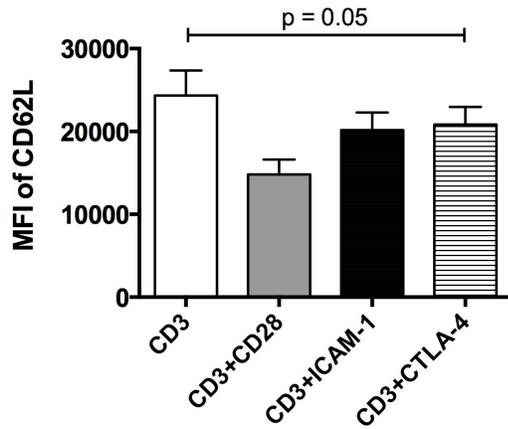


Fig. 2.54

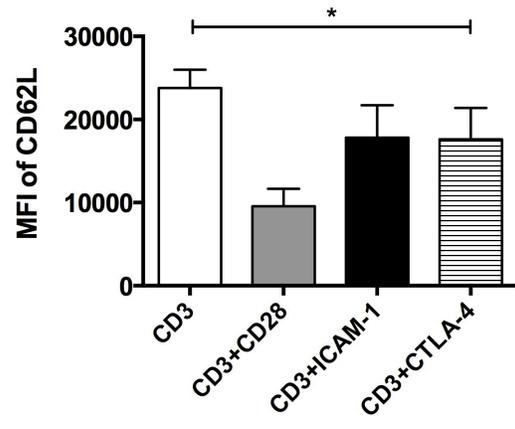


Fig. 2.55

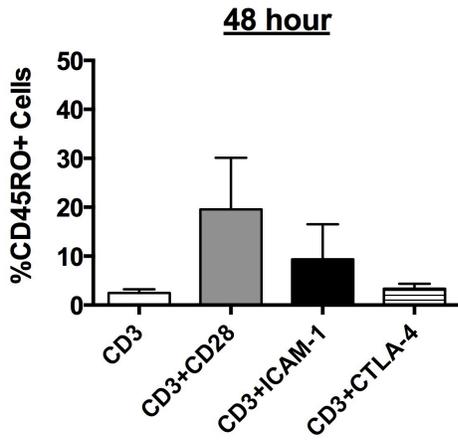


Fig. 2.56

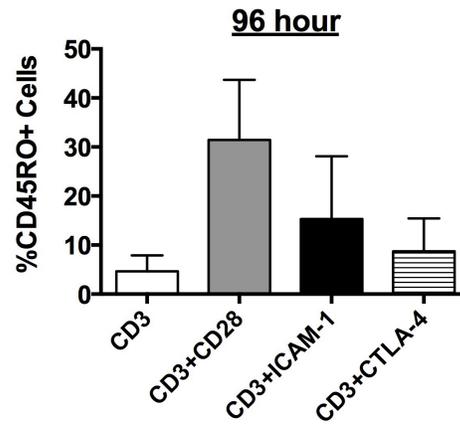


Fig. 2.57

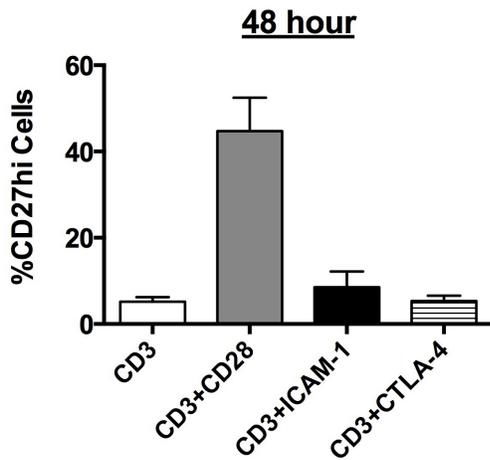
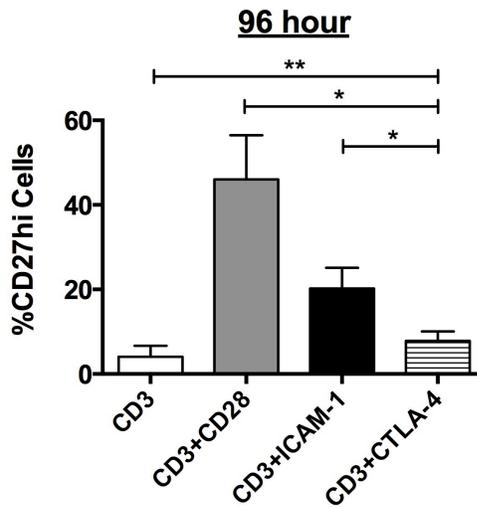


Fig. 2.58



**Figures 2.47-2.58. Expression of CD45RO and strong CD27 expression reflected the differences in activation, proliferation and differentiation induced by each costimulatory protein.** Human naïve CD4<sup>+</sup> T cells were stimulated for 48 or 96 hours with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 and analyzed for surface expression of CD69, CD25, CCR7, CD62L, CD45RO, and strong expression of CD27. Representative of 5 experiments for all surface markers except CCR7 (n = 3). **Fig. 2.47**, Average percentage of CD69<sup>+</sup> cells ± SEM at 48 hours. **2.48**, Average percentage of CD69<sup>+</sup> cells ± SEM at 96 hours. **Fig. 2.49**, Average percentage of CD25<sup>+</sup> cells ± SEM at 48 hours. **2.50**, Average percentage of CD25<sup>+</sup> cells ± SEM at 96 hours. **Fig. 2.51**, Average MFI of CCR7 ± SEM at 48 hours. **2.52**, Average MFI of CCR7 ± SEM at 96 hours. **Fig. 2.53**, Average MFI of CD62L ± SEM at 48 hours. **2.54**, Average MFI of CD62L ± SEM at 96 hours. **Fig. 2.55**, Average percentage of CD45RO<sup>+</sup> cells ± SEM at 48 hours. **2.56**, Average percentage of CD45RO<sup>+</sup> cells ± SEM at 96 hours. **Fig. 2.57**, Average percentage of CD27<sup>hi</sup><sup>+</sup> cells ± SEM at 48 hours. **2.58**, Average percentage of CD27<sup>hi</sup><sup>+</sup> cells ± SEM at 96 hours.

\* p < 0.05, \*\* p < 0.01

consistent with the kinetics of each stimulus. The population of CD27<sup>hi</sup> cells was gated above the median fluorescence intensity of the CD27<sup>+</sup> naïve T cell population to assess early activating and transitional T cells rather than naïve T cells. This CD27<sup>hi</sup> population also demonstrated a slight increase in CD45RO MFI (data not shown). The expression of strong CD27 expression at 96 hours statistically supported differences among the four stimuli early in the differentiation process and emulated the degree of proliferation and activation seen on day 7 caused by each stimulation (**Fig. 2.57-2.58**) with CD3+CD28 and CD3+ICAM-1 representing the stronger stimuli in contrast to CD3+CTLA-4 or CD3.

**Table 2.1**

	<b>CD3+CD28</b>	<b>CD3+ICAM-1</b>	<b>CD3+CTLA-4</b>
<b>% CD69+ cells</b>	48h: 71% 96h: 77%	48h: 32% 96h: 32%	48h: 30% 96h: 26%
<b>% CD25+ cells</b>	48h: 57% 96h: 62%	48h: 7% 96h: 15%	48h: 3% 96h: 8%
<b>CCR7 (MFI)</b>	48h: 3,859 96h: 2,211	48h: 2,460 96h: 1,673	48h: 2,433 96h: 1,754
<b>CD62L (MFI)</b>	48h: 14,810 96h: 9,563	48h: 20,152 96h: 17,819	48h: 20,825 96h: 17,624
<b>% CD45RO+ cells</b>	48h: 20% 96h: 31%	48h: 9% 96h: 15%	48h: 3% 96h: 9%
<b>% CD27<sup>hi</sup> cells</b>	48h: 45% 96h: 46%	48h: 9% 96h: 20%	48h: 5% 96h: 8%

**Table 2.1. Summary of the expression of surface proteins at 48 and 96 hours of stimulation through CD3+CD28, CD3+ICAM-1, and CD3+CTLA-4.** The percentage of CD45RO+ and CD27<sup>hi</sup> cells reflected the degree of activation and proliferation induced by each stimulus at later timepoints of differentiation.

*Costimulation through CTLA-4 differentially influenced expression of CD80 and CD86 on naïve T cells*

The counter receptors for CD28 and CTLA-4, CD80 and CD86, are typically found on APCs (antigen presenting cells); however, they can also be expressed to a lesser degree by activated T cells (64). It has been proposed that engagement of CD80:CTLA-4 may have a functionally distinct outcome to the engagement of CD86:CTLA-4 and that CTLA-4 favors interactions with CD80 (11). We examined whether costimulation through CTLA-4 would differentially upregulate or downregulate CD80 or CD86. Expression of CD80 and CD86 was highest at day 14 in all stimuli (**Fig. 2.59-2.60**). Costimulation through CD28 induced higher percentages of CD80+ and CD86+ cells on days 2 and 14 compared to ICAM-1 or CTLA-4. Costimulation through CTLA-4 maintained relatively lower expression of CD80 and CD86 through 10 days of stimulation (**Fig. 2.59-2.60**). CD3+ICAM-1 induced a significant increase in CD86+ cells on day 10 which may warrant future studies to determine whether this is a consistent finding. In general, MFI expression of CD86 was consistently higher than CD80 regardless of type or duration of stimulus (data not shown). The implications of T cells expressing CD80 and CD86 and the differential regulation of these counter receptors by CTLA-4 and CD28 offer an additional complexity to the function of CTLA-4 *in vivo* that will need to be investigated further.

Fig. 2.59

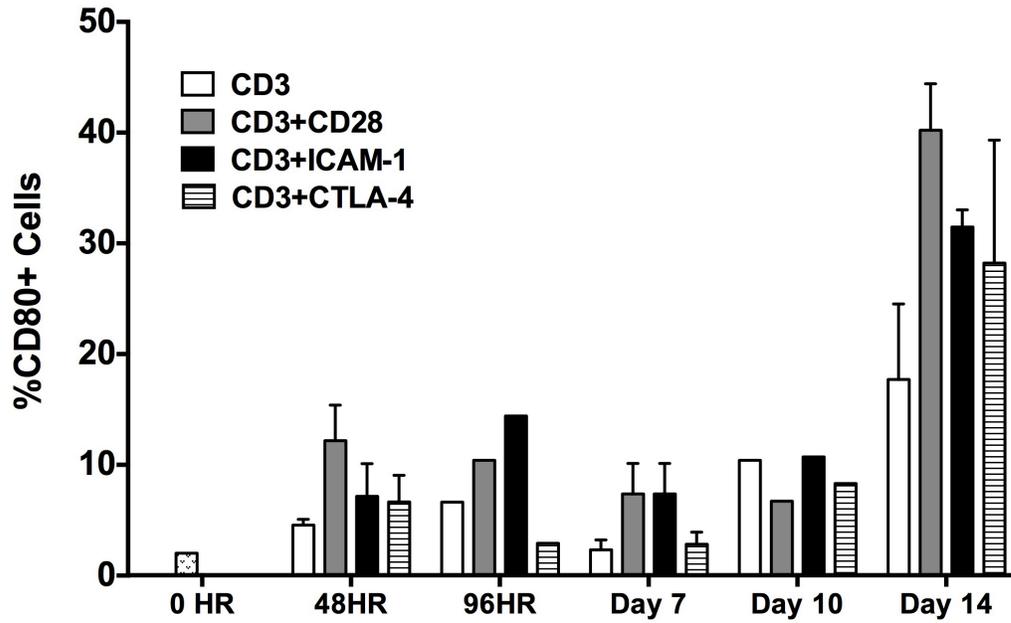
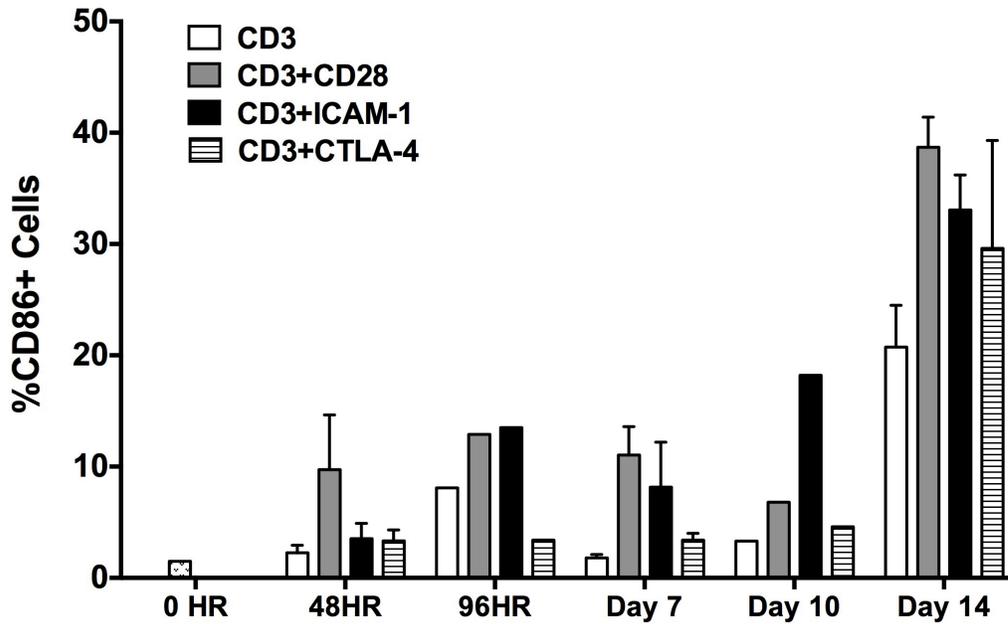


Fig. 2.60



**Figures 2.59-2.60. CD80 and CD86 expression were differentially regulated by costimulation through CD28, ICAM-1, and CTLA-4.** Human naïve CD4<sup>+</sup> T cells were stimulated for 48 or 96 hours or 7, 10 or 14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3 and analyzed for surface expression of CD80 and CD86. Representative of 3 (48h), 2 (7 or 14d), or 1 (96h or 10d) experiment(s). **Fig. 2.59,** Average percentage of CD80<sup>+</sup> T cells  $\pm$  SEM. **Fig. 2.60,** Average percentage of CD86<sup>+</sup> T cells  $\pm$  SEM.

*Part I-Interim Summary: Costimulation through CTLA-4 induced differentiation of naïve CD4+ T cells*

Our data from Part I of this chapter support a role for CTLA-4 as a stimulatory molecule by inducing naïve T cells to proliferate and differentiate to effector, memory, and regulatory T cells in the absence of exogenous cytokines. In contrast to the other two costimulatory molecules, CD28 and ICAM-1, costimulation through CTLA-4 induced overall less activation and slower kinetics of stimulation and favored differentiation to Treg cells, consistent with the role of CTLA-4 as a negative regulator. Also, unlike costimulation through either CD28 or ICAM-1, costimulation through CTLA-4 did not support generation of Th1 cells. Whether CTLA-4 can support Th2 function remains to be determined.

We also demonstrated how differentiation to Treg cells is influenced by the addition of CD28 stimulation to cells costimulated through either ICAM-1 or CTLA-4. These data revealed a difference between ICAM-1 and CTLA-4. Differentiation to Treg cells through CTLA-4 costimulation was inhibited by CD28, but Treg differentiation induced by ICAM-1 was resistant to inhibition by CD28. It appears that when both CD28 and CTLA-4 have equal opportunity to signal into a T cell, CD28 signaling is dominant to signaling through CTLA-4. In Part II, we continue this investigation of whether costimulation through CTLA-4 can influence differentiation when combined with stimulation through CD28. We also examine how the combined stimulation through ICAM-1 and CTLA-4 affects differentiation of naïve T cells.

## Part II

Here, we examined the participation of CTLA-4 as an additional stimulus to costimulation mediated through ICAM-1 or CD28. As observed in part I, both ICAM-1 and CTLA-4 induced differentiation of human naïve CD4<sup>+</sup> T cells to regulatory T cells (Treg cells). Whether these costimulatory proteins induce Treg cells by the same pathway remains to be determined. In the first section of Part II, the effect of combined stimulation through ICAM-1 and CTLA-4 (CD3+ICAM-1+CTLA-4) on Treg differentiation is compared to stimulation through each costimulatory molecule separately (CD3+ICAM-1 vs. CD3+CTLA-4).

Experiments in which anti-CD28 was added to CD3+ICAM-1 or CD3+CTLA-4 stimuli indicated a difference between the Treg populations induced by CTLA-4 and ICAM-1 (Part I). This also suggested that costimulation through CD28 may be dominant to stimulation through CTLA-4 and override signaling by CTLA-4 which seems contrary to earlier *in vitro* studies in murine T cells (14, 15, 65). We, therefore, tested the ability of anti-CTLA-4 to modulate costimulation through CD28 and ICAM-1 by examining other aspects of differentiation in the latter section of Part II.

### *Combined stimulation through ICAM-1 and CTLA-4 augmented differentiation to Treg cells*

We hypothesized that ICAM-1 and CTLA-4 may induce differentiation to Treg cells by distinct mechanisms since CD28 stimulation inhibited Treg differentiation by CTLA-4 but not ICAM-1 (Part I). If ICAM-1 and CTLA-4 use different pathways of Treg induction, then the number of Treg cells should increase when the stimuli are combined. If ICAM-1 and CTLA-4 utilize the same pathway, then the number of Treg cells may not increase when the stimuli are

combined. We examined how combined stimulation through both ICAM-1 and CTLA-4 would impact differentiation to Treg cells compared to each stimulation separately. Stimulation through CD3+ICAM-1+CTLA-4 increased differentiation to Treg cells compared to stimulation through each costimulatory protein separately and appeared to be additive (**Fig. 2.61** and **Fig. 2.62**). Although the number of Treg cells increased by 2- fold, the production of IL-10 by cells stimulated through both ICAM-1 and CTLA-4 was roughly equivalent to stimulation through CD3+ICAM-1 (**Fig. 2.63**).

The total population of CD25+ T cells (both activated and regulatory cells) also increased from  $48,070 \pm 13,140$  (CD3+ICAM-1) or  $27,810 \pm 7,057$  (CD3+CTLA-4) to  $72,710 \pm 10,120$  cells through CD3+ICAM-1+CTLA-4 ( $p < 0.05$ ) (**Fig. 2.64**). Functionally suppressive Treg cells express CD25 more strongly than activated T cells (66) so we also determined whether the MFI of CD25 within the three populations of Treg cells differed and therefore, may reflect functional differences. All three stimulations generated Treg populations that expressed equivalently high levels of CD25 (**Fig. 2.65**).

*Cells costimulated through CTLA-4 released soluble CD25 at a higher rate than cells costimulated through ICAM-1 or combined stimuli*

In part I, we found that the rate at which cells secreted soluble CD25 (sCD25) was greater with CTLA-4 costimulation than ICAM-1. We wondered whether cells stimulated through both ICAM-1 and CTLA-4 would reflect the distinct activity observed by one of these costimulatory proteins separately or an intermediate level of activity, reflecting some activity from both proteins. We analyzed the production of sCD25 by cells stimulated through CD3+ICAM-1+CTLA-4 compared to CD3+ICAM-1 or CD3+CTLA-4 (**Fig. 2.66**). Combined

Fig. 2.61

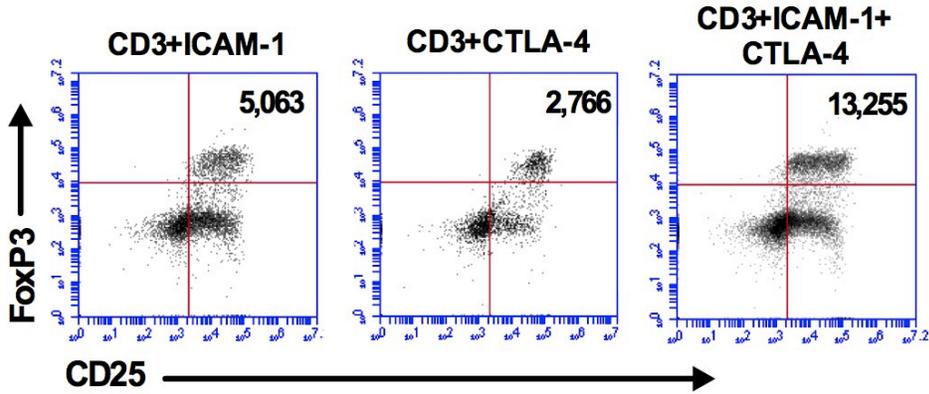


Fig. 2.62

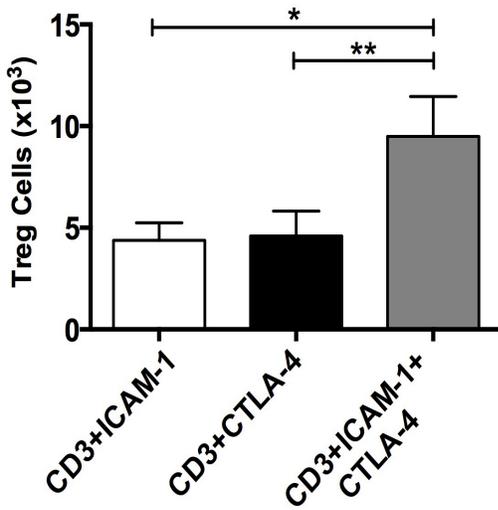
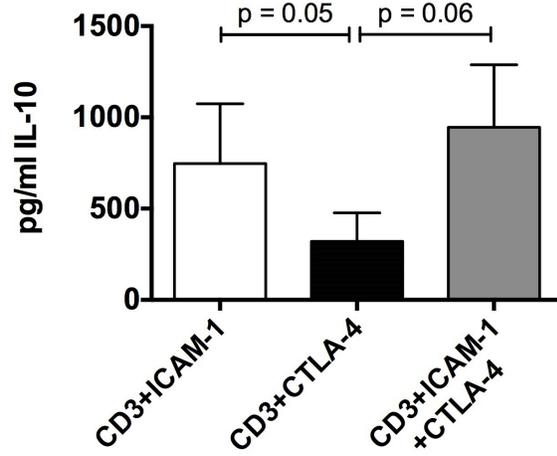


Fig. 2.63



**Figures 2.61-2.63. Combined stimulation through both ICAM-1 and CTLA-4 augmented differentiation to Treg cells.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 10 days with anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4 were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Regulatory T cells (Treg cells) are defined as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup>. Representative of thirteen experiments. Supernate was collected for analysis of IL-10 production by ELISA. **Fig. 2.61**, Upper right quadrant identifies regulatory T cells with cell numbers indicated. **Fig. 2.62**, Average number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup> T cells (Treg cells) ± SEM at days 7 or 10. **Fig. 2.63**, Average production of IL-10 pg/ml ± SEM at days 7 or 10. Representative of nine experiments.

\* p < 0.05, \*\* p < 0.01

Fig. 2.64

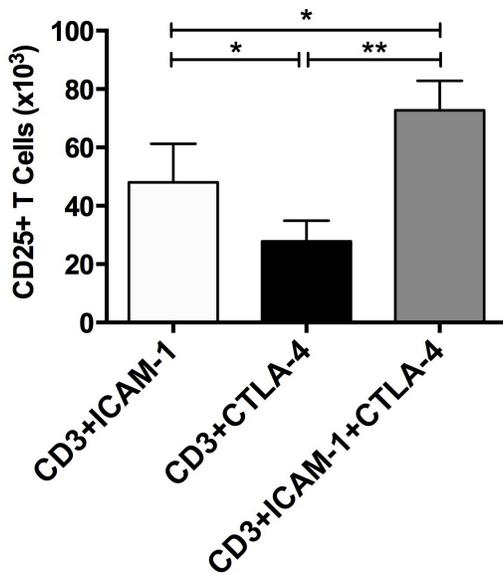
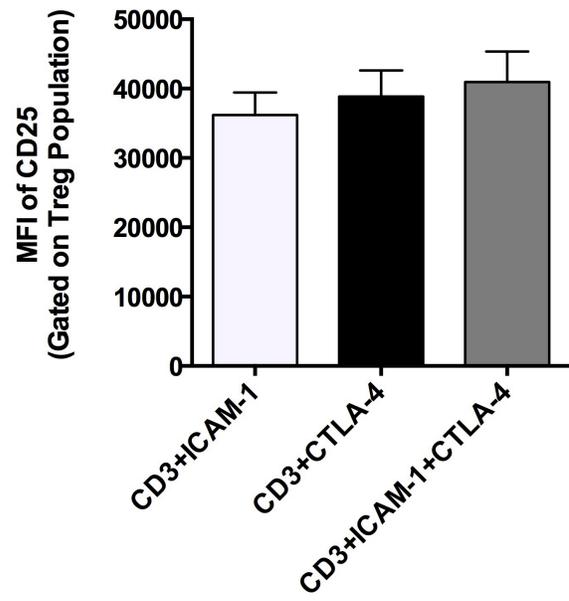


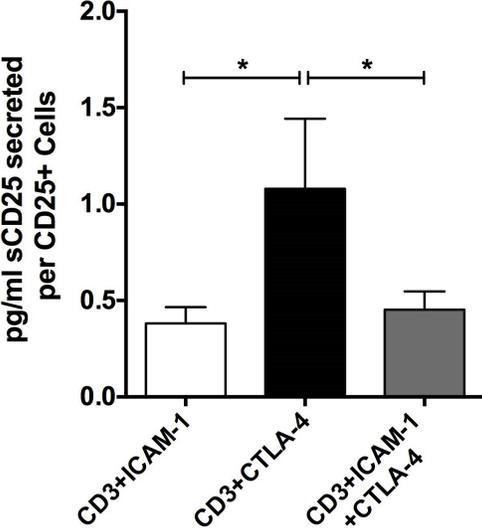
Fig. 2.65



**Figures 2.64-2.65. Combined stimulation through CTLA-4 and ICAM-1 augmented the total CD25+ population.** Human naïve CD4+ T cells stimulated for 7 or 10 days with anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4 were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Regulatory T cells (Treg cells) are defined as CD4+CD25+Foxp3<sup>hi</sup>. Representative of thirteen experiments. **Fig. 2.64**, Average number of CD25+ T cells ± SEM. **Fig. 2.65**, Average MFI ± SEM of the Treg population.

\* p < 0.05, \*\* p < 0.01

Fig. 2.66



**Figures 2.66. Stimulation through CD3+CTLA-4 induced the release of soluble CD25 at a higher rate than stimulation through CD3+ICAM-1 or CD3+ICAM-1+CTLA-4.** Human naïve CD4+ T cells were stimulated for 7 days with anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4, and anti-CD3+anti-ICAM-1+anti-CTLA-4. On day 7, supernate was collected and analyzed for sCD25 by ELISA. The cells were stained for CD25 expression and analyzed by flow cytometry to determine the number of CD25+ T cells. Production of sCD25 was normalized to the number of CD25+ T cells generated in each experiment. Representative of 7 experiments. **Fig. 2.66**, Average sCD25 (pg/ml) produced per CD25+ T cell  $\pm$  SEM.

\*  $p < 0.05$

stimulation through ICAM-1 and CTLA-4 generated 0.45 pg/ml sCD25/CD25+ T cell which resembled the rate seen in cells stimulated through CD3+ICAM-1 but was considerably less than cells stimulated through CD3+CTLA-4 (**Fig. 2.66**). Stimulation through the combined stimuli appeared to reflect only the activity seen when cells are costimulated through ICAM-1 rather than some activity of cells costimulated through each costimulatory protein.

*Differentiation to Treg cells by stimulation through CD3+ICAM-1+CTLA-4 was dependent on endogenous levels of IL-2 and resembled IL-2 production induced by ICAM-1*

As we observed in part I, Treg populations generated by costimulation through either ICAM-1 or CTLA-4 were dependent on IL-2, but cells stimulated through CTLA-4 produced minimal IL-2 compared to cells stimulated through ICAM-1 (**Fig. 2.26**). We examined how the combined stimuli would influence IL-2 production. High levels of IL-2 were produced in cells stimulated through both costimulatory proteins (**Fig. 2.67**). The activity of cells stimulated through both ICAM-1 and CTLA-4 again resembled the activity of ICAM-1 rather than a balance between CTLA-4 and ICAM-1 activity. We also tested whether Treg differentiation induced by the combined stimuli was dependent on endogenous levels of IL-2 similar to costimulation through the individual stimuli (**Fig. 2.68**). As expected, blocking endogenous IL-2 reduced the Treg population generated by CD3+ICAM-1+CTLA-4.

*Human donor cells varied in their responses to ICAM-1 or CTLA-4 costimulation*

In **Fig. 2.62**, the combined effect of stimulation through both CTLA-4 and ICAM-1 on Treg differentiation appeared additive. However, as seen in **Fig. 2.68** (“isotype control” bars), the effect of the combined stimuli appeared to be synergistic which was observed with certain

Fig. 2.67

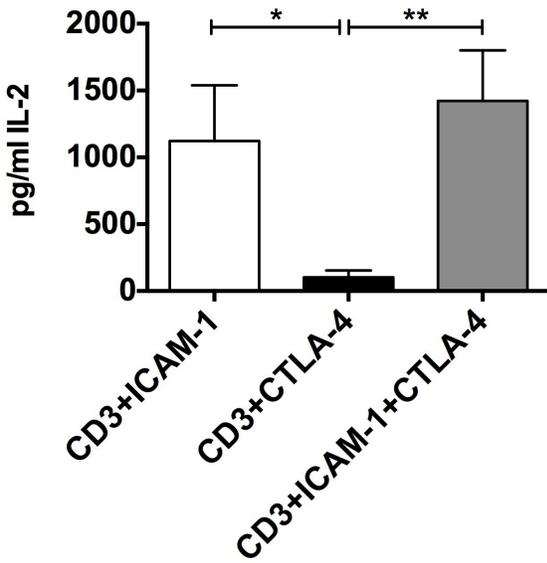
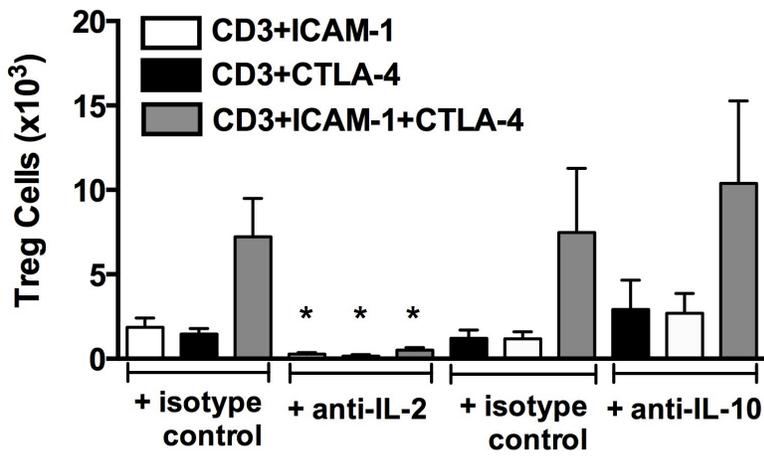


Fig. 2.68



**Figures 2.67-2.68. Stimulation through CD3+ICAM-1+CTLA-4 produced high levels of IL-2 and was dependent on endogenous levels of IL-2 for Treg differentiation.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4, or anti-CD3+anti-ICAM-1+anti-CTLA-4. Supernate was collected for analysis of IL-2 production by ELISA (**Fig. 2.72**). Isotype controls, anti-IL-2 antibody, or anti-IL-10 antibody were added to medium at the start of stimulations as indicated. On day 7, T cells stained for CD25 and strong Foxp3 expression were analyzed by flow cytometry. Treg cells were defined as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup>. Representative of 4 experiments. **Fig. 2.72**, Average pg/ml IL-2 ± SEM. **Fig. 2.73**, Average number of Treg cells ± SEM with added isotype control, anti-IL-2, or anti-IL-10.

\* p < 0.05

individuals. To further investigate these differences, we examined the type of dominance exerted by costimulation through either ICAM-1 or CTLA-4 to induce differentiation to regulatory T cells in every experiment where the three types of stimulation were used (CD3+ICAM-1, CD3+CTLA-4 and CD3+ICAM-1+CTLA-4). Donor cells were classified as ICAM-1-dominant, CTLA-4-dominant, or co-dominant depending on the number of Treg cells generated through CD3+ICAM-1 compared to CD3+CTLA-4. The algorithm used is outlined in **Fig. 2.69**. If the number of Treg cells generated by ICAM-1 was greater than 1.5 fold compared to the number of Treg cells generated through CTLA-4, that experiment was classified as ICAM-1-dominant and vice versa if CTLA-4 costimulation generated 1.5 fold more Treg cells than ICAM-1. If the number of Treg cells induced through ICAM-1 or CTLA-4 costimulation was less than 1.5 fold different, then the donor cells were classified as co-dominant (or dominant to both costimulatory proteins). Using this metric, out of 21 experiments analyzed, 9 were ICAM-1-dominant, 5 were CTLA-4-dominant, and 7 were co-dominant. Thus, human donor cells differed in their ability to respond to these costimulatory proteins.

*The consequence of combined stimulation through ICAM-1 and CTLA-4 on Treg induction differed greatly depending on the type of dominance exhibited by donor cells*

We compared the effect of stimulation through CD3+ICAM-1+CTLA-4 on the differentiation of naïve T cells to Treg cells among the three different types of dominance observed in donor cells. As described in **Fig. 2.69**, the fold change in Treg induction was determined in ICAM-1-dominant experiments by comparing the number of Treg cells seen with CD3+ICAM-1 versus CD3+ICAM-1+CTLA-4. Addition of anti-CTLA-4 to donor cells stimulated through CD3+ICAM-1 resulted in a nearly 6-fold increase in Treg cells (**Fig. 2.70**),

Fig. 2.69

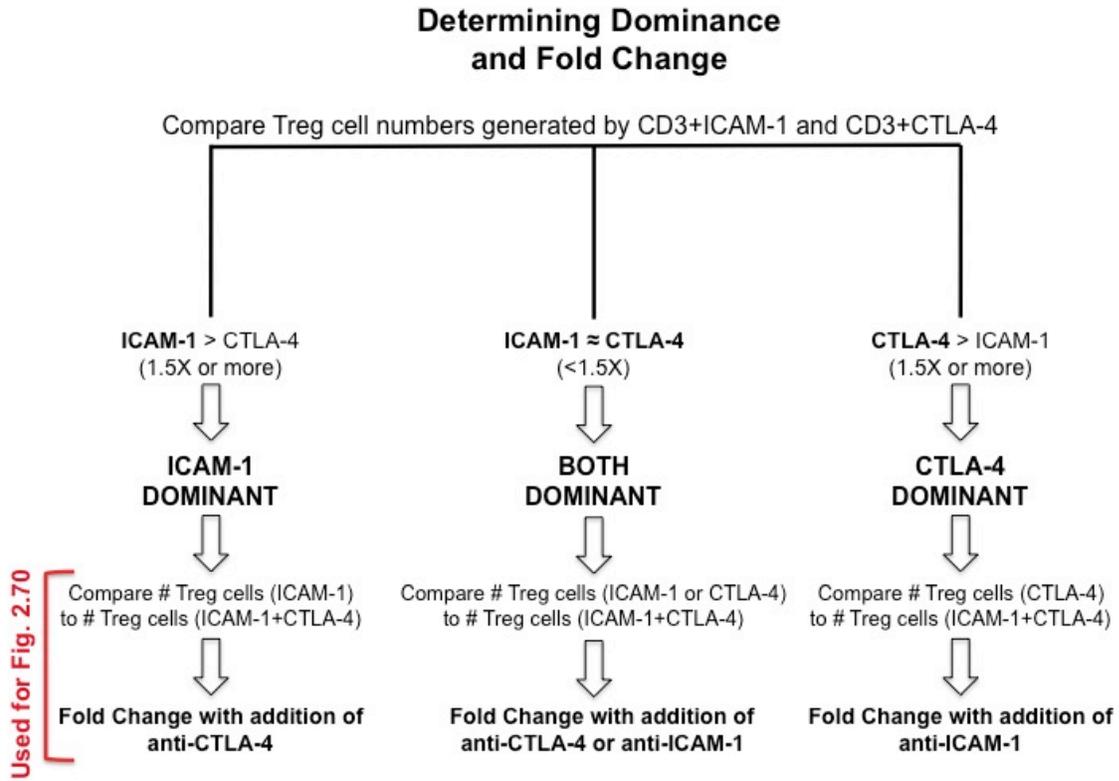
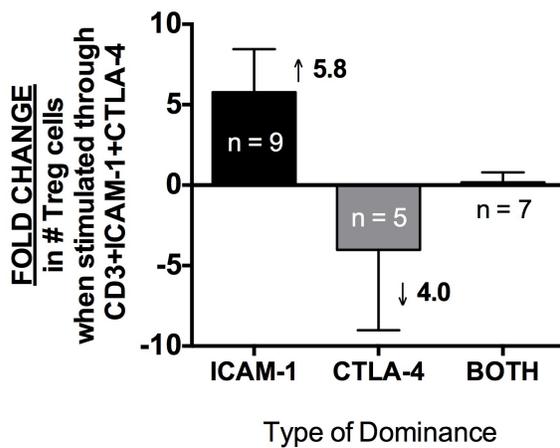


Fig. 2.70



**Figures 2.69-2.70. The effect of the addition of a third stimulus, either anti-CTLA-4 or anti-ICAM-1, on Treg cell induction depended on the type of dominance of donor cells.**

Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days with anti-CD3+anti-ICAM-1, anti-CD3+anti-CTLA-4, or anti-CD3+anti-ICAM-1+anti-CTLA-4. On day 7 or 10, T cells stained for CD25 and strong Foxp3 expression were analyzed by flow cytometry. Treg cells were defined as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup>. Representative of 21 experiments (n = 9, ICAM-1-dominant; n = 5, CTLA-4-dominant; n = 7, co-dominant or both dominant). **Fig. 2.69**, Algorithm developed to determine an individual's dominance in relation to Treg induction by either ICAM-1 or CTLA-4, and then subsequently, the fold change in Treg induction by the addition of a third stimulus provided by: anti-CTLA-4 (ICAM-1 dominant), anti-ICAM-1 (CTLA-4 dominant), or either anti-CTLA-4 or anti-ICAM-1 (BOTH or co-dominant). **Fig. 2.70**, Average fold change in Treg cell numbers  $\pm$  SEM induced by CD3+ICAM-1+CTLA-4 compared to either CD3+ICAM-1 or CD3+CTLA-4.

approximately three times greater than what was seen in **Fig. 2.62** when experiments were not separated by type of dominance. In comparison, addition of anti-ICAM-1 to CTLA-4-dominant donor cells stimulated through CD3+CTLA-4 reduced the number of Treg cells by four-fold compared to costimulation through only CTLA-4 (**Fig. 2.70**). If donor cells expressed co-dominance for Treg cell induction, then the combined effect of CD3+ICAM-1+CTLA-4 showed no overall change in the number of Treg cells (**BOTH, Fig. 2.70**) compared to costimulation through either ICAM-1 or CTLA-4 separately. Therefore, the additive effect seen when all experiments are combined does not accurately reflect the consequence of combined stimulation through both ICAM-1 and CTLA-4. ICAM-1 may impair costimulation through CTLA-4 whereas CTLA-4 may augment costimulation through ICAM-1 under certain circumstances. This is important to consider when interpreting data generated through CD3+ICAM-1+CTLA-4. These observations also suggest that the mechanism of differentiation to Treg cells by ICAM-1 and CTLA-4 differ.

*The ability of CTLA-4 to modulate differentiation induced by either ICAM-1 or CD28 is examined*

In the remaining section of part II, the functional consequence of adding anti-CTLA-4 to either ICAM-1 or CD28 costimulation is examined. Our first goal was to determine whether combined stimulation through ICAM-1 and CTLA-4 augments differentiation induced by ICAM-1 and thus, resembles the effect observed with Treg differentiation. Our second goal was to assess whether CTLA-4 stimulation can influence differentiation induced by CD28. In part I, stimulation through CD3+CD28+CTLA-4 did not generate Treg cells, indicating inhibition of CTLA-4 costimulation by CD28 which was unexpected. We evaluated other aspects of

differentiation to determine whether signaling through CTLA-4 can modulate the differentiation process induced by CD28. In general, costimulation through CTLA-4 was able to modulate the differentiation process induced by ICAM-1 and CD28 but did not influence differentiation by ICAM-1 and CD28 in the same manner.

*Costimulation through CTLA-4 differentially influenced memory and effector differentiation*

We examined differentiation of naïve T cells to effector and memory cells on days 7 and 14 to examine the questions regarding combined stimuli. Addition of CTLA-4 stimulation to CD28 costimulation enhanced differentiation to memory cells on both days 7 and 14 but suppressed differentiation to effector cells (representative figures in **Fig. 2.71** and **Fig. 2.74**, summarized in **Fig. 2.72-2.73** and **Fig. 2.75-2.76**), causing a 2.5-fold increase in memory differentiation and 2.3-fold decrease in effector differentiation at day 14 (**Fig. 2.75-2.76**). In contrast, addition of CTLA-4 stimulation to CD3+ICAM-1 enhanced both memory and effector differentiation observed on day 7 that disappeared by day 14 (**Fig. 2.71-2.73**), indicating an overall boost in differentiation but not modulation as was observed with CD28. The number of differentiated cells (CD45RO+; includes both memory and effector T cells) on day 7 was elevated with CD3+ICAM-1+CTLA-4 but unremarkable by day 14 (**Fig. 2.77-2.78**), further supporting an overall enhancement by CTLA-4 on differentiation induced by ICAM-1 but not regulation. CTLA-4 had no effect on the overall numbers of differentiated cells induced by CD28 on either day 7 or 14, indicating that stimulation through CTLA-4 is able to shift differentiation induced by CD28 to favor generation of memory cells. This also supports the ability of CTLA-4 to regulate stimulation through CD28, which was not evident in the Treg differentiation studies of these costimulatory proteins.

Fig. 2.71

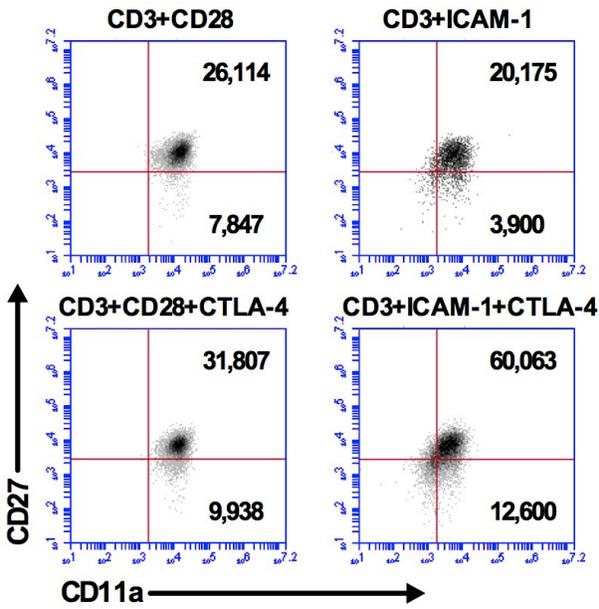


Fig. 2.72

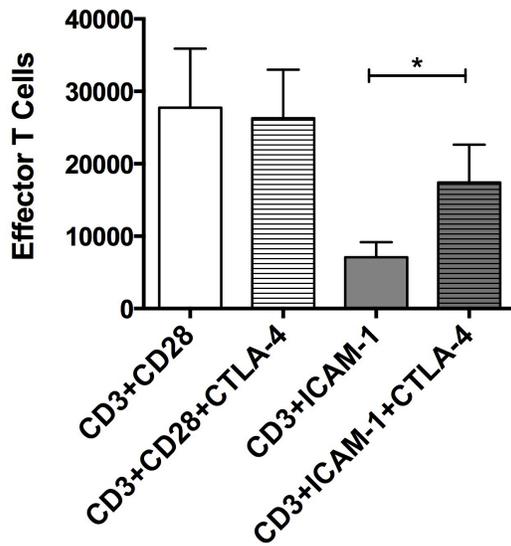


Fig. 2.73

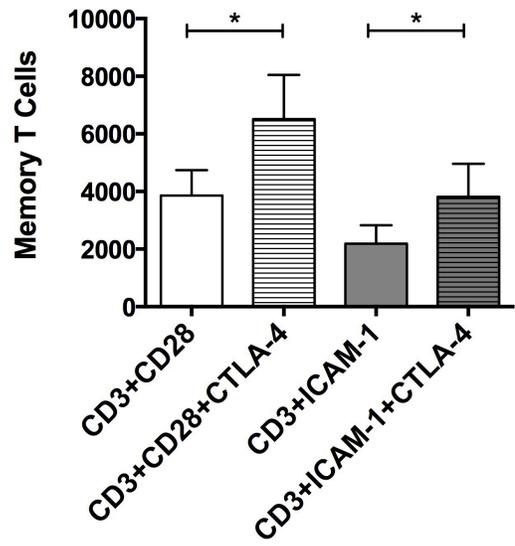


Fig. 2.74

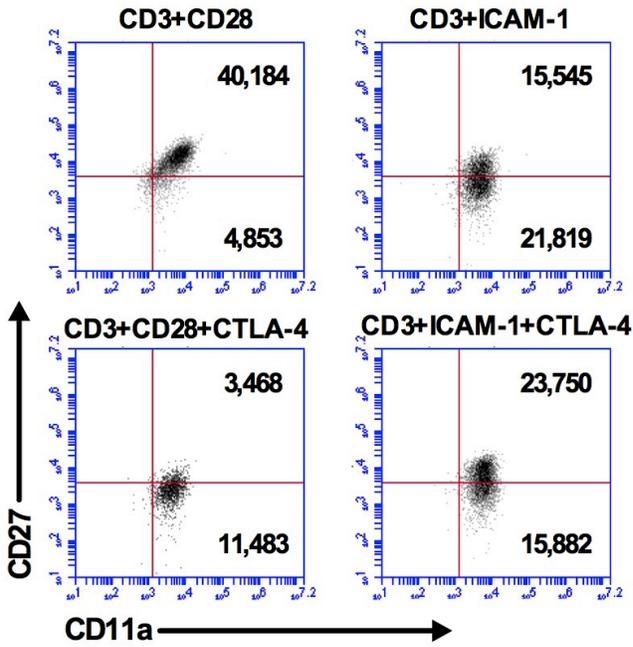


Fig. 2.75

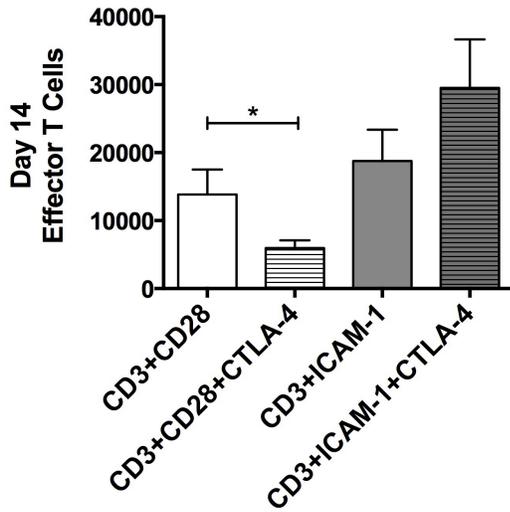
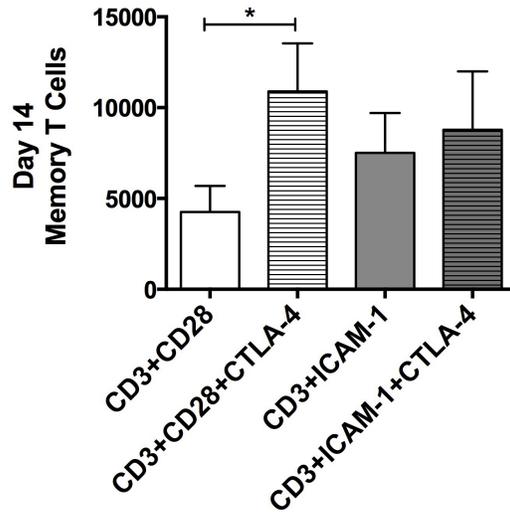


Fig. 2.76



**Figures 2.71-2.76. CTLA-4 stimulation enhanced differentiation to memory T cells by CD28 and augmented differentiation by ICAM-1.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4, or anti-CD3+anti-ICAM-1+anti-CTLA-4 were stained for CD11a, CD27 and CD45RO expression and analyzed by flow cytometry. Representative of fourteen (CD28) or thirteen (ICAM-1) experiments. **Fig. 2.71** and **2.74**, representative figures of the number of CD4<sup>+</sup>CD45RO<sup>+</sup>CD11a<sup>+</sup>CD27<sup>hi</sup> T cells (effector T cells, upper right quadrant) and CD4<sup>+</sup>CD45RO<sup>+</sup>CD11a<sup>+</sup>CD27<sup>lo</sup> T cells (memory T cells, lower right quadrant) on day 7 (**Fig. 2.71**) or day 14 (**Fig. 2.74**). **Fig. 2.72** and **2.75**, average number of effector T cells  $\pm$  SEM on day 7 (**Fig. 2.72**) or day 14 (**Fig. 2.75**). **Fig. 2.73** and **2.76**, average number of memory T cells  $\pm$  SEM at day 7 (**Fig. 2.73**) or day 14 (**Fig. 2.76**).

