Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Pablo Penaloza-MacMaster Date

Manipulation of Immune Regulatory Mechanisms During Chronic Infection

By
Pablo Penaloza-MacMaster
Doctor of Philosophy
Graduate Division of Biomedical and Biological Sciences
Immunology and Molecular Pathogenesis

_____
Rafi Ahmed
Advisor

_____
Brian Evavold
Committee Member

_____
James Zimring
Committee Member

_____
Rama Amara
Committee Member

_____
Robert Mittler
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Manipulation of Immune Regulatory Mechanisms During Chronic Infection
By

Pablo A. Penaloza-MacMaster
B.S., Florida International University, 2003

Advisor: Rafi Ahmed, Ph.D.

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Immunology and Molecular Pathogensis
2011

Abstract

Manipulation of Immune Regulatory Mechanisms During Chronic Infection

By Pablo Penaloza-MacMaster

Chronic infections affect millions of people worldwide, and herald significant decline in economic growth. A factor that contributes to the establishment of a persistent infection is the progressive dysfunction that takes place on antigen-specific T cells. A major focus in the field of immunology is the rescue of epitope-specific T cells by blocking or triggering specific receptors on the surface of immune cells. Here we analyze the contribution of different immune regulatory pathways during chronic viral infection in mice. We also show a role for CD4+ Foxp3+ T regulatory (Treg) cells in maintaining CD8 T cell exhaustion during chronic infection. Collectively, our data suggests that Treg-mediated suppression is dependent on antigen abundance, CD4 cells, and the B7 pathway. These findings show that immune regulatory pathways could be manipulated during chronic viral infection in order to rescue T cell function.

Manipulation of Immune Regulatory Mechanisms During Chronic Infection

By

Pablo A. Penaloza-MacMaster
B.S. Florida International University, 2003

Advisor: Rafi Ahmed, Ph.D.

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Immunology and Molecular Pathogensis
2011

Acknowledgements

I would like to dedicate this work to all the people who I call family.

Table of Contents

Chapter 4: Enhancing the T Cell restorative effect of PD-L1 blockade by depletion of CD4 cells

Chapter 5: T regulatory cells sustain CD8 T cell exhaustion during chronic infection

Chapter 6: Discussion

Table of Figures

Chapter 1: Introduction

Chronic Viral Infections and T Cell Exhaustion

Throughout million of years of natural selection, multi-cellular organisms have developed diverse abilities to protect their biomasses from the outer world. At the macroscopical level, fight-or-flight actions provide immediate defense from external insults. At the microscopical level, a more complex set of defense mechanisms takes place. Defense against microorganism that we do not usually see is the role of the immune system. Virtually every organism has immune defense systems, but of all species, the chordates have developed a very sophisticated army of immune cells with the ability to recombine their specificity receptors, and remember intruders upon secondary exposure(35). This stochastic process of producing different specificity receptors provides an ample opportunity for pathogen recognition. Vertebrate immune surveillance is divided into innate and adaptive immune systems. The innate immune sytem is comprised by dendritic cells, macrophages, and other cells that quickly recognize molecular patterns in pathogens, and instruct cells of the adaptive immune system to mount a response.

These cells that are capable of recombining their specificity receptors are collectively called B and T lymphocytes, are the main players of adaptive immunity. B cells are in charge of neutralizing pathogens, but they can also present antigen to prime T cell responses. T cells, on the other hand, recognize

pieces of intruders on the surface of cells, and proliferate furiously, undergoing extensive phenotypic changes that allow them to “remember” better. T cells can posses either a CD4 or CD8 correceptor that aids in T cell receptor binding to the major histocompatibility complex (MHC) molecules on the surface of cells(7). T cells require three independent signals for activation: TCR ligation, costimulation and cytokines (113). The TCR binds to the MHC:peptide complex providing direct recognition of foreign pathogen components. Then, costimulatory signals and cytokines such as IL-2 provide survival advantages on T cells(113). A more detailed description of costimulation will be included in Chapters 2 and 3 of this thesis.

The thesis work presented here focuses on the effect of manipulating costimulatory or inhibitory pathways during viral infection. T cells undergo well-characterized behaviors after acute or chronic infections. If the pathogen is cleared, T cell numbers contract down to 5% of the peak response, and a very stable population of fully functional memory cells survives for the rest of the host’s life(17, 70). On the other hand, if the pathogen persists, T cells progressively lose the ability to express interleukin 2 (IL-2), tumoral necrosis factor a (TNFα), and interferon gamma (IFNγ) expression(135, 141). This is collectively referred to as T cell functional exhaustion. T cell dysfunction occurs during chronic viral infections such as those with HIV, HBV, and HCV, in which the pathogen is not cleared from the system. Our lab and others have shown that during chronic viral infection, T cell exhaustion goes hand by hand with the expression of inhibitory receptors. These progressive defects in T cell function

have also been observed in human chronic viral infections such as human immunodeficiency virus (HIV)(39, 72), hepatitis B virus (HBV)(21), and hepatitis C virus (HCV)(99), in which the pathogen is not cleared from the host.

T cell exhaustion also is linked to very distinct phenotypic changes. Our lab and others have shown that during chronic viral infection, T cell exhaustion correlates with the expression of several inhibitory receptors(11, 16). The programmed death 1 (PD-1) inhibitory pathway has been the most extensively studied. Ligation of PD-1 to its known ligands, (PD-L1 and PD-L2) induces inhibition of CD8 T cell function (figure 1). At the molecular level, ligation of PD-1 to its ligands results in dephosphorylation of the T cell receptor (TCR) signalosome, resulting in decreased TCR signal transduction, and reduced T cell cytotoxic activity(116).

It was first shown in our lab that blockade of the PD-1 pathway, in a model of chronic lymphocytic choriomeningitis virus (LCMV) infection, restores CD8 T cell function and results in reduction of viral loads(11). Blocking the PD-1 pathway has also shown promising results during other established viral infections such as those with SIV(129) and HCV(97-99). There is a delicate balance between a protective T cell response and T cell mediated immunopathology. This threshold is usually the threat that persistent viruses impose to their hosts. In other words, an efficient T cell response could curtail pathogen replication, but it can also induce excessive cytolysis of infected or neighboring cells. This occurs because on the surface of infected cells T cells recognize peptides derived from pathogens. Once they recognize infected cells

from the host, T cell kill them, curtailing additional pathogen replication, but also killing the host cell that harbored the intruder. In addition, cytokines are released and they act on both infected ad uninfected cells, resulting in additional nonspecific effects that are often detrimental to healthy cells. The immune system could also undergo a phase of “molecular alert”, known as cytokine storm, which induces extreme discomfort to the host, and is characterized by pain, fever, inflammation, and sometimes even death(55).

Therefore, the immune response can act as a double-edged sword, and manipulation of immune regulatory pathways constitute an enticing area of investigation. It may be that temporal fine-tunning of regulatory pathways could provide a boost to the immune system in the pathogen-host battle. Perhaps by boosting T cell responses during a small period of time, it may be possible to induce significant curtailing of viral replication with minimal bystandard damage. This may affect the outcome of a chronic infection. I will describe in the final chapters of this thesis how it is critical to control the timing and duration of immune modulation. Throughout the pages of this thesis, the reader will understand the importance of immune regulation, and how its brief manipulation could result in benefit to the host.

We have taken advantage of the widely employed murine LCMV system to study chronic viral infections and address our experimental questions. LCMV is a non-cytopathic RNA virus (Arenaviridae) containing two ambisense RNA strands termed L and S (long and short segments). The L segment encodes for the polymerase, whereas the S segment codes for the glycoprotein (GP) and

nucleoprotein (NP). The GP is cleaved into GP1 and GP2, which folds to form the tetrameric spike that aids in cellular receptor binding(26). The NP is the internal constituent of the virus, and may be involved in transcription of viral nucleic acid.

LCMV is a great model to study immune responses to viral infections because depending on the viral strain used the infection can be acute (Armstrong strain) or chronic (Cl-13 strain)(1, 2). These two strains differ in only two point mutations in the GP and the polymerase genes. The chronic strain LCMV Cl-13 has a wider tropism, and its polymerase replicates RNA faster, compared to the acute LCMV Armstrong strain(109). Besides infecting dendritic cells (DC) more efficiently than the Armstrong strain, the Cl-13 strain has been shown to infect fibroblastic reticular cells (FRC) of the spleen(84). These two strains were isolated by my mentor (Rafi Ahmed) when he was a postdoctorate fellow at Michael Oldstone lab(2). The parental Armstrong strain was isolated from the central nervous system of Armstrong infected-mice (carriers), whereas the newly generated Cl-13 strain was a selected isolate from the spleen. An interesting question is how Cl-13 is selected in the spleen of Armstrong-neonatally infected mice, despite the absence of T cell responses that may select variants. Three possibilities may help to explain this interesting organ-specific selection of LCMV strains. First, type I interferons could shape selection of variants in different tissues. One may speculate that type I interferons could also act on cells of the innate immune system to induce viral control or expression of pathogen recognition molecules. The second possibility could be differences in cellular

factors that may favor replication of particular virus quasi-species. The third possibility may be that T cells control virus differently in particular organs, allowing for distinct selection bottlenecks. Until now, it is not clear what accounted for this selection of LCMV strains in carrier mice.

The LCMV model enabled us to follow epitope-specific T cell responses to a chronic or acute infection, since T cell specificities remain unchanged despite the two aminoacid substitutions between these two viral strains. T cell responses to these two LCMV strains is quite different(135). LCMV Armstrong infection is cleared within one week of infection, whereas infection with the more virulent CL-13 strain results in a viremia lasting 2-3 months, and lifelong viral replication in the brain and kidney(135). T cell responses after Armstrong infection are fully functional, expressing multiple cytokines, and conferring protection after Cl-13 challenge(132, 135, 141). The precursor frequency of an immunodominant viral-specific response (GP33-41) is estimated to be around 200 cells, and this number is expanded 10^4-10^5 fold by the peak of the immune response to LCMV(17). After several weeks, only 5-10% of these expanded cells (those that express the interleukin-7 receptor) survive(70), and retain the ability to proliferate extensively and produce cytokines upon antigen re-challenge(132).

Conversely, infection with LCMV Cl-13 results in T cell exhaustion, characterized by hypo-responsiveness (135), and expression of co-inhibitory receptors(16, 68). Exhausted CD8 T cells preferentially migrate to peripheral tissues(135). The use of MHC class I tetramers to identify antigen-specific CD8 T after chronic infection, allowed the detection of viral-specific CD8 T cells(3).

Intracellular cytokine staining misled some researchers in the field to think that there was total physical deletion of viral-specific T cells during chronic infection. We now know that antigen-specific CD8 T cells do not completely disappear during a chronic infection, but they fail to make detectable amounts of cytokines. The progression to exhaustion occurs in a hierarchical fashion, as CD8 T cells first lose the ability to make IL-2, then TNFα, and IFNγ(141). These cells are also different phenotypically from those generated after an acute infection. During persistent infection, CD8 T cells become CD127- and CD62L-, whereas cells generated after acute infections are positive for these memory markers. CD62L is a lymph node homing molecule, and CD127 is the IL-7 receptor a chain, important for memory conversion(70, 141).

Cells of different antigen-specificity also exhaust at different rates. Immunodominant epitope-specific responses are the first to exhaust, and subdominant responses usually preserve functionality for longer times(135). Even though the CD8 T cells from chronically infected mice are still able to kill targets, they require supraphysiological concentrations of peptide to respond, whereas those cells from acutely infected mice can kill at lower peptide concentrations(10). The LCMV Cl-13 infection model can also be subdivided into a milder chronic infection (viremia lasts 2-3 months), and a more stringent infection with lifelong viremia, induced by CD4 depletion prior to infection(81). Stringent Cl-13 infection results in a more pronounced T cell exhaustion and increased viral loads compared to CD4 undepleted, Cl-13 infected mice. These findings previously reported by Matloubian and Ahmed(81) highlight the

importance of CD4 help during priming of responses to chronic infection. Recently, in an issue of Science, two independent laboratories reported the essential role of IL-21 as a CD4 signal required for enhancing CD8 T cell function during chronic infection(43, 142).

Even though PD-1 blockade mediated CD8 T cell restoration can be achieved in both models of chronic infection (in the presence or absence of CD4 cells), the T cell expansion is greater in the most stringent CD4 helpless model(11), suggesting a role for antigen abundance in mediating this rescue of exhausted T cells after PD-1 blockade. This also suggests that not only PD-1 blockade is necessary to restore exhausted T cells, but also their T cell receptors (TCRs) need to be triggered considerably. However, even though T cell expansion is greater in the chronically viremic mice after PD-1 blockade, antiviral effect is reduced compared to the CD4 helped Cl-13 infection (11).

Regulation of Immune Responses: Role of T regulatory cells during homeostasis and immune responses

In a recent immunology review paper by Ronald Germain(50), the study of T regulatory cells (Tregs) was compared to the resurgence of the Old World mythological phoenix. The first emergence of the Treg field started back in 1970s when Gershon and Kondo postulated the presence of a suppressor cell population(51). Soon after their seminal paper, the field suffered from a collapse due to the lack of validating results and discrepancies with allo-antisera that was predicted to react with suppressor cells. Another reason for the early collapse of the Treg field was the inability to clone these cells (50), and this was perhaps due to the anergic nature of Tregs. The Treg field was considered risky and unscientific for several years, but finally resurrected after a paper by Sakaguchi in the Journal of Immunology(107). In this paper, Sakaguchi's group showed that transfer of CD25- CD4+ cells into nude mice results in autoimmunity. However, co-transfer of CD25- CD4+ cells together with CD25+ CD4+ cells prevented the onset autoimmunity, indicating a suppressive role for the CD25+ CD4+ cell fraction, which was quantified to be about 10% of total CD4+ cells(107). These studies were soon validated by experiments performed at Ethan Shevach's lab, and CD25+ CD4+ cells were shown to suppress TCR mediated CD8 and CD4 responses (125).

Tregs are known as the master regulators of immune tolerance (59, 75), and are characterized by their constitutive expression of the forkhead transcription factor FoxP3+(45, 46, 64). FoxP3+ CD4+ Tregs are indispensable for preventing catastrophic autoimmunity, as mice that are depleted of FoxP3+ cells succumb to rapid, lethal autoimmunity (75). These experiments were performed at the Rudenski lab using $FoxP3^{DTR}$ knock-in mice (diphtheria toxin receptor expressed on all FoxP3+ cells), in which specific ablation of FoxP3+ cells could be achieved by intraperitoneal administration of diphtheria toxin. Although their mechanisms of suppression have been widely explored, there are still many unanswered questions about the contexts in which Tregs suppress immune responses.

Even though many pathways have been shown to mediate Treg suppression, the CTLA-4 pathway has gained some special attention in the last 2 years after a research paper by the Sakaguchi group (138). In this paper, the authors showed that expression of CTLA-4 is required on Tregs in order to prevent autoimmunity. A possible mechanism is that CTLA-4 may act via transendocytosis, by binding B7 molecules on antigen presenting cells (APC) and inducing its internalization by Tregs(138). This could result in mitigation of the APC costimulatory capacity. Other inhibitory markers that have been explored and suggested as potential mediators of Treg function are GITR (glucocorticoid-induced tumour-necrosis-factor-receptor-related protein), ectonucleotidases CD39/CD73, LAG-3, IDO, among others (25, 96, 103, 117, 118). Another mechanism that has been proposed is the “CD25 sink”, in which

CD25 overexpression by Tregs allows them to soak all the IL-2 that could be otherwise used as an effector T cell growth factor(27).

There is also evidence for the role for suppressive cytokines produced by Tregs, such as IL-10(91), TGF-β(30) and more recently, IL-35(32-34). Although there is no universal way that Tregs work, they seem to be versatile and use different pathways to suppress depending on the context. Evidence also proposes that Tregs control DC maturation by modulating costimulatory molecule expression(110, 138). Under steady state, antigen presentation by immature DCs induces T cell tolerance. However, in the absence of Tregs, DCs upregulate costimulatory molecules (CD80 and CD86), and this together with antigen-presentation induces T cell responses(110, 138). As figure 2 shows, Tregs appear to utilize different pathways to suppress.

The role of Tregs during chronic infection still remains ill-defined, with most of the literature data showing correlations, but not direct causations. Increase of Tregs during chronic diseases such as HIV and HCV correlates with increased viral loads and worse prognosis, and Tregs isolated from HCV and HBV patients are highly suppressive (14, 20, 44, 77). Experiments involving depletion of CD25+ cells prior to infection have identified a role of Tregs in controlling virus-specific CD8 T cell responses during herpes simplex virus 1 (HSV-1) infection in mice (122). However, effector T cells also express CD25, and therefore, these studies are complicated to interpret. Until now, however, no one has explored the effect of specific Treg ablation during the persistent phase of an infection.

As mentioned earlier, Tregs suppress self-reactive T cells under normal homeostatic situations. During these circumstances, there is an abundance of self-antigens, yet the host does not normally mount a T cell response. It has been shown that Tregs induce tolerance in steady state dendritic cells by modulation of costimulatory potential (DCs) (56, 110). It is possible that during chronic infection, a situation in which exogenous antigen is also abundant, specific Treg:DC interactions are involved in suppression of CD8 T cell responses.

Overall, our findings go beyond chronic infections, and propose a universal mechanism for Treg mediated suppression of antigen-specific responses. In this thesis, I present new concepts that may enlighten our views on how the immune system works during antigen-persistence, and how manipulation of Treg homeostasis affects T cell exhaustion. We will first show how costimulatory and inhibitory signals could be manipulated during persistent infection. Then, on the later part of this thesis, we will demonstrate a role for Tregs at sustaining CD8 T cell exhaustion during chronic infection.

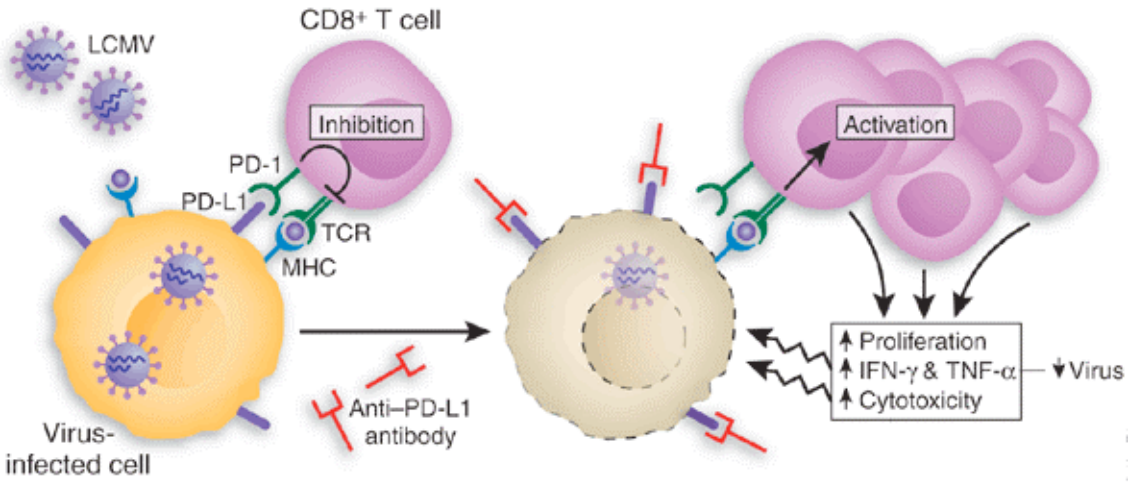


Fig 1. Inhibition of CD8 T cell function by PD-1/PD-L1 signaling. Adapted from Eui-Cheol Shin & Barbara Reherman, Nature Reviews of Immunology, 2008.

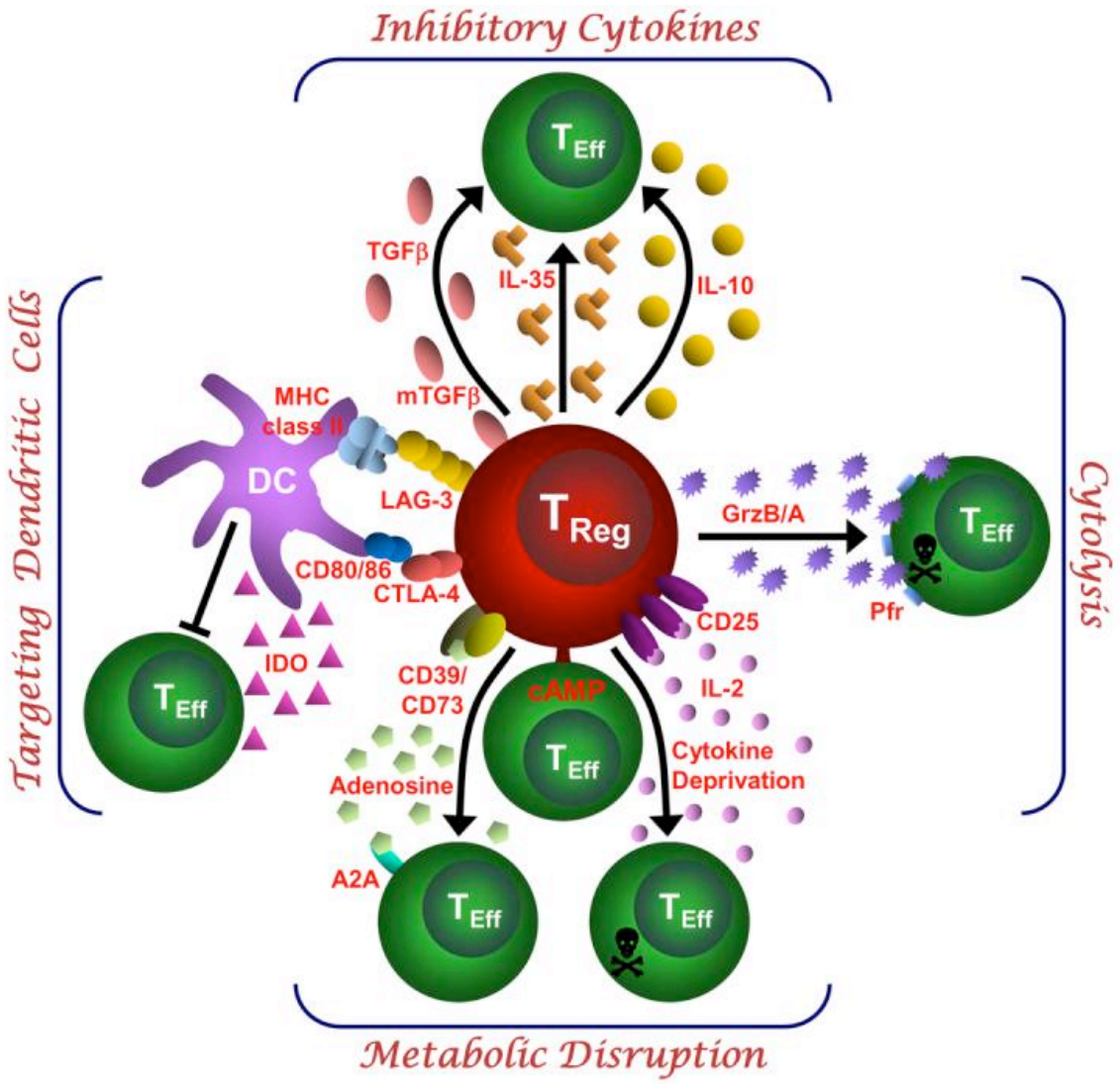


Figure 2. Mechanisms used by Tregs to suppress T cell responses. Adapted from Dario Vignali, Nature Reviews of Immunology, 2008.

Chapter 2: CD137 signaling synergizes with PD-L1 blockade to augment CD8 T cell responses during chronic viral infection

Abstract

Previous studies from our laboratory have identified the inhibitory role that the PD-1 pathway plays during chronic infection. Blockade of this pathway results in rescue of viral-specific CD8 T cells, and reduction of viral loads in mice chronically infected with lymphocytic choriomeningitis virus (LCMV). We tested the effect of combining standard PD-L1 blockade with an agonistic regimen that induces CD137 costimulation during chronic LCMV infection. There is substantial synergy between PD-L1 blockade and CD137 costimulation when the amount and timing of CD137 costimulation is carefully controlled. When blocking antibodies to PD-L1 are given together with a single low dose of anti-CD137 agonistic antibodies, there is synergistic and stable expansion of viral-specific CD8 T cells. Conversely, when blocking antibodies to PD-L1 are given with a repetitive high dose of anti-CD137 agonistic antibodies, there is an initial synergistic expansion of viral-specific CD8 T cell by day 7, followed by a dramatic apoptosis by day 14. Viral control paralleled CD8 T cell kinetics after dual treatment. By day 7 post-treatment, viral titers were lower in both of the combined regimens (compared to PD-L1 blockade alone). However, while the high dose of anti-4-1BB plus PD-L1 blockade resulted in rebound of viral titers to original levels, the low dose of anti-4-1BB plus PD-L1 blockade resulted in a stable reduction of viral loads. These findings demonstrate the importance of

carefully manipulating the balance between activating and inhibitory signals to enhance T cell responses during chronic viral infection.

Introduction:

Upon antigen challenge, naïve T cells undergo a rapid phase of proliferation that results in a great expansion of effector cells(135). If the antigen is cleared, T cells become bone fide memory cells able to respond to a secondary challenge, and express IFNγ, TNFα, and IL-2(135, 141, 143). However, if the antigen persists, there is a gradual loss of T cell function, resulting in progressive T cell exhaustion, and inability of T cells to respond to cognate antigen(135, 143). This is the case with chronic infections such as HIV, HBV, and HCV.

We, and many others, have previously shown that the programmed death-1 (PD-1) pathway plays an important role in directing T cell exhaustion caused by chronic viral infection(11, 39, 48, 126, 129). Decreased CD8 T-cell proliferative potential and high viral load are major obstacles that limit the effectiveness of therapeutic vaccination(134). Our lab and many others have shown that blockade of PD-1 ligand 1 (PD-L1) results in an increase of antigen-specific CD8 T cells, with enhanced functional capacity, and this treatment improves viral control(11, 129). In addition, blocking PD-1 inhibitory signals results in enhancement of therapeutic vaccination during chronic infection(53). Thus, the PD-1 pathway tightly regulates T cell responses during chronic infection(18, 115, 126).

It is unclear, however, which other immune pathways may synergize during PD-L1 blockade. Dual blockade of the inhibitory molecule LAG-3 and PD-L1 results in additive effects on T cell restoration and viral reduction during

chronic infection (16). We wanted to determine whether agonistic costimulatory signals would synergize with PD-L1 blockade and result in a more robust rescue of exhausted virus-specific CD8 T cells. CD137 (also known as 41BB), a TNF receptor family member(130), is expressed by activated T cells, NK cells, NKT cells, mast cells, and neutrophils, whereas its ligand (41BBL) is restricted mostly to APCs(38, 137). This CD137 interaction with its ligand has been shown to be important for T cell responses to bacterial and viral infections(85, 86, 119). Interestingly, the timing and dosing amount of CD137 stimulation can result in different outcomes during viral infections. During an acute LCMV Armstrong infection, if agonistic anti-CD137 antibodies are given before viral priming, suppression of immunity occurs(146).

Conversely, when agonistic antibodies to CD137 are administered,a few days after infection, antiviral T cell responses are enhanced(146). Such an increase in T cell responses could be beneficial during persistent infections, where severe decrease in function and absolute numbers of antigen-specific T cells is observed(135). Robertson et al. demonstrated that when mice chronically infected with Friend virus are treated with an agonistic anti-CD137 antibody, along with transfer of transgenic virus-specific CD8 T cells, there is a 99% reduction of viral loads, as well as increased numbers of transferred T cells(102). CD137 signaling can also regulate immune responses to allo- and auto-antigens and improves T cell mediated anti-tumor efficacy (31, 66, 76, 78, 83).

Because of the co-stimulatory role of the CD137 pathway, we wanted to determine if synergistic effects could be achieved by combining PD-L1 blockade with an anti-CD137 regimen during chronic LCMV infection. In this study, we show that combining PD-L1 blockade with anti-CD137 resulted in synergistic responses leading to augmentation of anti-viral CD8 T cell responses during chronic LCMV infection (compared to just PD-L1 blockade alone). The synergistic effect was dependent on the amount and duration of CD137 signaling. A high, constant dose of agonistic anti-CD137 given in combination with PD-L1 blockade resulted in a transient restoration of antigen-specific T cell responses, whereas a low, one-time dose of anti-CD137 resulted in a sustained increase of antigen-specific T cells numbers, which correlated with faster viral control early after initiation of treatment (compared to PD-L1 blockade alone at day 7 post-treatment).

Materials and Methods:

Mice and infections

4-8 week old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice received 2 x 10^6 pfu LCMV clone-13 intravenously (i.v.) via the lateral vein. To establish a stringent chronic infection model with lifelong viremia, CD4 T cells were depleted by administration of 500 μg GK1.5 antibody (BioXCell) given intraperitoneally (i.p.) one day before and at the day of infection with LCMV clone 13. For a milder LCMV Cl-13 infection model with transient viremia lasting only 2-3 months, mice were infected with Cl-13 without performing CD4 depletion prior to infection. All mice were used in accordance with National Institutes of Health and the Emory University Institutional Animal Care and Use Committee guidelines.

Viral titrations

LCMV titers were determined by plaque assays in 6-well plates of VERO cells grown in MEM media supplemented with 10% FBS. Samples were diluted in 1% FCS DMEM and aliquoted on top of VERO cell monolayers. Plates were then incubated for 1 hour, rocking them every 10 minutes. Wells were overlayed with a mixture of 1% agarose: 2x199 media (1:1), and 1/50 neutral red. Plaques were counted the day after (similar to method described previously) (135).

Antibody treatment regimens

For PD-L1 blockade, 200 μg of rat antimouse PD-L1 antibody (10F.9G2) or rat IgG2b isotype control (BioXcell) were administered i.p. every 3 days, five times. For the high dose CD137 stimulation, 200 μg of the agonist rat anti-mouse CD137 antibody (3H3) was administered i.p. together with PD-L1 antibody. For low dose CD137 stimulation, 50 μg of the agonist rat anti-mouse CD137 antibody was delivered only once i.p. along with the first dose of anti-PD-L1 antibody. Mice were analyzed at day 7 or 14 post-treatment.

