THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

THE EFFECT OF CTLA-4 ON EFFECTOR TO MEMORY DIFFERENTIATION OF CD8 T CELLS

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SPRING 2015

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Veterinary and Biomedical Sciences
with honors in Veterinary and Biomedical Sciences

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ABSTRACT

Acute viral infection leads to the formation of long lasting memory cells whose numbers and functionality are products of signals received throughout infection. Regulatory T cells (Tregs) modulate the extent of effector CD8 T cell proliferation and differentiation to prevent autoimmunity and overly exuberant immune responses that result in excessive tissue damage. Tregs express CTLA-4 as one mechanism to modulate the immune response; CTLA-4 binds to B7 molecules and decreases the sensitivity of effector cells to antigen. We hypothesized that administration of CTLA-4 *in vivo* during development of the effector response would mimic the effect of Tregs, limit proliferation of CD8 T cells, and improve memory quality by increasing the number of surviving memory precursors. Treatment with CTLA-4 during priming produced a defect in overall expansion. The number of memory precursors decreased, as did polyfunctionality and expression of Bcl-2. Treatment during pathogen clearance and peak expansion caused neither functional nor phenotypic differences. Treatment during contraction and early memory formation caused lower expression of GzmB and higher expression of Bcl-2 at day 15 and day 21 post infection, but these differences began to normalize by day 40 post infection. A peptide stimulation and recall demonstrated no functional differences in the memory CD8 T cells. Transfer of stimulated Tregs during contraction and early memory formation increased the survival of antigen specific CD8 T cells *in vivo*. Phenotypic and functional changes observed following various treatments during infection show that CTLA-4 modulation has important implications for the development of memory CD8 T cells. While treatment during effector contraction appeared to help memory formation, more work remains to learn to better exploit these differences. Optimized doses of CTLA-4, or similar agonists, could be co-administered with a vaccine regimen to increase memory cell production.

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ACKNOWLEDGEMENTS

Thanks are due to Dr. Vandana Kalia, Dr. Surojit Sarkar, Rebekah Dadey, and Florian Baumann for their guidance, criticism, and support. Special thanks are due to Yevgeniy Yuzefpolskiy for three years of invaluable assistance and tireless patience.

1. Introduction

1.1 The Immune System

The immune system consists of all of the body's defenses against invasion by infectious organisms and other foreign particles, known as pathogens. These defenses range from physical barriers to bloodborne molecules to specialized cells, all of which are divided into categories based on the nature of their function and their mechanism of action. All immune responses can be divided into two categories: innate or acquired.

1.2 The Innate Immune System

Innate immune responses are the mechanisms of defense present at the birth of an organism. They do not exhibit specificity for a particular pathogen, nor do they confer immunity to the organism.[1] Immunity is protection of variable length against reinfection by a pathogen to which the organism has been exposed previously. Mechanisms of innate immunity can be physical or chemical barriers, molecules present in the blood stream, or cells. Physical barriers include the skin and mucous membranes such as those that line the respiratory and digestive tracts. Chemical barriers include the low pH of stomach acid and enzymes such as lysozyme which are secreted in tears.[1] The goal of these barriers is to prevent pathogens from entering the body, while the goal of other branches of the immune system is to prevent infectious organisms from colonizing tissues and spreading throughout the body.

One of the most prominent acellular mechanisms of innate immunity is complement. Complement is a system comprised of a variety of plasma proteins that interact with one another to induce inflammatory responses, opsonize pathogens and, in some cases, damage pathogens directly. Opsonization is a process by which pathogens are coated with antibodies or complement to increase their susceptibility to phagocytosis by cells such as macrophages and neutrophils.[1] Many complement proteins are proteases which are secreted as zymogens, also known as pro-enzymes. When one protease in the complement pathway is activated by the presence of a pathogen, it cleaves other complement proteins that are in their precursor, zymogen forms. These newly activated proteases then cleave other complement proteins in their zymogen forms, causing a cascade of protein activation that amplifies the signal from the original protein.[1] The end result of complement activation is large quantities of complement proteins that covalently bind to pathogens resulting in opsonization or, if the pathogen is a bacterium, formation of pores in the bacterial membrane, causing lysis of the cell. Some of the complement fragments resulting from zymogen cleavage can also attract additional phagocytes to the site of infection, activate them, and cause an inflammatory response.[1]

The final components of the innate immune response are cellular. They include natural killer cells, mast cells, basophils, eosinophils, neutrophils, macrophages, and dendritic cells.[1] Natural killer cells are granular cells that recognize abnormal cells of the body and lyse them through the release of lytic granules.[1] The function of mast cells is not clearly defined, but they are known to participate in allergic reactions and are believed to participate in the body's response to parasitic worms.[1] Eosinophils, basophils, and neutrophils are classified as granulocytes because they all have densely staining cytoplasmic granules. Eosinophils and basophils are believed to participate in parasitic and allergic responses, although their function is

not as clearly defined as neutrophils. Neutrophils are the most numerous of the cells comprising the innate immune system. They phagocytize microorganisms and destroy them using degradative enzymes contained in cytoplasmic granules.[1] Macrophages have several roles in the innate immune response; they phagocytize pathogens, induce inflammation, and activate and recruit other immune cells through the release of signaling molecules. They also phagocytize cells that are infected with invading microorganisms and have been targeted for destruction by the acquired immune response.[1] Dendritic cells are the final type of cell in the innate immune system. They, like macrophages and neutrophils, phagocytose pathogens, but their main role is presenting antigen to cells of the acquired immune system.[1]

1.3 The Acquired Immune System

The acquired immune system is comprised of B lymphocytes (B cells), which participate in humoral immunity, and T lymphocytes (T cells), which participate in cell mediated immunity. B cells produce immunoglobulins, otherwise known as antibodies, which bind to pathogens. Antibody binding can opsonize pathogens by coating them with antibody or by activating complement.[1] B cells originate in the bone marrow from common lymphoid progenitors that arise from hematopoietic stem cells. Upon receipt of specific signals which initiate B cell differentiation, common lymphoid progenitors become pro-B cells and undergo immunoglobulin gene rearrangement.[1] Gene rearrangement is the basis for the range of antigen specificities exhibited by both B and T cells. Pro-B cells transition through several stages as they rearrange each locus of their immunoglobulin genes, migrate through the bone marrow and complete their maturation in peripheral lymphoid organs.[1] Mature B cells circulate in the blood and lymph until

they encounter antigen recognized by surface bound immunoglobulin molecules, also known as their B cell receptors. Several signals contribute to B cell activation, after which antibody-secreting cells, called plasma cells, and memory B cells are produced. The former produces antibodies to combat ongoing infections and the later provides memory to create a rapid response upon reinfection.[1]

1.4 T Cell Development

Similar to B cells, T cells originate from common lymphoid progenitors derived from hematopoietic stem cells in the bone marrow. Some lymphoid progenitors leave the marrow and migrate to the thymus where it is believed that stromal cells provide a signal to the progenitor cells, activating specific genes and causing the cells to commit to the T cell lineage.[1,2] Differentiation to the T cell lineage coincides with a brief period of proliferation at which time the cells are double-negative thymocytes, expressing neither CD4 nor CD8, coreceptors of TCR signaling.[1] These double-negative thymocytes undergo T cell receptor (TCR) gene rearrangement to produce $\gamma\delta$ or $\alpha\beta$ TCRs. $\alpha\beta$ TCRs are present on the majority of T cells and react to peptides presented by major histocompatibility complex (MHC) class I and II. To determine if the TCR rearrangement is successful, double-negative thymocytes must transmit a signal through a CD3:pre-TCR complex. This is the first checkpoint in the development of T cells, after which the double-negative thymocytes express both CD4 and CD8 and become double-positive thymocytes.[1]

Double-positive thymocytes undergo further TCR gene rearrangement, bringing them to the second checkpoint of T cell development. In a process called positive selection, the

assembled TCR must recognize self-peptides presented by MHC on the thymic epithelium; failure to do so causes the cell to undergo apoptosis.[2] Positive selection is critical to T cell development because it ensures that an organism's T cells are able to recognize peptides presented by its MHC molecules, an important survival signal for mature naïve CD8 T cells.[1]

Positively selected double-positive thymocytes lose expression of CD4 or CD8, becoming single-positive thymocytes. Single-positive thymocytes undergo negative selection, a process by which cells that bind too strongly to self-peptide:MHC undergo apoptosis.[2] Negative selection decreases the degree of self-reactivity in the T cell population in order to prevent autoimmune diseases, such as type I diabetes and multiple sclerosis .[2] In Type I Diabetes, CD4 and CD8 T cells attack the pancreatic beta cells, decreasing insulin production.[3] Multiple sclerosis is a disease of the central nervous system in which CD4 and CD8 T cells attack myelin-producing Schwann cells.[3] In both diseases, negative selection has failed because T cells bind strongly to self-peptide:MHC and initiate an immune response.