\* p < 0.05

Fig. 2.77

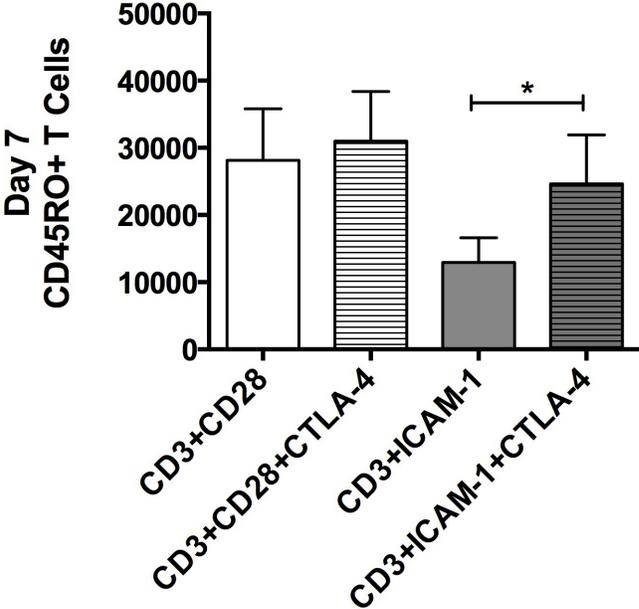
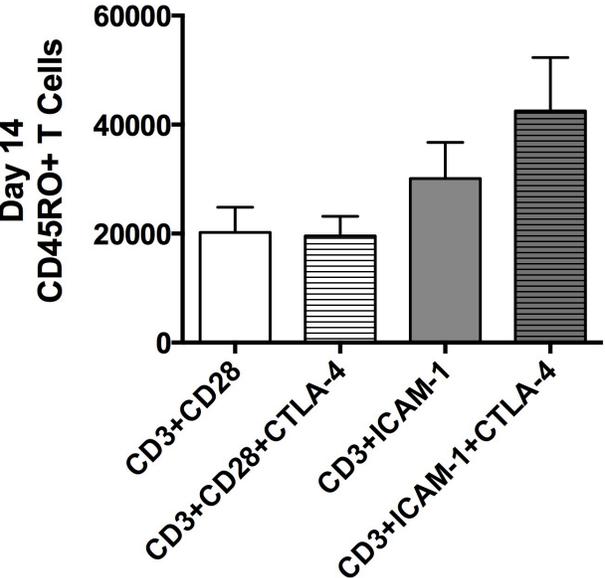


Fig. 2.78



**Figures 2.77-2.78. Costimulation through CTLA-4 augmented the overall number of differentiated induced by ICAM-1 on day 7 but not CD28.** Human naïve CD4+ T cells stimulated for 7 or 14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4, or anti-CD3+anti-ICAM-1+anti-CTLA-4 were stained for CD45RO expression and analyzed by flow cytometry. Representative of fourteen (CD28) or thirteen (ICAM-1) experiments. **Fig. 2.77.** Average number of CD45RO+ T cells  $\pm$  SEM on day 7. **Fig. 2.78,** Average number of CD45RO+ T cells  $\pm$  SEM on day 14.

\*  $p < 0.05$

*Costimulation through CTLA-4 enhanced proliferation of naïve T cells stimulated through ICAM-1 but not CD28 without affecting cell viability*

Next, we assessed the effect of CTLA-4 on naïve T cell proliferation induced by ICAM-1 or CD28. CTLA-4 augmented proliferation induced by costimulation through ICAM-1, demonstrating up to a three-fold increase in the percentage of divided cells in some cases (**Fig. 2.79** and **2.81**). Although the addition of anti-CTLA-4 to stimulation through CD3+CD28 resulted in a statistically significant increase in the percentage of cells that divided at least once, this was a modest increase and was not reflected in cells that had divided two more times (**Fig. 2.80**). Overall, CTLA-4 augmented proliferation induced by ICAM-1 but not CD28. We also determined the degree of cell death in these *in vitro* cultures, and addition of CTLA-4 did not affect cell viability with either type of stimulation (data not shown).

*CTLA-4 stimulation sustained surface expression of CTLA-4 in cells stimulated through CD28*

We examined the surface expression of the three costimulatory proteins (CD28, ICAM-1, and CTLA-4) to determine whether the surface expression of any was altered during combined stimulation and consequently, may affect the degree of stimulation through that protein. The only change in CTLA-4 expression was an increase at 96 hours of combined stimulation through CTLA-4 and CD28 (**Fig. 2.82**). Expression of CTLA-4 in cells stimulated through ICAM-1 with or without CTLA-4 stimulation did not differ (**Fig. 2.83**).

Fig. 2.79

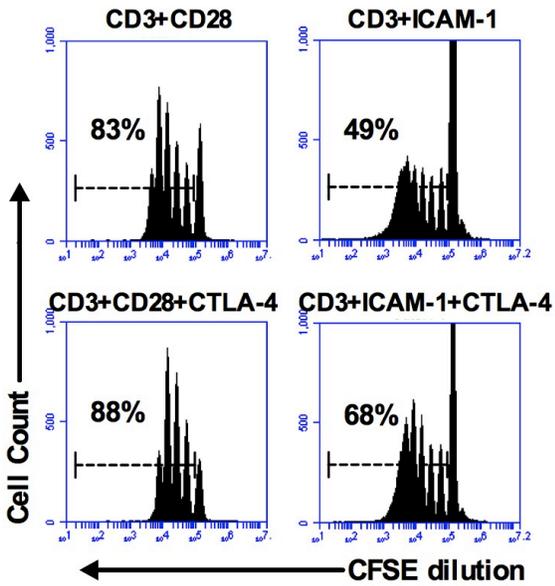


Fig. 2.80

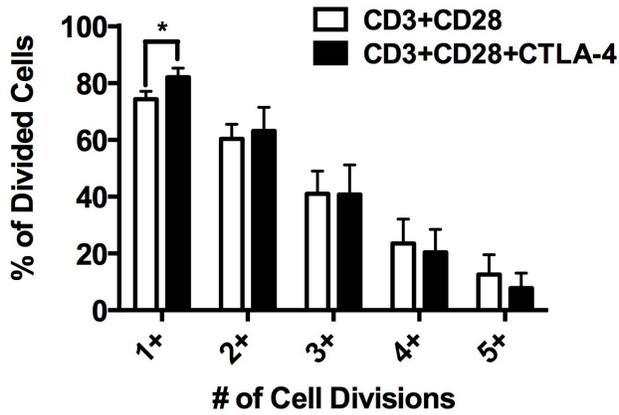
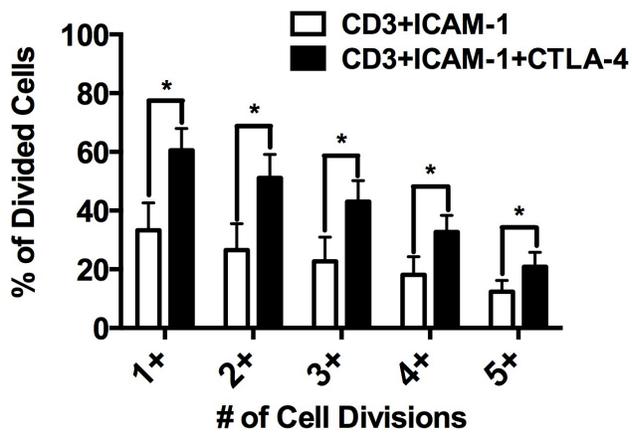


Fig. 2.81



**Figures 2.79-2.81. CTLA-4 augmented naïve T cell proliferation induced by costimulation through ICAM-1.** Human naïve CD4<sup>+</sup> T cells were stained with 2.5  $\mu$ M CFSE on day 0 and stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4. CFSE dilution was analyzed by flow cytometry. Representative of 4 experiments. The # of cells divisions along the x-axes indicates the percentage of cells that have divided 1 or more times (1+), 2 or more times (2+), 3 or more times (3+) and so on. **Fig. 2.79**, Percentage of divided cells on day 7 indicated. **Fig. 2.80**, Average percentage of divided cells  $\pm$  SEM comparing CD3+CD28 (open bars) and CD3+CD28+CTLA-4 (closed bars). **Fig. 2.81**, Average percentage of divided cells  $\pm$  SEM comparing CD3+ICAM-1 (open bars) and CD3+ICAM-1+CTLA-4 (closed bars).

\*  $p < 0.05$

Fig. 2.82

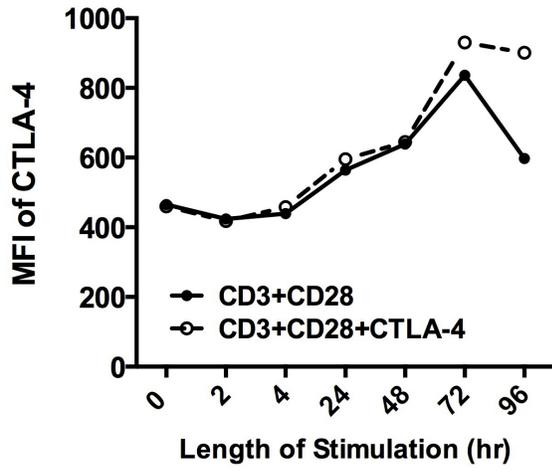
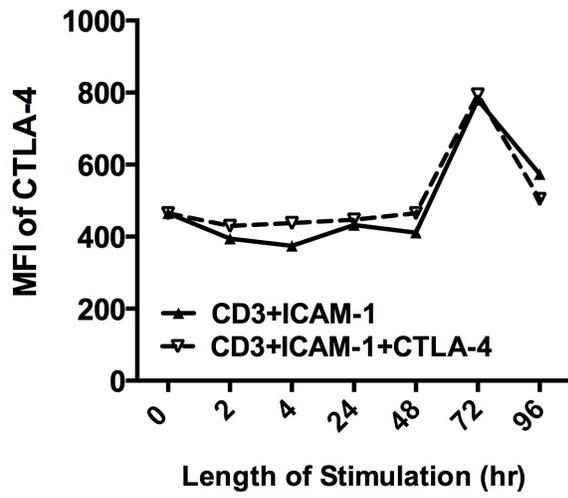


Fig. 2.83



**Figures 2.82-2.83. Costimulation through CTLA-4 sustained expression of CTLA-4**

**induced by CD28.** Human naïve CD4<sup>+</sup> T cells were stimulated with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4. At 2, 4, 24, 48, 72 or 96 hours, cells were analyzed for surface expression of CTLA-4.

Representative of 4-5 experiments for all timepoints except 96 hour (n = 2). **Fig. 2.82**, Average MFI of CTLA-4 comparing stimulation through CD3+CD28 and CD3+CD28+CTLA-4. **Fig.**

**2.83**, Average MFI of CTLA-4 comparing stimulation through CD3+ICAM-1 and CD3+ICAM-1+CTLA-4.

*The addition of CTLA-4 stimulation did not affect expression of ICAM-1 but did significantly modulate expression of CD28 in cells costimulated through CD28*

In **Fig. 2.84-2.90**, the effect of the addition of CTLA-4 stimulation on surface expression of ICAM-1 or CD28 was determined. Expression of ICAM-1 was unaffected by the addition of anti-CTLA-4 to either CD28 or ICAM-1 costimulation (**Fig. 2.84-2.85**). However, the addition of anti-CTLA-4 maintained higher levels of CD28 expression from 2-72 hours of stimulation in cells stimulated through CD28 compared to stimulation in the absence of CTLA-4 (**Fig. 2.86-2.88**). At 96 hours, cells stimulated through CD3+CD28+CTLA-4 exhibited lower CD28 expression than cells stimulated through CD3+CD28 (**Fig. 2.87**). The addition of anti-CTLA-4 to cells costimulated through ICAM-1 did not alter surface expression CD28 (**Fig. 2.89**).

At 96 hours, when CTLA-4 stimulation decreased expression of CD28, it concurrently enhanced expression of CTLA-4 in cells stimulated through CD28. The implications of these expression patterns modulated by costimulation through CTLA-4 and are likely to have stark functional consequences *in vivo*. The functional consequences of these differences in the expression of CD28 and CTLA-4 between CD3+CD28 and CD3+CD28+CTLA-4 stimulations warrant further investigation.

*Costimulation through CTLA-4 combined with either ICAM-1 or CD28 generally did not alter expression of activation, migration or differentiation markers at early timepoints*

The expression of CD69, CD25, CCR7, CD62L, CD45RO and strong CD27 expression was assessed at 48 and 96 hours of stimulation through CD3+CD28, CD3+CD28+CTLA-4, CD3+ICAM-1 or CD3+ICAM-1+CTLA-4 to determine whether there were any differences

Fig. 2.84

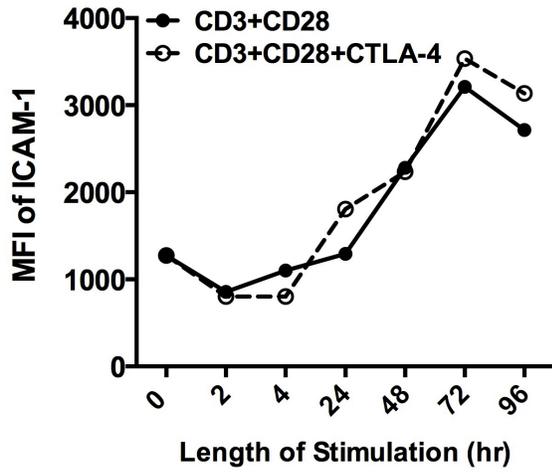
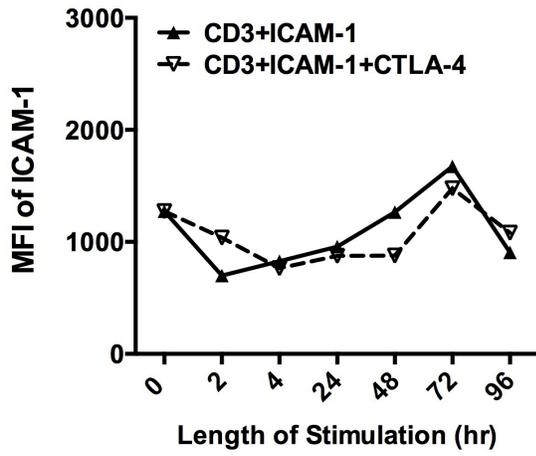


Fig. 2.85



**Figures 2.84-2.85. Costimulation through CTLA-4 did not alter expression of ICAM-1 induced by CD28 or ICAM-1.** Human naïve CD4<sup>+</sup> T cells were stimulated with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4. 2, 4, 24, 48, 72 or 96 hours after stimulation began, cells were analyzed for surface expression of ICAM-1. Representative of 4-6 experiments. **Fig. 2.84,** Average MFI of ICAM-1 comparing stimulation through CD3+CD28 and CD3+CD28+CTLA-4. **Fig. 2.85,** Average MFI of ICAM-1 comparing stimulation through CD3+ICAM-1 and CD3+ICAM-1+CTLA-4.

Fig. 2.86

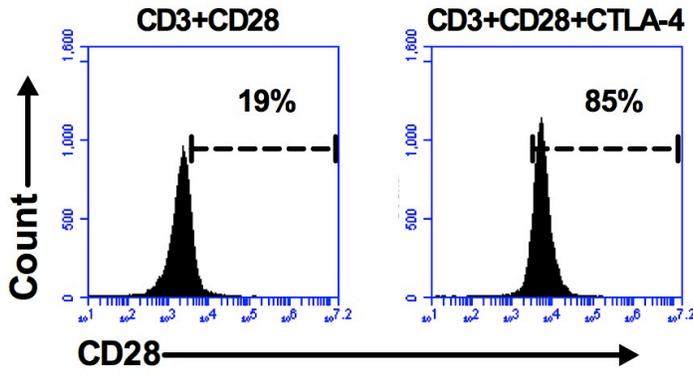


Fig. 2.87

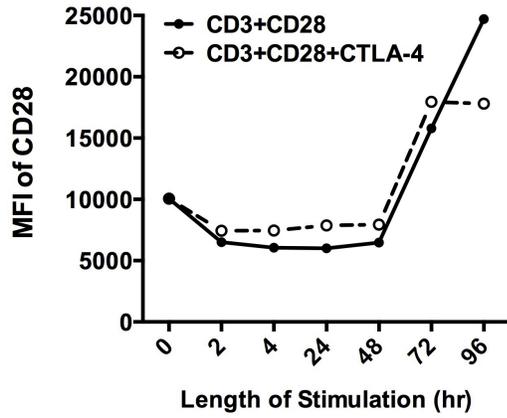


Fig. 2.88

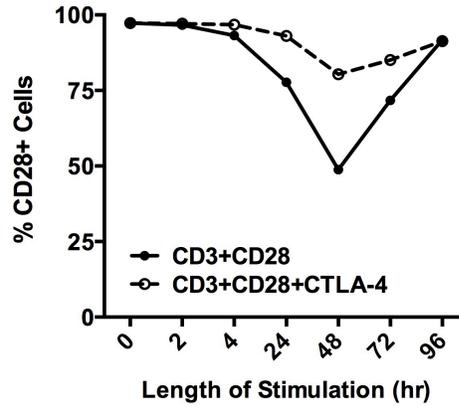
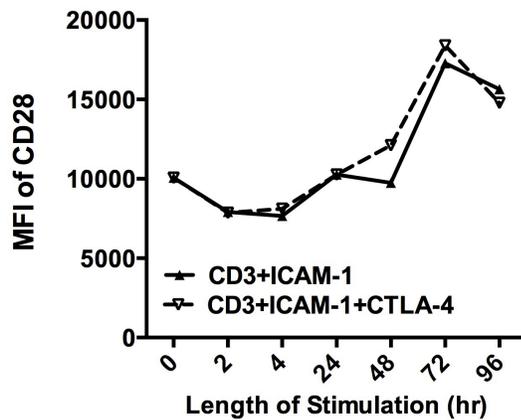


Fig. 2.89



**Figures 2.86-2.89. Stimulation through CD3+CD28+CTLA-4 maintained expression of CD28 compared to stimulation through CD3+CD28.** Human naïve CD4<sup>+</sup> T cells were stimulated with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4. 2, 4, 24, 48, 72 or 96 hours after stimulation began, cells were analyzed for surface expression of CD28. Representative of 3-4 experiments.

**Fig. 2.86,** Percentage of CD28<sup>+</sup> T cells at 48 hours induced through stimulation through CD3+CD28 or CD3+CD28+CTLA-4. **Fig. 2.87,** Average MFI of CD28 comparing stimulation through CD3+CD28 and CD3+CD28+CTLA-4. **Fig. 2.88,** Average percentage of CD28<sup>+</sup> T cells comparing stimulation through CD3+CD28 and CD3+CD28+CTLA-4. **Fig. 2.89,** Average MFI of CD28 comparing stimulation through CD3+ICAM-1 and CD3+ICAM+1+CTLA-4.

induced by the addition of CTLA-4 (summarized in **Table 2.2**). Expression of CCR7 was also determined on days 7 and 14. Overall, the addition of anti-CTLA-4 to either ICAM-1 or CD28 costimulation did not influence the expression of these various markers as summarized in **Table 2.2**. The only effect observed was a slight increase in the percentage of CD25+ T cells at 96 hours with the addition of anti-CTLA-4 to costimulation through CD28 (**Fig. 2.90**).

*Induction of CD80 and CD86 on T cells followed a similar pattern and level of expression and was moderately regulated by the addition of anti-CTLA-4 to costimulation through CD28*

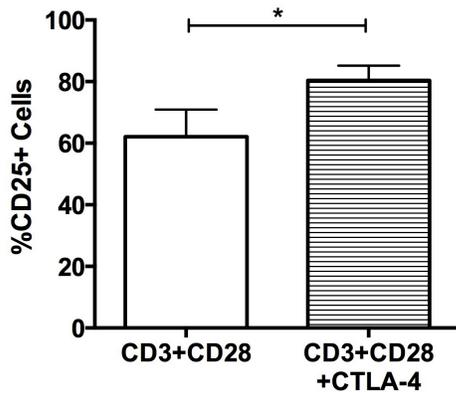
As mentioned earlier, expression of the counter receptors for CD28 and CTLA-4, CD80 and CD86, are typically associated with APCs but can be induced in activated T cells. We assessed whether the addition of CTLA-4 stimulation would modulate the expression of these ligands on cells stimulated through either CD28 or ICAM-1. The pattern and degree of CD80+ or CD86+ T cells demonstrated a similar pattern and level of expression across 2 to 14 days of stimulation regardless of stimulus (**Fig. 2.91-2.94**). Addition of anti-CTLA-4 to cells stimulated through CD28 induced slightly higher percentages of T cells expressing CD80 or CD86 at 4 and 10 days but lower percentages at day 14 (**Fig. 2.91** and **Fig. 2.93**). The addition of anti-CTLA-4 to cells costimulated through ICAM-1 did not influence expression of CD80 or CD86 (**Fig. 2.92** and **Fig. 2.94**). Addition of stimulation through CTLA-4 appeared to modulate the expression of the CD80 and CD86 in cells stimulated through CD28 but not ICAM-1.

**Table 2.2**

	<b>CD3+CD28+CTLA-4 (compared to CD3+CD28)</b>	<b>CD3+ICAM-1+CTLA-4 (compared to CD3+ICAM-1)</b>
<b>CD69</b>	No change	No change
<b>CCR7</b>	No change (includes days 7 and 14)	No change (includes days 7 and 14)
<b>CD62L</b>	No change	No change
<b>CD25</b>	Increased at 96 hr	No change
<b>CD45RO</b>	No change	No change
<b>CD27<sup>hi</sup></b>	No change	No change

Surface expression analyzed by flow cytometry at 48 and 96 hours of stimulation.

**Fig. 2.90**



**Table 2.1 and Figure 2.90. Costimulation through CTLA-4 combined with either ICAM-1 or CD28 generally did not alter expression of activation, migration or differentiation markers.** Human naïve CD4<sup>+</sup> T cells were stimulated for 48 or 96 hours with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4 and analyzed for surface expression of CD69, CD25, CCR7, CD62L, CD45RO, and strong expression of CD27. Expression of CCR7 was also assessed at days 7 and 14. Representative of 5 experiments for all surface markers except CCR7 (n = 3). **Table 2.1,** Summarizes observations of the effect on expression of indicated surface proteins by adding anti-CTLA-4 to either anti-CD3+anti-CD28 or anti-CD3+anti-ICAM-1. **2.90,** Average percentage of CD25<sup>+</sup> cells ± SEM at 96 hours comparing stimulation through CD3+CD28 to CD3+CD28+CTLA-4.

Fig. 2.91

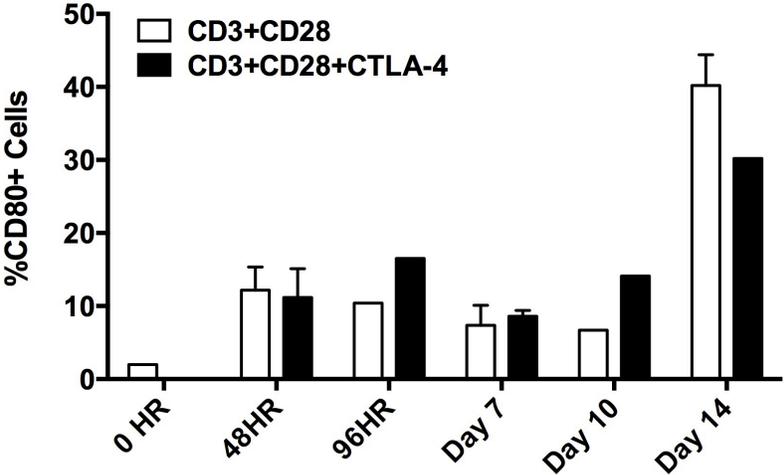


Fig. 2.92

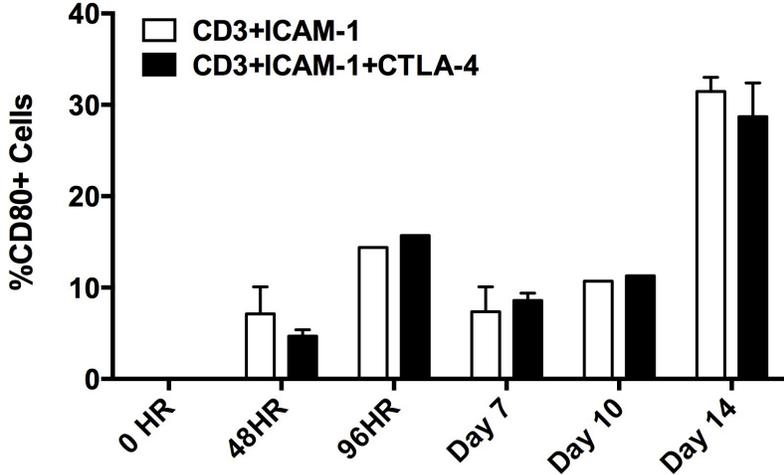


Fig. 2.93

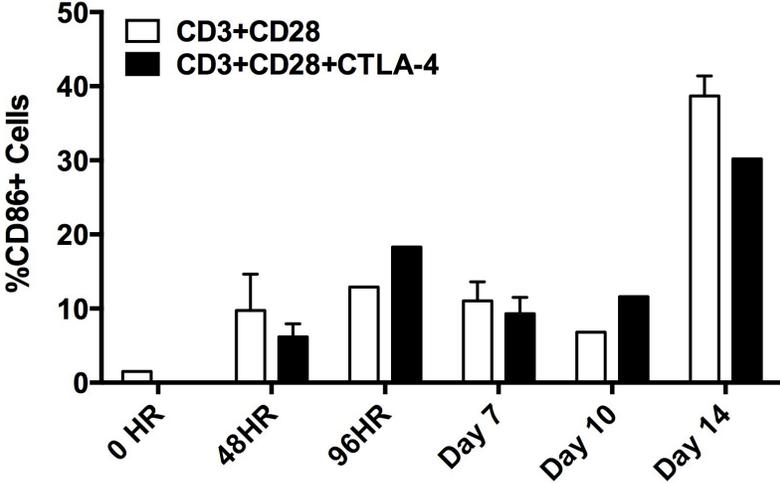
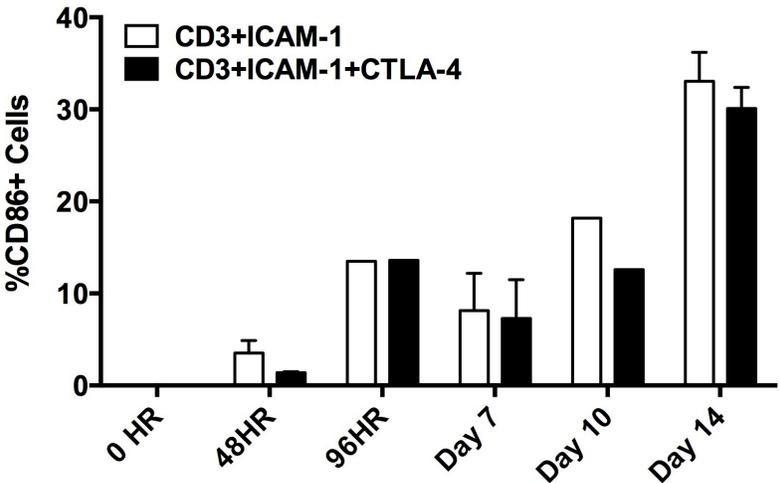


Fig. 2.94



**Figures 2.91-2.94. Induction of CD80 and CD86 expression on T cells followed a similar pattern and level of expression.** Human naïve CD4<sup>+</sup> T cells were stimulated for 48 or 96 hours or 7, 10 or 14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, anti-CD3+anti-CD28+anti-CTLA-4 or anti-CD3+anti-ICAM-1+anti-CTLA-4 and analyzed for surface expression of CD80 and CD86. Representative of 3 (48h), 2 (7 or 14d), or 1 (96h or 10d) experiment(s). **Fig. 2.91**, Average percentage of CD80<sup>+</sup> T cells  $\pm$  SEM comparing stimulation through CD3+CD28 and CD3+CD28+CTLA-4. **Fig. 2.92**, Average percentage of CD80<sup>+</sup> T cells  $\pm$  SEM comparing stimulation through CD3+ICAM-1 and CD3+ICAM-1+CTLA-4. **Fig. 2.93**, Average percentage of CD86<sup>+</sup> T cells  $\pm$  SEM comparing stimulation through CD3+CD28 and CD3+CD28+CTLA-4. **Fig. 2.94**, Average percentage of CD86<sup>+</sup> T cells  $\pm$  SEM comparing stimulation through CD3+ICAM-1 and CD3+ICAM-1+CTLA-4.

*Part II-Interim Summary: CTLA-4 regulated differentiation by ICAM-1 and CD28 differently*

Our data from Part II of this chapter addressed two questions: 1) whether CTLA-4 could modulate differentiation induced by CD28 and 2) whether the mechanisms by which CTLA-4 and ICAM-1 generate regulatory T cells differ. Although signaling through CD28 inhibited differentiation to Treg cells by CTLA-4 (Part I), costimulation through CTLA-4 regulated effector and memory differentiation induced by CD28 by shifting differentiation to favor memory cells at the cost of effector cells. This suggests that an activating cell that receives signals from both CD28 and CTLA-4 may be influenced by both stimuli. The degree to which each costimulatory molecule contributes to directing cell fate most likely will depend on the relative expression level of CD28, CTLA-4 and their counter receptors, CD80 and CD86. We show that under circumstances where both proteins have equal opportunity to signal, neither one was necessarily dominant. Both influenced differentiation. CD28 prevented differentiation to Treg cells by CTLA-4, and CTLA-4 prevented differentiation to effector T cells by CD28, producing a large memory population. As we have previously published (41, 42), CD28 directs cells to Th1 and Th2, and CTLA-4 directed cells to Treg but not Th1 cells in the present work. Whether stimulation through CD3+CTLA-4 induces Th2 cells remains to be determined. Future studies should also address whether CTLA-4 combined with CD28 shifts the Th1/Th2 balance.

Our observations from combined stimulation through ICAM-1 and CTLA-4 suggest distinct mechanisms of Treg induction. Responses to costimulation through ICAM-1 or CTLA-4 varied among donors resulting in three types of dominance: ICAM-1-dominance, CTLA-4-dominance or co-dominance. Although originally, it appeared that the combined stimulation through ICAM-1 and CTLA-4 was additive, separating experiments based on dominance resulted in a different interpretation. Under conditions of ICAM-1 dominance, addition of

CTLA-4 stimulation was synergistic, augmenting differentiation to Treg cells by ICAM-1. In CTLA-4-dominant donor cells, though, the addition of ICAM-1 inhibited differentiation to Treg cells by CTLA-4. In the discussion at the end of this chapter, we propose hypotheses for these distinct outcomes based on dominance.

### **Part III**

#### *Costimulation through CTLA-4 did not induce differentiation of murine CD4<sup>+</sup> T cells to Treg cells in the absence of exogenous cytokines*

In Dr. Kelli Williams's dissertation and published work (41, 57), she demonstrated that costimulation through ICAM-1 induced differentiation of human naïve CD4<sup>+</sup> T cells to Treg cells, but that ICAM-1 costimulation failed to induce differentiation of murine CD4<sup>+</sup> T cells to Treg cells. Here we tested whether costimulation through CTLA-4 either as a single costimulatory molecule or combined with CD28 or ICAM-1 stimulations could generate Treg cells in the absence of exogenous cytokines. **Table 2.3** describes the antibody clone and concentrations used for these experiments. Stimulation through CTLA-4 did not generate Treg cells regardless of the other stimulus or stimuli (**Fig. 2.95-2.97**). In fact, addition of anti-CTLA-4 to either ICAM-1 or CD28 costimulation was inhibitory (**Fig. 2.96-2.97**). Although Dr. Williams did not observe Treg induction by ICAM-1 in her murine studies, it did appear that both clones of anti-ICAM-1 generated small but recognizable Treg populations, 4% or 6% in the representative figure (upper right two panels in **Fig. 2.95** and summarized in **Fig. 2.97**). Although no one has demonstrated that costimulation through CTLA-4 in the absence of exogenous cytokines can induce Treg differentiation in mice, stimulation through CTLA-4 in conjunction with CD28+IL-2+TGF- $\beta$  has been shown to enhance differentiation of murine naïve T cells to Treg cells (38). This may warrant future studies in which murine naïve CD4<sup>+</sup> T cells are used instead of the total CD4<sup>+</sup> T cell population and multiple anti-mouse CTLA-4 antibodies are tested at varying concentrations.

**Table 2.3**

<b>Stimulating Antibody</b>	<b>Clone</b>	<b>Concentration (<math>\mu\text{g/ml}</math>)</b>
<b>Anti-CD3</b>	500A2	0.5
<b>Anti-CD28</b>	37.51	2.5
<b>Anti-ICAM-1(KAT)</b>	KAT-1	10
<b>Anti-ICAM-1(YN1)</b>	YN1/1.7.4	10
<b>Anti-msCTLA-4 (anti-murine CTLA-4)</b>	9H10	20
<b>Anti-huCTLA-4 (anti-human CTLA-4)</b>	BN13	20

Fig. 2.95

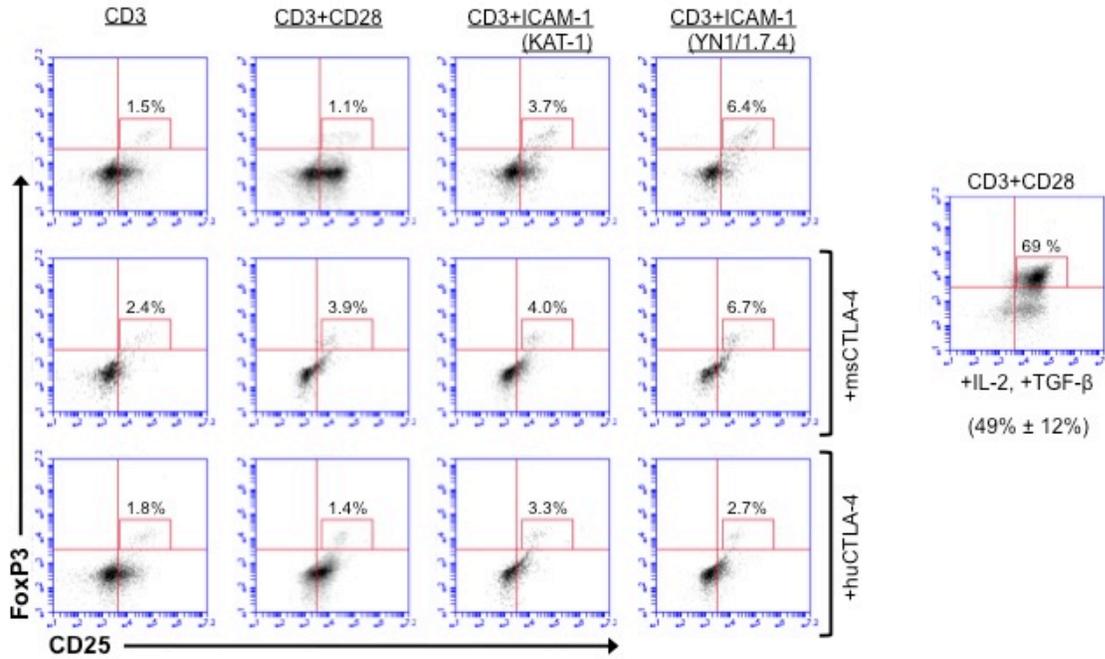


Fig. 2.96

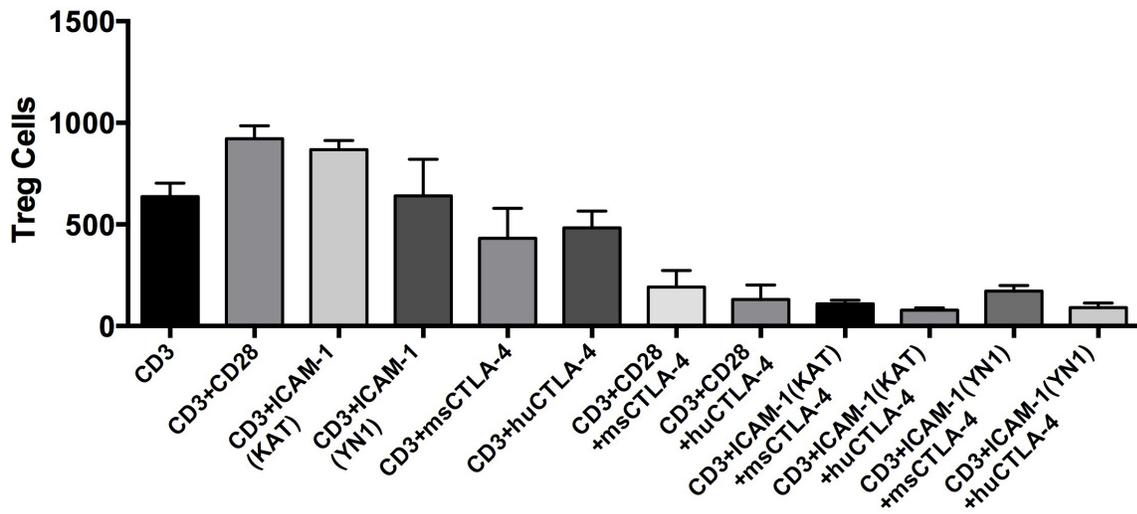
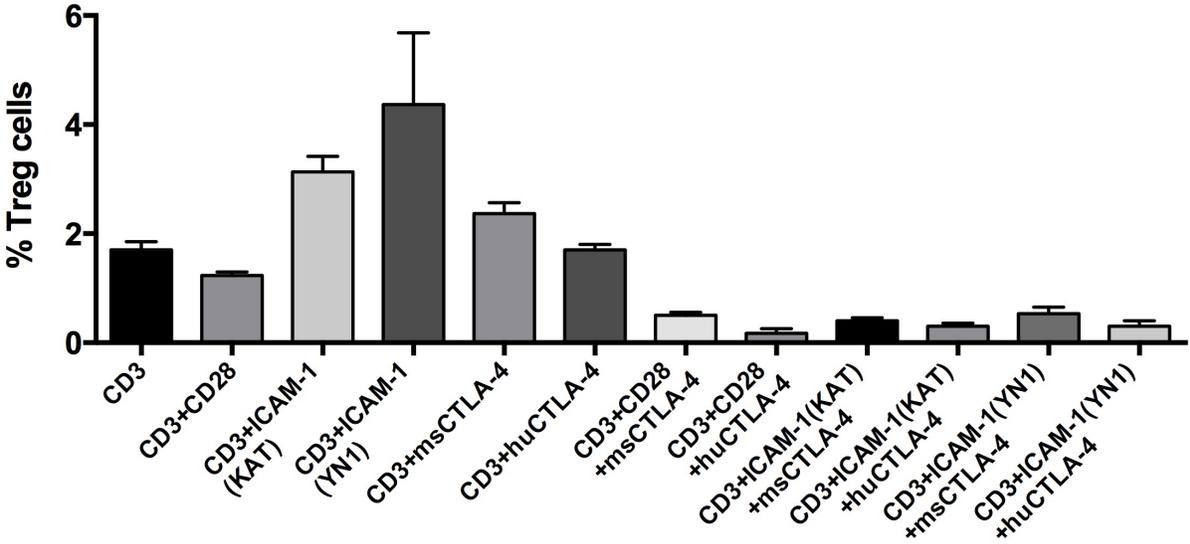


Fig. 2.97



**Table 2.3 and Figures 2.95-2.97. Costimulation through CTLA-4 did not induce differentiation of splenic CD4<sup>+</sup> T cells to regulatory T cells in mice.** CD4<sup>+</sup> T cells were isolated from the spleens of female C57Bl/6 mice 10 weeks of age and stimulated for 5 days using anti-CD3, anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1(KAT), anti-CD3+anti-ICAM-1(YN1), anti-CD3+anti-msCTLA-4, anti-CD3+anti-huCTLA-4, anti-CD3+anti-CD28+msCTLA-4, anti-CD3+anti-CD28+huCTLA-4, anti-CD3+anti-ICAM-1(KAT)+msCTLA-4, anti-CD3+anti-ICAM-1(KAT)+huCTLA-4, anti-CD3+anti-ICAM-1(YN1)+msCTLA-4, and anti-CD3+anti-ICAM-1(YN1)+huCTLA-4 in the absence of exogenous cytokines. T cells stained for CD25 and strong Foxp3 expression were analyzed by flow cytometry. Representative of 3 experiments each performed in triplicate. **Fig. 2.95,** Percentage of Treg cells indicated for each stimulation. Representative positive control (anti-CD3+anti-CD28+IL-2+TGF- $\beta$ ) to the right of panels with average percentage of Treg cells induced from all positive control experiments indicated parenthetically. **Fig. 2.96,** Average number of Treg cells  $\pm$  SEM on day 5. **Fig. 2.97,** Average percentage of Treg cells  $\pm$  SEM on day 5. **Table 2.3,** identifies the target protein, clone, and concentration used in all experiments.

## **Discussion:**

### *Costimulation through CTLA-4 induced differentiation of human naïve CD4+ T cells*

We demonstrate for the first time that costimulation through CTLA-4 in the absence of exogenous cytokines induced activation, differentiation and proliferation of human naïve CD4+ T cells. Stimulation through CD3+CTLA-4 generated a Treg population capable of producing IL-10 and releasing sCD25 at a high frequency. In general, costimulation through CTLA-4 was weaker than costimulation through CD28 or ICAM-1 on day 7 in the ability to induce proliferation, but appeared to be indistinguishable by day 10. This delay may partially be explained by the slower upregulation of CTLA-4 on the surface of activated naïve T cells compared to expression of CD28 or ICAM-1. However, costimulation through CTLA-4 also favored differentiation to Treg cells compared to CD28 or ICAM-1 costimulation. These data support a novel mechanism by which CTLA-4 functions as a stimulatory protein to induce Treg differentiation, which is consistent with the role of CTLA-4 as a negative regulator of the immune response.

### *CTLA-4 and ICAM-1 may drive differentiation to regulatory T cells by distinct mechanisms*

The mechanisms by which ICAM-1 and CTLA-4 drive naïve T cell differentiation to Treg cells may differ. Treg cells induced through CTLA-4 but not ICAM-1 were sensitive to inhibition by CD28 costimulation. Cultures stimulated through CD3+CTLA-4 released sCD25 at a higher frequency than those through CD3+ICAM-1. Exogenous IL-2 enhanced the number of Treg cells induced by costimulation through ICAM-1 but had no effect on the number of Treg cells induced by CTLA-4. Also, costimulation through ICAM-1 generated high levels of IL-2 and IFN- $\gamma$  production and greater numbers of activated cells, which was not observed in cells

costimulated through CTLA-4. These observations suggest different pathways of differentiation utilized by CTLA-4 and ICAM-1.

The outcome of naïve CD4<sup>+</sup> T cell differentiation when both ICAM-1 and CTLA-4 were engaged (CD3+ICAM-1+CTLA-4) further supports the hypothesis that the mechanism of differentiation by these two costimulatory proteins differs. Combined stimulation through both ICAM-1 and CTLA-4 induced higher numbers of activated, effector, memory and regulatory T cells compared to stimulation through either costimulatory protein alone. Cells stimulated through either CD3+ICAM-1 or CD3+ICAM-1+CTLA-4 produced roughly equivalent levels of IL-10 and IL-2 and released sCD25 at the same frequency. In contrast, differentiation outcomes from stimulation through CD3+CTLA-4 or CD3+ICAM-1+CTLA-4 were routinely not equivalent. Finally, we observed three types of responses by donor cells—ICAM-1 dominance, CTLA-4 dominance or co-dominance. Differentiation outcome from combined stimulation through CTLA-4 and ICAM-1 is dictated by responsiveness of donor cells to each costimulatory protein separately. Treg differentiation was greatly augmented by the addition of anti-CTLA-4 in ICAM-1 dominant cells but reduced by the addition of anti-ICAM-1 in CTLA-4 dominant donor cells. Thus, ICAM-1 appeared to inhibit differentiation to Treg cells by CTLA-4, but CTLA-4 augmented differentiation to Treg cells by ICAM-1. These opposing outcomes between the types of dominance support distinct mechanisms of differentiation utilized by each costimulatory protein.

*Hypotheses for the opposing outcomes on Treg differentiation by stimulation through CD3+ICAM-1+CTLA-4*

We hypothesize that ICAM-1 and CTLA-4 use distinct mechanisms to induce differentiation to regulatory T cells. First of all, the two costimulatory proteins may utilize different signaling pathways. Signaling events were not examined in the present study, however, so we are unable to determine if this occurs. Another hypothesis is that costimulation through ICAM-1 directs differentiation to Treg cells by inducing a stronger activating signal compared to CTLA-4. Weak costimulation is one proposed mechanism for induction of Treg cells in the periphery (67). We observed that costimulation through CTLA-4 tended to be weaker than ICAM-1 or CD28 costimulation, and thus, may induce low levels of activation to direct naïve T cells to differentiate into Treg cells.

The effects of stimulation through CD3+ICAM-1+CTLA-4 on Treg differentiation indicate distinct mechanisms utilized by ICAM-1 and CTLA-4. CTLA-4 can upregulate LFA-1 adhesion and clustering (68). One hypothesis to explain the disparate effects on Treg differentiation between the three types of dominance may be that CTLA-4 signaling added to donor cells that respond well to ICAM-1 costimulation (either ICAM-1 dominant or co-dominant) upregulates LFA-1. LFA-1 on the activating T cell may interact with other T cells in culture boosting activating signals by its own signaling or by enhancing adhesion, and thus, T cell:T cell interactions. In the ICAM-1 dominant individuals, this may explain the synergistic effect of stimulation through CD3+ICAM-1+CTLA-4. In donor cells that do not respond well with ICAM-1 but do respond to CTLA-4, costimulation through CTLA-4 may provide a weak costimulatory signal driving naïve T cell differentiation to Treg cells. However, when ICAM-1 signaling is present, the stronger activating signals override this weak costimulation preventing

differentiation to Treg cells by CTLA-4. In donor cells that respond well to either ICAM-1 and CTLA-4 costimulation, there is no overall change in the Treg numbers induced by CD3+ICAM-1+CTLA-4. The aforementioned hypotheses would also explain this observation in co-dominant donor cells. Both mechanisms may be at work in these cultures: CTLA-4 augmenting Treg induction by ICAM-1 and ICAM-1 suppressing Treg induction by CTLA-4. These effects counteract each other resulting an overall observation of no change in co-dominant cells.

*IL-2 was essential for the differentiation of Treg cells by costimulation through ICAM-1, CTLA-4 and combined stimulation through both ICAM-1 and CTLA-4*

Regulatory T cells require IL-2 for survival but produce very little IL-2 of their own (69), which is consistent with our observation that Treg differentiation was dependent on IL-2 regardless of stimulus. The high levels of IL-2 production observed by cells stimulated through CD3+ICAM-1 or CD3+ICAM-1+CTLA-4 may have been generated by other T cell populations in these cultures. Th1 cells produce significant amounts of IL-2, and as shown previously (40) and verified in chapters 2 and 3 of this dissertation, costimulation through ICAM-1 induces differentiation of naïve T cells to Th1 cells. Costimulation through CTLA-4 favored differentiation to Treg cells and did not induce Th1 function. This may explain the dependency of Treg differentiation on IL-2 but low level of IL-2 produced by cells costimulated through CTLA-4. In cultures stimulated through CTLA-4, the ratio of regulatory T cells to conventional T cells is higher so any IL-2 being produced is most likely being consumed but not replenished by the Treg cells. The higher frequency at which sCD25 was produced by cells stimulated through CD3+CTLA-4 may have also played a role in the low levels of IL-2 production. These

data support a role for CTLA-4 as a negative regulator of T cells by activating Treg differentiation.

*The balance and strength of CTLA-4 and CD28 signaling may influence cell fate*

Our findings concerning CD28 and CTLA-4 costimulation do not conflict with previous findings that CTLA-4 may regulate CD28 signaling (14-17). The addition of anti-CTLA-4 to stimulation through CD3+CD28 was unable to induce Treg differentiation, and therefore, CD28 appeared to suppress the effects of CTLA-4 signaling. However, if our hypothesis for the mechanism of Treg induction by CTLA-4 is correct, then this inhibition by CD28 may be explained similar to what happens in CTLA-4-dominant donor cells when anti-ICAM-1 is added. Stronger activating signals generated through CD28 costimulation may overcome weak costimulation through CTLA-4 that may be central to the mechanism by which CTLA-4 induces Treg differentiation. This would also explain why we observe an effect on memory and effector differentiation by CTLA-4 when combined with CD28 but not Treg differentiation. An increase in activating signals provided by CD28 would be unlikely to inhibit generation of memory and effector T cells. In addition, the signaling pathway of CTLA-4 rather than weak costimulation may play a greater role in influencing memory and effector differentiation.

CTLA-4 may regulate effector and memory differentiation. Stimulation through CTLA-4 shifting naïve T cell differentiation by CD28 to memory cells rather than activated, effector cells. Krummel and Allison proposed that inhibitory signaling through CTLA-4 may protect some T cells from activation-induced cell death, supporting the generation of memory T cells (70). Although CTLA-4 was initially thought to promote apoptosis (71), later findings revealed that CTLA-4 actually provides a protective mechanism against cell death (15, 72, 73), supporting this

theory. Blocking CTLA-4 function *in vivo* can inhibit the generation of memory cells (74). It appears that CTLA-4 may function in the participation of memory T cell differentiation.