Cell isolation and flow cytometry

Single cell suspensions were made from spleens and non-lymphoid organs via mechanical disruption. RBC were lysed to isolate PBL. MHC class I tetramers were produced and used as described previously(. All antibodies were purchased from BD Biosciences, except granzyme B (Invitrogen). Surface and intracellular staining protocols were followed as described previously(135). LCMV peptide stimulations were performed at 37°C, for five hours in a CO_2 incubator in the presence of GolgiPlug and GolgiStop (BD Biosciences). LCMV peptides were purchased from the Emory Microchemical Facility (Atlanta, GA). Cells were acquired using FACSCanto flow cytometer (Becton Dickinson), and analyzed using FlowJo (Treestar).

Statistical analysis

GraphPad (Prism) software was used for statistic analysis.

Results:

High and repetitive doses of anti-CD137 transiently augments LCMV-specific CD8 T cell responses in chronically infected animals during PD-L1 blockade. Our lab has previously reported that blockade of PD-1 signaling during chronic LCMV infection leads to the increase of LCMV-specific CD8 T cell numbers, increased effector function and decreased viral loads(11, 53, 68). We aimed to determine if further enhancement in immune function was possible by combining blockade of the PD-L1 inhibitory pathway, with an agonistic regimen for the CD137 costimulatory pathway. Mice chronically infected with LCMV were treated with a blocking antibody to PD-L1, as previously published(11).

As a combination modality, these chronically infected animals also received anti-CD137 (200 μg) every three days, five times (Fig 1A). During this high dose regimen, both anti-PD-L1 and anti-4-1BB antibodies were administered on the same days. Seven days after treatment, the percent of LCMV-specific CD8 T cells increased more than 3-fold in the dual treated mice, as compared to control (Fig 1B). In contrast, anti-PD-L1 alone only increased 2-fold by day 7 post-treatment. We continued to treat groups of mice using the same high antibody dosing protocol for another week to determine whether this constant stimulation could keep inflating the percentages of anti-viral T cells. After day 14 post-treatment, however, the percentages of antigen-specific cells in the dually treated animals returned to original pre-treatment values.

The percentages of antigen-specific CD8 T cells correlated with their absolute numbers at all the days tested. The total numbers of LCMV-specific CD8 T cells in anti-CD137 plus anti-PD-L1 treated mice increased ~8-fold by day 7 post-treatment, as compared to pre-treatment numbers (Fig 1C). This is a 2.5-fold increase over anti-PDL1 alone at day 7 post-treatment. The continuous addition of anti-CD137 and anti-PD-L1 resulted in a dramatic loss of cells, recoiling to original T cell numbers (Figure 1C). Indeed, the number of anti-viral T cells after 14 days of treatment was essentially the same as the starting time-point, indicating that this stimulation protocol transiently increased numbers of anti-viral T cells during chronic LCMV infection, compared to a stable population generated with PD-L1 blockade alone. Spleen data is shown, and similar results were obtained from liver, lung, and blood (not shown).

We next addressed whether such a transient increase in LCMV-specific CD8 T cell numbers impacted viral titers. At day 7, there was a five-fold drop in viral titers in animals treated with the dual antibody treatment (greater viral control than that by PD-L1 blockade alone at day 7) (Fig 1D). However, by day 14, virus rebounded to starting levels in the dual treated mice, mimicking the recoiled kinetics of the antiviral CD8 T cell response. Thus, the transient increase in antiviral CD8 T cells numbers by day 7 due to CD137 costimulation and PD-L1 blockade contributes to quick viral control, but this viral control is only transient. Viral titration was performed in several tissues with similar results (not shown).

Reducing the dose and frequency of anti-CD137 treatment changes T cell longevity. We wished to determine whether modulating the dosing of

CD137 costimulation would lead to better T cell longevity during a combined treatment with anti-PD-L1. We had the same experimental set-up as before, but we decreased the dosing of CD137 agonistic regimen. The new treatment consisted of only one injection of anti-CD137 (50 mg) along with the first dose of anti-PD-L1 antibody (Fig 2A). At day 7 after treatment, the percentages of LCMV-specific CD8 T cells were increased in the dual treated group, similar to what was seen with high dose of CD137 costimulation (Fig 2B). There was approximately an 8-fold increase compared to untreated mice. At day 14, animals receiving low dose CD137 costimulatory regimen plus PD-L1 blockade were able to maintain the high percentages of LCMV-specific CD8 T cells that were seen at day 7 (Fig 2B). Absolute numbers of antigen-specific CD8 T cells were also higher in the dual treated group (Fig 2C). Spleen data is shown, and similar results were obtained from liver, lung, and blood (not shown).

Acceleration of viral control with a reduced dose of anti-CD137 together with PD-L1 blockade. A significant drop in viral titer was detected in dual treated animals at day 7 (Fig 2D), similar to what was seen in the high dose protocol. This control of viremia was maintained to day 14 post treatment, correlating well with sustained numbers of anti-viral CD8 T cells. By day 14, viral titers were similar to PD-L1 blockade alone. Thus, the amount of stimulation is an important factor in longevity of T cells rescued from exhaustion and Activating signals should be carefully controlled during chronic infection to ensure T cell viability. Viral titration was performed in several tissues with similar results (not shown).

Functional and phenotypic changes in viral-specific CD8 T cells after reduced CD137 stimulation paired with PD-L1 blockade. Functional changes occurring in mice treated with combined CD137 and PD-L1 therapy were examined to determine how this stimulation was affecting T cell physiology. We evaluated the ability of virus-specific T cells to produce cytokines by stimulating cells with different LCMV CD8 T cell epitopes. For several T cells epitopes tested, the low dose CD137 stimulation with PD-L1 blockade enhanced cytokine expression of anti-viral CD8 T cells above the level of rescue seen with anti-PD-L1 alone (Fig 3A). Also, the percent CD8 T cells producing IFNg and TNF-a increased compared to control or PD-L1 blockade alone (anti-4-1BB alone was similar to IgG). In most cases, the percent of dual cytokine producing T cells increased in double treated group. Therefore, a reduced agonistic anti-CD137 regimen given concomitantly with PD-L1 blockade acts synergistically to augment CD8 T cell responses and function during chronic LCMV infection. Absolute numbers of functional cells were significantly enhanced for some LCMV epitope-specific responses (Fig 3B).

One difference between PD-L1 alone and combined regimen was the large increase in total activated CD8 T cells. There is a doubling in the percentages of CD8 T cells, and increased percentages of activated CD8 T cells in the dual treated mice, compared to control mice (Fig 4A, first row). In addition, the percentage of CD8 T cells that were PD-1 positive was dramatically

increased in the dual treatment, suggesting expansion of exhausted CD8 T cells of several specificities (Fig 4A, second row).

The dual treated group had a marked increase of granzyme B+ expression on CD8 T cells (Fig 4B, first row). Indeed, 50% of all CD8 T cells after dual treatment now produced granzyme B, compared to 8% in the untreated controls. Also, CD8 T cells from the dual group had lower CD62L and CD127 expression, suggesting higher overall activation compared to PD-L1 blockade alone (Fig 4B, second and fourth row). The dual treatment also induced higher T cell proliferation, as measured by expression of Ki67, which is associated with cell proliferation (111) (Fig 4B, third row). This correlates with the increase of T cell numbers seen in the dually treated mice, indicating that at least part of T cell rescue is coupled to T cell division.

CD8 T cell viability and apoptosis in either high or low dose CD137 stimulation combined with PD-L1 blockade. As a comparative study, we wished to determine how changing the amount and duration of CD137 stimulation during standard PD-L1 blockade affected antigen-specific CD8 T cell viability. Therefore, we examined apoptosis in LCMV-specific CD8 T cells in animals given a high (Fig 1A) or low (Fig 2A) dose of anti-CD137 together with PD-L1 blockade. We measured apoptosis by Annexin V / 7AAD staining at day 7 and 14 after treatment (Figure 5). As expected, a proportion of antigen-specific CD8 cells are apoptotic in untreated animals, in agreement with previous reports(131).

At day 7, there was a 2-fold increase in the percentages of viral-specific CD8 T cells that were apoptotic in either of the combined regimens (Fig 5A, first row). This suggested that even though there is expansion of viral-specific cells, there is also apoptosis occurring at the same time in the dual treated groups. By day 14, however, the low dose combined regimen equilibrated and had very few percentages of apoptotic cells, whereas the high combined regimen resulted in increased percentages of apoptotic cells (Fig 5A, second row, and 5B).

Our results show that the amount and duration of CD137 signaling is crucial, as over-stimulation will lead to apoptotic death, whereas an optimallevel of costimulation leads to a larger, more viable T cell population. The percentages of Annexin V– / 7AAD- cells, which represented viable cells were examined (Figure 5C). By day 7, most groups had similar percentages of live (Annexin V– / 7AAD-) viral-specific CD8 T cells. However, by day 14, the low dose anti-CD137 plus PD-L1 blockade group had a statistically higher percentage of live cells, compared to the high dose anti-CD137 plus PD-L1 blockade group (Fig 5C). This suggested a survival advantage after a low dose of anti-CD137 and anti-PD-L1. We have also looked at later time points (after day 30), and the low dose anti-CD137 plus PD-L1 blockade group still has increased levels of viral-specific cells after interruption of treatment (not shown).

Synergism of CD137 signaling and PD-L1 blockade is optimal during an infection resulting in chronic viremia. All experiments presented so far used mice depleted of CD4 T cells prior to infection with LCMV clone 13, in which we wait more than 45 days to allow for full CD8 T cell exhaustion. This infection

protocol provides a more stringent infection, which results in lifelong viremia(81). We were interested in understanding how CD8 T cells in a less stringent chronic infection model would respond to CD137 stimulation with PD-L1 blockade. To test this, mice were not depleted of CD4 T cell before infecting with LCMV clone 13 and then subjected to the low dose CD137 stimulation/PD-L1 blockade starting treatment anytime from day 15 to 21 post-infection (Fig 6A). The percent (Fig 6B) and total numbers (Fig 6C) of cytokine expressing cells were similar by day 14 post-treatment. Viral control was moderately impaired in the dual treated group compared to PD-L1 blockade alone (Fig 6D). Thus, during a less stringent chronic LCMV infection, additional CD137 costimulation during PD-L1 blockade does not result in additional increase of antigen-specific CD8 T cell responses or viral control.

Discussion:

We hypothesized that the rescue of exhausted CD8 T cells achieved with PD-L1 blockade could be enhanced by additional costimulation. So far, the combinatorial effects of blocking antibodies against multiple inhibitory receptors during chronic infection has been explored(16). However, to our knowledge, no one has tested the effect of blocking the PD-1 pathway and inducing additional T cell costimulation (in the form of CD137) at the same time. We have tested the effect of dual treatment with an agonistic anti-CD137 antibody 200 mg every three days, five times, along with anti-PD-L1 antibody in mice chronically infected with LCMV.

Our initial experiments showed that the inclusion of CD137 stimulation boosted numbers of LCMV-specific CD8 T cells 8-fold at a time-point when PD-L1 blockade alone only increased cell numbers less than 3-fold. This also resulted in a drop in viral titers in dual treated mice at an early timepoint, indicating the usefulness of this therapeutic protocol to quickly control virus. However, the large numbers of LCMV-specific CD8 T cells seen at day 7, were not maintained to day 14 if excessive costimulation is provided (Fig 1), indicating that this extra push of costimulation through CD137 was acting to overstimulate CD8 T cells, leading to apoptosis. No significant differences in viral-specific CD8 T cell frequencies, or viral control were observed between the high dose anti-4-1BB alone and IgG treated animals. A slight increase in total numbers was seen by day 7, but it was not statistically significant.

We reasoned that lowering the amount of CD137 costimulation may not drive the cells to eventual deletion by day 14 post-treatment. Thus, we adapted our protocol by lowering the amount and duration of CD137 signaling to a one time low dose of 50 mg of anti-CD137 given together only with the first day of our standard PD-L1 blockade. The decreased CD137 dosing resulted in a similar expansion of LCMV-specific CD8 T cells at day 7, as compared to mice receiving high dose CD137 signaling. In contrast to the high dose of anti-CD137 combined regimen, anti-viral cell numbers were stable with reduced co-stimulation (Fig 2). No significant differences were observed between the low dose anti-4-1BB alone and IgG treated animals.

Therefore, there is a delicate balance between a sufficient level of co-stimulation to potentiate T cell rescue and over-stimulation leading to cell death. The connection between excessive immune activation and exhaustion has been widely documented(105, 124, 136). Caution should be taken with therapies based on co-stimulatory regimens to ensure optimal cell longevity and function.

The dual low dose blockade therapy also potentiated the function of LCMV-specific CD8 T cells in the face of a stringent chronic infection. Not only were the numbers of T cells producing cytokines boosted, but the numbers of T cell producing both IFNγ and TNFα (dual producers) increased compared to PD-1 blockade alone (Fig 3). Even though we observed a permanent increase in the viral-specific CD8 T cell response in the low anti-CD137 plus anti-PD-L1 blockade group, viral control was only accelerated by day 7 (after day 14, viral titers were similar to anti-PD-L1 alone). One possibility could be viral escape

after excessive CTL attack. It is possible that LCMV variants may appear as a result of “selective bottlenecks” result of increased T cell mediated cytolytic control. Another possibility may be that increased T cell rescue above a set threshold (dictated by just PD-1 blockade) may not directly correlate to enhanced viral control. We are currently investigating the reasons for these results. Our findings may be important in therapeutic vaccination regimens that require fast clinical intervention resulting in accelerated decline in viral titers.

There were also significant phenotypic changes after co-administration of anti-4-1BB (low dose) and anti-PD-L1 antibodies. CD8 T cells downregulated CD62L and CD127, which may correlate with their overall activation status, and immediate cytotoxity capability(4, 8, 62). This phenotype may be a result of an expanded effector cell population (8). Anti-CD137 treatment also resulted in an increase of Ki67+ anti-viral CD8 T cells, as well as large change in the frequency of CD8 T cells producing granzyme BThere were increased percentages of PD-1+ CD44+ cells after dual treatment. This last subset seems to represent the total increase in antigen-specific cells after treatment, showing expansion of exhausted cells from different specificities that are contained within the PD-1+ CD44+ subset. This cell population contains exhausted, antigen-specific T cells with different proliferative capacities after PD-1 blockade, with PD-1^{int} $CD44^{hi}$ having a proliferative advantage over PD-1hi $CD44^{hi}$ (15).

We then wanted to know what would be the outcome of the immune response after dual treatment in the less stringent model of T cell exhaustion (Cl-13 infection without CD4 depletion). In this less stringent model chronic infection,

viremia lasts only 2-3 months and CD8 T cell exhaustion is less pronounced compared to the GK1.5 Cl-13 infection model.

Dual treatment with PD-L1 blockade and a low dose of CD137 agonistic signaling resulted in normal rescue of antigen-specific T cell responses similar to that seen with PD-L1 alone. However, viral control was slightly delayed compared to anti-PD-L1 alone. This is a proof a principle that under less stringent conditions, the threshold of T cell rescue and viral control achieved by PD-L1 blockade alone may obviate the need for any additional costimulation, and additional activating signals may hamper antiviral control.

One lesson from these studies would be that excessive T cell responses produced by providing additional costimulation do not always translate into enhanced viral control above a given threshold (in this case determined by just PD-1 blockade). Administration of additional activating signals may hamper antiviral function and overall T cell fitness if not carefully controlled and tailored to a particular infection status.

The dual nature of CD137 costimulatory pathway is well documented. If agonistic antibodies to CD137 are given after antigen priming, this results in augmentation of T cell responses(80, 146). Conversely, administration of antibodies agonistic for CD137 before or during priming results in suppression of antigen-specific responses (88, 121, 146). This blunting of pathogen-specific responses has been reported to be TNFa dependent (146). Even though our treatment started late after antigen challenge, and after chronic infection has ensued, we hypothesize that a similar mechanism of TNFα dependent

suppression may be occurring between day 7 and day 14 post-treatment in the mice that got the high dose anti-CD137 plus PD-1 blockade. One of our future directions is to test anti-TNF therapy to see if it prevents the drastic decline of the expanded cells after day 7 with the high dose dual regimen in the stringent infection LCMV model.

By further manipulating the kinetics of T cell responses following blockade of the inhibitory PD-1 pathway, it may be possible to accelerate viral clearance during established chronic infections. However, careful tailoring of the amount of activating signals and the status of infection should be taken into consideration to prevent overt stimulation above a physiologically accepted level. In conclusion, in a more stringent model of chronic infection, PD-1 blockade together with a low dose of CD137 costimulation induces acceleration of T cell restoration and viral control compared to PD-1 blockade alone. This may be important in clinical settings where immediate T cell restoration is wanted. However, in a less stringent model of chronic infection, PD-1 blockade alone seems optimal at rescuing T cell responses without additional costimulation.

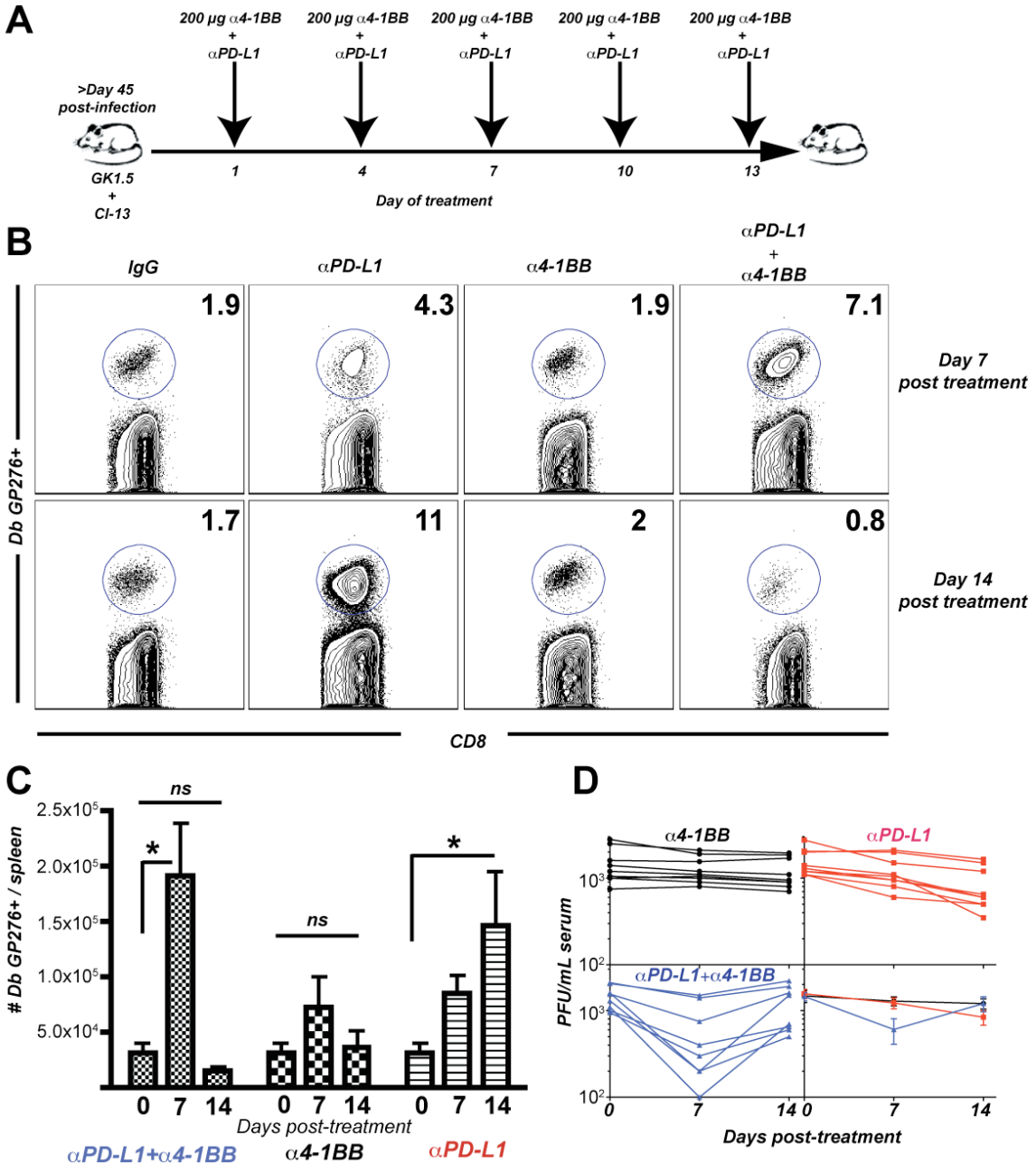


Figure 1. High dose of CD137 stimulation together with PD-L1 blockade results in transient increase in viral-specific CD8 T cell responses. A). Experimental set up: Mice received CD4 depleting antibody (GK1.5), and were infected the following day with LCMV Cl-13. After 45 days, mice received multiple doses of anti-CD137 agonistic antibodies (200 μg) together with standard PD-L1 blockade. B) Percentages of splenic CD8 T cells that are specific for the LCMV immunodominant Db GP276-286 epitope. C) Total numbers of splenic CD8 T cells that are specific for the LCMV immunodominant Db GP276-286 epitope. D) Serum viral control kinetics. *, P<0.05; ns=not significant.

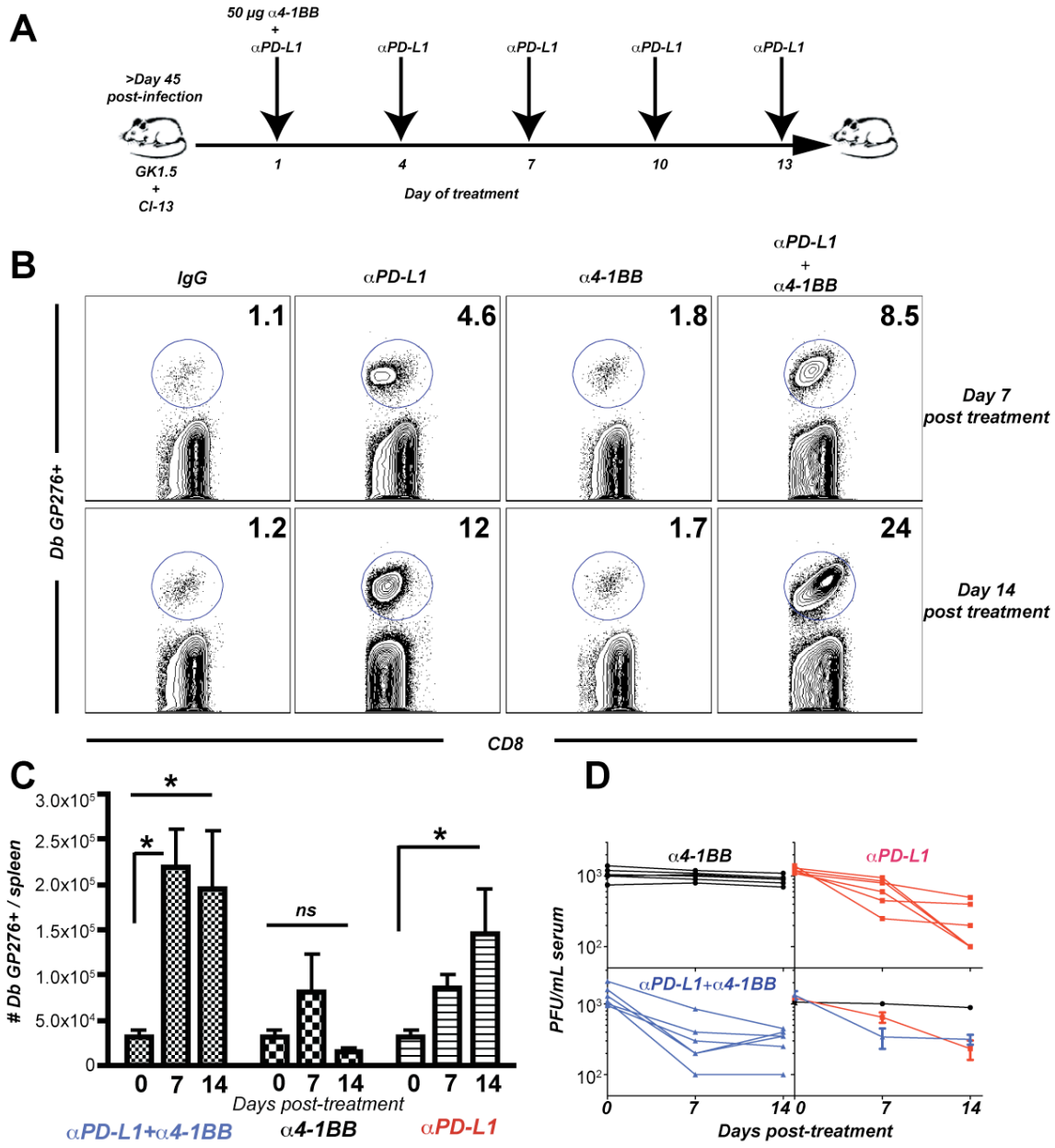


Figure 2. Low dose of CD137 stimulation together with PD-L1 blockade results in permanent increase in viral-specific CD8 T cell responses. A) Experimental set up: Mice received CD4 depleting antibody (GK1.5), and were infected the following day with LCMV Cl-13. After 45 days, mice received a single dose of anti-CD137 agonistic antibodies (50 μg) together with standard PD-L1 blockade. B) Percentages of splenic CD8 T cells that are specific for the LCMV immunodominant Db GP276-286 epitope. C) Total numbers of splenic CD8 T cells that are specific for the LCMV immunodominant Db GP276-286 epitope. D) Serum viral control kinetics. *, P<0.05; ns=not significant.

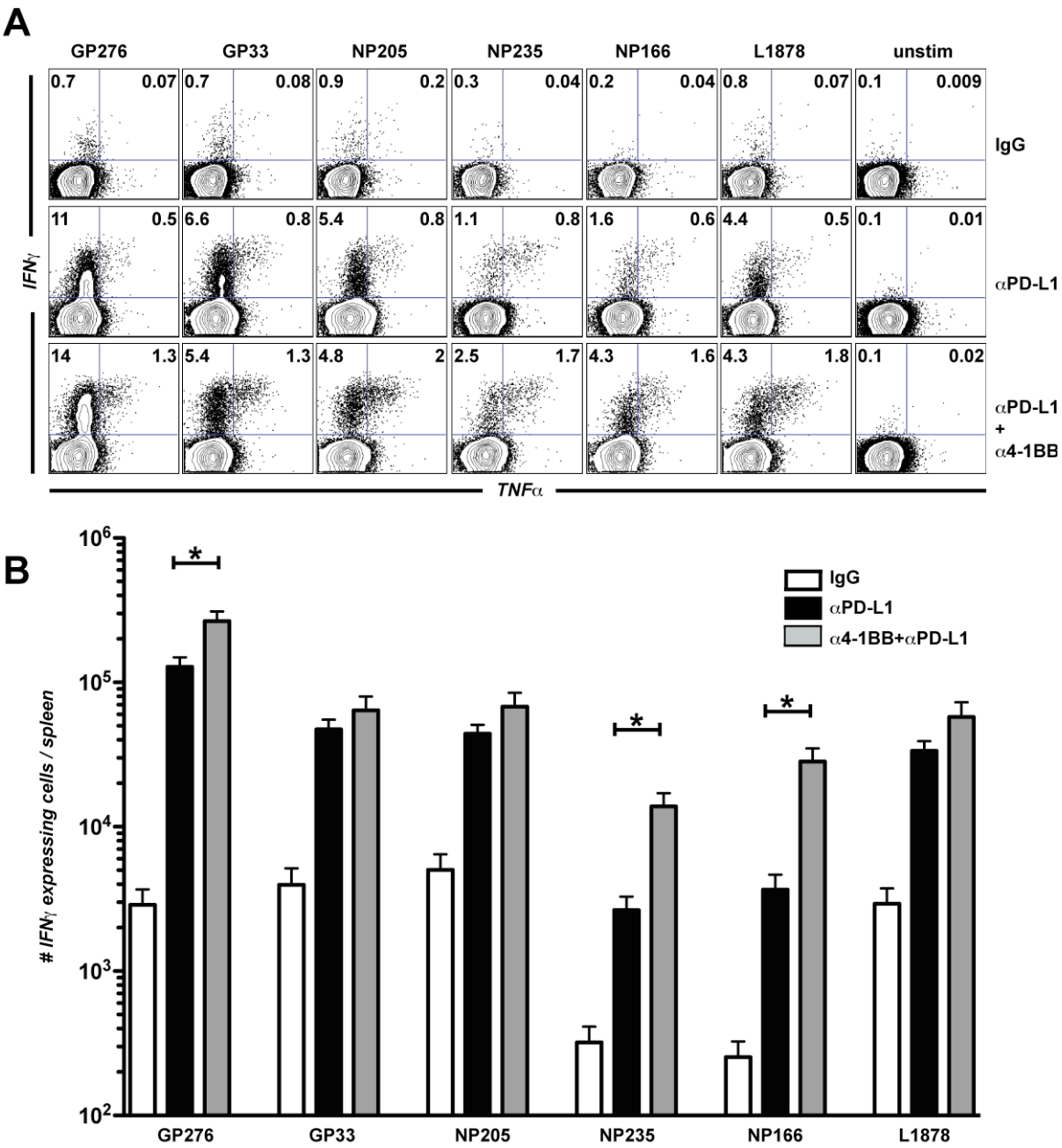


Figure 3. Low dose of CD137 stimulation together with PD-L1 blockade increases cytokine production in anti-viral CD8 T cells. A) Percentages of splenic cytokine expressing CD8 T cells after 5 hour stimulation with various LCMV peptides. B) Total numbers of cytokine expressing cells after 5 hour stimulation with several LCMV peptides. *, $P<0.05$.