1.5 Naïve T Cell Homeostasis

After successfully passing through positive and negative selection, mature, naïve T cells enter the blood and begin circulating through peripheral lymphoid organs.[1] Naïve CD8 T cells circulating through the blood must possess weak self-reactivity in order to receive homeostatic signals through their TCRs. Naïve T cells also receive survival signals from the cytokine IL-7. IL-7 is produced by stromal and epithelial cells in the bone marrow and thymus, and by secondary lymphoid organs. It binds to CD127, the IL-7 receptor, on mature, circulating memory and naïve T cells.[4] Naïve CD8 T cells express CD62L and CCR7 in order to enter

peripheral organs to receive homeostatic signals and search for antigen that will bind to their TCRs. CD62L is responsible for the adherence of T cells in the blood to the entry points of lymph nodes known as high endothelial venules (HEVs). CCR7 is a chemokine receptor on T cells that increases their responsiveness to the chemokines expressed at HEVs and aids their entry into lymph nodes.[5] Naïve T cells circulate through the blood and peripheral lymphoid organs continuously, unable to enter nonlymphoid tissues because they lack receptors for inflammatory chemokines that would draw them into the tissue.[5]

1.6 Activation of T Cells

Activation of T cells occurs under different circumstances depending on whether they express CD4 or CD8. CD4 T cells, also known as T helper cells, recognize MHC class II, which presents peptides from pathogens internalized by B cells and phagocytic cells, such as dendritic cells and macrophages.[1] Four classes of CD4 T cells have been identified: T_H1, T_H2, T_H17, and regulatory T cells (Tregs). T_H1 cells promote cell-mediated immune responses, while T_H2 cells primarily activate B cells.[1] T_H17 cells are believed to be responsible for stimulating neutrophils to respond to extracellular bacterial infections during the early portion of the adaptive immune response.[1] Tregs, contrary to the other types of CD4 T cells, suppress the responses of other T cells.[1]

1.7 Regulatory T Cells

Tregs can develop in the thymus or they can differentiate from naïve CD4 T cells in peripheral tissues. They characteristically express CD25, CD62L, CD45, Foxp3, and CTLA-4.[1,6]

CD25, also known as IL-2Rα, is believed to regulate effector T cells by binding to large amounts of available IL-2, preventing the effector T cells from using it to proliferate and differentiate.[6] CD62L is responsible for the initial adherence of T cells in the blood to HEVs for entrance into lymph nodes.[5] CD45 plays a role in the regulation of signaling through antigen receptors, such as the B and T cell receptors.[5] Foxp3 is a unique transcription factor expressed by Tregs.[7] Thus far, it is the most specific marker that has been identified as a means of identifying Tregs and is required for their development, maintenance, and function.[7,8] CTLA-4, cytotoxic T-lymphocyte-associated protein 4, regulates T cell signaling by binding to the costimulatory ligands B7.1 and B7.2 that are required to enhance T cell signaling. The affinity of CTLA-4 for B7 molecules is approximately twenty times stronger than that of the costimulatory molecule CD28.[1]

CTLA-4 limits the proliferative ability of T cells through cell to cell contact with Tregs; it decreases the sensitivity of T cells to antigen presenting cells and limits their production of IL-2, the cytokine most responsible for T cell expansion.[1,9] Another mechanisms of cell to cell suppression involves increasing T cell levels of cyclic adenosine monophosphate (cAMP) through direct delivery of cAMP or local generation of adenosine.[9] Increased levels of cAMP have been associated with inhibited proliferation and differentiation of lymphocytes by decreasing the expression of cytokines such as IL-2 and IFN-γ.[9] Secretion of TGF-β and IL-10, both of which play a role in T cell homeostasis, have been identified as additional mechanisms of Treg suppression.[9]

1.8 Activation of the CD8 T Cell Response

Tregs are responsible for suppressing the adaptive immune response, the main component of which is CD8 T cells. There are three main stages of the CD8 T cell response: activation and expansion, death, and stability or memory.[10] In order to be activated, CD8 T cells must be presented with antigen by antigen-presenting cells (APCs). APCs, such as macrophages and dendritic cells, phagocytize pathogens, process them to produce peptides, and then display the peptide antigens in a complex with MHC class I.[11] CD8 T cells bind to the peptide:MHC class I complexes through their TCRs and receive a stimulatory signal; however, this is not sufficient for activation. CD8 T cells must also receive a costimulatory signal through CD28, which binds to B7 molecules present on the surface of antigen-presenting cells.[11] CD28 is present at constant levels on both activated and resting T cells and promotes differentiation and cytokine production. IL-2, produced by CD4 T cells, is another important factor in the development and survival of effector CD8 T cells.[1] Optimal activation of T cells requires a third signal provided by IL-12 or type I interferons (IFN). IL-12 promotes the proliferation of T cells during the initial immune response and also plays a role in the development of cytotoxic CD8 T cells by promoting the production of IFN-γ.[12] Type I IFNs promote survival and effector differentiation of CD8 T cells.[12] Failure of a T cell to receive a costimulatory signal causes anergy, a nonresponsive state that eventually leads to cell death.[13] When all three signals have been received by the T cell, it acquires effector properties, some of which include the production of cytokines, such as IFN-γ and TNF-α, and downregulation of molecules involved in homing to the lymph nodes, such as CD62L and CCR7.[13] The acquisition of effector function is accompanied by rapid proliferation and differentiation to produce cytotoxic T lymphocytes (CTLs). The primary targets of CTLs

are virus infected cells; CTLs release cytokines, such as IFN-γ which can block viral replication, and granyzmes, such as granzyme B which kills virally infected cells.[1,14]

1.9 Formation of Memory CD8 T Cells

CD8 T cell expansion following exposure to antigen increases cell numbers by up to 50,000-fold, but 90-95% of effector CD8 T cells undergo apoptosis following pathogen clearance, leaving a population of long lived memory cells.[12] The transition from effector to memory T cells entails changes in phenotype, gene expression, and function of the surviving cells.[15] Characteristics of memory CD8 T cells include the ability to expand rapidly during secondary infection, the ability to produce IL-2 when restimulated by antigen, increased expression of Bcl-2, CD62L, and CCR7, and the ability to maintain their resting population via homeostatic proliferation.[16] They also exhibit increased expression of IL-7R and CD62L compared to effector cells.[12] Memory CD8 T cell precursors are present in the effector cell population during the peak of infection, but the mechanism that determines which cells will die following pathogen clearance and which will continue as memory cells remains unclear.[16] One hypothesis states that sustained proliferation and effector function during infection leads to a decreased potential for effector CD8 T cells to survive as memory cells once the infection is cleared.[16]

1.10 Effector and Central Memory CD8 T Cells

Two main subsets of memory CD8 T cells exist. The first is called central memory and expresses high levels of CD62L and CCR7, both of which aid the entrance of T cells into lymph

nodes. These cells are found in lymphoid organs and do not exhibit lytic function until they are activated by secondary exposure to antigen.[12] The second subset is called effector memory and expresses high levels of CD62L and low levels of CCR7. These cells reside in nonlymphoid tissues and demonstrate lytic activity without a delay following reexposure to antigen.[12] While the fates of effector cells may be predetermined, it is believed that memory CD8 T cells differentiate along a spectrum that produces memory cells with an intermediate lifespan between effector cells and memory cells. This spectrum allows cells to move between states of differentiation depending on the signals they are receiving from their surroundings; this provides memory cells with the potential to differentiate into effector cells upon secondary exposure to antigen.[14] Entrance of memory cells into nonlymphoid organs is a continuous process, but the population of central and effector memory cells remains constant due to homeostatic proliferation.[12] Homeostatic proliferation stimulates mild expansion of memory CD8 T cells through IL-7 and IL-15 signaling. These signals allow memory cells to survive without constant restimulation from antigen, causing low rates of proliferation that match rates of cell death to maintain the overall memory T cell population.[4]

Long lasting memory CD8 T cells provide a higher quality of immune response compared to naïve CD8 T cells because they respond faster than naïve cells upon secondary exposure to antigen, mounting a more efficient effector response.[11] Additionally, memory CD8 T cells localize in nonlymphoid and mucosal tissues where they are capable of responding to pathogens immediately.[11] By contrast, naïve cells must be activated in lymph nodes before traveling to the site of infection to combat invading pathogens.[11] Finally, for a given epitope, memory CD8 T cells are found at higher frequencies than naïve T cells, increasing the number of cells capable of recognizing and responding to a specific antigen.[11]

1.11 Experimental Purpose

The quality of the memory T cell population produced following infection is influenced by the duration of antigen stimulation to which the memory precursors are exposed. Lengthy antigen stimulation can lead to functional exhaustion and eventual deletion in CTLs. This, in turn, decreases the quality of memory because fewer memory precursors are available to differentiate into memory T cells following antigen clearance.[12] As mentioned previously, Tregs regulate effector T cells by decreasing their proliferation and differentiation.[6] We hypothesized that limiting the extent of effector function of the CTL population would lead to improved memory formation by decreasing functional exhaustion of the memory precursors. We simulated the effect of Tregs by administering CTLA-4 *in vivo* during priming of the effector population, clearance of pathogen, and peak of effector expansion and analyzed the memory T cell population to determine if there were resulting functional or phenotypic differences.