Our data also refute the conclusion originally drawn from our Treg differentiation studies that CD28 is strictly dominant to CTLA-4 since costimulation through CTLA-4 was able to modulate effector and memory differentiation by CD28. Whether stimulation through CTLA-4 when combined with costimulation through CD28 influences T helper subsets, particularly the balance between Th1 and Th2, may warrant further study. The differentiation outcome of an activating naïve T cell may vary depending on the relative expression levels of CTLA-4, CD28 and the B7 ligands (CD80 and CD86) in the microenvironment because this will influence the degree of CD28 and CTLA-4 signaling. Differences between the engagement of CD80-CTLA-4 and CD86-CTLA-4 (52) may also alter the type or strength of signaling by CTLA-4, causing distinct differentiation outcomes.

### *Summary*

Costimulation through CTLA-4 induced differentiation of human naïve CD4<sup>+</sup> T cells and differentially regulated differentiation induced by CD28 and ICAM-1 costimulation. CTLA-4 suppressed effector and enhanced memory differentiation induced by CD28, consistent with its known ability to regulate the function of CD28. Our data also suggest a novel stimulatory role for CTLA-4 in cells stimulated through CD3+ICAM-1+CTLA-4. We also show for the first time that costimulation of human naïve CD4<sup>+</sup> T cells through CTLA-4 induced differentiation to regulatory T cells in the absence of exogenous cytokines. It will be important to further define and clarify the function of CTLA-4 in human T cells. Such studies will be extremely valuable predicting efficacy and unwanted side effects of therapeutic treatments used in the clinical

setting involving CTLA-4. Collectively, these findings support a novel role for CTLA-4 in human Treg differentiation.

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## **Chapter 3**

**Costimulation of naïve CD4<sup>+</sup> T cells through ICAM-1, CD28 or LFA-1 did not induce differentiation to Th17 cells**

## Introduction

A principal hypothesis under investigation in this laboratory is that the nature of the costimulatory signal delivered during naïve T cell activation guides the outcome of differentiation to specific cell fates. T helper 17 (Th17) cells represent an important component of host defense against bacterial and fungal pathogens. These cells promote inflammatory responses and are particularly important for host defense in tissues with mucosal epithelia. Although many studies have examined the cytokines involved in generating this subpopulation from naïve T cells, differential response to costimulatory signaling has not been examined. In this chapter, we examined whether costimulatory proteins in the absence of exogenous cytokines could induce the differentiation of naïve T cells into Th17 cells.

The role of cytokines in differentiation of naïve T cells to Th17 cells has been well studied and is more complex in humans compared to mice. The addition of TGF- $\beta$  + IL-6 or IL-21 to *in vitro* stimulation generates murine Th17 cells from naïve T cells (1-3). In humans, cytokine polarization of naïve T cells to Th17 cells is less defined, suggesting possibly multiple pathways to Th17 differentiation. Naïve T cells isolated from cord blood can be induced to Th17 cells by stimulation and addition of exogenous TGF- $\beta$ +IL-21, TGF- $\beta$ +IL-23+IL-1 $\beta$ , IL-6 or TNF- $\alpha$ , or finally, TGF- $\beta$ +IL-1 $\beta$ +IL-6, IL-21, or IL-23 (4-6). Stimulated naïve T cells isolated from peripheral blood differentiate to Th17 cells in presence of exogenous IL-1 $\beta$ +IL-6 or IL-1 $\beta$ +IL-23 (7-8).

According to several studies, Th17 cells appear to have a protective role against both bacterial and fungal infections beyond that of Th1 and Th2 responses (reviewed in 9). Th17 cells secrete IL-17 which is considered the hallmark of this T helper subset (1, 2). They secrete various effector cytokines, including IL-17A, IL-17F, IL-21 and IL-22, which activate numerous

cell types to secrete proinflammatory cytokines and chemokines resulting in a robust inflammatory response (10-12). Th17 cells also may play a significant role in autoimmune diseases and have been implicated in rheumatoid arthritis (13), multiple sclerosis (14), and inflammatory bowel disease (15). By understanding the biological factors that guide Th17 generation and function, improved therapies can be developed for treating autoimmunity driven by Th17 cells. Although much work has been done to elucidate the cytokines responsible for Th17 differentiation, the influence of costimulatory proteins has not been investigated. In the present study, we found that none of the costimulatory proteins assayed induced differentiation of naïve CD4<sup>+</sup> T cells to Th17 cells in the absence of exogenous cytokines.

## **Materials and Methods**

### *Antibodies and reagents*

Antibodies used to stain cells for flow cytometry were: anti-IL-17A-PE (eBioscience), anti-IL-17A-APC (eBioscience), anti-CD4-FITC (Invitrogen), anti-CD45RO-FITC (Invitrogen), and anti-ROR $\gamma$ t-APC. eBioscience FoxP3 buffers and protocol were used for intracellular staining of ROR $\gamma$ t and various modifications of this protocol were used for intracellular staining of IL-17A. Monensin or brefeldin A (BD Biosciences) was added to cultures 5-6 hours before staining for IL-17A. Flow cytometry was performed using an Accuri C6 (BD Accuri Cytometers, Ann Arbor, MI) and data analysis using CFlow (Accuri).

### *Human Subjects*

Peripheral blood cells (240 ml into heparin) were obtained after informed consent from healthy adult volunteers of both genders, ages 20-30 years, who had been free of infection for 14 days and did not have immune disorders. Procedures were approved by the University of Kansas Institutional Review Board.

### *Human naïve CD4<sup>+</sup> T cell purification*

Human naïve CD4<sup>+</sup> T cells were isolated as described in Chapter 2.

### *Human Naïve CD4<sup>+</sup> T cell stimulation*

Human naïve CD4<sup>+</sup> T cells were stimulated through plate-bound antibodies as we have previously described (16, 17) and described in Chapter 2. Anti-LFA-1 (HB202, purified from

hybridoma) used at 5  $\mu\text{g/ml}$ . PBMCs were stimulated with PDB ( $10^{-6}$  M) and ionomycin (1  $\mu\text{g/ml}$ ) for 6 hours.

### *ELISA*

Supernates from stimulated cells were collected for analysis of IL-17A production using the Ready-Set-Go! ELISA kit and protocol (eBioscience). Plates were read at 450 nm using the EL311 Microplate Autoreader (Bio-Tek Instruments, Inc., Winooski, VT).

### *Statistical Analysis*

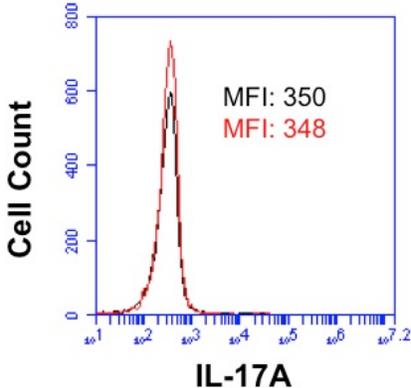
The one-way, paired student's *t*-test was used to determine statistical differences for the IL-17A ELISA using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

## Results

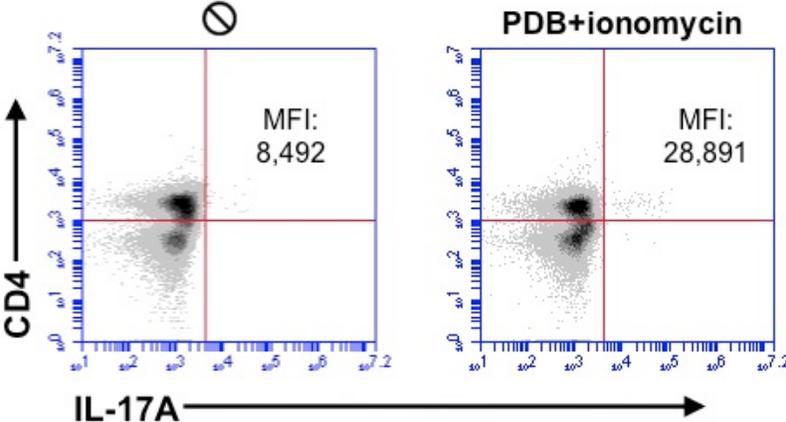
A multiplex assay (n = 1) performed by Drs. Abby Dotson and Kelli Williams revealed that naïve T cells costimulated through CD28 produced 120 and 180 pg/ml IL-17 on days 7 and 14, respectively (18). Costimulation through ICAM-1 generated less IL-17: 80 and 60 pg/ml on days 7 and 14. Based on this experiment, we examined the possibility that costimulation of naïve CD4<sup>+</sup> T cells through CD28, ICAM-1 or LFA-1 may induce differentiation to Th17 cells by examining the production of IL-17A, the hallmark cytokine of Th17 function. As expected, freshly isolated human naïve CD4<sup>+</sup> T cells did not express IL-17A (**Fig. 3.1**), but we were able to induce intracellular expression of IL-17A in PBMCs stimulated with PDB and ionomycin for 6 hours (**Fig. 3.2**), indicating that intracellular production of IL-17A could be detected by flow cytometry.

We tested whether costimulatory proteins could induce differentiation of naïve CD4<sup>+</sup> T cells to Th17 cells by examining production of IL-17 by two methods: flow cytometry and ELISA. Several days were examined for IL-17A production by differentiating naïve T cells: days 1 (data not shown, n = 2), 3, 5, 7, 9-10, and 12-14. As seen in **Fig. 3.3-3.5**, none of the costimulatory proteins (CD28, ICAM-1, or LFA-1) was able to induce production of IL-17A at any timepoint during differentiation. Costimulation through CTLA-4 (n = 1) was also not able to generate Th17 cells (data not shown). Supernates from cultures that were not used for intracellular staining were collected and analyzed for IL-17A by ELISA to verify the results obtained by flow cytometry. The highest average IL-17A production observed from any costimulatory regimen was 43 pg/ml by cells costimulated through LFA-1 on day 7 (**Fig. 3.6**). No statistically significant differences were observed among the various stimuli.

**Fig. 3.1**



**Fig. 3.2**



**Figures 3.1-3.2. Expression of IL-17A in naïve CD4+ T cells and stimulated PBMCs.**

Freshly isolated naïve CD4+ T cells or PBMCs were stained for expression of IL-17A. PBMCs were also stained for CD4 and stimulated with PDB and ionomycin for 6 hours to induce production of IL-17A. **Fig. 3.1**, MFI of IL-17A in naïve CD4+ T cells comparing isotype control (black) to staining for IL-17A (red). Representative of 5 experiments. **Fig. 3.2**, Comparison of IL-17A expression between freshly isolated PBMCs and PBMCs stimulated through PDB. MFI of IL-17A is indicated for the CD4+IL-17A+ population (upper right quadrant). Representative of two experiments.

Fig. 3.3

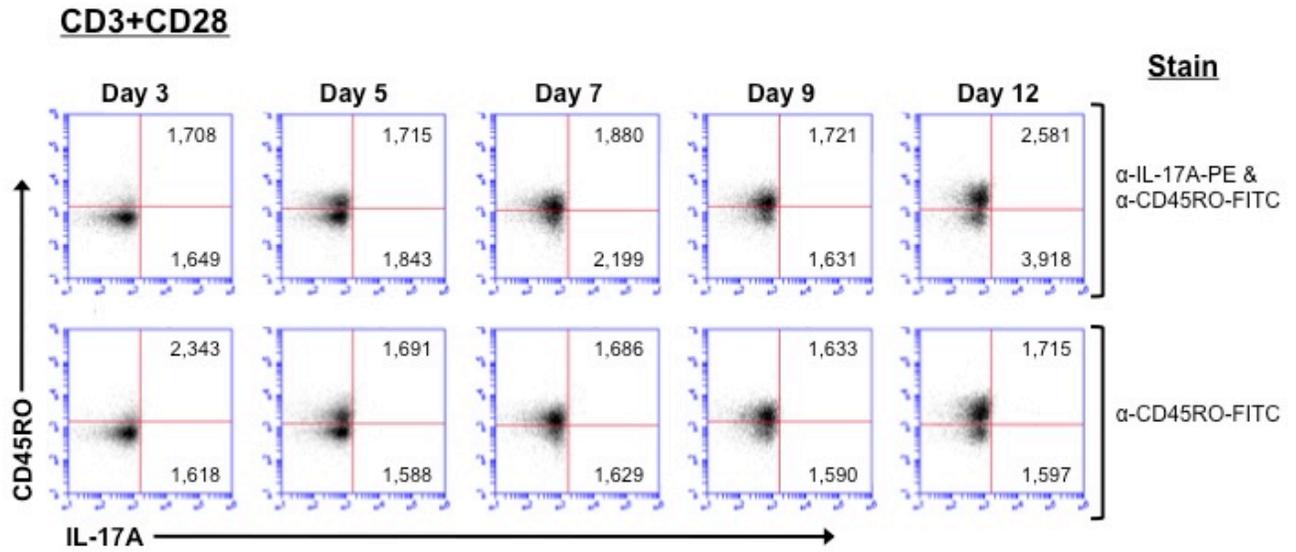


Fig. 3.4

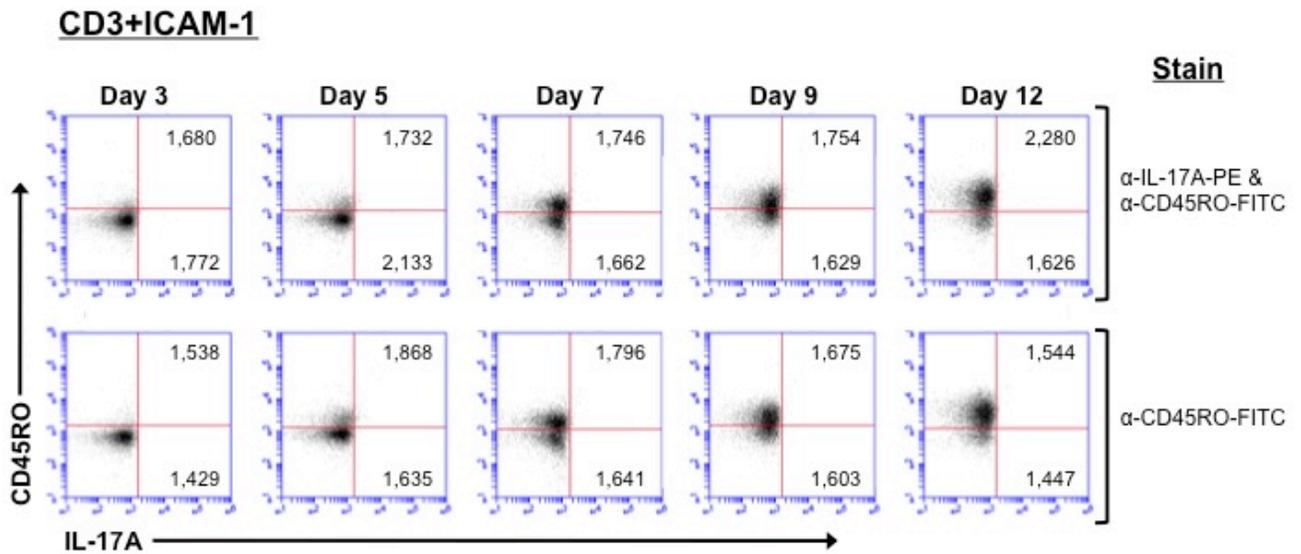
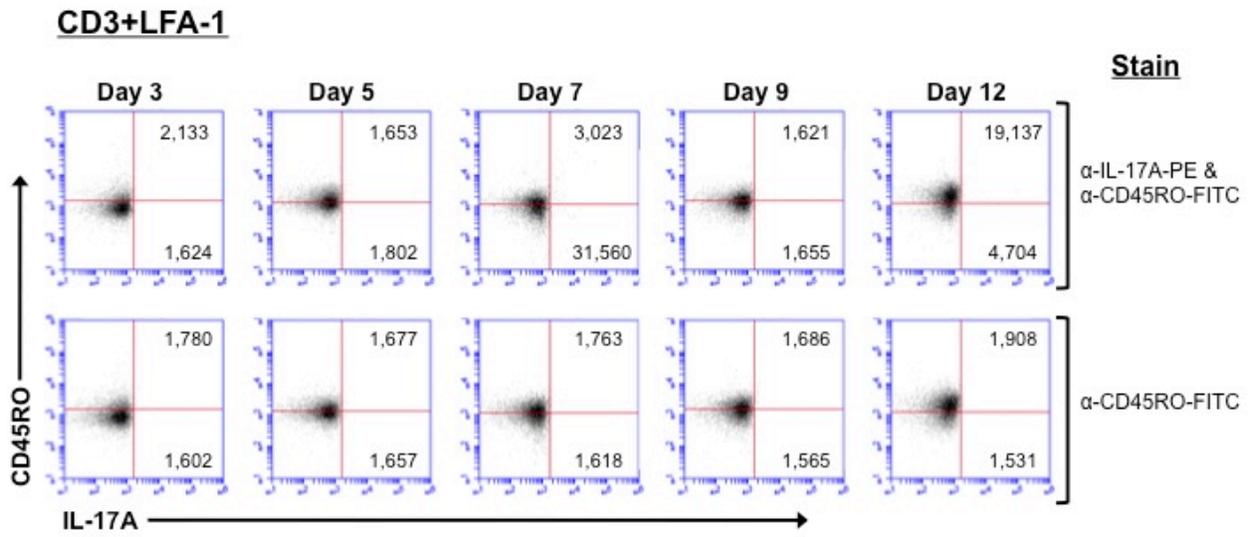
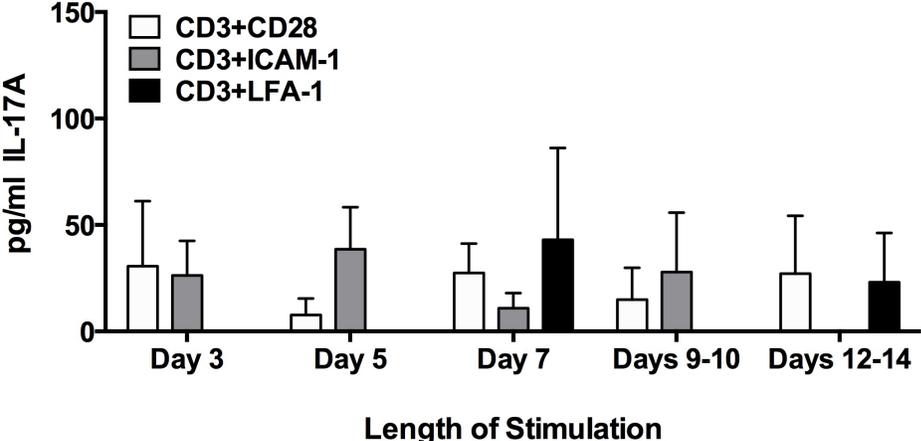


Fig. 3.5



**Figures 3.3-3.5. Costimulation of naïve CD4+ T cells by CD28, ICAM-1 or LFA-1 did not induce differentiation to Th17 cells in the absence of exogenous cytokines.** Human naïve CD4+ T cells stimulated for 3, 5, 7, 9-10, or 12-14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-LFA-1 were stained for expression of CD45RO and IL-17A and analyzed by flow cytometry. Representative of 5-6 experiments (CD28 or ICAM-1) or 3-4 experiments (LFA-1). **Fig. 3.3**, MFI of IL-17A in CD45RO+IL-17A+ T cells (upper right quadrant) or CD45RO(-)IL-17A+ T cells (lower right quadrant) stimulated through CD3+CD28. **Fig. 5.4**, MFI of IL-17A in CD45RO+IL-17A+ T cells (upper right quadrant) or CD45RO(-)IL-17A+ T cells (lower right quadrant) stimulated through CD3+ICAM-1. **Fig. 3.5**, MFI of IL-17A in CD45RO+IL-17A+ T cells (upper right quadrant) or CD45RO(-)IL-17A+ T cells (lower right quadrant) stimulated through CD3+LFA-1.

Fig. 3.6



**Figure 3.6. Naïve CD4+ T cells stimulated through CD28, ICAM-1 or LFA-1 produced minimal amounts of IL-17A.** Supernates from human naïve CD4+ T cells stimulated for 3, 5, 7, 9-10, or 12-14 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-LFA-1 were collected and analyzed for production of IL-17A by ELISA. Representative of 3-10 experiments (CD28 or ICAM-1; n = 5: day 3; n = 6: day 5; n = 10: day 7; n = 3: days 9-10; n = 4: days 12-14) or 2 experiments (LFA-1). Average pg/ml IL-17A  $\pm$  SEM with each costimulatory regimen as indicated.

## **Discussion**

In this chapter, we extended our ongoing investigation of how the choice of costimulatory signal guides differentiation of naïve T cells to various cell fates by examining Th17 differentiation. Our data indicate the costimulatory proteins tested (CD28, ICAM-1, LFA-1 and CTLA-4) cannot induce differentiation of naïve T cells to Th17 cells in the absence of exogenous cytokines. This could suggest that Th17 differentiation relies more heavily on cytokines for polarization than costimulatory signaling. Alternatively, we have only examined a few costimulatory proteins in this study so it is possible that another type of costimulation may have a different outcome such as OX40, ICOS, or 4-1BB. Whether costimulatory proteins can induce activation or expansion of Th17 cells from mixed or mature T cell populations in the absence of exogenous cytokines could be examined in future studies. In **Chapter 7 (Summary)**, we summarize the data collected from our published and unpublished work on how the various costimulatory proteins influence T cell subsets.

## **Acknowledgements**

Previous lab members, Dr. Kelli Williams, Dr. Abby Dotson, Dr. Jacob Kohlmeier, Dr. Chintana Chirathaworn, and Dr. Scott Tibbetts, have all contributed to the findings summarized in **Table 7.1 of Chapter 7 (Summary)**. Their previous work laid the foundation for all my projects on naïve T cell differentiation.

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## **Chapter 4**

### **Expression and function of CD23 in naïve and mature T cells**

## Introduction

According to a recent CDC report (1), 25.7 million people in the U.S. suffer with asthma, and it is estimated that 60% of these cases are due to allergic asthma (2). The humoral immune response is mediated by Th2 cells that induce B cells to make antibody (Ab) against the target antigen. IgE is the predominant isotype produced by B cells during a parasitic infection. In individuals with type I hypersensitivity (allergy), this anti-parasitic program is thought to be misdirected against non-self antigens, or allergens, that are not generally recognized by the rest of the human population (3).

CD23, the low affinity IgE receptor, is expressed by a number of cells including: monocytes, macrophages, eosinophils, intestinal epithelia, follicular dendritic cells, B cells and T cells (4-7). CD23 is upregulated on B cells in response to IL-4 and influences the IgE response by regulating class-switching and production of IgE by B cells through a complex system that is still being elucidated (8-10). Dr. Marcia Chan (Children's Mercy Hospital, Kansas City, MO) has been investigating the role of CD23 in B cell function in pediatric patients with allergic asthma. Single nucleotide polymorphisms (SNPs) in the *fcεr2* gene that encode CD23 have been associated with atopy and high IgE levels in serum (11-13). One particular SNP in the *fcεr2* gene that is associated with a relatively high incidence of atopy causes a tryptophan residue to replace an arginine residue at amino acid 62 (R62W) (13). Dr. Chan is currently investigating the participation of this SNP (R62W) in IgE-mediated allergic responses in asthmatics and how this SNP influences the function of B cells in these patients.

Despite the upregulation of CD23 on T cells in response to mitogenic stimuli, exogenous IL-4 or allergens (5, 6, 14-16), neither the function of CD23 on T cells nor the expression of CD23 on human naïve CD4<sup>+</sup> T cells has been determined. Based on the role of Th2 cells in

mediating the IgE response and the regulation of IgE synthesis by CD23, we hypothesized that engagement of CD23 by IgE on T cells may function to induce the Th2 response. Therefore, we propose that IgE:CD23 complexes on T cells enhance Th2 responses to either appropriately targeted parasites or inappropriately targeted allergens in atopic individuals. We used our *in vitro* system of activation and differentiation (17-19) to model how stimulation through CD23 would modulate naïve and mature T cells in healthy individuals (part I) compared to atopic individuals with asthma (part II). We hypothesize that CD23 may function to exaggerate the Th2 response in patients with allergic asthma compared to healthy controls. Much of the data presented in this chapter are preliminary, and additional studies should clarify any tentative conclusions drawn.

## **Materials and Methods**

### *Antibodies and reagents*

Antibodies used to stain cells for flow cytometry were: anti-CD11a-FITC (BD Biosciences), anti-CD27-PE (Invitrogen), anti-CD27-PECy5 (eBioscience), anti-CD45RO-APC (Invitrogen), anti-CD45RO-Tri Color (Invitrogen), anti-Foxp3-PE (Miltenyi Biotec), anti-Gata3-PE (eBioscience), anti-Tbet (eBioscience), anti-phosphoERK1/2-Alexa Fluor 488 (Cell Signaling), anti-CD23-APC (eBioscience) and anti-CD25-Tri Color (Invitrogen). eBioscience Foxp3 buffers and protocol were used for intracellular staining of CD23, Foxp3, Tbet, and Gata3. Staining cells with CFSE and flow cytometry were performed as described in Chapter 2.

### *Human Subjects*

Peripheral blood (240 ml into heparin) was obtained after informed consent from healthy adult volunteers of both genders, ages 20-30 years, who had been free of infection for 14 days and did not have immune disorders for Part I. Peripheral blood cells (15-20 ml into heparin) were obtained after informed consent from allergic asthma patients, ages 16-17 years for Part II. Procedures were approved by the University of Kansas Institutional Review Board.

### *Human naïve CD4<sup>+</sup> T cell purification (240 ml)*

Human naïve CD4<sup>+</sup> T cells were isolated as described in Chapter 2.

### *Human CD45RO<sup>+</sup> T cell purification*

PBMCs were isolated as described in Chapter 2. T cells were e-rosetted with sheep red blood cells as previously described (20), and then negatively selected for CD45RO<sup>+</sup> T cells by

combining the use of Human CD45RA Tetramer (15 minutes at RT) followed by magnetic colloid (15 minutes at RT) (STEMCELL Technologies).

*Human T cell stimulation (for cells from 240 ml peripheral blood)*

Human naïve CD4<sup>+</sup> naïve T cells or mature CD45RO<sup>+</sup> T cells were stimulated through plate-bound antibodies as we have previously described (17, 19). Same clones and concentrations were used as described in Chapter 2. Anti-CD23 (Bu38) was used at 10 µg/ml (Ancell). Exogenous cytokines were added at the start of stimulation for select experiments. Recombinant human cytokine TGF-β1 (R&D Systems) was used at 10 ng/ml, IL-2 (Boehringer Mannheim) at 10 U/ml, and IL-4 (R&D Systems) at 1 ng/ml.

*Modified T cell stimulation for cells from 15-20 ml peripheral blood*

The same concentration and method of stimulation was used as described above with some modifications for a low blood volume. Flat-bottom 384-well plates were used instead of 96-well plates. Immediately after plating antibodies (60 µl/well), plates were centrifuged at 800 RPM briefly and then incubated at 37°C for 2-3 hours. After washing the plates with sterile PBS, cells were plated at 1.25 x10<sup>6</sup> cells/ml (75,000 cells/well) in 60 µL complete RPMI 1640. Plates were centrifuged again at 800 RPM briefly prior to addition of exogenous cytokines and incubation.

*Intracellular staining of phosphorylated ERK*

At the indicated timepoints, 4% formaldehyde in staining buffer (0.5% BSA in PBS) was added to wells for a final concentration of 2% formaldehyde. Cells were incubated for 10

additional minutes at 37°C. Once transferred to Eppendorf tubes for intracellular staining, cells were washed and permeabilized in 90% methanol for 30 minutes on ice. Cells were washed twice with staining buffer and resuspended in 100 µl staining buffer. Cells were incubated in staining buffer with anti-phospho-ERK-Alexa Fluor 488 (2.5 µl/test) for 1 hour at RT in the dark, washed and finally resuspended in staining buffer for collection by flow cytometry.

### *Statistical Analysis*

The one-way, paired student's *t*-test was used to determine statistical differences unless otherwise indicated. All statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

## Results

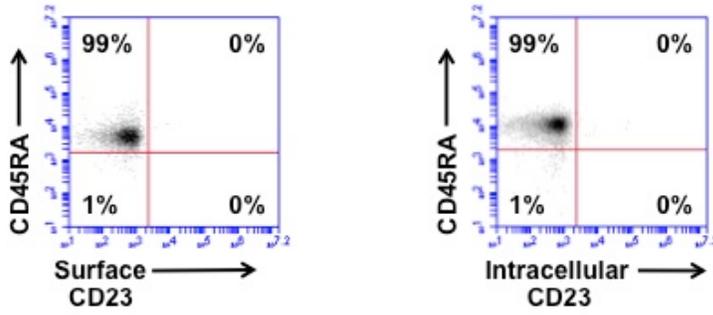
### Part I: Characterization and function of CD23 in T cells from healthy subjects

*Costimulatory proteins induced moderate levels of CD23 expression that is enhanced by exogenous IL-4*

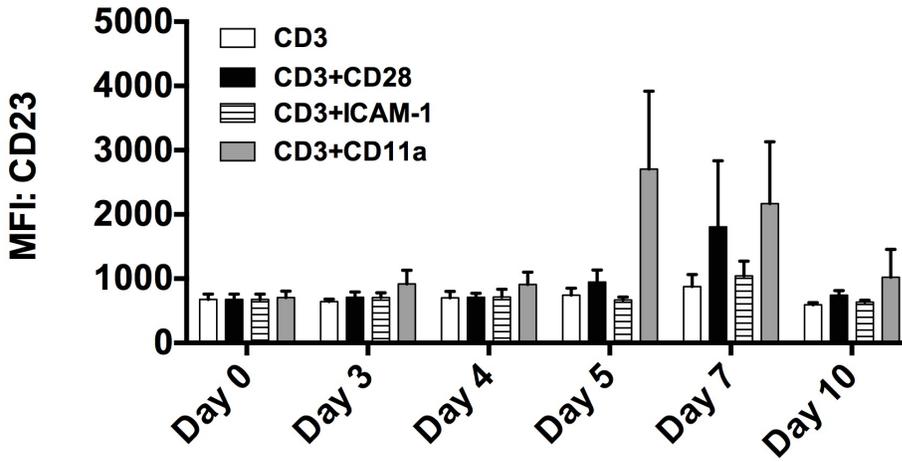
We first sought to determine the expression level and patterns of CD23 using our *in vitro* model of naïve T cell differentiation (17, 19). Resting T cells do not express CD23 (5, 6). As expected, freshly isolated naïve CD4<sup>+</sup> T cells did not express surface or intracellular CD23 (**Fig. 4.1**), but expression could be induced by stimulation through either CD3+CD28 or CD3+LFA-1 (CD11a) in the absence of exogenous cytokines (**Fig. 4.2**). Costimulation through CD28 or LFA-1 induced moderate expression of CD23 on naïve CD4<sup>+</sup> T cells with highest expression at day 7 (CD28) or day 5 (CD11a). Stimulation through the CD3 or CD3+ICAM-1 did not increase expression of CD23 above baseline (day 0) levels.

Studies on mature T cells populations have shown that the addition of IL-4 can augment expression of CD23 (5). In naïve CD4<sup>+</sup> T cells, with all stimuli except CD3+LFA-1, addition of IL-4 increased the degree of CD23 expression with most robust upregulation of CD23 observed on day 7 (**Fig. 4.3-4.7**). Although costimulation through LFA-1 appeared to induce high levels of CD23 expression (**Fig. 4.2**), results were inconsistent with subsequent experiments and LFA-1 routinely resulted in fewer cells possibly to increased cell death (**Fig. 4.3**). Therefore, for the remaining experiments, we eliminated stimulation through CD3+LFA-1. We also examined expression of CD23 on cells stimulated through CD3+CD23, CD3+ICAM-1+CD23 or CD3+CD28+CD23, but were unable to detect any surface expression of CD23 when stimulating

#### 4.1



#### 4.2



**Figures 4.1-4.2. Stimulation of human naïve CD4+ T cells in the absence of exogenous cytokines was sufficient to induce moderate levels of CD23 expression.** Human naïve CD4+ T cells stimulated for 3, 4, 5, 7 or 10 days with anti-CD3, anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-CD11a were analyzed for expression of CD23 by flow cytometry. CD23 expression was also assessed on freshly isolated naïve CD4+ T cells (Day 0). **Fig. 4.1**, Surface (left panel, n = 6) or intracellular (right panel, n = 3) expression of CD23 in human naïve CD4+ T cells. **Fig. 4.2**. Average MFI of CD23  $\pm$  SEM. Representative of 3-7 independent experiments.

Fig. 4.3

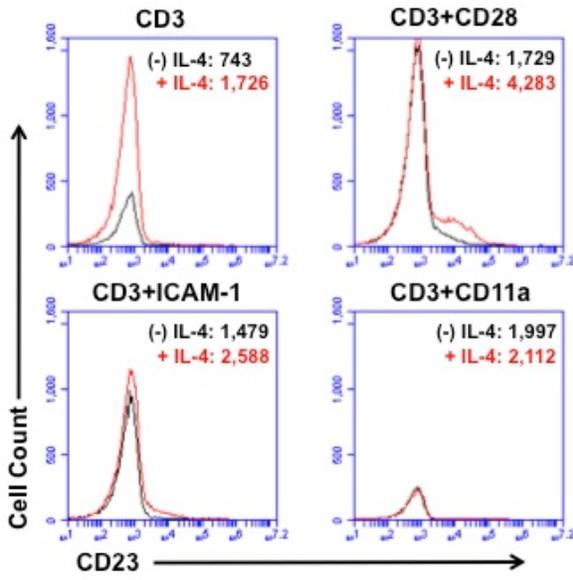


Fig. 4.4

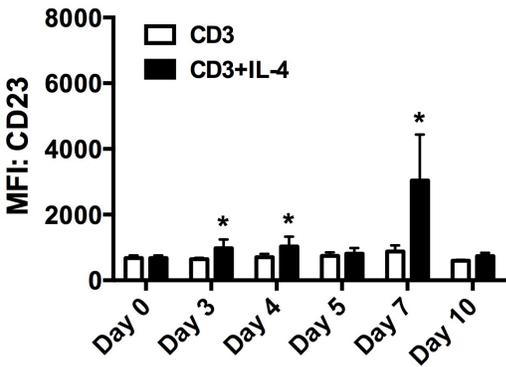


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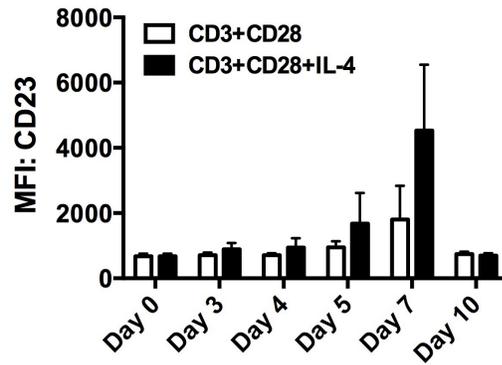


Fig. 4.6

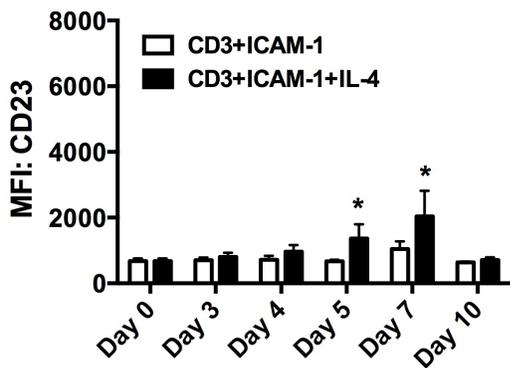
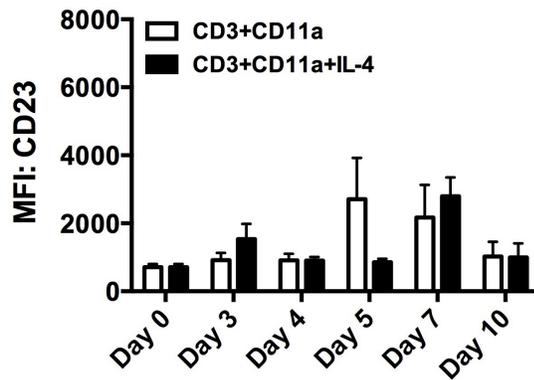


Fig. 4.7



**Figures 4.3-4.7. Addition of exogenous IL-4 enhanced expression of CD23 by differentiating naïve T cells.** Human naïve CD4<sup>+</sup> T cells stimulated for 3, 4, 5, 7 or 10 days with anti-CD3, anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-CD11a in the presence or absence of IL-4 (1 ng/ml) were analyzed for expression of CD23. Cells were also analyzed at day 0 for CD23 expression. Representative of 3-7 independent experiments. **Fig. 4.3**, histogram plots of CD23 expression of cells stimulated as indicated without exogenous IL-4 (black line) or with exogenous IL-4 (red line) on day 7. **Fig. 4.4-4.7** average MFI  $\pm$  SEM at 3, 4, 7 and 10 days with each costimulatory regimen with or without the addition of exogenous IL-4.

through CD23 (data not shown). This may reflect an inhibition of CD23 expression by CD23 stimulation or that the stimulating antibody engaging CD23 interferes with the binding of the detection anti-CD23 antibody. Future studies examining mRNA expression may help resolve this issue.

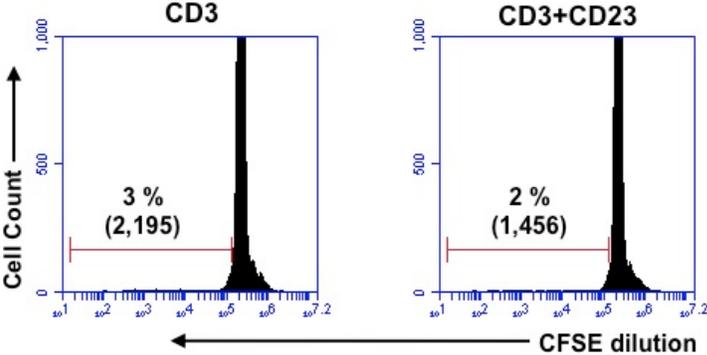
*Costimulation through CD23 did not provide a second signal for proliferation or differentiation of human naïve CD4+ T cells*

The ability of naïve T cells to express CD23 upon activation and differentiation suggests that CD23 will serve a functional purpose in these cells. We tested whether CD23 can function as a costimulatory molecule in human naïve CD4+ T cells when combined with stimulation through the TCR. Naïve T cells stimulated through CD3+CD23 did not proliferate (**Fig. 4.8-4.9**), differentiate to effector or memory T cells (**Fig. 4.10-4.12**), express Th1 or Th2 transcription factors, Tbet and Gata3, respectively (**Fig. 4.13-4.16**), or differentiate to regulatory T cells (**Fig. 4.17-4.18**). Overall stimulations through CD3 or CD3+CD23 were unremarkable in all assays. CD23, therefore, did not function independently as a costimulatory protein to induce human naïve T cell proliferation or differentiation.

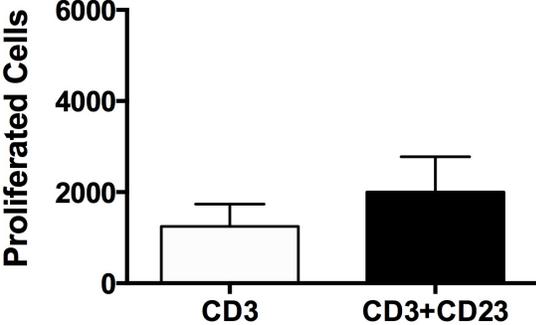
*Addition of stimulation through CD23 enhanced proliferation through ICAM-1 but not CD28 costimulation*

Next, we tested our hypothesis that CD23 regulates naïve T cell differentiation by supporting Th2 function but not Th1, thus stimulating cells to produce IL-4 and promote IgE-driven responses in an allergic reaction. We compared the effects of adding stimulation through CD23 to two models of differentiation: CD3+CD28 and CD3+ICAM-1. Addition of anti-CD23

**Fig. 4.8**

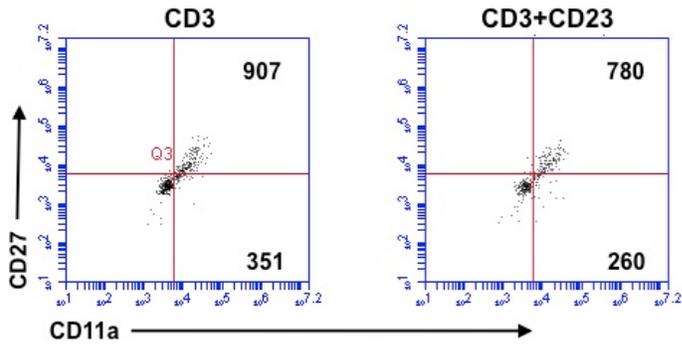


**Fig. 4.9**

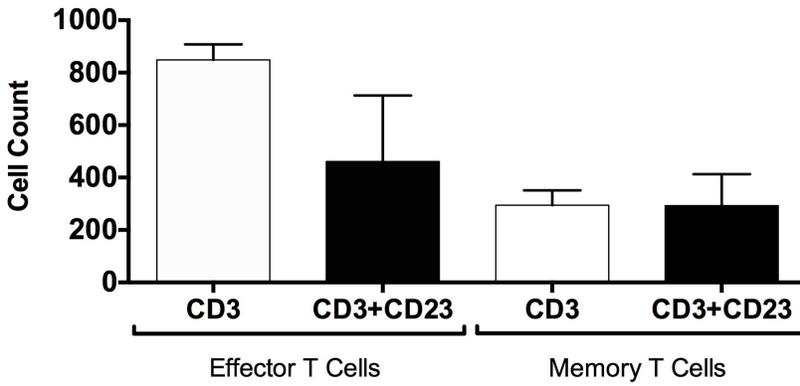


**Figures 4.8-4.9. Costimulation through CD23 did not stimulate proliferation of naïve T cells.** Human naïve CD4<sup>+</sup> T cells were stained with 2.5  $\mu$ M CFSE on day 0 and stimulated for 7 days with anti-CD3 or anti-CD3+anti-CD23 were analyzed for CFSE dilution. Representative of four experiments. **Fig. 4.8**, Percentage of divided cells with number of divided cells indicated parenthetically. **Fig. 4.9**, Average number of divided cells  $\pm$  SEM.

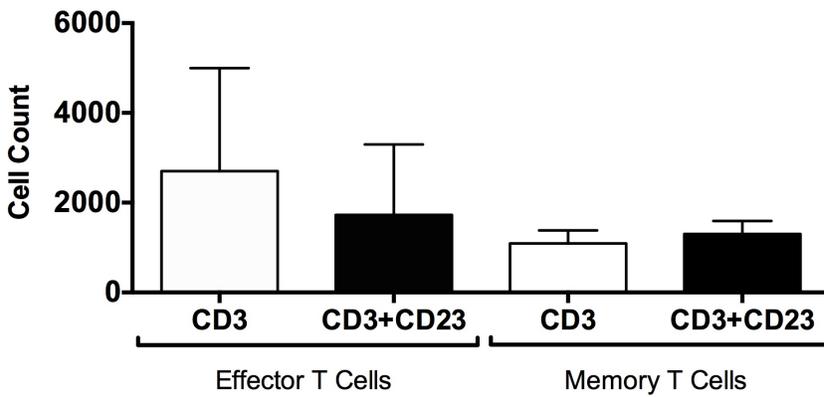
**Fig. 4.10**



**Fig. 4.11**

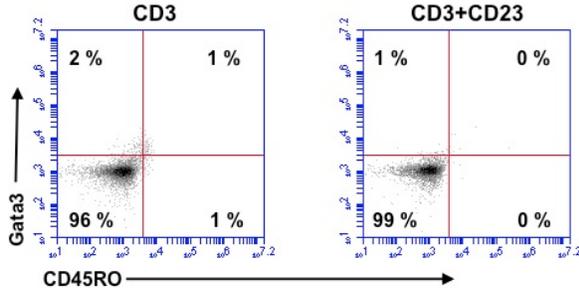


**Fig. 4.12**

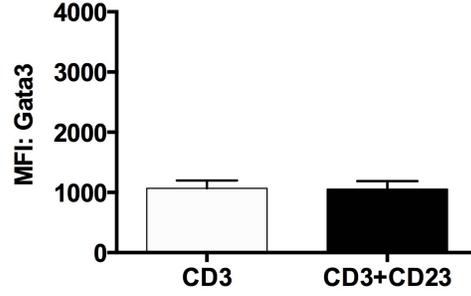


**Figures 4.10-4.12. Costimulation through CD23 did not induce naïve CD4+ T cells to differentiate to effector or memory cells.** Human naïve CD4+ T cells stimulated for 7 or 14 days with anti-CD3 or anti-CD3+anti-CD23 were stained for expression of CD45RO, CD27, and CD11a and analyzed by flow cytometry. Effector T cells were defined as CD4+CD45RO+CD11a+CD27<sup>hi</sup>, and memory T cells as CD4+CD45RO+CD11a+CD27<sup>lo</sup>. Representative of two experiments. **Fig. 4.10**, representative figure of the number of effector T cells (upper right quadrant) and memory T cells (lower right quadrant) on day 7. **Fig. 4.11**, Average number of effector or memory T cells  $\pm$  SEM on day 7. **Fig. 4.12**, Average number of effector or memory T cells  $\pm$  SEM on day 14.

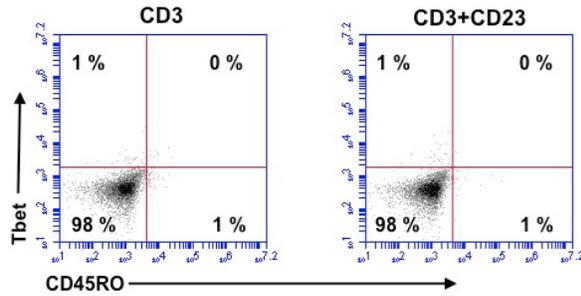
**Fig. 4.13**



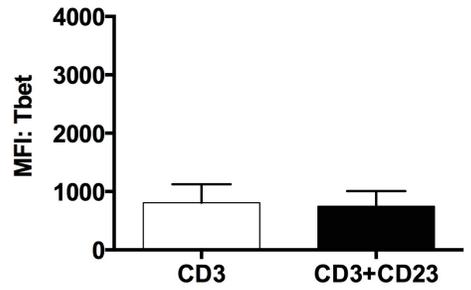
**Fig. 4.14**



**Fig. 4.15**

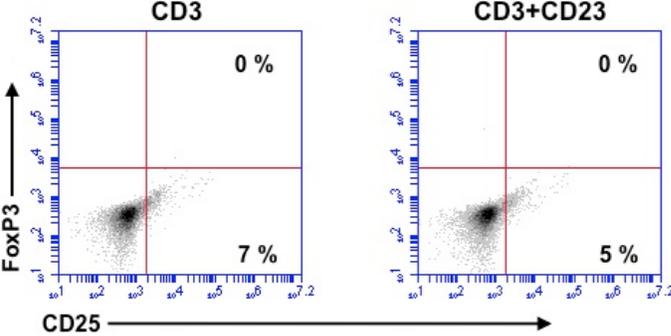


**Fig. 4.16**

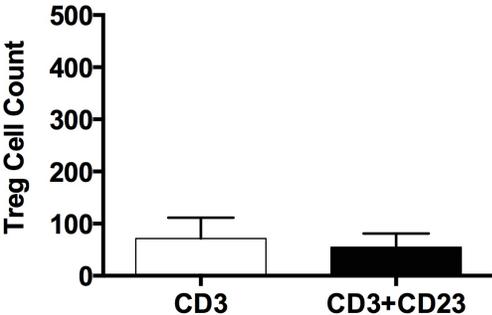


**Figures 4.13-4.16. Costimulation through CD23 did not induce expression of the transcription factors, Gata3 (Th2) or Tbet (Th1).** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 10 days with anti-CD3 or anti-CD3+anti-CD23 were stained for expression of CD45RO and Gata3 (**Fig. 4.13-4.14**) or CD45RO and Tbet (**Fig. 4.15-4.16**) and analyzed by flow cytometry. Representative of 5 (**Fig. 4.13-4.14**) or 3 (**Fig. 4.15-4.16**) experiments. **Fig. 4.13** and **Fig. 4.15**, Representative figure indicating the percentages of each population. **Fig. 4.14**, Average MFI of Gata3  $\pm$  SEM. **Fig. 4.15**, Average MFI of Tbet  $\pm$  SEM.