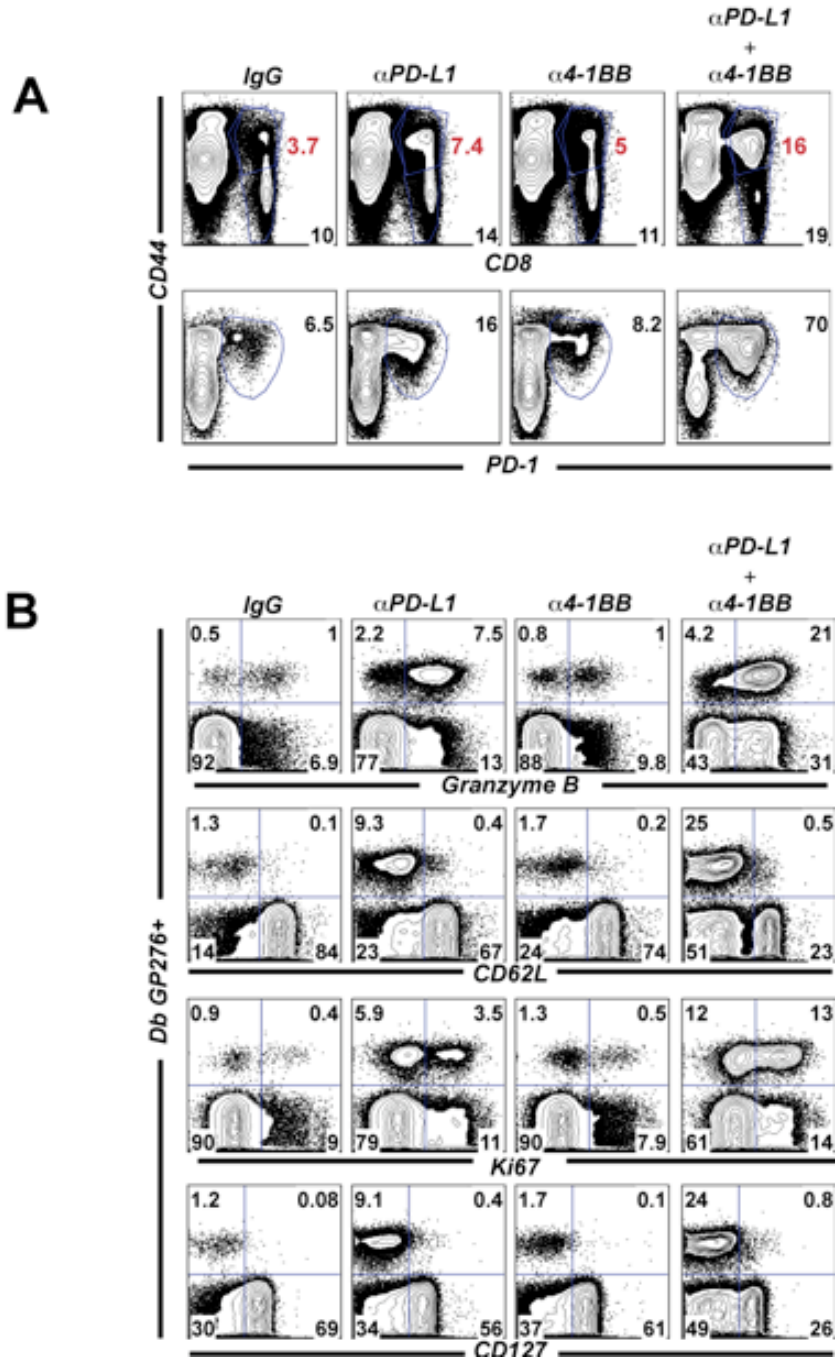


Figure 4. Low dose of CD137 stimulation together with PD-L1 blockade results in marked phenotypic and functional differences. A) Fist row: Percentages of live splenic T lymphocytes that are CD8+ (black number), or activated CD8+ CD44+ (red number). Second row: Percentages of CD8 T cells that are PD-1+. B) Granzyme B, CD62L, Ki67, and CD127 expression on immunodominant Db GP276-286 specific CD8 T cells in spleen.

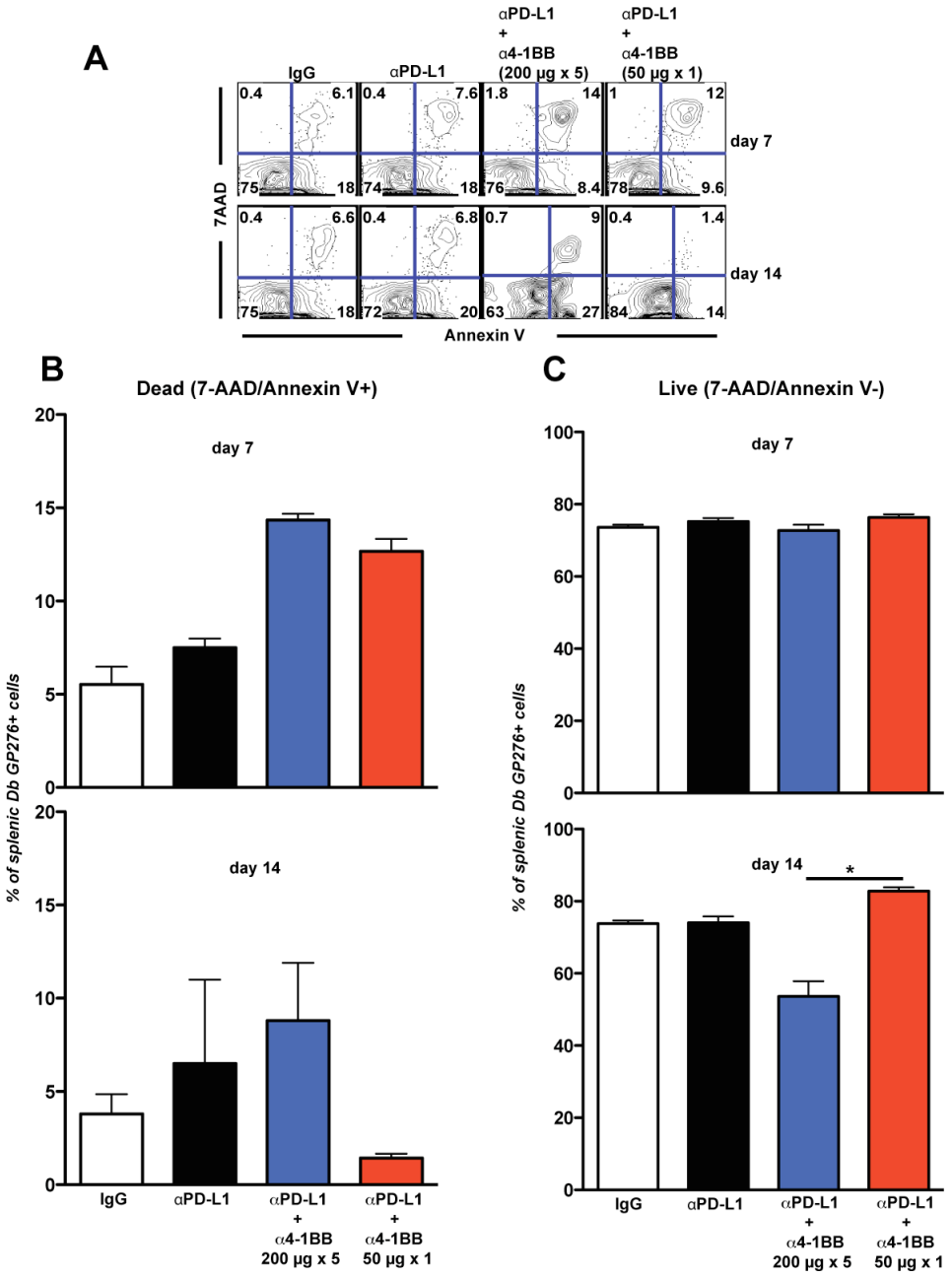


Figure 5. Viral-specific CD8 T cell viability after co-treatment with high or low dose of CD137 together with PD-L1 blockade. A) Percentages of splenic Db GP276-286 specific CD8 T cells that are apoptotic or live by Annexin V / 7AAD staining in spleen at day 7 and 14 post-treatment. B) Percentages of Db GP276-286 specific CD8 T cells that are positive for both Annexin V and 7AAD (apoptotic population). C) Percentages of Db GP276-286 specific CD8 T cells that are negative for both Annexin V and 7AAD (live population). *, $P<0.05$.

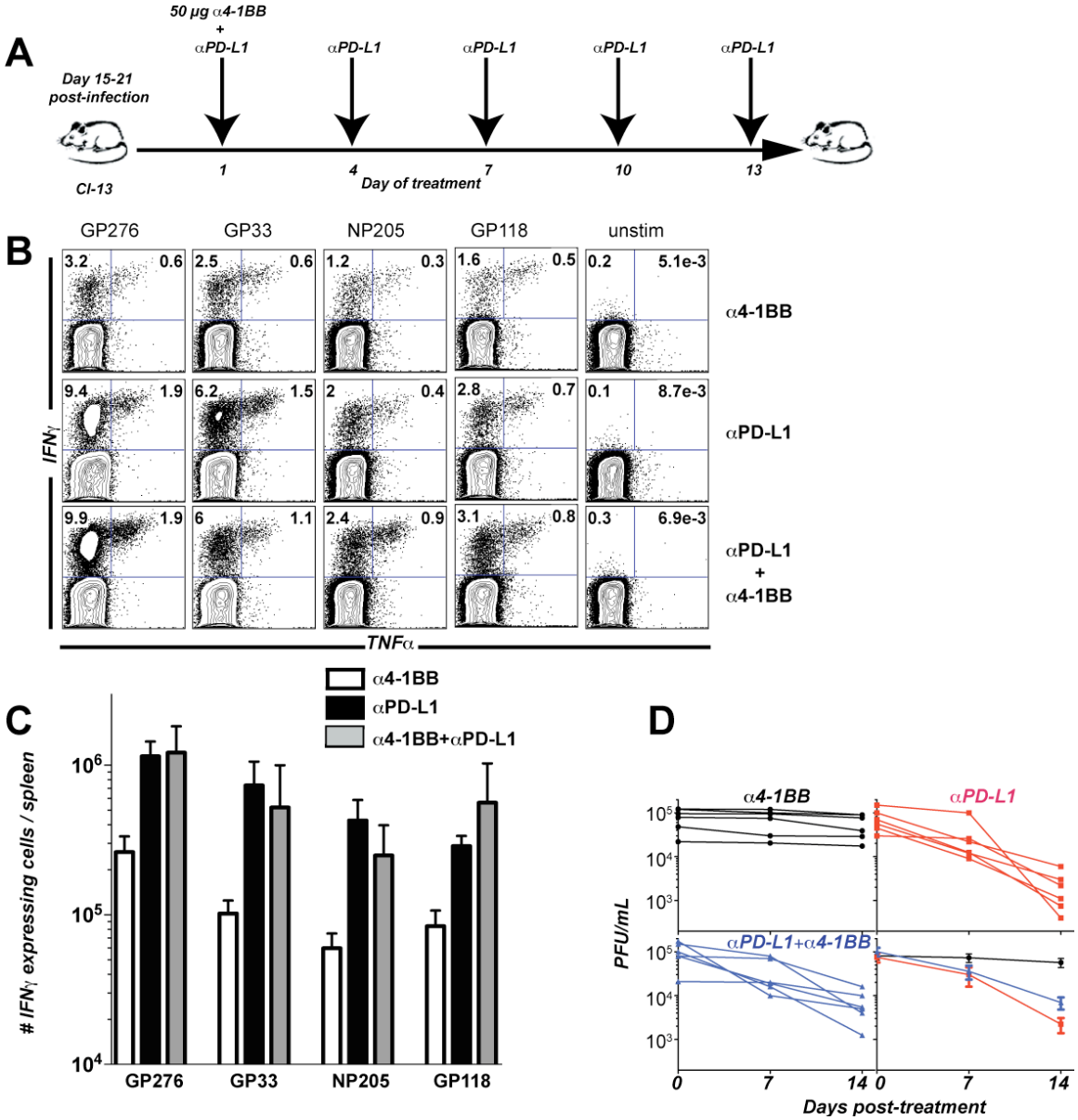


Figure 6. Low dose of CD137 stimulation together with PD-L1 blockade during a milder chronic infection does not result in enhanced CD8 T cell rescue compared to PD-L1 blockade alone. A) Experimental set up: mice were infected with LCMV Cl-13 (without prior CD4 depletion). Around day 15-21 post-infection, mice received a single dose of anti-CD137 agonistic antibodies (50 μg) together with standard PD-L1 blockade. B) Percentages of splenic cytokine expressing CD8 T cells after 5 hour stimulation with several LCMV peptides. C) Total numbers of cytokine expressing cells after 5 hour stimulation with several LCMV peptides. D) Serum viral control kinetics. IgG treatment was similar to anti-4-1BB alone.

Chapter 3. Opposing effects of CD70 costimulation during acute and chronic viral infection

Abstract:

T cell costimulation is important for T cell activation. The CD27/CD70 pathway contributes to effector and memory T cell development, and is involved in T cell and B cell activation. CD27/CD70 is known for having opposing roles during different models of antigenic challenges. During primary T cell responses to influenza infection or during tumor challenges, CD27/CD70 costimulation has a positive role on T cell responses. However, during some chronic infections, constitutive triggering of this signaling pathway has a negative role on T cell responses. Therefore, it is currently unclear what specific characteristic of an antigen determines the outcome of CD27/CD70 costimulation. We investigated the effect of a transient CD70 blockade during an acute or a chronic lymphocytic choriomeningitis virus (LCMV) infection in mice. Blockade of this pathway during acute LCMV infection (Armstrong strain) resulted in delayed T cell responses, and decreased CD127 (IL-7Rα chain) conversion. Upregulation of CD127 is an important event in T cell differentiation that heralds the passage of an effector T cell to a long-lived memory T cell. In contrast to the reduced CD8 T cell responses after CD70 blockade during acute infection, CD70 blockade during chronic LCMV infection resulted in increased CD8 T cell responses. The present findings show the dual roles of this costimulatory pathway in acute versus persistent antigen challenge. Our findings suggest that antigen persistence

determines the effect of CD27/CD70 signaling on CD8 T cell responses. Tailored triggering or blockade of this costimulatory pathway may be important in vaccination regimens against acute or chronic pathogens.

Introduction:

T cell responses are pivotal for pathogen clearance and control. Normal activation and differentiation of T cells occurs after two signals. First, the T cell receptor (TCR) binds to its bona fide MHC peptide complex. Second, costimulatory signals provide a necessary stimulus to drive T cell activation and differentiation (47, 52, 100). Costimulation signals include that from CD28/B7 (114), 4-1BB/4-1BBL (123), OX-40/OX-40L (29), and CD27/CD70 (128) interactions, among others.

Interestingly, there is independently generated evidence that suggests the opposing roles of the CD27/CD70 costimulatory pathway. Several groups have shown that induction of this signaling pathway results in improved anti-tumor T cell responses(5, 36, 74). In addition, signaling through CD27, the receptor for CD70, is required for the generation and long-term maintenance of influenza-specific T cell responses (57). Recent evidence also shows that CD11b+ DCs enhance influenza-specific CD8 T cell responses via CD70 costimulation, highlighting the importance of this costimulatory pathway (9). These models could be categorized as acute challenges, in which the antigen is usually resolved within a few days.

Acute viral infections promote the formation of functional memory T cells capable of clearing virus. These cells are able to quickly respond to a secondary challenge, and confer protection to the host. In contrast, chronic infection usually results in progressive exhaustion of viral-specific T cell responses. During some

chronic infections, expression of CD70 is a marker of poor T cell responses, and undesirable prognosis. HIV-positive patients who undergo chronic immune activation and progress to AIDS have increased expression of CD70 on CD8 T cells (24, 139). This raises the question of whether CD27/CD70 signaling could be harmful during a chronic infection, such as HIV. In this regard, HIV-1+ patients seem to accumulate an atypical population of CD27hi CD8 T cells, which positively correlates with HIV-1 viremia (63). Chronic viral diseases also come hand in hand with bystander immunosupression. Persistent CD27/CD70 costimulation has been proposed to contribute to this process, as constitutive triggering of CD27 induces lethal T cell immunodeficiency reminiscent to that seen on AIDS patients (124). Moreover, in a mouse model of chronic LCMV infection (Docile strain), long-term blockade of this pathway has been shown to significantly enhance viral control (82), suggesting that signaling via the CD70/CD27 pathway during chronic infection intereferes with the clearance of a chronic viral infection.

All the aforementioned literature with influenza, tumors models, and chronic infections suggested that the CD27/CD70 pathway may have different roles depending on the persistence or perhaps the type of the antigen. In order to resolve these observations, we took advantage of the LCMV system. This model of infection has both an acute and a chronic infection model with identical T cell epitope specificities. We show here that after acute infection with LCMV Armstrong, CD27/CD70 signaling contributes to the induction of an efficient T cell response, since blockade of this pathway results in delayed T cell responses.

This was evidenced by reduced absolute number of antigen-specific CD8 T cells at the peak of the antiviral T cell response, and slower CD127 conversion, a marker for memory commitment (70). Conversely, after chronic infection with the chronic LCMV Cl-13 strain, CD27/CD70 costimulation negatively regulates T cell responses, since blockade of this pathway results in increased CD8 T cell responses after 3 weeks post-infection. Our data suggests that context-dependent regimens to manipulate the CD27/CD70 pathway may be of interest in the design of T cell-based vaccines depending on the persistence of the pathogen.

Materials and Methods:

Mice and infections

6-8 week old, female C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, Mayne). Mice were infected intraperitoneally (i.p.) with LCMV Armstrong (2x10^5 pfu) or intravenously (i.v.) with Clone 13 (2x10^6 pfu) via lateral tail vein injection.

Virus Titration

Titration of LCMV was performed on Vero cell monolayers plated on 6-well plates as described previously (2). Vero cells were grown on MEM media supplemented with 10% FCS, 1% L-Glutamine, and PenStrep. Samples were diluted in 1% FCS DMEM and aliquoted on top of cell monolayers. Plates were incubated for 1 hour, rocking them every 10 mintutes. Wells were then overlayed with a 1:1 mixture of 1% agarose and 2x199 media. After 4 days, wells were overlaid with a 1:1 mixture of 1% agarose and 2x199 media containing 1:50 neutral red. Plaques were counted the following day.

In vivo antibody blockade

300 μg of rat anti-mouse CD70 (FR70), or rat IgG2b isotype control (diluted in PBS) were administered i.p. every three days starting on day 0 (days 0, 3, 6, 9, and 12 after LCMV infection). This regimen was similar to that used by

Matthias Matter (82), in which mice were treated twice a week. FR70 monoclonal antibody was prepared as described previously (93).

Intracellular cytokine and surface staining

Intracellular cytokine, and surface staining were performed as described previously (135). LCMV peptide stimulations were performed at 37°C, for five hours in a CO_2 incubator in the presence of GolgiPlug and GolgiStop (BD Biosciences). LCMV peptides were obtained from the Emory Microchemical Facility (Atlanta, GA). All monoclonal antibodies for flow cytometry were purchased from BD Biosciences (San Jose, CA). Stained cells were acquired using FACSCanto flow cytometer (Becton Dickinson), and analyzed using FlowJo (Treestar).

Antibody titration

Serum LCMV-specific IgG titers were titrated by a solid-phase direct ELISA as previously described (2), using HRP-conjugated antibodies against mouse IgG on ELISA 96-well microplates. Serum from Armstrong infected mouse day 40 (positive control), or naïve mouse (negative control) were used tested at the same time.

Statistical analysis

GraphPad (Prism) Software was used for statistical analysis. Mann-Whitney test was used for calculating statistical significance.

Results

Role of CD27/CD70 signaling on CD8 T cell responses during acute viral infection. We first decided to analyze the effect of blocking CD27/CD70 signaling during an acute viral infection. We injected C57BL/6J mice i.p. with CD70 blocking antibodies on the day of LCMV Armstrong infection, and every 3 days, for a total of five times, prior to the analysis of acute cellular responses at day 7 and 15 (Fig. 1A). The frequencies (Fig. 1B) and absolute numbers (Fig. 1C) of LCMV-specific CD8 T cell responses at day 7 post-infection were reduced compared to control. This result suggested a positive role for CD70 costimulation in primary responses. Although the percentages (Fig. 1D) of CD8 T cells that were LCMV-specific were similar by day 15 post-infection, there was still a modest, but consistent reduction in their absolute numbers (Fig. 1E) after CD70 blockade. Percentages of TNFα-expressing CD8+ T cells are also lower in anti-CD70 treated mice, as shown on the representative plots, especially at early time points (day 7).

Memory conversion after CD70 blockade during acute viral infection. In addition to reduced primary CD8 T cell responses after CD70 blockade, viral-specific (Db GP33-41+) CD8 T cells displayed a delayed CD127 conversion by day 15 post-infection, evidenced by reduced per cell expression of CD127, and diminished percentages of CD127+ CD8 T cells (Fig. 1F). These data suggested not only defects in the magnitude of the viral-specific T cell response, but also in memory conversion. Functionality of antigen-specific CD8 T cells was also

compromised, especially at the peak of the antiviral CD8 T cell response. The absolute number of IFNγ and TNFα coexpressing CD8 T cells was significantly reduced after CD70 blockade (Fig. 2), but by day 15, cytokine co-expressing cells were just slightly decreased (not shown).

Role of CD27/CD70 signaling on CD4 cell responses during acute LCMV infection. In contrast to the reduced percentages of viral-specific CD8 T cells after CD70 blockade during acute LCMV infection, the percentages of viral-specific CD4 cells (expressing IFNγ after GP61-80 stimulation) were not different from those of control mice (Fig. 3). Nevertheless, there was a modest, but consistent reduction of their absolute numbers at days 7 and 15 post-infection. This is because of a reduced spleen count in anti-CD70 treated mice.

Effect of CD27/CD70 signaling on antibody responses during acute LCMV infection. The effect of anti-CD70 monoclonal antibody blockade on LCMV-specific IgG antibody responses was opposite to that on CD8 T cells. Even though the CD8 T cell response was delayed after CD70 blockade, the LCMV-specific IgG antibody titers were slightly enhanced at day 15 after LCMV Armstrong infection (Fig. 4). This suggested a positive effect of CD70 costimulation on CD8 T cell responses, but negative role on antibody responses.

Role of CD27/CD70 signaling on CD8 T cell responses during chronic LCMV infection. In order to evaluate the effects of CD70 blockade in a chronic LCMV infection, we took advantage of the chronic LCMV strain. For this, we injected C57BL/6J mice with CD70 blocking antibodies on the day of LCMV Cl-13 infection, and every 3 days, for five times, and analyzed immune responses at

day 7 and 21 post-infection (Fig. 5A). In contrast to acute LCMV Armstrong infection, CD70 blockade during chronic LCMV Cl-13 infection results in no change in the frequencies (Fig. 5B) and numbers (Fig. 5C) of LCMV-specific CD8 T cell responses at day 7 post-infection. However, during the later stage of chronic infection (day 21), even though the frequencies (Fig. 5D) of antigen-specific CD8 T cell responses were similar between CD70 blockade and control mice, there was an almost 2-fold increase in the absolute number (Fig. 5E) of viral-specific CD8 T cell responses after CD70 blockade. This clearly suggested a negative role for CD27/CD70 during chronic infection.

Role of CD27/CD70 signaling on CD4 cell responses during chronic LCMV infection. At day 7 post-infection, there was not difference in the percentages or the absolute numbers of viral-specific CD4 T cells. However, by day 21 post-infection, even though the percentages of viral-specific CD4 T cells were similar, the absolute numbers were increased in anti-CD70 treated mice (Fig. 6). This is in sharp contrast to acutely infected mice, in which there is a reduction in LCMV-specific CD4 T cells (GP61-80 specific).

Effect of CD27/CD70 signaling on antibody responses and viral control during chronic LCMV infection. Transient CD70 blockade had a moderately positive effect on antibody responses at day 21 post-infection during chronic LCMV infection (Fig. 7A). This pattern of increased antibody responses after CD70 blockade was similar to that during acute infection. Short-term blockade of CD70 signaling during chronic LCMV infection results in modest and transient reduction in viral loads that was only noticeable at day 15 post-infection, but

returned to normal values after interruption of treatment (Fig. 7B).

Discussion:

T cell costimulation is an important event during T cell activation, and provides an additional requirement after TCR triggering (38). Signals from CD27 and the TCR mediate CD8 T cell clonal expansion independently of IL-2, and contributes to promoting T cell survival (28). CD70 is usually expressed on activated APCs, but both CD27 and CD70 can be expressed on T cells (58, 60, 82). Therefore, this pathway has been suggested to be involved in not only DC-T interactions, but also in a T-T cell network (79). Expression of CD70 on immune cells is tightly regulated. CD70 is upregulated at 1-2 weeks after infection, and gradually decreases over time after an antigenic challenge, highlighting the importance of its proper regulation on immune cells (82). The duration and cell type involved in CD27/CD70 costimulation also determines the outcome of immune responses. For example, mice that overexpress CD70 constitutively on B cells display increased effector T cell differentiation, and decreased numbers of B cells, suggesting opposite effects of constitutive CD27/CD70 signaling on T and B cell responses (6).

Previous reports have shown that CD27/CD70 signaling enhances anti-tumor (5, 37, 41, 57, 90), and primary influenza-specific T cell responses (1, 9, 40, 57, 127). Consistent with these reports, we found delayed primary response kinetics after blockade of the CD27/CD70 pathway after acute Armstrong LCMV infection. At day 7 post-infection, the peak of the antiviral T cell response, there were fewer absolute numbers of antigen-specific T cells by intracellular cytokine

staining, and by day 15 post-infection (IFNg-expressing after LCMV peptide stimulation). Also, percentages and absolute numbers of CD8 T cells that co-expressed IFNγ and TNFα were reduced after CD70 blockade at the peak of the primary response. This suggested functional defects after CD70 blockade during an acute viral infection.

However, viral clearance kinetics was similar in both treated and control groups by day 8 (data not shown). This suggested that even if the numbers of T cells that recognize Armstrong gets curtailed 3-fold, this reduced fraction of the response is more than enough to completely clear this virus with similar kinetics (within a week this acute infection is cleared in either group). CD127 conversion was also impaired. CD127, the IL-7 receptor α chain is a bona-fide marker for memory T cells, as those T cells that survive the contraction phase after the peak of the T cell response are usually CD127hi, and are the ones that survive to become long-lived cells and respond to secondary challenges (54, 70). Our results with the acute LCVM model were in agreement with previous reports using either CD70 antibody blockade or CD27-/- mice (28, 57). These studies have shown that CD27/CD70 signaling is necessary for the generation and maintenance of primary CD8 T cell responses. Primary clonal T cell expansion to influenza (an acute pathogen) is impaired in CD27-/- mice indepdendent of CD28 and IL-2 (57), highlighting the importance of this pathway. The aforementioned studies, and those from our acute LCMV infection showed a positive role for this costimulatory pathway on T cell responses. Clearly, abrogation of CD27/CD70 signaling either with receptor knockout mice or antibody-mediated blockade

results in reduced numbers of antigen-specific T cell responses during primary acute responses

However, there is also evidence of an opposing role of CD27/CD70 costimulation during chronic infection, suggesting a negative effect of constitutive CD27/CD70 signaling on immune responses (82, 124, 127). Constitutive expression of CD70 on T cells induces a T cell differentiation pathway that is similar to that observed during chronic infections such as HIV, including upregulation of PD-1 and downregulation of CD127 (127). Mice that overexpress CD70 on B cells succumb to lethal immunodeficiency after 6-8 months due to overt immune activation and spontaneous conversion of naïve T cells into effector memory cells (124).

These results, together with those in the aforementioned models, suggested context-dependent outcomes of CD27/CD70 costimulation. In order to consolidate these previous data, we decided to use the LCMV system, which has both an acute and a chronic model of infection. This helped us to determine whether the persistence, or the nature of an antigen plays a role in the outcome of CD70 costimulation. LCMV Armstrong and Cl-13 strains differ in only 2 point mutations in the polymerase and the receptor binding genes (108, 109). These subtle differences enable the Cl-13 strain to persist and cause progressive T cell exhaustion (135, 141). Thus, the nature of the antigen is virtually identical, with all T cell epitopes unchanged, and the only significant difference between these 2 LCMV strains is their persistence within the host.

As a comparative study, we decided to analyze the effect of blocking CD27/CD70 interactions during chronic LCMV infection (comparing effects seen on acute LCMV infection). A previous report has demonstrated a negative role of CD70 signaling during chronic viral infection, showing that absence of signaling through CD27 during a chronic LCMV Docile viral challenge results in elimination of chronic viral infection (82).

In our studies, we only performed transient CD70 antibody blockade, and we noticed increased absolute numbers of antigen-specific T cells in the anti-CD70 treated mice after day 21 post-infection with LCMV Cl-13. Before day 21, there were no apparent differences in the T cell response (data not shown). This increased numbers of LCMV-specific CD8 T cells was a reflection of the higher spleen cellularity in anti-CD70 treated mice (~31 x10^6 splenocytes in control IgG treated mice, versus ~52x10^6 splenocytes in anti-CD70 treated mice). This suggested a partial role of CD70 signaling in mediating reduction of lymphoid cellularity during chronic infection. In addition, mice treated with anti-CD70 displayed visible lymph nodes by day 21 (data not shown), and this is noteworthy because mice infected with Cl-13 display noticeable reduction in lymph node size.

Consistent with the results from Matter et al. (82), we found some increase in B cell responses after CD70 blockade during chronic LCMV infection (blockade in these reports was performed from day 4 to day 35, post-infection). We did not notice significant reduction in viral titers after 12 days of anti-CD70 antibody treatment. The studies from Matter et al. (82) were performed using

CD27-/- mice as well as with longer blocking antibody regimens. Our more short-term blockade approach may account for the reduced and only transient effect on viral titers, and the more modest increase in antibody responses that we observed during CD70 blockade after either chronic or acute infection.

Collectively, our data, and that of others independently with various models of antigen challenge, show that it may be possible to enhance cellular responses in a context-dependent manner by manipulating the CD27/CD70 pathway. During an acute antigen challenge, this pathway provides a transient and beneficial costimulatory signal that promotes T cell expansion, and memory conversion. On the other hand, during a chronic infection, constitutive triggering of this pathway may be detrimental to immune cell homeostasis. Our results with either the Armstrong or Cl-13 strains of LCMV suggest that the effect of CD70 costimulation is dependent on antigen persistence, and not on the intrinsic nature of the antigen. These findings may have implications for vaccination regimens to enhance responses to either acute or chronic pathogens.