1.12 Experimental Results

In previous experiments, we demonstrated that mice treated with CTLA-4 following Treg ablation did not develop an inflammatory response. These results justified the use of CTLA-4 as a method of Treg supplementation. Treatment with CTLA-4 during priming produced a defect in CD8 T cell expansion, reducing the number of memory precursors, expression of Bcl-2, and polyfunctionality. Treatment during pathogen clearance and peak expansion did not produce any functional or phenotypic differences. Treatment during contraction and early memory formation demonstrated a decrease in the expression of GzmB and an increase in the expression of Bcl-2,

but these differences normalized by day 40 post infection. Adoptive transfer of stimulated Tregs during contraction and early memory formation improved the survival of CD8 T cells.

2. Materials and Methods

2.1 Mice

Three strains of mice were used for these experiments, all of which were purchased from the Jackson Laboratory and bred in-house. The first, B6 mice (C57BL/6), are wild type mice used to study the behavior of the immune system in a natural environment. These mice are Thy1.2$^{+/+}$ (homozygous), allowing them to be distinguished from adoptively transferred regulatory T cells. The second strain, CD4$^{-/-}$ mice (B6.129S2-Cd4^{tm1Mak}/J), have a mutation that prevents development of CD4 T cells in the thymus. They are used to study the effect of CD4 T cell ablation on the kinetics of the immune response. The third strain, MHCII$^{-/-}$ mice (B6.129S2-H2$^{dlAb1\text{-}Ea}$/J), lack MHCII genes in all of their cells and are used to study the immune response in the absence of CD4 T cells because they cannot mature in the absence of MHCII.

All animals were used in accordance with University Institutional Animal Care and Use Committee guidelines.

2.2 Basic Experimental Setup

Naïve mice were infected with lymphocytic choriomeningitis virus Armstrong ($LCMV_{Arm}$), and a given treatment was administered for a duration specified for each experiment. Mice were bled longitudinally every seven days, taking 50 μL of blood mandibularly, and the samples were analyzed for changes in CD8 T cell responses. At the end of each experiment, the mice were

sacrificed and their spleens collected. Splenocytes were stimulated with peptide to compare cytokine production or transferred into new mice to test recall potential.

2.3 Intraperitoneal Injections

Intraperitoneal injections were used to deliver 500μg CTLA-4 Ig per dose or 1 μg of murine IL-2 and 5 μg of anti-IL-2 (JES61A12) per dose. Each mouse was restrained and tilted downwards, exposing the abdomen. The needle was inserted on the right side of the mouse, close to the midline. The injection site was aspirated to ensure the needle had not punctured the small intestine or the bladder. The fluid in the syringe was injected.

2.4 $LCMV_{Arm}$ Infection

Lymphocytic choriomeningitis virus Armstrong ($LCMV_{Arm}$) produces an acute infection that elicits a CD8 T cell response, resulting in the formation of memory CD8 T cells. $LCMV_{Arm}$ was administered intraperitoneally by Yevgeniy Yuzefpolskiy at a dose of $2x10^5$ pfu per mouse from a $4x10^5$ pfu per mL stock solution.

2.5 Treg Isolation and Purification

In order to transfer Tregs to a recipient mouse, they first were stimulated in a donor mouse. Tregs in the donor mouse were stimulated with 1 μg of murine IL-2 and 5 μg of anti-IL-2 (JES61A12) that was incubated for 30 minutes at 37°C, placed on ice for 10 minutes, and

injected intraperitoneally, as described previously, on days 1, 2, and 3 of treatment. On day 5, the mice were bled to confirm the presence of Tregs.

On day 6 post treatment, the donor mice were euthanized and their spleens collected according to the protocol described below. Tregs were isolated according to the EasySep CD25 Regulatory T Cells Positive Selection Kit. The isolated splenocytes were suspended at a concentration of 1 x 10^8 cells per mL in Rosewell Park Memorial Institute medium (RPMI) supplemented with 1% fetal bovine serum (FBS) in a 14 mL round bottom tube. EasySep Mouse FcR Blocker at a concentration of 50 μL per mL of cells was added to the tube and incubated at room temperature for 5 minutes. EasySep CD25 Regulatory T Cell Positive Selection Cocktail at a concentration of 50 μL per mL of cells was added to the tube, mixed, and incubated at room temperature for 10 minutes. EasySep PE Selection Cocktail at 20 μL per mL of cells was added, mixed, and incubated for 5 minutes at room temperature. EasySep Dextran RapidSpheres were added to the tube at a concentration of 20 μL per mL of cells, mixed, and incubated at room temperature for 5 minutes. The test tube was placed in the EasySep magnet for 5 minutes, the supernatant was poured off, the tube was removed from the magnet, and the cells were resuspended. Separation using the magnet was repeated three more times, and the cells were resuspended in RPMI supplemented with 10% FBS in preparation for adoptive transfer.

2.6 Adoptive Transfer

In an adoptive transfer, cells from a donor mouse are transferred into a recipient mouse in order to study the transferred cells in an environment that could not be created in the donor mouse. For these experiments, regulatory T cells (Tregs) and CD8 T cells were collected from

the spleens of donor mice according to the spleen isolation and processing protocol detailed below and transferred into the recipient mice intravenously by Yevgeniy Yuzefpolskiy. To administer intravenous injections, mice were warmed beneath a heat lamp until they began grooming their whiskers, a behavior indicative of elevated body temperature. The mice were placed in a tailvein restrainer, the tail was sprayed with ethanol, and the cells were injected into the tail vein.

2.7 Blood Collection and Processing

Blood samples were collected from the mandibular vein throughout the experiments to monitor the progression of CD8 T cells from naïve cells to effector cells and memory precursors to memory cells. The mice were anesthetized using 5% isoflurane administered at 0.3L per minute and restrained by grasping the skin along the back. The hairless region along the mandible was cleaned with ethanol and a needle was inserted at this point. Approximately 100 μL of blood was collected in a capillary tube and emptied into a 5 mL polystyrene test tube containing 500 μL of 4% sodium citrate to prevent clotting. Two milliliters of RPMI containing 1% FBS was added to the tube, which was subsequently vortexed. The solution was underlaid with 1 mL of histopaque-1077 using a 9" glass Pasteur pipette. The tube was centrifuged for 20 minutes at 2,000 rpm and 20°C with no brake to pellet the red blood cells at the bottom of the tube and suspend the peripheral blood mononuclear cells (PBMCs) at the interface. The PBMCs were removed from the interface using a 5 ¾" glass Pasteur pipette and placed into another 5 mL polystyrene test tube containing 2 mL of RPMI supplemented with 1% FBS. The solution was centrifuged for 10 minutes at 2,000 rpm and 4°C with brake to pellet the PBMCs at the bottom of

the tube. The media was dumped without disturbing the pellet and 130 μL of fluorescence assisted cell sorting (FACS) buffer was added to the tube. Approximately 0.5 x 10^6 cells in 200 μL were transferred to a 96-well U-bottom plate and placed on ice in preparation for flow cytometry analysis.

2.8 Spleen Isolation and Processing

In order to analyze the quality of memory cells in the spleen, mice were euthanized using gaseous carbon dioxide administered at a rate of 2 L per minute followed by cervical dislocation. The spleens were removed from the mouse, cut in half, and added to 4 mL of RPMI supplemented with 1% FBS on ice in a 15 mL tube. The spleen and RPMI were poured into a small petri dish on ice. The spleen was rubbed gently between two frosted glass slides to extract the cells. The cells were washed off of the slides using a 5 mL syringe filled with 1% FBS supplemented RPMI. The cells were returned to the 15 mL tube and centrifuged for 10 minutes at 1,200 rpm and 4°C to pellet the splenocytes. The excess media was poured off without disturbing the pellet. Pelleted red blood cells were lysed using 0.83% ammonium chloride, resuspended, and centrifuged for 10 minutes at 1,200 rpm and 4°C. The excess media was poured off and the cells were resuspended in RPMI supplemented with 10% FBS.

2.9 Peptide Stimulation

Peptide stimulation is used to test the quality of memory cells when challenged with antigen. Splenocytes were resuspended in RPMI supplemented with 10% FBS at a concentration of 20 million cells per mL, 100 μl of which were added to a 96-well flat-bottom plate. A

negative control was given 100 μl of 10% FBS supplemented RPMI and Brefeldin A (BFA) at a concentration of 2 μg per mL. Stimulated samples were given 100 μL of RPMI supplemented with 10% FBS, as well as 2 μg per mL of BFA and 1.3 ng of peptide. The peptides used to stimulate the splenocytes were glycoprotein or nuclear protein fragments of LCMV and included GP33, NP396, GP276, GP118, NP205, GP92, and GP61. The cells were incubated for five hours at 37°C in 5% carbon dioxide. The cells were stained for CD8, CD44, IL-2, TNF-α, and IFN-γ according to the following protocol.

2.10 Secondary Heterologous Challenge

Secondary heterologous challenges test the functional properties of memory cells. Splenocytes from memory mice were isolated, normalized to 10,000 cells per mouse, and transferred into recipient mice. Recipient mice were infected by Yevgeniy Yuzefpolskiy with *Listeria monocytogenes* expressing glycoprotein 33-41 (rLM-GP33), bled longitudinally to assess the kinetics of the memory response, and sacrificed to enumerate memory CD8 T cells.