**Fig. 4.17**



**Fig. 4.18**



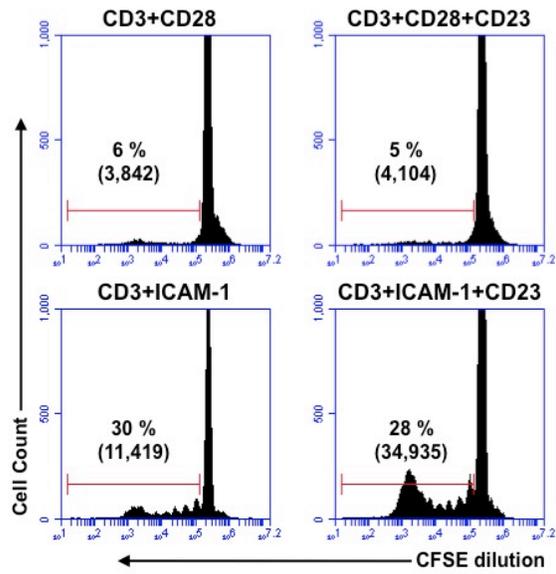
**Figures 4.17-4.18. Costimulation through CD23 did not support the differentiation of naïve T cells to regulatory T cells.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 days with anti-CD3 or anti-CD3+anti-CD23 were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Representative of 4 experiments. **Fig. 4.17**, percentage of regulatory T cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup>, upper right quadrant) generated at day 7 with each stimulation. **Fig. 4.18**, average number of Treg cells  $\pm$  SEM at day 7.

to cells costimulated through CD3+ICAM-1 enhanced the number of cells that had proliferated from  $7,107 \pm 2,199$  to  $18,726 \pm 8,050$  but did not influence the degree of proliferation by cells stimulated through CD3+CD28 (**Fig. 4.19-4.20**). Responses of donor cells to costimulatory proteins can vary greatly. The degree of proliferation induced by CD28 in these two experiments was uncharacteristically low so further studies are needed to determine how addition of CD23 stimulation influences cells that exhibit the typical proliferation activated by CD3+CD28. We also examined the amount of cell death in cultures where anti-CD23 was added to either CD3+CD28 or CD3+ICAM-1. In this one experiment (**Fig. 4.21**), stimulation through CD23 enhanced cell death in cells costimulated through CD28 but not ICAM-1. Thus far, CD23 differentially regulated costimulation through CD28 and ICAM-1 resulting in distinct outcomes on proliferation and cell viability.

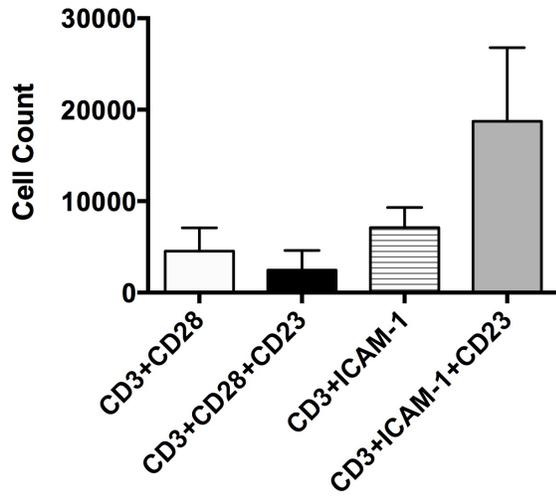
*Addition of stimulation through CD23 exerted opposing effects on differentiation induced through CD28 and ICAM-1 costimulation*

As we have frequently observed in our lab, both CD28 and ICAM-1 induce differentiation of human naïve CD4<sup>+</sup> T cells to effector and memory T cells. We tested whether stimulation CD23 would shift the differentiation process by these costimulatory proteins to favor either effector or memory cells. On day 7, addition of CD23 to cells stimulated through CD3+CD28 decreased differentiation to both effector and memory cells from  $6,846 \pm 1,635$  to  $2,027 \pm 453$  effector T cells and from  $3,534 \pm 2,256$  to  $754 \pm 325$  memory T cells (**Fig. 4.22-4.24**). CD23 did not appear to inhibit effector and memory differentiation at 14 days to the degree observed on day 7 which may be a result of the two experiments of day 14 not generating robust differentiation by CD28 costimulation (**Fig. 4.25-4.26**). Additional experiments should

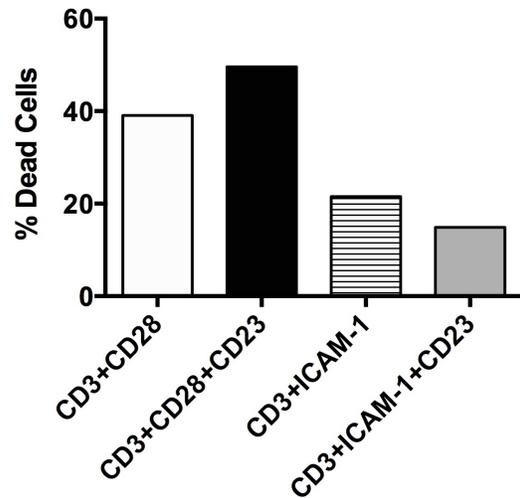
**Fig. 4.19**



**Fig. 4.20**

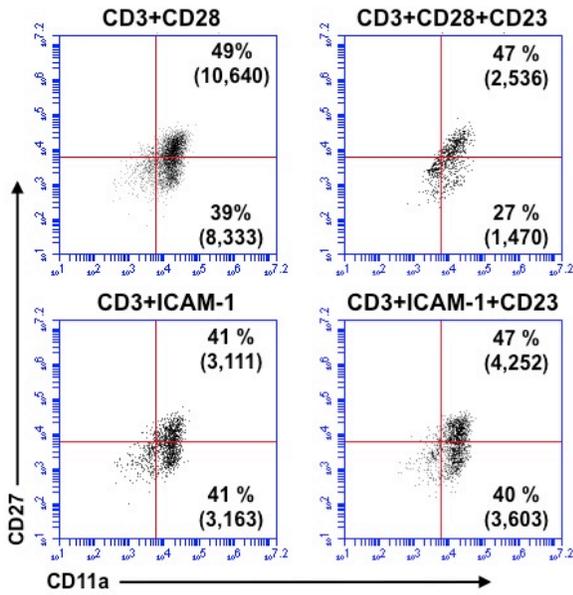


**Fig. 4.21**

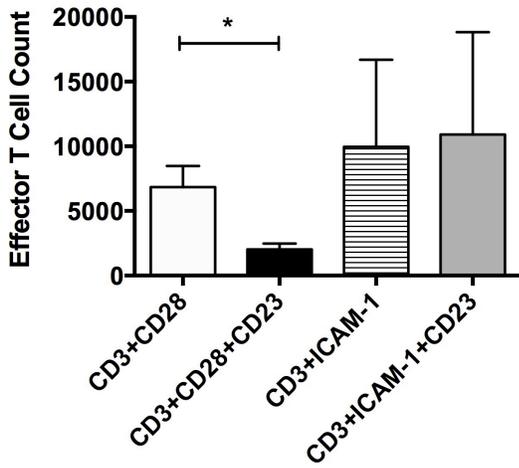


**Figures 4.19-4.21. Stimulation through CD23 enhanced proliferation of naïve T cells costimulated through ICAM-1 but not CD28. CD23 may enhance cell death in cells costimulated through CD28.** Human naïve CD4<sup>+</sup> T cells were stained with 2.5  $\mu$ M CFSE on day 0 and stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 and analyzed for CFSE dilution or expression of Annexin V and 7-AAD on day 7. Representative of three (ICAM-1) or two (CD28) experiments (**Fig. 4.19-4.20**) or 1 experiment (**Fig. 4.21**). **Fig. 4.19**, Percentage of divided cells with number of divided cells indicated parenthetically. **Fig. 4.20**, Average number of divided cells  $\pm$  SEM. **Fig. 4.21**, Percentage of Annexin V+7-AAD<sup>+</sup> cells on day 7.

**Fig. 4.22**



**Fig. 4.23**



**Fig. 4.24**

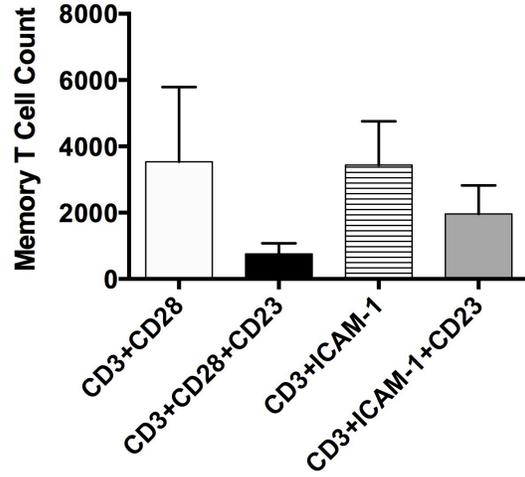


Fig. 4.25

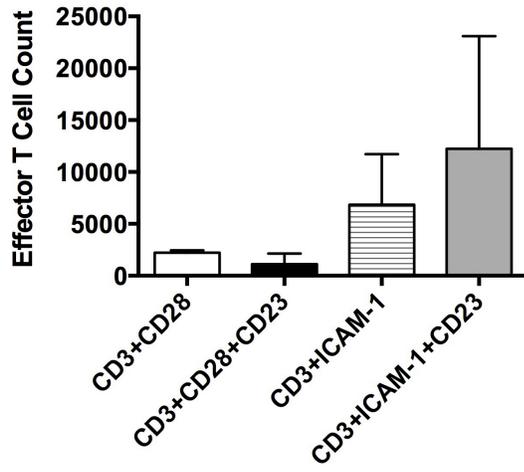
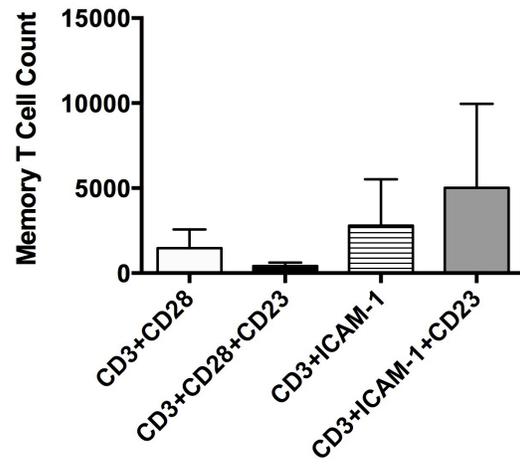


Fig. 4.26



**Figures 4.22-4.26. Costimulation through CD23 suppressed effector and memory differentiation induced by CD28 costimulation but not ICAM-1 costimulation.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 were stained for expression of CD45RO, CD27, and CD11a and analyzed by flow cytometry. Effector T cells were defined as CD4<sup>+</sup>CD45RO<sup>+</sup>CD11a<sup>+</sup>CD27<sup>hi</sup>, and memory T cells as CD4<sup>+</sup>CD45RO<sup>+</sup>CD11a<sup>+</sup>CD27<sup>lo</sup>. Representative of two (day 14) or three (day 7) experiments. **Fig. 4.22**, representative figure of the number of effector T cells (upper right quadrant) and memory T cells (lower right quadrant) on day 7. **Fig. 4.23**, Average number of effector T cells  $\pm$  SEM on day 7. **Fig. 4.24**, Average number of memory T cells  $\pm$  SEM on day 7. **Fig. 4.25**, Average number of effector T cells  $\pm$  SEM on day 14. **Fig. 4.26**, Average number of memory T cells  $\pm$  SEM on day 14.

\*  $p < 0.05$

resolve this issue. In contrast to CD28 costimulation, the addition of CD23 did not inhibit effector and memory differentiation stimulated through CD3+ICAM-1 (**Fig. 2.22-2.26**). CD23 appears to exert inhibitory effects on costimulation induced by CD28 but not ICAM-1.

*Stimulation through CD23 inhibited expression of both Gata3 and Tbet in cells costimulated through CD3+CD28*

Our data on proliferation and differentiation of effector and memory cells suggest that CD23 inhibits differentiation induced by CD28 signaling but not ICAM-1. In our *in vitro* model of differentiation, costimulation through CD28 generates both Th1 and Th2 cells whereas costimulation through ICAM-1 induces Th1 and Treg cells but not Th2 (17-19). The inhibitory effects of anti-CD23 when combined with CD28 costimulation may be due to a selective inhibition of Th2 differentiation that is not seen in cultures stimulated through ICAM-1 because stimulation through ICAM-1 does not induce Th2 cells. We tested whether the inhibition by CD23 was selective for Th1 or Th2 function by staining for the transcription factors, Gata3 (Th2) and Tbet (Th1). The addition of anti-CD23 to stimulation through CD3+CD28 caused significant decreases in the expression of both Gata3 and Tbet (**Fig. 4.27-4.30**). Gata3 (Th2) and Tbet (Th1) expression were reduced by nearly 2-fold and 3-fold, respectively, comparing stimulation through CD3+CD28 to CD3+CD28+CD23 (**Fig. 4.28** and **Fig. 4.30**). Addition of anti-CD23 did not affect expression of Gata3 or Tbet in cells stimulated through ICAM-1, though (**Fig 4.27-4.30**). Thus, inhibition by CD23 is selective to costimulation through CD28 and may suppress both Th1 and Th2 differentiation. We plan to analyze supernates for production of Th1 and Th2 cytokines in these cultures to verify modulation of Th1 and Th2 cells.

Fig. 4.27

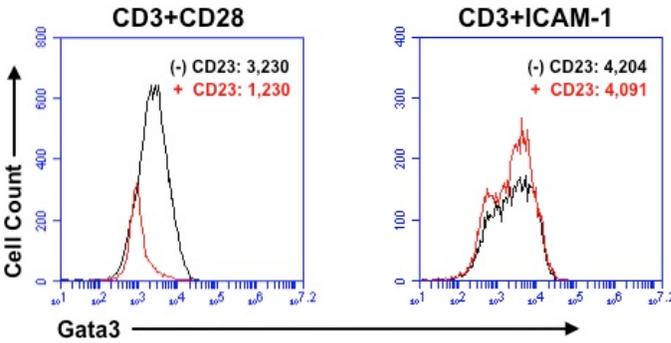
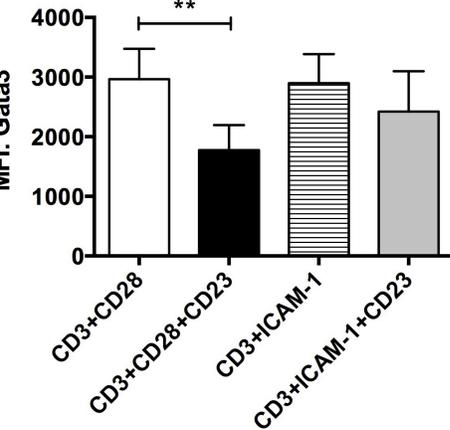


Fig. 4.28



**Figures 4.27-4.28. Costimulation through CD23 inhibited expression of the Th2 associated transcription factor, Gata3 (Th2), in cells costimulated through CD28 but not ICAM-1.**

Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 10 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 were stained for Gata3 expression and analyzed by flow cytometry. Representative of 5 (CD28) or 4 (ICAM-1) experiments. **Fig. 4.27**, MFI of Gata3 in cells stimulated through CD3+CD28 or CD3+ICAM-1 without stimulation through CD23 (black lines) or with stimulation through CD23 (red lines) at day 7. **Fig. 4.28**, Average MFI of Gata3  $\pm$  SEM on days 7 or 10.

\*\* p < 0.01

Fig. 4.29

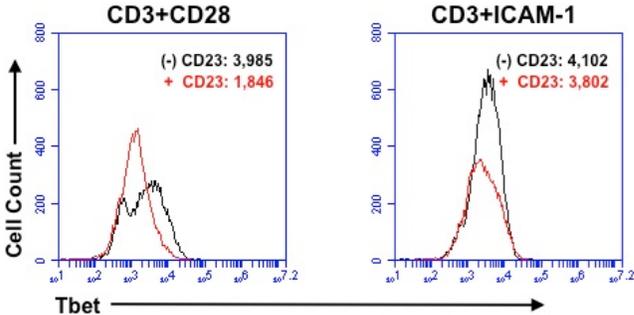
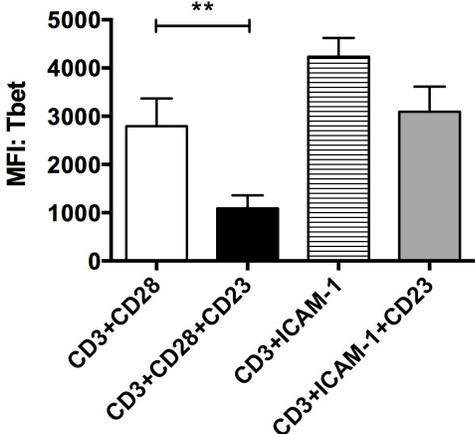


Fig. 4.30



**Figures 4.29-4.30. Costimulation through CD23 inhibited expression of the Th1 associated transcription factor, Tbet, in cells costimulated through CD28.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 10 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 were stained for Tbet expression and analyzed by flow cytometry. Representative of 4 (CD28) or 3 (ICAM-1) experiments. **Fig. 4.29,** MFI of Tbet of cells stimulated through CD3+CD28 or CD3+ICAM-1 without stimulation through CD23 (black lines) or with stimulation through CD23 (red lines) at day 7. **Fig. 4.30,** Average MFI of Tbet  $\pm$  SEM on days 7 or 10.

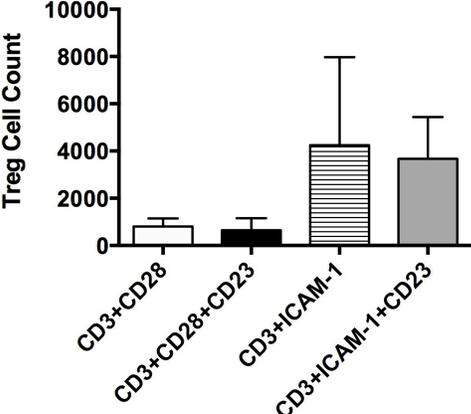
\*\*  $p < 0.01$

*Costimulation through CD23 may support differentiation to Treg cells*

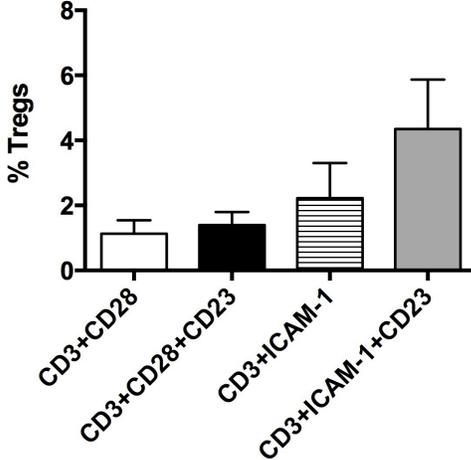
Next, we tested whether the other subset of T cells generated through ICAM-1 costimulation would be affected by the addition of stimulation through CD23. At 7 days, stimulation through CD3+ICAM-1+CD23 did not enhance the number of regulatory T cells (**Fig. 4.31**); however, the percentage of Treg cells was slightly increased by the addition of CD23 ( $p = 0.17$ ) (**Fig. 4.32**). Repeated experiments will be required to determine if CD23 can enhance differentiation to Treg cells induced by costimulation through ICAM-1 due to donor variability.

IL-4 supports differentiation to Th2 cells, and the combination of IL-2 and TGF- $\beta$  induces differentiation to Treg cells. We examined the effect of CD23 stimulation on differentiation to Treg cells under these two different polarizing conditions, adding either exogenous IL-4 ( $n = 2$ ) or the combination of IL-2 and TGF- $\beta$  ( $n = 1$ ). The addition of exogenous IL-4 when CD23 stimulation is combined with CD28 appears to augment or induce differentiation of naïve T cells to Treg cells (**Fig. 4.33**). This observation that CD23 may induce Treg cells in the presence of high levels of IL-4 is intriguing and seems consistent with the inhibitory effects observed when combined with CD3+CD28 stimulation. In the absence of exogenous cytokines, addition of CD23 enhanced Treg differentiation by ICAM-1, but adding IL-4 to this stimulation inhibited Treg differentiation (**Fig. 4.33**). Stimulation through CD23 does not influence Treg differentiation in the presence of exogenous IL-2 and TGF- $\beta$  (data not shown). We also examined the expression of CD23 and the receptor for IL-4 (IL-4R) on peripheral T cells stimulated in the presence of IL-2 and TGF- $\beta$  to determine if CD23 or IL-4 may be involved in the induction of Treg cells under classic polarizing cytokines. Stimulation of total peripheral T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) through either CD3+CD28 or CD3+ICAM-1 in the

**Fig. 4.31**

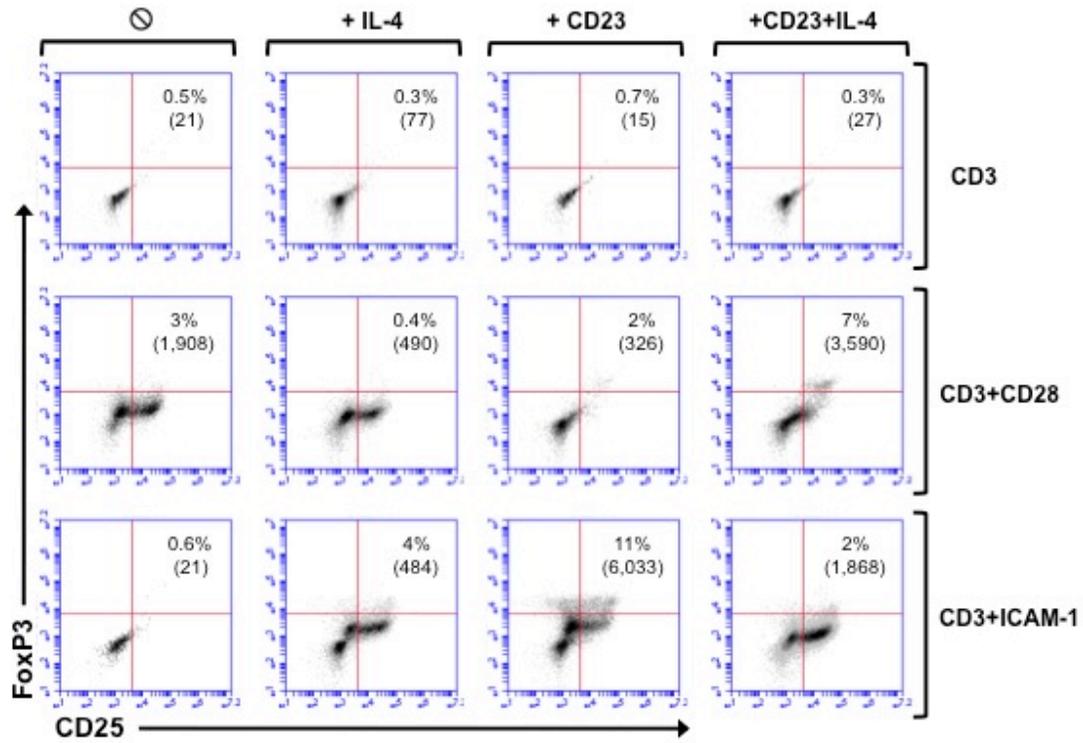


**Fig. 4.32**



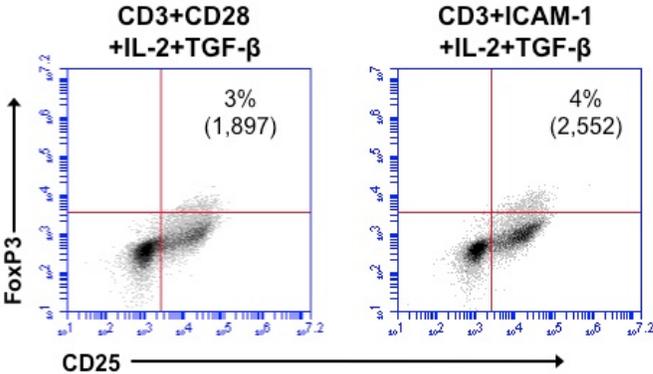
**Figures 4.31-4.32. Costimulation through CD23 did not alter the differentiation of naïve T cells to Treg cells by either CD28 or ICAM-1 costimulation in the absence of exogenous cytokines.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 10 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Representative of 3 (CD28) or 4 (ICAM-1) experiments. **Fig. 4.31**, average number of Treg cells  $\pm$  SEM at day 7. **Fig. 4.32**, average percentage of Treg cells  $\pm$  SEM at day 7.

Fig. 4.33

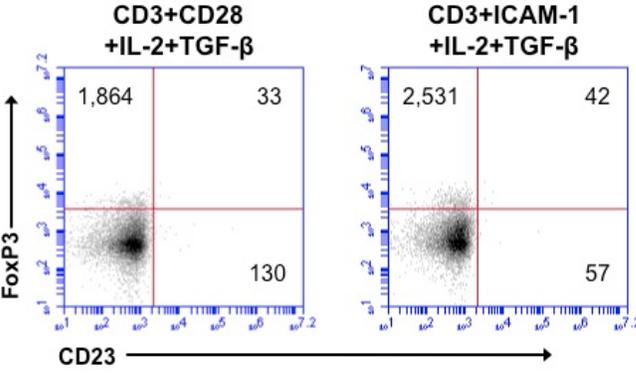


**Figure 4.33. Stimulation through CD23 may induce Treg differentiation in the presence of high levels of IL-4.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 in the presence of absence of IL-4 (1 ng/ml). On day 7, cells were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Representative of 2 experiments. Percentage of Treg cells indicated with Treg numbers parenthetically.

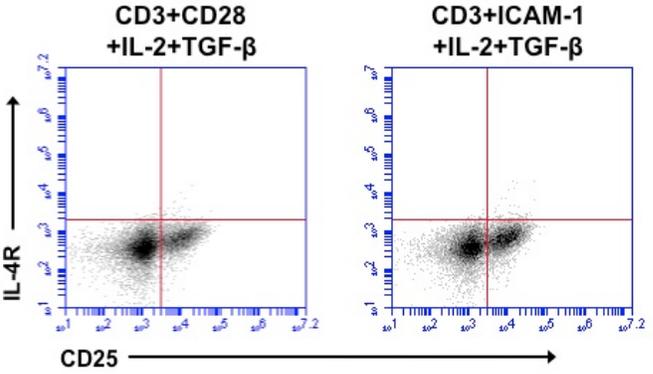
**Fig. 4.34**



**Fig. 4.35**



**Fig. 4.36**



**Figure 4.34-4.36. Treg expansion induced by classical polarizing conditions did not yield CD23+ or IL-4R+ cells.** E-rosetted peripheral T cells were stimulated for 48 hours with either anti-CD3+anti-CD28 or anti-CD3+anti-ICAM-1 in the presence of exogenous IL-2 (10 U/ml) and TGF- $\beta$  (10 ng/ml) to expand the Treg population. At 48 hours, cells stained for CD25, CD23, IL-4R and strong Foxp3 expression were analyzed by flow cytometry. Representative of 1 experiment. **Fig. 4.34**, Number of Treg cells with cell numbers indicated. **Fig. 4.35**, Number of Foxp3<sup>hi</sup>CD23(-) T cells (upper left quadrant), Foxp3<sup>hi</sup>CD23+ T cells (upper right quadrant), or Foxp3<sup>lo-intermediate</sup>CD23+ T cells (lower right quadrant). **Fig. 4.36**, Dot plot of CD25 vs. IL-4R expression.

presence of exogenous IL-2 and TGF- $\beta$  did not generate CD23<sup>+</sup> or IL-4R<sup>+</sup> T cells in the Treg or total T cell population (**Fig. 4.34-4.36**).

*Signaling through CD23 may induce a transient activation of ERK in cells costimulated through ICAM-1*

We examined whether early signaling events were affected by stimulation through CD23 and may account for the differential effects on differentiation outcome observed between CD28 and ICAM-1 costimulation. Phosphorylation of ERK was determined at 5, 10, 30, and 60 minutes of stimulation with and without anti-CD23. CD23 did not alter phosphorylation events induced by CD28 (**Fig. 4.37-4.39**). In contrast, combined with costimulation through ICAM-1, CD23 stimulated an increase in ERK activation at 30 minutes and possibly a modest increase at 5 minutes (**Fig. 4.37-4.39**). Repeated studies will determine whether this is a consistent finding, but the increase in phosphorylation of ERK in cells costimulated through CD3+ICAM-1+CD23 is consistent with the supportive role of CD23 in ICAM-1 costimulation observed in other experiments.

*CD23 was expressed by both CD45RA and CD45RA(-) T cell populations through 7-10 days of stimulation*

We examined the expression of CD23 and the CD45 isoforms, CD45RO (mature T cells) and CD45RA (naïve T cells), to determine what type of cells may be responding to stimulation through CD23 in our differentiation model. CD45RA<sup>+</sup>CD23<sup>+</sup> and CD45RA(-)CD23<sup>+</sup> T cells were determined at 3, 4, 5, 7, and 10 days of stimulation in the presence and absence of exogenous IL-4 (**Fig. 4.40-4.42**). CD23<sup>+</sup> T cells included both CD45RA<sup>+</sup> and CD45RA(-)

Fig. 4.37

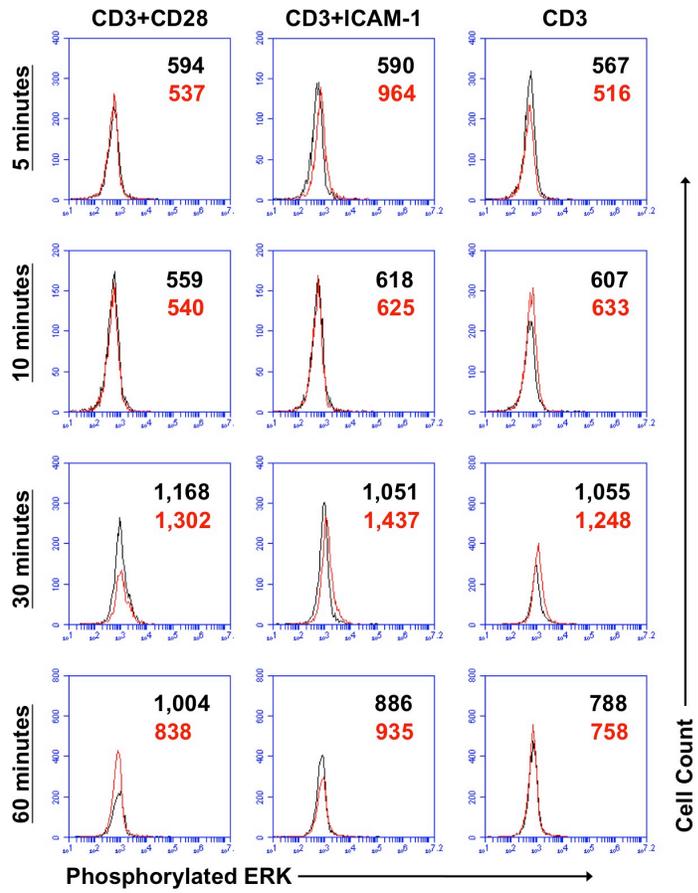


Fig. 4.38

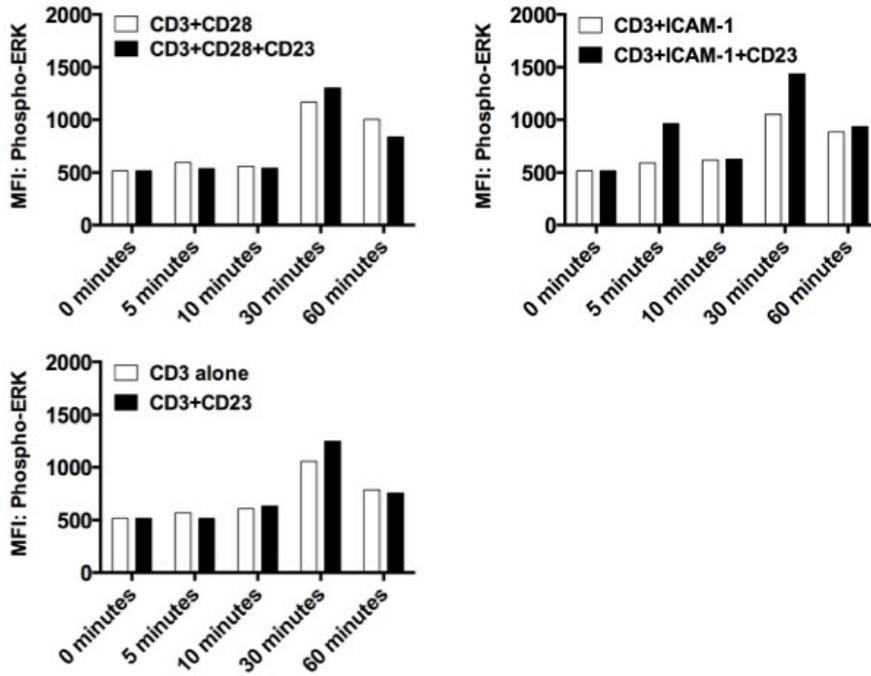
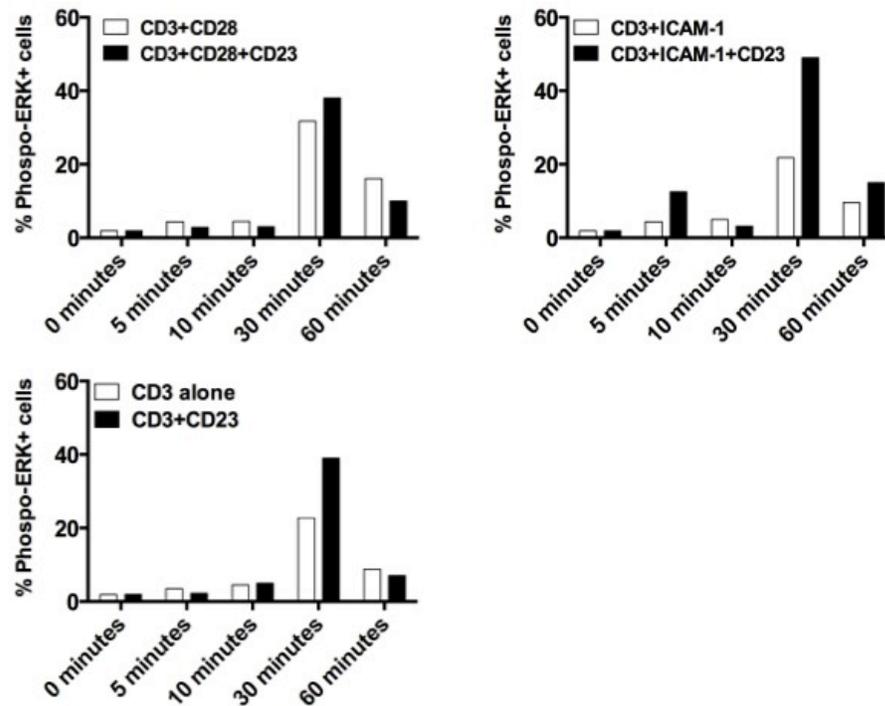


Fig. 4.39



**Figures 4.37-4.39. Signaling through CD23 may induce a transient activation of cells costimulated through ICAM-1 by the phosphorylation of ERK.**

Human naïve CD4<sup>+</sup> T cells stimulated for 5, 10, 30 or 60 minutes with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1, anti-CD3+anti-ICAM-1+anti-CD23, anti-CD3 or anti-CD3+anti-CD23 were analyzed for phosphorylated ERK. Cells were also analyzed at 0 minutes before stimulations began (MFI: 515). Representative of 1 experiment.

**Fig. 4.37**, histogram plots of phosphorylated ERK at 5, 10, 30 or 60 minutes of cells stimulated through CD3+CD28, CD3+ICAM-1 or CD3 (black lines) or CD3+CD28+CD23, CD3+ICAM-1+CD23 or CD3+CD23 (red lines). MFI for cells stimulated through CD23 are indicated in red and those not stimulated through CD23 are indicated in black. **Fig. 4.38**, MFI of phosphorylated ERK in cells with each costimulatory regimen. **Fig. 4.39**, Percentage of cells containing phosphorylated ERK with each costimulatory regimen.

**Fig. 4.40**

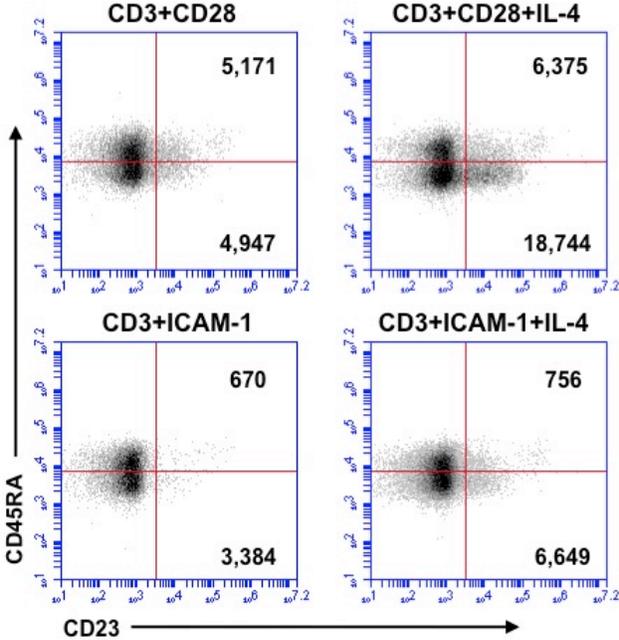


Fig. 4.41

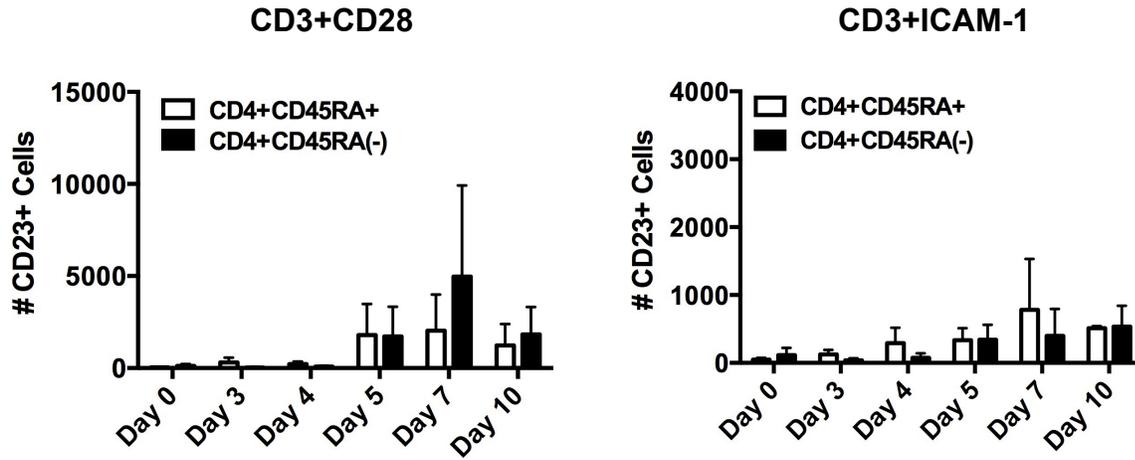
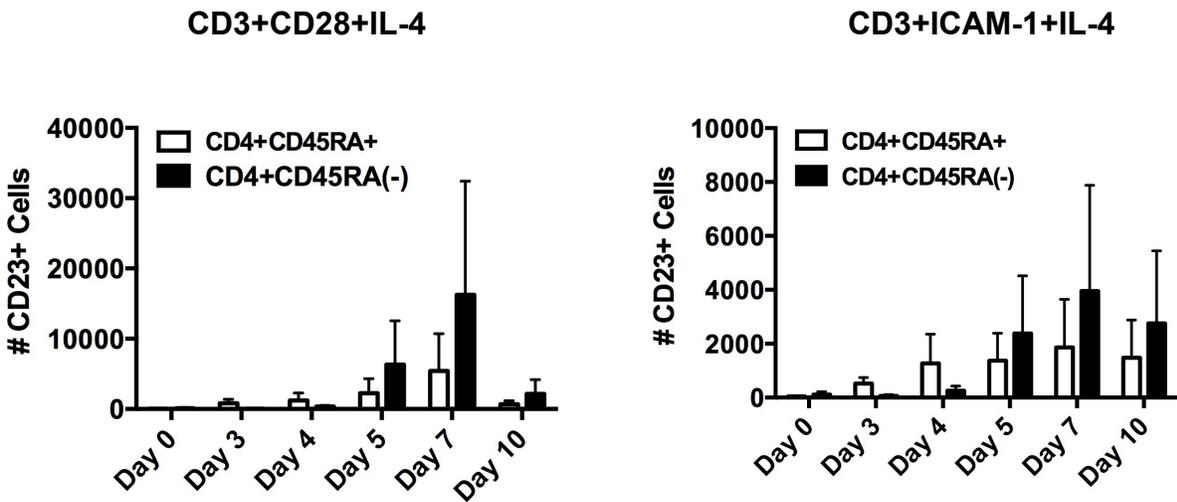


Fig. 4.42



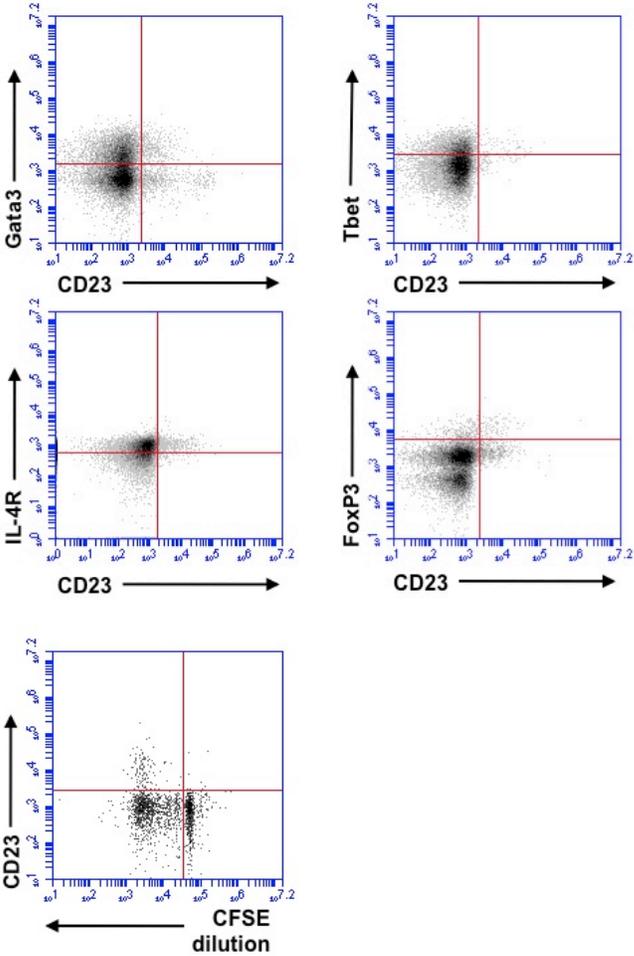
**Figures 4.40-4.42. Both CD45RA+ and CD45RA(-) T cells expressed CD23 with peak expression for both populations on day 7.** Human naïve CD4+ T cells stimulated for 3, 4, 5, 7 or 10 days with anti-CD3+anti-CD28 or anti-CD3+anti-ICAM-1 in the presence or absence of IL-4 (1 ng/ml) were analyzed for expression of CD23 and CD45RA. Cells were also analyzed at day 0 for CD23 expression before stimulations began. Representative of 3-7 independent experiments. **Fig. 3.40**, number of CD4+CD23+CD45RA+ (upper right quadrant and CD4+CD23+CD45RA(-) (lower right quadrant) T cells at day 5 with each costimulatory regimen. **Fig., 4.41**, average number  $\pm$  SEM of CD4+CD23+CD45RA+ cells (open bars) and CD4+CD23+CD45RA(-) cells (closed bars) generated with stimulation through CD3+CD28 or CD3+ICAM-1. **Fig. 4.42**, average number  $\pm$  SEM of CD4+CD23+CD45RA+ cells (open bars) and CD4+CD23+CD45RA(-) cells (closed bars) generated with stimulation through CD3+CD28 or CD3+ICAM-1 in the presence of IL-4 (1 ng/ml).

populations (**Fig. 4.40**) that increased over time peaking at day 7 (**Fig. 4.41-4.42**). The peak in CD23<sup>+</sup> T cells at day 7 is consistent with our observations of MFI expression at day 7 (**Fig. 4.5-4.6**). Cells costimulated through ICAM-1 did not induce sizeable CD23<sup>+</sup> populations unless exogenous IL-4 was added (**Fig. 4.41-4.42**). Costimulation through CD28 regardless of exogenous IL-4 induced more CD23<sup>+</sup> T cells than ICAM-1. Differences in the number CD45RA(-)CD23<sup>+</sup> T cells generated by these costimulatory proteins were more dramatic. Stimulation through CD3+CD28 and CD3+ICAM-1 generated 4,963 and 399 CD45RA(-)CD23<sup>+</sup> T cells in the absence of exogenous IL-4 and 16,250 and 3,958 CD45RA(-)CD23<sup>+</sup> T cells in the presence of exogenous IL-4, respectively (**Fig. 4.42**). Thus, costimulation through CD28 generated at the least 2.5-fold and at the most 12-fold greater numbers of CD23<sup>+</sup> T cells than costimulation through ICAM-1 which may account for some of the differences in CD23 regulation of these costimulatory proteins.

#### *Characterization of CD23<sup>+</sup> T cells*

One of our goals was to characterize the phenotype of CD23<sup>+</sup> T cells observed in our differentiation studies, which may assist in determining the role of CD23 function in T cells. Expression of Gata3 (Th2), Tbet (Th1), Foxp3 (Treg), and IL-4R (IL-4 receptor) was determined in the context of CD23 expression. Although all data are preliminary, it appears the CD23<sup>+</sup> T cells express intermediate and high levels of Gata3 (Th2) and Foxp3 (Treg) and high levels of Tbet (Th1) and IL-4R (**Fig. 4.43**). The CD23<sup>+</sup> population predominantly expresses intermediate levels of Foxp3 represents the activated T cell population (middle right panel, **Fig. 4.43**). We also tested whether CD23<sup>+</sup> T cells proliferate. As seen in the bottom panel of **Fig. 4.43**, cells that express CD23 undergo multiple rounds of

Fig. 4.43



**Figure 4.43. Characterization of CD23+ T cells.** Human naïve CD4+ T cells stimulated for 7 or 10 days with anti-CD3+anti-CD28 or anti-CD3+anti-ICAM-1 in the presence or absence of IL-4 (1 ng/ml) were analyzed for expression of CD23 and coexpression of Gata3, Tbet, IL-4R, or Foxp3. CD23 and dilution of CFSE was also determined on day 7. **Top left panel**, CD23+ cells are found on Gata3+ and Gata3(-) T cells (upper left panel), representative of 3 experiments. **Top right panel** Tbet+ T cells express CD23. Representative of 3 experiments. **Middle left panel**, CD23+ T cells express higher MFI for IL-4R. Representative of 1 experiment. **Middle right panel**, Foxp3<sup>hi</sup> and Foxp3<sup>intermediate</sup> T cells express CD23. Representative of 1 experiment. **Bottom panel**, CD23+ T cells at day 7 have undergone multiple cellular divisions. Representative of 1 experiment.

division. The functional consequences of the expression profile by CD23<sup>+</sup> T cells remains to be determined.

*Mature (CD45RO<sup>+</sup>) T cells from healthy subjects did not increase expression of CD23 in response to IL-4*

We have found that naive and mature T cells do not always respond similarly in our *in vitro* system of stimulation (21, 22), and it is well recognized that there are many functional differences in the responses of these populations *in vivo*. Therefore, we also examined the expression and function of CD23 in mature (differentiated) T cells as seen by expression of CD45RO. E-rosetted T cells were negatively selected for CD45RO expression. Isolated mature T cells averaged 100%, 89%, 12%, and 97% for CD3, CD4, CD8 and CD45RO expression (data not shown). Average MFI of CD23 in this population prior to stimulation was 1,219 (with a variable range of 560 to 2,507 depending on the donor), and intracellular CD23 was not detected. Cells were stimulated through CD3, CD3+CD28, or CD3+ICAM-1 in the absence or presence of exogenous IL-4 and analyzed for expression of CD23. The addition of IL-4 did not enhance CD23 expression as observed in the naïve CD4<sup>+</sup> T cell population (**Fig. 4.44-4.46**). The increase at 48 hours in cells costimulated through CD3+CD28 occurred in one of the two experiments performed so it remains to be determined if this is a consistent finding (**Fig. 4.44**).

*Stimulation of human CD45RO<sup>+</sup> T cells through CD23 did not influence expression of Gata3*

We observed that stimulation through CD23 when combined with costimulation through CD28 but not ICAM-1 inhibited differentiation and decreased both Gata3 (Th2) and Tbet (Th1)

Fig. 4.44

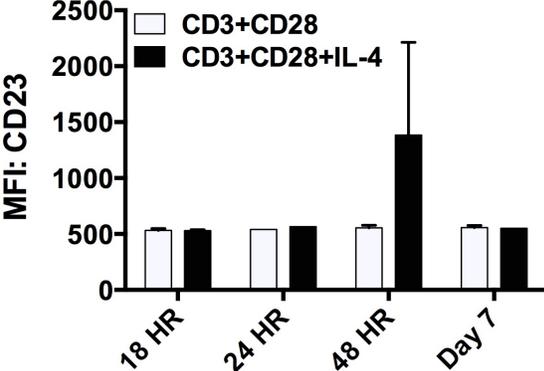


Fig. 4.45

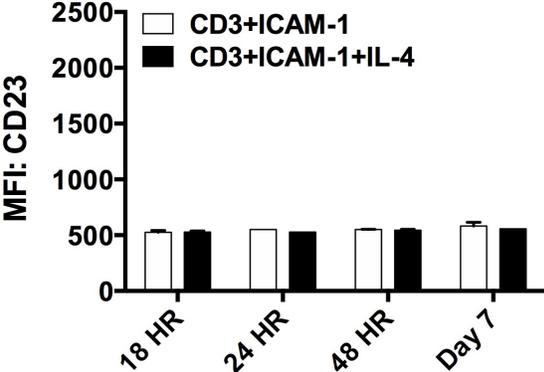
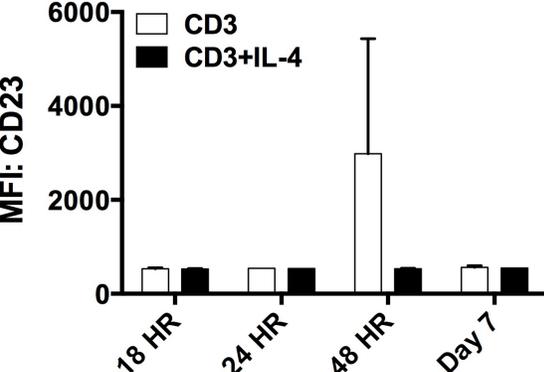


Fig. 4.46



**Figures 4.44-4.46. IL-4 did not induce CD23 expression in mature CD45RO+ T cells.**

CD45RO expressing T cells stimulated with anti-CD3, anti-CD3+anti-CD28 or anti-CD3+anti-ICAM-1 in the presence or absence of IL-4 (1 ng/ml) were analyzed for expression of CD23.