Acknowledgments

I would like to thank Ata Ur Rasheed, Smita Smita Iyer, Hideo Yagita, Bruce Blazar and my mentor Rafi Ahmed for their help and intellectual input. This work was funded by NIH grant 30048 and P01 AI056299.

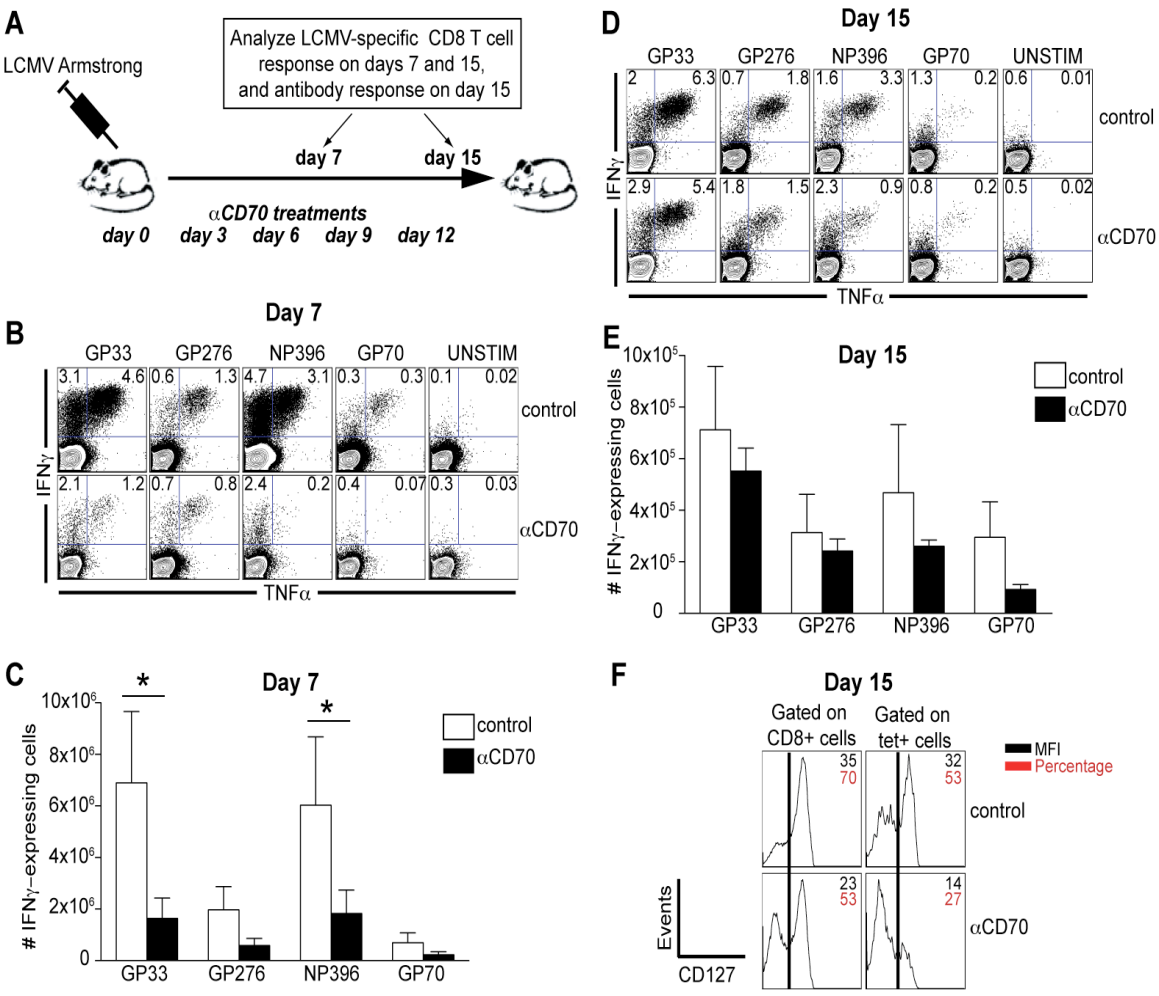


Fig. 1. Role of CD27/CD70 signaling during acute viral infection. A) Experimental outline. 6-8 week old C57BL/6J mice were treated with anti-CD70 at the indicated time points, and infected with LCMV Armstrong. Mice were sacrificed at days 7 and 15 for analysis of cellular responses. B) Intracellular cytokine staining after stimulation with several LCMV peptides at day 7 post-infection (FACS data from one representative experiment). C) Absolute numbers of IFNγ-expressing CD8 T cells after LCMV peptide stimulation at day 7 post-infection. D) Intracellular cytokine staining after stimulation with several LCMV peptides at day 15 post-infection (data from one representative experiment). E) Absolute numbers of IFNγ-expressing CD8 T cells after LCMV peptide stimulation at day 15 post-infection. F) Memory conversion by CD70 blockade during acute viral infection (expression of CD127 on H-2D(b) GP33-41 specific CD8 T cells from spleen at day 15 post-infection). Black= Mean Fluorescence Intensity; Red= Average percentage. Data are from spleens, 2 separate experiments, each n=6. *P<0.5.

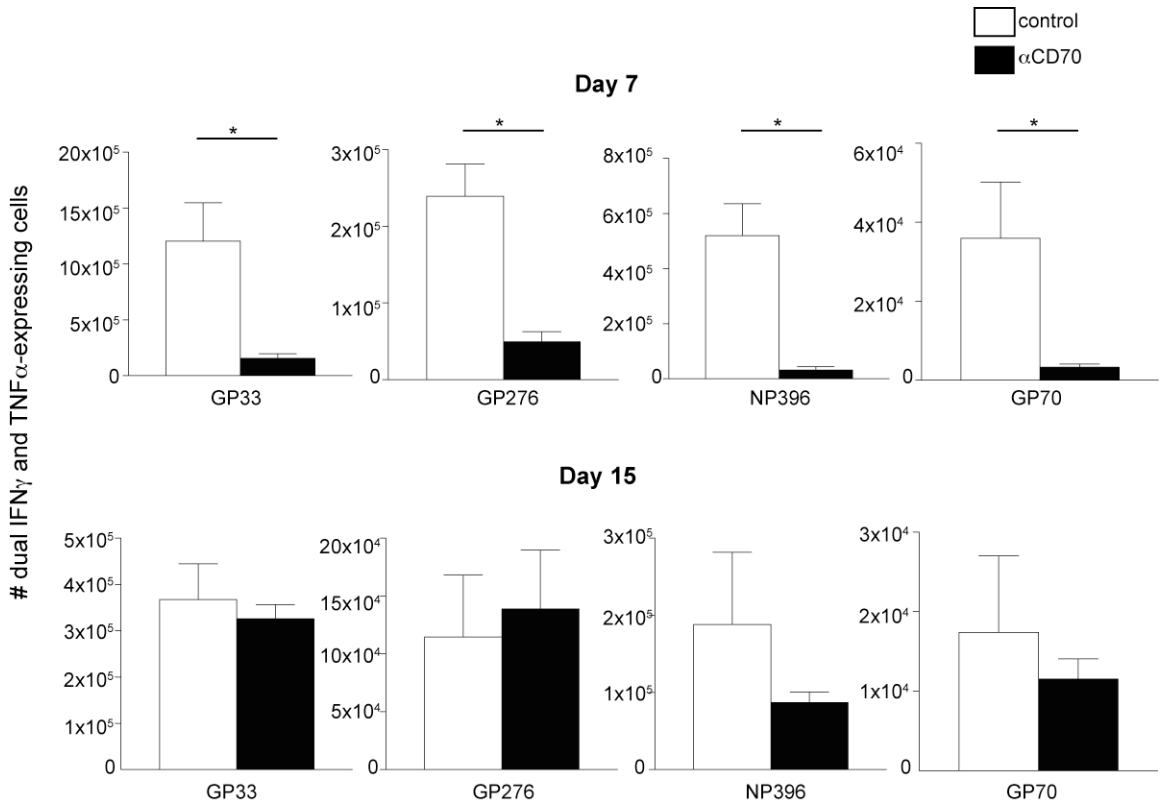


Fig. 2. Dual IFNγ and TNFα coexpression after CD70 blockade during acute viral infection. *P<0.05.

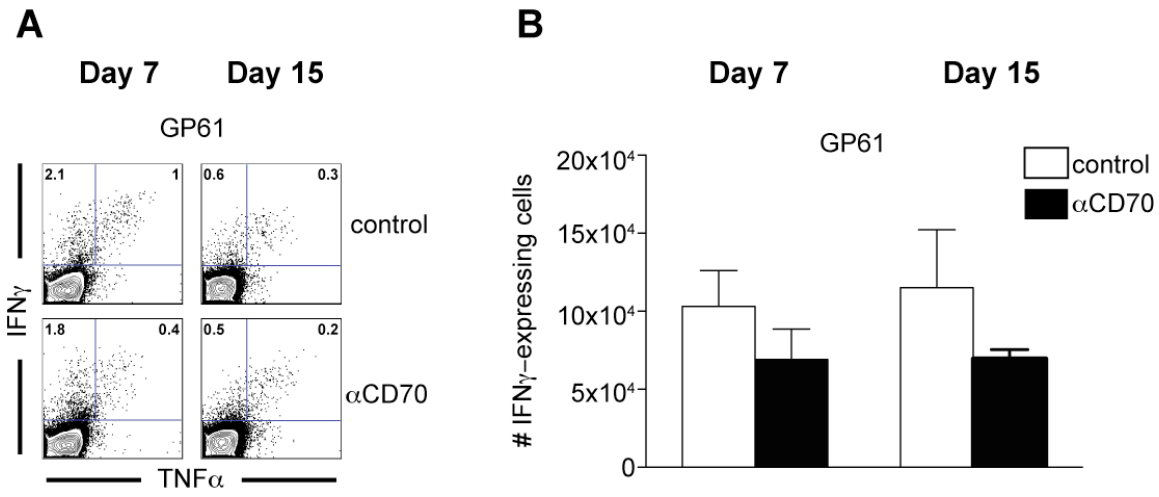


Fig. 3. LCMV-specific CD4 response after CD70 blockade during acute viral infection. A) Intracellular cytokine staining after stimulation with immunodominant LCMV GP61-80 at days 7 and 15 post-infection (FACS data from one representative experiment). C) Absolute numbers of IFNγ-expressing CD4 T cells after LCMV peptide stimulation at days 7 and 15 post-infection.

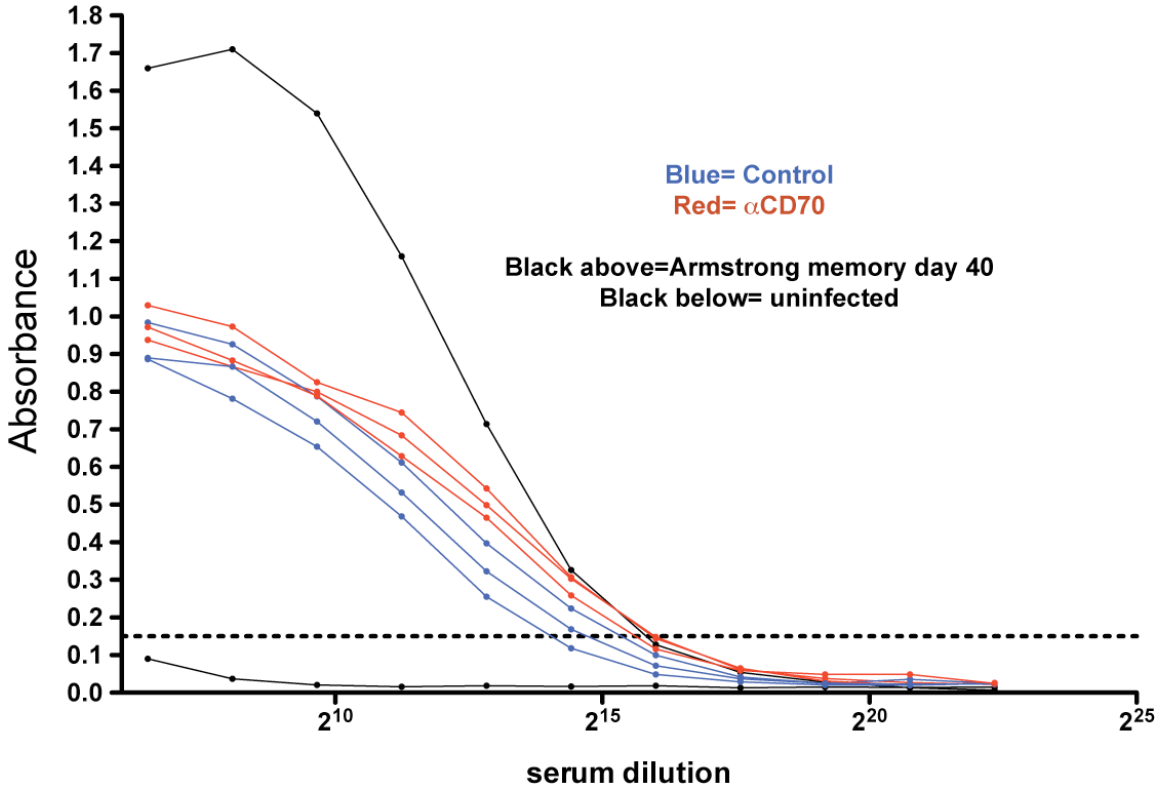


Fig. 4. Viral-specific antibody responses after CD70 blockade during acute viral infection. ELISA was performed for detection of LCMV-specific IgG titers from serum at day 15 post-infection. Armstrong memory at day 40 post-infection (black line above), and naïve (black line below) were included as controls. Data are from one representative experiment.

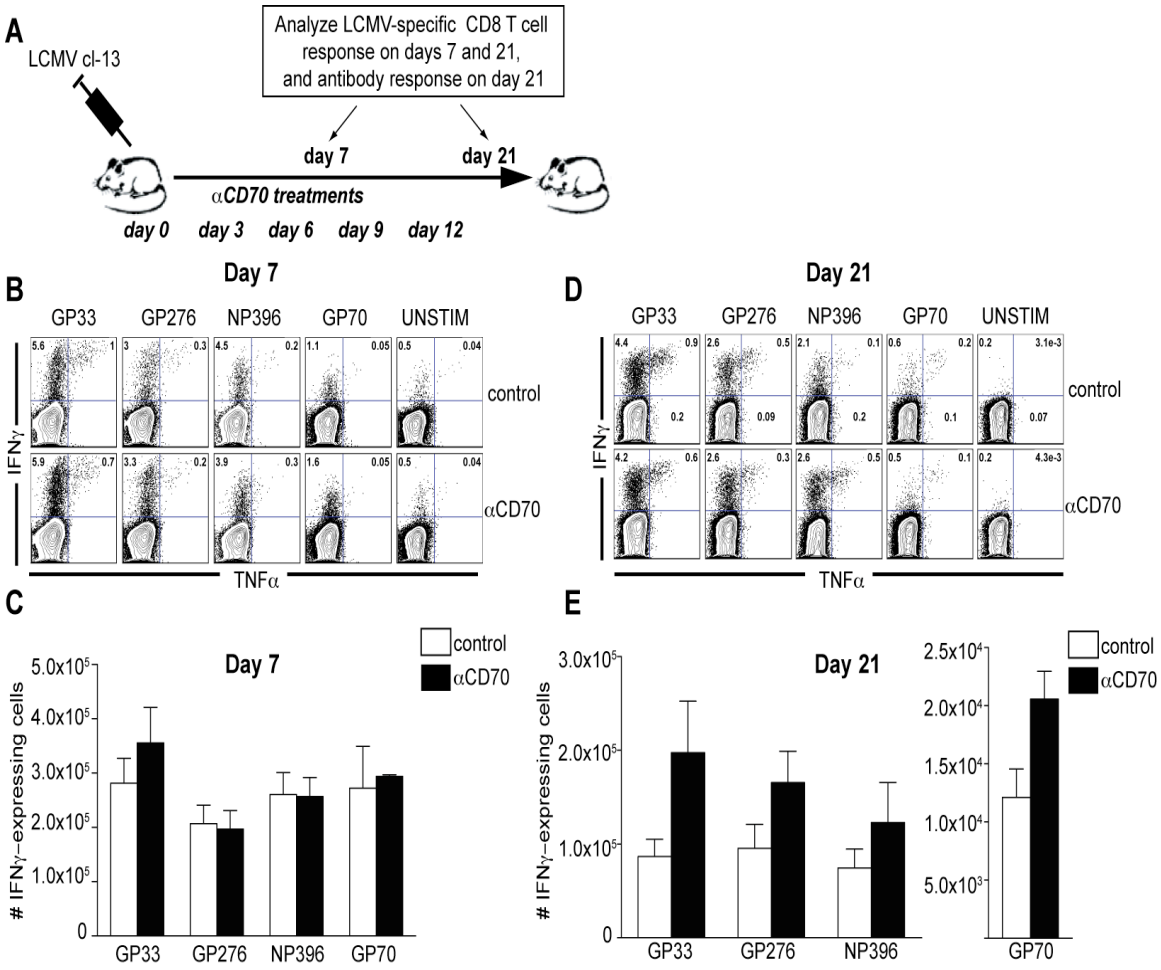


Fig. 5. Role of CD27/CD70 signaling during chronic viral infection. A) Experimental outline. 6-8 week old C57BL/6J mice were treated with anti-CD70 at the indicated time points, and infected with LCMV Cl-13. Mice were sacrificed at days 7 and 21 for analysis of cellular responses. B) Intracellular cytokine staining after stimulation with several LCMV peptides at day 7 post-infection (data from one representative experiment). C) Absolute numbers of IFNγ-expressing CD8 T cells after LCMV peptide stimulation at day 7 post-infection. D) Intracellular cytokine staining after stimulation with several LCMV peptides at day 21 post-infection (FACS data from one representative experiment). E) Absolute numbers of antigen-specific CD8 T cells at day 21 post-infection. Data are from spleen, 2 separate experiments, each n=6.

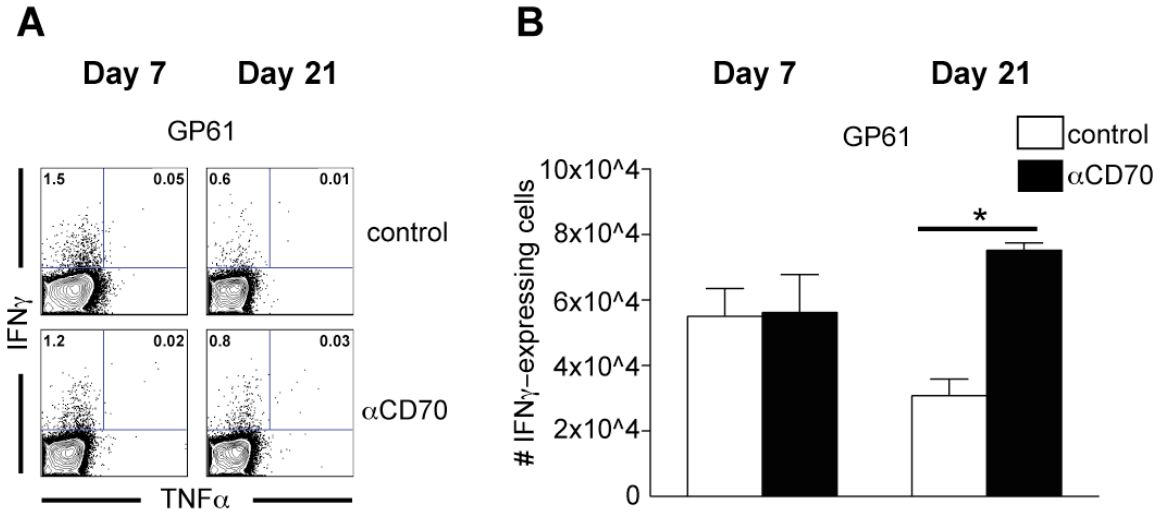


Fig. 6. LCMV-specific CD4 response after CD70 blockade during chronic viral infection. A) Intracellular cytokine staining after stimulation with immunodominant LCMV GP61-80 at days 7 and 21 post-infection (FACS data from one representative experiment). C) Absolute numbers of IFNγ-expressing CD4 T cells after LCMV peptide stimulation at days 7 and 21 post-infection.

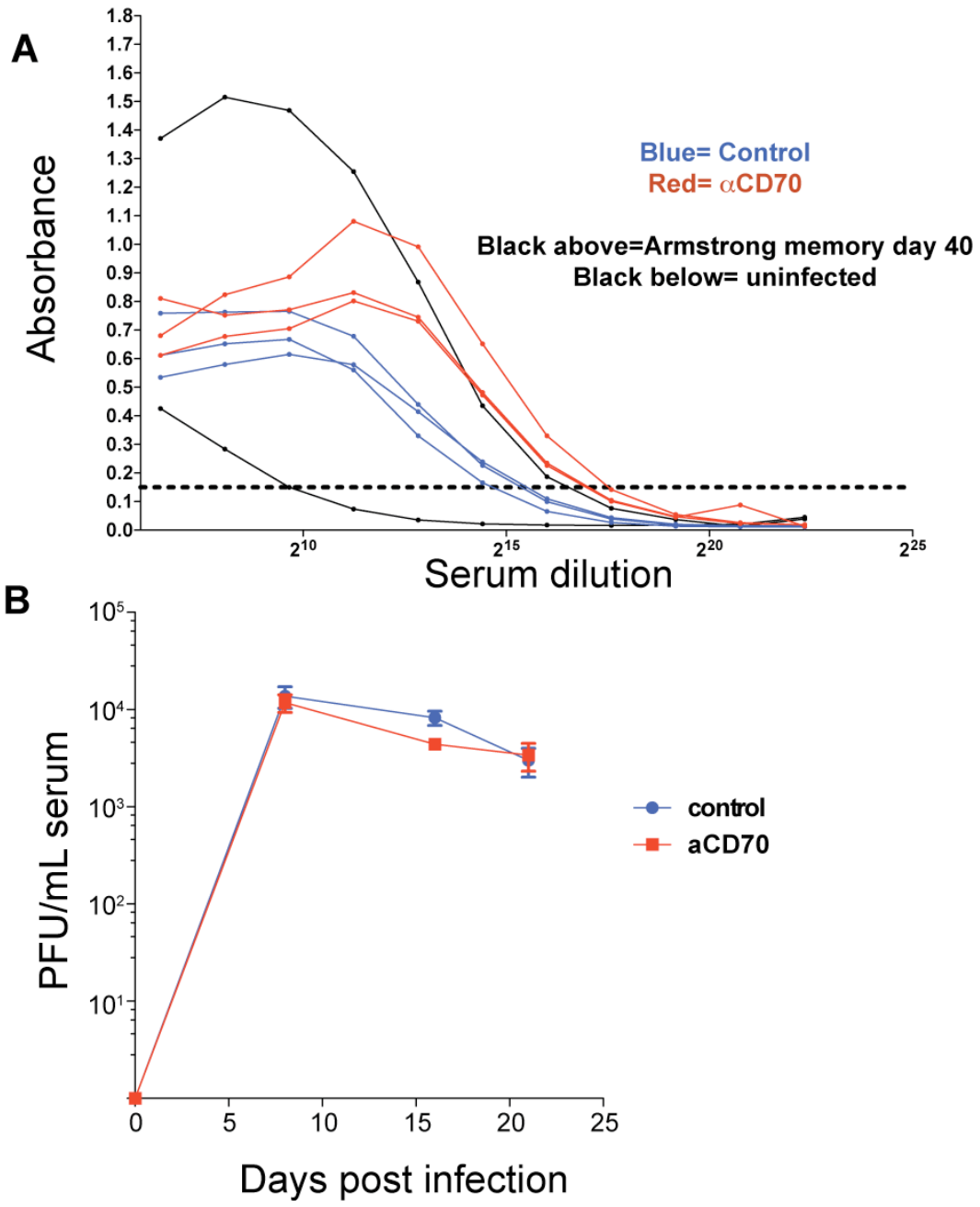


Fig. 7. Viral-specific antibody responses and viral control after CD70 blockade during chronic viral infection. ELISA was performed for detection of LCMV-specific IgG titers from serum at day 21 post-infection. Armstrong memory at day 40 post-infection (black line above), and naïve (black line below) were included as controls. Data are from one representative experiment.

Chapter 4: Enhancing the T Cell Restorative Effect of PD-L1 blockade by transient depletion of CD4 cells

Abstract

Chronic infections result from the inability of the host to clear a pathogen. Soon after the onset of a chronic infection, antiviral CD8 T cells undergo significant changes in their function and phenotype, phenomena that is collectively called exhaustion. Previously, we showed that specific ablation of Tregs during a chronic viral infection results in explosive restoration of viral-specific CD8 T cell function. The mechanism of Treg-mediated viral-specific CD8 T cell suppression appeared to be dependent on B7 modulation, CD4 cells, and cognate antigen. These experiments were performed in FoxP3^{DTR} knock in transgenic mice. However, it remains a goal to be able to restore CD8 T cell function in wild type hosts by transient and milder modulation of T regulatory cell activity. We have combined our standard PD-1 blockade with a transient CD4 depletion, and have observed synergy at rescuing CD8 T cell responses during chronic infection. In addition, CD4+ FoxP3+ cells are reconstituted faster in animals that received a PD-1 blockade regimen, suggesting a role for the PD-1 pathway in controlling homeostatic expansion of Tregs.

Introduction:

In the earlier parts of this thesis we have covered the different regulatory pathways involved in immune control during chronic infection. We explained the role of the PD-1 pathway, and the therapeutic effect of blocking it and addition to inducing costimulatory receptors. We also showed that specific ablation of Tregs, in a model of inducible Foxp3+ cell depletion results in dramatic rescue of exhausted CD8 T cell responses. This significant restoration of CD8 T cell responses after Treg depletion occurs only in mice that are chronically infected with LCMV, whereas those that have cleared virus do not experience any increase. This suggests a role for antigen in determining the suppressive potential on Tregs (background on Tregs has been covered extensively in earlier chapters). In addition, FoxP3- CD4+ cells, and the B7 pathway play a pivotal role in determining CD8 T cell increase after Treg depletion, as depletion of CD4 cells, or blockade of B7 abrogates viral-specific CD8 T cell rescue after Treg depletion.

However, these later studies were performed using the $Foxp3^{DTR}$ knock-in mice, highlighting the importance of developing more physiological therapies in wild type (not transgenic) hosts. For this, we performed transient, antibody mediated CD4 depletions, followed by PD-1 blockade in chronically viremic mice infected with LCMV Cl-13. We then analyzed LCMV-specific CD8 T cell responses, and Treg reconstitution kinetics in mice after this dual regimen. We noticed increase in LCMV-specific CD8 T cell responses in all tissues (compared

to PD-1 blockade alone). We also noticed faster CD4+ Foxp3+ cell recovery after CD4 depletion followed by PD-1 blockade. These findings suggest an important role of PD-1 pathway and Tregs in mediating CD8 T cell exhaustion during chronic infection.

Materials and Methods:

Mice and viral infections

6-8 week old, C57BL/6 female mice were purchased from the Jackson Laboratories (Bar Harbor, Mayne). In order to generate chronically viremic mice, we performed CD4 cell depletions prior to infection (day before and infection, and day of infection). We infected intravenously (i.v.) with LCMV Clone 13 (2x10^6 pfu). Mice started antibody treatments after 45 days of infection. Plaque assay for titration of virus was performed on Vero cells as previously described (2).

In vivo antibody treatments

200 μg of rat anti-mouse PD-L1 (10F.9G2), or rat IgG isotype control were injected i.p. on days 0, 3, 6, 9, and 12 of treatment. 10F.9G2 monoclonal antibody was provided by Gordon Freeman. CD4 depletions were performed with 500 μg of GK1.5 (BioXcell) on days -1 and 0. Each mouse received a volume of 500 μL of diluted antibody (in PBS).

Surface and intracellular staining

Intracellular cytokine staining was performed as described previously (135). Peptide stimulations were at 37°C, for five hours in a CO_2 incubator. All fluore-conjugated antibodies for flow cytometry were from BD Biosciences (San Jose, CA), except for anti-Foxp3 APC, which was from ebiosciences (San Diego, CA).

Results:

CD4 cell depletion followed by PD-1 blockade results in enhanced CD8 T cell rescue during chronic viral infection. LCMV chronically viremic mice started transient CD4 depletion at day 45 post-infection, and received a PD-1 blockade regimen the day after (Fig 1A). This resulted in an increased percentage of CD8 T cells that were LCMV-specific, which was even better than PD-1 blockade alone (Fig 1B). In addition, the percentage of CD8 T cells that were PD-1+ was increased in the mice that received transient CD4 depletion followed by PD-1 blockade (lower right quadrant of FACS plots). This suggested expansion of additional viral-specific CD8 T cells different than the immunodominant GP276-286. There was also a 2-3 fold expansion in the absolute numbers of GP276-286+ CD8 T cells compared to PD-1 blockade alone (Fig 1C).

Increased numbers of PBMCs that are viral-specific after CD4 depletion followed by PD-1 blockade. There number of Db GP276-286 specific CD8 T cells was moderately increased in mice that got CD4 depletion prior to PD-1 blockade (Fig 2).

CD4 cell depletion followed by PD-1 blockade results in increased cytokine expression by viral-specific CD8 T cells. There was an increase in the frequency of single IFNγ and dual IFNγ/TNFα expressing CD8 T cells in mice that received CD4 depletion followed by PD-1 blockade (Fig 3A). The absolute numbers of single IFNγ expressing (Fig 3B) and dual IFNγ/TNFα expressing (Fig

3C) CD8 T cells were also increased after CD4 depletion and PD-1 blockade, compared to PD-1 blockade alone.

Phenotypic changes on peripheral and thymic T cell populations. There was an increased percentage of activated (CD44hi) CD8+ lymphocytes in multiple tissues after CD4 depletion followed by PD-1 blockade (Fig 4A). Viral-specific CD8 T cells upregulated the proliferation marker Ki67 (Fig 4B). In the thymus, there was a consistent reduction in the percentages of double positive thymocytes, and increase in single positive CD4+ or CD8+ populations (Fig 4C).