2.11 Antibody Labeling

Fluorescent antibodies were used to stain isolated PBMCs and splenocytes in order to follow protein markers and identify populations of cells. The following antibodies were used throughout the experiments: KLRG-1-FITC (MAFA), Thy1.1-PerCP (OX-7), CD8-PerCP (53-6.7), CD3-PerCP (145-2C11), FoxP3-PE (FJK-16a), CD127-PE (A7R34), Bcl-2-PE (BCL/10C4), CD4-PE-Cy7 (RM4-5), CD127-PE-Cy7 (A7R34), CD8-PE-Cy7 (53-6.7), GP33-

APC, GP276-APC, NP396-APC, CD8-APC (53-6.7), CD25-APC (PC61), Thy1.1-A700 (OX-7), CD8-A700 (53-6.7), CD62L-A700 (MEL-14), CD44-APC-Cy7 (IM7), GzmB-PacBlue (GB11), CD62L-PacBlue (MEL-14), IL-2-PE (JES6-5H4), TNF-α-APC (MP6-XT22), IFN-γ-FITC (XMG1.2), FoxP3-A488 (MF-14).

PBMCs and splenocytes suspended in FACS buffer were placed in a 96-well U-bottom plate on ice. The plate was centrifuged for 2 minutes at 1,800 rpm and 4°C to pellet the cells. The plate was inverted, flicked to remove the excess media, and blotted on a paper towel to remove the excess media. The plate was tapped to resuspend the pelleted cells. To perform a surface stain, 50 μL of FACS buffer containing fluorescent antibodies was added to each well, and the plate was incubated for 45 minutes on ice in a dark condition. The cells were washed with 150 μL of FACS buffer, centrifuged for 2 minutes at 1,800 rpm and 4°C, and inverted to flick off the excess media. The cells were washed twice more with 200 mL of FACS, fixed with 70 μL of 1X Cytofix/Cytoperm, and incubated for 20 minutes on ice in a dark condition in order to perform an intracellular stain. The cells were washed with 150 μL of 1X Perm/Wash buffer and centrifuged for 2 minutes at 2,200 rpm at 4°C. The cells were washed twice more with 200 μL of Perm/Wash and stained with 50 μL of Perm/Wash containing fluorescent antibodies. The cells were incubated for 45 minutes on ice in a dark condition, washed with 150 μL of Perm/Wash, centrifuged, washed twice more with 200 μL of Perm/Wash, and washed twice with 200 μL of FACS buffer. The cells were fixed with 100 μL of FACS buffer and 100 μL of paraformaldehyde dissolved in PBS and incubated for 30 minutes on ice in a dark condition.

2.12 Flow Cytometry

Samples labeled with fluorescent antibodies were analyzed using a flow cytometer. The injection probe acquired the samples and narrowed them into a stream one cell wide using laminar flow. The cells passed in front of lasers that struck the surface of the cell and the attached fluorescent antibodies. The flow cytometer recorded the forward scatter, which identified the size of the cell, the 90° side scatter, which identified the granularity of the cell, and the amount and wavelength of light produced by the antibodies. This information was analyzed using FlowJo, a flow cytometry analysis program that gated cells based on characteristics such as size, granularity, and the surface proteins they express.

2.13 Statistical Analysis

An unpaired Student t-test was used as indicated to evaluate differences between sample means of two groups. Protein mean fluorescence intensities (MFIs), frequencies of memory precursor effector cells (MPECs), frequencies of short lived effector cells (SLECs), and total CD8 T cell numbers in spleens were compared between untreated and treated groups. All statistical analyses were performed using Prism 5 and P values of statistical significance are depicted by an asterisk as per the Michelin guide scale: * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$); ($P > 0.05$) was considered not significant (ns).

3. Results

3.1 Characterization of Tregs during Homeostasis and Infection

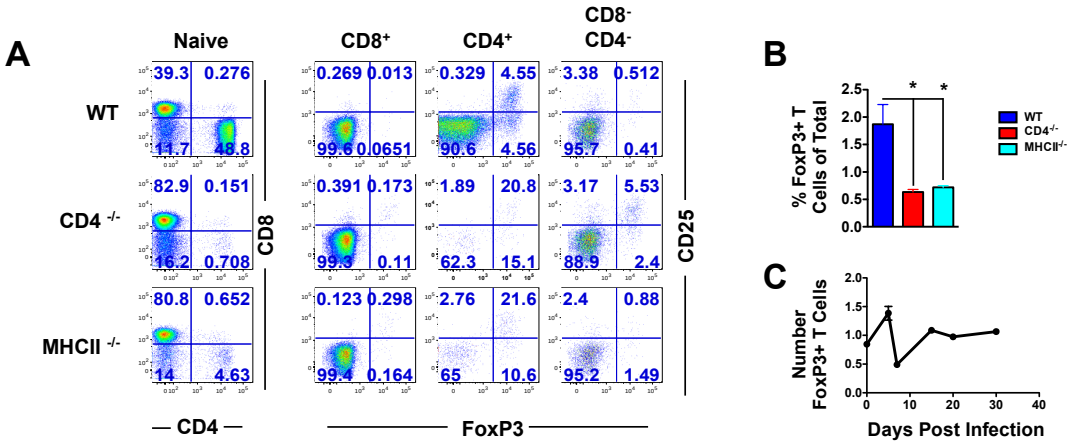

Figure 1: Characterization of Tregs in WT, CD4$^{-/-}$, and MHCII$^{-/-}$ Mice
A. Peripheral blood mononuclear cells (PBMCs) taken from WT, CD4$^{-/-}$, and MHCII$^{-/-}$ mice were stained for CD8, CD4, CD25, and FoxP3. CD4 was plotted against CD8, and the cells from each quadrant (CD8+, CD4+, and CD8- CD4-) were plotted on CD25 versus FoxP3 to identify the presence of FoxP3+ regulatory T cells. **B.** The percentage of FoxP3+ T cells in the blood of each genotypic group. **C.** Longitudinal plot of the number of FoxP3+ T cells in PBMC following infection with $LCMV_{Arm}$ in WT mice (generated by Yevgeniy Yuzefpolskiy). Experiment had three mice per group and was performed once.

We hypothesized that administration of CTLA-4 *in vivo* would mimic the effects of endogenous Tregs. The first step in evaluating this hypothesis was characterization of the Tregs found in WT, CD4$^{-/-}$, and MHCII$^{-/-}$ mice. Peripheral blood mononuclear cells were taken from naïve mice and analyzed using FoxP3, the transcription factor unique to Tregs. WT mice exhibited typical endogenous CD4+ FoxP3+ Tregs (Figures 1A-B). CD4$^{-/-}$ mice, despite the fact that they were incapable of producing CD4+ T cells, exhibited some FoxP3+ T cells that may have served the function of regulatory T cells in the absence of standard endogenous Tregs

(Figure 1B). Compared to WT mice, MHCII$^{-/-}$ mice produced a small amount of FoxP3+ CD4+ T cells (Figure 1B). Theoretically, MHCII$^{-/-}$ mice should not produce any CD4+ T cells because there is no MHC molecule for developing thymocytes to bind to in order to become CD4+ cells; however, this regulatory mechanism is imperfect and, as a result, some CD4+ FoxP3+ T cells developed. These Tregs found in mice lacking CD4 or MHCII could explain the absence of autoimmunity observed in these mice. As expected, mice hindered in their ability to generate CD4+ T cells were deficient in FoxP3+ T cell numbers compared to WT mice. Further analysis needs to be performed to enumerate FoxP3+ T cells in splenocytes of WT, CD4$^{-/-}$, and MHCII$^{-/-}$ mice.

To complete our characterization of endogenous Tregs, we analyzed fluctuations in the population of FoxP3+ T cells in WT mice following infection (Figure 1C). The level of Foxp3+ T cells remained fairly constant throughout the duration of the immune response, increasing slightly around day fifteen when the population of effector T cells began to contract.

3.2 Administration of CTLA-4 at Day 7 Post Infection Exerts Minimal Impact on the Contraction of Antigen Specific CD8 T Cells

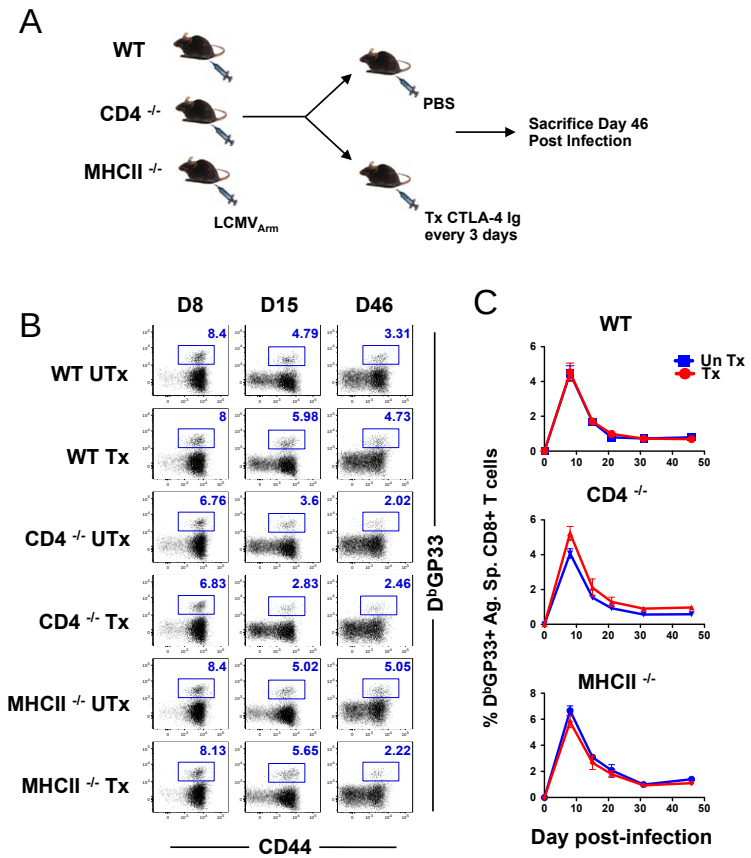

Figure 2: Effect of Day 7 Post Infection CTLA-4 Administration on Antigen Specific CD8 T Cell Numbers in WT, CD4$^{-/-}$, and MHCII$^{-/-}$ Mice