Representative of 1 or 2 experiments. **Fig. 4.44**, average MFI of CD23  $\pm$  SEM comparing stimulation through CD3+CD28 with or without exogenous IL-4. **Fig. 4.45**, average MFI of CD23  $\pm$  SEM comparing stimulation through CD3+ICAM-1 with or without exogenous IL-4.

**Fig. 4.46**, average MFI of CD23  $\pm$  SEM comparing stimulation through CD3 with or without exogenous IL-4.

expression in naïve CD4<sup>+</sup> T cells. We examined whether stimulation through CD23 could also modulate Th1 and Th2 responses in mature T cells. Overall stimulation of CD45RO<sup>+</sup> T cells through CD23 did not influence expression of Gata3 (**Fig. 4.47-4.49**). Thus, CD23 does not seem to activate or inhibit the Th2 response of mature T cell populations.

*Stimulation of human mature CD45RO<sup>+</sup> T cells through CD23 differentially influenced activation and expression of Tbet*

We tested the effect of stimulation through CD23 on the activation of T cells and expression of Tbet. In contrast to naïve T cell responses, addition of CD23 stimulation to mature T cells costimulated through CD28 augmented the percentage of activated cells from 41% to 74% ( $p < 0.01$ ) (**Fig. 4.50**) with a concomitant increase in the percentage of CD25<sup>+</sup>Tbet<sup>+</sup> T cells (30% to 53%,  $p < 0.05$ ) (**Fig. 4.51**). Cells costimulated through ICAM-1 were unaffected by the additional CD23 stimulus. Stimulation through CD3 and CD23 impaired the percentage of CD25<sup>+</sup>Tbet<sup>+</sup> T cells compared to stimulating mature T cells through only the TCR ( $p < 0.01$ ) (**Fig. 4.51**). Thus, the function of CD23 in mature T cells may differ from its function in naïve CD4<sup>+</sup> T cells.

The data from Part I of this chapter are summarized in **Table 4.1**.

Fig. 4.47

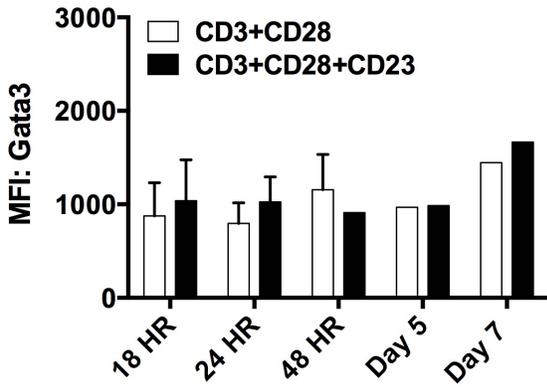


Fig. 4.48

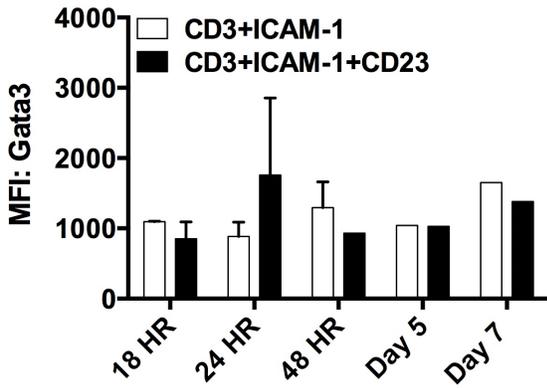
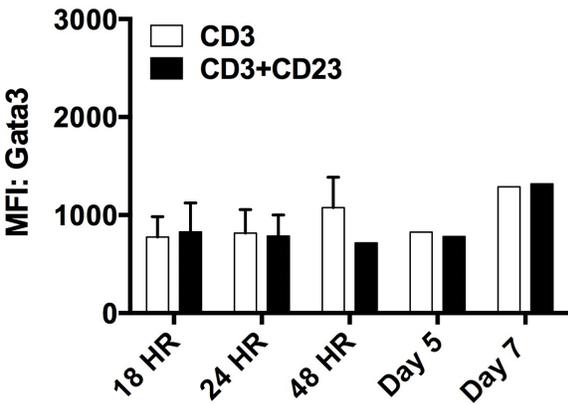


Fig. 4.49



**Figures 4.47-4.49. Simulation of human mature CD45RO+ T cells through CD23 did not influence expression of Gata3.** CD45RO+ T cells stimulated with anti-CD3, anti-CD3+anti-CD23, anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 were analyzed for expression of Gata3 by flow cytometry. Representative of 1 (day 5 and 7) or 2 experiments (18, 24, and 48 hours). **Fig. 4.47**, average MFI of Gata3  $\pm$  SEM comparing stimulation through CD3+CD28 and CD3+CD28+CD23. **Fig. 4.48**, average MFI of Gata3  $\pm$  SEM comparing stimulation through CD3+ICAM-1 and CD3+ICAM-1+CD23. **Fig. 4.49**, average MFI of Gata3  $\pm$  SEM comparing stimulation through CD3 and CD3+CD23.

Fig. 4.50

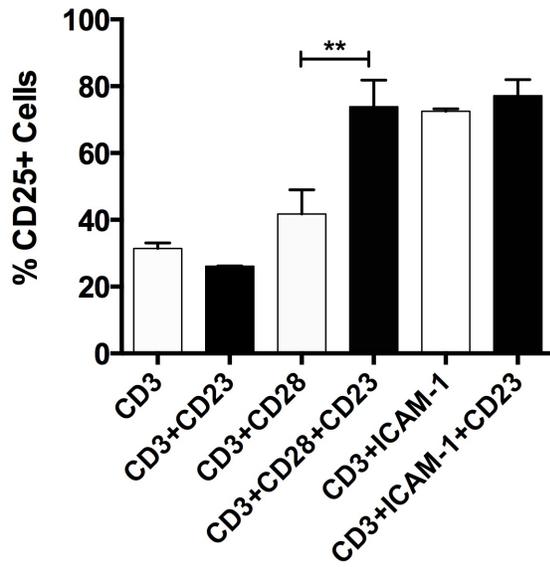
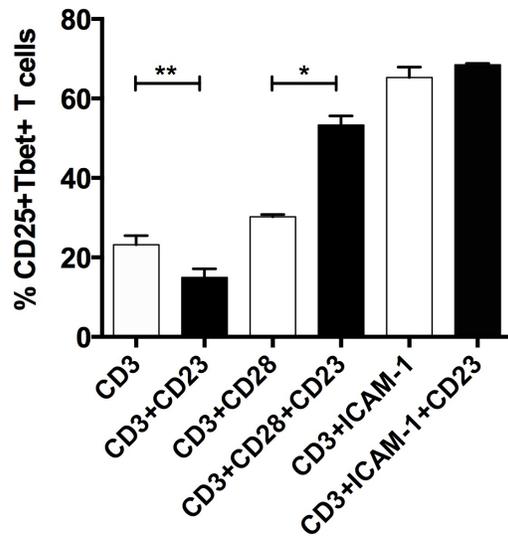


Fig. 4.51



**Figures 4.50-4.51. Stimulation through CD23 enhanced activation and Tbet expression in mature cells costimulated through CD28.** Mature CD45RO<sup>+</sup> T cells stimulated through anti-CD3, anti-CD3+anti-CD23, anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 were analyzed for expression of CD25 and Tbet on day 7. Representative of 2 experiments. **Fig. 4.50**, average percentage CD25<sup>+</sup> T cells  $\pm$  SEM. **Fig. 4.51**, average percentage of CD25<sup>+</sup>Tbet<sup>+</sup> T cells  $\pm$  SEM.

\*  $p < 0.05$ , \*\*  $p < 0.01$

**Table 4.1**

**Naïve CD4+ T Cells (Healthy subjects)**

**Mature (CD45RO+) T cells**

	<b>CD3+CD28 (+CD23)</b>	<b>CD3+ICAM-1 (+CD23)</b>		<b>CD3+CD28 (+CD23)</b>	<b>CD3+ICAM-1 (+CD23)</b>
<b>Proliferation</b>	No effect	↑	<b>Th1 function</b>	↑	No effect
<b>Protection from apoptosis</b>	No	Yes	<b>Th2 function</b>	No effect	No effect
<b>Memory T cell Differentiation</b>	↓	No effect	<b>Activation (CD25 expression)</b>	↑	No effect
<b>Effector T cell Differentiation</b>	↓	No effect			
<b>Differentiation to Th1 cells</b>	↓	No effect			
<b>Differentiation to Th2 cells</b>	↓	No effect			
<b>Differentiation to Treg cells</b>	No effect	↑			
<b>+ IL-4: Differentiation to Treg cells</b>	↑	↓			
<b>+ IL-2&amp;TGF- β: Differentiation to Treg cells</b>	No effect	No effect			
<b>Activation of ERK</b>	No effect	↑			

**Table 4.1. Summarized results of addition of anti-CD23 to costimulation through CD28 or ICAM-1 on naïve CD4+ T cells (left) or mature (CD45RO+) T cells (right) from healthy individuals.**

## **Part II- Characterization and function of CD23 in naïve CD4+ T cells from subjects with allergic asthma**

Our original hypothesis regarding CD23 function in atopic (allergic) individuals is that stimulation through CD23 during an allergic reaction would exacerbate Th2 responses. However, our studies of naïve CD4+ T cells from healthy subjects indicate that CD23 normally does not support Th2 function; in fact, CD23 may be inhibitory and downregulate T cell responses, particularly those mediated through CD28 costimulation. Therefore, we modified our hypothesis and proposed that the natural function of CD23 in T cells may be impaired or altered in allergic asthmatics resulting in less inhibition of T cell responses during an allergic response and thus, a heightened Th2 response. First we characterized the expression of CD23 and then the functional sequelae of stimulating naïve T cells through CD23 in these allergic patients.

*Expression of CD23 by naïve CD4+ T cells from allergic asthmatics was detected earlier after stimulation than cells from healthy subjects*

Naïve CD4+ T cells from subjects with allergic asthma were routinely  $\geq 98\%$  pure [CD45RO(-)CD11a<sup>lo</sup>CD27+] and did not express IL-4R or CD23 (data not shown). Our results from healthy subjects were that expression of CD23 peaked at day 7, was enhanced by exogenous IL-4, and was higher in cells costimulated through CD28 than ICAM-1. So we next examined expression of CD23 at days 5 and 7 on naïve CD4+ T cells from allergic asthmatics. Expression of CD23 by naïve CD4+ T cells from allergic asthmatics peaked earlier, at day 5 instead of day 7 (**Fig. 4.52-4.53**). Exogenous IL-4 only enhanced the CD23 expression in cells costimulated through CD28 and only on day 5 (**Fig. 4.52**). As seen in **Fig. 4.54**, stimulation

Fig. 4.52

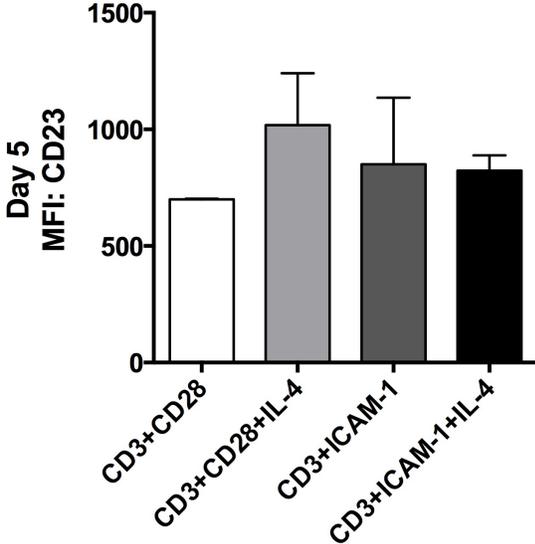
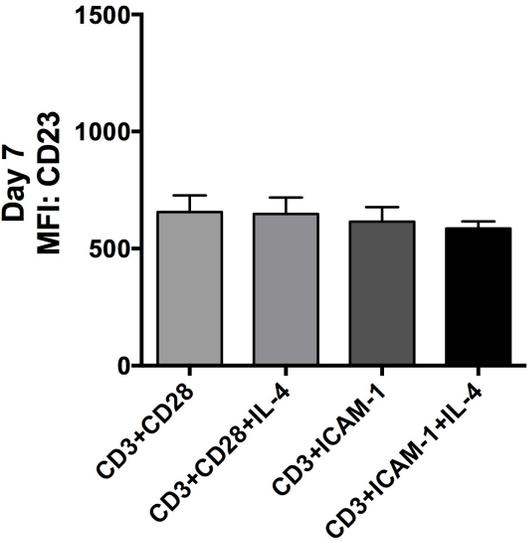


Fig. 4.53



**Figures 4.52-4.53. Expression of CD23 by naïve CD4+ T cells from allergic asthmatics was detected earlier after stimulation than cells from healthy subjects.** Human naïve CD4+ T cells stimulated for 5 or 7 days with anti-CD3+anti-CD28 or anti-CD3+anti-ICAM-1 in the presence of absence of IL-4 (1 ng/ml) were stained for CD23 and analyzed by flow cytometry. Representative of 2 experiments. **Fig. 4.52**, Average MFI of CD23  $\pm$  SEM on day 5. **Fig. 4.53**, Average MFI of CD23  $\pm$  SEM on day 7.

Fig. 4.54

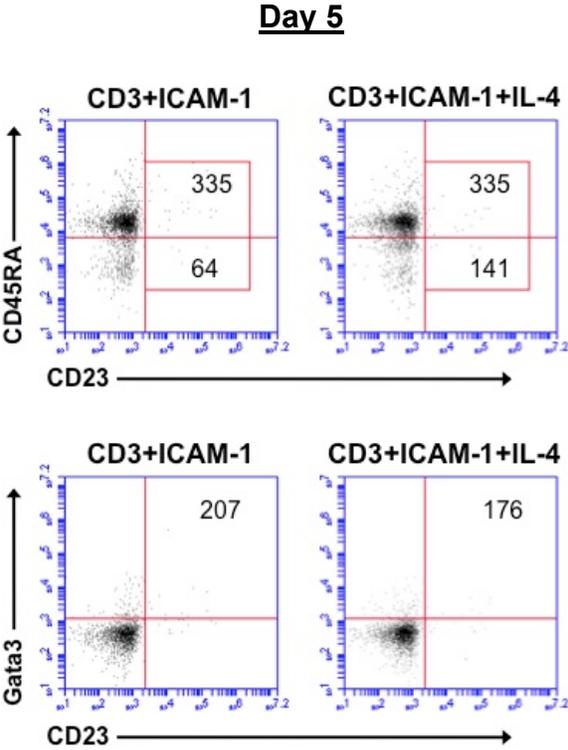


Fig. 4.55

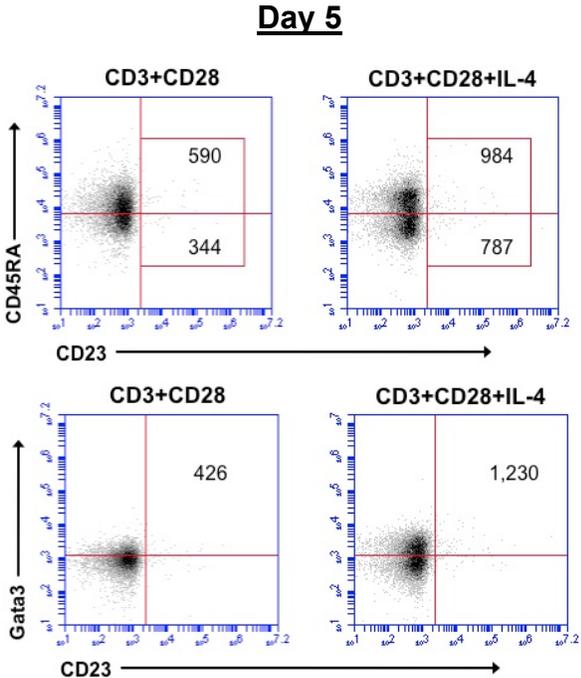


Fig. 4.56

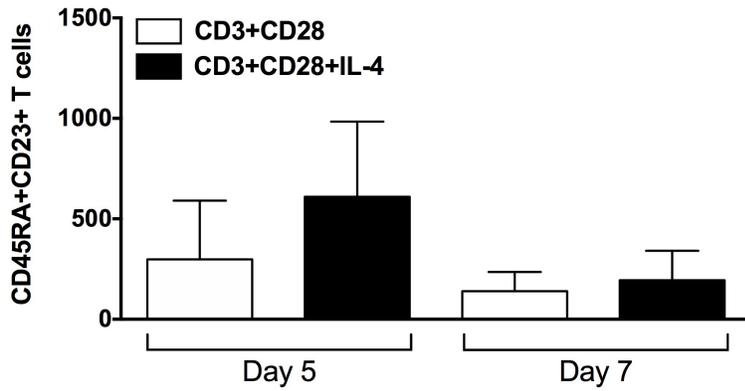


Fig. 4.57

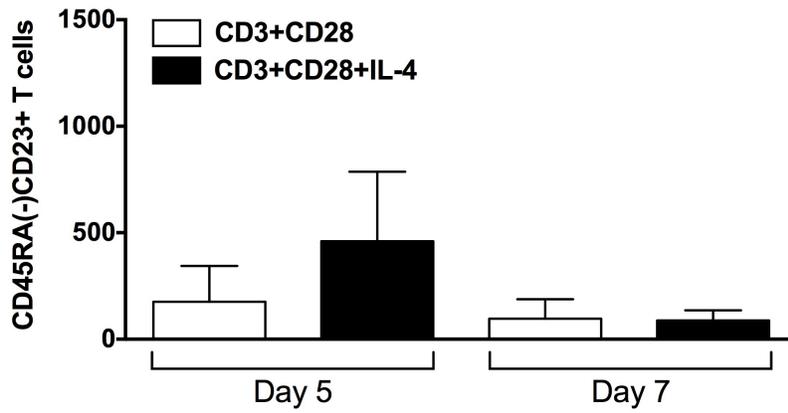
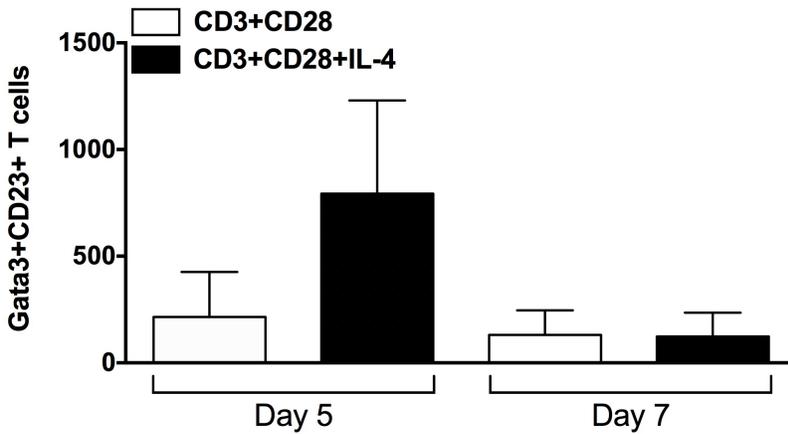


Fig. 4.58



**Figures 4.54-4.58. Exogenous IL-4 enhanced expression of CD23 by naïve CD4+ T cells from allergic asthmatics on day 5.** Human naïve CD4+ T cells stimulated for 5 or 7 days with anti-CD3+anti-CD2 or anti-CD3+anti-ICAM-1 in the presence or absence of IL-4 (1 ng/ml) were stained for expression of CD23, CD45RA, and Gata3 (Th2) and analyzed by flow cytometry. Representative of 2 experiments. **Fig. 4.54**, Number of CD45RA+CD23+ T cells (upper right quadrant, top panels), CD45RA(-)CD23+ T cells (lower right quadrant, top panels), or Gata3+CD23+ T cells (upper right quadrant, bottom panels) generated by CD3+ICAM-1 ± IL-4 on day 5. **Fig. 4.55**, Number of CD45RA+CD23+ T cells (upper right quadrant, top panels), CD45RA(-)CD23+ T cells (lower right quadrant, top panels), or Gata3+CD23+ T cells (upper right quadrant, bottom panels) generated by CD3+CD28 ± IL-4 on day 5. **Fig. 4.56**, Average number of CD45RA+CD23+ T cells ± SEM. **Fig. 4.57**, Average number of CD45RA(-)CD23+ T cells ± SEM. **Fig. 4.58**, Average number of Gata3+CD23+ Th2 cells ± SEM.

through CD3+ICAM-1 with or without IL-4 did not activate differentiation of naïve T cells from these atopic subjects. Addition of IL-4 to cultures costimulated through CD28 enhanced the number of both CD45RA+CD23+ T cells and CD45RA(-)CD23+ T cells by approximately two-fold (**Fig. 4.55-4.57**) and enhanced the number of CD23+ cells expressing Gata3 (Th2 cells) by nearly four-fold (**Fig. 4.58**) on day 5.

*Stimulation through CD23 may have supported the differentiation of naïve T cells to Treg cells in allergic patients*

In healthy subjects, stimulation through CD23 may induce Treg cells by working in concert with exogenous IL-4 depending on the other costimulatory protein engaged during naïve T cell activation. We tested whether CD23 would also enhance differentiation to Treg cells in atopic donor cells. In healthy subjects, costimulation through ICAM-1 but not CD28 stimulates naïve CD4+ T cells to differentiate to regulatory T cells (17). Unfortunately, cells from subjects with allergic asthma did not stimulate through CD3+ICAM-1 so we could not assay the effect on Treg induction by ICAM-1. However, we observed (**Fig. 4.59-4.60**) that the addition of anti-CD23 or the combination of anti-CD23 and exogenous IL-4 induced Treg differentiation in cells stimulated through CD3+CD28. CD25+Foxp3<sup>hi</sup> T cells increased, and the level of CD25 expression (MFI) was higher in cells stimulated through CD3+CD28+CD23 with or without IL-4. Thus, naïve T cells from either healthy or allergic subjects seem to respond to stimulation through CD23 in the presence of IL-4 to induce Treg cells.

Fig. 4.59

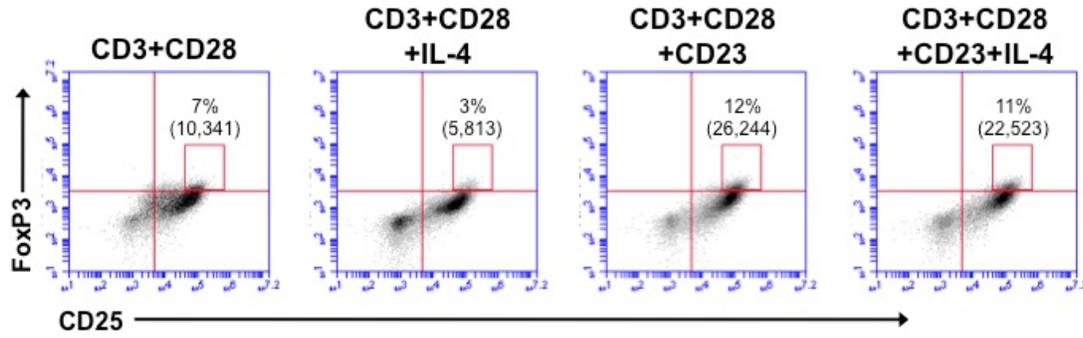
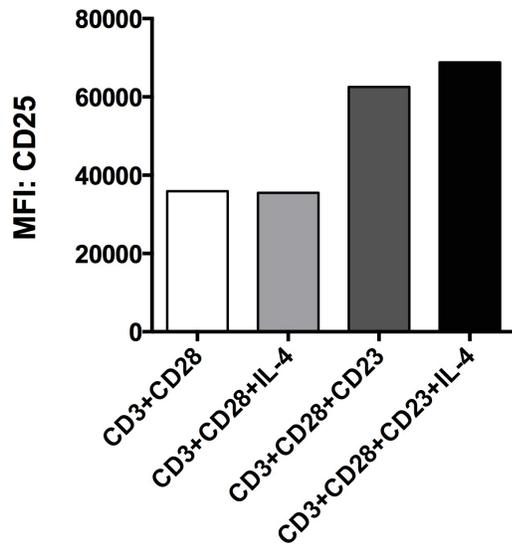


Fig. 4.60

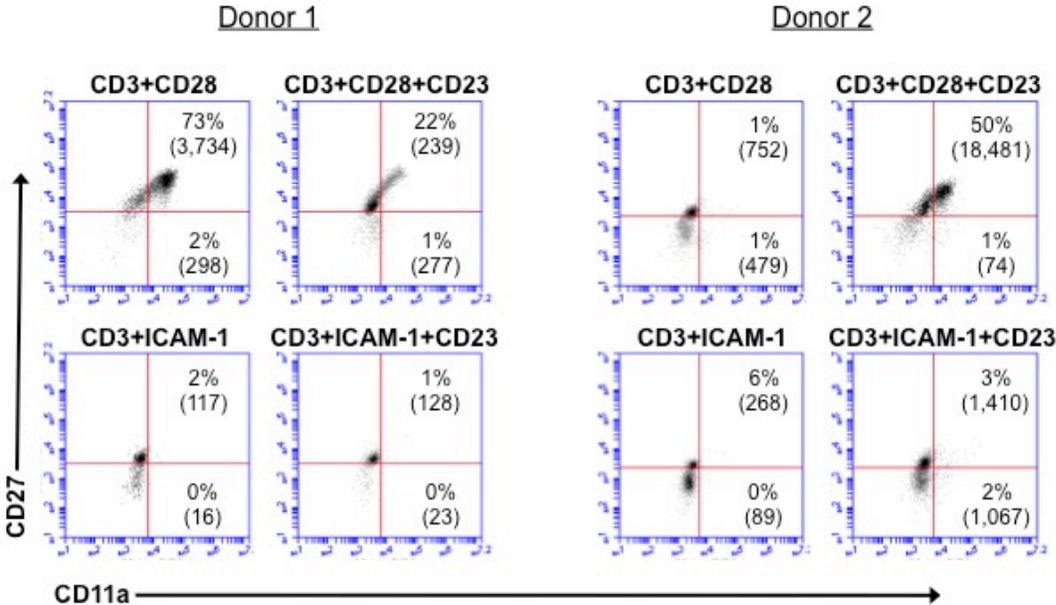


**Figures 4.59-4.60. Stimulation through CD23 seemed to induce differentiation of naïve CD4+ T cells from allergic patients to regulatory T cells.** Human naïve CD4+ T cells stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 in the presence or absence of IL-4 (1 ng/ml) were stained for CD25 and strong Foxp3 expression and analyzed by flow cytometry. Representative of 1 experiment. **Fig. 4.59**, Percentage of Treg cells indicated with numbers of Treg cells parenthetically. **Fig. 4.60**, MFI of CD25 indicated for each stimulation.

*Regulation of effector and memory differentiation by CD23 may vary among subjects with allergic asthma*

In our studies of human naïve T cells from healthy subjects, stimulation through CD23 reduced the number of effector and memory T cells induced by costimulation through CD28 but not ICAM-1. We tested whether CD23 would do the same thing in naïve T cells from allergic asthmatics. Naïve CD4<sup>+</sup> T cells from all three subjects with allergic asthma did not stimulate well through CD3+ICAM-1 and thus, failed to differentiate to effector or memory cells (**Fig. 4.61**, bottom panels). The cells from one of these subjects also did not stimulate through CD28 and was excluded from the final analysis. **Fig. 4.61** shows the expression of CD11a and CD27 on day 7 from the two subjects that were stimulated either through CD3+CD28 or CD3+CD28+CD23. The results of CD23 stimulation on effector and memory differentiation in these two experiments conflict. Addition of anti-CD23 to stimulation through CD3+CD28 inhibited differentiation to effector and memory cells in donor 1 but enhanced differentiation to effector and memory cells in donor 2 (**Fig. 4.61**). We are unable to conclude the effect of CD23 stimulation on effector and memory differentiation. It is possible that with more experiments, we may notice two distinct groups of patients with allergic asthma based on their response to stimulation through CD23.

Fig. 4.61

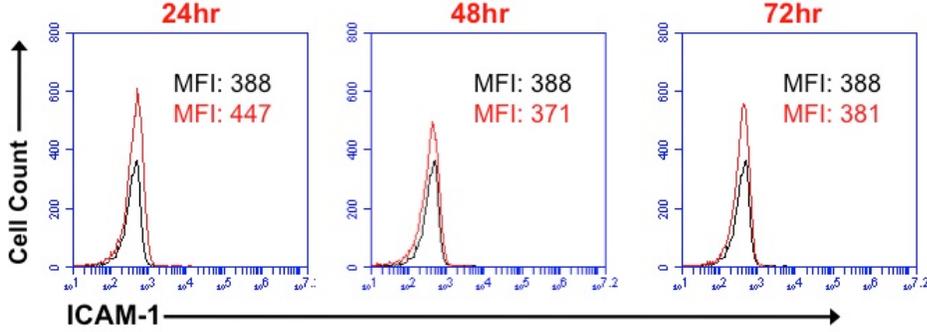


**Figure 4.61. Regulation of effector and memory differentiation by CD23 seemed to vary among subjects with allergic asthma.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-CD28+anti-CD23, anti-CD3+anti-ICAM-1 or anti-CD3+anti-ICAM-1+anti-CD23 were stained for expression of CD45RO, CD27 and CD11a and analyzed by flow cytometry. Representative of 2 experiments. The plots are gated on the live population rather than the CD45RO<sup>+</sup> T cell population typical for effector and memory dot plots so that the naïve T cell population (upper left quadrant) can be visualized. Upper right quadrants identify the percentage of effector T cells with cell numbers parenthetically. Lower right quadrants identify the percentage of memory T cells with cell numbers parenthetically.

*Naïve CD4+ T cells from allergic asthmatics upregulated expression of ICAM-1 after stimulation through the TCR at 24 hours but did not sustain expression of ICAM-1 at 48-72 hours*

Normally, stimulation of human naïve CD4+ T cells through the TCR increases the existing level of expression of ICAM-1 within 24-48 hours, peaking at 72 hours (Chapter 2). We hypothesized that the naïve T cells from these atopic individuals may not have responded to stimulation through ICAM-1 because of a failure to upregulate expression of ICAM-1 within the first few days of stimulation. TCR stimulation upregulated ICAM-1 expression at 24 hours (MFI of 447) by 15% (**Fig. 4.62**) but did not continue to sustain expression, returning to baseline levels (day 0, MFI of 388) at 48 and 72 hours (MFI of 371 and 381, respectively). This expression pattern of ICAM-1 in allergic asthmatics differs from what we observed in our other studies of naïve T cells from healthy subjects (Chapter 2) where peak expression of ICAM-1 is seen at 72 hours of stimulation by all stimuli including stimulation through the TCR. The failure of these naïve T cells to sustain ICAM-1 expression past 24 hours after activation may account for the inability of costimulation through ICAM-1 to induce differentiation. We also stained naïve CD4+ T cells for CD28 prior to stimulation and then at 24, 48, and 72 hours of stimulation. High levels of CD28 were expressed by cells at all timepoints (data not shown) as expected.

**Fig. 4.62**



**Figure 4.62. TCR stimulation of naïve CD4+ T cells from allergic individuals upregulated expression of ICAM-1 at 24 hours but not 48 or 72 hours.** Human naïve CD4+ T cells from allergic subjects stimulated with anti-CD3 for 24, 48 or 72 hours were stained for ICAM-1 expression and analyzed by flow cytometry. Representative of 1 experiment. MFI of ICAM-1 expression is indicated before stimulation began on day 0 (black lines) or 24, 48 or 72 hours of stimulation through CD3 (red lines).

## **Discussion**

### *Expression of CD23 in T cells from healthy subjects*

Our goals for this project included determining the expression of CD23 in mature and naïve T cells and the phenotype of cells that express CD23. Stimulation induced greater expression of CD23 in naïve T cells than in mature T cells regardless of the type of stimulus. Naïve CD4<sup>+</sup> T cells also responded to IL-4 by upregulating CD23 in contrast to mature T cells that were mostly unresponsive to the addition of exogenous IL-4. This suggests that CD23 may function to regulate the activation and differentiation of naïve T cells more than the responses of mature T cells.

The phenotype of CD23<sup>+</sup> cells generated from the naïve CD4<sup>+</sup> T cell population is variable, and CD23 can be detected on many T cell subsets. CD23 was detected on both CD45RA<sup>+</sup> (mostly naïve) and CD45RA<sup>-</sup> (mature) T cells. Also, activated T cells (based on CD25 and the intermediate levels of Foxp3 and Tbet expression) may express CD23 more frequently than other populations. This suggests that CD23 expression and thus, function, may be relevant in transiently activated T cells, possibly influencing the differentiation of naïve T cells and responses of T helper cells during a primary immune response.

### *Functional outcomes of CD23 stimulation of T cells from healthy subjects differed between naïve and mature subsets and between ICAM-1 and CD28 costimulation*

Stimulation through CD23 models a situation where a T cell receives a signal through resident CD23 with a captured IgE Ab that is engaging its cognate Ag. We examined potential ramifications of this in combination with other signals on T cell subsets.

### Naïve CD4+ T cells

CD23 did not function as an independent costimulatory protein to induce differentiation of naïve CD4+ T cells. Thus, any positive function of CD23 on naïve T cells appears to be limited to situations in which another costimulatory protein such as CD28 or ICAM-1 cooperates with Ag stimulation through TCR(CD3)

### Mature T cells

Stimulation of mature T cells through CD3+CD23 was inhibitory compared to stimulation through only CD3. However, CD23 stimulation enhanced activation and the percentage of Tbet+ cells when combined with stimulation through CD28, suggesting that CD23 stimulation of mature T cells may enhance Th1 function in the presence of CD28 signaling. Consistent with naïve T cells, cells costimulated through ICAM-1 were less responsive to the effects of CD23 stimulation.

### *Overall Function of CD23*

Our hypothesis prior to beginning this project was that *CD23 regulates T helper responses through supporting the generation and function of Th2 cells*. However, our data suggest a different role for CD23 as a negative regulator of Th2 responses. Th1 cells oppose Th2 cells by directly inhibiting Th2 cell differentiation or function by cytokine secretion. In mature T cells, CD23 may oppose Th2 function by supporting the expansion of activated, Th1 cells that can suppress Th2 cells. In naïve T cells, the effect of CD23 depended on the traditional costimulatory molecule that also was engaged. In cells costimulated through ICAM-1, CD23 enhanced activation of ERK and proliferation and modestly enhanced differentiation to Treg

cells. Stimulation through CD3+CD28+CD23 compared to CD3+CD28 resulted in fewer activated, effector and memory cells and reduced expression of Gata3 (Th2) and Tbet (Th1). The effects of stimulation through CD23 on naïve T cells and mature T cells when combined with CD3+CD28 differed. CD23 inhibited naïve T cells and activated mature T cells. Overall, though, these effects on naïve and mature populations are consistent with a role for CD23 as a negative regulator of Th2 responses because it suppressed Th2 function (naïve T cells) and supported Th1 function (mature T cells). Th1 and Th2 cells suppress each other; by enhancing Th1 function in mature T cells, the Th2 response is likely to be inhibited. Costimulation through CD28 but not ICAM-1 generates Th2 cells (17-19). Therefore, it seems reasonable that costimulation through CD28 but not ICAM-1 would be inhibited by CD23 based on the proposed inhibition of Th2 responses by CD23. In mature T cells, CD23 promoted Th1 cell expansion.

*CD23 expression and functional outcomes of CD23 stimulation in subjects with allergic asthma*

Conclusions herein regarding CD23 expression and function in allergic asthmatics are based on preliminary data with the hope that the project continues. Experiments from only two of the three subjects were usable so continued studies are certainly needed. Similar to naïve T cells from healthy subjects, costimulation through CD28 plus exogenous IL-4 induced higher levels of CD23 expression in cells from allergic asthmatics. However, the peak of CD23 expression in the donor cells from these atopic individuals peaked two days earlier than in cells from non-atopic individuals. Differences in gene regulation between day 5 and 7 in these activating naïve T cells may result in different functional outcomes when CD23 is engaged. Stimulation of naïve CD4+ T cells through CD3+ICAM-1 did not induce differentiation in the cells from allergic asthmatics, but expression of ICAM-1 was also lower at 48-72 hours

compared to naïve T cells from healthy subject. This reduced expression of ICAM-1 may limit the amount of ICAM-1 signaling received by these cells. This may suggest that expression and participation of costimulatory proteins such as ICAM-1 in allergic asthmatics may differ from the general population. The ability of CD23 stimulation to induce Treg differentiation was consistent, though, between cells from allergic and healthy subjects in the presence or absence of IL-4. It appears that contrary to our hypothesis, CD23 may function in allergic asthmatics and healthy individuals in a similar manner. Whether CD23 also promotes Th1 expansion from a mature T cell population in these patients remains to be determined.

*Proposed hypothesis for function of CD23 in vivo (both healthy and allergic individuals)*

We propose an *in vivo* function for CD23 during an elevated Th2-driven immune response based on our data from both healthy and allergic subjects. CD23 binds to IgE and IgE:Ag complexes one-tenth of the affinity of the high affinity receptor (4). CD23 may not be engaged and regulate T cell responses when IgE levels are low to moderate depending on the expression of the high affinity IgE receptor in the microenvironment. Under conditions in which IgE levels are high such as during a parasitic infection or allergic response, sufficient IgE is available to maintain binding to the low affinity receptor which serves as a sensor to the heightened Th2 response. CD23, therefore, seems to function to balance the immune response by inducing differentiation of naïve T cells to Treg cells, inhibiting differentiation of naïve T cells to Th2 cells, and promoting Th1 expansion of mature T cells.

### *Summary*

Thus far, we have characterized the expression and function of CD23 in naïve CD4+ T cells from healthy subjects and plan to continue these studies using subjects with allergic asthma. We have preliminary evidence regarding the function and expression of CD23 in mature T cells from healthy individuals. In future studies, we will examine the function of CD23 in mature T cells from allergic asthmatics and determine whether the function of CD23 differs between allergic asthmatics with or without the R62W SNP mutation in CD23. Overall, our data refute our original hypothesis for increased Th2 function and have led to the development of a new proposal in which CD23 functions as a negative regulator of Th2 responses.

### **Acknowledgements**

This project is part of a collaboration with Dr. Marcia Chan that is funded through the Patton Trust Research Development Grant Program through the Kansas City Area Life Sciences Institute. I would like to thank Dr. Marcia Chan for collaborating with the Benedict lab and inspiring this work in addition to her expertise in CD23 and IgE.

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## **Chapter 5**

**Oxidized low density lipoprotein (oxLDL) promotes human naïve CD4+ T cell differentiation to Th1 cells: a novel mechanism to exacerbate atherosclerosis.**

## **Introduction**

Atherosclerosis, a chronic inflammatory condition of the major arteries, contributes significantly to cardiovascular disease, the leading cause of death worldwide (1). Oxidized low-density lipoprotein (oxLDL) promotes atherogenesis through several known mechanisms whereas high-density lipoprotein (HDL) opposes atherogenesis. Pathogenic Th1 cells represent a major cell population within atherosclerotic plaque. We investigated the hypothesis that selective influence on naïve T cell differentiation by oxLDL and HDL may identify novel mechanisms by which oxLDL promotes and HDL inhibits atherosclerosis.

### *LDL, HDL and the immune response in atherosclerotic plaque*

It is widely theorized that insult to the endothelium, which can be physical, immune-related or both, initiates atherogenesis. Atherosclerotic lesion formation typically occurs at sites where the arteries bifurcate causing a greater fluid shear force on the endothelial cells and increasing their permeability to LDL (2). Accumulation of LDL in the arterial wall is accompanied by oxidation to oxLDL, which is atherogenic. OxLDL induces vascular endothelial cells to express adhesion molecules and secrete cytokines and chemokines that attract immune cells (3-7). Activated macrophages ingest oxLDL to become lipid rich foam cells, release proinflammatory cytokines and contribute to the necrotic core of the lesion (8-10). In addition, platelets activated by oxLDL adhere to the lesion and promote thrombus formation (11, 12). HDL opposes atherogenesis by inhibiting LDL oxidation, endothelium activation, macrophage activation, and platelet aggregation (13-17) and by removing cholesterol from existing plaque (13).

### *T cells in atherosclerosis*

T cells comprise 10-20% of cells in early atherosclerotic plaque and continue to be found in lesions throughout plaque development (18-20). However, the mechanisms by which pathogenic T cells participate in atherogenesis have not been fully elucidated. In the absence of T cells, atherosclerotic plaque develops less efficiently reaching 10% of potential size, and adoptive transfer of CD4<sup>+</sup> T cells into *ApoE*<sup>(-/-)</sup> *scid/scid* mice accelerates lesion development (21-27). T cells resident in plaque predominantly exhibit a Th1 phenotype secreting IFN- $\gamma$  (19, 20, 28, 29). Deficiency in expression of the IFN- $\gamma$  receptor and reduced production of IFN- $\gamma$  diminish plaque development, whereas exogenously added IFN- $\gamma$  exacerbates lesion development (30, 31). Thus, Th1 cells contribute to plaque evolution. A subset of mature effector T cells recognizes modified LDL using the T cell antigen receptor (25-27). In addition to this mature T cell antigen response, we hypothesize that oxLDL interacts with specific LDL receptors on immature naïve T cells to promote selective differentiation that favors atherosclerosis. We also hypothesize that HDL opposes the proposed oxLDL effect on naïve T cell differentiation.

### *Naïve T cell differentiation in plaque*

Naïve T cells have emigrated recently from the thymus carrying a half-life of 6 weeks and requiring engagement with cognate antigen to stimulate activation and differentiation leading to effector T cells, and to the increased longevity of memory T cells. Naïve T cells are not limited to the lymph node as a site of differentiation. Under chronic inflammatory conditions, naïve T cells can migrate, activate, and proliferate in lymph node-like aggregates within non-lymphoid tissues (32-35). Perhaps the most notable alternative site described thus far is the islets

of Langerhans in developing diabetes. The chronic inflammatory conditions prevailing in plaque seem likely to provide a hospitable microenvironment for extra-lymphoid activation of naïve T cells. Although not as abundant as in peripheral blood, CD45RA expressing T (naïve) cells have been found in human atherosclerotic plaque (36, 37), suggesting that naïve T cells can migrate to plaque and once there, may become activated by a number of cells that express HLA Class II (HLA II). Dendritic cells are found at very early stages of lesion development and co-localize with T cells in atherosclerotic plaque (20-22). Activated macrophages populate plaque, and smooth muscle cells and CD8+ T cells in these atherosclerotic lesions also express HLA Class II (37, 38). All these cell types are capable of activating naïve CD4+ T cells.

#### *Model Differentiation System*

Previously we reported different effects of HDL and oxLDL on peripheral blood-derived total T cell proliferation, cell death, and cytokine secretion (39) using a mixed population of naïve and mature, differentiated T cells. Here, we examined whether the lipoproteins could affect human naïve CD4+ T cell differentiation in response to different cellular microenvironments. Because intercellular interactions engage dozens of T cell surface counter receptors, we use antibodies to stimulate only through surface proteins of interest in a reductionist approach to model specific events (40, 41) that participate individually. This approach has the added benefit of limiting control of differentiation to a targeted microenvironment. Thus it involves cytokines created only by the differentiating naïve T cells without the classic addition of polarizing cytokines provided by other cells, for example, in the lymph node. We compared (40-43) classic costimulation through the T cell antigen receptor, TCR(CD3)+CD28 with less well-studied costimulation through CD3+ICAM-1 and CD3+CTLA-

4. We have observed that costimulation through CD3+CD28 induces human naïve CD4+ T cells to differentiate to Th1 and Th2 effector and memory cells in our system but not to become regulatory T cells (Treg cells) without exogenously added cytokines. In contrast, costimulation through CD3+ICAM-1 induces the same naïve CD4+ T cell population to differentiate to effector and memory Th1 cells and to Treg cells without exogenously added cytokines but not to become Th2 cells. CD3+CTLA-4 induces differentiation of naïve T cells to effector, memory and regulatory T cells (See Chapter 2). Here, we compared effects of oxLDL and HDL on human naïve T cell differentiation in the context of our three different, modeled microenvironments.

#### *Hypotheses examined*

The working hypothesis was that oxLDL promotes differentiation of human naïve CD4+ T cells to plaque-favoring effector cells and that HDL opposes this process. On any day, the percentage of naïve T cells with specificity for a particular antigen is expected to be very low. Hence, use of naïve T cells here assures us that any reasonably robust response to LDL or HDL would be due to non-TCR mediated interaction. In support of the hypotheses, oxLDL promoted human naïve CD4+ T cell differentiation and survival of activated, effector T cells with a Th1 activity, suggesting a novel mechanism by which oxLDL could enhance atherogenesis. In contrast, HDL inhibited proliferation and decreased cell survival suggesting opposition to atherogenesis at the level where naïve T cells differentiate.

## **Materials and methods**

### *Antibodies and reagents*

Antibodies used to stain cells for flow cytometry were: anti-CD11a-FITC (clone: G43-25B, BD Biosciences, San Jose, CA), anti-CD27-PE (clone: CLB-27/1, Invitrogen, Carlsbad, CA), anti-CD27-PECy5 (clone: 0323, eBioscience, San Diego, CA), anti-CD45RO-APC (clone: UCHL1, Invitrogen), anti-CD45RO-PerCP (clone: UCHL1, Invitrogen), anti-Foxp3-PE (clone: 3G3, Miltenyi Biotec, Auburn, CA), and anti-CD25-Tri Color (clone: 3G10, Invitrogen). Intracellular staining of Foxp3, CFSE staining and flow cytometry analysis were performed as describe in Chapter 2.

### *Human Subjects*

Peripheral blood (240 ml into heparin) was obtained after informed consent from healthy adult volunteers of both genders, ages between 20 and 30y, who had been free of infection for 14 days. No other information was collected. Procedures were approved by the University of Kansas Institutional Review Board.

### *Human naïve CD4<sup>+</sup> T cell purification*

Human naïve CD4<sup>+</sup> T cells were isolated as described in Chapter 2.

### *Naïve CD4<sup>+</sup> T cell stimulation*

Human naïve CD4<sup>+</sup> T cells were stimulated using plate-bound antibodies as we have described (40). Clones and concentrations of Ab were used as describe in Chapter 2. Either HDL (200 µg/ml) or oxLDL (25 µg/ml) from Intracel (Frederick, MD) was added immediately

after plating cells. Concentrations of lipoproteins were based on our previously published work (39) and attained roughly equivalent molar concentrations between the largely disparate molecular masses of these lipoproteins (45).

### *ELISA*

Supernate from stimulated cells was collected at day 4 or 7 and stored at  $-70^{\circ}\text{C}$  until analysis of IL-2, IFN- $\gamma$ , and IL-4 production using Quantikine Colorimetric Sandwich ELISA kits (R&D Systems, Minneapolis, MN) and The Ready-Set-Go! ELISA kit (eBioscience) for IL-17A. Assays were performed in duplicate. Plates were washed with the ELx50 Auto Strip Washer (Bio-Tek Instruments Inc., Winooski, VT) and read at 450 nm using an EL311 Microplate Autoreader (Bio-Tek Instruments Inc.).

### *Migration Assay*

400  $\mu\text{l}$  serum-free RPMI 1640 was added to the wells of 24-transwell plates with a pore size of 5  $\mu\text{m}$ . OxLDL (25  $\mu\text{g}/\text{ml}$ ), LDL (25  $\mu\text{g}/\text{ml}$ ), HDL (200  $\mu\text{g}/\text{ml}$ ) were added to the wells and mixed prior to securing transwell inserts. 200  $\mu\text{l}$  freshly isolated human naïve CD4<sup>+</sup> T cells were plated at  $5 \times 10^6$  cells/ml in the inserts and allowed to migrate for 5 hr at  $37^{\circ}\text{C}$ . Inserts were removed, and migrated cells collected and analyzed by flow cytometry.