Viral titers are reduced in mice that get CD4+ cell depletion followed by PD-1 blockade. LCMV Cl-13 viral titers were measured in spleen, liver, and serum of chronically infected mice following antibody treatments(Fig 5).

Effect of PD-1 blockade on Treg populations. Mice that were CD4 depleted, and treated with anti-PD-L1 (similar results for anti-PD-1 blocking antibodies) experienced a greater CD4+ Foxp3+ cell replenishment, compared to CD4 depleted alone. (Fig 6A). Tregs had higher PD-1 expression after PD-1 blockade. Tregs were also higher for CD44 in mice that received CD4 depletion plus PD-1 blockade, compared to the mice that only received CD4 depletion. This suggested higher reconstitutive proliferation in CD4 depleted mice that are treated with PD-1 blocking antibodies. CD127 expression was also increased in the newly generated Treg cell pool, suggesting survival advantage after PD-1 blockade. Total numbers of splenic CD4+ Foxp3+ cells was also higher in mice that received CD4 depletion followed PD-1 blockade, compared to CD4 depletion alone (Fig 6B).

Discussion:

We have shown that CD4+ cell depletion followed by PD-1 blockade (Fig 1A) results in an enhanced CD8 T cell rescue compared to PD-1 blockade alone. The percentages of viral-specific CD8 T cells were increased, suggesting a role for CD4+ Tregs in mediating additional inhibitory functions during chronic infection (independent of the PD-1 pathway) (Fig 1B). The percentages of CD8 T cells that were PD-1+ were also higher in the dual treated group, suggesting expansion of other exhausted viral-specific CD8 T cells (Fig 1B). The absolute numbers of immunodominant Db GP276-286 CD8 T cells was also increased in the CD4 depleted, PD-L1 blockade group (Fig 2C). Slight increase in total numbers of viral-specific CD8 T cells was also observed in CD4 depleted compared to CD4 undepleted. However, the differences were not statistically significant.

This synergy was also evident, although more modestly, in the blood in which there was an increase in the number of tetramer+ cells by the day of analysis (Fig 2). Number of cytokine-expressing cells was also increased further after dual CD4 depletion and PD-1 blockade (Fig 3A, 3B, 3C), suggesting not only physical expansion of viral-specific CD8 T cells, but also functional improvement. This synergy between Treg depletion and PD-1 blockade was previously explained in earlier chapters of this thesis, when we depleted Foxp3+ cells and performed PD-1 depletions. However, we did not have a more physiological read-out, as all of Foxp3+ cell depleted mice succumb to lethal

autoimmunity by day 14. In addition, this was in a knock in Foxp3^{DTR} mouse model, so a more therapeutically feasible model was needed.

We also analyzed the effect of CD4 depletion followed by PD-1 blockade on total CD8 T activation. Splenic CD8 T cells upregulated CD44 activation marker (Fig 4A). This increased CD44 upregulation was also seen in CD8 T cells from liver, lung, blood, and gut (not shown). Db GP276-286 specific cells also upregulated Ki67, suggesting enhanced proliferation of viral-specific CD8 T cells after CD4 depletion plus PD-1 blockade (Fig 4B). Within the thymus, there were also interesting differences. There was a reduction in the frequency of double positive thymocytes, and emergence of more single positive thymocytes after CD4 depletion followed by PD-1 blockade (Fig 4C). One possibility is that CD4+ Tregs and the PD-1 pathway could modulate maturation of T cell thymic precursors. Another possibility is that migration of peripheral T cells into the thymus is controlled by the CD4+ Foxp3+ Tregs and the PD-1 pathway. Additional experiments are needed to elucidate which possibility is correct.

In addition to increased CD8 T cell restoration, mice that received CD4+ cell depletions followed by PD-1 blockade also controlled virus better compared to PD-1 blockade alone.

The effect of PD-1 blockade on Tregs after whole CD4 depletion was also noteworthy. PD-1 blockade induced a faster CD4+ FoxP3+ cell reconstitution after CD4 depletion (40% in PD-1 blockade group versus 15% in control group) (Fig 6). Tregs in the CD4 depleted plus PD-1 blockade group looked more activated as evidenced by increased PD-1 and CD44 (Fig 6). It is believed that

Treg specificity is biased for self-antigens(65, 69, 106), so it is possible that PD-1 blockade may have some effects on these cells, as PD-1 signaling is known to act downstream of the TCR(73, 116).

We have shown that transient CD4 depletion optimizes CD8 T cell rescue after PD-1 blockade during a stringent chronic viral infection. Considering our previous data with $Foxp3^{DTR}$ mice, it is considerable to attribute this enhancement in viral-specific CD8 T cells to transient Treg ablation. The increase in antigen-specific CD8 T cells was only 2-3 fold, and not a log or higher as in our $FoxP3^{DTR}$ experiments. A possibility for this reduced rescue effect could be that conventional CD4 cells are also ablated by our standard CD4 depletion regimen. We showed previously that CD4+ Foxp3- cells are needed for the CD8 T cell rescue after specific CD4+ Foxp3+ depletion. It is possible that antibody mediated CD4 cell depletion does not result in 100% ablation in the mouse, so the CD4+ cells leftover could be involved in rescuing exhausted CD8 T cells.

Even though the transient, whole CD4 depletion followed by PD-1 blockade resulted in a more modest CD8 T cell rescue, compared to specific Treg depletion followed by PD-1 blockade, the mice did not experience lethal autoimmunity. This is an advantage of temporarily ablating all CD4 cells and not only CD4+Foxp3+.

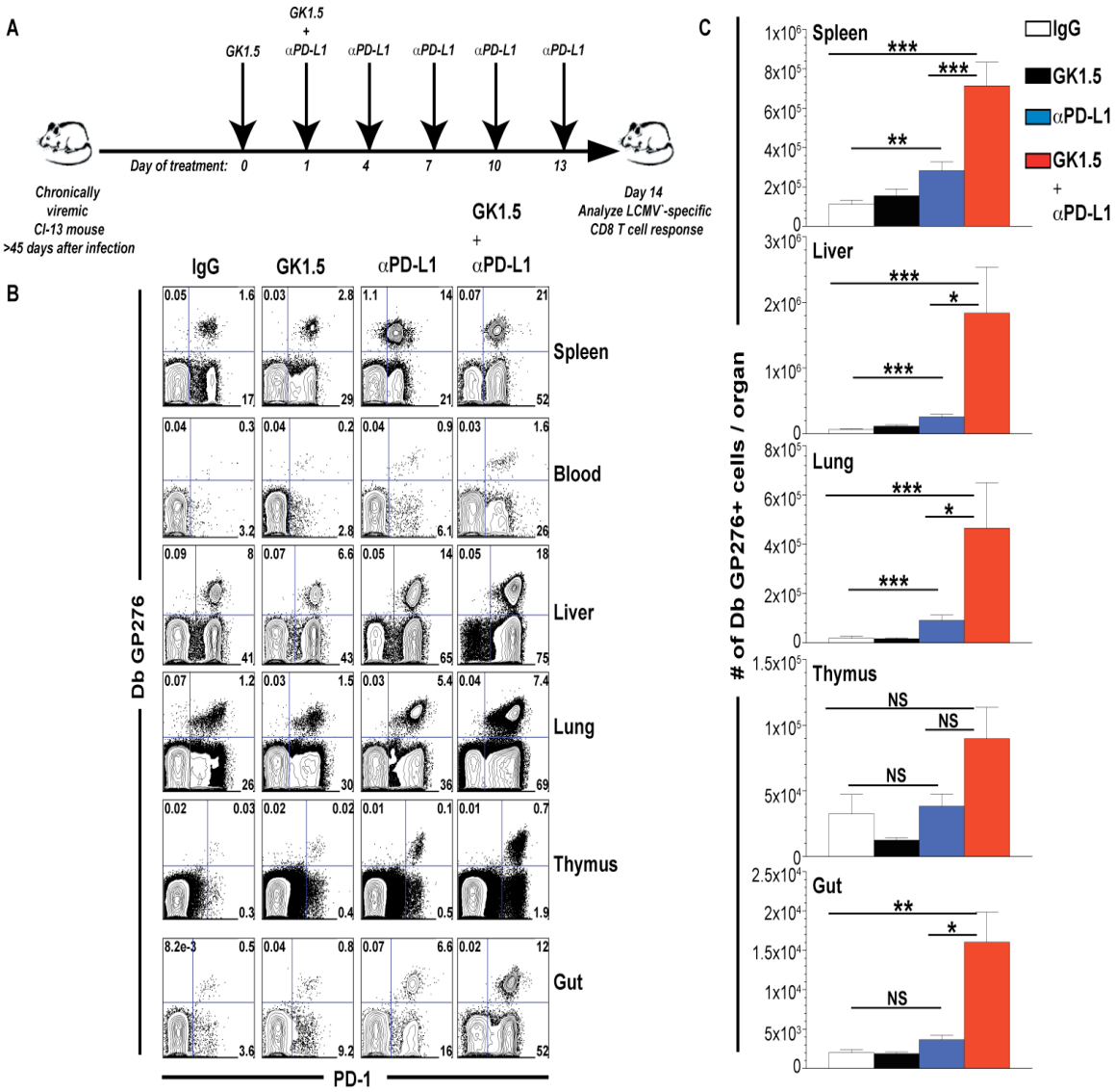


Fig 1. CD4 depletion followed by PD-1 blockade results in greater CD8 T cell restoration, compared to PD-L1 blockade alone. A) Experimental set-up for CD4 depletion (GK1.5) followed by PD-1 blockade during chronic LCMV infection. B) Percentages of CD8+ lymphocytes that are Db GP276-286 specific in multiple tissues. C) Absolute number of CD8+ lymphocytes that are Db GP276-286 specific in multiple tissues. FACS plots were gated from live CD8+ lymphocytes.

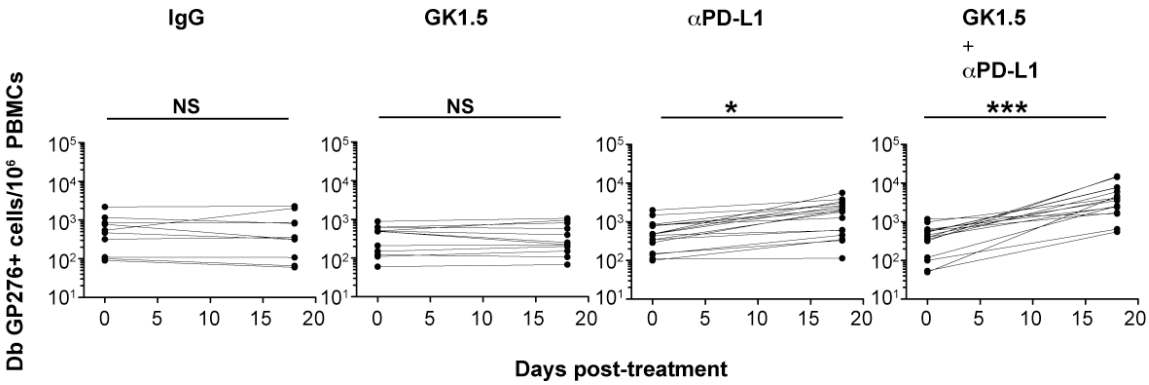


Fig 2. Increase in numbers of Db GP276-286 specific CD8 T cells after CD4 depletion and PD-L1 blockade. Mice were bled before treatment (day 0), and at the end of the treatment (day 14), and PBMCs were stained for Db GP276-286 tetramer.

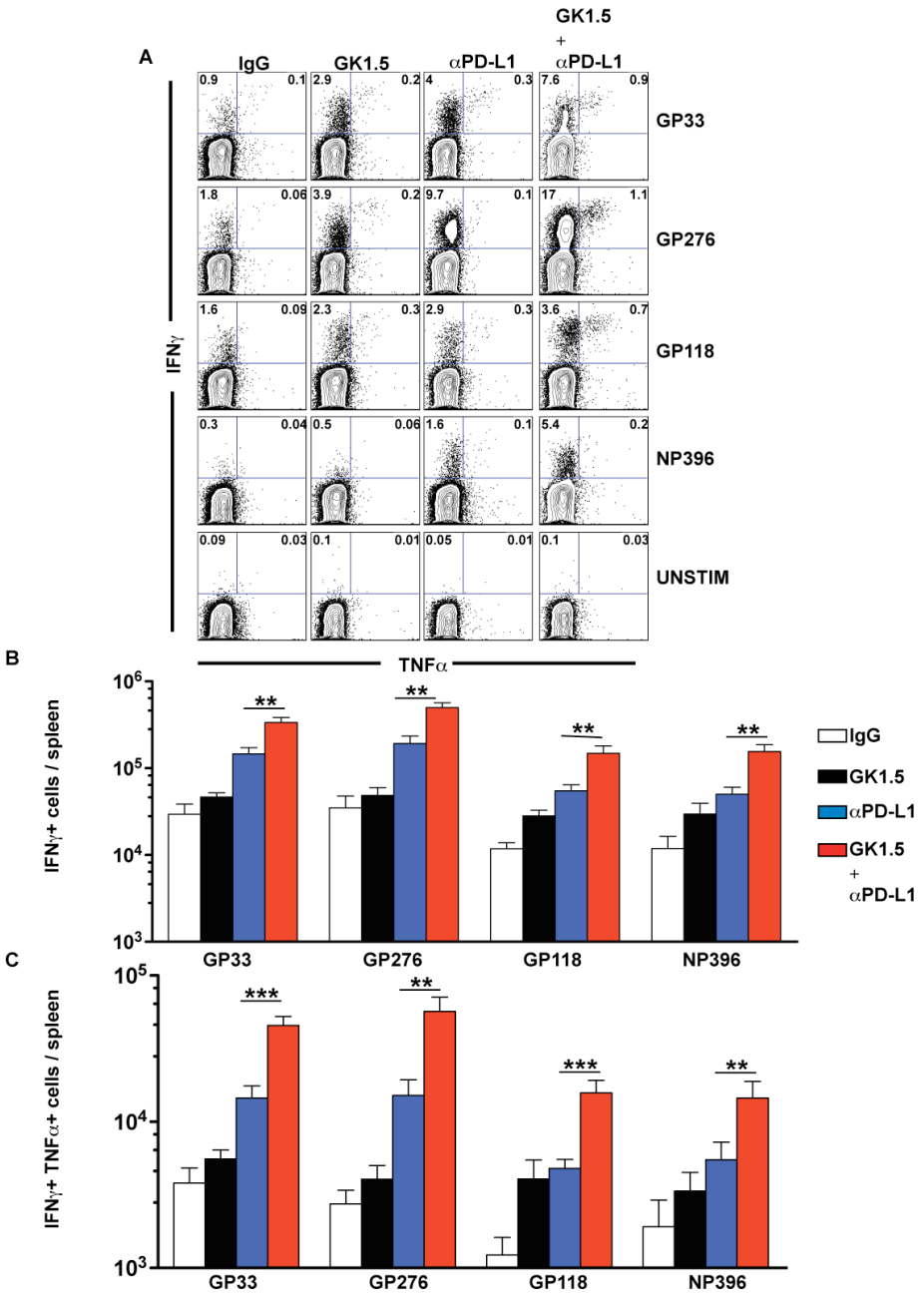


Fig 3. CD4 depletion followed by PD-1 blockade results in greater functional rescue of exhausted CD8 T cells, compared to PD-L1 blockade alone. A) Intracellular IFNγ and TNFα cytokine staining after 5 hr stimulations with LCMV peptides, showing percentages of LCMV specific CD8 T cells. B) Absolute numbers of IFNγ expressing CD8 T cells. C) Absolute numbers of IFNγ and TNFα co-expressing CD8 T cells. FACS plots were gated from live CD8+ lymphocytes.

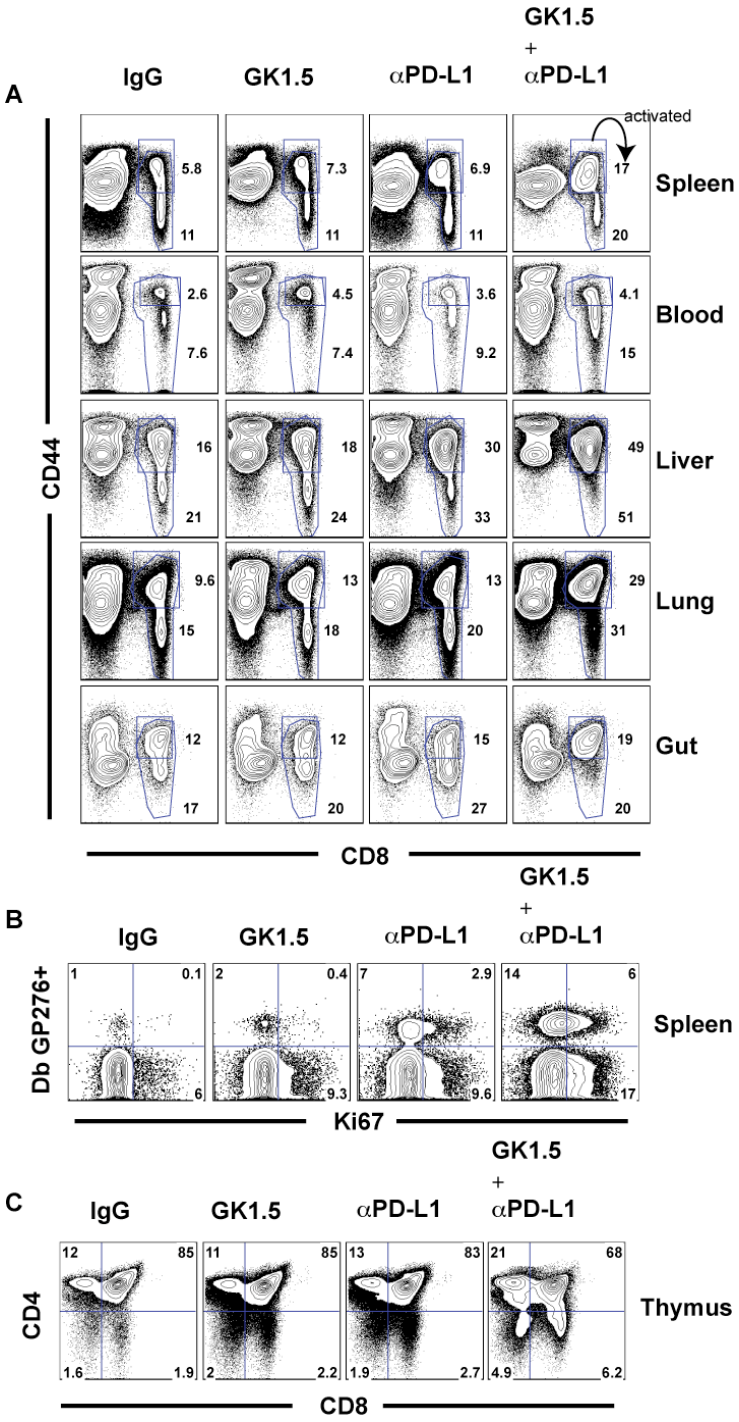


Fig 4. Phenotypic changes after CD4 depletion followed by PD-1 blockade. A) Percentages of CD8 T cells that are CD44hi in multiple tissues. Top number represents the percentage of CD8+ cells that are CD44hi, whereas the lower number represents total CD8 cell percentages. B) Measuring proliferation of exhausted CD8 T cells after treatment by Ki67 staining. FACS plots were gated from live lymphocytes. C) Changes in the frequencies of thymic precursors.

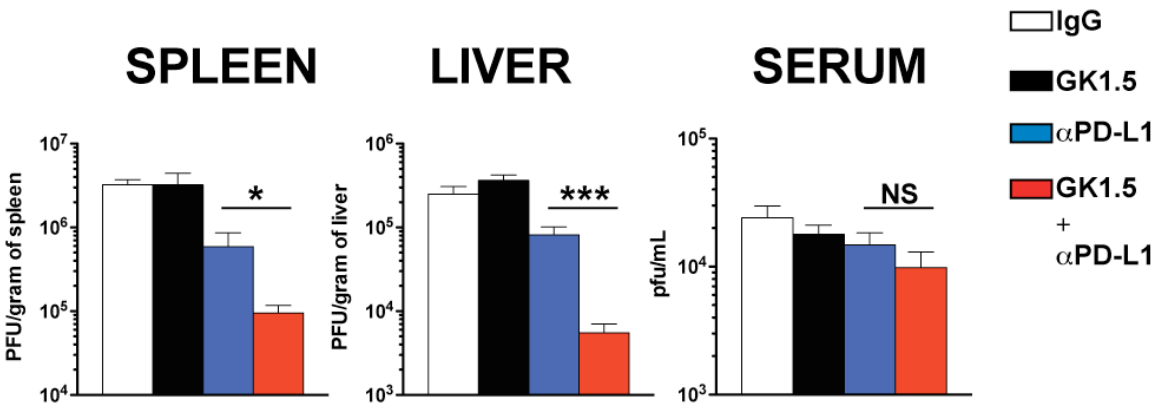


Fig 5. Viral titers in spleen, liver and lung. LCMV titration in tissues after treatment.

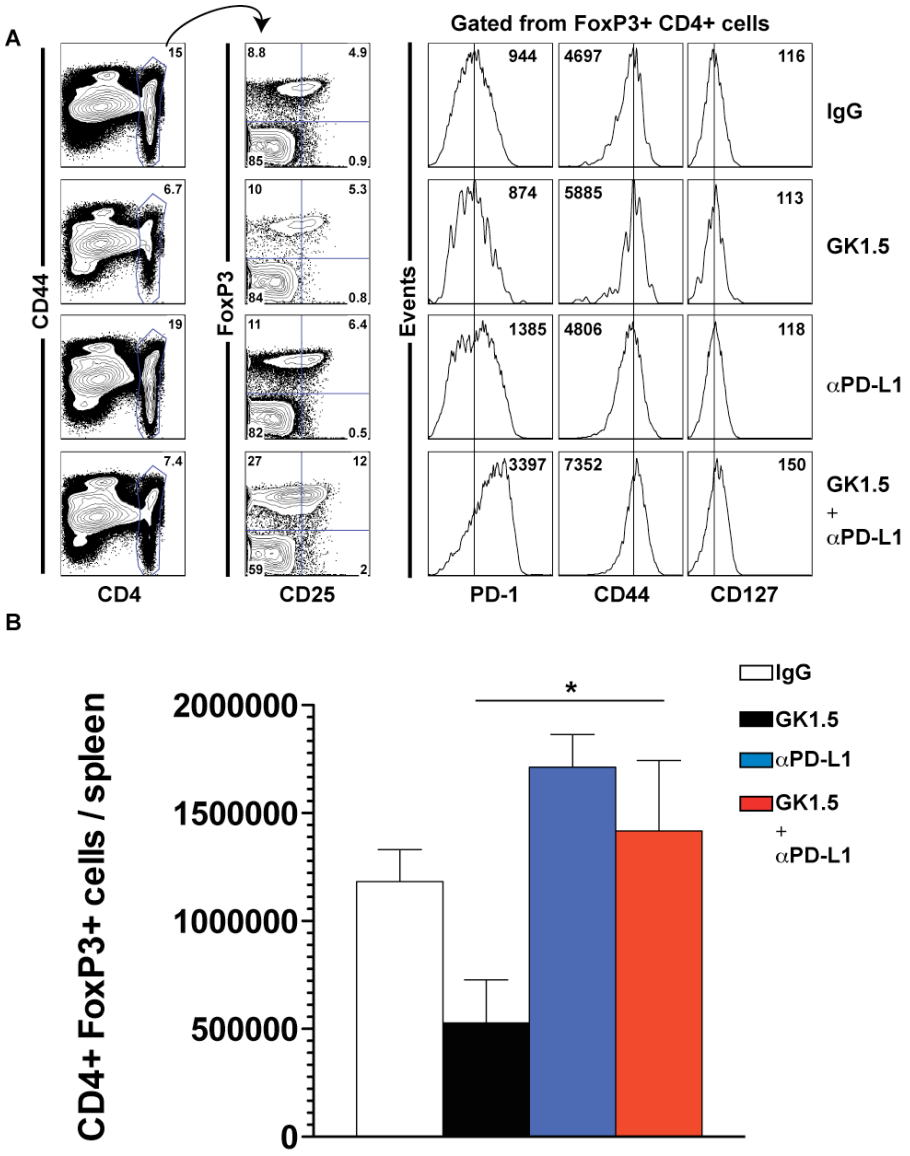


Fig 6. PD-1 blockade enhances CD4+ Foxp3+ reconstitution. A) Frequency of CD4+ Foxp3+ cells after treatment (spleen). Expression of PD-1, CD44, and CD127 on CD4+ Foxp3+ cells after treatment. B) Total numbers of CD4+ Foxp3+ cells (spleen). FACS plots were gated from live lymphocytes (first column), and then on live CD4+lymphocytes (second column).

Acknowledgments

I would like to thank Koichi Araki, Alice Kamphorst, Smita Iyer, Erin West, and Bogna Konieczny for their help with this work.

Chapter 5: T regulatory cells sustain CD8 T cell exhaustion during chronic viral infection

Abstract

CD8 T cell exhaustion is a hallmark of chronic infections, and is characterized by decrease in proliferation, cytokine expression, and distinctive phenotypic changes. Understanding the factors that guide this process may provide opportunities to modulate T cell responses during persistent infections. We analyzed the contribution of T regulatory cells (Tregs), the master regulators of immune tolerance, to the process of CD8 T cell exhaustion during murine chronic lymphocytic choriomeningitis virus (LCMV) infection, using $Foxp3^{DTR}$ mice, in which Foxp3+ cells can be selectively depleted with diphtheria toxin (DT). Normally, these chronically viremic mice experience a pronounced CD8 T cell exhaustion. However, specific Treg ablation resulted in a significant rescue of viral-specific CD8 T cells. The mechanism of Treg mediated suppression is dependent on B7 modulation, CD4 T cells, and the presence of viral antigen, suggesting a general role for these factors in maintaining CD8 T cell exhaustion during chronic infections.

Introduction

Chronic infections result from the inability of the immune system to clear a pathogen. There is a clear association between persistent infections and T cell exhaustion, which is defined by characteristic changes in phenotype and function. Changes in the phenotype of antigen-specific CD8 T cells involve downregulation of the memory marker CD127, and upregulation of co-inhibitory receptors, such as PD-1, whereas changes in function involve reduction of cytokine expression (16, 39, 132, 141). It is critical to determine the factors that guide and sustain T cell exhaustion during chronic infections, as this may provide novel therapies to rescue immune function. Our lab and others, have demonstrated the association of inhibitory receptors (especially PD-1) with T cell exhaustion (11, 16, 39, 129), but it is currently unknown if Foxp3+ CD4+ cells (herein referred as Tregs) play a role in sustaining this dysfunctional conversion.

Tregs are a specialized subpopulation of CD4 T cells characterized by expression of Foxp3+ (45, 64), and their remarkable ability to modulate immune responses. They are involved in mediating immune homeostasis by suppressing responses to self-antigens. However, knowledge about their role during the late phases of chronic infections is still limited. It remains unanswered if Tregs are involved in maintaining the developmental process of CD8 T cell exhaustion that is associated with persistent infections. Although curtailing excessive pathogen-specific T cell responses may prevent immunopathology, it may also facilitate the establishment and maintenance of chronic infections. Therefore, it is important to

determine the specific role that Tregs play during established infections. Seminal studies in parasitic and viral infections showed that Tregs dampen antigen-specific T cell responses, and affect pathogen replication (12-14, 61, 122). Studies in retroviral infections have also demonstrated Treg expansion after infection (67), and a critical role of these cells in suppressing retroviral-specific T cell responses (87, 89, 101, 144, 145). Treg in vitro suppressive activity have also been demonstrated during several human chronic infections. During hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, a high frequency of circulating and liver-localized Tregs is associated with chronic active hepatitis, decreased viral-specific T cell function, and viral persistence (20, 42, 94, 120, 140).

During simian and human immunodeficiency virus (SIV and HIV) infections, the presence of Tregs at sites of viral replication (lymph nodes, and gut-associated lymphoid tissue) is directly correlated to increased viral loads and poor viral control by viral-specific T cells (44, 77). These independent results suggested an association (yet not a causation) between Tregs and the outcome of CD8 T cell responses during chronic infections. Also, most Treg suppression experiments were performed in vitro, and did not provide a complete readout of pathogen-specific responses. In addition, even though the interesting correlation between Tregs and chronic infections was established, there is no mechanistic explanation for Treg suppression of antigen-specific responses during persistent infections. To understand the precise in vivo role that they play during chronic infections, it is necessary to use a chronic infection system in which one can

follow multiple antigen-specific responses of known phenotype and numbers, with a transgenic system that would allow for specific, complete, and transient ablation of Tregs.

Materials and Methods

Mice and infections

6-8 week old, Foxp3^{DTR} female mice on a C57BL/6 background were sent as part of a collaborative study with Alexander Rudenski (mice were bred at Taconic Farms). Mice were infected intraperitoneally (i.p.) with LCMV Armstrong (2x10^5 pfu) for acute infection analysis. For chronic infection analysis, mice were CD4 depleted, and infected intravenously (i.v.) with LCMV Clone 13 (2x10^6 pfu) via lateral tail vein injection.

Virus Titration

Viral Titration of LCMV was performed by plaque assay on Vero cell monolayers as described previously (2), and also in Chapter 2 and 3 of this thesis.

In vivo Treg depletions

Diphteria toxin (DT) was administered i.p. at days 0, 1, 4, and 7 at a dose of 50 μg/kg, and diluted in 500 μL PBS. DT was purchased from SIGMA, and stock was reconstituted at 1 mg/mL, and stored according to manufacturer's recommendations.

Intracellular and surface staining

Stains were performed as previously described (135). Five hour peptide

stimulations were performed at 37°C with GolgiPlug and GolgiStop (BD Biosciences). All staining antibodies were purchased from BD Biosciences (San Jose, CA). Cells were acquired using FACSCanto (Becton Dickinson), and analyzed with FlowJo (Treestar).

Statistical analysis

GraphPad (Prism) was used for statistical analysis. Mann-Whitney test was used for calculating statistical significance.