A. Each genotypic group (WT, CD4$^{-/-}$, and MHCII$^{-/-}$) was divided into a control and treatment group. The control group received injections of PBS intraperitoneally every three days, beginning at day 7 post infection. The treatment group received injections of CTLA-4 every three days, beginning at day 7 post infection. Mice were sacrificed at day 46 post infection for tissue analysis. **B.** Peripheral blood mononuclear cells (PBMCs) were taken from each control and treatment group and stained for CD8, CD44, and D^{b}GP33. PBMCs were plotted on CD44 versus CD8 (not shown). CD8+ T cells were gated and plotted on D^{b}GP33 versus CD44. D^{b}GP33+ CD44+ cells were gated, and their percentage of CD8+ T cells calculated. **C.** Longitudinal plot of the percentage of D^{b}GP33+ CD8 T cells for untreated versus treated groups for each genotype of total PBMCs. Experiment had three mice per group and was performed once.

To examine the effect of CTLA-4 administration *in vivo* beginning at the peak of expansion, six mice of each of the three genotypes, WT, $CD4^{-/-}$, and $MHCII^{-/-}$, were infected with $LCMV_{Arm}$. Within each genotypic group, three mice received injections of CTLA-4 intraperitoneally every three days beginning at day seven post infection, while the remaining three mice received intraperitoneal injections of PBS every three days beginning at day seven post infection (Figure 2A). At day 46 post infection, the mice were sacrificed for tissue analysis.

The kinetics of memory formation did not demonstrate any significant changes between the treated and untreated genotypic groups (Figure 2B). However, differences in the frequencies of D^bGP33 specific cells were seen between the phenotypic groups (Figure 2C). $CD4^{-/-}$ mice exhibited decreased expansion of antigen specific CD8 T cells compared to WT and $MHCII^{-/-}$ groups (Figure 2B). $MHCII^{-/-}$ mice seemed to have the highest expansion of D^bGP33 antigen specific CD8 T cells of total PBMCs (Figure 2C).

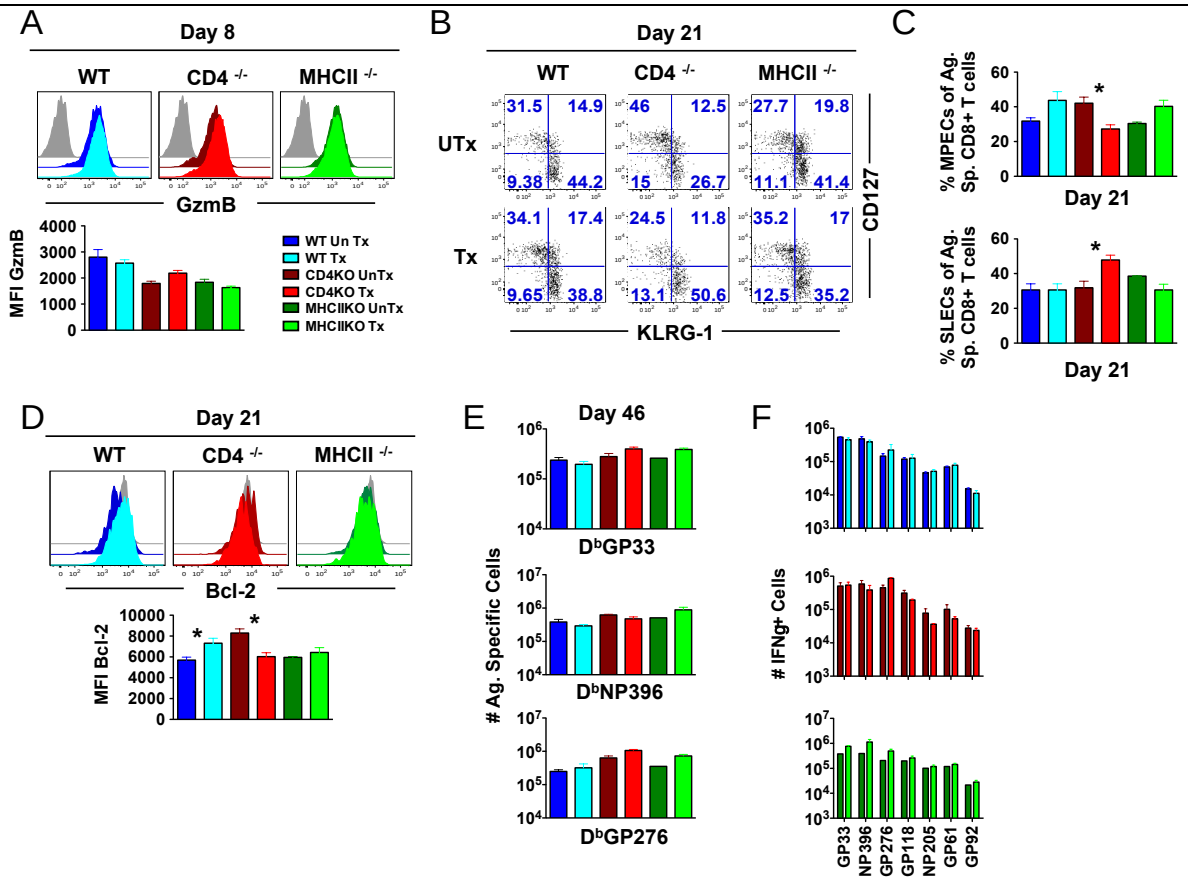

Figure 3: Effector Differentiation of CD8+ T Cells Treated with CTLA-4 Beginning at Day 7 Post Infection in WT, CD4$^{-/-}$, and MHCII$^{-/-}$ Mice
A. Histogram and bar graph of mean fluorescence intensity of GzmB of D^{b}GP33 CD8+ T cells at day 8 post infection. **B.** CD8+ CD44+ D^{b}GP33+ T cells plotted on CD127 versus KLRG-1 at day 21 post infection. **C.** Bar graph of the frequency of MPECs (CD127+ KLRG-1-) and SLECs (KLRG-1+ CD127-) of D^{b}GP33+ CD8+ T cells at day 21 post infection. **D.** Histogram and bar graph of mean fluorescence intensity of Bcl-2 of D^{b}GP33 CD8+ T cells at day 21 post infection. **E.** Number of D^{b}GP33+, D^{b}GP276+, and D^{b}NP396+ CD8+ T cells in the spleen at day 46 post infection. **F.** Number of IFN-γ+ antigen specific CD8+ T cells in the spleen following *ex vivo* peptide stimulation at day 46 post infection. Experiment had three mice per group and was performed once.

Treatment with CTLA-4 at day seven post infection did not produce any differences in granzyme B expression between treated and untreated groups in WT, CD4$^{-/-}$, or MHCII$^{-/-}$ mice (Figure 3A). Unsurprisingly, WT mice were higher for granzyme B overall, suggesting a more effective effector CD8 T cell population due to the presence of functional CD4 T cells (Figure

3A). By day 21 post infection, during the contraction phase of effector CD8 T cell differentiation, WT and MHCII$^{-/-}$ mice exhibited increased MPEC formation in CLTA-4 treated groups while CD4$^{-/-}$ mice treated with CLTA-4 exhibited decreased MPEC formation and increased SLEC formation compared to the untreated controls (Figure 3B-C).

In accordance with the increase in MPEC formation, WT mice treated with CTLA-4 demonstrated increased expression of the prosurvival molecule Bcl-2 (Figure 3D). Conversely, CD4$^{-/-}$ mice, in accordance with their decreased frequency of MPECs, demonstrated decreased Bcl-2 expression in CTLA-4 treated mice compared with the untreated control (Figure 3D). However, there were no significant changes in the expression of Bcl-2 for MHCII$^{-/-}$ mice treated with CTLA-4 (Figure 3D).

Despite the differences in MPEC and SLEC formation for all of the genotypic groups, by day 46 post infection, no significant differences were observed in the number of D^{b}GP33, D^{b}GP276, or D^{b}NP396 specific cells following splenocyte enumeration (Figure 3E). Additionally, peptide stimulation using seven different epitopes of LCMV glycoproteins (GP) and nuclear proteins (NP) (GP33, NP396, GP276 [immunodominant], GP118, NP205, GP92, and GP61 [immunorecessive]) did not produce any significant differences in the number of IFN-γ+ CD8+ T cells (Figure 3F). In summary, treatment with CTLA-4 at day seven post infection during peak expansion appeared to accelerate memory formation, but only during the contraction phase.