### *Statistical Analysis*

The one-way ANOVA was used to determine statistical confidence for all analyses except analysis of IFN- $\gamma$  and IL-17A ELISA data for which the two-way ANOVA was performed. All

statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

## Results

### *Phenotypic characterization of naïve, effector, and memory T cell populations*

Throughout this work, naïve T cells were CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO(-)CD11a<sup>lo</sup>CD27<sup>+</sup> and averaged 98% purity as we have observed many times (40, 41) and as seen in **Fig. 5.1A**. Because a subpopulation of effector T cells recognizes modified LDL using the TCR (25-27), use of naïve T cells reduces the probability that oxLDL-specific cells will represent a significant population of responding cells and allows study of more direct effects on cell differentiation induced by oxLDL or HDL. **Figures 5.2-5.5** ask whether a microenvironment rich in lipoproteins relevant to atherosclerosis might influence differentiation of human naïve CD4<sup>+</sup> T cells. Human T cells were defined phenotypically some time ago by others (44), and we verified these observations (40) in our own system. Effector T cells reside in the upper right quadrant of the flow dot plots as diagrammed in **Fig. 5.1B** and memory T cells in the lower right quadrant. The memory CD4<sup>+</sup> T cells observed here as CD45RA(-)CD45RO<sup>+</sup>CD11a<sup>hi</sup>CD27(-) cells (lower right quadrant) routinely respond to suboptimal TCR stimulation (alone) with more robust proliferation and higher Th1 cytokine secretion than naïve T cells from the same individual (40). Thus, these phenotypic distinctions were used to study the influence on differentiation to memory and effector T cells.

### *oxLDL augmented differentiation of human naïve CD4<sup>+</sup> T cells to T effector cells*

As we observed previously (40), the majority of human naïve CD4<sup>+</sup> T cells costimulated through CD3<sup>+</sup>CD28 (Fig. 5.2-5.3) shifted to the upper right quadrant and thus differentiated to an effector cell phenotype [CD45RA(-)CD45RO<sup>+</sup>, CD11a<sup>hi</sup>, CD27<sup>hi</sup>]. Addition of oxLDL (third

column) significantly increased the percentage of effector cells regardless of the choice of costimulatory molecule or timepoint of differentiation (representative figures in **Fig. 5.2-5.3**, summarized in **Fig. 5.4**). OxLDL caused effector T cells induced by stimulation through CD3+CD28 to increase from  $63 \pm 3\%$  to  $79 \pm 3\%$  ( $p < 0.005$ ) at day 7 and from  $62 \pm 8\%$  to  $80 \pm 4\%$  ( $p < 0.05$ ) at day 14 (**Fig. 5.4**). Differentiation to effector cells by costimulation through CD3+ICAM-1 or CD3+CTLA-4 was affected in a similar manner by oxLDL. With ICAM-1 costimulation, oxLDL enhanced differentiation to effector T cells from  $34 \pm 6\%$  to  $58 \pm 4\%$  ( $p < 0.005$ ) at day 7 and from  $38 \pm 5\%$  to  $66 \pm 4\%$  ( $p < 0.005$ ) at day 14. The effector T cell population generated through CD3+CTLA-4 also increased from  $34 \pm 8\%$  to  $65\% \pm 6\%$  at day 7 and from  $51\% \pm 7\%$  to  $76 \pm 4\%$  at day 14. Overall, oxLDL augmented the effector T cell population in all stimuli. In contrast, HDL (column 2) exerted no consistent effect on differentiation of naïve CD4+ T cells to effector cells with any costimulatory regimen.

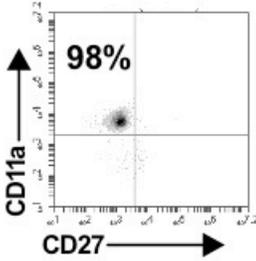
#### *oxLDL suppressed differentiation to memory T cells*

Human naïve CD4+ T cells differentiated to memory T cells in response to all costimuli as expected (36, 37 and Chapter 2). The relative kinetics and memory cell numbers were consistent with our previous (30) parameters (**Fig. 5.2-5.3** summarized in **Fig. 5.5**). OxLDL inhibited differentiation to memory phenotype by the naïve CD4+ T cells regardless of the costimulation. On day 7, memory T cells arising from CD28 costimulation decreased from  $13 \pm 2\%$  to  $6 \pm 1\%$  ( $p < 0.005$ ) (summarized in **Fig. 5.5**), and by 14 days the difference with or without oxLDL was no longer detectable. ICAM-1 and CTLA-4 costimulated cells demonstrated a similar response to oxLDL except the decrease in memory cells was delayed to day 14 (from

**Figure 5.1**

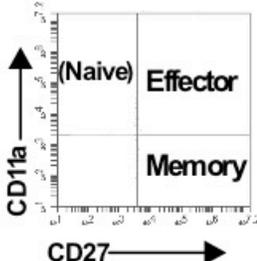
**A**

Gated on CD45RA+RO(-) Cells



**B**

Gated on CD45RA(-)RO+ Cells



**Figure 5.1A-B. Highly pure populations of naïve CD4+ T cells are used in the *in vitro* differentiation studies.** **Fig. 5.1A**, Representative dot plot of naïve T cell purity, gated on CD4+CD45RA+CD45RO(-) population. **Fig. 5.1B**, Diagram indicating location of naïve [CD45RA+CD45RO(-)], effector and memory T cell populations [CD45RA(-)CD45RO+]. Effector (upper quadrant) and memory (lower quadrant) populations are gated on the CD4+CD45RA(-)CD45RO+ population.

Figure 5.2

**Day 7**

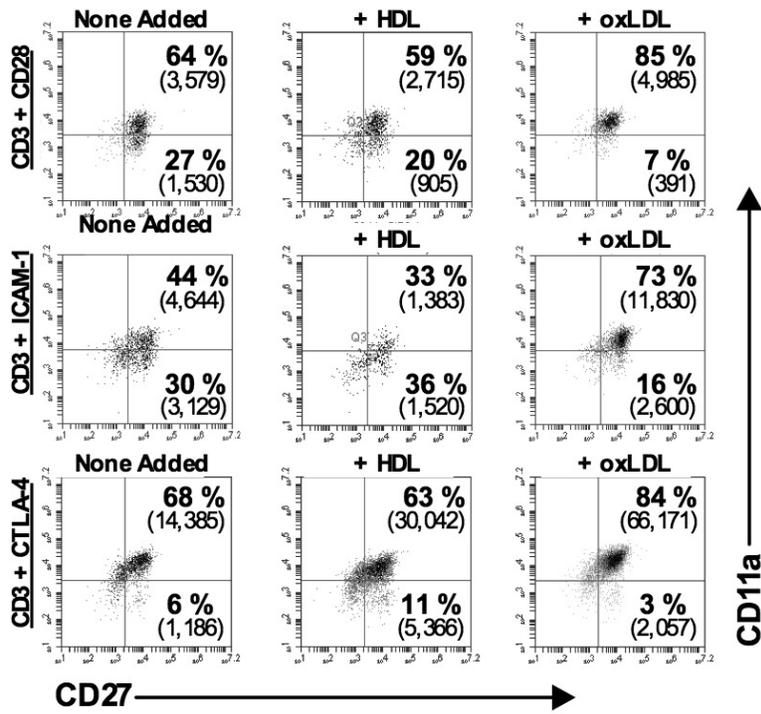
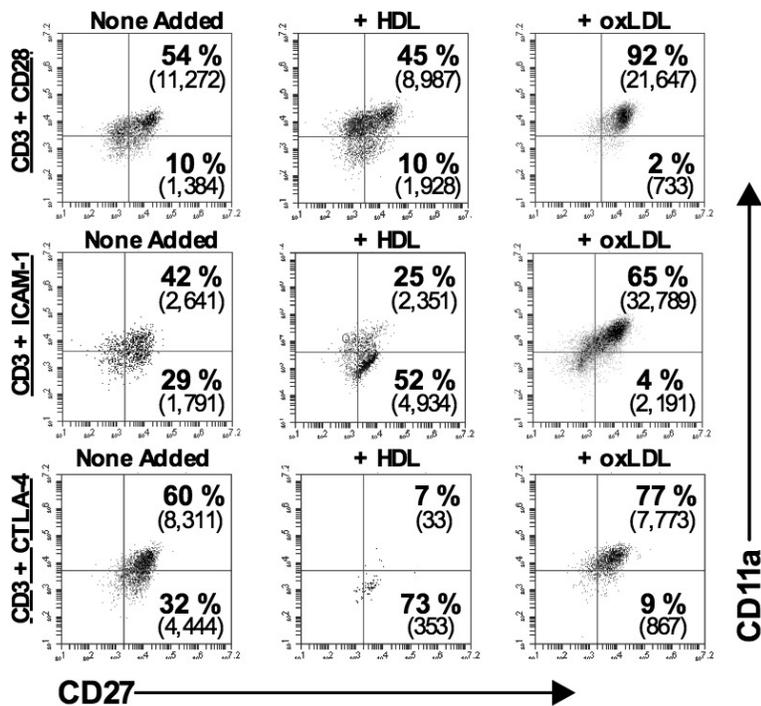


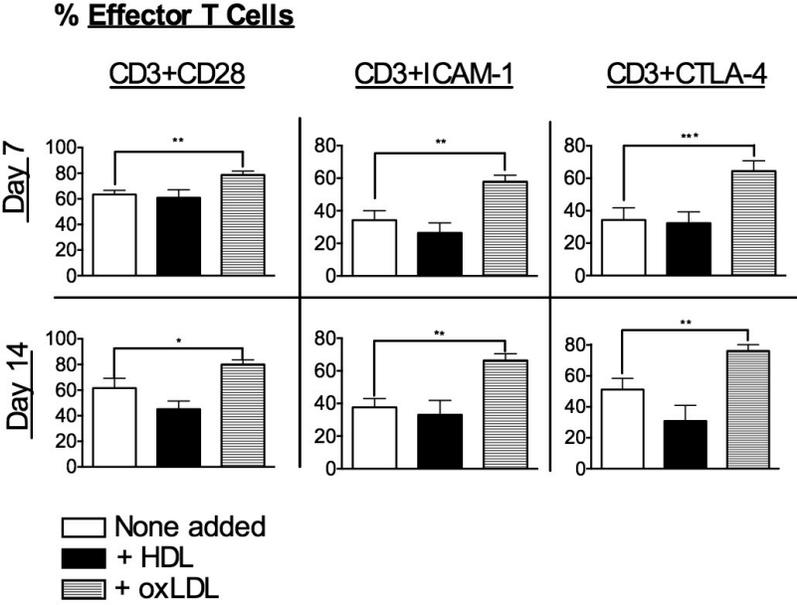
Figure 5.3

**Day 14**



**Figures 5.2-5.3. OxLDL augmented T cell differentiation to effector T cells but not memory cells.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days using anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4 were analyzed for CD45RO, CD27, and CD11a expression by flow cytometry. HDL (200 µg/ml) or oxLDL (25 µg/ml) was added on day 0. Dot plots were gated on the CD45RO<sup>+</sup> population. Effector T cells are defined as CD45RO<sup>+</sup>CD11a<sup>hi</sup>CD27<sup>hi</sup> and memory T cells as CD45RO<sup>+</sup>CD11a<sup>hi</sup>CD27<sup>lo</sup>. Effector (upper right quadrant) and memory (lower right quadrant) T cell populations after 7 days (**Fig. 5.2**) and 14 days (**Fig. 5.3**) of stimulation through CD3+CD28 (upper panels), CD3+ICAM-1 (middle panels), or CD3+CTLA-4 (lower panels). Additions: medium (column 1), HDL (column 2), or oxLDL (column 3). Percentage of each is indicated, with cell numbers parenthetically. Representative of eight (CD3+CD28, 7d and 14d), eight (CD3+ICAM-1, 7d), nine (CD3+ICAM-1, 14d), seven (CD3+CTLA-4, 7d) or eight (CD3+CTLA-4, 14d) separate experiments.

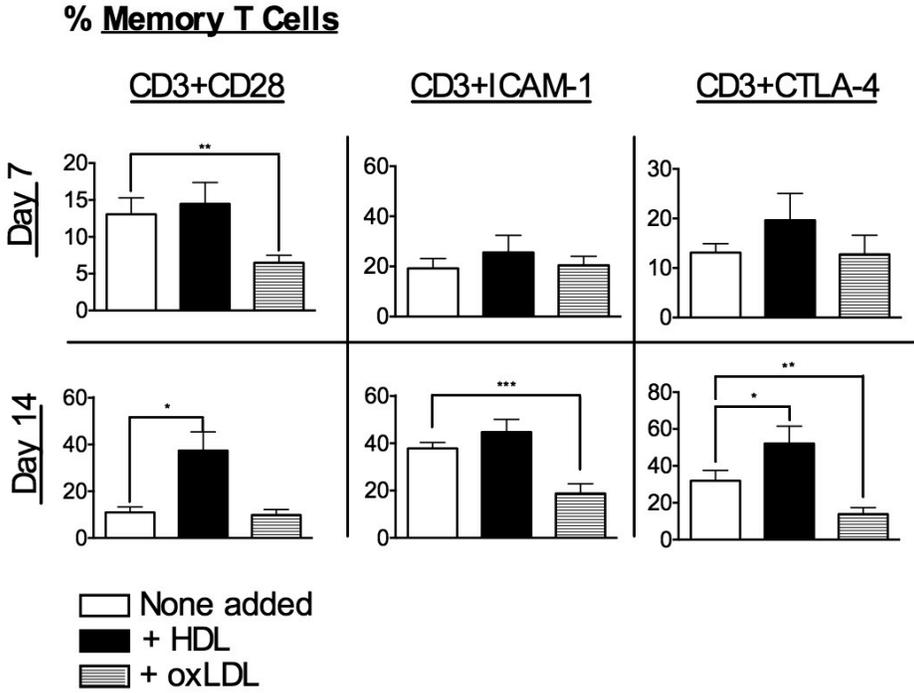
Figure 5.4



**Figures 5.4. OxLDL augmented T cell differentiation to effector T cells.** Human naïve CD4+ T cells stimulated for 7 or 14 days using anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4 were analyzed for CD45RO, CD27, and CD11a expression by flow cytometry. HDL (200 µg/ml) or oxLDL (25 µg/ml) was added on day 0. Effector T cells are defined as CD45RO+CD11a<sup>hi</sup>CD27<sup>hi</sup>. **Fig. 5.4,** Average percentage of CD4+CD45RO+CD11a<sup>hi</sup>CD27<sup>hi</sup> cells (effector cells) ± SEM at 7d and 14d with each costimulatory regimen (open bars) and costimulation plus HDL (closed bars) or oxLDL (striped bars).

\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

Figure 5.5



**Fig. 5.5. OxLDL suppressed differentiation to memory T cells.** Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days using anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4 were analyzed for CD45RO, CD27, and CD11a expression by flow cytometry. HDL (200 µg/ml) or oxLDL (25 µg/ml) was added on day 0. Memory T cells are defined as CD45RO<sup>+</sup>CD11a<sup>hi</sup>CD27<sup>lo</sup>. **Fig. 5.5,** Average percentage of CD4<sup>+</sup>CD45RO<sup>+</sup>CD11a<sup>hi</sup>CD27<sup>lo</sup> cells (memory cells) ± SEM at 7 and 14d with each costimulatory regimen and indicated lipoprotein. Representative of eight (CD3+CD28, 7d and 14d), eight (CD3+ICAM-1, 7d), nine (CD3+ICAM-1, 14d), seven (CD3+CTLA-4, 7d) or eight (CD3+CTLA-4, 14d) separate experiments.

\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

38 ± 3% to 19 ± 4% with ICAM-1 and from 32 ± 6% to 14 ± 3% with CTLA-4;  $p < 0.001$  and  $p < 0.01$ ) (**Fig. 5.5**). HDL either had little effect on differentiation to memory cells or favored an increase in memory (day 14, CD3+CD28 and CD3+CTLA-4) over several experiments. As seen in **Fig. 5.3**, the percentage of memory T cells (lower right quadrant) produced by CTLA-4 costimulation in the presence of HDL is high, 73%, but the number of cells is considerably low. Although statistically significant, the increase in the percentage of memory cells by HDL was likely the result of either a decrease in cell viability or proliferation because we often observed fewer cells in the presence of HDL as will be discussed later in Chapter 5.

In support of the working hypothesis, a cellular microenvironment providing elevated oxLDL during costimulation through CD3+CD28, CD3+ICAM-1 or CD3+CTLA-4 favored increased effector T cells capable of causing damage. In contrast, elevated HDL favored no increase in effector cells and a possible increase in memory T cells.

#### *oxLDL and HDL did not alter expression of CD45RO*

During activation, naïve T cells upregulate CD45RO and typically downregulate CD45RA as they differentiate (54-56). To verify that oxLDL induced a shift in favor of effector but not memory T cell differentiation, we assessed the percentage of total CD4+CD45RO+ cells at days 7 and 14. Neither oxLDL nor HDL altered the percentage of CD45RO+ cells at either timepoint through any of the costimulatory regimen (representative plots in **Fig. 5.6-5.7**, summarized in **Fig. 5.8**). However, the addition of HDL resulted in fewer CD45RO+ cells for all costimuli (**Fig. 5.6-5.7**, middle columns), suggesting a generalized suppressive effect on differentiation by HDL. Possible explanations for this observation may be inhibition of proliferation or cell viability by HDL. Thus, the increase in the effector T cell population and

Figure 5.6

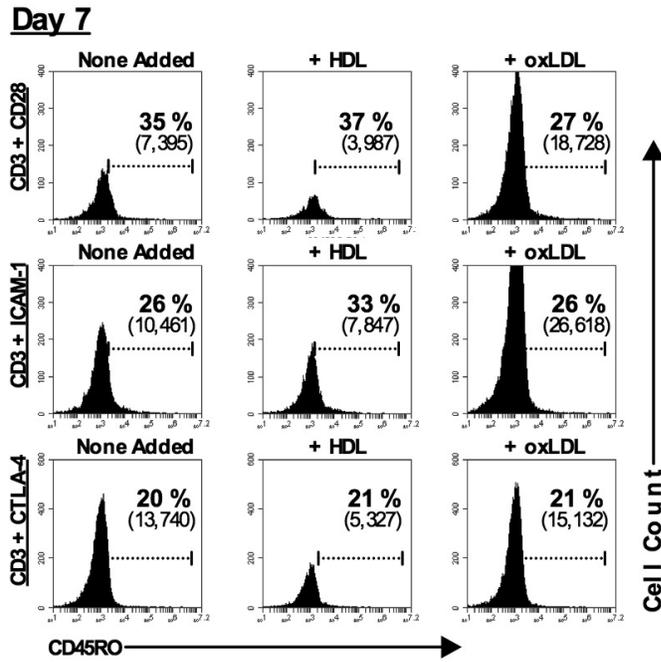


Figure 5.7

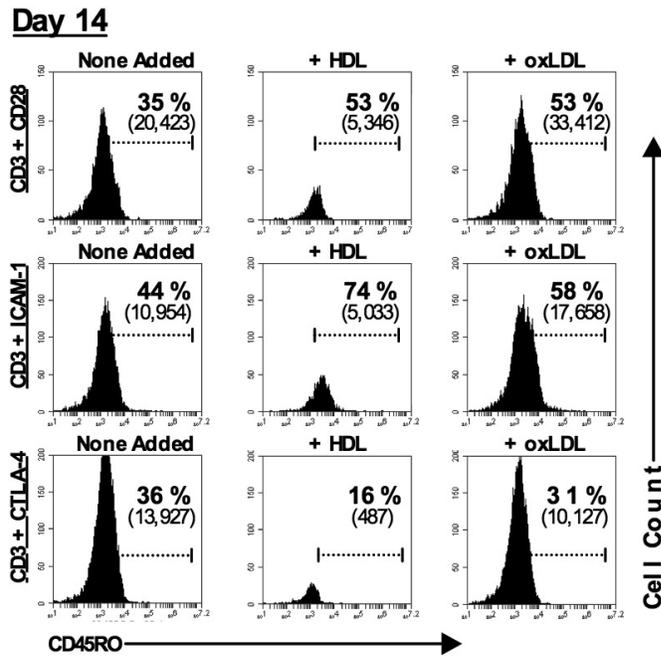
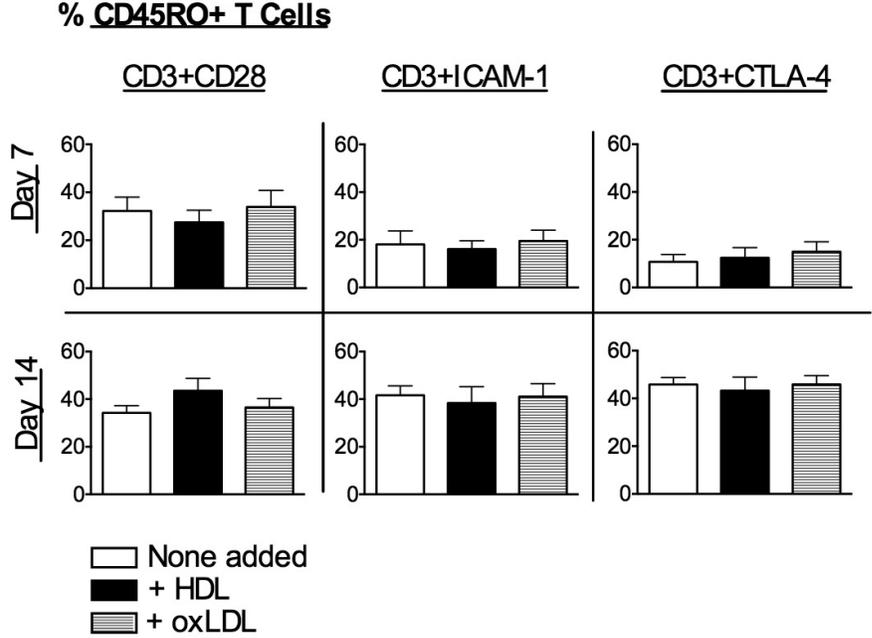


Figure 5.8



**Figures 5.6-5.8. Lipoproteins did not alter the percentage of cells expressing CD45RO.**

Human naïve CD4<sup>+</sup> T cells stimulated for 7 or 14 days using anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4 were analyzed for CD45RO expression by flow cytometry. HDL (200 µg/ml) or oxLDL (25 µg/ml) was added on day 0. Representative histogram plots are shown for day 7 (**Fig. 5.6**) and day 14 (**Fig. 5.7**) of stimulation through CD3+CD28 (upper panels), CD3+ICAM-1 (middle panels), or CD3+CTLA-4 (lower panels). Additions: medium (column 1), HDL (column 2), or oxLDL (column 3). Percentage of each is indicated, with cell numbers parenthetically. **Fig. 5.8, Fig. 5.5**, Average percentage of CD4<sup>+</sup>CD45RO<sup>+</sup> cells ± SEM at 7 and 14d with each costimulatory regimen and indicated lipoprotein. Representative of eight (CD3+CD28, 7d and 14d), eight (CD3+ICAM-1, 7d), nine (CD3+ICAM-1, 14d), seven (CD3+CTLA-4, 7d) or eight (CD3+CTLA-4, 14d) separate experiments.

decrease in memory T cell population is a result of oxLDL shifting naïve T cell differentiation to effector cells. The increase in the memory population by HDL (CD28 and CTLA-4 costimulations, 14d) may be a result of analyzing a smaller sample size which can readily skew percentages.

*Effector T cells generated in the presence of oxLDL exhibited a Th1 phenotype*

We investigated whether oxLDL promoted differentiation to a particular T helper subset. Th1 or Th17 were likely candidates since they foster cell mediated immunity and inflammatory conditions (57, 58). Effector T cell functions were assessed by determining secretion of IFN- $\gamma$  and IL-2 (Th1), IL-4 (Th2), and IL-17A (Th17) using ELISA. On day 4 or 7, neither lipoprotein altered secretion of IL-2 with any stimulus (not shown). As seen in **Fig. 5.9**, oxLDL had little effect on secretion of IFN- $\gamma$  by cells costimulated through CD28 at day 4, but enhanced secretion from  $273 \pm 127$  pg/ml to  $844 \pm 317$  pg/ml by day 7 ( $p < 0.001$ ). With ICAM-1 costimulation, oxLDL enhanced IFN- $\gamma$  production (**Fig. 5.9**) at day 4 from  $21 \pm 21$  pg/ml to  $1,251 \pm 625$  pg/ml ( $p < 0.001$ ) fully a 60 fold increase that had diminished by day 7. There was no increase in IFN- $\gamma$  production by cells costimulated through CTLA-4 at day 7; however, there was great variation (up to 500 fold for the same experimental condition) among the three samples analyzed, and no other days or other cytokines were examined for this costimulus. Thus, oxLDL (striped bars) caused an increase in the Th1 cytokine (IFN- $\gamma$ ) with either CD28 or ICAM-1 costimulation, although the timing was consistently different. In contrast, HDL (closed bars) had no effect on IFN- $\gamma$  secretion by the differentiating cultures.

Secretion of the Th2 cytokine IL-4 by CD28-costimulated cells (**Fig. 5.10**) decreased from  $639 \pm 173$  pg/ml to  $148 \pm 45$  pg/ml on day 7 in the presence of oxLDL ( $p < 0.05$ ).

Figure 5.9

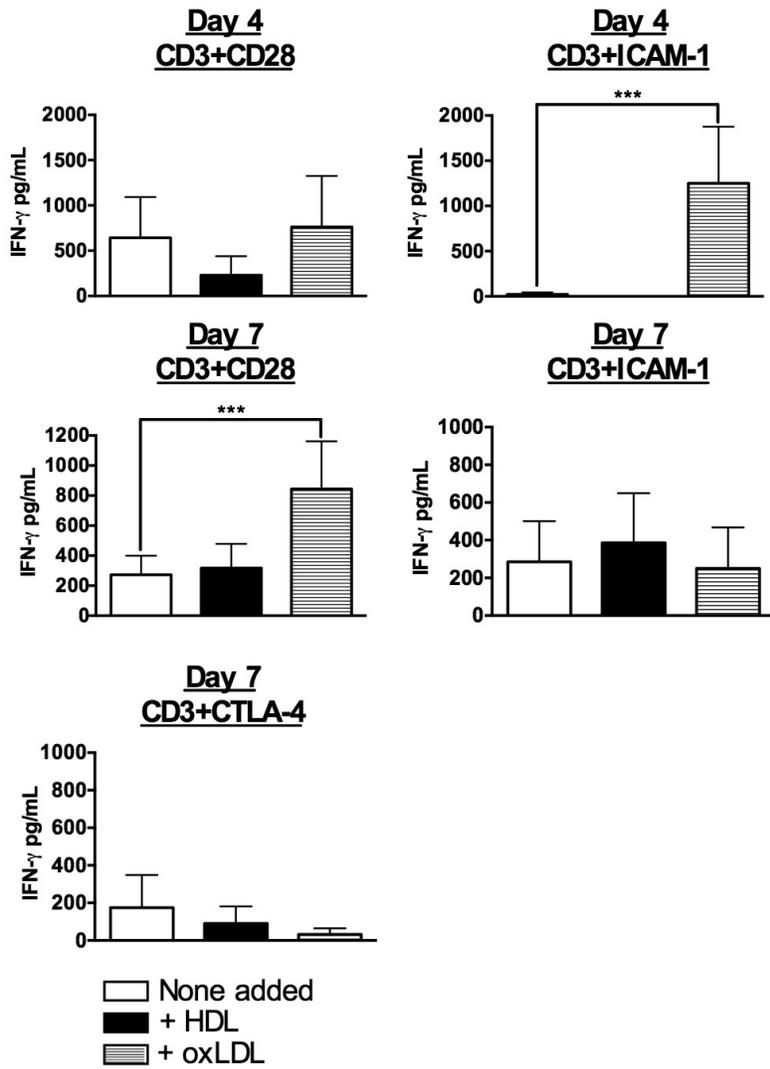


Figure 5.10

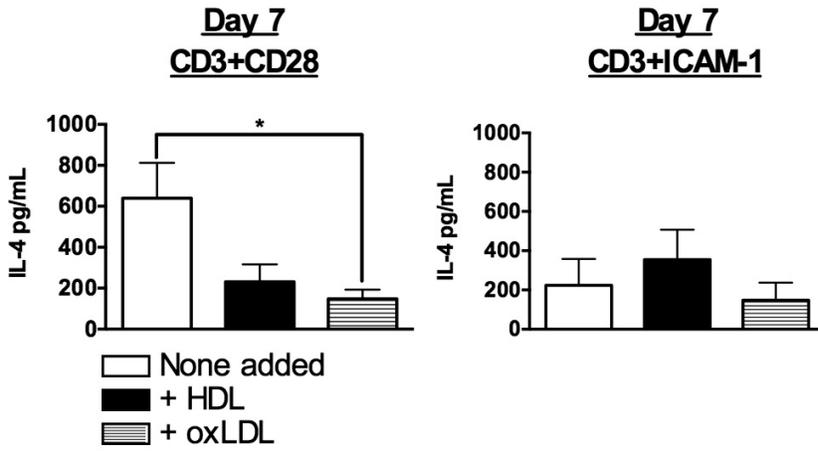
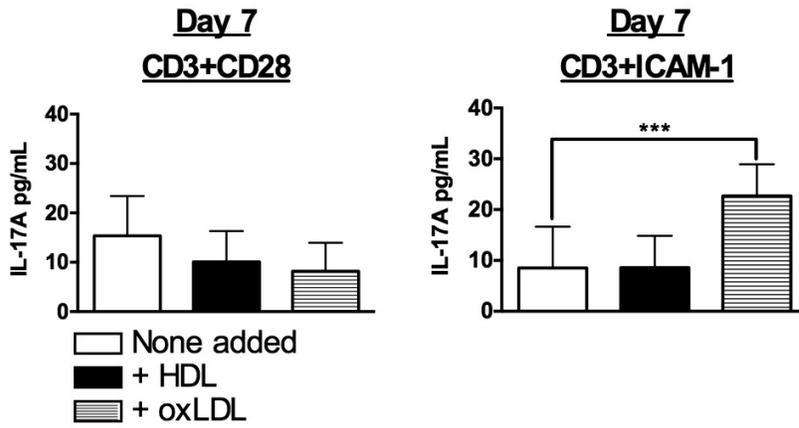


Figure 5.11



**Figures 5.9-5.11. OxLDL enhanced Th1 function, modestly increased Th17 activity and impaired Th2.** Supernates collected from human naive CD4<sup>+</sup> T cells stimulated for 4 or 7d with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1 or anti-CD3+anti-CTLA-4 in medium alone (open bars), medium+HDL (closed bars), or medium+oxLDL (striped bars). Secretion of IFN- $\gamma$ , IL-4, or IL-17 was determined by ELISA. **Fig. 5.9**, Average IFN- $\gamma$  secretion  $\pm$  SEM on 4 and 7d. Representative of five (CD3+CD28 and CD3+ICAM-1) or three (CD3+CTLA-4) experiments, each conducted in duplicate. **Fig. 5.10**, Average IL-4 secretion  $\pm$  SEM on day 7. Representative of five experiments each conducted in duplicate. **Fig. 5.11**, Average IL-17 secretion  $\pm$  SEM on day 7. Representative of four (CD3+CD28) or six experiments (CD3+ICAM-1) each in duplicate.

\*  $p < 0.05$ , \*\*\*  $p < 0.001$

Interestingly, HDL exerted a similar effect. When applied to cells undergoing ICAM-1 costimulation, oxLDL had no effect on IL-4 production on day 7 (**Fig. 5.10**) and HDL exerted the same lack of effect. So neither lipoprotein favored differentiation to Th2 cells, and both reduced the Th2 cytokine activity induced by costimulation through CD28.

Human naïve T cells costimulated through CD3+CD28 or CD3+ICAM-1 produce minimal differentiation to Th17 cells in the absence of exogenous cytokines (A. Newton, unpublished). However, whether the two lipoproteins could directly influence differentiation to a Th17 phenotype has not been tested. Neither lipoprotein affected production of IL-17A by cells costimulated through CD3+CD28 (**Fig. 5.11**). Cells stimulated through CD3+ICAM-1 demonstrated a modest increase in IL-17A production from  $9 \pm 8$  pg/ml to  $23 \pm 6$  pg/ml in the presence of oxLDL ( $p < 0.001$ ) but not HDL.

Thus, oxLDL augmented Th1 effector function while inhibiting Th2 effector function during CD28 costimulation. With ICAM-1 costimulation, oxLDL promoted Th1 effector function and slightly augmented Th17 effector function.

#### *oxLDL enhanced activation of naïve CD4+ T cells*

When naïve T cells encounter appropriately presented cognate antigen, they undergo activation prior to differentiation. The alpha subunit (CD25) of the IL-2 receptor is expressed by activated T cells, and by regulatory T cells (59-61). CD25 expression was assessed to learn whether oxLDL or HDL influenced the normal activation of naïve T cells induced by the three types of costimulation. In the representative experiment, (**Fig. 5.12**) 41% (~37,000 cells) of CD28 costimulated cells expressed CD25 and addition of oxLDL increased this to 73% (~60,000 cells). Results were similar in the representative experiment either using ICAM-1 or CTLA-4

costimulated cells. Several experiments are summarized in **Fig. 5.13**. In comparison with CD28 costimulation (open bars), oxLDL (striped bars) increased the expression of CD25 from  $44 \pm 3\%$  to  $63 \pm 5\%$  ( $p < 0.005$ ). Cells stimulated through CD3+ICAM-1 in the presence of oxLDL demonstrated a statistically non-supported trend increasing from  $33 \pm 8\%$  to  $53 \pm 9\%$  ( $p = 0.058$ ) on day 7 as did cells stimulated through CD3+CTLA-4 with an increase from  $27 \pm 7\%$  to  $41 \pm 8\%$  ( $p = 0.07$ ). Thus, oxLDL seemed to augment the percentage of activated CD4+ T cells with all sets of costimuli. HDL (closed bars) did not affect the percentage of activated CD4+ T cells with either CD28 or ICAM-1 costimulation, but overall cell numbers were reduced (**Fig. 5.12**) consistent with earlier observations of decreased cell numbers involving CD45RO+ and memory cells (**Fig. 5.2, 5.3, 5.6, 5.7**). Although not statistically significant ( $p = 0.055$ ), HDL seemed to reduce the percentage of activated CD4+ T cells in cells costimulated through CTLA-4 from  $27 \pm 7\%$  to  $14 \pm 5\%$ .

CD25 also functions as a Treg cell (regulatory T cell) marker. We recently observed that *in vitro* stimulation of human naïve CD4+ T cells through CD3+ICAM-1 (41) or CD3+CTLA-4 (See Chapter 2) generated a functionally inhibitory inducible Treg (Treg) cell population in the absence of exogenously added cytokines, and this was in contrast to CD28 costimulation where no Treg cells were observed. We next asked whether the increased CD4+CD25+ T cells in **Fig. 5.13**, was an effect on the Treg cell population. Cells were assessed for CD25 plus strong Foxp3 expression at day 7 (summarized in **Fig. 5.14**). Neither lipoprotein altered the percentage of CD4+CD25+Foxp3<sup>hi</sup> T (Treg) cells with CD28 or ICAM-1 costimulation. Interestingly, HDL reduced the percentage of Treg cells generated through CTLA-4 costimulation, and oxLDL exerted no consistent effect. Thus, the increase in CD4+CD25+ T cells caused by the addition of oxLDL represent activated T cells and not an inducible Treg cell population and further supports

Figure 5.12

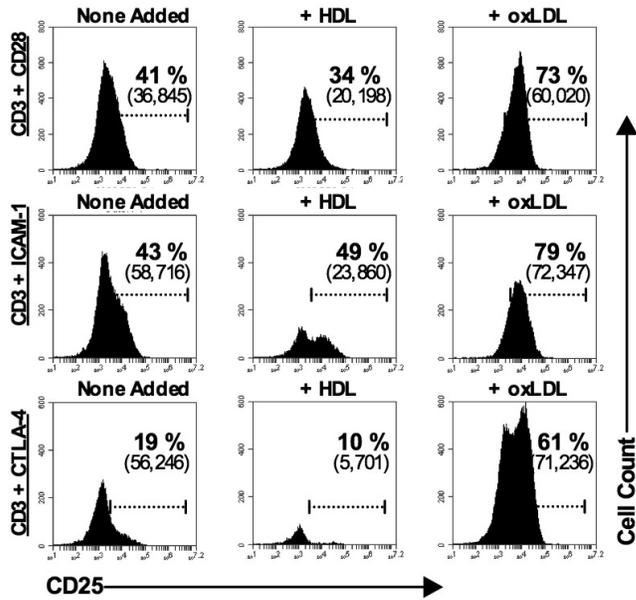


Figure 5.13

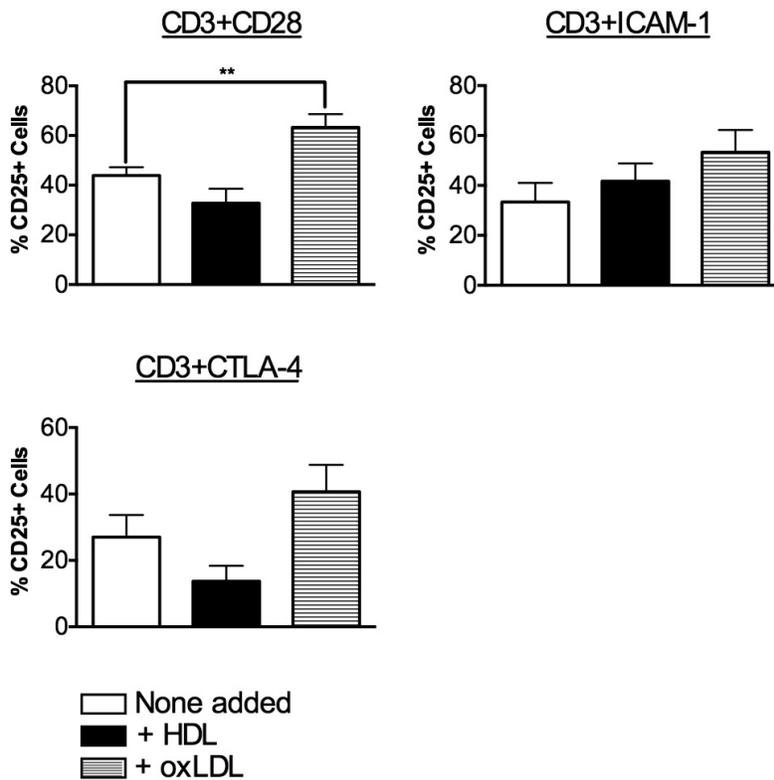
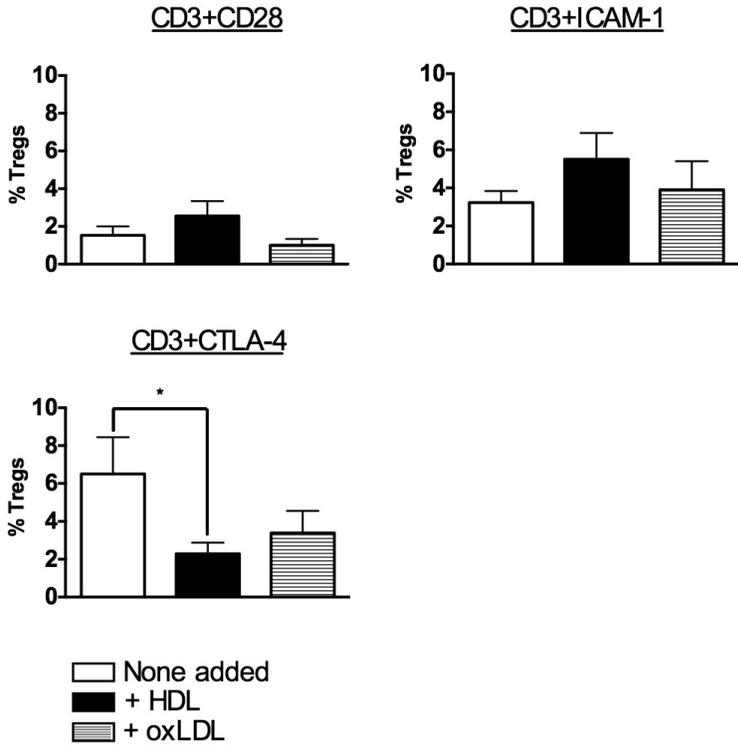


Figure 5.14



**Figures 5.12-5.14. OxLDL enhanced T cell activation without expanding the Treg population.** Human naïve CD4<sup>+</sup>T cells stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, or anti-CD3+anti-CTLA-4 were analyzed for CD25 or strong Foxp3 expression by flow cytometry. Medium (none added), oxLDL or HDL was added on day 0. **Fig. 5.12**, Representative figure, cells stimulated through CD3+CD28 (upper panels), CD3+ICAM-1 (middle panels), or CD3+CTLA-4 (lower panels). Percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells at 7d are indicated, with cell numbers in parentheses. **Fig. 5.13**, Average percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells (activated cells) ± SEM at day 7, with medium (open bars), HDL (closed bars) or oxLDL (striped bars). Representative of eight (CD3+CD28), or seven (CD3+ICAM-1 and CD3+CTLA-4) experiments. **Fig. 5.14**, Percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>hi</sup> T cells (Treg cells) ± SEM at day 7. Representative of seven experiments.

\* p < 0.05

the earlier observation that oxLDL augments differentiation to effector cells, a highly activated (CD25+) population.

*oxLDL supported T cell proliferation whereas HDL inhibited proliferation*

As demonstrated in **Fig. 5.4**, oxLDL increased the number of effector cells in our cell culture model of differentiation for all three stimuli. Increased effector cell numbers could be explained by increased differentiation of naïve T cells, by increased proliferation of newly differentiating effector cells or by decreased death rates of effector T cells in the population. As previously discussed, the percentage of overall differentiation did not change with the addition of either lipoprotein (**Fig. 5.8**). In **Fig. 5.15**, human naïve CD4+ T cells were stimulated through either CD3+CD28, CD3+ICAM-1, or CD3+CTLA-4 in the absence or presence of HDL or oxLDL and assessed for proliferation on day 7 using CFSE dilution. The percentage of T cells with at least one division did not change with addition of oxLDL to either CD28-, or ICAM-1-costimulated cells (representative data in **Fig. 5.15**, summarized in **Fig. 5.16**). In addition, the form and number of cell divisions remained essentially the same with oxLDL for each of these costimulatory regimens, as did the overall numbers of dividing cells (parenthetically in **Fig. 5.15**). However, oxLDL did enhance proliferation of cells stimulated through CD3+CTLA-4 from  $25 \pm 11\%$  to  $56\% \pm 9\%$  ( $p < 0.01$ ) as well as the number of dividing cells, suggesting a possible mechanism by which oxLDL enhances differentiation of naïve T cells stimulated through CD3+CTLA-4 to activated, effector cells.

HDL inhibited proliferation from  $75 \pm 4\%$  to  $57 \pm 10\%$  ( $p < 0.05$ ) during costimulation through CD3+CD28 and from  $48 \pm 7\%$  to  $26 \pm 8\%$  ( $p < 0.05$ ) during differentiation with CD3+ICAM-1 (**Fig. 5.16**). The form and number of divisions remained similar, but the number

Figure 5.15

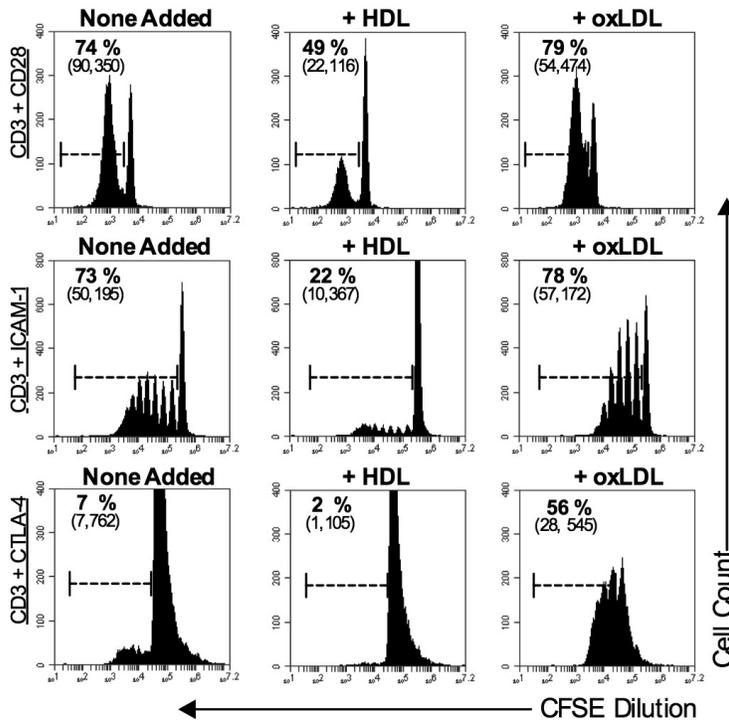
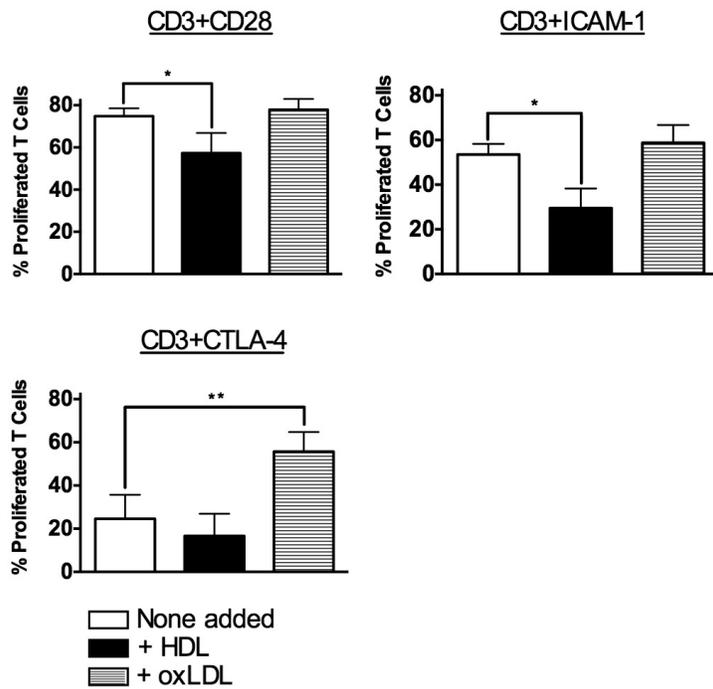


Figure 5.16



**Figures 5.15-5.16. OxLDL allowed or enhanced while HDL suppressed naïve T cell**

**proliferation.** Naïve CD4<sup>+</sup> T cells stained with 2.5 µM CFSE at day 0 were analyzed for CFSE dilution after stimulation for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, or anti-CD3+anti-CTLA-4, in medium, oxLDL or HDL. Representative of seven (CD3+CD28 and CD3+ICAM-1) or five (CD3+CTLA-4) experiments. **Fig. 5.15.** Cells costimulated through CD3+CD28 (upper panels), CD3+ICAM-1 (middle panels), or CD3+CTLA-4 (lower panels). Histogram plots indicate total percentage of divided cells with number of cells in parentheses. **Fig. 5.16.** Average percentage of total divided T cells ± SEM at day 7 with added medium (open bars) or HDL (closed bars) or oxLDL (striped bars).

\* p < 0.05, \*\* p < 0.01

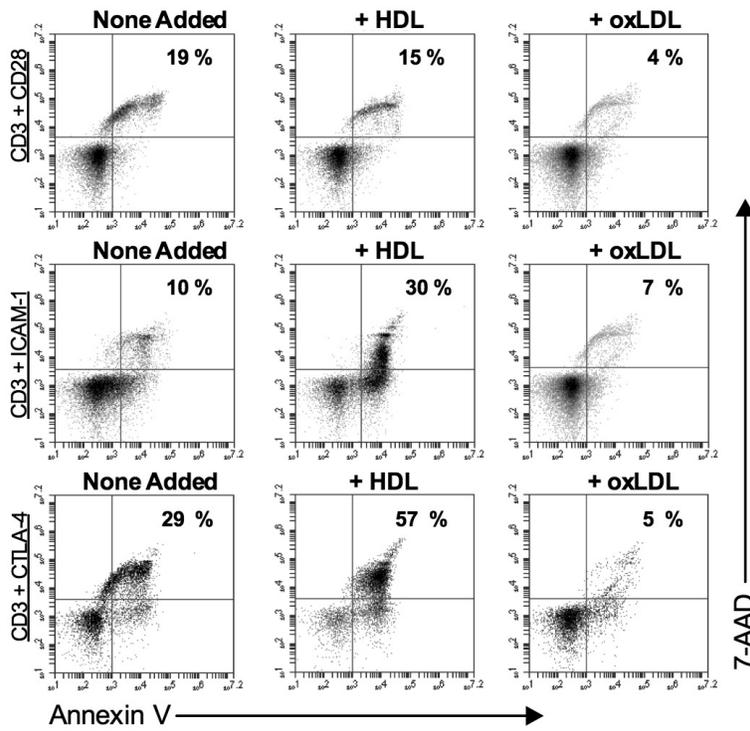
of participating cells was decreased. HDL did not inhibit the overall percentage of proliferated cells through CTLA-4 costimulation, but reduced cell numbers were observed (**Fig. 5.15**).

Hence, depending on the choice of costimulation, oxLDL either had no effect on proliferation (CD28 and ICAM-1) or enhanced proliferation (CTLA-4) of naïve CD4<sup>+</sup> T cells. HDL reduced division and resulted in fewer cells at day 7 for all costimuli.