Results

Chronically infected mice have Tregs that are phenotypically different compared to normal mice. In our preliminarily observations we noticed that Tregs display a distinct phenotype in LCMV chronically infected mice, compared to uninfected or acute memory mice (which had efficiently cleared LCMV). Tregs of mice with chronic LCMV cl-13 infection showed decreased CD25 expression (Fig 1A). It is possible that Tregs in these persistently viremic mice may be more resistant to IL-2 deprivation, suggesting a survival advantage. Also, we noticed increased expression of CTLA-4, CD103, GITR, CD39, granzyme B, ICOS, PD-1, and PD-L1, CD44, CD69, and KLRG-1 (Fig 1B). Conversely, CD62L was downregulated on Tregs of chronically infected mice, whereas CD127 was slightly upregulated. These phenotypic differences suggested a possible functional difference in Tregs of chronically infected mice.

Ablation of Tregs during a persistent viral infection results in rescue of CD8 T cell responses. To explore this hypothesis, we used a chronic LCMV cl-13 infection model, in which mice are viremic for life. The LCMV system provides an excellent opportunity to study the precise effects that Tregs exert on exhausted viral-specific CD8 T cells of multiple specificities, with well-characterized phenotypes. We depleted Foxp3+ cells in $Foxp3^{DTR}$ knock-in mice (75) after the onset of a chronic LCMV infection, and analyzed the response after 11 days without Foxp3+ cells (Fig. 2A). This transient Treg depletion resulted in a 10-20 fold increase in the percentage of LCMV-specific CD8 T cells (Db GP276+)

in multiple tissues (Fig. 2B). The fold-increase of antigen-specific CD8 T cells in the blood was also remarkable, as normally these chronically infected mice have very few to negligible numbers of viral-specific cells in blood (81), but upon Treg depletion, there is >50-fold average fold increase (Fig. 2C). This drastic increase in viral-specific responses occurs between day 5 and 7 after Treg depletion (Fig. 2D). There was also a significant increase in the total numbers of viral-specific CD8 T cells (Db GP276+) in multiple tissues after 11 days of Treg depletion (Fig. 2E). The phenotype of antigen-specific CD8 T cells was also different after Treg ablation (Fig. 2F). Viral-specific CD8 T cells upregulated CD127, the IL-7 receptor α chain, which is an important memory marker that enhances survival of antigen-specific cells (70). A hallmark of CD8 T cell exhaustion and poor viral control is their seemingly irreversible CD127 downregulation (19, 71, 133, 141), which correlates with decreased viral control and inadequate CD4 help in chronic infections, such as that with HIV or HBV (19, 49, 104).

Therefore, It was surprising to observe CD127 re-expression on viral-specific CD8 T cells, as this suggested de novo memory conversion (or survival advantage) of these expanded CD8 T cells after Treg depletion. Viral-specific CD8 T cells also exhibited a significant increase in granzyme B and CD44 expression, suggesting enhanced cytocidal function and increased activation after depletion of Foxp3+ cells. Viral-specific cells had proliferated extensively after 11 days without Tregs, as measured by Ki67 staining. These expanded CD8 T cells were highly functional, expressing IFNγ and TNFα upon LCMV peptide stimulation (Fig. 2G). Even undetectable CD8 T cell responses, such as

that for the NP396-404 epitope (135), re-appear after Treg ablation, resulting in enhanced breadth of CD8 T cell responses. Surprisingly, 70-90% of all CD8 T cells in Treg depleted mice were specific for the 5 LCMV epitopes tested (Fig.2G), suggesting strong suppression of viral-specific CD8 T cell responses by Tregs. In addition, cytokine expression on a per cell basis was also increased after Treg depletion, as evidenced by higher mean fluorescence intensity after intracellular cytokine staining (Fig. 2H), and higher percentage of IFNγ+ cells that were tetramer positive (Fig 3).

B7 costimulation, CD4 cells, and viral antigen are required for viral-specific CD8 T cell rescue after Treg depletion. We then aimed to elucidate the mechanism by which CD8 T cell function is restored after transient Treg depletion during chronic infection. It has been reported that Tregs keep autoreactive T cells in check by downregulating DC expansion, and costimulatory molecule expression under steady-state conditions in naïve mice (75, 110, 138). We noticed significantly higher expression of B7.1 (Fig 4A) and B7.2 (Fig 4B) on splenic DCs after Treg depletion in chronically infected mice. This suggested a role for B7 costimulation in the rescue of exhausted viral-specific CD8 T cell responses after Treg ablation. In addition, modest increase was observed for CD40 on DCs of Treg depleted mice (Fig 4C). Another striking difference was a significant increase in the percentage of activated CD4 cells after Treg depletion (Fig 5). This CD4+ CD44hi subset was not LCMV-specific, and these may self-reactive clones that play a role in the viral-specific CD8 T cell rescue after Treg ablation.

To explore the precise roles of B7 costimulation and CD4 cells on the massive expansion of viral-specific CD8 T cell responses after Treg depletion, we continuously treated Treg depleted mice with *CTLA-4 Ig* (which precludes B7 costimulation), or CD4 depleting antibody (which abrogates any effect from CD4+ cells). Chronically viremic mice that were Treg depleted and received CTLA-4 Ig or CD4 depleting antibody did not experience increase in virus-specific CD8 T cells, and their percentages (Fig. 6A) and total numbers (Fig. 6B) were similar to chronically infected control mice. Their phenotypes were also similar to that of untreated mice (Fig. 6C). These results indirectly suggested a decisive role for B7 modulation and CD4 cells during Treg mediated suppression of viral-specific CD8 T cell responses during chronic infection. It was also noteworthy that mice treated with either CTLA-4 Ig or CD4 depleting antibody during Treg ablation did not develop typical signs of morbidity that are associated with sustained absence of Tregs (our unpublished observations).

We then explored the role of viral antigen in influencing Treg mediated suppression during chronic viral infection. To test this, we examined whether Treg depletion would also result in increase of viral-specific memory CD8 T cell responses in acutely infected mice that had cleared virus (Fig. 7A). For this comparative study, we infected naïve mice with LCMV Armstrong, which results in an acute infection that is cleared within 8 days (132, 135). Treg depletion ~3 months after acute infection only resulted in increase of total activated CD44+ T cells (Fig. 7B). This is consistent with previous studies in naïve mice, in which most of the expanded clones after Treg depletion are self-reactive (75). However,

even though the total number of T cells was expectedly increased, there was no change in the numbers of LCMV-specific CD8 T cells (Fig. 7C). Phenotype of viral-specific CD8 T cells after Treg depletion in memory mice was also unchanged (not shown). This is in sharp contrast to chronically infected mice that experienced >10-fold increase in the absolute numbers of viral-specific CD8 T cells, and changes in their phenotype after Treg depletion. This suggested a decisive role for cognate antigen persistence in determining CD8 T cell suppression by Tregs. Our data also suggested that Tregs are involved in coordinating the process of T cell exhaustion and deletion in situations in which antigen is ubiquitous, perhaps as part of a dominant tolerance mechanism that occurs extrathymically. During either immune homeostasis or chronic infection, there may be mechanistic overlaps. Persistent antigen, whether self or non-self, appears to induce a common Treg suppressive response.

Upregulation in PD-L1 after Treg depletion abrogates viral control by expanded viral-specific CD8 T cells. We then questioned whether the observed changes in number and phenotype of viral-specific CD8 T cells after Treg depletion during chronic infection was correlated with increased viral control. Surprisingly, there was no statistically significant reduction in viremia after 11 days without Tregs, compared to undepleted controls (3.5x10^4 PFU/mL in Treg depleted mice, compared to 4.3x10^4 PFU/mL in undepleted mice). This apparent discrepancy could be due to two non-mutually exclusive possibilities.

The first possibility could be that even though Treg depletion induces upregulation of costimulatory molecules on DCs, it also induces upregulation of co-inhibitory receptor PD-L1 (Fig. 8A). This is in agreement with previous Treg depletion studies in uninfected animals (75, 110, 138). Therefore, we reasoned that by blocking the PD-1 pathway, we could enhance even further the T cell rescue observed after Treg ablation alone, and induce significant reduction in viremia. Depletion of Tregs followed by PD-L1 blockade resulted in increased percentages of co-expressing IFNγ+ TNFα+ cells (Fig. 8B). The absolute numbers of viral-specific CD8 T cells were comparable to Treg depletion alone (Fig. 8C), but the total numbers of co-expressing cells were slightly increased in Treg depleted mice that also received PD-L1 blockade, compared to Treg depletion alone (Fig. 8D). Consistent with our expectations, there was a five-fold drop in viremia, which was higher than either Treg depleted, or PD-L1 blockade alone (Fig. 8E). This additive effect suggested independent Treg pathways mediating exhaustion that are different from PD-1. It is possible that Treg and co-inhibitory receptor pathways differ in their induction of CD8 T cell exhaustion.

Discussion

Our results show that in vivo depletion Foxp3+CD4+ cells results in significant increase in the numbers, cytokine expression, phenotype, and killing efficiency of viral-specific CD8 T cells during a chronic vial infection. The fact that viral loads were not reduced in the Treg depleted mice could be explained by the increased expression of PD-L1 on viral targets. This is consistent with our results with Treg depletion plus PD-L1 blockade, which results in synergistic reduction in viral loads. Therefore, upregulation of PD-L1 after Treg depletion is most likely the cause for the lack of viral control. Another non-mutually exclusive possibility for the observed discrepancy in viral titers may be an LCMV model specific bias. The chronic strain LCMV cl-13 replicates in several subsets of DCs (112). and these cells undergo 5-10 fold expansion after Treg depletion (75, 110).

It is reasonable to think that even though viral control by expanded viral-specific CD8 T cells is occurring, constant expansion of DCs would result in expanded niches for LCMV cl-13 replication despite the increased T cell expansion. This also suggested that viruses that replicate in DCs, or any other cell population that expands after Treg ablation (75), may experience an increase in viral replication followed by T cell control after expansion of viral-specific cells. It is of interest to test Treg depletion regimens in other chronic infection models, and to modify the Treg depletion protocols in order to test viral control at later times after depletion. We were only able to detect viral loads at day 11 post depletion (our usual time of sacrificing) because the mice also developed signs of

autoimmunity, and some usually died after 11 days without Tregs. The data, however, does not exclude also the contribution of additional factors involved in viral control, such as the integrity of all physiological components within the host. Sustained Treg depletion provokes considerable activation of self-reactive cells, and this may induce profound alterations in homeostasis that may be vital for pathogen control. Milder regimens to modulate specific Treg pathways may be of interest.

We propose a model in which exhausted viral-specific CD8 T cells are kept in a quiescent state by Tregs, via B7 modulation on DC, signals from CD4 cells, and persistent antigen (Fig 9). There is an additional synergistic role of the PD-1 pathway in mediating exhaustion, which seems independent from that of Tregs. It is possible that there are mechanistic overlaps between T cell exhaustion during chronic infections, and suppression of self-reactive responses during immune homeostasis, in which self-antigen is also ubiquitously present. A possibility that could explain the specificity of Treg suppression to self-antigens or chronic pathogens may be that signal 1 (TCR recognition) without signal 2 (costimulation) results in potent antigen-specific suppression. This possibility is consistent with data from other groups that show that steady-state DCs that present antigen are tolerizing (56, 95, 110). Therefore, constant antigen-presentation, together with Treg modulation of DC costimulatory potential, and signals from CD4 T cells may determine whether suppression or activation of CD8 T cell responses occurs. Understanding Treg mechanisms may be useful to fine-tune immune responses during cancer, chronic infections such as HIV or

HCV, and autoimmunity. Brief manipulation of Tregs or their pathways, may provide clinical utility.

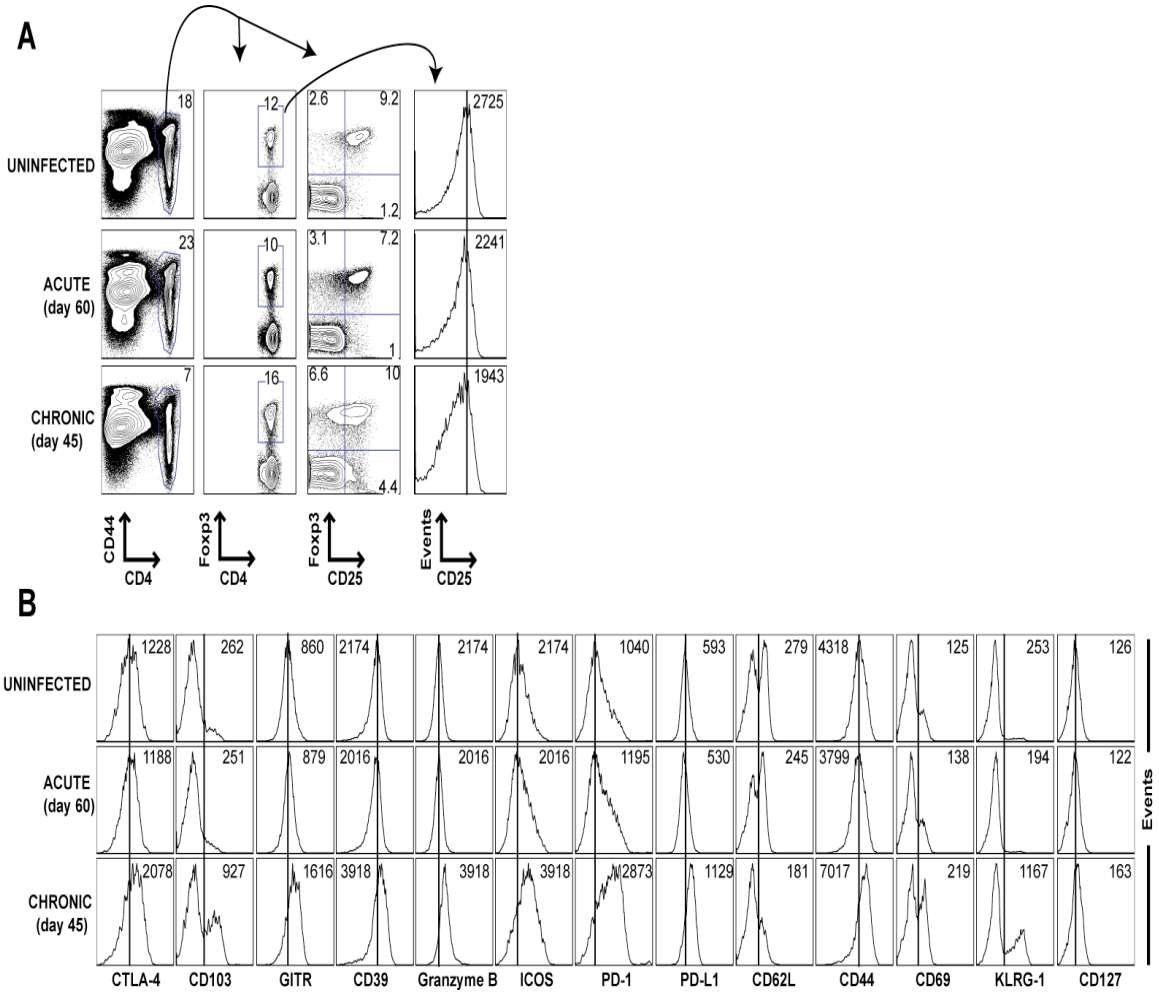
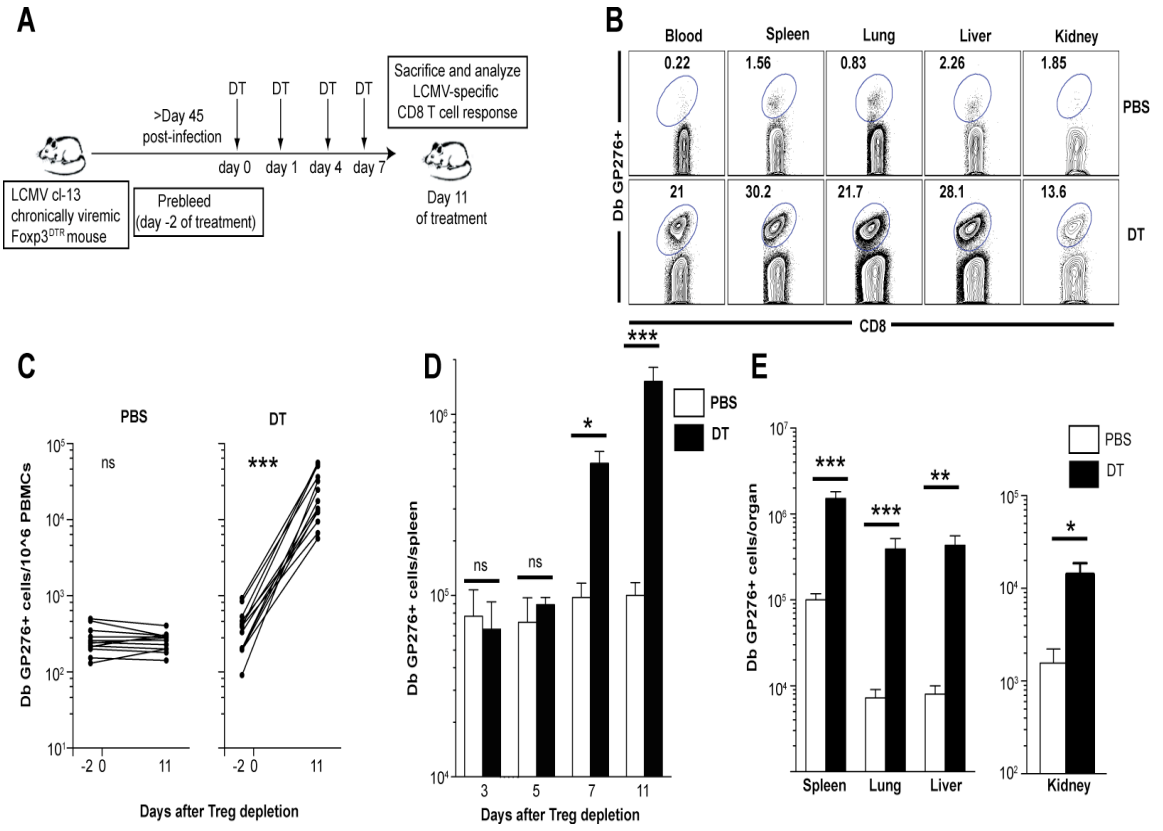


Figure 1. Tregs of chronically infected mice (day 45 post-infection) are phenotypically distinct from those of uninfected, or acutely infected memory mice. A) Decreased CD25 expression on Tregs of chronically infected mice. B) Expression of different Treg markers and activation markers on Tregs of chronically infected mice. Gated from live CD4+ Foxp3+ lymphocytes from spleen.


A
DT DT DT DT
>Day 45 post-infection
day 0 day 1 day 4 day 7
Sacrifice and analyze LCMV-specific CD8 T cell response
LCMV cl-13 chronically viremic Foxp3DTR mouse
Prebleed (day -2 of treatment)
Day 11 of treatment
B
Blood Spleen Lung Liver Kidney
0.22 1.56 0.83 2.26 1.85
PBS
21 30.2 21.7 28.1 13.6
DT
Db GP276+
CD8
***
C
PBS DT
ns ***
Db GP276+ cells/10^6 PBMCs
-2 0 11
Days after Treg depletion
D
PBS DT
ns ns * ***
Db GP276+ cells/spleen
3 5 7 11
Days after Treg depletion
E
PBS DT
*** *** ** *
Db GP276+ cells/organ
Spleen Lung Liver Kidney

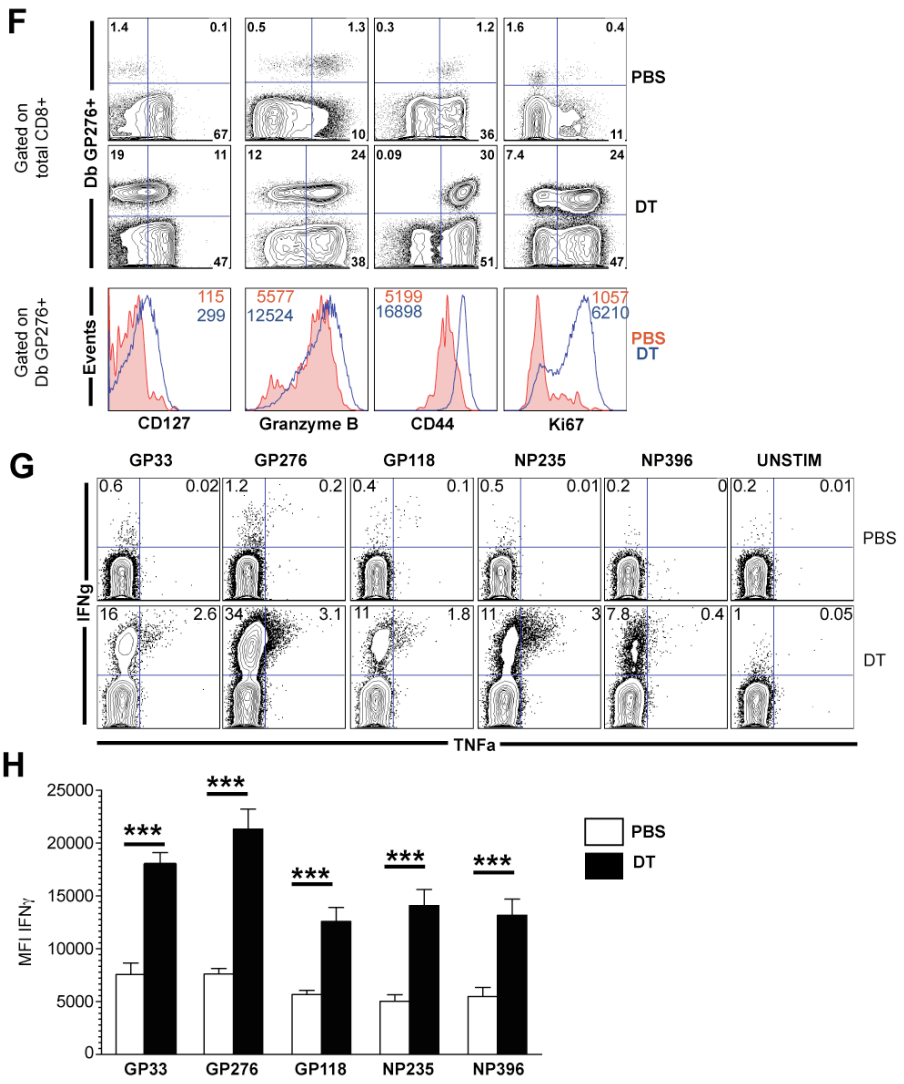


Fig. 2. Rescue of exhausted viral-specific CD8 T cells after Treg depletion during chronic viral infection. A) Experimental model for specific Treg ablation in LCMV chronically viremic Foxp3^{DTR} knock-in mice by intraperitoneal (i.p.) injection of Diphtheria toxin (DT). B) Increase in the percentages of Db GP276+ CD8 T cells in multiple tissues. C) Increase in the number of Db GP276+ CD8 T cells in blood (pre is 2 days before, and post is 11 days after treatment). D) Progressive expansion of Db GP276+ CD8 T cells in spleen. E) Increase in the numbers of Db GP276+ cells in multiple tissues after Treg depletion in chronically viremic mice. F) Phenotypic changes on LCMV Db GP276-specific CD8 T cells in spleen. G) IFNγ and TNFα cytokine expression of viral-specific CD8 T cells. H) IFNγ expression on a per cell basis. Plots B, F, and G are gated on live CD8+ lymphocytes. *, P=0.0357; **, P=0.0006; ***, P<0.0001. Data is representative from 5 experiments, n=3-5 mice per group. In G and H, peptide stimulations were performed for 5 hours with various LCMV peptides at 37 °C. Panel B, E, F, G, and H are at day 11 post-Treg depletion.

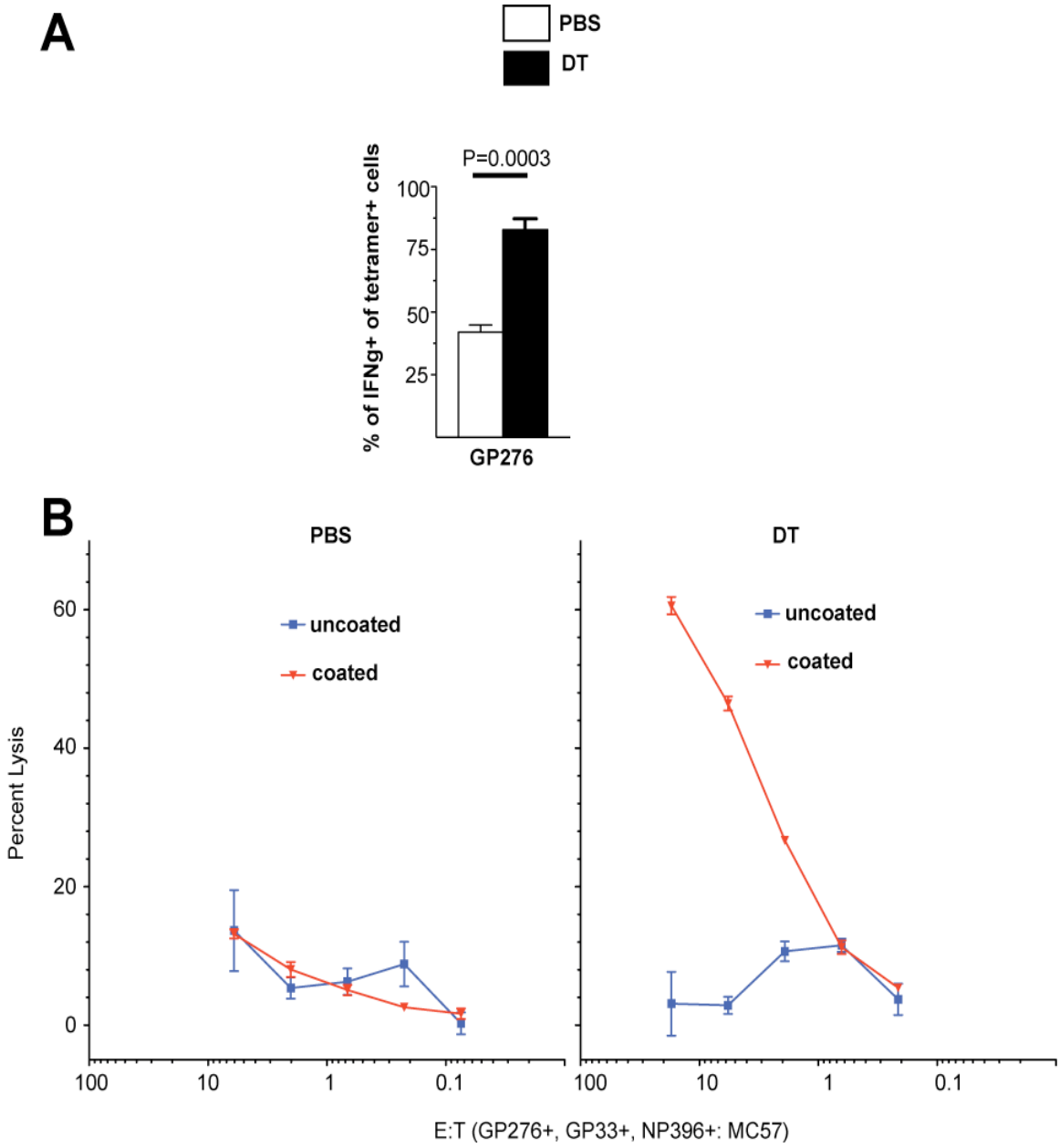


Figure 3. A) Percentage of tetramer+ cells (Db GP276+) that express IFNγ (spleen data). B) 6 hour chromium release assay showing per cell killing activity of effector CD8 T cells (GP33, GP276 and NP396 specific). All wells received 10^4 MC57 mouse fibroblast targets coated with a mix of GP33, GP276, and NP396 peptides or no peptide, and labeled with 350 μCi. Data from n=3-4, mouse spleens of each group were pooled.

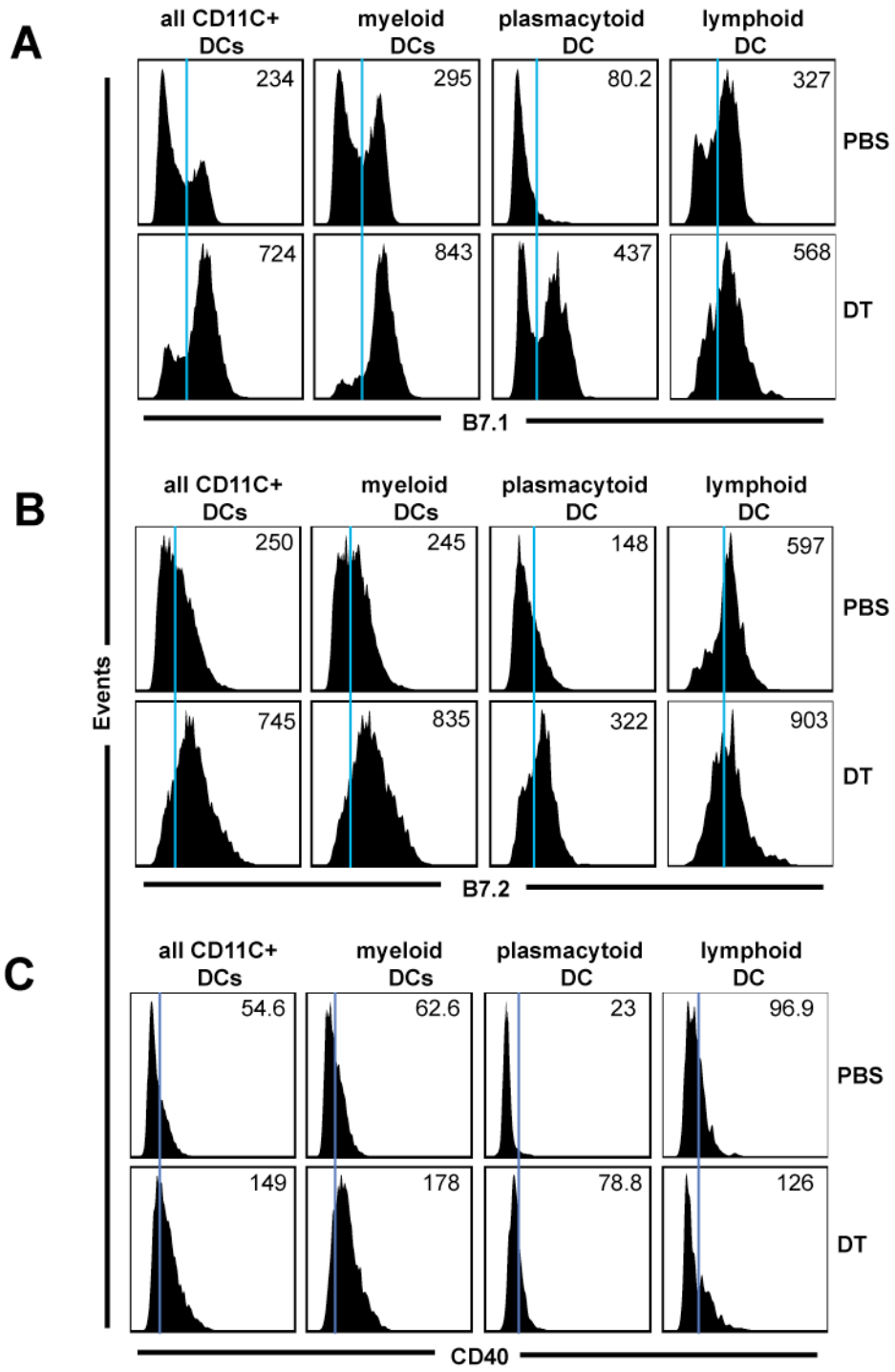


Figure 4. Increase in costimulatory molecule expression on splenic DCs after Treg ablation during chronic viral infection. Expression B7.1 (A), B7-2 (B) and CD40 (C) on CD3- CD19- CD11c+ DCs, myeloid DCs, plasmacytoid DCs, and lymphoid DCs.