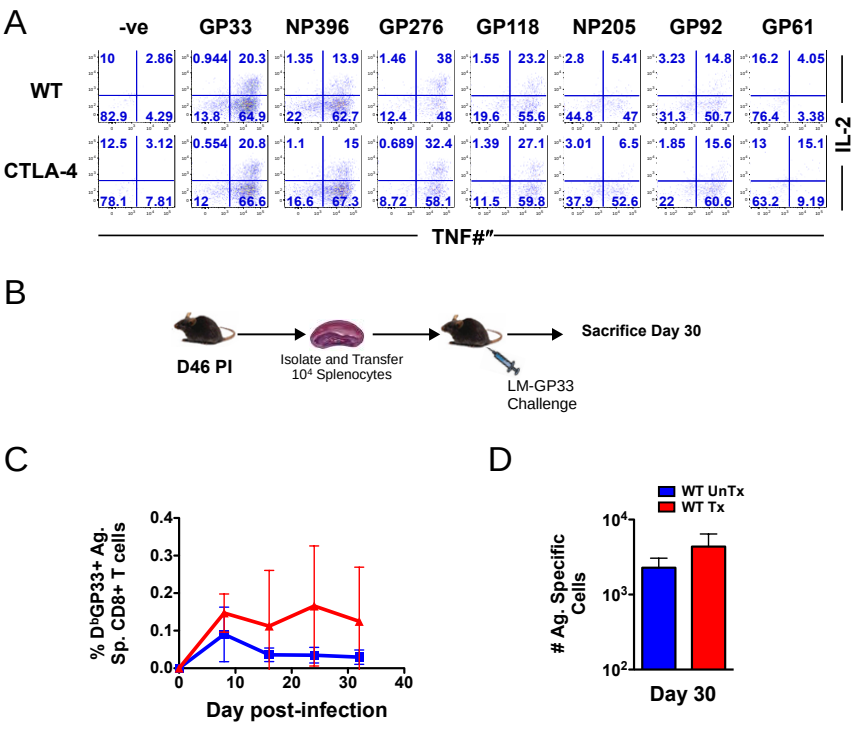

Figure 4: Effect of CTLA-4 Administration Beginning at Day 7 Post Infection on Effector Differentiation and Memory Recall of WT Mice

A. Plot of IL-2 versus TNF-α for antigen specific CD8+ T cells stimulated with seven epitopes of the LCMV glycoprotein (GP) and nuclear protein (NP) gated on IFN-γ producing CD8 T cells. **B.** WT mice treated with PBS or CTLA-4 starting at day 7 post infection were sacrificed at day 46 post infection. Splenocytes from each group were adoptively transferred into two groups of WT mice, one of which received untreated splenocytes while the other received treated splenocytes. Both groups were infected with LM-GP33 and sacrificed at day 30 post infection for tissue analysis. **C.** Longitudinal pot of the percentage of total PBMCs of D^bGP33+ CD8+ T cells present in the blood of recipient mice. **D.** Number of D^bGP33+ CD8+ T cells in the spleen at day 30 post infection. Experiment had three mice per group and was performed once.

We used peptide stimulation with seven different epitopes of the LCMV glycoprotein (GP) and nuclear protein (NP) to evaluate the polyfunctionality of the memory cells formed following CTLA-4 treatment of WT mice begun at day seven post infection. There were no differences in the functionality of treated versus untreated cells in any of the analyzed peptides for WT (Figure 4A) or $CD4^{-/-}$ or $MHCII^{-/-}$(data not shown).

In order to evaluate the recall response of memory cells that had been treated with CTLA-4 starting at peak expansion, we collected the splenocytes from CTLA-4 treated and untreated WT mice sacrificed at day 46 post infection. We adoptively transferred 10^4 splenocytes from CTLA-4 treated and untreated WT mice into naïve mice (Figure 4B). These mice were then infected with LM-GP33 as a secondary challenge, bled longitudinally, and sacrificed at day 30 post infection to evaluate the quality of their response.

Memory cells treated with CLTA-4 during their first challenge did not show a significant difference in the expansion or maintenance of their numbers following secondary challenge (Figure 4C). Additionally enumeration of splenocytes failed to demonstrate any significant differences in the number of antigen specific memory cells following secondary infection (Figure 4D). In conclusion, the quality of the memory formed in the presence or absence of CTLA-4 treatment begun during peak expansion at day seven post infection failed to exhibit any differences beyond the early contraction time point.

3.3 Administration of CTLA-4 at Day 2 Post Infection in WT Mice

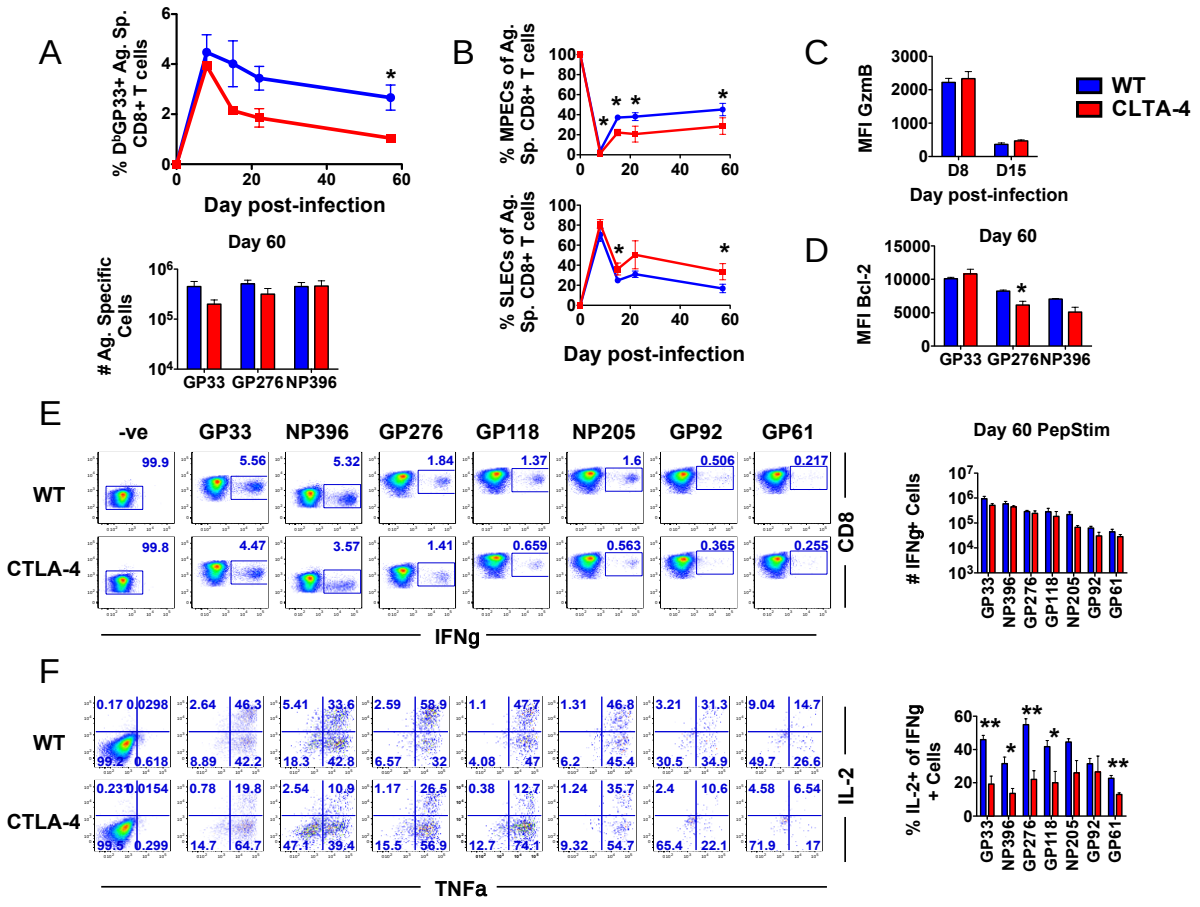

Figure 5: Effector Differentiation of CD8+ T Cells Treated with CLTA-4 at Day 2 Post Infection in WT Mice

A. Longitudinal plot of the percentage of D^bGP33+ cells of CD8+ T cells in the blood. Bar graph of the number of D^bGP33+, $D^bGP276+$, and $D^bNP396+$ CD8+ T cells in the spleen at day 60 post infection. **B.** Longitudinal plot of frequency of MPECs (CD127+ KLRG-1-) and SLECs (KLRG-1+ CD127-) of D^bGP33+ CD8+ T cells. **C.** Bar graph of mean fluorescence intensity of GzmB of D^bGP33+ T cells at days 8 and 15 post infection. **D.** Bar graph of mean fluorescence intensity of Bcl-2 of D^bGP33+, $D^bGP276+$, and $D^bNP396+$ CD8+ T cells in the spleen at day 60 post infection. **E.** Plot and bar graph of CD8 versus IFN-γ+ T cells stimulated *ex vivo* with seven epitopes of LCMV glycoprotein and nuclear protein at day 60 post infection. **F.** Plot and bar graph of IL-2 versus TNF-α for antigen specific CD8+ T cells stimulated with seven epitopes of the LCMV glycoprotein (GP) and nuclear protein (NP). Experiment had three mice per group and was performed once.