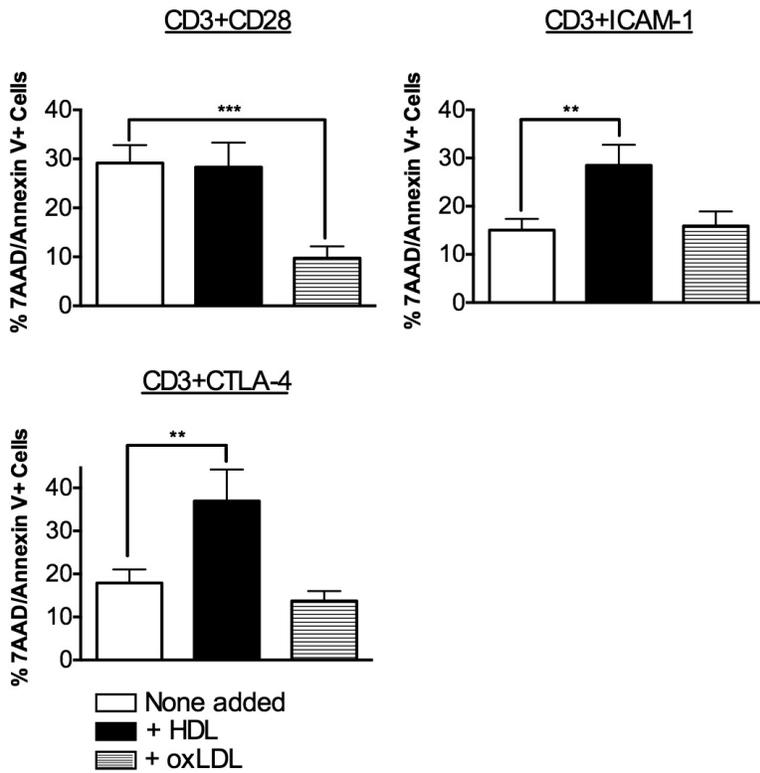
*oxLDL and HDL exerted opposing and differential effects on cell death depending on the choice of costimulatory molecule*

Thus far, oxLDL led to increased T effector cells without increasing proliferation in CD28 and ICAM-1 costimulated cells. One possible explanation was that oxLDL decreased the rate of cell death. Similarly, HDL reduced proliferation and the number of proliferating cells undergoing differentiation. A possible additional explanation is that HDL increased cell death rates, and this contributed to decreased T cells in the differentiating populations. We analyzed effects of the lipoproteins on the moderate level of cell death that is common in this model of cell differentiation. Costimulated cells were stained for Annexin V and 7-AAD and different effects of the lipoproteins were observed based on the costimulatory molecule used. In cells costimulated through CD3+CD28, oxLDL inhibited the percentage of Annexin V<sup>+</sup>,7-AAD<sup>+</sup> dying cells at day 7 from  $29 \pm 4\%$  to  $10 \pm 2\%$  ( $p < 0.0001$ ). Representative data are in **Fig. 5.17** and data are summarized in **Fig. 5.18**. Inhibition of cell death would help explain an increase in viable cells. The baseline level of death was lower in cells costimulated through CD3+ICAM-1 than CD3+CD28, and oxLDL (striped bars) did not inhibit cell death as it did with CD28

**Figure 5.17**



**Figure 5.18**



**Figures 5.17-5.18. OxLDL decreased cell death whereas HDL enhanced cell death.** Naïve CD4<sup>+</sup> T cells stimulated for 7 days with anti-CD3+anti-CD28, anti-CD3+anti-ICAM-1, or anti-CD3+anti-CTLA-4, in medium, or with added oxLDL, or HDL, were analyzed for expression of Annexin V and 7AAD by flow cytometry. **Fig. 5.17**, Cells stimulated through CD3+CD28 (upper panels), CD3+ICAM-1 (middle panels), or CD3+CTLA-4 (lower panels). Upper right quadrant identifies the percentage of Annexin V<sup>+</sup>, 7AAD<sup>+</sup> cells (percentage of cell death). **Fig. 5.18**, Average percentages of Annexin V<sup>+</sup>, 7AAD<sup>+</sup> T cells ± SEM (day 7) with added medium (open bars), HDL (closed bars) or oxLDL (striped bars). Representative of ten (CD3+CD28 and CD3+ICAM-1) or eight (CD3+CTLA-4) separate experiments.

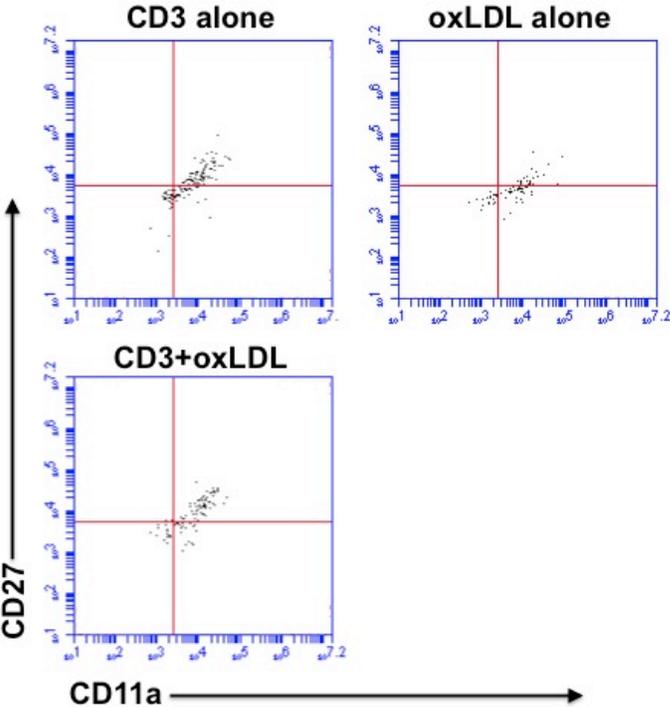
\*\* p < 0.01, \*\*\* p < 0.001.

costimulation nor did it in cells undergoing CTLA-4 costimulation (**Fig. 5.18**). Due to the robust effects observed with oxLDL, we tested whether oxLDL could induce activation and differentiation of naïve T cells in the absence of TCR or costimulatory protein signaling (**Fig. 5.19**). OxLDL did not induce proliferation or differentiation to effector, memory or regulatory T cells. As shown in **Fig. 5.19**, stimulating through the TCR in the presence of oxLDL did not generate effector or memory cells at day 7 or day 14. This lack of effect was also observed when proliferation and differentiation to regulatory T cells was assessed at day 7 (not shown). HDL (closed bars) exerted no effect on cell death in cells costimulated through CD3+CD28 (**Fig. 5.18**). However, HDL enhanced the percentage of Annexin V<sup>+</sup>,7-AAD<sup>+</sup> cells from  $15 \pm 2\%$  to  $28 \pm 4\%$  ( $p < 0.01$ ) in cells costimulated through CD3+ICAM-1 and from  $17 \pm 3\%$  to  $37 \pm 7\%$  ( $p < 0.01$ ) in cells costimulated through CD3+CTLA-4. Overall, these data suggest that oxLDL can enhance CD4<sup>+</sup> T cell survival whereas HDL reduces CD4<sup>+</sup> T cell survival under certain circumstances. OxLDL-induced survival of T effector cells is consonant with the hypothesis that increased activity of effector T cells promotes atherosclerosis. Increased death of T effector cells in the presence of HDL agrees with the hypothesis that HDL inhibits atherosclerosis in part by decreasing the function of attacking T cells.

*oxLDL and HDL induced migration of naïve T cells more strongly than native LDL*

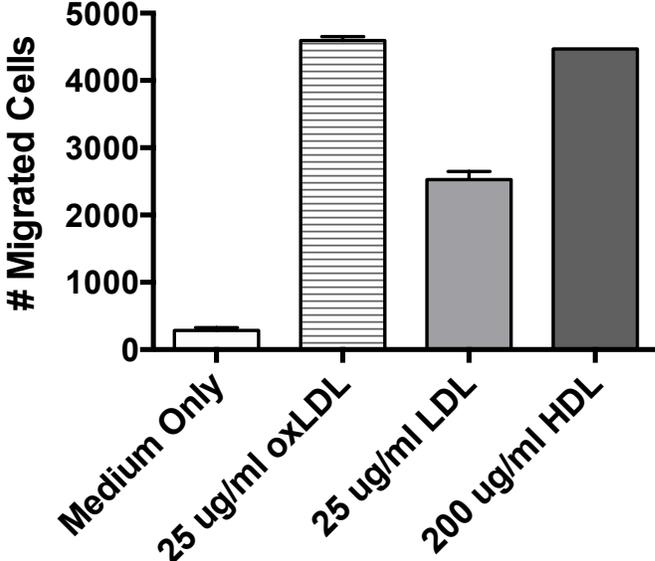
To determine whether freshly isolated human naïve CD4<sup>+</sup> T cells could migrate in response to the presence of the lipoproteins examined in the differentiation studies, we assessed the number of naïve T cells that migrated within 5 hours in response to medium only and medium supplemented with oxLDL (25 µg/ml), LDL (25 µg/ml) or HDL (200 µg/ml) (**Fig. 5.20**). Although all lipoproteins induced migration of naïve T cells, oxLDL and HDL caused

Figure 5.19



**Figures 5.19. OxLDL did not induce differentiation of naïve T cells in the absence of a second signal through a costimulatory molecule.** Human naïve CD4<sup>+</sup> T cells were stimulated for 7 days using anti-CD3 in the presence or absence of oxLDL (25 µg/ml) or alternatively, were exposed to oxLDL in the absence of any stimulation through the TCR. Cells were analyzed for expression of CD45RO, CD27, and CD11a by flow cytometry. Representative of three separate experiments.

Figure 5.20



**Figure 5.20. OxLDL and HDL induced migration of naïve T cells more strongly than native LDL.** Freshly isolated human naïve CD4<sup>+</sup> T cells were added to inserts of 24 transwell plates at  $5 \times 10^6$  cells/ml in serum-free RPMI 1640 exposed to medium only, oxLDL (25 µg/ml), LDL (25 µg/ml) or HDL (200 µg/ml) and allowed to migrate at 37°C for 5 hr. Representative of one experiment performed in duplicate.

nearly a 2-fold increase in migrated cells beyond that induced by native LDL (**Fig. 5.20**). This suggests that both HDL and oxLDL can attract naïve CD4<sup>+</sup> T cells and may compete for regulation of T cell processes in atherosclerosis.

## Discussion

Elevated LDL and lower HDL levels increase the risk of cardiovascular disease. As LDL accumulates within the arterial wall, it is oxidized to oxLDL, recruiting monocytes and T cells, generating pro-inflammatory foam cells, and activating platelets, endothelial cells, and macrophages (3-6, 9, 10) exacerbating lesion development. HDL opposes atherogenesis, inhibiting plaque development and progression (13-17). Macrophages in atherosclerotic lesions have been well studied, but although T cells have been established as important for atherogenesis, mechanisms of T cell function in atherogenesis are still being defined. Mature T cells participate in perpetuation and development of atherosclerotic plaque and can specifically recognize by TCR plaque related antigens including oxLDL. Participating T cells are predominantly Th1 cells that produce IFN- $\gamma$  and promote inflammation. Whether naïve T cells might participate early in the process is not as clear. For example, it has not been addressed whether a microenvironment rich in LDL can influence the ability of naïve T cells to differentiate in favor of atherogenic phenotypes such as Th1 or Th17. Here we used a model system for differentiation of human naïve CD4<sup>+</sup> T cells to demonstrate for the first time that oxidized LDL can influence naïve cell differentiation to Th1. The data suggest a novel mechanism in which oxLDL can perpetuate atherogenesis by tuning the differentiation process to favor Th1 cells. In analogous experiments, HDL opposes the activity of oxLDL on human naïve T cell differentiation.

### *Effects of oxLDL*

Here, oxLDL promoted naïve CD4<sup>+</sup> T cell differentiation to effector cells, augmented secretion of Th1 derived IFN- $\gamma$  and inhibited appearance of memory T cells. OxLDL inhibited

Th2 function, reducing secretion of IL-4 by cells differentiating in response to CD28 costimulation. Th2 differentiation is not usually observed with ICAM-1 costimulation in this system and the lipoproteins did not alter this result. The role of CTLA-4 as a costimulatory molecule in guiding human naïve T cell differentiation is still being defined (See Chapter 2), and further studies are needed to determine how costimulation through CTLA-4 influences T helper function. Th17 function was modestly enhanced by oxLDL in cells costimulated through ICAM-1. Thus, in general, oxLDL promoted Th1 but not Th2 during differentiation, and suppressed appearance of memory T cells. A decrease in memory T cells plus increased Th1 and Th17 function support the hypothesis that oxLDL promotes generation of pathogenic Th1 cells capable of exacerbating plaque progression.

Effector T cells have a shorter lifespan and higher rates of apoptosis than resting memory T cells. After clonal expansion, 90% or more of the effector population undergoes apoptosis resulting in effector T cells only living a few days as opposed to the decades that memory T cells survive *in vivo* (62, 63). Th1 effector cells exhibit a higher rate of apoptosis and begin undergo AICD (activation-induced cell death) 3-5 days earlier than other subsets such as Th2 cells (64, 65). The increase in T cell activation and differentiation of naïve T cells to Th1 cells by oxLDL was not accompanied with higher rates of cell death as may be expected with expansion of Th1 effector cells. In fact, in cells costimulated through CD28, oxLDL actually improved cell viability. This protection by oxLDL from cell death is evident even at 14 days of *in vitro* stimulation when the effector population is expected to contract as we have previously observed (41, See Chapter 2). In the microenvironment of atherosclerotic plaque, naïve T cell differentiation to Th1 cells that are resistant to apoptosis and thus, may persist much longer than a few days, may exacerbate and prolong inflammatory processes. The present data suggest a

novel mechanism by which oxLDL may increase the number of pathogenic T cells within plaque by favoring differentiation of naïve T cells into Th1 cells and by enhancing activation and reducing the rate of death as modeled in **Fig. 5.21**. Consistent with its other roles in atherogenesis, this would provide an additional avenue by which oxLDL may promote atherosclerosis.

### *Effects of HDL*

HDL exerted little effect on activation or differentiation of human naïve CD4<sup>+</sup> T cells in the various costimulatory systems. HDL did decrease proliferation and survival of potentially pathogenic cells. Memory T cells generated in the presence of HDL differed according to costimulatory regimen where increase was observed with costimulation through CD3+CD28 and CD3+CTLA-4 at day 14 but not through CD3+ICAM-1. However, overall there were fewer CD45RO<sup>+</sup>, CD25<sup>+</sup> cells, memory and regulatory T cells, indicating an overall inhibition of proliferation or viability rather than a favored induction of memory differentiation by HDL. We have often observed a similar effect when naïve T cells are stimulated through only CD3 (TCR), which does not induce differentiation or proliferation. The decrease in cell viability and proliferation by HDL was limited to naïve CD4<sup>+</sup> T cells and was not detected in our previous work using mature peripheral blood T cells (39). Although HDL decreased proliferation with all costimulatory regimens, it selectively decreased survival in both ICAM-1 and CTLA-4 costimulated cells but not CD28-costimulated cells. As mentioned previously, ICAM-1 or CTLA-4 costimulation induce differentiation to Treg cells which can suppress T cell responses such as inducing apoptosis. The presence of regulatory T cells in these populations may have influenced the ability of HDL to induce higher rates of cell death, and in fact, there was a slight

increase in Treg cells generated by stimulation through CD3+ICAM-1. However, HDL reduced the percentage of Treg cells induced by CTLA-4 warranting a different explanation. As discussed in Chapter 2, costimulation through CTLA-4 induces slightly delayed and weaker T cell activation and proliferation in comparison to CD28 or ICAM-1, and therefore, may exhibit increased sensitivity to inhibition by HDL. Overall, HDL inhibited proliferation and differentiation of naïve T cells suggesting a novel mechanism in which in the context of the microenvironment of plaque, HDL may inhibit or slow the progression of atherosclerosis as modeled in **Fig. 5.22**. The effects on naïve T cells by HDL and oxLDL in the present study seem to oppose each other and are consistent with the accepted and more general roles of these lipoproteins in atherosclerosis. Interestingly, both lipoproteins induced migration of naïve T cells much higher than levels produced in response to native LDL. This suggests a possible competition between HDL and oxLDL attracting T cells and subsequently, regulating them to oppose or favor atherogenesis.

*Differential costimulation implies influence of microenvironment.*

In our *in vitro* human T cell system, costimulation through CD3+CD28, CD3+ICAM-1, and CD3+CTLA-4 induce memory and effector differentiation (40, 43). Costimulation through either CD28 or ICAM-1 induces Th1 function (40). ICAM-1 costimulation and CTLA-4 costimulation generate functional regulatory T cell populations in the absence of exogenous cytokines (41, 43), and only CD28 induces Th2 responses (40, 42). Thus, although all costimulatory regimens are capable of inducing naïve T cell differentiation to mature, functional T cell populations, they differ in their influence on differentiation outcome. These inherent distinctions may account for the variations among CD28-, ICAM-1-, and CTLA-4-costimulated

cells when the lipoproteins were added and suggest that we may be modeling differential influence by various cellular microenvironments.

### *Summary*

We suggest an additional mechanism by which HDL and oxLDL modulate plaque development (Proposed models in **Fig. 5.21** and **Fig. 5.22**). This is by influencing human CD4+ naïve T cell activation, proliferation, differentiation, or survival, depending on the nature of the microenvironment and the lipoprotein involved. Consistent with its broader, more recognized anti-atherogenic roles, HDL may reduce disease severity by inhibiting T cell proliferation during differentiation, and inducing death of disease-exacerbating T cells that appear with differentiation. In contrast, oxLDL may exacerbate the chronic inflammatory microenvironment of plaque by guiding the differentiation process to favor an effector Th1 population that provides IFN- $\gamma$  production and experiences increased survival or proliferation. Thus, the present data suggest that the two opposing lipoproteins could function in opposition to regulate naïve T cell differentiation and survival and influence the extent of T cell participation in atherosclerosis. This would provide an additional and novel mechanism by which atherosclerotic plaque can be regulated and should provide new avenues for investigation of atherosclerotic plaque formation and possible therapeutic approaches.

### **Notes**

Some data presented in this chapter are included in a paper accepted for publication:

A.H. Newton and S.H. Benedict. Low density lipoprotein promotes human naïve T cell differentiation to Th1 cells. *Human Immunology*, 2014.

Fig. 5.21

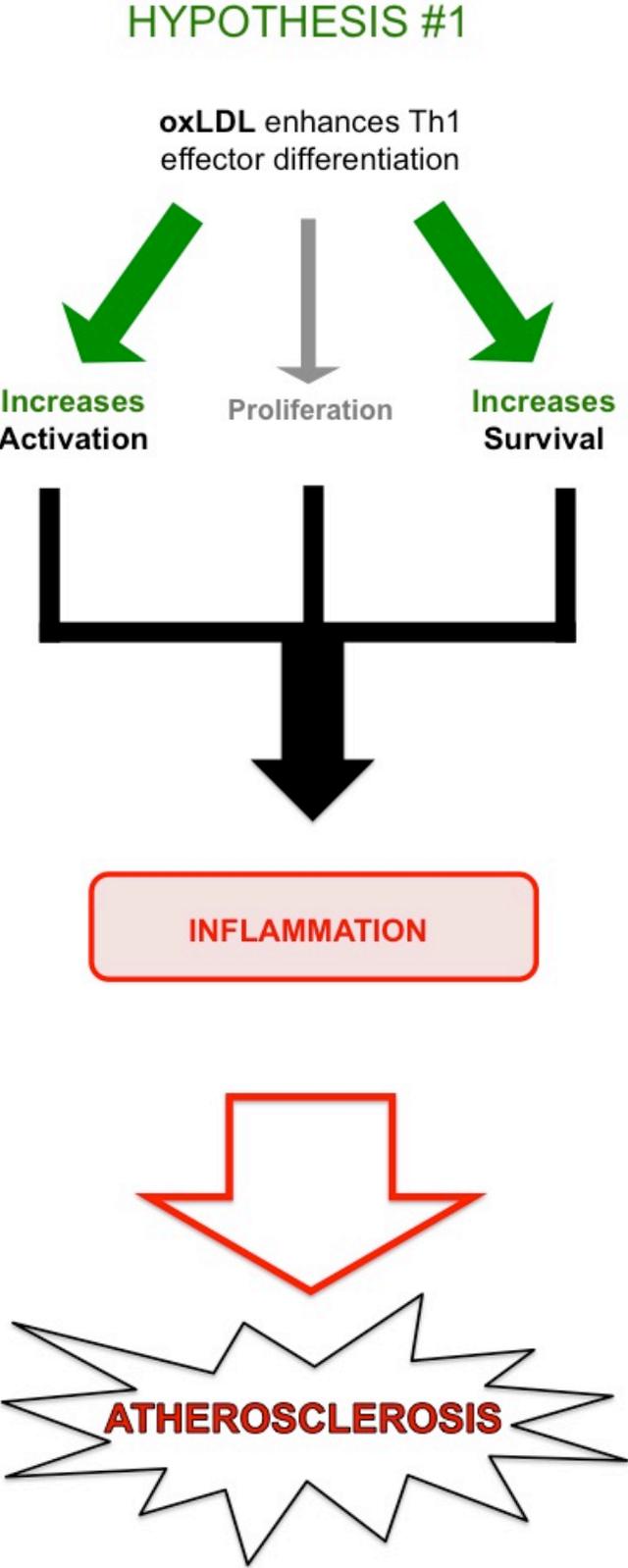
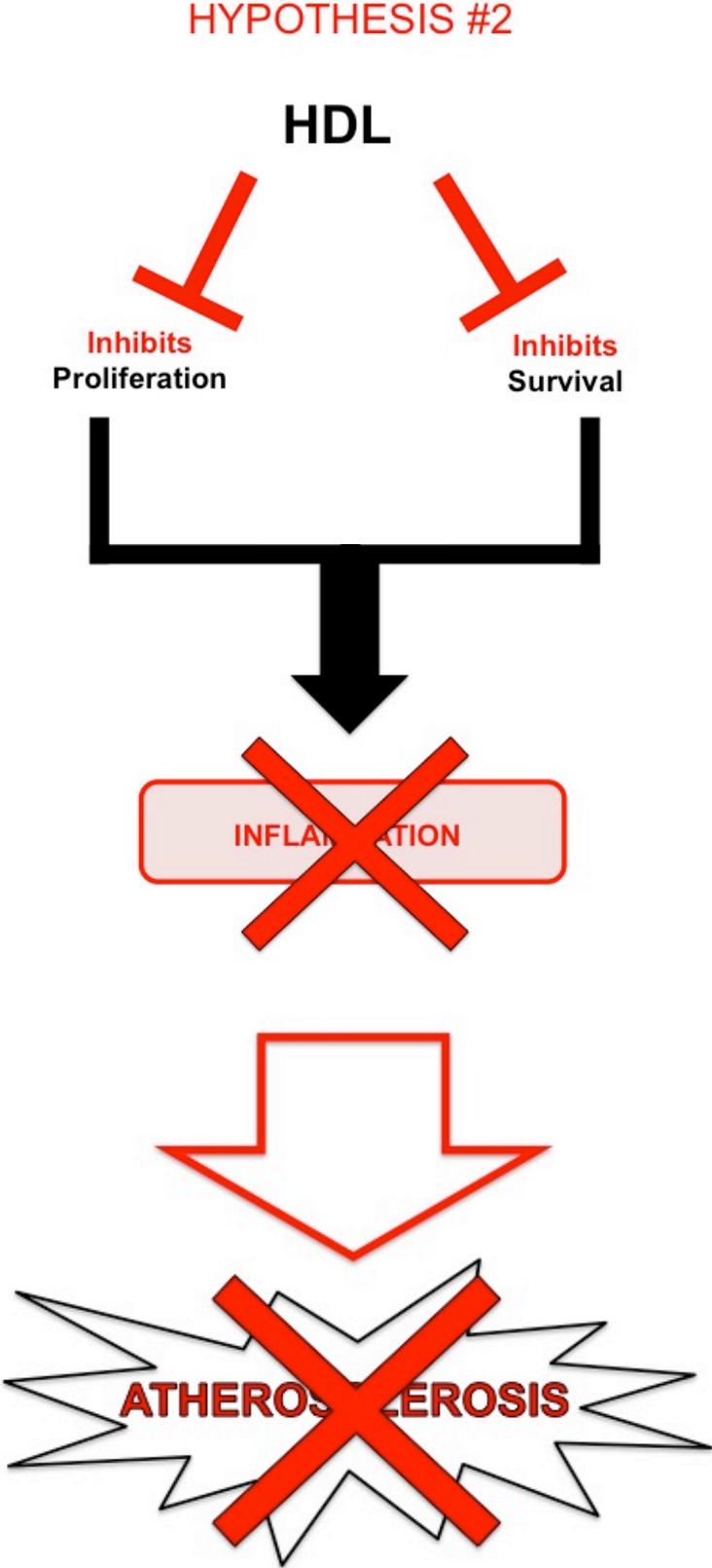


Fig. 5.22



**Fig. 5.21-5.22. Model of proposed hypotheses for lipoprotein regulation of naïve T cells and implications in the pathogenesis of atherosclerosis. Fig. 5.21,** modulation of activation, proliferation, survival, and differentiation of human naïve CD4+ T cells by oxLDL. **Fig. 5.22,** modulation of proliferation and survival of human naïve CD4+ T cells by HDL.

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## **Chapter 6**

**Blocking ICAM-1:LFA-1 interactions attenuates severity of disease in a mouse model of emphysema that is driven by T cells**

## Introduction

Chronic obstructive pulmonary disease (COPD), a leading cause of morbidity and death worldwide. Two major forms of COPD occur: chronic bronchitis and emphysema. We address the emphysematous form here, which is characterized by a progressive decline in respiration as a result of destruction to the alveoli and narrowing of the airways. Inflammatory processes mediated primarily by neutrophils and macrophages contribute significantly to this destruction. Recent evidence also supports an autoimmune component driven by lymphocytes (1, 2), but whether COPD can be considered an autoimmune disease remains to be determined. Here we tested the ability of T cells to transfer disease in an elastase-induced model of emphysema (Part I) and the therapeutic treatment of elastase-induced emphysema using peptides to block ICAM-1:LFA-1 interactions (Part II).

While the innate immune response is recognized as an integral part of disease development in COPD, elucidating the role of the adaptive immune response has been more challenging. Macrophages and neutrophils propagate inflammation and damage in the lungs by releasing various inflammatory mediators such as proteases, cytokines, chemokines, and reactive oxygen species (reviewed in 3). The degree to which adaptive immune cells such as T lymphocytes contribute to pathogenesis has been more controversial as emphysema can be induced in T cell deficient mice (4). However, elevated numbers of CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes in the lungs and periphery of COPD patients correlate to disease severity (5-8), and recent studies indicate that T cells transfer disease in both immunocompetent and immunodeficient mice (1, 2). Furthermore, an autoimmune model of emphysema in rats mediated by antigen-specific CD4<sup>+</sup> T lymphocytes has been developed, and oligoclonal T cells that respond to elastin fragments have been isolated from patients with emphysema (9, 10).

These studies suggest that T cells may contribute to pathogenesis of COPD by directing an antigen-specific immune response reminiscent of an autoimmune disease.

Animal models are essential tools in elucidating these complex mechanisms of disease development and progression and for testing potential therapies. The elastase- and cigarette-smoke-induced models are the most commonly used animal models of emphysema, each with its advantages and limitations (reviewed in 11, 12). In the elastase model, elastase (commonly porcine pancreatic elastase or PPE) is directly introduced to the lungs to initiate pathogenesis resulting in robust inflammation and alveolar destruction within a few weeks. In the cigarette-smoke model, exposure to cigarette smoke for several months causes mild to moderate destruction of the lungs. T cells from cigarette-smoke induced models of emphysema are capable of transferring disease (1, 2). However, the ability of T cells from the elastase-induced model to transfer disease to naïve animals has not been investigated.

Thus, in Part I of this chapter, we tested the hypothesis that T cells could transfer disease from mice with PPE-induced emphysema to immunodeficient mice. SCID or NOD SCID mice received CD3<sup>+</sup> T cells from wild-type mice with elastase-induced emphysema. T cells initiated leukocyte infiltration in the lungs of both immunodeficient strains but the degree of leukocyte infiltration and alveolar destruction in the NOD SCID mice was more severe. These data suggest that T cells can transfer disease using the PPE-model of emphysema, supporting to the utility of this model in studies of autoimmunity in emphysema.

Because our evidence from Part I suggested autoimmune involvement in this model of emphysema, we tested whether a therapeutic intervention previously found to be effective in treating autoimmunity (13) would attenuate disease severity here. In this therapy, the combined use of two cyclic peptides block the interactions between LFA-1:ICAM-1 and have been shown

to inhibit homotypic adhesion and function of T cells in mixed lymphocyte reactions without affecting cell viability by blocking the second signal generated through costimulatory proteins on the T cells (14). In our study of autoimmune diabetes using the NOD mouse, these peptides delayed onset of diabetes, reduced leukocyte infiltration, and suppressed antigen-specific responses of autoreactive T cells (13). Because adoptively transferred T cells from mice with elastase-induced emphysema caused leukocyte infiltration and alveolar destruction (Part I), we hypothesized that these peptides would suppress disease in the elastase model of emphysema by inhibiting autoreactive T cells. We demonstrate that peptide therapy attenuates severity of emphysema if begun by day 7 of the 21-day model but only partially inhibits infiltration, suggesting other possible mechanisms of action by the peptides (Part II).

## Materials and Methods

### Part I

#### *Antibodies and reagents*

Antibodies used for flow cytometry were: anti-CD3 $\epsilon$ -FITC (145-2C11, eBioscience), anti-CD4-PE (RM4-5, BD Biosciences), and anti-CD8 $\alpha$ -PE-Cy5 (53.6.7, BD Biosciences). Flow cytometry was performed on an Accuri C6 (BD Accuri Cytometers, Ann Arbor, MI) and data analysis using CFlow (Accuri). Porcine pancreatic elastase was purchased from Elastin Products Co. (EC134GI, Owensville, MO).

#### *Mice*

Female BALB/cByJ mice 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME) received either saline (saline control; n = 9) or elastase (elastase/donor mice; n = 8) intratracheal instillations. Female CBySmn.CB17-*Prkdc*<sup>scid</sup>/J (SCID; n = 8) or NOD.CB17-*Prkdc*<sup>scid</sup>/J (NOD SCID; n = 10) mice 8 weeks of age (The Jackson Laboratory) were recipients in the adoptive transfer experiments. In both immunodeficient strains of mice, a genetic mutation in the *prkdc* gene that encodes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) causes severe combined immunodeficiency syndrome (*scid*) and consequently, a lack of functional T and B lymphocytes (15, 16). The SCID mice are on the BALB/cByJ background and the NOD (non-obese diabetic) SCID mice on the NOD/ShiLtSz background. The University of Kansas Institutional Animal Review Board approved all animal experiments.

### *Intratracheal administration of PPE or saline (elastase or saline controls only)*

Mice were anesthetized with open-drop exposure to 20% isoflurane as previously described (17). Intratracheal instillations of either 25  $\mu$ l sterile saline or 25  $\mu$ l elastase solution (1 mg/ml) were introduced to the lungs of female BALB/cByJ as previously described (18, 19). 21 days later, mice were euthanized, lungs harvested for histological analysis, and spleens harvested for T cell isolation.

### *T cell isolation and adoptive transfer*

Spleens from elastase control or saline control mice were harvested, minced and pressed through a sterile 70  $\mu$ m nylon mesh cell strainer. Splenocytes were incubated at room temperature for 5 minutes in ACK lysis buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , 0.1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.2) to remove erythrocytes.  $\text{CD}3^+$  T cells were negatively selected using the Mouse T Cell Enrichment Kit from STEMCELL Technologies (Vancouver, BC) and were routinely  $\geq 97\%$  pure. T cells from donor mice with elastase-induced emphysema were injected by tail vein ( $5 \times 10^6$  T cells/100  $\mu$ l saline) into either SCID or NOD SCID mice with 2-3 recipients per donor. SCID and NOD SCID mice were euthanized and lungs harvested for histological analysis 21 days after adoptive transfer. See **Fig. 6.1** for a model of experimental design.

### *Tissue processing*

After exsanguination, the heart was perfused with saline, and lungs were inflated by instilling 10% neutral-buffered formalin at constant pressure. Inflated lungs were fixed for 24 hr in 10% formalin at room temperature before embedding in paraffin.

### *Morphometry and imaging*

Mid-sagittal sections (6  $\mu\text{m}$ ) were stained with hematoxylin and eosin and photographed at 20X using a Zeiss Axioskop photomicroscope (Carl Zeiss Microimaging, LLC, Thornwood, NY). The mean linear intercept (Lm) was determined by light microscopy as previously described (20) by taking 6 fields of view for each mouse, avoiding fields containing blood vessels or conducting airways. Mean linear intercept measurements were determined by single-blinded investigators (D. Danahy and A. Newton). Degree of infiltration was determined by counting the number of leukocytes in three separate, random fields of view for each mouse using a BH-2 Olympus microscope (Olympus America Inc., Center Valley, PA) by single-blinded investigators (M. Chan and A. Newton).

### *Statistical analysis*

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Statistical tests and significance for individual figures are indicated in figure legends. Data are expressed as the mean value  $\pm$  SEM.

## **Part II**

### *Antibodies and reagents*

Antibodies used for flow cytometry were: anti-CD3 $\epsilon$ -FITC (145-2C11, eBioscience), anti-CD4-PE (RM4-5, BD Biosciences), anti-CD8 $\alpha$ -PE-Cy5 (53.6.7, BD Biosciences), and anti-IFN- $\gamma$ -PE (BD Biosciences). For intracellular cytokine staining, transcription factor buffers and monensin from eBioscience were used. Flow cytometry was performed on an Accuri C6 (BD Accuri Cytometers, Ann Arbor, MI) and data analysis using CFlow (Accuri). Porcine pancreatic

elastase and murine elastin peptides were purchased from Elastin Products Co. (Owensville, MO). For antigen recall assays, cells were cultured immediately at 37°C in complete RPMI 1640 (Mediatech, Herndon, VA) plus 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (Invitrogen), 50 units/ml penicillin, and 50 µg/ml streptomycin (P/S, Invitrogen). Stimulating antibodies, anti-CD3 (500A2) and anti-CD28 (37.51) were purchased from BD Biosciences.

### *Mice*

Female BALB/cByJ mice 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME) received either saline (saline control; n = 9) or elastase (elastase and peptide groups) tracheal instillations. Mice were monitored weekly for changes in weight. The University of Kansas Institutional Animal Review Board approved all animal experiments.

### *Intratracheal administration of PPE or saline*

Mice were anesthetized with open-drop exposure to 20% isoflurane as previously described (17). Intratracheal instillations of either 25 µl sterile saline (saline control) or 25 µl elastase solution (1 mg/ml) (all other treatment groups) were introduced to the lungs of female BALB/cByJ as previously described (18, 19). 21 days later, mice were euthanized, lungs harvested for histological analysis, and spleens harvested for T cell isolation.

### *Peptides*

Cyclic peptides (cLAB-L and cIE-L) were designed as we have previously described (14), and purchased from American Peptide Company, Inc. (Sunnyvale, CA). Lyophilized

peptides were stored at -20°C in a desiccator. Each peptide was resuspended in sterile saline at 1 mg/ml and stored separately as aliquots at -70°C until used. For intravenous injections, equal volumes of cLAB-L and cIE-L were mixed thoroughly immediately before use, and mice received 100 µl solution containing 50 µg of each peptide by tail vein. Amino acid sequences are DQPKLLGIET for cIE-L and ITDGEATDSG for cLAB-L.

#### *Intravenous injections of saline or peptides*

Each mouse received 100 µl of either saline (saline or elastase control groups) or peptides (all peptide treatment groups) by tail vein for 5 consecutive days. Peptide therapy began at different timepoints in the disease model with the day of intratracheal instillations marking day 0 of this 21-day model (See **Fig. 6.11** for model of experimental design).

#### *Tissue processing*

Procedures were performed as described in Part I of this chapter.

#### *Morphometry and imaging*

Procedures were performed as described in Part I of this chapter.

#### *Antigen recall by proliferation or IFN- $\gamma$ production*

Spleens from elastase control or saline control mice were harvested, minced and pressed through a sterile 70 µm nylon mesh cell strainer. Splenocytes were incubated at room temperature for 5 minutes in ACK lysis buffer (0.15 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA, pH 7.2) to remove erythrocytes. Remaining leukocytes (1.5x10<sup>6</sup> cells/ml) were

incubated in the presence or absence of murine elastin peptide (Elastin Products Co.), human elastin peptide (Elastin Products Co.), or homogenized lung tissue from 11 week old syngeneic mice (age matched for saline and elastase control mice at time of euthanasia). Splenocytes were stimulated through anti-CD3+anti-CD28 (clone 500A2 at 0.5  $\mu\text{g}/\text{ml}$  and clone 37.51 at 2.5  $\mu\text{g}/\text{ml}$ ) as a positive control for proliferation and IFN- $\gamma$  production. For assessing antigen recall response by proliferation, splenocytes from elastase or saline control mice were stained with 2.5  $\mu\text{M}$  CFSE (from 5 mM CFSE stock in DMSO) in serum-free RPMI 1640 for 7 minutes at 37°C at the start of *in vitro* culture as described earlier (Chapter 2) and assessed for CFSE dilution at 3 and 5 days. IFN- $\gamma$  production was determined by intracellular flow cytometry at days 3 and 5 by adding monensin at 1:1000 and stimulating cells with PDB and ionomycin for 5 hours prior to intracellular staining.

### *Statistical analysis*

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Statistical tests and significance for individual figures are indicated in figure legends. Data are expressed as the mean value  $\pm$  SEM.

## Results

### Part I

*Emphysema induced in donor mice for either the NOD SCID or SCID mice was indistinguishable*

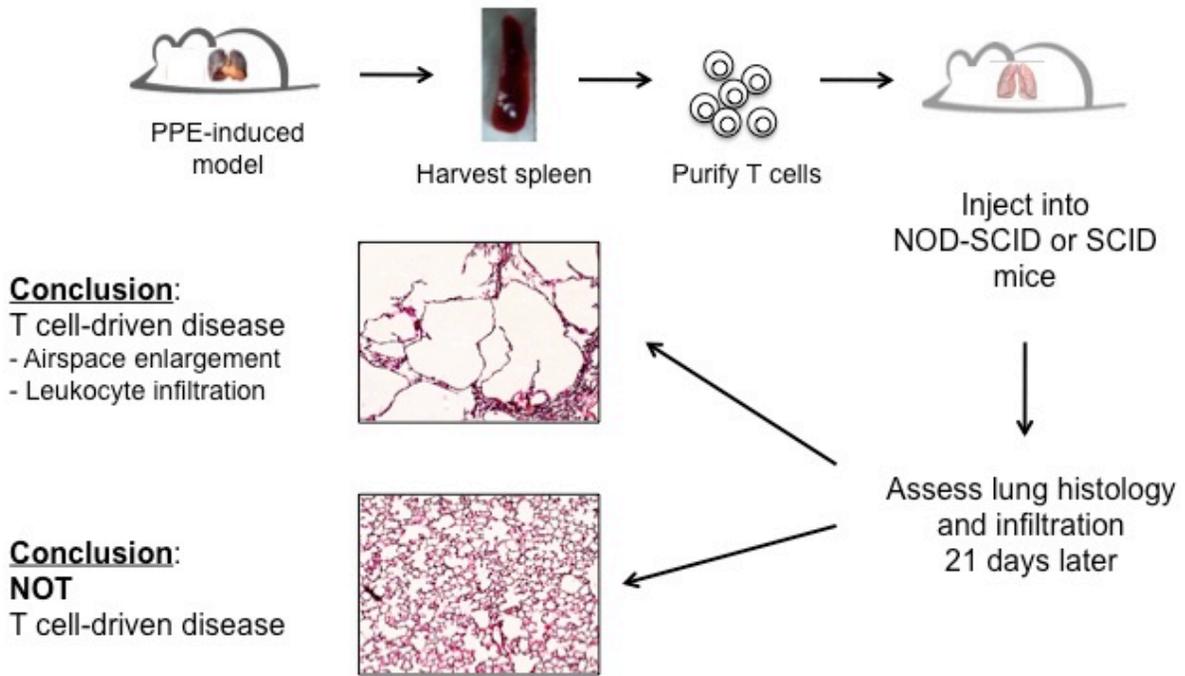
Although studies have implicated autoimmunity in the cigarette smoke-induced model of emphysema (1, 2), autoimmunity in the elastase-induced model has not been investigated despite its widespread use. To determine whether an autoimmune response may be involved in the elastase-induced model, T cells from the spleens of mice with elastase-induced emphysema were isolated and injected intravenously into either NOD SCID or SCID mice. Lungs of recipient mice were harvested and assessed for indications of emphysema 21 days later (See **Fig. 6.1** for model of experimental design). To verify that emphysema was induced in donor mice used for the adoptive transfers, the degree of airspace enlargement and infiltration were compared between elastase and saline control mice (representative images in **Fig. 6.2**). Lungs from elastase control mice exhibited marked enlargement and infiltration of airspaces (Lm:  $69 \pm 15 \mu\text{m}$ ) in comparison to saline control mice (Lm:  $23 \pm 1 \mu\text{m}$ ) (**Fig. 6.2-6.3**). The degree of alveolar destruction did not differ between the two groups of donor mice averaging  $67 \pm 15 \mu\text{m}$  and  $73 \pm 37 \mu\text{m}$  for the donor mice used for the adoptive transfer into NOD SCID and SCID recipients, respectively.

*Peripheral CD8<sup>+</sup> T cells were elevated in mice with elastase-induced emphysema*

CD8<sup>+</sup> T cells represent the major T cell population in the lungs of patients with COPD, and their numbers within the lung correlate with disease severity (5-8). Pulmonary and peripheral CD4:CD8 ratios in patients with emphysema frequently is reduced (7, 21, 22)

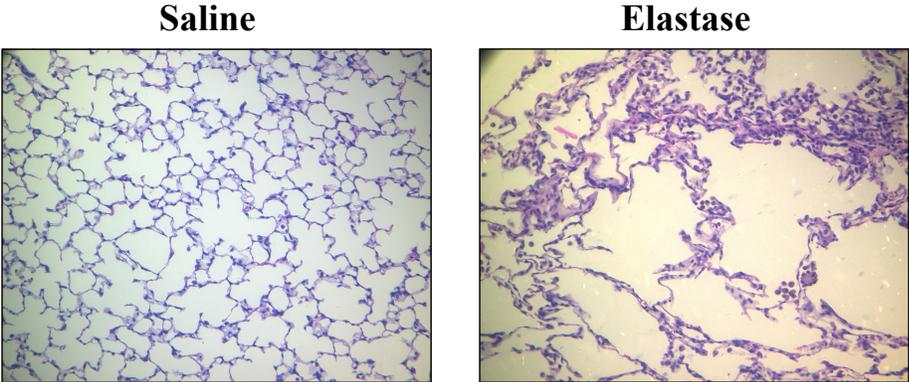
**Fig. 6.1**

Can purified T cells from sick mice transfer emphysema to immunodeficient mice?

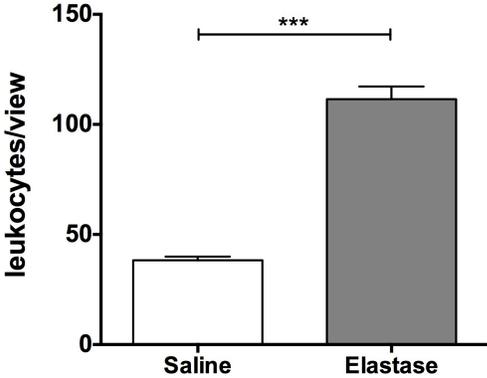


**Figure 6.1, Model of adoptive transfer experimental design.** CD3<sup>+</sup> T cells ( $5 \times 10^6$  cells/100  $\mu$ l saline) isolated from the spleens of donor mice (BALB/cByJ) with elastase-induced emphysema (21 days after intratracheal instillation of elastase) were injected intravenously into either NOD SCID or SCID mice. 21 days following adoptive transfer, recipient mice were euthanized and lungs harvested for histological analysis of alveolar destruction (by mean linear intercept) and leukocyte infiltration. Evidence of airspace enlargement and infiltration will indicate that the elastase-induced model of emphysema can be driven by T cells.

**Fig. 6.2**



**Fig. 6.3**



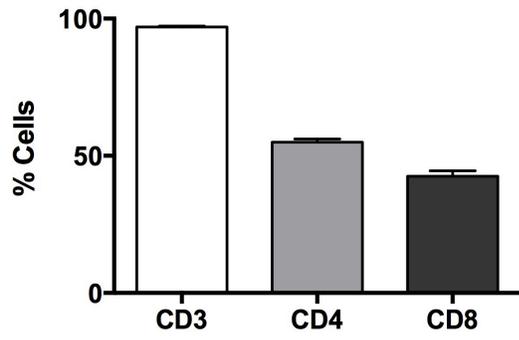
**Figures 6.2-6.3. Elastase induced emphysema in donor mice.** Lungs were harvested from female BALB/cByJ mice 21 days after either elastase (donor mice) or saline (saline control) intratracheal instillations. Airspace enlargement was measured by mean linear intercept (Lm). Infiltration scores were determined as described in Materials and Methods for all groups of mice. Representative of 2 separate experiments. **Fig. 6.2,** Micrographs of lungs showing the degree of airspace enlargement and infiltration at 100X. **Fig. 6.3,** Average leukocytes/field of view  $\pm$  SEM. Statistical significance was determined using a Mann-Whitney test. **\*\*p < 0.01**

although in some individuals, the ratio in the periphery remains unchanged (23). Furthermore, CD8<sup>+</sup> T cell-deficient mice exposed to cigarette smoke do not develop emphysema (24), and MHC I-deficient mice are protected from emphysema induced by the adoptive transfer of CD3<sup>+</sup> T cells from cigarette smoke-exposed mice (1). Despite the contribution of CD8<sup>+</sup> T cells to pathogenesis, the peripheral level of CD8<sup>+</sup> T cells has not been established in the elastase-induced model. Therefore, we characterized the splenic T cells isolated from donor mice with elastase-induced emphysema (**Fig. 6.4** and **Fig. 6.5**). Splenic T cells were 97%, 55%, and 43% positive for CD3, CD4, and CD8 expression, respectively (**Fig. 6.4**). The peripheral CD4:CD8 ratio in mice with emphysema ( $1.34 \pm 0.07$ ) was significantly lower than their saline control counterparts ( $2.0 \pm 0.04$ ) (**Fig. 6.5**), demonstrating an elevated frequency of CD8<sup>+</sup> T cells in the periphery of mice with elastase-induced emphysema supported by elevated numbers of CD8<sup>+</sup> T cells but constant numbers of CD4<sup>+</sup> T cells. This implies that the CD8<sup>+</sup> T cell population has activated and expanded in this model.