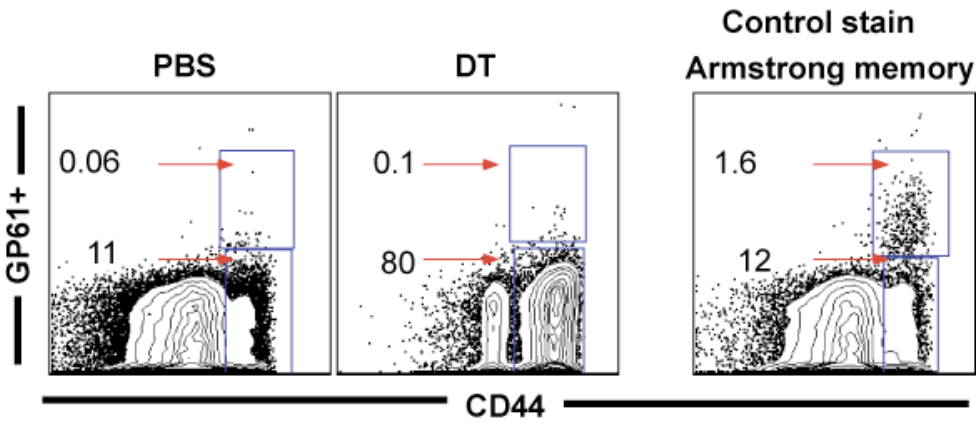


Figure 5. Increase in the numbers of activated non-viral specific CD4 T cells (I-Ab GP61-80 negative) after 11 days of Treg ablation during chronic infection. Gated on CD4+ live lymphocytes.

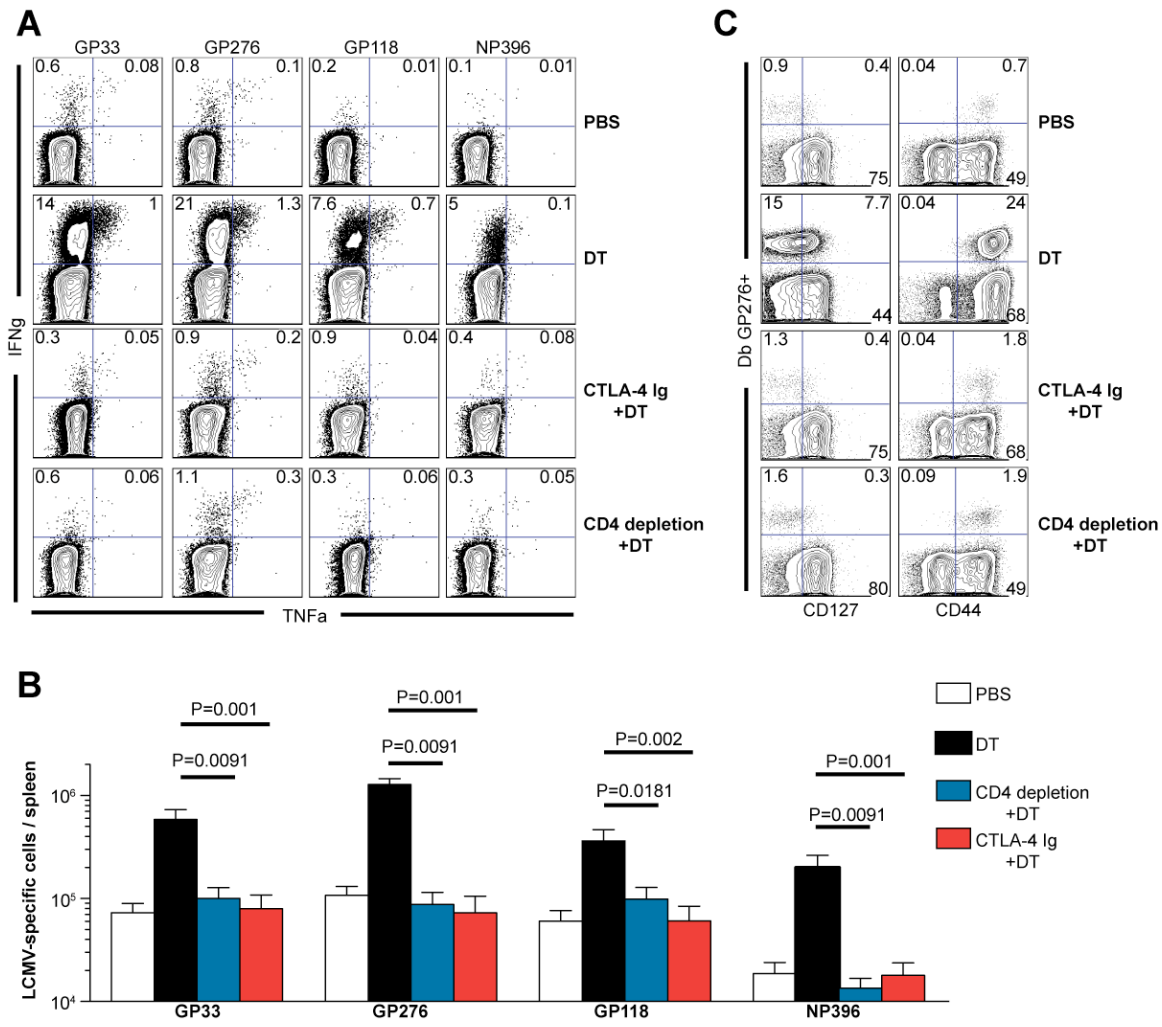


Figure 6. Ablation of Tregs together with CTLA-4 Ig treatment, or whole CD4 depletion, abrogates CD8 T cell rescue. A) IFNγ and TNFα cytokine expression of viral-specific CD8 T cells. B) Number of LCMV-specific CD8 T cells (IFNγ+ after peptide stimulation). C) Phenotype of LCMV Db GP276+ CD8 T cells. Spleen data is shown, and is representative of 2 experiments, each n=3-5 mice per group. In A and B, peptide stimulations were performed for 5 hours with various LCMV peptides at 37 °C. 500 μg of CTLA-4 Ig was injected i.p., on days -3, 0, 3, 6, and 9 of Treg ablation. CD4 depletion was performed by i.p. injection with 500 μg of GK1.5, on days -2 and -1 of Treg ablation.

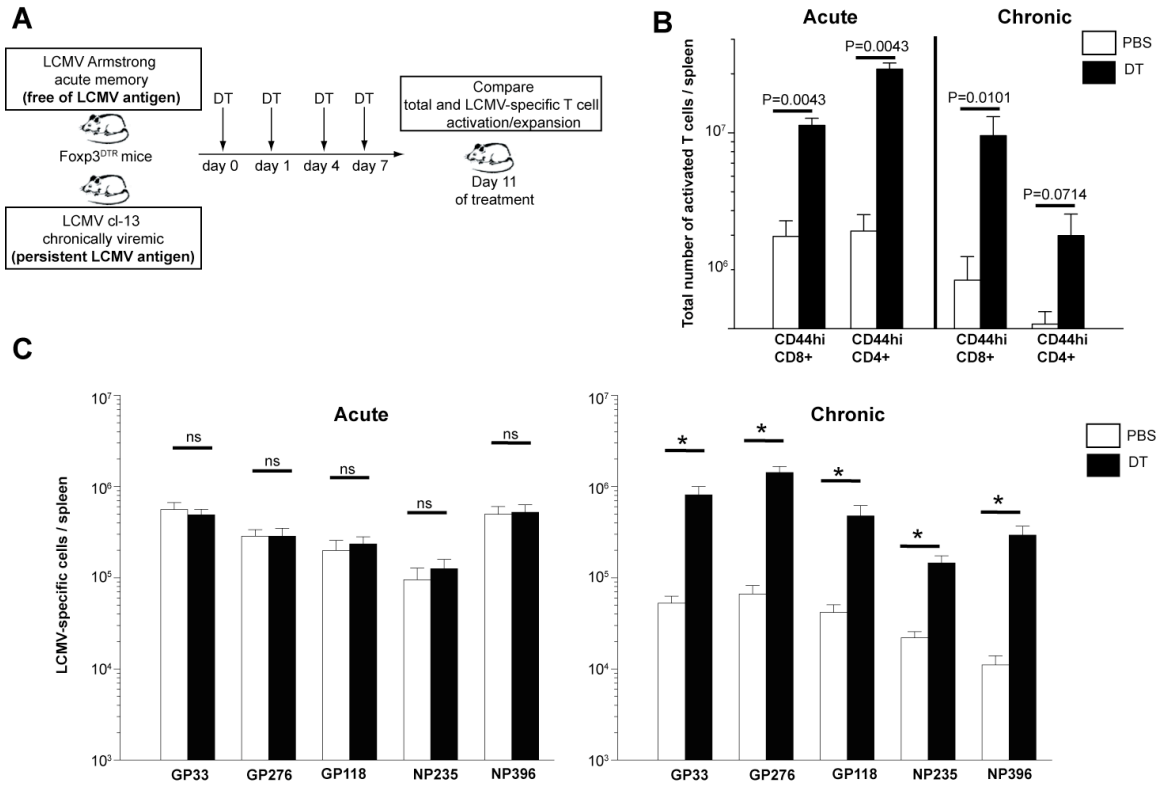


Fig. 7. Viral antigen persistence plays a role in determining suppression of CD8 T cell responses by Tregs during chronic infection. A) Experimental model for comparing the effects of Treg ablation on antigen-specific CD8 T cell responses in either LCMV Armstrong memory mice, or LCMV cl-3 chronically viremic mice. B) Overall increase in the number of activated T cells after Treg depletion in either acute memory or chronically viremic mice. C) Number of LCMV-specific CD8 T cells (IFNγ+ after peptide stimulation). Spleen data representative from 1 experiment (Armstrong acute) with n=5 mice per group, and 5 experiments (cl-13 chronic) with n=3-5 mice per group. *, P<0.0001

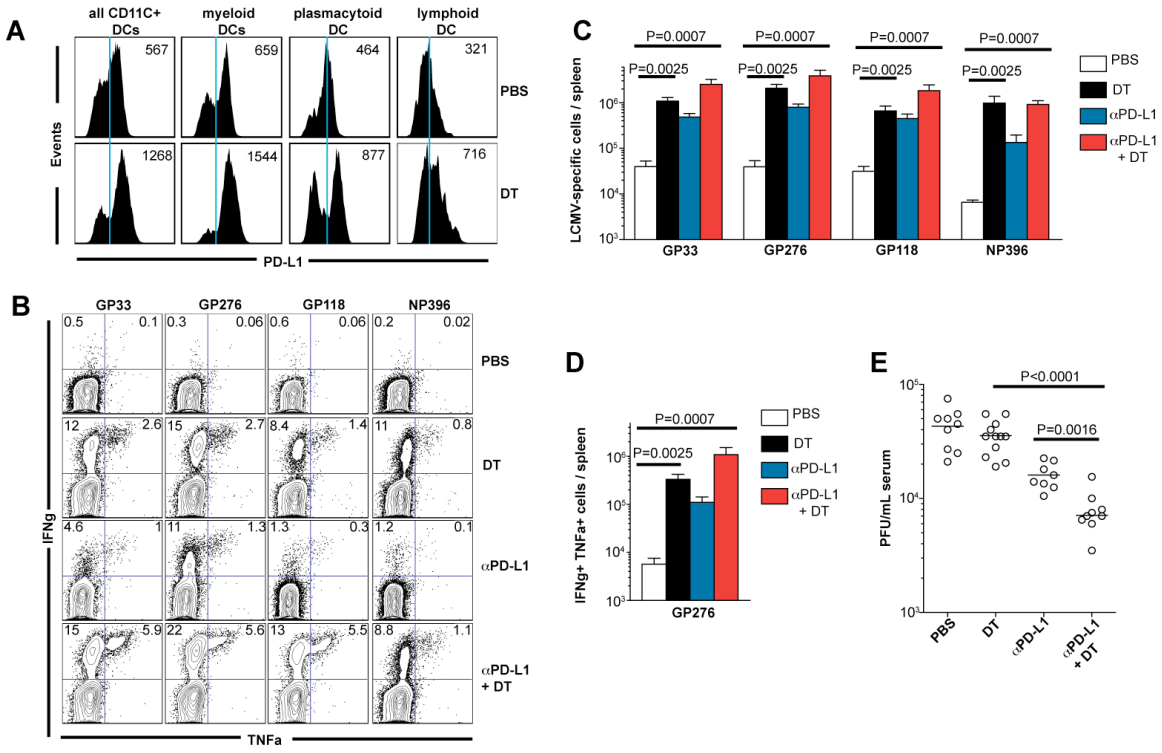


Fig. 8. Role of the PD-1 pathway in determining viral control after Treg ablation during chronic LCMV infection. A) PD-L1 is upregulated on CD3- CD19- CD11c+ DCs, myeloid DCs (CD11b+), plasmacytoid DCs (CD45RA+ B220+), and lymphoid DCs (CD8α) after Treg depletion during chronic viral infection. B) Increased percentages of IFNγ and TNFα co-expressing cells after Treg depletion followed by PD-1 blockade. C) Numbers of LCMV-specific CD8 T cells (IFNγ after peptide stimulation). D) Increased absolute numbers of IFNγ and TNFα co-expressing cells by Treg depletion and PD-L1 blockade. E) Enhanced viral control after Treg depletion and PD-L1 blockade (serum). Representative of 2 experiments, with n=4-5 mice per group. All flow plots are from spleen. 200 μg PD-L1 blocking antibody (10F.9G2) was administered i.p. on days 1, 4, and 7 after Treg ablation.

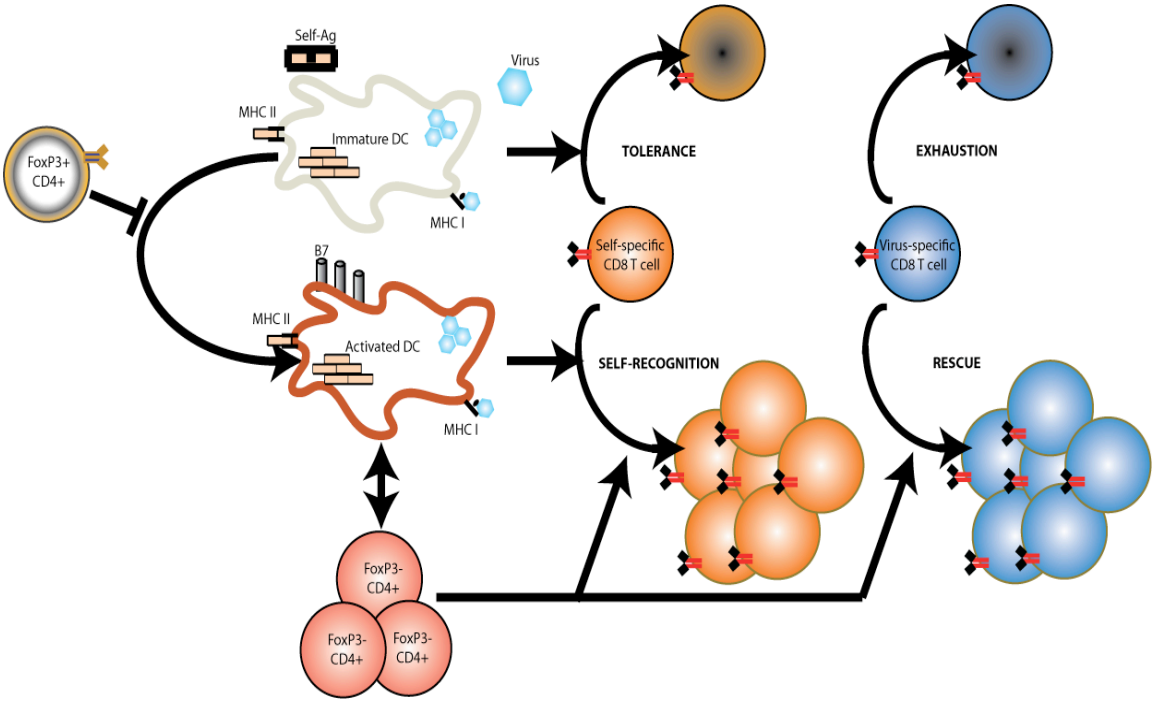


Fig. 9. Proposed model of T cell responses during immune homeostasis or chronic infection. Tregs inhibit DC activation (defined mainly by upregulation of B7 molecules). Under normal circumstances, DCs present ubiquitous self or viral antigen to their cognate antigen-specific CD8 T cells, but in the context of low costimulation resulting in CD8 T cell tolerance (self-specific) or exhaustion (viral-specific). Nevertheless, after Treg ablation, DCs present these same antigens in the context of high costimulation resulting in self-recognition or rescue of CD8 T cell responses. This model is a modification of Peter Bretscher two-signal hypothesis(22, 23). Signal 1 (TCR recognition) without signal 2 (costimulation) results in anergy, whereas signal 1 with signal 2 results in T cell activation. A cryptic signal 3 (perhaps provided by CD4 cells) acts concertedly with signals 1 and 2, resulting in activation of T cell responses. Therefore, antigen abundance, B7 costimulation, and CD4 cells are universally involved in these regulatory circuits during immune homeostasis or chronic infections.

Acknowledgments

I would like to thank Koichi Araki, Alice Kamphorst, Smita Iyer, Erin West, and Bogna Konieczny for their help and input with this work.

Chapter 6. Discussion

Until now, we have covered two main immune regulatory pathways that appear to act in concert during chronic viral infection: the PD-1 pathway and Treg suppression pathway. The PD-1 pathway is involved in downmodulation of CD8 T cell responses, especially during antigen persistence. We showed how we could modulate immune responses during chronic infection, not only by blocking PD-1, but also by triggering costimulatory receptors, such as 4-1BB and CD70.

We have learned that upon antigen challenge, naïve T cells experience a rapid phase of proliferation (135). If the antigen is cleared, those T cells become memory cells that are able to respond quickly to a secondary challenge. If the antigen persists, however, there is a hierarchical loss of T cell function, resulting in T cell exhaustion (135, 143). This is the case with chronic infections such as those with HIV, HBV, and HCV. The PD-1 pathway plays an important role in directing T cell exhaustion caused by chronic viral infection(11, 39, 126, 129).

A question of interest is which other immune pathways could synergize with PD-L1 blockade. It was shown by John Wherry's group that dual blockade of the inhibitory molecule LAG-3 and PD-L1 results in additive effects on viral-specific T cell rescue during a chronic LCMV infection (16). Instead of blocking additional inhibitory receptors, we wanted to determine if agonistic costimulatory signals could synergize with PD-L1 blockade and result in an enhanced rescue of exhausted virus-specific CD8 T cells. As mentioned earlier, CD137, a TNF receptor family member(130), has been shown to be important for T cell

activation. Interaction with its ligand has been shown to be important for T cell responses to bacterial and viral infections(85, 86, 119).

We have shown that induction of 4-1BB in combination with PD-1 blockade results in acceleration of CD8 T cell restoration and viral control. By day 7 post-treatment, there is a higher number of viral-specific CD8 T cells in the dual treated group, compared to PD-1 blockade alone. However, by day 14 post-treatment, the number of antigen-specific CD8 T cells “catch up” and viral titers look similar to PD-1 blockade. We also showed that at a high dose (200 μg every 3 days, five times) of anti-4-1BB agonistic antibody, viral-specific CD8 T cells increase by day 7, but crash down to the numbers seen in control mice. However, with a small dose (50 μg on the first day of treatment), not only the increase of antiviral response is higher by day 7 compared to PD-1 blockade alone, but also these numbers are maintained. Viral control is also accelerated, but by day 14, titers are identical to PD-1 blockade alone. These results corroborate that there’s a fine line between T cell costimulation and T cell death. Providing excessive activating signals may renders cells more prone to death.

Then, we covered the effect of blocking the CD70/CD27 costimulatory pathway with blocking antibodies. Even though the results were modest compared to those from our 4-1BB and PD-1 blockade experiments, the data consistently showed that during acute infection, CD70 blockade results in mitigation of immune responses compared to control (by day 7 and day 15 post-treatment). Signaling through CD70/CD27 aids in CD127 conversion of antigen-specific CD8 T cells. Conversely, during chronic infection, CD70 blockade

promoted a slight increase in the absolute numbers of viral-specific CD8 T cells. The data for this chapter was more modest compared to that from other chapters, but provided a proof of principle about the duality of the CD70/CD27 costimulatory axis. The data in this part was consistent with that other groups (e.g. Van Lier; Ochsenbein) in which it has been shown the role of CD70/CD27 signaling in enhancing memory responses during acute influenza challenge, but inducing immune overativation reminiscent to AIDS during chronic triggering of this receptor.

However, the most interesting data of this thesis is perhaps that regarding Treg mediated suppression of antiviral CD8 T cell responses. We showed that CD4+ cell depletion followed by PD-1 blockade results in improved viral-specific CD8 T cell restoration compared to PD-1 blockade alone. This suggested that CD4+ mediate inhibition of CD8 T cell responses during chronic infection. There was a significant and consistent increase of viral-specific CD8 T cells on multiple tissues.

This synergy was also evident, although more modestly, in the blood in which there was an increase in the number of tetramer+ cells. Number of IFNg -expressing cells was also increased after dual CD4 depletion and PD-1 blockade. At the thymic level, there were also differences, such as reduction in the frequency of CD4+ CD8+ double positives after CD4 depletion followed by PD-1 blockade. It could be that CD4+ Tregs and the PD-1 pathway control maturation and exit of thymic precursors. Another possibility is that migration of

peripheral T cells into the thymus is controlled by the CD4+ Tregs and the PD-1 pathway.

Mice that received CD4+ cell depletions followed by PD-1 blockade also resulted in lower viral titers compared to PD-1 blockade alone. In addition, the effect of PD-1 blockade on Treg reconstitution after whole CD4 depletion was also intriguing. PD-1 blockade accelerated CD4+ FoxP3+ cell reconstitution after CD4 depletion. Tregs in the CD4 depleted plus PD-1 blockade group looked more activated as evidenced by increased PD-1 and CD44. There was an issue with the GK1.5 (CD4 depleting antibody) that may be worth mentioning. When these experiments were first performed, our GK1.5 batch resulted in long-lasting, and almost 99% depletion. By day 14-16 post-depletion, only about < 1% of lymphocytes were CD4+ cells.

In other words, the antibody batch seemed to have been working optimally. In three different experiments, there was a greater whole CD4 reconstitution, although we never stained for Foxp3 during these initial experiments. However, when we purchased a different batch, we started to notice suboptimal depletions. Around 7-10% of lymphocytes were CD4+ cells at day 14-16 post-depletion, and PD-1 blockade resulted in enhanced reconstitution of only Foxp3+ CD4+ cells, but not total CD4+ cells, as mentioned earlier. Since these last experiments are repeating with the two antibody batches that we have purchased since the initial experiments, we have decided to focus on these results more.

After CD4 depletion and PD-1 blockade, the increase in antigen-specific CD8 T cells was only 2-3 fold, and not a log or higher as in our Foxp3^{DTR} mice that are Treg depleted. A possibility for this reduced CD8 T cell restoration could be that conventional CD4 cells are also needed. We know this is the case, because whole CD4 depletion with GK1.5 results in virtually no changes on viral-specific CD8 T cell responses.

An interesting observation is that even though transient, whole CD4 ablation followed by PD-1 blockade results in a more modest CD8 T cell rescue, compared to specific Treg depletion, the mice did not experience lethal autoimmflammatory disease. This is a trade-off from only performing temporary depletion of all CD4 cells and not only the CD4+FoxP3+ population, which results in a fulminant and lethal autoimmunity.

The Treg mediated suppression pathway, has been shown to be involved in immune homeostasis, but recently is starting to be considered as a pathway controlling also immune responses to chronic infection. As explained before, earlier models with *Leishmania major* almost a decade ago heralded a new avenue for this field. The work in our lab suggested not only a role for Tregs in maintaining CD8 T cell exhaustion, but also sparked hints about the way that Tregs suppress immune responses. The data suggest that antigen abundance is required to mediate cognate suppression by Tregs, and modulation costimulatory molecule expression on APCs may be what determines immune response versus tolerance. It still remains unanswered what additional signals determine suppression by Tregs. It seems that PD-1 blockade acts synergistic to Treg

depletion (in the specific Treg depletion or whole CD4 depletion experiments). This suggests independent pathways that are used to sustain CD8 T cell exhaustion. The data also provides evidence for the PD-1 pathway controlling Treg proliferation or survival during situations of lymphopenia induced proliferation. We showed that after whole CD4 depletion, PD-1 blockade increases reconstitution of FoxP3+ CD4+ cells compared to CD4 depleted without PD-1 blockade.

Why don't Tregs suppress primary acute viral responses as much as they suppress self-specific or severely exhausted T cell responses? This is a main question that has enticed effervescent discussions in our lab. It may be that naïve T cells are less sensitive to Treg mediated suppression, or that Tregs behave differently under different immune situations. Perhaps, they constantly manipulate DC phenotype and activation depending on the context of immune responses (normal uninfected environment, primary responses, or antigen persistence). Location or activation status of Tregs may hold the answer to this intriguing question. We are starting to look at the numbers and activation status of Tregs at different times of the immune response, and at different sites. This may hold the answer to this basic question that has perplexed immunologists for decades.

Overall, we have analyzed the roles of immune regulatory pathways that act in concert during viral infections. It is known that costimulation provides an activating signal, that together with TCR triggering, induces robust T cell responses instead of anergy{Bretscher, 1992 #260}. Several members of the

TNFRS are involved in potentiating T cell responses, and they do so by providing survival signals to T cells{Sharpe, 2002 #131}. Another important aspect of immune regulation that has been extensively covered in this thesis is the role of Tregs, as defined by CD4+ and Foxp3+ expression. Tregs could be either innate (thymus derived) or adaptive (derived from conventional CD4+ cells that become suppressive in the presence of TGF-β or IL-10). It is unclear from our observations, what is the main origin of Tregs during chronic infection. It may as well be a combination of natural and adaptive Tregs that make up the pool of suppressive cells during a chronic infection. There are distinct phenotypic differences between Tregs from uninfected or chronically infected mice.

Understanding these differences may elucidate the role of Tregs during the onset and maintenance of a chronic infection, and may provide therapeutic opportunities to modulate immune function. We noticed increased expression of CTLA-4, CD103, GITR, CD39, granzyme B, ICOS, PD-1, and PD-L1, CD44, CD69, KLRG-1, and a modest increase in CD127. Conversely, CD62L was downregulated on Tregs of chronically infected mice. These phenotypic patterns first suggested to us that there were functional differences between Tregs of normal and chronically infected mice. Some differences such as increased CTLA-4, CD103, GITR, CD39, granzyme B, ICOS, PD-1, and PD-L1 may mean that Tregs from chronically infected animals are more suppressive. Other differences, such as increased CD44 and CD69 may be because Tregs of chronically infected mice may be more activated. The significance of the observed upregulation of KLRG-1 remains a question of interest. It may be that

the more suppressive Tregs are, the more effector-like they become. My thesis project rendered me with more questions than answers. It would be important to continue these Treg experiments, in order to answer additional questions, such as what is the role of each of these aforementioned receptors in mediating viral specific CD8 T cell suppression by Tregs during chronic infection.

A question that remains from these studies is why Tregs suppress self-reactive T cell responses during immune homeostasis and antiviral responses during an established infection, but not the onset of a primary T cell response. Preliminary data from my studies have shown that at the peak of the T cell response (day 8), there is a systemic collapse of Foxp3+ CD4+ cells. The percentages and absolute numbers are reduced 5-fold compared to naïve hosts (data not shown), and perhaps this Treg reduction is necessary for early antiviral control. Similar findings have been reported during a parasite infection(92), suggesting that downregulation of Treg function during the initiation of an acute response may be necessary for pathogen control at the early phase of the response. It is also of interest what factors control Treg homeostasis, as a way to understand what regulates the regulators of the immune response. This will be a focus of immunology in the next decade.

References:

1. **Ahmed, R., and M. B. Oldstone.** 1988. Organ-specific selection of viral variants during chronic infection. J Exp Med **167:**1719-24.

2. **Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. Oldstone.** 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. J Exp Med **160:**521-40.

3. **Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science **274:**94-6.