We wanted to further characterize the effect of CTLA-4 on effector CD8 T cells and determine if there is a critical time point of administration. Thus, we next administered CTLA-4

in vivo to WT mice beginning at day two post infection during priming of the CD8 T cell response. CD8 T cells specific for the D^bGP33 epitope of $LCMV_{Arm}$ slightly decreased expansion and significantly increased contraction compared to untreated populations (Figure 5A). This trend continued until day 60 post infection when enumerated splenocytes demonstrated lower numbers of cells specific for the D^bGP33 and D^bGP276 epitopes of $LCMV_{Arm}$ (Figure 5A).

Following effector expansion, CD8 T cells undergo contraction and memory differentiation. During this phase of the immune response, the frequency of memory precursor effector cells (CD127+ KLRG-1-; MPECs) increases, while the frequency of short lived effector cells (KLRG-1+ CD127-; SLECs) declines. Treatment with CTLA-4 during priming decreased the frequency of MPECs and increased SLEC formation (Figure 5B). This decrease in the number of memory precursors and increase in the number of effector cells could be responsible for the overall decrease in the number of antigen specific splenocytes demonstrated in Figure 5A. The level of granzyme B expression was similar between treated and untreated CD8 T cell populations, indicating that effector function was not impacted (Figure 5C). SLECs are a pro-apoptotic population while MPECs differentiate into long-lived cells, thus they differentially express prosurvival markers such as Bcl-2. Expression of Bcl-2 was lower in cells treated with CLTA-4, a finding consistent with the increased frequency of SLECs and the decreased total population of antigen specific memory cells (Figure 5D).

Using peptide stimulation, we evaluated the effect of CTLA-4 on the diversity of the immune response. Seven different epitopes of the LCMV glycoprotein (GP) and nuclear protein (NP) were considered, most of which exhibited lower numbers of antigen specific cells when primed in the presence of CLTA-4 (Figure 5E). Polyfunctionality for each of the peptides also

was considered; each set of antigen specific cells treated with CTLA-4 demonstrated significantly decreased polyfunctionality (Figure 5F).

Overall, administration of CTLA-4 *in vivo* at day two post infection during priming of the immune response appears to increase the formation of terminal effector cells and decrease the formation of memory precursors. These changes are reflected in the number of cells present at memory and in the polyfunctionality of those cells.

3.4 Administration of CTLA-4 at Day 4 Post Infection in WT Mice

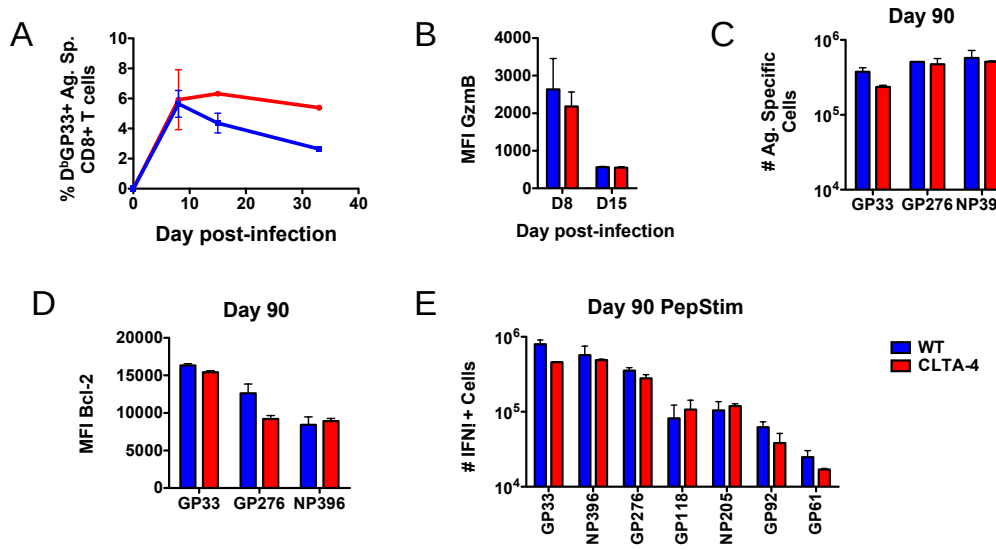

Figure 6: Effector Differentiation of CD8+ T Cells Treated with CTLA-4 at Day 4 Post Infection in WT Mice
A. Longitudinal plot of the percentage of D^bGP33+ cells CD8+ T cells of total PBMCs. **B.** Bar graph of mean fluorescence intensity of granzyme B of D^bGP33+ CD8+ T cells at days 8 and 15 post infection. **C.** Bar graph of the number of D^bGP33+, $D^bGP276+$, and $D^bNP396+$ CD8+ T cells in the spleen at day 90 post infection. **D.** Bar graph of mean fluorescence intensity of Bcl-2 of D^bGP33+, $D^bGP276+$, and $D^bNP396+$ CD8+ T cells in the spleen at day 90 post infection. **E.** Bar graph of CD8 versus IFN-γ+ T cells for seven epitopes of LCMV glycoprotein and nuclear protein at day 90 post infection. Experiment had three mice per group and was performed once.

We next administered CTLA-4 *in vivo* during antigen clearance, beginning at day four post infection. Peak numbers of D^bGP33 specific T cells did not exhibit any differences between treated and untreated groups but, throughout the course of treatment, there was a slight increase in the number of D^bGP33 cells in the blood (Figure 6A). The effector function of the CD8 T cells in the two groups was evaluated using the level of granzyme B production which did not demonstrate any differences in mean fluorescence intensity between the two groups (Figure 6B).

Enumeration of splenocytes at day 90 post infection did not exhibit significant differences between treated and untreated D^bGP33, D^bGP276, or D^bNP396 specific memory CD8 T cells (Figure 6C). Consistently there were no significant differences in the expression of Bcl-2 between treated or untreated cells in any of the three antigen specific groups (Figure 6D). An examination of seven epitopes of $LCMV_{Arm}$ did not produce any significant differences between the numbers of antigen specific cells in treated versus untreated groups (Figure 6E). TNF-α and IL-2 also showed no significant differences (data not shown). In short, treatment with CTLA-4 *in vivo* during antigen clearance does not seem to have a significant effect on memory CD8 T cell differentiation.