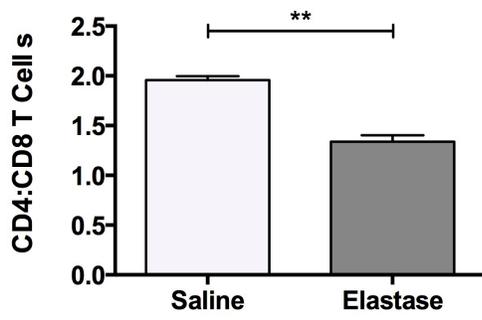
*Adoptively transferred T cells induced alveolar destruction in NOD SCID but not SCID mice*

The basic hypothesis under investigation was that T cells could transfer disease and thus, support a role for autoimmunity in an elastase-induced model of emphysema. The degree of alveolar destruction and infiltration in the lungs of recipient mice (NOD SCID and SCID) was measured to address this question (**Fig. 6.6-6.9**). SCID strains of mice lack functional T and B cells and readily accept allogeneic or xenogeneic grafts. Therefore, these immunodeficient mice are often used for models of autoimmunity (25, 26). To determine whether T cells can transfer emphysema, we measured the degree of airspace enlargement in the two strains of SCID mice. Alveolar destruction occurred in NOD SCID mice (Lm:  $27.72 \pm 0.56 \mu\text{m}$ ) but not SCID mice

**Fig. 6.4**

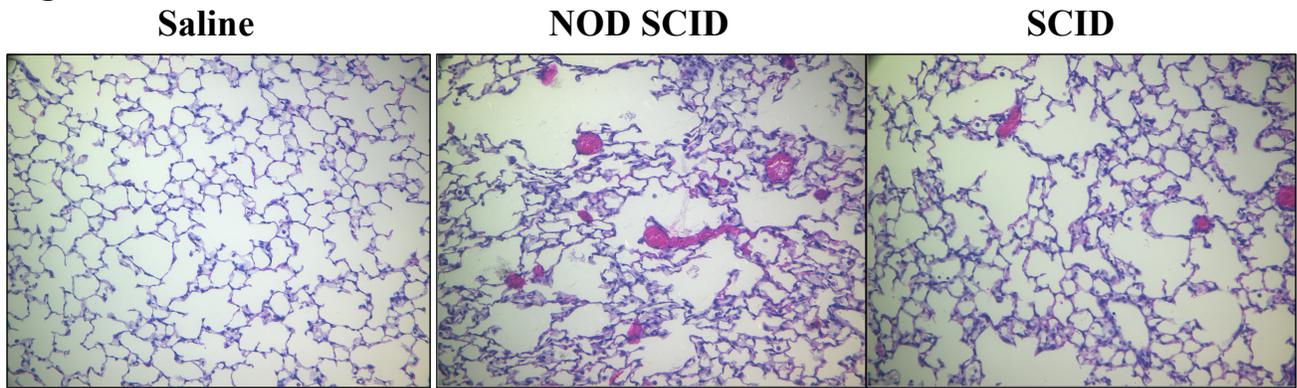


**Fig. 6.5**

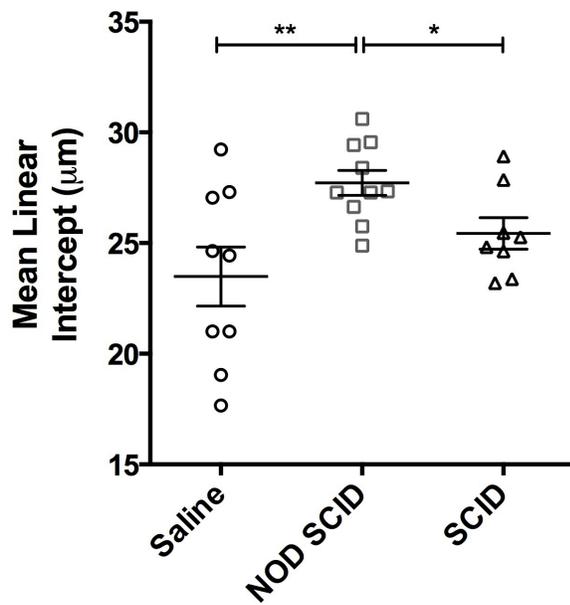


**Figures 6.4-6.5. Peripheral CD8<sup>+</sup> T cells were elevated in mice with elastase-induced emphysema.** Spleens were harvested from female BALB/cByJ mice 21 days after either elastase (donor mice) or saline (saline control) intratracheal instillations. T cells isolated from spleens were analyzed for CD3, CD4, and CD8 expression. Representative of 2 separate experiments. **Fig. 6.4.** Percentage  $\pm$  SEM of CD3<sup>+</sup>, CD4<sup>+</sup>, or CD8<sup>+</sup> T cells from donor mice. **Fig. 6.5.** Ratio of CD4:CD8 T cells  $\pm$  SEM comparing saline control mice (open bar) to donor mice with elastase-induced emphysema (gray bar). Statistical significance was determined using a Mann-Whitney test. \*\*p < 0.01

**Fig. 6.6**



**Fig. 6.7**



**Figures 6.6.-6.7. T cells induced alveolar destruction in NOD SCID but not SCID mice.**

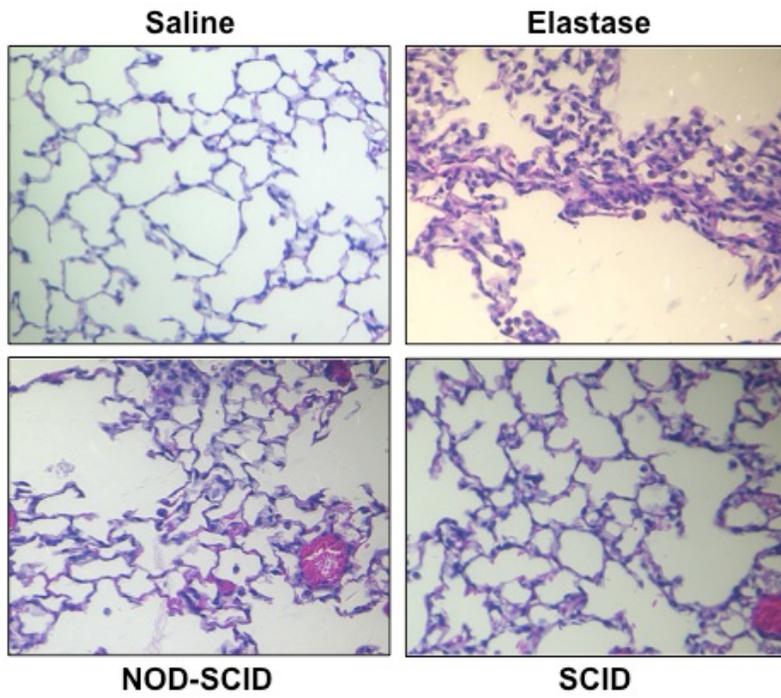
Lungs were harvested from BALB/cByJ mice 21 days after saline (saline control) intratracheal instillations. Purified T cells from mice with elastase-induced emphysema were injected into either NOD SCID or SCID mice. Lungs were harvested from the NOD SCID and SCID mice 21 days after the adoptive transfer of T cells. Airspace enlargement was determined by mean linear intercept (Lm). Representative to 2 separate experiments. **Fig. 6.6**, Micrographs of lungs showing the degree of airspace enlargement at 100X. **Fig 6.7**, Mean linear intercept  $\pm$  SEM. Statistical significance was determined using a Mann-Whitney test. \*  $p < 0.05$ , \*\* $p < 0.01$

(Lm:  $25.44 \pm 0.71 \mu\text{m}$ ) as determined by comparing mean linear intercept values to those of saline control mice,  $p < 0.01$  and  $p = 0.16$ , respectively (representative images in **Fig. 6.6** and summarized in **Fig. 6.7**). Adoptive transfer of T cells into NOD SCID but not SCID mice causes destruction of the alveoli.

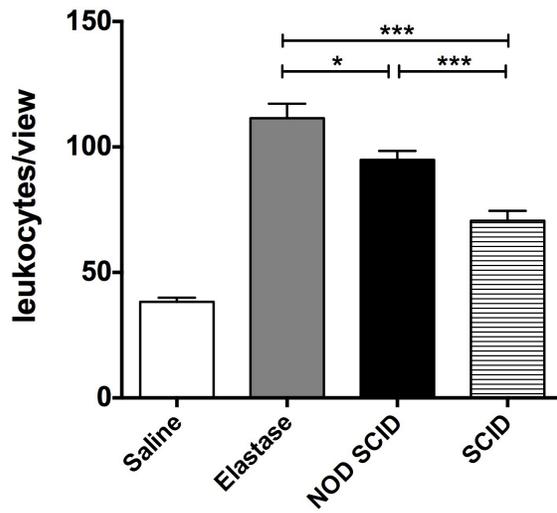
*Adoptively transferred T cells induced greater infiltration in the lungs of NOD SCID than in SCID mice*

Next, we determined the degree of leukocyte infiltration induced by adoptive transfer of CD3<sup>+</sup> T cells in each strain of SCID mice. Infiltration was determined for saline control, elastase control, NOD SCID, and SCID mice by counting the number of leukocytes from three fields of view per mouse (**Fig. 6.8**). Both types of immunodeficient strains exhibited signs of enhanced infiltration in comparison to the saline control ( $p < 0.001$ ) (representative images in **Fig. 6.8**, summarized in **Fig. 6.9**). However, the degree of infiltration was significantly greater in the NOD SCID mice averaging  $95 \pm 5$  leukocytes/view compared to  $71 \pm 5$  leukocytes/view in the SCID mice ( $p < 0.001$ ) but less than  $112 \pm 5$  leukocytes/view observed in the elastase control mice ( $p < 0.05$ ). Collectively, these data provide evidence that T cells can transfer emphysema from mice with elastase-induced emphysema, implicating autoimmunity in this widely used model.

**Fig. 6.8**



**Fig. 6.9**



**Figures 6.8-6.9. T cells infiltrated the lungs of immunodeficient mice and promote inflammation.** Lungs were harvested from BALB/cByJ mice 21 days after either elastase (elastase control) or saline (saline control) intratracheal instillations. Purified T cells from mice with elastase-induced emphysema were injected into either NOD SCID or SCID mice. Lungs were harvested from the NOD SCID and SCID mice 21 days after the adoptive transfer of T cells. Infiltration scores were determined as described in Materials and Methods for all groups of mice. Representative of 2 separate experiments. **Fig. 6.8,** Micrographs of lungs showing the degree of infiltration at 200X. **Fig. 6.9,** Average leukocytes/field of view  $\pm$  SEM. Statistical significance was determined using a Mann-Whitney test. \*  $p < 0.05$ , \*\*\* $p < 0.001$

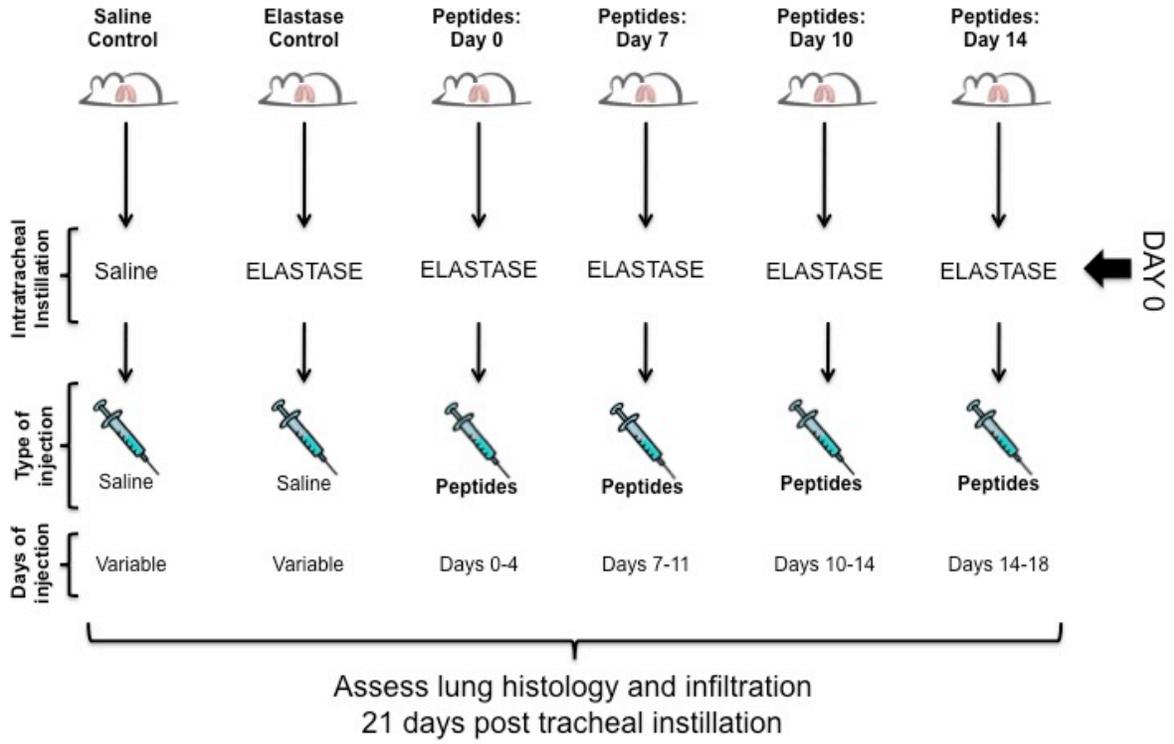
## Part II

### *Peptide therapy protected mice from elastase-induced emphysema*

The ability of CD3<sup>+</sup> T cells to transfer emphysema in the elastase-induced model (Part I) suggests that autoimmunity is involved. Cyclic peptides, cIE-L and cLAB-L, designed previously by our lab inhibit mixed lymphocyte reactions and homotypic adhesion of T cells through blockade of second signaling (stimulated through LFA-1 or ICAM-1 in T cells) (14, 27). We also recently demonstrated that these peptides suppress autoreactive T cells and delay onset of diabetes using the NOD mouse model (13). Because these peptides can suppress T cell function *in vitro* and T cell-driven autoimmune disease in mice, we tested whether these peptides could protect mice from elastase-induced emphysema. Intratracheal instillations of saline (saline control) or elastase (all other treatment groups) were given at the start (Day 0) of the 21-day model (See **Fig. 6.10** for model of experimental design). All mice were given 5 daily intravenous injections of either saline (saline and elastase control groups) or peptides (Day 0, Day 7, Day 10, and Day 14 peptide groups). For the peptide treatment groups, the timing of when peptide therapy began differed, starting at day 0 (same day of intratracheal instillations), day 7 (7 days after intratracheal instillations and thus, a third of the way into the model), day 10 (10 days after intratracheal instillations or the midpoint of the model) or day 14 (14 days after intratracheal instillations during the last third of the model) for Day 0, Day 7, Day 10 and Day 14 mouse groups, respectively. Lungs from all groups were harvested and assessed for alveolar destruction and leukocyte infiltration 21 days after intratracheal instillations. Mice that began peptide therapy at either day 0 or day 7 were protected from emphysema (**Fig. 6.11-6.13**). Mean linear intercept values from Day 0 or Day 7 groups were similar to the saline control mice ( $p =$

**Fig. 6.10**

Experimental design to test peptide therapy in 21-day model of emphysema



**Figure 6.10. Model of peptide experiment to test the therapeutic efficacy ICAM-1 and LFA-1 derived peptides in an elastase-induced model of emphysema.** Lungs were harvested from female BALB/cByJ mice 21 days after saline (saline control) or elastase (all other groups) intratracheal instillations. Airspace enlargement was determined by mean linear intercept (Lm) and leukocyte infiltration as described in Materials and Methods. Saline and elastase control mice received 5 daily intravenous injections of saline compared to the peptide treatment groups that were given peptide injections. Day 0 treatment group began their first peptide injection the same day of intratracheal instillations and thus, received injections on days 0-4. Day 7 treatment group began peptide injections 7 days after intratracheal instillations, receiving injections on days 7-11. Day 10 treatment group began peptide injections 10 days after intratracheal instillations so on days 10-14. Day 14 treatment group began peptide injections 14 days after intratracheal instillations and thus, received peptide treatment on days 14-18.

Fig. 6.11

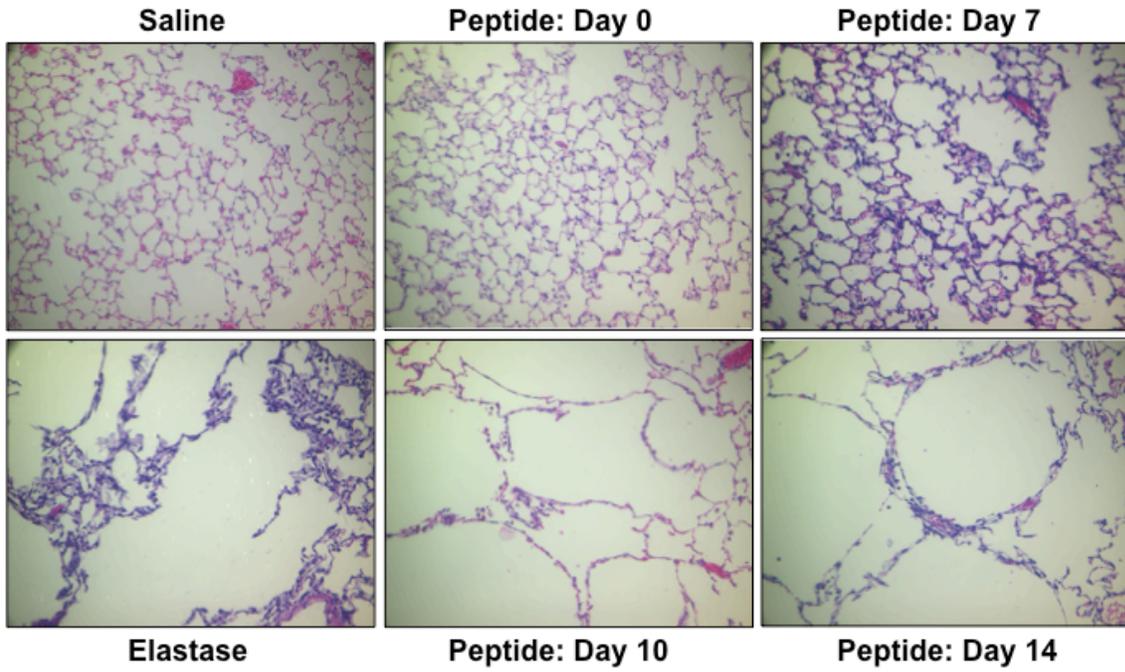


Fig. 6.12

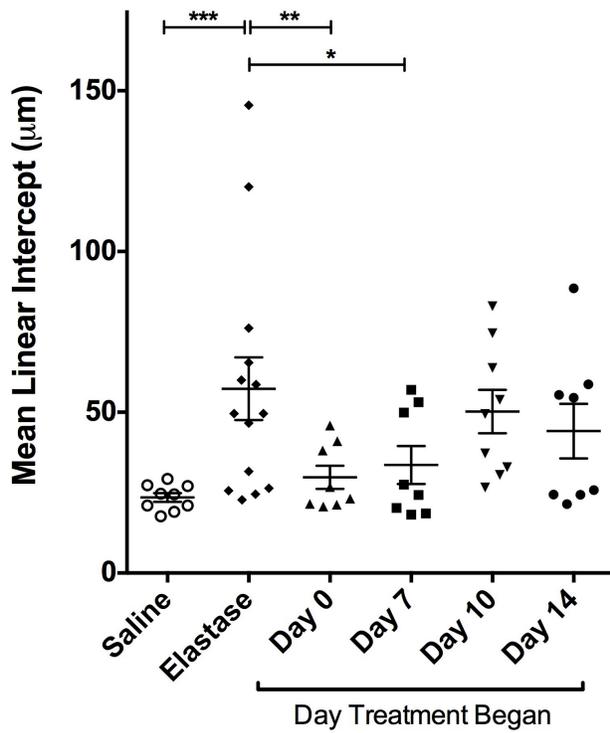
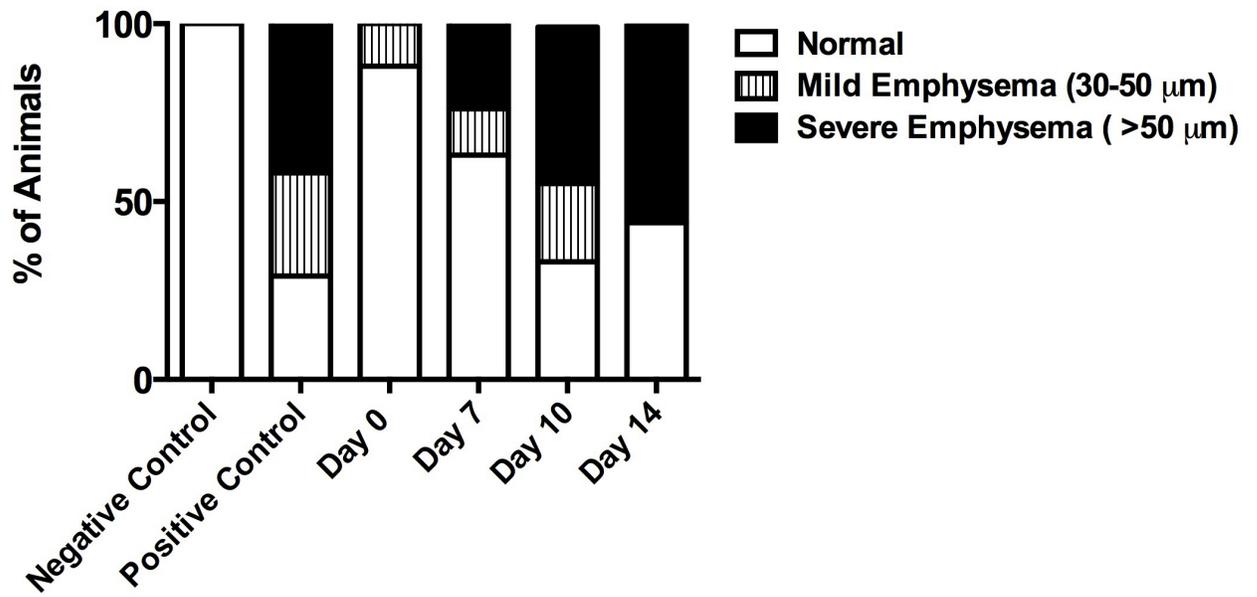


Fig. 6.13



**Figures 6.11-6.13. Treatment with peptides attenuated disease severity in an elastase-induced model of emphysema.** Lungs were harvested from BALB/cByJ mice 21 days after saline (saline control) or elastase (all other groups) intratracheal instillations. Airspace enlargement was determined by mean linear intercept ( $L_m$ ). Representative to 3 separate experiments. **Fig. 6.11**, Micrographs of lungs showing the degree of airspace enlargement at 100X. **Fig 6.12**, Mean linear intercept  $\pm$  SEM. **Fig. 6.13**, The percentage of animals exhibiting no signs of emphysema (open bars), mild emphysema (striped bars:  $L_m = 30-50 \mu\text{m}$ ) or severe emphysema (closed bars:  $L_m > 50 \mu\text{m}$ ) was determined for each group of mice. Statistical significance was determined using a Mann-Whitney test. \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

or 14, peptide therapy was ineffective against airspace enlargement. The percentage of mice exhibiting any indications of emphysema were 0% (saline), 72% (elastase), 12% (Day 0), 38% (Day 7), 66% (Day 10), and 56% (Day 14) (**Fig. 6.13**). As peptide therapy began at later timepoints after disease initiation, the percentage of mice affected with severe emphysema (Lm: > 50  $\mu$ m), increased from 0% (Day 0), 25% (Day 7), 44% (Day 10), to finally 56% (Day 14) (**Fig. 6.13**).

*Peptide therapy prevented a reduced CD4:CD8 ratio in the spleen and partially inhibited leukocyte infiltration*

In our previous study (13), peptides significantly reduced the degree of infiltration in a mouse model of autoimmune diabetes. Leukocyte infiltration particularly by neutrophils, macrophages and T cells is commonly observed in the elastase-induced model (12). In part I, we observed that splenic T cells from mice with elastase-induced emphysema exhibited a skewed CD4:CD8 ratio with an elevated frequency of CD8<sup>+</sup> T cells (**Fig. 6.5**). We assessed the degree of infiltration for each treatment group and the CD4:CD8 ratio in the spleen (**Fig. 6.14-6.16**). The peptides significantly reduced leukocyte infiltration in the mice treated with peptides beginning on days 0 and 7 (**Fig. 6.14-6.15**) but only by 21%. There was no therapeutic effect on leukocyte infiltration in the day 10 and 14 treatment groups, and all groups exhibited elevated infiltration compared to saline mice (**Fig. 6.15**). Regardless of the time at which peptide treatment began, the mice treated with peptide therapy possessed a normal CD4:CD8 ratio in the periphery ( $2.0 \pm 0.04$ ) in contrast to the elastase control mice ( $1.6 \pm 0.10$ ) (**Fig. 6.16**). The mechanism of protection by peptide therapy in the elastase-induced model of emphysema could not be entirely explained by inhibiting leukocyte infiltration. We expect a

Fig. 6.14

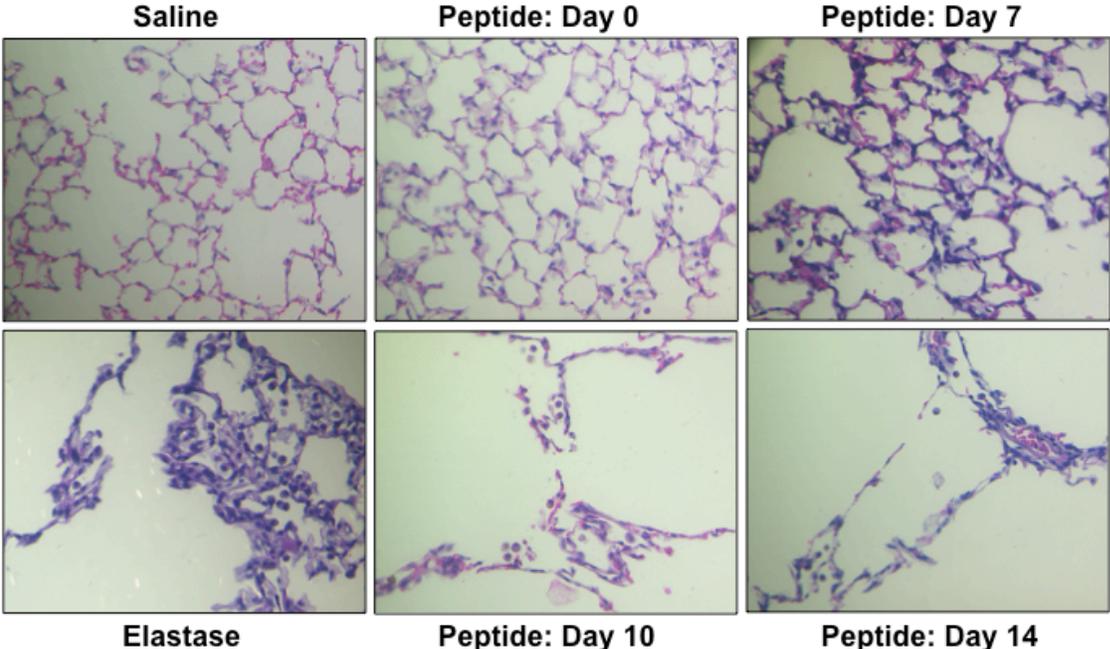
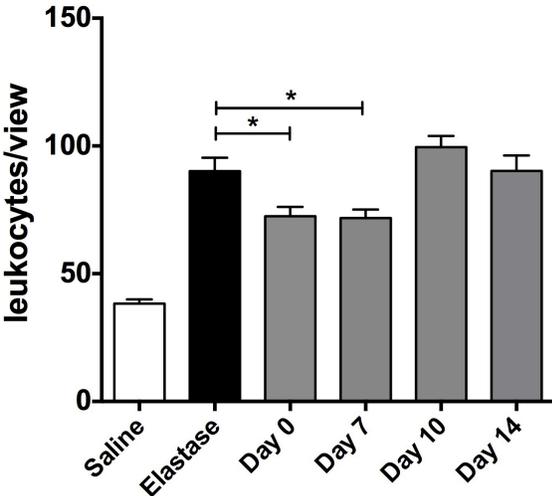
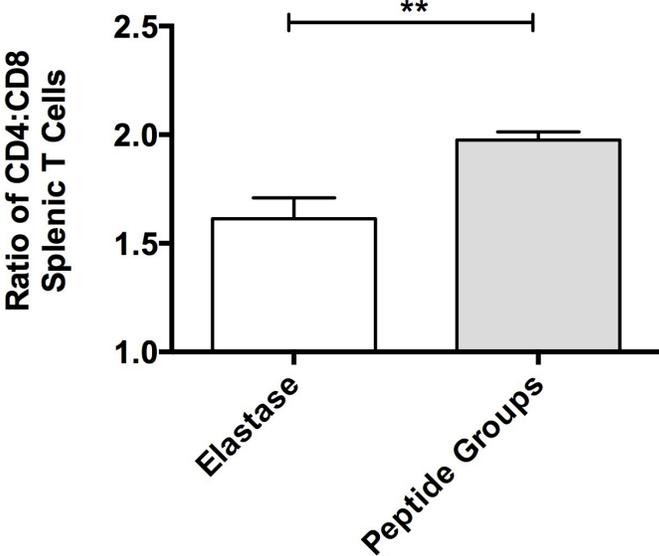


Fig. 6.15



**Figures 6.14-6.15. Peptide therapy partially inhibited leukocyte infiltration.** Lungs were harvested from BALB/cByJ mice 21 days after saline (saline control) or elastase (all other groups) intratracheal instillations. Leukocyte infiltration was determined as described in Materials and Methods. Representative to 3 separate experiments. **Fig. 6.14**, Micrographs of lungs showing infiltration at 200X. **Fig. 6.15**, Average leukocytes/field of view  $\pm$  SEM. Statistical significance was determined using a Mann-Whitney test. \*  $p < 0.05$

Fig. 6.16



**Figure 6.16. Peptide therapy prevented the reversal of the CD4:CD8 ratio in the periphery.** Splenic T cells were isolated on day 21 from elastase or peptide groups and analyzed by flow cytometry for CD4 and CD8 expression. Representative of 1 experiment. Average CD4:CD8 T cell ratio  $\pm$  SEM comparing elastase control mice to all the peptide groups. Statistical significance was determined using a Mann-Whitney test. \*\* $p < 0.01$ ,

direct inhibitory effect on T cells as we have previously observed (13) that results in either anergy or apoptosis due to blockade of second signaling by the peptides. The partial inhibition of infiltration is not surprising because these peptides also function to suppress autoreactive T cells.

*Homogenized whole lung lysate and commercially prepared murine elastin peptides failed to induce an antigen-specific T cell response*

In our previous work, peptide therapy inhibited antigen-specific responses of both CD4+ and CD8+ T cells in a murine model of autoimmune diabetes (13). Although COPD has not been formally established as an autoimmune disease, there is growing evidence both in COPD patients and animal models of disease to support the hypothesis of autoimmune involvement (1, 2, 10, 28-31). Part I of this chapter also supported a role for autoimmunity in emphysema. In many autoimmune diseases such as type I diabetes and multiple sclerosis, the autoantigens involved in the disease pathogenesis of have been clearly identified. In the case of COPD, these are still being defined along with the contribution of autoimmunity. One group has identified autoreactive CD4+ T cells in patients with COPD specific for elastin peptides, and elastin fragments induce monocyte recruitment to the lungs in a murine model of emphysema (30, 32). Therefore, we tested whether the peptide treatment would inhibit an antigen-specific response to either homogenized whole lung lysate or commercially prepared murine elastin peptides. However, two separate experiments comparing T cell proliferation (6 mice/group) or one experiment comparing the production of IFN- $\gamma$  (3 mice/group, data not shown) between elastase and saline control groups failed to identify an antigen-specific response by either the CD4+ or CD8+ T cells from the elastase control group (**Fig. 6.17-6.18**). Thus, we could not assess the

Fig. 6.17

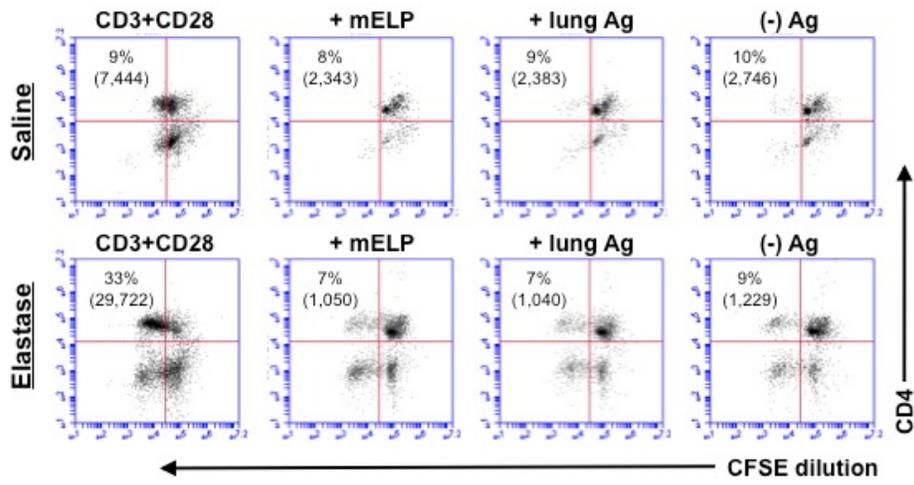
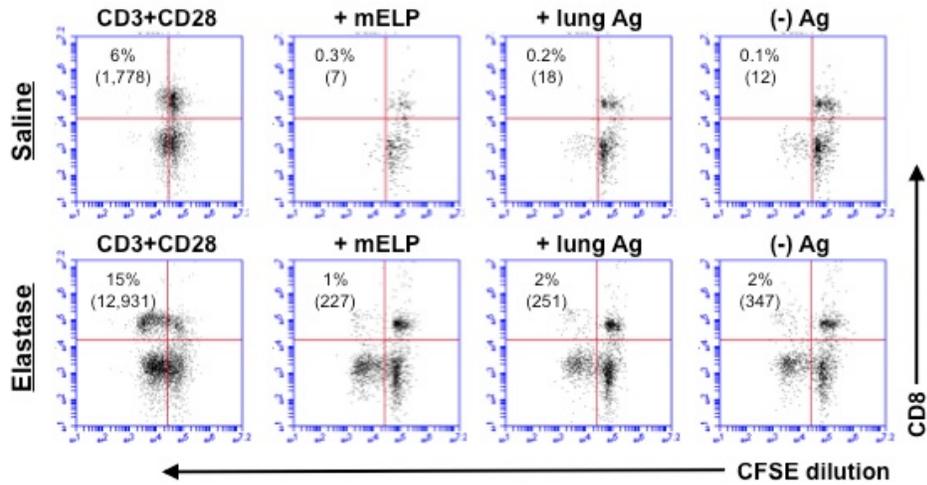


Fig. 6.18



**Figures 6.17-6.18. T cells from mice with emphysema did not elicit an antigen-specific response to commercially prepared elastin peptides or homogenized lung lysate.**

Splenocytes were isolated from elastase treated (n = 6) or saline (n = 6) control mice 21 days after intratracheal instillations and stained with 2.5  $\mu$ M CFSE. Cells stimulated through anti-CD3+anti-CD28 served as a positive control (left panels). All other treatments were not stimulated using antibody but rather, murine elastin lung peptides (+ mELP), whole homogenized lung lysate (+ lung Ag) or saline ((-) Ag) were added to the wells at day 0. 5 days later, cells were analyzed for CFSE dilution and CD4 and CD8 expression by flow cytometry. Representative of 2 experiments. **Fig. 6.17**, Percentage of CD4+ T cells that divided cells are indicated with cell numbers parenthetically. **Fig. 6.18**, Percentage of CD8+ T cells that divided cells are indicated with cell numbers parenthetically.

ability of the peptides to inhibit antigen recall responses. The non-specific proliferative responses by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells stimulated through CD3+CD28 in the absence of antigen were routinely heightened in the elastase mice compared to saline mice (**Fig. 6.17-6.18**, CD3+CD28 panels). This observation may indicate an overall elevated T cell activation state in the periphery of mice with emphysema.

*Mouse weight did not correlate with disease severity or differ among treatment groups*

To test whether disease severity was associated with the weight of the mouse, we examined the weight of the mice at the start of the model in comparison to the mean linear intercept value (**Fig. 6.19**). No correlation was found between the weight of the mouse and the degree of airspace enlargement. We also compared the weights among the various treatment groups and found no significant differences (**Fig. 6.20**), indicating that the variances in mouse weight did not impact our results.

Fig. 6.19

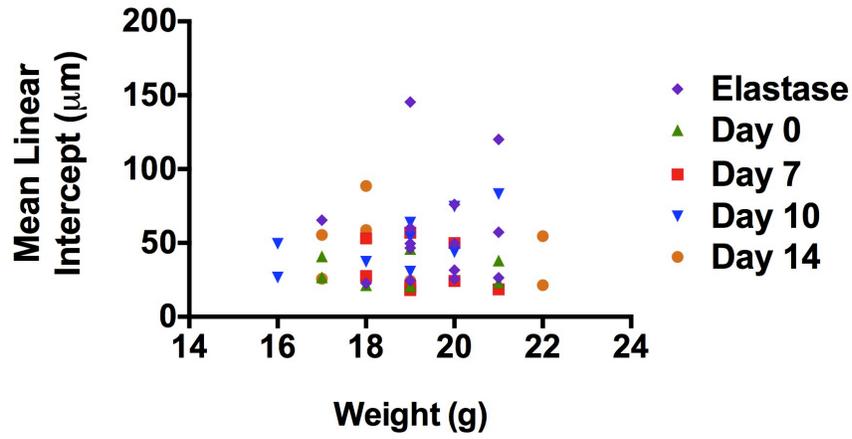
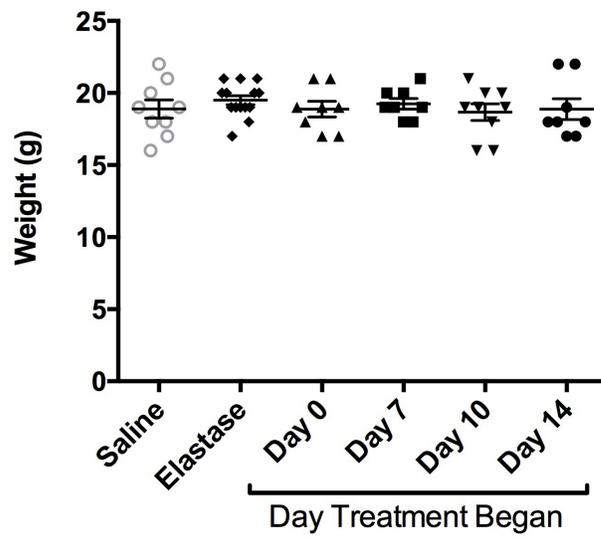


Fig 6.20



**Figures 6.19-6.20. Mouse weight did not influence the severity of emphysema.** Mice from all treatment groups were weighed on day 0, immediately before intratracheal instillations of either saline (saline control) or elastase (all other groups). **Fig. 6.19**, Plots of the weight of the mouse (g) versus its mean linear intercept value ( $\mu\text{m}$ ). Pearson correlation coefficients show no correlation between  $L_m$  and weight. **Fig. 6.20**, Average weight  $\pm$  SEM of mice for each group as indicated. One-way ANOVA indicates no significant differences in the weights among the different treatment groups of mice.

## **Discussion**

### *Evidence for autoimmunity in COPD in human studies*

COPD predominantly affects current or past smokers. Although smoking is the primary risk factor, only a fraction of smokers develop COPD (33), suggesting a complex pathogenesis involving the participation of genetic factors in addition to environmental ones. Several polymorphisms have been associated with COPD; some of these affect genes that encode important mediators of the immune response such as TNF- $\alpha$ , TGF- $\beta$  and IL-13 (34). Furthermore, atopic individuals have a heightened risk for developing COPD, experience more severe respiratory symptoms, and represent 18-42% of COPD cases by some reports (35, 36). Pulmonary inflammation and subsequent destruction persists in COPD patients even after smoking cessation (28, 37, 38). Additionally, CD4<sup>+</sup> T cell responses to elastin peptides and the presence of anti-elastin antibodies correlate with disease severity; several CD4<sup>+</sup> T cell clones from patients with emphysema have been characterized and produce elevated levels of IL-6 and IFN- $\gamma$  in response to elastin fragments (31, 39, 40). These observations and the various genetic risk factors associated with immune dysfunction suggest that autoimmunity is involved in the development and progression of emphysematous disease.

### *Evidence of autoimmunity in the elastase-induced model of emphysema*

Evidence for autoimmunity in the pathogenesis of COPD is also supported by various studies in animal models of emphysema. Mice deficient in either MHC I or CD8<sup>+</sup> T cells are protected from emphysema (1, 24), and CD3<sup>+</sup> T cells can transfer emphysema to naïve rodents (1, 2, 9). Although both the cigarette smoke- and elastase-induced models are widely used to study emphysema in mice, the ability of T cells to transfer disease using the elastase-induced

model has not been investigated until now. Additionally previous studies using other models have transferred (1, 2, 9) disease by injecting T cells intraperitoneally whereas we introduced T cells intravenously. We demonstrate that CD3+ T cells from elastase induced emphysema can induce leukocyte infiltration and alveolar destruction in naïve immunodeficient mice and that the use of therapeutic peptides can inhibit pathogenesis. The ability of these T cells to transfer disease suggests that they recognize autoantigen(s) within the lungs, home to the lungs or draining lymph nodes, and initiate disease, supporting a role for autoimmunity driven by T cells in the elastase-induced model. Collectively, these observations suggest the involvement of an autoimmune response to pulmonary autoantigen released by the introduction of elastase to the lungs.

#### *Mechanism of action by peptide therapy in elastase-induced model*

The peptide therapy attenuated the severity of alveolar destruction but not to the same degree that leukocyte infiltration was reduced, supporting the participation of more than one mechanism of action by the peptide therapy. These peptides can suppress T cells by blocking the induction of second signals by the costimulatory molecules, LFA-1 and ICAM-1. Therefore, it is likely that the peptides are also suppressing T cell responses in this model. In support of this, we observed a normal CD4:CD8 ratio in the spleens of all peptide treatment groups in contrast to the elastase-treated mice that did not receive peptide therapy. We were unable to identify an antigen-specific response to a murine elastin peptides or homogenized lung lysate. However, this does not exclude the possibility of an antigen-specific response since we were able to effectively transfer emphysema driven by T cells into naïve mice. Whether COPD is a form of autoimmunity is still an ongoing discussion, and although many candidate autoantigens have

been identified, no autoantigen has officially been established in contrast to other autoimmune diseases. Recently, one group was able to identify elastin-reactive CD4<sup>+</sup> T cells from patients with emphysema by synthesizing 78 overlapping peptide sequences (20 amino acids in length) encompassing the entire elastin sequence (40). In this study, elastin-specific responses were confined to only 2 areas of the elastin molecule (786 amino acids). Thus, it is possible that the lung homogenate and commercial elastin peptides did not include the correct amino acid sequence for an antigen-specific response.

Alternatively, the commercial peptides and lung lysate may have included the correct autoantigen, but the levels of the autoantigen were too low within the two types of antigen preparations to be detected by a highly heterogeneous population of peripheral T cells. The lungs or draining lymph nodes may contain oligoclonal expansions of autoreactive T cells specific to pulmonary antigens, which may provide a more robust response than splenic T cells. The presence of many other biological factors in the whole lung lysate may have interfered with the identification of an antigen-specific response.

Therefore, we were unable to determine the other possible mechanisms of action of the peptide therapy in this model. However, based on our other studies (13, 14), these peptides inhibit homotypic adhesion of T cells and antigen-specific T cell responses and most likely function to suppress T cell responses in the present model either by anergy induction or apoptosis. The combined use of L-ABL and L-IEL peptides was able to reduce infiltration and destruction of pancreatic islets, delay onset of disease, and inhibit antigen-specific T cell responses to pancreatic lysate in an autoimmune model of diabetes (13). In comparison, the peptide therapy in the emphysema model inhibited disease severity, reduced infiltration and

prevented an elevation of splenic CD8<sup>+</sup> T cells in the peptide-treated mice compared to their elastase control counterparts.

The timing of peptide treatment and efficacy is also consistent with T cell modulation since treatment was only effective if begun by 7 days after the start of inflammation within the lungs. It is well understood that during a primary immune response, clonal expansion of T cells occurs during the first week, generating a large number of effector T cells that peaks around 7 days followed by a significant reduction in cell number by the second week (41). Peptide treatment that began 10 or 14 days after initiation of disease may have been too late to modulate the T cell response since T cells have already entered the contraction phase of the response. It is likely that modulation of the T cell response such as inhibition of autoreactive cells by peptide therapy occurred in the day 10 and 14 treatment groups, but the timing of therapy was begun too late in the disease process to inhibit the destruction that already had ensued. In future studies, adoptive transfer of T cells from peptide-treated mice at the various timepoints may determine whether the peptide therapy effectively inhibits the T cell response even after pulmonary inflammation and destruction have occurred. The ability to suppress the driving factor of disease progression in COPD patients combined with a regenerative therapy to repair damaged lung epithelia may reverse disease and provide long-term protection in the future.

#### *NOD SCID mice were more susceptible to development of emphysema*

Although we observed enhanced leukocyte infiltration in both strains of immunodeficient mice, airspace enlargement was only evident in the NOD SCID mice 21 days after the adoptive transfer. Additional mutations in the NOD SCID compared to the SCID mice may account for variation in disease susceptibility. In comparison to other SCID strains, the NOD SCID mouse

accepts spleen engraftments donor cells at a four-fold increased frequency (42). In the present study, leukocyte infiltration of the lungs in the NOD SCID mice was approximately three-fold higher than the SCID mice, demonstrating a similar degree of enhanced engraftment in the NOD SCID mouse. The SCID deficiency in the NOD SCID mouse is approximately 10 fold less leaky than the SCID mouse (42), which may also influence disease susceptibility. The increased leakiness of the SCID mouse (as defined by serum Ig levels  $> 1 \mu\text{g/ml}$ ) (42), may result in sufficient T and B cell populations to interfere with the establishment of donor T cells. Additionally, the degree of emphysematous disease can vary among immunocompetent strains of mice (43). In a smoke-induced model of emphysema, T cells were able to transfer disease to naïve immunocompetent mice (1). Whether T cells from the elastase-induced model can transfer disease to immunocompetent mice remains to be determined.

### *Summary*

The controversy over whether an autoimmune response is involved in the pathogenesis of COPD may be attributed to the complexity of mechanisms involved in this disease, significant variables among COPD patients including the use of corticosteroids and differences in susceptibility to disease among mouse strains. Overall, combined observations from patients with COPD and animal models of smoke-induced emphysema have led to the hypothesis that COPD may involve an autoimmune component and led us to investigate this in an elastase-induced model of emphysema. We demonstrate for the first time the ability of T cells from mice with elastase-induced emphysema to transfer disease to naïve immunodeficient mice, supporting the utility of this model in future studies of autoimmunity in emphysema. We also show that blocking ICAM-1:LFA-1 interactions by peptide therapy attenuates disease severity by inhibiting

leukocyte infiltration and suppressing a skewed CD4:CD8 ratio. As evidence for autoimmunity in COPD continues to grow, inevitably more studies will be needed to establish the mechanisms by which T cells contribute to pathogenesis and develop therapeutic strategies to inhibit these responses.

### **Acknowledgments**

I would like to thank Dr. John Hartney from National Jewish Health for providing the procedure for intratracheal instillations, Dr. Jake Kohlmeier from Emory for advice on lung isolation, and Dr. Matthew Buechner for the extensive use of his microscope and trouble-shooting a microscopy issue. I would also like to thank Dr. Marcia Chan from Children's Mercy Hospital for developing the method for quantifying leukocyte infiltration and training me how to identify infiltrating cells in addition to her extensive work determining leukocyte infiltration in the adoptive transfer experiments. Derek Danahy was instrumental in scoring at least 60% or more of the alveolar destruction in the mice from all experiments. I am also extremely grateful for Derek's and Dr. Abby Dotson's assistance with the intratracheal instillations. This project was funded by the Patricia Watkins Emphysema Research Fund.

### **Notes**

Some of the work presented in this chapter is included in two separate papers in preparation.

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## Chapter 7: Summary of Dissertation

This dissertation addresses how costimulatory proteins such as CTLA4, and other biological factors such as biologically relevant lipoproteins LDL and HDL can tune the differentiation of human naïve CD4<sup>+</sup> T cells to various T cell subsets. In this way, the work contributes to the overall work by the Benedict lab in establishing the differential influence of costimulatory proteins on cell fate (See **Table 7.1** for a summary of our collective data in support of this) Costimulation through CTLA-4 in the absence of exogenous cytokines activated proliferation and differentiation of naïve CD4<sup>+</sup> T cells to effector, memory, and regulatory T cells. CTLA-4 favored differentiation to Treg cells more aggressively than any of the stimuli studied thus far, supporting its other known roles as an overall negative regulator of the immune response. As a an additional stimulus to either costimulation through ICAM-1 or CD28, CTLA-4 augmented differentiation to Treg cells induced by ICAM-1 and shifted differentiation induced by CD28 in favor of memory T cells.

Th17 cells represent an important T helper subset that had not been evaluated using our *in vitro* system of differentiation until now. Costimulation through CD28, ICAM-1, LFA-1, or CTLA-4, did not induce differentiation to Th17 in the absence of exogenous cytokines (also summarized in **Table 7.1**).

The function of CD23, the low affinity receptor for IgE, was evaluated in naïve and mature T cells in healthy subjects. I found that the addition of CD23 to costimulation through CD28 opposed Th2 functions by inhibiting differentiation of naïve T cells to Th2 cells and by activating Th1 cells in mature T cells.

The influence of other biological factors potentially present in the microenvironment during naïve T cell activation also was addressed. I found that similar to their opposing roles in atherosclerosis, LDL and HDL exerted opposing effects on naïve T cell differentiation and proliferation. LDL enhanced the generation of effector T cells with a Th1-phenotype, the T cell type most predominant in atherosclerotic plaque, supporting a novel hypothetical role by which LDL regulates T cell differentiation to Th1 to exacerbate atherosclerosis. HDL, in contrast, inhibited proliferation and cell survival and this hypothetically would slow the progression of atherosclerosis by limiting the number of Th1 cells in plaque.

Finally, I investigated whether autoimmunity plays a role in a murine model of emphysema. I determined that purified T cells transferred from a mouse in which emphysema had been induced by an enzyme treatment, carried the disease emphysema to naïve mice when injected intravenously. I also demonstrated the ability of a peptide therapy to attenuate disease severity in this murine model of emphysema.

It is well studied and well accepted that regulation of naïve T cell differentiation is guided by cytokines located in the intercellular microenvironment. Collectively, my data support our ongoing hypothesis that costimulatory proteins and other relevant factors within the T cell microenvironment also can influence the outcome of cell differentiation. The implications of these findings may support the development of future therapies in which specific T cell responses are targeted for inhibition or activation depending on the disease.

Table 7.1

	CD3+CD28	CD3+ICAM-1	CD3+LFA-1	CD3+CTLA-4
<b>Proliferation</b>	✓	✓	✓	✓
<b>Protection from apoptosis</b>	✓	✓	×	✓
<b>Memory T cell Differentiation</b>	✓	✓	×	✓
<b>Effector T cell Differentiation</b>	✓	✓	✓	✓
<b>Differentiation to Th1 cells</b>	✓	✓	✓	×
<b>Differentiation to Th2 cells</b>	✓	×	×	?
<b>Differentiation to Tregs</b>	×	✓	×	✓
<b>Differentiation to Th17 cells</b>	×	×	×	×

**Table 7.1. Summary of the differential effects of costimulatory proteins on T cell differentiation and function.** Black text reflects our published work determined by previous lab members: Drs. Jacob Kohlmeier, Scott Tibbetts, Chintana Chirathaworn, Kelli Williams, and Abby Dotson. Orange text reflects my contribution to this ongoing study of how specific resident T cell surface proteins can participate in regulating T cell fate during differentiation (Chapters 2 and 3 of this dissertation).