4. **Appasamy, P. M.** 1999. Biological and clinical implications of interleukin-7 and lymphopoiesis. Cytokines Cell Mol Ther **5:**25-39.

5. **Arens, R., K. Schepers, M. A. Nolte, M. F. van Oosterwijk, R. A. van Lier, T. N. Schumacher, and M. H. van Oers.** 2004. Tumor rejection induced by CD70-mediated quantitative and qualitative effects on effector CD8+ T cell formation. J Exp Med **199:**1595-605.

6. **Arens, R., K. Tesselaar, P. A. Baars, G. M. van Schijndel, J. Hendriks, S. T. Pals, P. Krimpenfort, J. Borst, M. H. van Oers, and R. A. van Lier.** 2001. Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFNgamma-mediated B cell depletion. Immunity **15:**801-12.

7. **Artyomov, M. N., M. Lis, S. Devadas, M. M. Davis, and A. K. Chakraborty.** CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. Proc Natl Acad Sci U S A **107:**16916-21.

8. **Bachmann, M. F., P. Wolint, K. Schwarz, P. Jager, and A. Oxenius.** 2005. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol **175:**4686-96.

9. **Ballesteros-Tato, A., B. Leon, F. E. Lund, and T. D. Randall.** Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. Nat Immunol **11:**216-24.

10. **Barber, D. L., E. J. Wherry, and R. Ahmed.** 2003. Cutting edge: rapid in vivo killing by memory CD8 T cells. J Immunol **171:**27-31.

11. **Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed.** 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature **439:**682-7.

12. **Belkaid, Y.** 2007. Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol **7:**875-88.

13. **Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks.** 2002. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature **420:**502-7.

14. **Belkaid, Y., and B. T. Rouse.** 2005. Natural regulatory T cells in infectious disease. Nat Immunol **6:**353-60.

15. **Blackburn, S. D., H. Shin, G. J. Freeman, and E. J. Wherry.** 2008. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. Proc Natl Acad Sci U S A **105:**15016-21.

16. **Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali, and E. J. Wherry.** 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol **10:**29-37.

17. **Blattman, J. N., R. Antia, D. J. Sourdive, X. Wang, S. M. Kaech, K. Murali-Krishna, J. D. Altman, and R. Ahmed.** 2002. Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med **195:**657-64.

18. **Blattman, J. N., and P. D. Greenberg.** 2006. PD-1 blockade: rescue from a near-death experience. Nat Immunol **7:**227-8.

19. **Boettler, T., E. Panther, B. Bengsch, N. Nazarova, H. C. Spangenberg, H. E. Blum, and R. Thimme.** 2006. Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection. J Virol **80:**3532-40.

20. **Boettler, T., H. C. Spangenberg, C. Neumann-Haefelin, E. Panther, S. Urbani, C. Ferrari, H. E. Blum, F. von Weizsacker, and R. Thimme.** 2005. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. J Virol **79:**7860-7.

21. **Boni, C., P. Fisicaro, C. Valdatta, B. Amadei, P. Di Vincenzo, T. Giuberti, D. Laccabue, A. Zerbini, A. Cavalli, G. Missale, A. Bertoletti, and C. Ferrari.** 2007. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. J Virol **81:**4215-25.

22. **Bretscher, P.** 1992. The two-signal model of lymphocyte activation twenty-one years later. Immunol Today **13:**74-6.

23. **Bretscher, P., and M. Cohn.** 1970. A theory of self-nonself discrimination. Science **169:**1042-9.

24. **Brugnoni, D., P. Airo, R. Marino, L. D. Notarangelo, R. A. van Lier, and R. Cattaneo.** 1997. CD70 expression on T-cell subpopulations: study of normal individuals and patients with chronic immune activation. Immunol Lett **55:**99-104.

25. **Buckner, J. H.** Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol **10:**849-59.

26. **Burns, J. W., and M. J. Buchmeier.** 1991. Protein-protein interactions in lymphocytic choriomeningitis virus. Virology **183:**620-9.

27. **Busse, D., M. de la Rosa, K. Hobiger, K. Thurley, M. Flossdorf, A. Scheffold, and T. Hofer.** Competing feedback loops shape IL-2 signaling between helper and regulatory T lymphocytes in cellular microenvironments. Proc Natl Acad Sci U S A **107:**3058-63.

28. **Carr, J. M., M. J. Carrasco, J. E. Thaventhiran, P. J. Bambrough, M. Kraman, A. D. Edwards, A. Al-Shamkhani, and D. T. Fearon.** 2006. CD27

mediates interleukin-2-independent clonal expansion of the CD8+ T cell without effector differentiation. Proc Natl Acad Sci U S A **103:**19454-9.

29. **Chen, A. I., A. J. McAdam, J. E. Buhlmann, S. Scott, M. L. Lupher, Jr., E. A. Greenfield, P. R. Baum, W. C. Fanslow, D. M. Calderhead, G. J. Freeman, and A. H. Sharpe.** 1999. Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. Immunity **11:**689-98.

30. **Chen, W., and J. E. Konkel.** TGF-beta and 'adaptive' Foxp3(+) regulatory T cells. J Mol Cell Biol **2:**30-6.

31. **Choi, B. K., Y. H. Kim, W. J. Kang, S. K. Lee, K. H. Kim, S. M. Shin, W. M. Yokoyama, T. Y. Kim, and B. S. Kwon.** 2007. Mechanisms involved in synergistic anticancer immunity of anti-4-1BB and anti-CD4 therapy. Cancer Res **67:**8891-9.

32. **Collison, L. W., V. Chaturvedi, A. L. Henderson, P. R. Giacomin, C. Guy, J. Bankoti, D. Finkelstein, K. Forbes, C. J. Workman, S. A. Brown, J. E. Rehg, M. L. Jones, H. T. Ni, D. Artis, M. J. Turk, and D. A. Vignali.** IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol **11:**1093-101.

33. **Collison, L. W., M. R. Pillai, V. Chaturvedi, and D. A. Vignali.** 2009. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. J Immunol **182:**6121-8.

34. **Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg, and D. A. Vignali.** 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature **450:**566-9.

35. **Cooper, M. D., and B. R. Herrin.** How did our complex immune system evolve? Nat Rev Immunol **10:**2-3.

36. **Cormary, C., E. Hiver, B. Mariame, G. Favre, and A. F. Tilkin-Mariame.** 2005. Coexpression of CD40L and CD70 by semiallogenic tumor cells induces anti-tumor immunity. Cancer Gene Ther **12:**963-72.

37. **Couderc, B., L. Zitvogel, V. Douin-Echinard, L. Djennane, H. Tahara, G. Favre, M. T. Lotze, and P. D. Robbins.** 1998. Enhancement of antitumor immunity by expression of CD70 (CD27 ligand) or CD154 (CD40 ligand) costimulatory molecules in tumor cells. Cancer Gene Ther **5:**163-75.

38. **Croft, M.** 2009. The role of TNF superfamily members in T-cell function and diseases. Nat Rev Immunol **9:**271-85.

39. **Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker.** 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature **443:**350-4.

40. **Dolfi, D. V., A. C. Boesteanu, C. Petrovas, D. Xia, E. A. Butz, and P. D. Katsikis.** 2008. Late signals from CD27 prevent Fas-dependent apoptosis of primary CD8+ T cells. J Immunol **180:**2912-21.

41. **Douin-Echinard, V., J. M. Peron, V. Lauwers-Cances, G. Favre, and B. Couderc.** 2003. Involvement of CD70 and CD80 intracytoplasmic domains in the

co-stimulatory signal required to provide an antitumor immune response. Int Immunol **15:**359-72.

42. **Ebinuma, H., N. Nakamoto, Y. Li, D. A. Price, E. Gostick, B. L. Levine, J. Tobias, W. W. Kwok, and K. M. Chang.** 2008. Identification and in vitro expansion of functional antigen-specific CD25+ FoxP3+ regulatory T cells in hepatitis C virus infection. J Virol **82:**5043-53.

43. **Elsaesser, H., K. Sauer, and D. G. Brooks.** 2009. IL-21 is required to control chronic viral infection. Science **324:**1569-72.

44. **Epple, H. J., C. Loddenkemper, D. Kunkel, H. Troger, J. Maul, V. Moos, E. Berg, R. Ullrich, J. D. Schulzke, H. Stein, R. Duchmann, M. Zeitz, and T. Schneider.** 2006. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. Blood **108:**3072-8.

45. **Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky.** 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol **4:**330-6.

46. **Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky.** 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity **22:**329-41.

47. **Freeman, G. J., C. M. Disteche, J. G. Gribben, D. A. Adler, A. S. Freedman, J. Dougery, and L. M. Nadler.** 1992. The gene for B7, a costimulatory signal for T-cell activation, maps to chromosomal region 3q13.3-3q21. Blood **79:**489-94.

48. **Freeman, G. J., E. J. Wherry, R. Ahmed, and A. H. Sharpe.** 2006. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. J Exp Med **203:**2223-7.

49. **Fuller, M. J., D. A. Hildeman, S. Sabbaj, D. E. Gaddis, A. E. Tebo, L. Shang, P. A. Goepfert, and A. J. Zajac.** 2005. Cutting edge: emergence of CD127high functionally competent memory T cells is compromised by high viral loads and inadequate T cell help. J Immunol **174:**5926-30.

50. **Germain, R. N.** 2008. Special regulatory T-cell review: A rose by any other name: from suppressor T cells to Tregs, approbation to unbridled enthusiasm. Immunology **123:**20-7.

51. **Gershon, R. K., and K. Kondo.** 1971. Infectious immunological tolerance. Immunology **21:**903-14.

52. **Guerder, S., S. R. Carding, and R. A. Flavell.** 1995. B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. J Immunol **155:**5167-74.

53. **Ha, S. J., S. N. Mueller, E. J. Wherry, D. L. Barber, R. D. Aubert, A. H. Sharpe, G. J. Freeman, and R. Ahmed.** 2008. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. J Exp Med **205:**543-55.

54. **Hand, T. W., M. Morre, and S. M. Kaech.** 2007. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. Proc Natl Acad Sci U S A **104:**11730-5.

55. **Harrison, C.** Sepsis: calming the cytokine storm. Nat Rev Drug Discov **9:**360-1.

56. **Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J. V. Ravetch, R. M. Steinman, and M. C. Nussenzweig.** 2001. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med **194:**769-79.

57. **Hendriks, J., L. A. Gravestein, K. Tesselaar, R. A. van Lier, T. N. Schumacher, and J. Borst.** 2000. CD27 is required for generation and long-term maintenance of T cell immunity. Nat Immunol **1:**433-40.

58. **Hendriks, J., Y. Xiao, and J. Borst.** 2003. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. J Exp Med **198:**1369-80.

59. **Hill, J. A., C. Benoist, and D. Mathis.** 2007. Treg cells: guardians for life. Nat Immunol **8:**124-5.

60. **Hintzen, R. Q., R. de Jong, S. M. Lens, M. Brouwer, P. Baars, and R. A. van Lier.** 1993. Regulation of CD27 expression on subsets of mature T-lymphocytes. J Immunol **151:**2426-35.

61. **Hisaeda, H., Y. Maekawa, D. Iwakawa, H. Okada, K. Himeno, K. Kishihara, S. Tsukumo, and K. Yasutomo.** 2004. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. Nat Med **10:**29-30.

62. **Hofmeister, R., A. R. Khaled, N. Benbernou, E. Rajnavolgyi, K. Muegge, and S. K. Durum.** 1999. Interleukin-7: physiological roles and mechanisms of action. Cytokine Growth Factor Rev **10:**41-60.

63. **Hoji, A., N. C. Connolly, W. G. Buchanan, and C. R. Rinaldo, Jr.** 2007. CD27 and CD57 expression reveals atypical differentiation of human immunodeficiency virus type 1-specific memory CD8+ T cells. Clin Vaccine Immunol **14:**74-80.

64. **Hori, S., T. Nomura, and S. Sakaguchi.** 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science **299:**1057-61.

65. **Hsieh, C. S., Y. Liang, A. J. Tyznik, S. G. Self, D. Liggitt, and A. Y. Rudensky.** 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. Immunity **21:**267-77.

66. **Ito, F., Q. Li, A. B. Shreiner, R. Okuyama, M. N. Jure-Kunkel, S. Teitz-Tennenbaum, and A. E. Chang.** 2004. Anti-CD137 monoclonal antibody administration augments the antitumor efficacy of dendritic cell-based vaccines. Cancer Res **64:**8411-9.

67. **Iwashiro, M., R. J. Messer, K. E. Peterson, I. M. Stromnes, T. Sugie, and K. J. Hasenkrug.** 2001. Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection. Proc Natl Acad Sci U S A **98:**9226-30.

68. **Jin, H. T., A. C. Anderson, W. G. Tan, E. E. West, S. J. Ha, K. Araki, G. J. Freeman, V. K. Kuchroo, and R. Ahmed.** Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc Natl Acad Sci U S A **107:**14733-8.

69. **Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton.** 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol **2:**301-6.

70. **Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed.** 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol **4:**1191-8.

71. **Kaech, S. M., E. J. Wherry, and R. Ahmed.** 2002. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol **2:**251-62.

72. **Kaufmann, D. E., D. G. Kavanagh, F. Pereyra, J. J. Zaunders, E. W. Mackey, T. Miura, S. Palmer, M. Brockman, A. Rathod, A. Piechocka-Trocha, B. Baker, B. Zhu, S. Le Gall, M. T. Waring, R. Ahern, K. Moss, A. D. Kelleher, J. M. Coffin, G. J. Freeman, E. S. Rosenberg, and B. D. Walker.** 2007. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. Nat Immunol **8:**1246-54.

73. **Keir, M. E., Y. E. Latchman, G. J. Freeman, and A. H. Sharpe.** 2005. Programmed death-1 (PD-1):PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes. J Immunol **175:**7372-9.

74. **Keller, A. M., Y. Xiao, V. Peperzak, S. H. Naik, and J. Borst.** 2009. Costimulatory ligand CD70 allows induction of CD8+ T-cell immunity by immature dendritic cells in a vaccination setting. Blood **113:**5167-75.

75. **Kim, J. M., J. P. Rasmussen, and A. Y. Rudensky.** 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol **8:**191-7.

76. **Kim, Y. H., B. K. Choi, H. S. Oh, W. J. Kang, R. S. Mittler, and B. S. Kwon.** 2009. Mechanisms involved in synergistic anticancer effects of anti-4-1BB and cyclophosphamide therapy. Mol Cancer Ther **8:**469-78.

77. **Kinter, A., J. McNally, L. Riggin, R. Jackson, G. Roby, and A. S. Fauci.** 2007. Suppression of HIV-specific T cell activity by lymph node CD25+ regulatory T cells from HIV-infected individuals. Proc Natl Acad Sci U S A **104:**3390-5.

78. **Ko, E., W. Luo, L. Peng, X. Wang, and S. Ferrone.** 2007. Mouse dendritic-endothelial cell hybrids and 4-1BB costimulation elicit antitumor effects mediated by broad antiangiogenic immunity. Cancer Res **67:**7875-84.

79. **Kobata, T., S. Jacquot, S. Kozlowski, K. Agematsu, S. F. Schlossman, and C. Morimoto.** 1995. CD27-CD70 interactions regulate B-cell activation by T cells. Proc Natl Acad Sci U S A **92:**11249-53.

80. **Laderach, D., M. Movassagh, A. Johnson, R. S. Mittler, and A. Galy.** 2002. 4-1BB co-stimulation enhances human CD8(+) T cell priming by augmenting the proliferation and survival of effector CD8(+) T cells. Int Immunol **14:**1155-67.

81. **Matloubian, M., R. J. Concepcion, and R. Ahmed.** 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J Virol **68:**8056-63.

82. **Matter, M., B. Odermatt, H. Yagita, J. M. Nuoffer, and A. F. Ochsenbein.** 2006. Elimination of chronic viral infection by blocking CD27 signaling. J Exp Med **203:**2145-55.

83. **McNamara, J. O., D. Kolonias, F. Pastor, R. S. Mittler, L. Chen, P. H. Giangrande, B. Sullenger, and E. Gilboa.** 2008. Multivalent 4-1BB binding

aptamers costimulate CD8+ T cells and inhibit tumor growth in mice. J Clin Invest **118:**376-86.

84. **Mueller, S. N., K. A. Hosiawa-Meagher, B. T. Konieczny, B. M. Sullivan, M. F. Bachmann, R. M. Locksley, R. Ahmed, and M. Matloubian.** 2007. Regulation of homeostatic chemokine expression and cell trafficking during immune responses. Science **317:**670-4.

85. **Munks, M. W., D. V. Mourich, R. S. Mittler, A. D. Weinberg, and A. B. Hill.** 2004. 4-1BB and OX40 stimulation enhance CD8 and CD4 T-cell responses to a DNA prime, poxvirus boost vaccine. Immunology **112:**559-66.

86. **Myers, L., S. W. Lee, R. J. Rossi, L. Lefrancois, B. S. Kwon, R. S. Mittler, M. Croft, and A. T. Vella.** 2006. Combined CD137 (4-1BB) and adjuvant therapy generates a developing pool of peptide-specific CD8 memory T cells. Int Immunol **18:**325-33.

87. **Myers, L., R. J. Messer, A. B. Carmody, and K. J. Hasenkrug.** 2009. Tissue-specific abundance of regulatory T cells correlates with CD8+ T cell dysfunction and chronic retrovirus loads. J Immunol **183:**1636-43.

88. **Myers, L., C. Takahashi, R. S. Mittler, R. J. Rossi, and A. T. Vella.** 2003. Effector CD8 T cells possess suppressor function after 4-1BB and Toll-like receptor triggering. Proc Natl Acad Sci U S A **100:**5348-53.

89. **Nair, S. R., G. Zelinskyy, S. Schimmer, N. Gerlach, G. Kassiotis, and U. Dittmer.** Mechanisms of control of acute Friend virus infection by CD4+ T helper cells and their functional impairment by regulatory T cells. J Gen Virol **91:**440-51.

90. **Nieland, J. D., Y. F. Graus, Y. E. Dortmans, B. L. Kremers, and A. M. Kruisbeek.** 1998. CD40 and CD70 co-stimulate a potent in vivo antitumor T cell response. J Immunother **21:**225-36.

91. **O'Garra, A., P. L. Vieira, P. Vieira, and A. E. Goldfeld.** 2004. IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. J Clin Invest **114:**1372-8.

92. **Oldenhove, G., N. Bouladoux, E. A. Wohlfert, J. A. Hall, D. Chou, L. Dos Santos, S. O'Brien, R. Blank, E. Lamb, S. Natarajan, R. Kastenmayer, C. Hunter, M. E. Grigg, and Y. Belkaid.** 2009. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. Immunity **31:**772-86.

93. **Oshima, H., H. Nakano, C. Nohara, T. Kobata, A. Nakajima, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, T. Muto, H. Yagita, and K. Okumura.** 1998. Characterization of murine CD70 by molecular cloning and mAb. Int Immunol **10:**517-26.

94. **Peng, G., S. Li, W. Wu, Z. Sun, Y. Chen, and Z. Chen.** 2008. Circulating CD4+ CD25+ regulatory T cells correlate with chronic hepatitis B infection. Immunology **123:**57-65.

95. **Probst, H. C., K. McCoy, T. Okazaki, T. Honjo, and M. van den Broek.** 2005. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nat Immunol **6:**280-6.

96. **Puccetti, P., and U. Grohmann.** 2007. IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-kappaB activation. Nat Rev Immunol **7:**817-23.

97. **Radziewicz, H., R. M. Dunham, and A. Grakoui.** 2009. PD-1 tempers Tregs in chronic HCV infection. J Clin Invest **119:**450-3.

98. **Radziewicz, H., H. L. Hanson, R. Ahmed, and A. Grakoui.** 2008. Unraveling the role of PD-1/PD-L interactions in persistent hepatotropic infections: potential for therapeutic application? Gastroenterology **134:**2168-71.

99. **Radziewicz, H., C. C. Ibegbu, H. Hon, M. K. Osborn, K. Obideen, M. Wehbi, G. J. Freeman, J. L. Lennox, K. A. Workowski, H. L. Hanson, and A. Grakoui.** 2008. Impaired hepatitis C virus (HCV)-specific effector CD8+ T cells undergo massive apoptosis in the peripheral blood during acute HCV infection and in the liver during the chronic phase of infection. J Virol **82:**9808-22.

100. **Reiser, H., G. J. Freeman, Z. Razi-Wolf, C. D. Gimmi, B. Benacerraf, and L. M. Nadler.** 1992. Murine B7 antigen provides an efficient costimulatory signal for activation of murine T lymphocytes via the T-cell receptor/CD3 complex. Proc Natl Acad Sci U S A **89:**271-5.

101. **Robertson, S. J., R. J. Messer, A. B. Carmody, and K. J. Hasenkrug.** 2006. In vitro suppression of CD8+ T cell function by Friend virus-induced regulatory T cells. J Immunol **176:**3342-9.

102. **Robertson, S. J., R. J. Messer, A. B. Carmody, R. S. Mittler, C. Burlak, and K. J. Hasenkrug.** 2008. CD137 costimulation of CD8+ T cells confers resistance to suppression by virus-induced regulatory T cells. J Immunol **180:**5267-74.

103. **Rudensky, A. Y., and D. J. Campbell.** 2006. In vivo sites and cellular mechanisms of T reg cell-mediated suppression. J Exp Med **203:**489-92.

104. **Sabbaj, S., S. L. Heath, A. Bansal, S. Vohra, J. M. Kilby, A. J. Zajac, and P. A. Goepfert.** 2007. Functionally competent antigen-specific CD127(hi) memory CD8+ T cells are preserved only in HIV-infected individuals receiving early treatment. J Infect Dis **195:**108-17.

105. **Sachdeva, M., M. A. Fischl, R. Pahwa, N. Sachdeva, and S. Pahwa.** Immune exhaustion occurs concomitantly with immune activation and decrease in regulatory T cells in viremic chronically HIV-1-infected patients. J Acquir Immune Defic Syndr **54:**447-54.

106. **Sakaguchi, S.** 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol **22:**531-62.

107. **Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda.** 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol **155:**1151-64.

108. **Salvato, M., P. Borrow, E. Shimomaye, and M. B. Oldstone.** 1991. Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. J Virol **65:**1863-9.

109. **Salvato, M., E. Shimomaye, P. Southern, and M. B. Oldstone.** 1988. Virus-lymphocyte interactions. IV. Molecular characterization of LCMV Armstrong (CTL+) small genomic segment and that of its variant, Clone 13 (CTL-). Virology **164:**517-22.

110. **Schildknecht, A., S. Brauer, C. Brenner, K. Lahl, H. Schild, T. Sparwasser, H. C. Probst, and M. van den Broek.** FoxP3+ regulatory T cells essentially contribute to peripheral CD8+ T-cell tolerance induced by steady-state dendritic cells. Proc Natl Acad Sci U S A **107:**199-203.

111. **Schonk, D. M., H. J. Kuijpers, E. van Drunen, C. H. van Dalen, A. H. Geurts van Kessel, R. Verheijen, and F. C. Ramaekers.** 1989. Assignment of the gene(s) involved in the expression of the proliferation-related Ki-67 antigen to human chromosome 10. Hum Genet **83:**297-9.

112. **Sevilla, N., D. B. McGavern, C. Teng, S. Kunz, and M. B. Oldstone.** 2004. Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion. J Clin Invest **113:**737-45.

113. **Sharpe, A. H., and A. K. Abbas.** 2006. T-cell costimulation--biology, therapeutic potential, and challenges. N Engl J Med **355:**973-5.

114. **Sharpe, A. H., and G. J. Freeman.** 2002. The B7-CD28 superfamily. Nat Rev Immunol **2:**116-26.

115. **Sharpe, A. H., E. J. Wherry, R. Ahmed, and G. J. Freeman.** 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol **8:**239-45.

116. **Sheppard, K. A., L. J. Fitz, J. M. Lee, C. Benander, J. A. George, J. Wooters, Y. Qiu, J. M. Jussif, L. L. Carter, C. R. Wood, and D. Chaudhary.** 2004. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett **574:**37-41.

117. **Shevach, E. M.** 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity **30:**636-45.

118. **Shevach, E. M., and G. L. Stephens.** 2006. The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity? Nat Rev Immunol **6:**613-8.

119. **Shuford, W. W., K. Klussman, D. D. Tritchler, D. T. Loo, J. Chalupny, A. W. Siadak, T. J. Brown, J. Emswiler, H. Raecho, C. P. Larsen, T. C. Pearson, J. A. Ledbetter, A. Aruffo, and R. S. Mittler.** 1997. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. J Exp Med **186:**47-55.

120. **Smyk-Pearson, S., L. Golden-Mason, J. Klarquist, J. R. Burton, Jr., I. A. Tester, C. C. Wang, N. Culbertson, A. A. Vandenbark, and H. R. Rosen.** 2008. Functional suppression by FoxP3+CD4+CD25(high) regulatory T cells during acute hepatitis C virus infection. J Infect Dis **197:**46-57.

121. **Sumi, T., W. Ishida, R. S. Mittler, H. Yagita, O. Taguchi, and A. Fukushima.** 2009. Regulatory T cells participate in 4-1BB-mediated suppression of experimental allergic conjunctivitis. Int Arch Allergy Immunol **148:**305-10.

122. **Suvas, S., U. Kumaraguru, C. D. Pack, S. Lee, and B. T. Rouse.** 2003. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. J Exp Med **198:**889-901.

123. **Takahashi, C., R. S. Mittler, and A. T. Vella.** 1999. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. J Immunol **162:**5037-40.

124. **Tesselaar, K., R. Arens, G. M. van Schijndel, P. A. Baars, M. A. van der Valk, J. Borst, M. H. van Oers, and R. A. van Lier.** 2003. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. Nat Immunol **4:**49-54.

125. **Thornton, A. M., and E. M. Shevach.** 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med **188:**287-96.

126. **Trautmann, L., L. Janbazian, N. Chomont, E. A. Said, S. Gimmig, B. Bessette, M. R. Boulassel, E. Delwart, H. Sepulveda, R. S. Balderas, J. P. Routy, E. K. Haddad, and R. P. Sekaly.** 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nat Med **12:**1198-202.

127. **van Gisbergen, K. P., R. W. van Olffen, J. van Beek, K. F. van der Sluijs, R. Arens, M. A. Nolte, and R. A. van Lier.** 2009. Protective CD8 T cell memory is impaired during chronic CD70-driven costimulation. J Immunol **182:**5352-62.

128. **van Lier, R. A., J. Borst, T. M. Vroom, H. Klein, P. Van Mourik, W. P. Zeijlemaker, and C. J. Melief.** 1987. Tissue distribution and biochemical and

functional properties of Tp55 (CD27), a novel T cell differentiation antigen. J Immunol **139:**1589-96.

129. **Velu, V., K. Titanji, B. Zhu, S. Husain, A. Pladevega, L. Lai, T. H. Vanderford, L. Chennareddi, G. Silvestri, G. J. Freeman, R. Ahmed, and R. R. Amara.** 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. Nature **458:**206-10.

130. **Watts, T. H.** 2005. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol **23:**23-68.

131. **Welsh, R. M., K. Bahl, and X. Z. Wang.** 2004. Apoptosis and loss of virus-specific CD8+ T-cell memory. Curr Opin Immunol **16:**271-6.

132. **Wherry, E. J., and R. Ahmed.** 2004. Memory CD8 T-cell differentiation during viral infection. J Virol **78:**5535-45.

133. **Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed.** 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc Natl Acad Sci U S A **101:**16004-9.

134. **Wherry, E. J., J. N. Blattman, and R. Ahmed.** 2005. Low CD8 T-cell proliferative potential and high viral load limit the effectiveness of therapeutic vaccination. J Virol **79:**8960-8.

135. **Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed.** 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol **77:**4911-27.

136. **Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed.** 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. Immunity **27:**670-84.

137. **Wilcox, R. A., A. I. Chapoval, K. S. Gorski, M. Otsuji, T. Shin, D. B. Flies, K. Tamada, R. S. Mittler, H. Tsuchiya, D. M. Pardoll, and L. Chen.** 2002. Cutting edge: Expression of functional CD137 receptor by dendritic cells. J Immunol **168:**4262-7.

138. **Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi.** 2008. CTLA-4 control over Foxp3+ regulatory T cell function. Science **322:**271-5.

139. **Wolthers, K. C., S. A. Otto, S. M. Lens, D. N. Kolbach, R. A. van Lier, F. Miedema, and L. Meyaard.** 1996. Increased expression of CD80, CD86 and CD70 on T cells from HIV-infected individuals upon activation in vitro: regulation by CD4+ T cells. Eur J Immunol **26:**1700-6.

140. **Yang, G., A. Liu, Q. Xie, T. B. Guo, B. Wan, B. Zhou, and J. Z. Zhang.** 2007. Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. Int Immunol **19:**133-40.

141. **Yi, J. S., M. A. Cox, and A. J. Zajac.** T-cell exhaustion: characteristics, causes and conversion. Immunology **129:**474-81.

142. **Yi, J. S., M. Du, and A. J. Zajac.** 2009. A vital role for interleukin-21 in the control of a chronic viral infection. Science **324:**1572-6.

143. **Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed.** 1998. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med **188:**2205-13.

144. **Zelinskyy, G., K. Dietze, T. Sparwasser, and U. Dittmer.** 2009. Regulatory T cells suppress antiviral immune responses and increase viral loads during acute infection with a lymphotropic retrovirus. PLoS Pathog **5:**e1000406.

145. **Zelinskyy, G., K. K. Dietze, Y. P. Husecken, S. Schimmer, S. Nair, T. Werner, K. Gibbert, O. Kershaw, A. D. Gruber, T. Sparwasser, and U. Dittmer.** 2009. The regulatory T-cell response during acute retroviral infection is locally defined and controls the magnitude and duration of the virus-specific cytotoxic T-cell response. Blood **114:**3199-207.

146. **Zhang, B., C. H. Maris, J. Foell, J. Whitmire, L. Niu, J. Song, B. S. Kwon, A. T. Vella, R. Ahmed, J. Jacob, and R. S. Mittler.** 2007. Immune suppression or enhancement by CD137 T cell costimulation during acute viral infection is time dependent. J Clin Invest **117:**3029-41.