3.5 Effect of Treg Expansion on Effector Differentiation in WT Mice

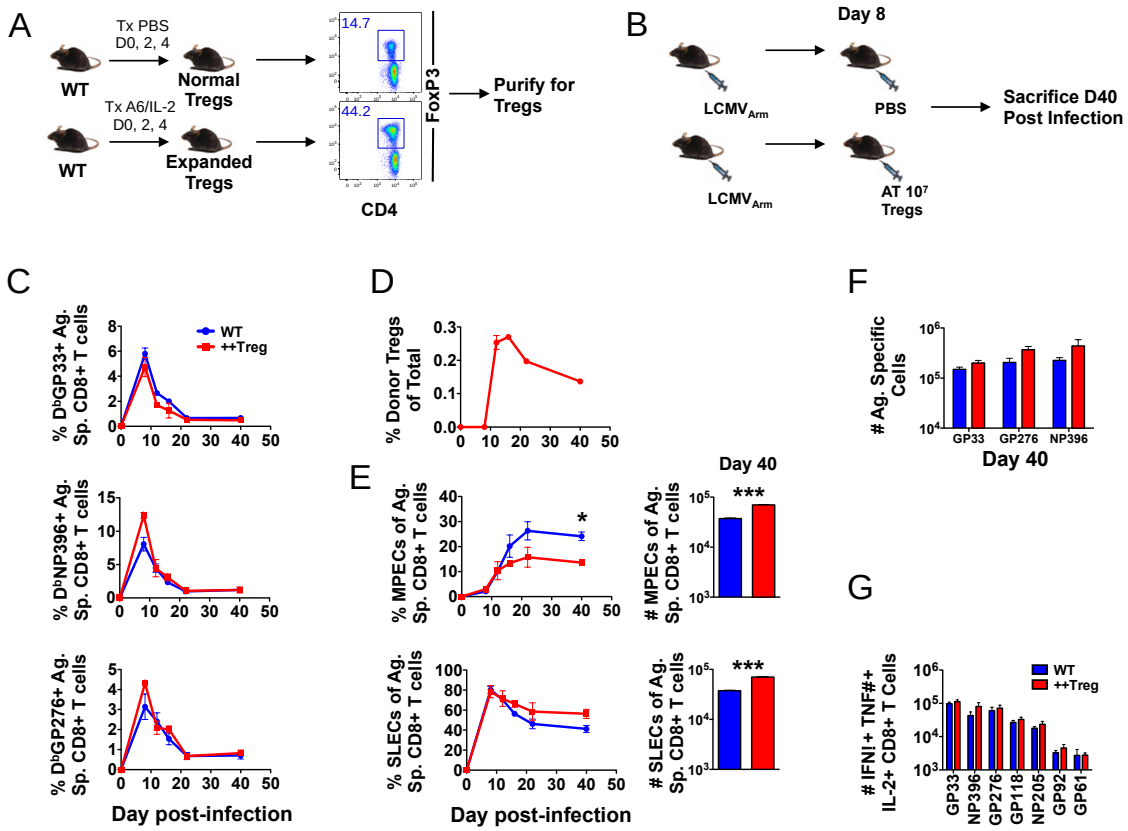

Figure 7: Effector Differentiation of Adoptively Transferred Expanded Tregs in WT Mice
A. To expand the Tregs of naïve WT mice, IL-2/αIL-2 was administered at day 0, 2, and 4. Peripheral blood mononuclear cells (PBMCs) were stained for CD4 and FoxP3 and compared to untreated, naïve WT. Expanded Tregs were then purified. **B.** Two groups of WT mice were infected with $LCMV_{Arm}$. At day 8 post infection, 10^7 Tregs were adoptively transferred to one group of mice. Both groups were sacrificed at day 40 post infection for tissue analysis. **C.** Longitudinal plot of the frequency of D^bGP33+, $D^bGP276+$, and $D^bNP396+$ CD8+ T cells of total PBMCs. **D.** Longitudinal plot of the frequency of donor Tregs in recipient mice. **E.** Longitudinal plot of frequency of MPECs (CD127+ KLRG-1-) and SLECs (KLRG-1+ CD127-) of D^bGP33+ CD8+ T cells. Bar graph of the number of MPECs (CD127+ KLRG-1-) and SLECs (KLRG-1+ CD127-) of D^bGP33+ CD8+ T cells at day 40 post infection. **F.** Bar graph of the number of D^bGP33+, $D^bGP276+$, and $D^bNP396+$ CD8+ T cells in the spleen at day 40 post infection. **G.** Bar graph of CD8 versus IFN-γ+ T cells for seven epitopes of LCMV glycoprotein and nuclear protein at day 90 post infection. Experiment had three mice per group and was performed once.

Tregs have the highest expression of CTLA-4 compared to all other somatic cells. We propose that CLTA-4 supplementation during infection mimics the effect of increased endogenous Tregs. In order to test this hypothesis, we administered an IL-2/αIL-2 mix treatment on days zero, two and four to a WT mouse in order to expand their endogenous Treg population (Figure 7A). PBS was administered to a control mouse on the same schedule. Both mice were bled and their peripheral blood mononuclear cells stained with FoxP3 to ascertain the presence of additional Tregs in the IL-2 treated mouse. The expanded Tregs were collected from the treated mouse and adoptively transferred at day eight post infection to mice that had been infected with $LCMV_{Arm}$. Control mice were given PBS at day eight in order to serve as a control for the Treg transfer (Figure 7B). The presence of the transferred Treg population was monitored in longitudinal bleeds (Figure 7D).

Neither treatment with CTLA-4 (Figure 2C) nor adoptive transfer with expanded Tregs influenced the kinetics of memory formation of D^bGP33, D^bGP26, or D^bNP396 specific cells (Figure 7C). Unlike the CLTA-4 treatment (Figure 3B-C), longitudinal bleeds of mice receiving adoptively transferred Tregs seemed to exhibit a decreased frequency of MPEC formation (Figure 7E). However, upon enumeration of MPECs and SLECs in the splenocytes at day 40 post infection, overall increases in the numbers of MPECs and SLECs were observed in mice receiving a Treg transfer compared to WT mice (Figure 7E). Enumeration of splenocytes at day 40 also demonstrated an increase in the number of D^bGP33, D^bGP276, and D^bNP396 specific CD8 T cells in recipients of expanded Tregs compared to mice treated with CTLA-4 (Figure 7F).

Polyfunctional cells capable of producing TNF-α, IFN-γ, and IL-2 (triple producers) are considered high quality memory cells. Peptide stimulation of splenocytes for seven epitopes of $LCMV_{Arm}$ demonstrated an increase in the number of triple producing cells for all peptides in

mice receiving expanded Tregs compared to WT mice (Figure 7G). In summary, similar to treatment with CTLA-4, transferring Tregs increased the formation of MPECs. Additionally, transfer of expanded Tregs also increased the formation of SLECs in a manner that CTLA-4 alone did not. Thus, while CTLA-4 alone does mimic some of the regulatory effects of Tregs, Tregs still have additional mechanisms of action that facilitate memory differentiation.

4. Discussion

Vaccines are an important and active field of study. One aspect of vaccine development being explored is the use of modulators to improve the efficacy of vaccination. The potential for more effective vaccines extends as far as cancer vaccination; currently, blockers of PD-1 and LAG-3 are being explored in an attempt to rescue T cells exhausted by chronic infections[15,16,17,18,19]. As a result of their role in regulating autoimmunity and T cell function during the course of an infection, Tregs are a vital component of the immune system. They express CTLA-4 at a higher level than any other cell type in the body[20,21]. CTLA-4 is a surface molecule that binds to CD80 and CD86 more efficiently than CD28, a costimulator of T cells. By binding to CD80 and CD86, Tregs prevent CD28 from doing so, thus limiting the extent of T cell activation.

Early during infection, CD28 is upregulated highly on CD8 T cells. When CD28 binds to CD80 and CD86 on the surface of antigen presenting cells, it acts as a secondary signal initiator, activating the T cell and preparing it to combat an infection. When we added CTLA-4, beginning at day two, contraction of antigen specific cells was increased significantly, leading to lower numbers of memory cells. It is possible that, by administering CTLA-4, we were hindering the costimulation of CD8 T cells and impacting their ability to differentiate into long lived memory precursor cells. Furthermore, CD8 T cells upregulate CTLA-4 at the peak of the immune response. Adding CTLA-4 in vivo could decrease CD8 T cell intrinsic signaling through CTLA-4 by acting as a competitive inhibitor. Elucidation of this mechanism will require examining differences in the effector function of treated versus wild type cells and performing a

recall to compare their memory quality. Additionally, we could test for viral persistence during days six through eight post infection in CTLA-4 treated mice. It is possible that blocking the costimulatory functions of CD28 caused increased exposure of CD8 T cells to antigen, negatively impacting the quality of the memory cells.

Treatment with CTLA-4 beginning at day four post infection failed to demonstrate any significant differences in effector differentiation or cell numbers. Kinetic studies demonstrate that the number of Tregs decreases following initial infections, decreasing the amount of CTLA-4 present in wild type mice. Administration of CTLA-4 during this time period could be ineffective because Treg mediated control of the immune response is not utilized by the body at this point in time, as indicated by the decreased number of Tregs.

Administration of CTLA-4 during peak expansion appears to participate in accelerating the conversion of CD8 T cells from effector to memory. This is evidenced by an increase in the number of memory precursor cells and expression of Bcl-2 at day 21 post infection. In terms of vaccine supplementation, this is the most promising aspect of CTLA-4 treatment because it could accelerate the generation of long lasting memory cells, an important consideration for vaccine time points. Further avenues of research would include administration of CLTA-4 crosslinked with antibody to determine if increasing the concentration of CTLA-4 that cells recognize increases the observed phenotype. Additionally, we could block CD28 on memory CD8 T cells to evaluate whether CTLA-4 acts directly on T cells or blocks normal T cell interactions with antigen presenting cells expressing CD80 and CD86.

Expansion of endogenous Tregs is one therapeutic method being investigated as a means of treating autoimmune diseases such as multiple sclerosis. Expansion of Tregs at day eight, after antigen clearance, increased the number of memory precursor cells, but also increased the

survival of short lived effector cells. This observation suggests that Tregs exert additional prosurvival effects directly on effector CD8 T cells or on their environment.

Understanding the effect of CTLA-4 on T cell expansion and memory maintenance must be scrutinized to determine the range of its effects on the immune response when administered as vaccine supplement. Recent studies from our lab suggest that CTLA-4 administration in certain instances can sustain the regulator effect of Tregs, despite the fact that they may be entirely absent. The role of CTLA-4 on effector differentiation and its mode of action, whether it is through antigen presenting cells or direct interaction with antigen specific CD8 T cells, remains to be determined. Results of these enquiries will guide further therapeutic endeavors regarding the administration of CTLA-4.

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Orbital sinus and submandibular blood sample collections in mice
Flow cytometry, microscopy, and data analysis using FlowJo software

Honors and Awards

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Undergraduate Faculty Award American Association of Immunologists	Spring 2014
The Evan Pugh Scholar Award	April 2014
The President Sparks Award	April 2013
The President's Freshman Award	April 2012

Volunteer Activities

Honey Brook Animal Hospital, Honey Brook, PA November 2011 to present

- Observe general appointments, emergency cases, and surgeries for over 400 hours
- Restrain animals, prepare equipment, and sanitize facilities

Ryerss Farm for Aged Equines, Pottstown, PA Summer 2014

- Groom, feed, walk, and administer medications to 30 horses
- Clean stalls and barns, maintain property appearance and cleanliness

Employment

Kennel Assistant, Tymberwyck Kennel, Pottstown PA Summer 2008 to 2009

- Clean, feed, walk, and groom 50 animals each week
- Perform administrative tasks, including file organization and fielding telephone calls
- Sanitation of facilities